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ARTICLE

Methodology matters: IVF versus ICSI and embryonic gene expression

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After completing her PhD in Biomedical Sciences with research in embryology, Dr Baker focused on education for reproductive laboratory personnel and established the first curricular-based graduate programme in the USA at the University of Kentucky, where she is professor and director of graduate studies in reproductive sciences. She conducts research in reproductive microbiology and policy issues related to assisted reproduction treatment. Her interest in the long-term clinical outcomes associated with assisted reproduction, especially assisted fertilization, has led to collaborations with basic scientists with established expertise in gene expression databases to study gene expression in murine blastocysts derived by different assisted fertilization techniques.

Abstract The use of assisted reproduction treatment, especially intracytoplasmic sperm injection (ICSI), is now linked to a range of adverse consequences, the aetiology of which remains largely undefined. Our objective of this study was to determine differences in gene expression of blastocysts generated by ICSI as well as ICSI with artificial oocyte activation (ICSI-A) versus the less manipulative IVF, providing fundamental genetic information that can be used to aid in the diagnosis or treatment of those adversely affected by assisted reproduction treatment, as well as stimulate research to further refine these techniques. Murine blastocysts were generated by ICSI, ICSI-A and IVF, and processed for a microarray-based analysis of gene expression. Ten blastocysts were pooled for each procedure and three independent replicates generated. The data were then processed to determine differential gene expression and to identify biological pathways affected by the procedures. In blastocysts derived by ICSI versus IVF, the expression of 197 genes differed ($P < 0.01$). In blastocysts derived by ICSI-A versus IVF and ICSI-A versus ICSI, the expression of 132 and 65 genes differed respectively ($P < 0.01$). Procedural-induced changes in genes regulating specific biological pathways revealed some consistency to known adverse consequences. Detailed investigation of procedure-specific dysfunction is therefore warranted.
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KEYWORDS: activation, blastocyst, gene expression, ICSI, IVF, mouse

Introduction

The use of assisted reproductive technology has increased dramatically over the last 30 years, providing an unprecedented opportunity for infertile couples to conceive a child. Unfortunately, the use of techniques such as intracytoplasmic sperm injection (ICSI) has become increasingly linked to adverse consequences that can affect both the mother and her child. In the mother, this includes an increased incidence of placental abruption, pre-eclampsia and stillbirths (Arav et al., 2010; Aytoz et al., 1998; Blumenfeld et al., 1992; Bonduelle et al., 2005; Buckett et al., 2008; Devroey and

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Van Steirteghem, 2004; Katalinic et al., 2004; Lidegaard et al., 2005; Lucifero et al., 2004; Osmanagaoglu et al., 2004; Pinborg et al., 2005; Poret et al., 2010; Unger et al., 2004; Woldringh, 2005). In children, dysfunction can range from the development of tumours and carcinomas (Katalinic et al., 2004; Lightfoot et al., 2004; Moll et al., 2003a,b; Niemitz and Feinberg, 2004; Odone-Filho et al., 2002; Owen and Segars, 2009; Toren et al., 1995; White et al., 1990) to congenital anomalies such as septal heart defects and cleft lip or palate (Anthony et al., 2002; Bertelsmann and de Carvalho, 2008; Bonduelle et al., 2005; Hansen et al., 2005; Kallen et al., 2005; Karpman et al., 2005; Katalinic et al., 2004; Olson et al., 2005; Orstavik, 2003; Poret et al., 2010; Reefhuis et al., 2009; Rimm et al., 2004; Schieve et al., 2005; Sutcliffe and Derom, 2006; Wennerholm et al., 2000; Williams et al., 2010), as well as neurological problems that may result in an intellectual lag (Bonduelle et al., 2005; Katalinic et al., 2004; Lidegaard et al., 2005). Unfortunately, the aetiology of these unwanted effects has proven difficult to determine, in part due to the patient-, clinic- and doctor-specific practices that are associated with the use of these reproductive techniques.

The extent to which assisted reproduction treatment-induced defects can be traced back to an altered pattern of embryonic gene expression is unknown. However, aberrant gene expression within the blastocyst, the first differentiated stage of development that occurs after any manipulation of the gametes, could increase the incidence of, or predisposition to, the multitude of defects that have now been associated with the use of techniques such as intracytoplasmic sperm injection (ICSI). The objective of this study was to identify the genes and the biological pathways that they regulate, which differ in blastocysts generated by ICSI versus IVF. Because chemical activation of the oocyte is now included in some ICSI protocols, aiming to mimic the events within the oocyte that are induced by the penetrating spermatozoon, the experimental design was expanded to include ICSI with chemical activation (ICSI-A) as an independent procedure in itself. The hypothesis was that gene expression would differ in blastocysts derived by ICSI versus IVF, and that these differences would be negated by the inclusion of the chemical activation procedure to the ICSI protocol. In effect, artificial activation of the oocyte would overcome some of the differences in gene expression induced by the ICSI procedure. Determining how these methodologies affect gene expression in the blastocyst should stimulate research that will translate to advances in the diagnosis, treatment and/or management of patients and offspring adversely affected by these technologies, as well as in the refinement of these practices that benefit so many. To facilitate this, the data generated are provided in a manner as complete as possible, relying heavily upon the use of supplementary tables (available online only) that will allow the identification of specific gene targets for further investigation by others.

Materials and methods

Animals

All mice were purchased from Samtaco (IcrTacSam; Samtaco, Seoul, Korea), housed under a 12:12 h light/dark cycle in a temperature- and humidity-controlled room and provided with food and water *ad libitum*. The protocol for the use of these animals was approved by the Institutional Animal Care and Use Committee of CHA University, Seoul, Korea.

Sperm collection and preparation

Epididymal spermatozoa were obtained from male BDF1 mice at 8–10 weeks of age. Spermatozoa used for IVF were collected from the cauda epididymis in 200 μl drops of Quinn's Advantage Medium with Hepes (SAGE In-Vitro Fertilization; Pasadena, CA, USA) and capacitated by incubation for 1.5 h at 37°C under 5% CO_2 in air. Spermatozoa used for ICSI and ICSI-A were squeezed from the cauda epididymis and placed in the bottom of 1.5-ml tubes containing 500 μl of the same Quinn's Advantage medium with Hepes. Spermatozoa were then allowed to 'swim up' for 5 min at room temperature before being collected for the intracytoplasmic injections.

Oocyte collection

Six-week old female B6D2F1 mice were treated with 5 IU pregnant mare's serum gonadotrophin (PMSG; Sigma–Aldrich, St. Louis, MO, USA) to induce follicular development and 48 h later with 5 IU human chorionic gonadotrophin (Sigma–Aldrich) to induce ovulation. At 12–15 h after human chorionic gonadotrophin, ovulated cumulus–oocyte-complexes were retrieved from the ampullary region of each oviduct and placed in Quinn's Advantage Medium with Hepes. Cumulus–oocyte-complexes to be used for IVF were then transferred into fertilization drops of the same medium. Cumulus–oocyte-complexes to be used for ICSI and ICSI-A were first incubated in Quinn's medium with the addition of 0.1% bovine testicular hyaluronidase (Tokyo Kasei Kogyo, Tokyo, Japan), effectively dispersing cumulus cells. The cumulus-free oocytes were then washed in fresh Quinn's Advantage Medium with Hepes and used immediately for ICSI.

IVF

Cumulus-intact oocytes in 200 μl drops of Quinn's Advantage Fertilization medium were incubated with capacitated spermatozoa at a final concentration of 100 spermatozoa/μl medium. Gametes were co-incubated for 6 h at 37°C under 5% CO_2 in air. After the 6 h co-incubation, oocytes were washed several times with fresh Quinn's Advantage Medium with Hepes. This protocol is based on the well-established methodology of Hogan et al. (1994).

Intracytoplasmic sperm injection

A small volume of the sperm suspension was mixed with Quinn's Advantage Medium with Hepes containing 12% polyvinyl pyrrolidone. Injections were performed immediately thereafter using a micromanipulator with a Piezo-electric actuator (PMM Controller, model PMAS-CT150; Prima Tech, Tsukuba, Japan). The head of each spermatozoa was separated from the tail by applying pulses to the head-tail junction by means of the Piezo-driven pipette. Only the head of one spermatozoon was injected into the cytoplasm of each

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metaphase II-stage oocyte and the oocytes were injected in groups of 10—15 (on a particular day), overall taking less than 10 min per group. A photographic time-course of the procedure is provided as **Figure 1**. Spermatozoa-injected oocytes were then transferred into KSOM medium and cultured at 37°C, 5% CO_2 in air. To activate the oocytes after ICSI (ICSI-A treatment), injected oocytes were placed in Ca^{2+}-free CZB medium containing 10 mmol/l $SrCl_2$ at 60 min after the injection and cultured for 1 h. ICSI-A oocytes were then returned to culture in KSOM medium at 37°C, 5% CO_2 in air. This protocol is based on the original work of Kimura and Yanagimachi (1995).

Embryo culture

Following ICSI, ICSI-A and IVF, oocytes were placed in 50 μl drops of KSOM medium and incubated at 37°C, 5% CO_2 in air. The culture drops were contained in plastic culture dishes and overlaid with mineral oil. Cultured embryos were then evaluated for developmental progress after 24 and 96 h.

Table 1 Development of embryos obtained by IVF, intracytoplasmic sperm injection (ICSI) and ICSI with artificial oocyte activation (ICSI-A).

Technique	*Fertilized oocytes cultured*	*2-Cell embryos*	*Blastocysts*
IVF	108	101 (93.52)	79 (78.22)
ICSI	130	111 (85.38)	57 (51.35)
ICSI-A	80	74 (92.50)	40 (54.05)

Data are the sum results from four independent experiments. Values are *n* or *n* (%).

Embryos were generated by the hands of a single experienced embryologist, with the rates of fertilization and development shown in **Table 1**. A comparison of cell numbers (total, inner cell mass and trophectoderm) for blastocysts generated by ICSI, ICSI-A and IVF is presented in **Table 2**.

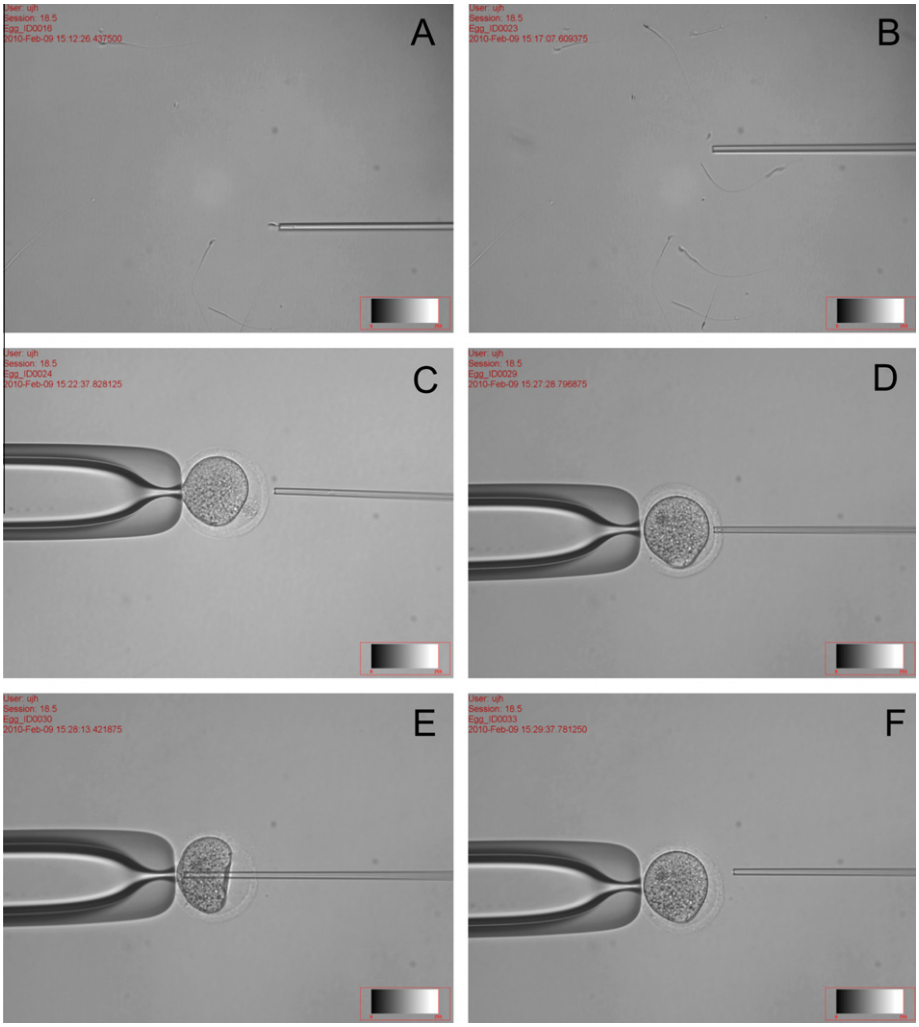


Figure 1 Representative images taken during intracytoplasmic sperm injection. (**A**) Loading of an epididymal spermatozoa. (**B**) Separation of head and tail. (**C**) The metaphase II-staged oocyte ready for injection. (**D**) Penetration of the zona pellucida. (**E**) Injection of the spermatozoa into the oocyte. (**F**) The injected oocyte.

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Table 2 Comparison of cell numbers in blastocysts derived by IVF, intracytoplasmic sperm injection (ICSI) and ICSI with chemical activation (ICSI-A).

Technique (no. of blastocysts)	*Mean cells/ blastocyst (n)*	*Cells in trophectoderm*		*Cells in inner cell mass*	
		n	%	n	%
IVF (n = 10)	39.7	25.9 ± 1.2[a]	65.3 ± 2.1[b]	13.8 ± 1.0[b]	34.7 ± 2.1[b]
ICSI (n = 10)	39.4	29.2 ± 1.4[a]	74.2 ± 1.7[a]	10.2 ± 0.9[a]	25.8 ± 1.7[a]
ICSI-A (n = 9)	38.7	28.3 ± 3.5[a]	72.6 ± 2.7[a]	10.3 ± 1.1[a]	28.0 ± 2.7[a]

Values are mean ± SD.
Values in columns with different superscripts differ ($P < 0.05$).

Sample processing and microarray hybridization

Embryos that developed to the blastocyst stage *in vitro* were collected for the microarray analysis, chosen because at that stage of early development, blastocysts have differentiated to have an inner cell mass and trophectoderm and have a high chance of continuing in their development. Blastocysts were not collected from naturally bred mice *in vivo* as embryo-oviductal interactions that affect embryonic gene expression would confound the results. Ten blastocysts were pooled for each treatment group (ICSI, ICSI-A and IVF) and three independent replicates for each treatment procedure were collected. Pooling was required in order to obtain sufficient RNA for the analysis and also to reduce the overall chance of generating errors. Total RNA was extracted using Trizol and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA was then amplified and labelled using the Nugen WT Ovation One-Direct RNA Amplification and Nugen FL-Ovation cDNA Biotin Module V2 labelling kits, respectively (NuGen Technologies, San Carlos, CA, USA). Microarray hybridization was then performed using Affymetrix Mouse 430—2.0 whole genome arrays (Affymetrix, Santa Clara, CA, USA) by the Microarray Core Facility at the University of Kentucky, as previously described (Jeoung et al., 2010; Jo et al., 2004). Three independent microarray chips were hybridized for each treatment procedure.

Data analysis

Data from the microarray hybridization were sorted for each analysis, excluding probe sets that consistently exhibited absent or marginal detection calls (i.e. at least two of the three detection calls for both treatment groups in a particular analysis were absent or marginal). The resultant datasets were then processed using Pathways Studio 7.1 software (Ariadne Genomics, Rockville, MD, USA) to: (i) identify probe sets that differed among two treatment groups; and (ii) sort the data into biological pathways regulated by treatment. We have successfully used this software to analyse microarray datasets in the past (Jeoung et al., 2010). For the analysis to identify probe sets that differed among two treatment groups, differential expression was defined as those transcripts that exhibited a difference in expression of 2-fold or greater and were statistically different ($P < 0.01$), as determined by the software package that utilizes a statistical algorithm with a one-sided Mann—Whitney U-test to determine P-values indicating significance. To sort the data into biological processes regulated by treatment, the statistical algorithm was relaxed ($P < 0.05$) and a Gene Set Enrichment performed by the software package. The comparisons performed were ICSI versus IVF, ICSI-A versus IVF and ICSI-A versus ICSI, with only biological processes that exhibited at least five overlapping entities (genes) presented in the results.

Results

Gene expression I: ICSI versus IVF

Analysis of the microarray dataset identified 236 probes reflecting 197 known genes that differed in blastocysts generated by ICSI versus IVF ($P < 0.01$). For each of these genes, its probe set and Entrez gene identification number, fold-change in level of expression and P-value comparing the two treatment techniques are listed in Supplementary Table 1 (available online only). The dataset was also processed to reveal biological pathways that differed when blastocysts were generated by these two techniques (**Table 3**). Consistent with some of the developmental anomalies that have been associated with the use of assisted reproduction treatment (Anthony et al., 2002; Bertelsmann and de Carvalho, 2008; Bonduelle et al., 2005; Hansen et al., 2005; Kallen et al., 2005; Karpman et al., 2005; Katalinic et al., 2004; Olson et al., 2005; Orstavik, 2003; Poret et al., 2010; Reefhuis et al., 2009; Rimm et al., 2004; Schieve et al., 2005; Sutcliffe and Derom, 2006; Wennerholm et al., 2000; Williams et al., 2010), the analysis identified development as a primary biological process affected by technique (ICSI versus IVF). Inclusive to this were several genes regulating structural and organ-specific processes, as well as those that were classified into more general cellular categories. Several metabolic pathways were identified to differ by procedure as well as a variety of response processes, signalling and transport mechanisms. As the purpose of this study is to provide the background genetic information required to stimulate further research, the results are presented without overt explanation or likely bias to any one physiological mechanism. The complete list of individual genes that were grouped into each biological function is also presented as an expanded version of **Table 3** (Supplementary Table 2, available online only).

Gene expression II: ICSI-A versus IVF

A total of 146 probe sets representing 132 known genes were found to differ in blastocysts generated by ICSI-A versus IVF ($P < 0.01$). Again, the individual probes and associated

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Table 3 Functional classification of genes that differed in blastocysts derived by intracytoplasmic sperm injection (ICSI) versus IVF ($P < 0.05$).

Type	*Function*	*Name*	*Entities*	P-*value*
Development	Cellular	Multicellular organismal development	550	0.00062
		Cell differentiation	332	0.00160
		Ureteric bud branching	17	0.00264
		Cell development	11	0.00627
		Metanephros development	10	0.00767
		Morphogenesis of a branching structure	5	0.00911
	Growth	Growth	18	0.00091
	Neural	Nervous system development	219	0.00419
	Organ	Organ morphogenesis	95	0.00008
		Pancreas development	12	0.00142
		Sex differentiation	11	0.00427
		Endocrine pancreas development	9	0.00898
		Inner ear morphogenesis	30	0.00905
		Forebrain development	44	0.00979
		Sex determination	9	0.00993
	Structural	Anatomical structure development	9	0.00047
		Embryonic gut development	7	0.00061
		Proximal—distal pattern formation	14	0.00119
	Other	Pattern specification process	27	0.00294
Metabolism		Lipid catabolic process	57	0.00018
		Xenobiotic metabolic process	14	0.00059
		Lipid glycosylation	6	0.00155
		Cholesterol metabolic process	42	0.00581
		Taurine metabolic process	5	0.00958
Response	Cellular	Negative regulation of chondrocyte differentiation	5	0.00442
		Positive regulation of ossification	10	0.00781
		Negative regulation of erythrocyte differentiation	5	0.00854
		Positive regulation of cell proliferation	209	0.00065
		Negative regulation of angiogenesis	17	0.00089
		Positive regulation of vasodilation	10	0.00223
		Chemotaxis	57	0.00309
		Proteoglycan biosynthetic process	6	0.00315
		Positive regulation of survival gene product expression	7	0.00521
		Neurotransmitter secretion	21	0.00580
		Positive regulation of mitosis	11	0.00942
		Response to glucocorticoid stimulus	58	0.01000
	Immune	Inflammatory response	111	0.00060
		Immune response	202	0.00263
		Response to wounding	31	0.00630
		Positive regulation of T cell-mediated cytotoxicity	8	0.00637
	Mechanical	Response to mechanical stimulus	30	0.00023
		Sperm motility	12	0.00102
		Ciliary or flagellar motility	5	0.00812
		Positive regulation of smooth muscle contraction	8	0.00868
	Other	Visual perception	93	0.00095
		Response to stimulus	80	0.00139
		Digestion	18	0.00204
		Synaptic transmission	93	0.00014
		Response to external stimulus	5	0.00568

(*Table 3 continued on next page*)

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Table 3 (*continued*)

Type	*Function*	*Name*	*Entities*	*P-value*
Signalling		G-protein coupled receptor protein signalling pathway	210	0.00000
		Signal transduction	789	0.00022
		Cell–cell signalling	104	0.00069
		Inositol phosphate-mediated signalling	5	0.00399
		Wnt receptor signalling pathway, calcium modulating pathway	5	0.00455
		Cytokine-mediated signalling pathway	41	0.00590
		Elevation of cytosolic calcium ion concentration	38	0.00867
Transport		Iron ion transport	22	0.00460
		Ion transport	281	0.00902
Miscellaneous		Feeding behaviour	11	0.00016

information are listed in full (Supplementary Table 3, available online only). The biological process classification for differentially expressed genes in blastocysts derived by ICSI-A versus IVF ($P < 0.05$) is presented in **Table 4** and expanded to include gene listings in Supplementary Table 4 (available online only). Interestingly, while differences in developmental processes were still found to differ in blastocysts generated by ICSI-A versus IVF, the number of classes identified was approximately halved. A large number of response-type pathways were identified that ranged from the specific (e.g. the regulation of ossification) to the general (e.g. immune response) that should be evaluated in the future in concert with the specific probes listed in the supplementary tables.

Gene expression III: ICSI or ICSI-A versus IVF

From the 197 genes that were found to differ when blastocysts were derived by ICSI versus IVF, only 18 probe sets representing 17 genes were common to those identified when ICSI-A and IVF were compared. These 17 genes, their average intensities, fold changes and individual *P*-values are identified in **Table 5**.

Gene expression IV: ICSI versus ICSI-A

The analysis identified 74 probe sets representing 65 known genes that differed between the ICSI and ICSI-A treatment groups ($P < 0.01$). Each of these 65 genes, its identity, fold-change in level of expression and *P*-value comparing the two treatment techniques are listed in Supplementary Table 5 (available online only). The method of ICSI (i.e. with or without artificial activation of the oocytes) used to generate blastocysts was also found to affect developmental, metabolic and response processes in the biological pathway analysis ($P < 0.05$; **Table 6**) with this information expanded to include gene information in Supplementary Table 6 (available online only).

Discussion

Recent data from the US Centres for Disease Control and Prevention indicate that ~7.3 million US couples suffer from infertility and that ~140,000 assisted reproduction cycles are performed per year (Wright et al., 2007; http://www.cdc.gov/ART/). The benefit from the development and use of these techniques is unquestionable; however assisted reproduction treatment and especially ICSI have become increasingly linked to a broad range of unwanted and often serious consequences to the mother and/or her offspring. The objective of this study was to determine, in the developing mouse embryo, genetic pathways affected by ICSI versus the less manipulative IVF. In addition to this, this study evaluated whether artificial activation of oocytes would overcome some of the differences in gene expression attributed specifically to the ICSI procedure. Mice were used to generate the embryos as they represent a population of fertile, genetically homogenous and healthy subjects. Determination of procedural-specific changes in embryonic gene expression should stimulate research that will translate into advances in clinical practice, both in the management of prior treatment-induced effects, as well as in the refinement of these techniques in the future. Immediate advances could include identifying an array of treatment-induced genes as genetic markers, signalling the need for planned intervention and management of certain diseases in the newborn, or increasing the breadth of preimplantation genetic diagnosis for the most serious consequences associated with these treatments for infertility. The first live births after IVF and ICSI were reported in 1978 (Steptoe and Edwards, 1978) and 1992 (Palermo et al., 1992), respectively. The field of assisted reproduction treatment has grown at an unprecedented pace, making this analysis of treatment-induced gene function a timely and necessary report.

The majority of treatment cycles performed in the USA utilize IVF and/or ICSI (>50% ICSI) with the other treatment types, gamete and zygote intra-Fallopian transfer (GIFT and ZIFT) accounting for <1% of all procedures (Wright et al., 2007; http://www.cdc.gov/ART/). However, the data from these reports also indicate that ICSI is proportionally increasing in its use and routinely prescribed over IVF, regardless of the aetiology of infertility; i.e. even when the cause of infertility would indicate success with IVF alone. This is extremely pertinent given the data presented herein; 236 probe sets accounting for 197 genes differed in blastocysts generated by ICSI versus IVF, suggesting procedural-driven changes in embryonic gene expression

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Table 4 Functional classification of genes that differed in blastocysts derived by ICSI with chemical activation (ICSI-A) versus IVF ($P < 0.05$).

Type	*Function*	*Name*	*Entities*	*P-value*
Development	Cellular	Ureteric bud branching	16	0.00469
	Neural	Peripheral nervous system development	14	0.00398
		Neural crest cell development	5	0.00798
	Organ	Organ morphogenesis	98	0.00007
		Thyroid gland development	5	0.00147
		Inner ear morphogenesis	29	0.00197
		Middle ear morphogenesis	6	0.00324
		Embryonic gut development	5	0.00792
		Pattern specification process	28	0.00890
	Structural	Cartilage development	28	0.00659
		Embryonic skeletal system morphogenesis	26	0.00720
Metabolism		Lipid glycosylation	7	0.00147
		Retinol metabolic process	10	0.00297
Response	Cellular	Glial cell differentiation	10	0.00072
		Elevation of cytosolic calcium ion concentration	40	0.00005
		Cell adhesion	265	0.00036
		Chemotaxis	59	0.00217
		Regulation of ossification	7	0.00378
		Positive regulation of cellular protein metabolic process	5	0.00605
		Positive regulation of mitosis	12	0.00678
		NAD biosynthetic process	6	0.00710
		Cell–cell adhesion	52	0.00812
		Cellular calcium ion homeostasis	36	0.00877
		Cholesterol biosynthetic process	26	0.00889
		Protein amino acid N-linked glycosylation	18	0.00963
	Immune	Inflammatory response	112	0.00000
		Immune response	203	0.00002
		Defence response to bacterium	26	0.00055
		Antigen processing and presentation	18	0.00128
		Complement activation, alternative pathway	5	0.00259
		Complement activation, classical pathway	15	0.00723
	Mechanical	Response to mechanical stimulus	33	0.00154
		Vasoconstriction	5	0.00188
	Neural	Axon guidance	59	0.00678
		Synaptic transmission	98	0.00005
	Other	Detection of chemical stimulus involved in sensory perception of smell	5	0.00647
		Visual perception	93	0.00001
		Response to stimulus	84	0.00006
		Digestion	17	0.00629
		Acute-phase response	15	0.00840
Signalling		G-protein coupled receptor protein signalling pathway	216	0.00000
		Signal transduction	805	0.00008
		Cell surface receptor linked signal transduction	115	0.00014
		G-protein signalling, coupled to cyclic nucleotide second messenger	14	0.00026
		Cell–cell signalling	103	0.00115
		Inositol phosphate-mediated signalling	5	0.00651
		G-protein signalling, coupled to cAMP nucleotide second messenger	10	0.00783

(*Table 4 continued on next page*)

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Table 4 (*continued*)

Type	*Function*	*Name*	*Entities*	*P-value*
Transport		Ion transport	286	0.00006
		Calcium ion transport	68	0.00078
		Iron ion transport	22	0.00197
Miscellaneous		Feeding behaviour	11	0.00024
		Axonal fasciculation	7	0.00188
		Memory	21	0.00749

Table 5 Identification of the 17 genes that differed when blastocysts were derived by IVF versus intracytoplasmic sperm injection (ICSI) and IVF versus ICSI with chemical activation (ICSI-A).

Name	*IVF versus ICSI*		*IVF versus ICSI-A*		*Probe set ID*	*Intensity average*		
	Fold change	*P-value*	*Fold change*	*P-value*		*IVF*	*ICSI*	*ICSI-A*
Unknown	−3.5516	0.0020	−2.334	0.0035	1439599_at	598.6	172.9	259.6
ABHD14B	−2.2566	0.0087	−2.0973	0.0055	1451326_at	1313.4	582.5	619.9
C10orf32	2.3728	0.0013	2.4465	0.0013	1419299_at	137.2	327.6	337.9
CA12	−6.8079	0.0026	−3.9913	0.0059	1428485_at	186.6	29.0	48.9
CUBN	−2.1933	0.0041	−2.0282	0.0018	1452270_s_at	1921.2	886.1	949.9
DDA1	−12.5618	0.0100	−12.0678	0.0019	1429039_s_at	239.0	22.1	19.6
EPAS1	3.2595	0.0005	2.5005	0.0011	1449888_at	173.2	567.6	434.7
FNTB	4.7982	0.0061	4.1234	0.0057	1459043_at	82.3	381.3	321.4
GRB10	−5.5633	0.0004	−4.8841	0.0005	1425457_a_at	755.8	135.6	154.3
GYLTL1B	−2.162	0.0065	−2.2273	0.0027	1434007_at	671.5	315.6	304.4
HAS2	−14.8595	0.0064	−24.3663	0.0051	1449169_at	272.3	20.7	13.0
HMGA2	2.2213	0.0022	2.1215	0.0048	1450780_s_at	1068.8	2346.3	2250.7
IL6	−3.569	0.0017	−4.072	0.0054	1450297_at	1257.0	355.5	321.3
LARGE	−2.0839	0.0075	−4.2598	0.0027	1417435_at	1017.6	478.2	240.3
NR3C1	2.8113	0.0007	2.1535	0.0007	1460303_at	414.5	1174.6	895.2
RAB17	2.6639	0.0005	2.1843	0.0010	1422178_a_at	702.5	1868.9	1531.4
Sco2	−5.5218	0.0026	−3.4909	0.0046	1432181_s_at	450.7	81.4	126.5
TBX20	3.6076	0.0000	3.5279	0.0004	1453351_at	686.4	2477.4	2451.8

and, potentially, the unwanted consequences that have been associated with assisted reproduction treatment.

Of the birth defects related to the use of these techniques, the manifestation of aberrant growth and development appears a common consequence (Anthony et al., 2002; Bertelsmann and de Carvalho, 2008; Bonduelle et al., 2005; Hansen et al., 2005; Kallen et al., 2005; Karpman et al., 2005; Katalinic et al., 2004; Olson et al., 2005; Orstavik, 2003; Poret et al., 2010; Reefhuis et al., 2009; Rimm et al., 2004; Schieve et al., 2005; Sutcliffe and Derom, 2006; Wennerholm et al., 2000; Williams et al., 2010). One of the most readily recognizable of these defects includes cleft lip and palate, condition(s) reflecting an asymmetry in development. A septal heart defect is less overtly recognizable, although a serious defect that is also related to symmetry and assisted reproduction treatment. Interestingly, this study identified the differential expression of 14 genes (ICSI versus IVF, **Table 3** and Supplementary Table 2) that were categorized to regulate proximal–distal pattern formation (i.e. symmetry) in these early embryos. That being said, developmental problems associated with assisted reproduction treatment are not confined to those manifested from asymmetry. Several specific syndromes that include Beckwith–Wiedemann (Filippi and McKusick, 1970; Neelanjana and Sabaratnam, 2008) and Silver–Russell (Abu-Amero et al., 2008; Neelanjana and Sabaratnam, 2008), which are characterized by overgrowth and intrauterine growth retardation, respectively, are also well recognized (DeBaun et al., 2003; Gicquel et al., 2003; Gosden et al., 2003; Halliday et al., 2004; Katalinic et al., 2004; Lidegaard et al., 2005; Lucifero et al., 2004; Maher et al., 2003a,b; Niemitz and Feinberg, 2004; Niemitz et al., 2004; Owen and Segars, 2009; Sutcliffe et al., 2006). In addition to this, aberrant development extends to the manifestation of neurological defects (Bonduelle et al., 2005; Katalinic et al., 2004; Lidegaard et al., 2005) and the present study identified procedural-induced changes in several genes regulating neural development when the expression profiles of blastocysts derived from IVF and ICSI were compared.

Similar to the wide scope of developmental disorders implicated with assisted reproduction treatment, a range of metabolic consequences have been described including

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Table 6 Functional classification of genes that differed in blastocysts derived by intracytoplasmic sperm injection (ICSI) versus ICSI with chemical activation (ICSI-A) ($P < 0.05$).

Type	*Function*	*Name*	*Entities*	*P-value*
Development	Cellular	Somitogenesis	25	0.00686
	Neural	Peripheral nervous system development	12	0.00474
		Central nervous system development	72	0.00824
		Hindbrain development	28	0.00687
	Structural	Embryonic forelimb morphogenesis	6	0.00478
		Negative regulation of striated muscle development	10	0.00266
		Anatomical structure development	8	0.00717
Metabolism		Insulin secretion	15	0.00180
		Fucose metabolic process	6	0.00192
Response	Cellular	Receptor clustering	10	0.00151
		Fertilization	19	0.00266
		Protein localization	49	0.00039
		Synaptic vesicle endocytosis	7	0.00847
		Vesicle organization	7	0.00901
	Immune	Cytokine production	15	0.00822
	Neural	Synaptonemal complex assembly	8	0.00598

neonatal hypoglycaemia (Beckwith–Wiedemann syndrome (Hussain et al., 2005; Kapoor et al., 2009; Palladino et al., 2009)) and childhood-onset obesity (Prader–Willi and MatUPD14 syndromes, (Bouchard, 2009; Butler et al., 2009; O'Neill et al., 2005)). Analysis of the present data indicated differences in the expression of genes regulating several metabolic pathways, including cholesterol and lipid metabolism/catabolism, in blastocysts derived from ICSI versus IVF. The concomitant change in genes regulating organ morphogenesis, as an example, pancreatic development, is consistent with a metabolic consequence to assisted reproduction treatment and suggests an underlying genetic cause for the development of certain metabolic diseases. Overall, the changes in gene expression that this study has identified between ICSI and IVF indicate that further investigation into these pathways is vital. Although the refinement of techniques to circumvent adverse consequences is obviously a primary goal for the future, the development of genetic markers indicating susceptibility to disease could have a dramatic impact on the diagnosis and management of several of the adverse consequences, including those of a metabolic origin, that are associated with assisted reproduction treatment.

A more recent modification to the procedure of ICSI is the inclusion of a chemical activation step (Dozortsev et al., 1995), reviewed by Nasr-Esfahani et al., 2010, which aims to mimic the events within the oocyte that occur during the normal fertilization process; i.e. under normal conditions, spermatozoon–oocyte fusion is followed by the incorporation of, and interaction between, a demembranated sperm nucleus and the cytoplasm of the oocyte. The nucleus of the spermatozoa is readily accessible to ooplasmic factors, with fusion of the gametes stimulating pivotal intracellular calcium oscillations. Modifications to the zona pellucida and release from meiotic arrest ensue. Artificial methods to activate the oocyte have now been developed, that improve the rate of fertilization (especially in cases of low fertility) and can be performed using chemical agents such as strontium chloride (Kumagai, 2006) and calcium ionophores (Borges et al., 2009; Murase et al., 2004), or by mechanical (Dirican et al., 2008) or electrical (Yanagida et al., 1999) stimulation. In the experiments described herein, strontium chloride was used. Although this technique has proven very effective (Murase et al., 2004; Suttner et al., 2000), the utilization of activation after ICSI (ICSI-A) appears to be clinic-specific with only a very limited number of clinics activating oocytes after microinjection.

It is very interesting that when compared with IVF, the inclusion of this chemical activation step to the ICSI protocol brought the sample clustering of the probe sets closer together; i.e. ICSI-A clustered closer to IVF than to ICSI alone, suggesting that this artificial activation step effectively mimics, at the genetic level, a proportion of the events initiated by sperm penetration. When gene expression in blastocysts derived by ICSI-A and IVF were analysed independently, 146 probe sets were identified to differ, 90 fewer than the 236 probes identified to differ in the ICSI versus IVF analysis. In itself, this is a striking reduction in differential gene expression and further illustrative of the alignment of IVF and ICSI-A observed in the clustering analysis. When gene expression in blastocysts derived by ICSI was compared directly to those generated with the optional activation step (ICSI-A), 74 probe sets reflecting the differential expression of 65 known genes was revealed. The classification of development was highlighted by the biological pathway grouping and differences in the expression of genes regulating structural and neural developmental pathways uncovered, consistent with known treatment-induced dysfunction.

Overall, the expression of genes in blastocysts was affected by the procedure used to generate the embryo and classification of differentially expressed genes into biological pathways revealed consistency to known treatment-induced adverse consequences. Obviously, further

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investigation is needed before these findings can be translated into clinical advances and the reader must remain cognizant that these results were generated using blastocysts of a murine origin, and not those obtained from an assisted reproduction clinic. However, with the use of ICSI now appearing to be dominant over IVF, regardless of the aetiology of infertility, and ICSI-A not yet standardized into the operating procedures of the majority of assisted reproduction clinics, deadlines for teasing out procedure-specific consequences cannot be delayed.

The field of assisted reproduction treatment has grown exponentially over the last 30 years, providing an unprecedented opportunity for infertile couples to conceive a child. This genetic analysis was performed to stimulate research in this field with the overall goal of advancing understanding (and performance) of these most valuable treatment options for infertility. It is hoped that future investigation into the genes and pathways uncovered in this report will prove fruitful and that advances made will be timely.

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References

Abu-Amero, S., Monk, D., Frost, J., Preece, M., Stanier, P., Moore, G.E., 2008. The genetic aetiology of Silver—Russell syndrome. J. Med. Genet. 45, 193—199.

Anthony, S., Buitendijk, S.E., Dorrepaal, C.A., Lindner, K., Braat, D.D., den Ouden, A.L., 2002. Congenital malformations in 4224 children conceived after IVF. Hum. Reprod. 17, 2089—2095.

Arav, A., Gavish, Z., Elami, A., Natan, Y., Revel, A., Silber, S., et al., 2010. Ovarian function 6 years after cryopreservation and transplantation of whole sheep ovaries. Reprod. Biomed. Online 20, 48—52.

Aytoz, A., De Catte, L., Camus, M., Bonduelle, M., Van Assche, E., Liebaers, I., et al., 1998. Obstetric outcome after prenatal diagnosis in pregnancies obtained after intracytoplasmic sperm injection. Hum. Reprod. 13, 2958—2961.

Bertelsmann, H., de Carvalho, G., 2008. The risk of malformation following assisted reproduction. Dtsch. Arztebl. Int. 1, 105.

Blumenfeld, Z., Dirnfeld, M., Abramovici, H., Amit, A., Bronshtein, M., Brandes, J.M., 1992. Spontaneous fetal reduction in multiple gestations assessed by transvaginal ultrasound. Br. J. Obstet. Gynaecol. 99, 333—337.

Bonduelle, M., Wennerholm, U.B., Loft, A., Tarlatzis, B.C., Peters, C., Henriet, S., et al., 2005. A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. Hum. Reprod. 20, 413—419.

Borges Jr., E., de Almeida Ferreira Braga, D.P., de Sousa Bonetti, T.C., Iaconelli Jr., A., Franco Jr., J.G., 2009. Artificial oocyte activation with calcium ionophore A23187 in intracytoplasmic sperm injection cycles using surgically retrieved spermatozoa. Fertil. Steril. 92, 131—136.

Bouchard, C., 2009. Childhood obesity: are genetic differences involved? Am. J. Clin. Nutr. 89, 1494S—1501S.

Buckett, W.M., Chian, R.C., Dean, N.L., Sylvestre, C., Holzer, H.E., Tan, S.L., 2008. Pregnancy loss in pregnancies conceived after in vitro oocyte maturation, conventional in vitro fertilization, and intracytoplasmic sperm injection. Fertil. Steril. 90, 546—550.

Butler, J.V., Whittington, J.E., Holland, A.J., McAllister, C.J., Goldstone, A.P., 2009. The transition between the phenotypes of Prader—Willi syndrome during infancy and early childhood. Dev. Med. Child Neurol. 52, e88—e93.

DeBaun, M.R., Niemitz, E.L., Feinberg, A.P., 2003. Association of in vitro fertilization with Beckwith—Wiedemann syndrome and epigenetic alterations of LIT1 and H19. Am. J. Hum. Genet. 72, 156—160.

Devroey, P., Van Steirteghem, A., 2004. A review of ten years experience of ICSI. Hum. Reprod. Update 10, 19—28.

Dirican, E.K., Isik, A., Vicdan, K., Sozen, E., Suludere, Z., 2008. Clinical pregnancies and livebirths achieved by intracytoplasmic injection of round headed acrosomeless spermatozoa with and without oocyte activation in familial globozoospermia: case report. Asian J. Androl. 10, 332—336.

Dozortsev, D., Rybouchkin, A., De Sutter, P., Qian, C., Dhont, M., 1995. Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. Hum. Reprod. 10, 403—407.

Filippi, G., McKusick, V.A., 1970. The Beckwith—Wiedmann syndrome. Medicine (Baltimore) 49, 279—298.

Gicquel, C., Gaston, V., Mandelbaum, J., Siffroi, J.P., Flahault, A., Le Bouc, Y., 2003. In vitro fertilization may increase the risk of Beckwith—Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. Am. J. Hum. Genet. 72, 1338—1341.

Gosden, R., Trasler, J., Lucifero, D., Faddy, M., 2003. Rare congenital disorders, imprinted genes, and assisted reproductive technology. Lancet 361, 1975—1977.

Halliday, J., Oke, K., Breheny, S., Algar, E., D,, J.A., 2004. Beckwith—Wiedemann syndrome and IVF: a case-control study. Am. J. Hum. Genet. 75, 526—528.

Hansen, M., Bower, C., Milne, E., de Klerk, N., Kurinczuk, J.J., 2005. Assisted reproductive technologies and the risk of birth defects — a systematic review. Hum. Reprod. 20, 328—338.

Hogan, B., Beddington, R., Constantini, F., Lacy, E., 1994. Manipulating the Mouse Embryo: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, pp. 146—150.

Hussain, K., Cosgrove, K.E., Shepherd, R.M., Luharia, A., Smith, V.V., Kassem, S., et al., 2005. Hyperinsulinemic hypoglycemia in Beckwith—Wiedemann syndrome due to defects in the function of pancreatic beta-cell adenosine triphosphate-sensitive potassium channels. J. Clin. Endocrinol. Metab. 90, 4376—4382.

Jeoung, M., Lee, S., Hawng, H.K., Cheon, Y.P., Jeong, Y.K., Gye, M.C., et al., 2010. Identification of a novel role for endothelins within the oviduct. Endocrinology 151, 2858—2867.

Jo, M., Gieske, M.C., Payne, C.E., Wheeler-Price, S.E., Gieske, J.B., Ignatius, I.V., et al., 2004. Development and application of a rat ovarian gene expression database. Endocrinology 145, 5384—5396.

Kallen, B., Finnstrom, O., Nygren, K.G., Olausson, P.O., 2005. In vitro fertilization (IVF) in Sweden: infant outcome after different IVF fertilization methods. Fertil. Steril. 84, 611—617.

Kapoor, R.R., James, C., Hussain, K., 2009. Hyperinsulinism in developmental syndromes. Endocr. Dev. 14, 95—113.

Karpman, E., Williams, D.H., Lipshultz, L.I., 2005. IVF and ICSI in male infertility: update on outcomes, risks, and costs. Scientific WorldJournal 5, 922—932.

Katalinic, A., Rosch, C., Ludwig, M., 2004. Pregnancy course and outcome after intracytoplasmic sperm injection: a controlled, prospective cohort study. Fertil. Steril. 81, 1604—1616.

Kimura, Y., Yanagimachi, R., 1995. Intracytoplasmic sperm injection in the mouse. Biol. Reprod. 52, 709—720.

Kumagai, S.T.A., 2006. Three successful pregnancies and deliveries after oocyte. Fertil. Steril. 86, S156—S157.

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Lidegaard, O., Pinborg, A., Andersen, A.N., 2005. Imprinting diseases and IVF: Danish National IVF cohort study. Hum. Reprod. 20, 950–954.

Lightfoot, T., Bunch, K., Ansell, P., Murphy, M., 2004. Ovulation induction, assisted conception and childhood cancer. Eur. J. Cancer 41, 715–724.

Lucifero, D., Mann, M.R., Bartolomei, M.S., Trasler, J.M., 2004. Gene-specific timing and epigenetic memory in oocyte imprinting. Hum. Mol. Genet. 13, 839–849.

Maher, E.R., Afnan, M., Barratt, C.L., 2003a. Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs? Hum. Reprod. 18, 2508–2511.

Maher, E.R., Brueton, L.A., Bowdin, S.C., Luharia, A., Cooper, W., Cole, T.R., et al., 2003b. Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). J. Med. Genet. 40, 62–64.

Moll, A.C., Imhof, S.M., Cruysberg, J.R., Schouten-van Meeteren, A.Y., Boers, M., van Leeuwen, F.E., 2003a. Incidence of retinoblastoma in children born after in-vitro fertilisation. Lancet 361, 309–310.

Moll, A.C., Imhof, S.M., Schouten-van Meeteren, A.Y., van Leeuwen, F.E., 2003b. In-vitro fertilisation and retinoblastoma. Lancet 361, 1392.

Murase, Y., Araki, Y., Mizuno, S., Kawaguchi, C., Naito, M., Yoshizawa, M., 2004. Pregnancy following chemical activation of oocytes in a couple with repeated failure of fertilization using ICSI: case report. Hum. Reprod. 19, 1604–1607.

Nasr-Esfahani, M.H., Deemeh, M.R., Tavalaee, M., 2010. Artificial oocyte activation and intracytoplasmic sperm injection. Fertil. Steril. 94, 520–526.

Neelanjana, M., Sabaratnam, A., 2008. Malignant conditions in children born after assisted reproductive technology. Obstet. Gynecol. Surv 63, 669–676.

Niemitz, E.L., Feinberg, A.P., 2004. Epigenetics and assisted reproductive technology: a call for investigation. Am. J. Hum. Genet. 74, 599–609.

Niemitz, E.L., DeBaun, M.R., Fallon, J., Murakami, K., Kugoh, H., Oshimura, M., et al., 2004. Microdeletion of LIT1 in familial Beckwith-Wiedemann syndrome. Am. J. Hum. Genet. 75, 844–849.

O'Neill, M.A., Farooqi, I.S., Wevrick, R., 2005. Evaluation of Prader–Willi Syndrome gene MAGEL2 in severe childhood-onset obesity. Obes. Res. 13, 1841–1842.

Odone-Filho, V., Cristofani, L.M., Bonassa, E.A., Braga, P.E., Eluf-Neto, J., 2002. In vitro fertilization and childhood cancer. J. Pediatr. Hematol. Oncol. 24, 421–422.

Olson, C.K., Keppler-Noreuil, K.M., Romitti, P.A., Budelier, W.T., Ryan, G., Sparks, A.E., et al., 2005. In vitro fertilization is associated with an increase in major birth defects. Fertil. Steril. 84, 1308–1315.

Orstavik, K.H., 2003. Intracytoplasmic sperm injection and congenital syndromes because of imprinting defects. Tidsskr. Nor Laegeforen. 123, 177.

Osmanagaoglu, K., Kolibianakis, E., Tournaye, H., Camus, M., Van Steirteghem, A., Devroey, P., 2004. Cumulative live birth rates after transfer of cryopreserved ICSI embryos. Reprod. Biomed. Online 8, 344–348.

Owen, C.M., Segars Jr., J.H., 2009. Imprinting disorders and assisted reproductive technology. Semin. Reprod. Med. 27, 417–428.

Palermo, G., Joris, H., Devroey, P., Van Steirteghem, A.C., 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340, 17–18.

Palladino, A.A., Bennett, M.J., Stanley, C.A., 2009. Hyperinsulinism in infancy and childhood: when an insulin level is not always enough. Ann. Biol. Clin. (Paris) 67, 245–254.

Pinborg, A., Lidegaard, O., la Cour Freiesleben, N., Andersen, A.N., 2005. Consequences of vanishing twins in IVF/ICSI pregnancies. Hum. Reprod. 20, 2821–2829.

Poret, H., Blanchard, M., Lemseffer, M., Royere, D., Guerif, F., 2010. Conjoined twins after intracytoplasmic sperm injection and transfer of a single day 2 embryo: case report. Fertil. Steril. 93, 268, e267–e269.

Reefhuis, J., Honein, M.A., Schieve, L.A., Correa, A., Hobbs, C.A., Rasmussen, S.A., 2009. Assisted reproductive technology and major structural birth defects in the United States. Hum. Reprod. 24, 360–366.

Rimm, A.A., Katayama, A.C., Diaz, M., Katayama, K.P., 2004. A meta-analysis of controlled studies comparing major malformation rates in IVF and ICSI infants with naturally conceived children. J. Assist. Reprod. Genet. 21, 437–443.

Schieve, L.A., Rasmussen, S.A., Reefhuis, J., 2005. Risk of birth defects among children conceived with assisted reproductive technology: providing an epidemiologic context to the data. Fertil. Steril. 84, 1320–1324, discussion 1327.

Steptoe, P.C., Edwards, R.G., 1978. Birth after the reimplantation of a human embryo. Lancet 2, 366.

Sutcliffe, A.G., Derom, C., 2006. Follow-up of twins: health, behaviour, speech, language outcomes and implications for parents. Early Hum. Dev. 82, 379–386.

Sutcliffe, A.G., Peters, C.J., Bowdin, S., Temple, K., Reardon, W., Wilson, L., et al., 2006. Assisted reproductive therapies and imprinting disorders – a preliminary British survey. Hum. Reprod. 21, 1009–1011.

Suttner, R., Zakhartchenko, V., Stojkovic, P., Muller, S., Alberio, R., Medjugorac, I., et al., 2000. Intracytoplasmic sperm injection in bovine: effects of oocyte activation, sperm pretreatment and injection technique. Theriogenology 54, 935–948.

Toren, A., Sharon, N., Mandel, M., Neumann, Y., Kenet, G., Kaplinsky, C., et al., 1995. Two embryonal cancers after in vitro fertilization. Cancer 76, 2372–2374.

Unger, S., Hoopmann, M., Bald, R., Foth, D., Nawroth, F., 2004. Monozygotic triplets and monozygotic twins after ICSI and transfer of two blastocysts: case report. Hum. Reprod. 19, 110–113.

Wennerholm, U.B., Bergh, C., Hamberger, L., Lundin, K., Nilsson, L., Wikland, M., et al., 2000. Incidence of congenital malformations in children born after ICSI. Hum. Reprod. 15, 944–948.

White, L., Giri, N., Vowels, M.R., Lancaster, P.A., 1990. Neuroectodermal tumours in children born after assisted conception. Lancet 336, 1577.

Williams, C., Sutcliffe, A., Sebire, N.J., 2010. Congenital malformations after assisted reproduction: risks and implications for prenatal diagnosis and fetal medicine. Ultrasound Obstet. Gynecol. 35, 255–259.

Woldringh, G., 2005. Intracytoplamsmic sperm injection: a review of risks and complications. Br. J. Urol. Int. 96, 749–753.

Wright, V.C., Chang, J., Jeng, G., Chen, M., Macaluso, M., 2007. Assisted reproductive technology surveillance–United States, 2004. MMWR Surveill. Summ. 56, 1–22.

Yanagida, K., Katayose, H., Yazawa, H., Kimura, Y., Sato, A., Yanagimachi, H., et al., 1999. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Hum. Reprod. 14, 1307–1311.

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human reproduction

ORIGINAL ARTICLE *Infertility*

Assisted oocyte activation does not overcome recurrent embryo developmental problems

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STUDY QUESTION: Can recurrent embryo developmental problems after ICSI be overcome by assisted oocyte activation (AOA)?

SUMMARY ANSWER: AOA did not improve blastocyst formation in our patient cohort with recurrent embryo developmental problems after ICSI.

WHAT IS KNOWN ALREADY: The use of AOA to artificially induce calcium (Ca^{2+}) rises by using Ca^{2+} ionophores (mainly calcimycin and ionomycin) has been reported as very effective in overcoming fertilization failure after ICSI, especially in patients whose Ca^{2+} dynamics during fertilization are deficient. However, there is only scarce and contradictory literature on the use of AOA to overcome embryo developmental problems after ICSI, and it is not clear whether abnormal Ca^{2+} patterns during fertilization disturb human preimplantation embryo development. Moreover, poor embryo development after ICSI has also been linked to genetic defects in the subcortical maternal complex (SCMC) genes.

STUDY DESIGN, SIZE, DURATION: This prospective cohort single-center study compared ICSI-AOA cycles and previous ICSI cycles in couples with normal fertilization rates (≥60%) but impaired embryonic development (≤15% blastocyst formation) in at least two previous ICSI cycles. In total, 42 couples with embryo developmental problems were included in this study from January 2018 to January 2021.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Of the 42 couples included, 17 underwent an ICSI-AOA cycle consisting of $CaCl_2$ injection and double ionomycin exposure. Fertilization, blastocyst development, pregnancy, and live birth rates after ICSI-AOA were compared to previous ICSI cycles. In addition, the calcium pattern induced by the male patient's sperm was investigated by mouse oocyte calcium analysis. Furthermore, all 42 couples underwent genetic screening. Female patients were screened for SCMC genes (*TLE6*, *PADI6*, *NLRP2*, *NLRP5*, *NLRP7*, and *KHDC3L*) and male patients were screened for the sperm–oocyte-activating factor *PLCZ1*.

MAIN RESULTS AND THE ROLE OF CHANCE: We compared 17 AOA cycles to 44 previous ICSI cycles from the same patient cohort. After AOA, a total fertilization rate of 68.95% (131/190), a blastocyst development rate of 13.74% (18/131), a pregnancy rate of 29.41% (5/17), and a live birth rate of 23.53% (4/17) were achieved, which was not different from the previous ICSI cycles (76.25% (321/421, *P*-value = 0.06); 9.35% (30/321, *P*-value = 0.18), 25.00% (11/44, *P*-value = 0.75), and 15.91% (7/44, *P*-value = 0.48), respectively). Calcium analysis showed that patient's sperm induced calcium patterns similar to control sperm samples displaying normal embryo developmental potential. Genetic screening revealed 10 unique heterozygous variants (in *NLRP2*, *NLRP5*, *NLRP7*, *TLE6*, and *PADI6*) of uncertain significance (VUS) in 14 females. Variant *NLRP5* c.623-12_623-11insTTC (p.?) was identified in two unrelated individuals and variant *NLRP2* c.1572T>C (p.Asp524=) was identified in four females. Interestingly, we identified a previously reported homozygous mutation *PLCZ1*, c.1499C>T (p.Ser500Leu), in a male patient displaying impaired embryonic development, but not showing typical fertilization failure.

†The authors consider that the first two authors should be regarded as joint First Authors.

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LIMITATIONS, REASONS FOR CAUTION: Our strict inclusion criteria, requiring at least two ICSI cycles with impaired embryo development, reduced cycle-to-cycle variability, while the requirement of a lower blastocyst development not influenced by a poor fertilization excluded couples who otherwise would be selective cases for AOA; however, these criteria limited the sample size of this study. Targeted genetic screening might be too restricted to identify a genetic cause underlying the phenotype of poor embryo development for all patients. Moreover, causality of the identified VUS should be further determined.

WIDER IMPLICATIONS OF THE FINDINGS: Strong evidence for AOA overcoming impaired embryonic development is still lacking in the literature. Thus far, only one article has reported a beneficial effect of AOA (using calcimycin) compared to previous ICSI cycles in this patient population, whilst two more recent sibling-oocyte control studies (one using calcimycin and the other ionomycin) and our research (using ionomycin) could not corroborate these findings. Although no major abnormalities have been found in children born after AOA, this technique should be reserved for couples with a clear Ca^{2+}-release deficiency. Finally, genetic screening by whole-exome sequencing may reveal novel genes and variants linked to embryo developmental problems and allow the design of more personalized treatment options, such as wild-type complementary RNA or recombinant protein injection.

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Key words: embryo developmental problems / intracytoplasmic sperm injection / assisted oocyte activation / mouse oocyte calcium analysis / genetic screening

Introduction

Intracellular calcium (Ca^{2+}) release in the oocyte is a hallmark event during mammalian fertilization. The exact Ca^{2+} pattern induced during fertilization is species-specific, and it is characterized by the frequency, amplitude, and duration of the Ca^{2+} discharge (Stein *et al.*, 2020). Long-lasting Ca^{2+} oscillations originate right after the sperm delivers the phospholipase C zeta (PLCζ) protein during gamete fusion. Once PLCζ is present in the oocyte cytoplasm, it promotes the production of inositol trisphosphate (IP_3), which stimulates the release of Ca^{2+} through its receptor, IP_3R, located in the endoplasmic reticulum of the oocyte (Kashir *et al.*, 2010; Yeste *et al.*, 2016). Free intracellular Ca^{2+} molecules activate several oocyte kinases in a time-dependent manner, which trigger all events necessary to complete fertilization, including cortical granule exocytosis, meiotic resumption, extrusion of second polar body, pronuclei formation, and recruitment of maternal mRNAs (Ducibella *et al.*, 2002; Stein *et al.*, 2020). Although these events require different numbers of Ca^{2+} transients for their completion (Ducibella *et al.*, 2002), oocytes show a high degree of tolerance toward aberrant Ca^{2+} signals and can also be activated when the total amount of Ca^{2+} released reaches a minimum threshold (Ozil *et al.*, 2005; Tóth *et al.*, 2006). Insufficient or abnormal Ca^{2+} release, on the other hand, has been reported to cause low fertilization or total fertilization failure after ICSI (Yoon *et al.*, 2008; Ferrer-Buitrago *et al.*, 2018b), which can be rescued by assisted oocyte activation (AOA) (Rybouchkin *et al.*, 1997). By AOA, artificial Ca^{2+} oscillations are induced in the oocyte cytoplasm using mainly Ca^{2+} ionophores (ionomycin or calcimycin) (Vanden-Meerschaut *et al.*, 2014b). These Ca^{2+} ionophores, although very efficient, induce only one or two Ca^{2+} spikes and thus, do not mimic the physiological Ca^{2+} oscillatory pattern observed during human fertilization (Ferrer-Buitrago *et al.*, 2018a). AOA has been repetitively used to rescue reduced fertilization rates after ICSI (Bonte *et al.*, 2019; Shan *et al.*, 2022), resulting in normal embryo development and healthy live births (Vanden-Meerschaut *et al.*, 2014a; Mateizel *et al.*, 2018) and has been shown to be very efficient in patients with abnormal PLCζ protein content, where adequate Ca^{2+} dynamics are deficient (Torra-Massana *et al.*, 2019; Meng *et al.*, 2020; Mu *et al.*, 2020).

The effect of aberrant Ca^{2+} patterns on human preimplantation embryonic development is still not clear. Some studies performed in mouse and rabbit have shown that reduced or excessive Ca^{2+} influx affects both pre- and post-implantation events, as well as development to term (Ozil and Huneau, 2001; Ozil *et al.*, 2005, 2006). Since these studies have highlighted the importance of Ca^{2+} in post-fertilization events, AOA was suggested as a possible treatment to overcome embryo developmental problems after ICSI (Ebner *et al.*, 2015; Mateizel *et al.*, 2022; Yin *et al.*, 2022). In 2015, Ebner *et al.* (2015) reported a significant benefit of AOA treatment for these couples, as blastocysts rates were restored to normal values (~50%). In addition, there was a significant increase in live birth rates in comparison to their previous ICSI cycles without AOA (Ebner *et al.*, 2015). However, two more recent oocyte sibling studies reported that AOA treatment was not able to improve embryo development neither live birth rates in this patient population (Mateizel *et al.*, 2022; Yin *et al.*, 2022). It is important to highlight that the inclusion criteria for 'embryo developmental problems after ICSI' was different between these three studies. While Ebner *et al.* (2015) included patients with decreased blastocyst formation (≤15%), Yin *et al.* (2022) included patients with a low rate of good quality Day 3 (D3) embryos (≤30%), and Mateizel *et al.* (2022) included patients with a low rate of good quality Day 5 (D5) embryos. The differences in inclusion criteria, as well as study design and Ca^{2+} ionophore used during AOA may explain these contradictory findings (Ebner and Shebl, 2022). In addition, none of these studies investigated the underlying cause of the embryo developmental problems which could have been caused both by sperm- or oocyte-related factors. In particular, it would have been of interest to know whether the patient's sperm-induced normal or disturbed fertilization-related Ca^{2+} dynamics, in order to determine whether AOA would be beneficial.

Embryo developmental arrest (EDA) is a term used to define a multifactorial infertility condition where embryo development is stopped at any developmental stage (zygote, cleavage stage, or morula) before reaching the blastocyst stage (Mohebi and Ghafouri-Fard, 2019; Sfakianoudis et al., 2021). Alternatively, the embryos of some couples can achieve the blastocyst stage but at lower rates of success. The later condition is generally described under the category 'embryo developmental problems' (Ebner et al., 2015; Ebner and Shebl, 2022; Mateizel et al., 2022; Yin et al., 2022). Embryo arrest is a common event observed after ICSI, as 40% of the total zygote cohort always stop development before reaching the blastocyst stage (Sfakianoudis et al., 2021). Moreover, half of the patients treated with ICSI always show one arrested embryo in each cycle (Mohebi and Ghafouri-Fard, 2019). Nonetheless, when all embryos show EDA, sometimes during repeated cycles, treatment options become very limited, and patients often require gamete donation.

Until embryo genome activation occurs (in humans between the four- and eight-cell stage), early cleavages are almost exclusively dependent on oocyte-derived proteins and mRNAs, which are encoded by maternal effect genes (MEGs) (Paonessa et al., 2021). Many knockout mice studies have demonstrated the occurrence of embryo arrest at distinct stages after the interference of certain MEGs (Condic, 2016). However, research in human is still limited and only a few MEGs that form the subcortical maternal complex (SCMC) have been associated with EDA (Paonessa et al., 2021). The SCMC is a multiprotein complex, composed of at least six proteins, that participates in embryo genome activation and thus is crucial for correct embryonic development (Bebbere et al., 2016). Recently, mutations in the SCMC-related MEGs (*TLE6*, *PADI6*, *NRLP2*, *NRLP5*, *NRLP7*, and *KDHC3L*) have been detected in patients suffering from EDA, showing different degrees of embryo arrest depending on the protein affected and the type of mutation (Xu et al., 2016; Wang et al., 2018; Mu et al., 2019; Lin et al., 2020; Zheng et al., 2021).

The present study aimed to investigate whether AOA using ionomycin can overcome embryo developmental problems (≤15% blastocyst formation) as the literature shows contradictory results. In addition, to understand the underlying cause of impaired embryonic development seen in our patient cohort better, we investigated the cause for the observed EDA. First, we studied whether the male partner's sperm were able to induce a normal Ca^{2+} pattern during fertilization by mouse oocyte calcium analysis (MOCA). Next, we performed genetic screening of female SCMC components (*NLRP2, NLRP5, NLRP7, TLE6, PADI6, KHDC3L*), which are critical for embryo development and of *PLCZ1* to exclude a possible male factor.

Materials and methods

Ethical approval

Ghent University Hospital Ethical Committee approved the conducted study (EC/2017/0819). All included patients signed informed consent for the participation in this study and agreed to undergo AOA treatment, to donate saliva samples for genetic screening, and to donate sperm samples for MOCA. The use of laboratory animals for performing MOCA was approved by the Ghent University Hospital Ethical Committee for Laboratory Animals (ECD no. 15/56 and no. 19/66).

Study design, setting, and participants

A prospective cohort study was performed between January 2018 and January 2021, including 42 couples with a history of embryo developmental problems. Patients were only included when they underwent at least two fresh ICSI cycles at the Centre for Reproductive Medicine at Ghent University Hospital. Furthermore, high fertilization (≥60% 2 pronuclei (2PN)) with a minimum of 10 zygotes but low blastocyst rates on D5 (≤15%) (Ebner et al., 2015) (Supplementary Table SI) were required before inclusion in the study (Supplementary Table SII). All couples were included for genetic screening, while 17 out of the 42 couples also underwent a fresh ICSI-AOA cycle from February 2018 until August 2019 at Ghent University Hospital (Supplementary Table SIII). The time from the last ICSI cycle to the ICSI-AOA cycle was on average 5 months. Except for two patients who used donor sperm (P3 and P7) and one where testicular sperm cells were used (P12), the couple's sperm samples were used to assess their Ca^{2+} releasing capacity by means of MOCA. Therefore, male partners donated an extra sperm sample which was frozen until MOCA was performed. The mean maternal age was 33.8 ± 3.6 years (range: 28–38 years). Most of the couples were diagnosed with unexplained female infertility (n = 6; P3, P5, P7, P9, P16, P17) or male factor infertility (due to poor sperm quality determined as oligozoospermia, asthenozoospermia, or teratozoospermia) (n = 6; P6, P8, P11, P13, P14, P15). The remaining patients were diagnosed with polycystic ovarian syndrome (PCOS) (n = 1; P1), genetic disease (n = 1; P4), tubal factor (n = 1; P10), a combination of endometriosis and tubal factor (n = 1; P2), or obstructive azoospermia (n = 1; P12).

MOCA procedure

MOCA was performed as previously described (Vanden-Meerschaut et al., 2013). Metaphase II (MII) oocytes were collected from 8- to 12-week-old B6D2F1 female mice (Janvier Labs, France) 12–14 h after hCG administration and cultured in protein-free potassium simplex optimized medium (KSOM) supplemented with 0.4% bovine serum albumin (BSA) (Calbiochem, USA) at standard conditions (37°C in 6% CO_2 and 5% O_2). Frozen-warmed human patient and control sperm were washed by centrifugation (315×*g*, 10 min) using Sydney IVF Gamete Buffer (Cook Ltd, Ireland). Mouse oocytes were first preloaded in 7.5 µM of Ca^{2+}-sensitive dye Fura-2 acetoxymethyl ester (Fura 2-AM, Teflabs, USA) diluted in KSOM-BSA for 30 min at 37°C. Afterwards, they were transferred to KSOM-HEPES supplemented with 20% fetal bovine serum and gradually cooled (10 min at room temperature and 10 min at 15–17°C). Motile spermatozoa were selected after swim-up, mechanically immobilized, demembranated with 0.2% lysolecithin (Instruchemie, The Netherlands) for 1 min, and immediately microinjected into mouse MII oocytes using piezoelectrical pulses at 15–17°C. Within 30 min after the first microinjection, oocytes were placed in KSOM-BSA medium on a glass-bottom dish (cat.no. P35G-0-14-C, MatTek Corporation, USA). Imaging was performed on an inverted epifluorescence microscope (Olympus IX71, Olympus Soft Imaging Solutions GmBH, Belgium) with a 10× objective and a filter switch (Lambda DG-4 filter switch, Sutter Instrument Company, USA), enabling excitation alternating between 340 and 380 nm wavelengths. Ca^{2+} imaging was performed on a heated stage and images were acquired every 5 s over a period of 2 h. All Ca^{2+} patterns were analyzed using Clampfit 10.2 software. The total amount of

Ca^{2+} released was calculated as the product of the mean amplitude (A) per mean frequency (F) of all oocytes injected per patient (from at least 15 surviving oocytes). The AxF score has been described earlier to detect oocyte activation deficiencies in the sperm of patients with low or total failed fertilization after ICSI (Vanden-Meerschaut *et al.*, 2013). AxF scores ≤ 9 indicate sperm-related deficiencies during oocyte activation, whereas AxF scores >9 exclude this involvement.

ICSI and ICSI-AOA procedures

Female partners were stimulated according to a short agonist (35.3%; n = 6), a long agonist (17.6%; n = 3), or an antagonist (47.1%; n = 8) protocol. Ovulation was triggered by administering hCG. Only two couples changed the stimulation protocol for the ICSI-AOA procedure, compared to their previous ICSI cycle. At 36-h post-hCG administration, oocytes were aspirated from the follicles in the ovaries by transvaginal ultrasound-guided puncture in HTF-HEPES medium (Cooper Surgical). Cumulus cells were removed from the oocyte complexes by means of enzymatic (Cumulase; Cooper Surgical) and subsequent mechanic denudation. Oocytes were cultured in Sydney IVF Fertilization Medium (Cook Ltd, Ireland). For both treatments (ICSI and ICSI-AOA), fresh ejaculated patient sperm or frozen-warmed donor sperm (P3, P17, and P31) were used. For both, fresh and frozen-warmed sperm samples, sperm selection was performed by density gradient centrifugation using Sydney IVF Spermient (Cook Ltd, Ireland), after which the sample recovered was washed by means of centrifugation (315×*g*, 10 min) with Sydney IVF Gamete Buffer (Cook Ltd, Ireland). The pellet was resuspended in Sydney IVF Gamete Buffer (Cook Ltd, Ireland) and directly used for sperm immobilization. Only motile spermatozoa were immobilized before injection into the patient's oocytes (standard ICSI technique).

The AOA procedure was performed as previously described (Bonte *et al.*, 2019). Briefly, fresh mature oocytes were injected with the patient's sperm together with 0.1 mol/l $CaCl_2$ (Sigma-Aldrich, Belgium). After a 30-min incubation period, oocytes were exposed twice to 10 µmol/l Ca^{2+} ionophore ionomycin (I9657, Sigma-Aldrich, Belgium) for 10 min with a 30-min time interval, followed by extensive washing. For both ICSI and ICSI-AOA cycles, the injected oocytes were cultured in Sydney IVF Cleavage Medium (Cook Ltd, Ireland) until D3 and in Sydney IVF Blastocyst Medium (Cook Ltd, Ireland) from D3 to D5 under standard conditions (37°C in 6% CO_2 and 5% O_2). Embryo transfers were performed exclusively on D5, in fresh embryos or after warming the cryopreserved embryos.

Embryo grading

D3 and D5 embryos were classified into four quality categories (excellent: A; good: B; moderate: C; and poor: D) in accordance with European Society for Human Reproduction and Embryology guidelines (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) (Supplementary Table SI). Good quality embryo formation rate refers to embryos classified with Qualities A and B.

Genetic screening

Saliva samples from the 42 couples were collected in Oragene OG-500 saliva kits (DNA Genotek, Canada). Genomic DNA was extracted from saliva and purified according to the manufacturer's instructions, using the ethanol precipitation protocol and the prepIT•L2P reagent (DNA Genotek, Canada). The extracted DNA was quantified using the Qubit™ 1× dsDNA HS Assay Kit (ThermoFisher, USA). Coding exons and flanking intronic sequences of *PLCZ1* (NM_033123.4), *NLRP2* (NM_001174081.1), *NLRP5* (NM_153447.4), *NLRP7* (NM_001127255.1), *TLE6* (NM_001143986.1), *PADI6* (NM_207421.4), and *KHDC3L* (NM_001017361.2) genes were amplified via singleplex PCR (Lefever *et al.*, 2017) and subjected to targeted next-generation sequencing (MiSeq, Illumina, San Diego, CA, USA) at the Centre for Medical Genetics Ghent (De Leeneer *et al.*, 2015). The primers used for genetic screening are described in Supplementary Table SIV. Identified variants were classified according to the guidelines of the American College of Medical Genetics (Richards *et al.*, 2015) employing Varsome (https://varsome.com) (Kopanos *et al.*, 2019). The identified variants were confirmed by bidirectional Sanger sequencing (ABI3730XL, Applied Biosystems, Foster City, CA, USA). The mutations were named according to the Human Genome Variation Society (HGVS) standards. Four different *in silico* predictive algorithms scores, also implemented in the Varsome classification tool, were reported: PolyPhen-2 (Polymorphism Phenotyping v2) (http://genetics.bwh.harvard.edu/pph2/), SIFT (sorts intolerant from tolerant) (http://sift.jcvi.org/), and PROVEAN (Protein Variation Effect Analyzer) (http://provean.jcvi.org/index.php) assess the potential pathogenic effects of missense mutations according to protein structure, protein function, evolutionary conservation, and sequence homology (Richards *et al.*, 2015), while the Grantham score quantifies the biochemical difference between two amino acids (Grantham, 1974). The higher the Grantham score the greater evolutionary distance.

Statistics

Fisher's exact test was used for statistical analysis with the use of the Statistical Package for Social Sciences (SPSS Statistics 25; IBM). Fertilization (2PN/MII injected oocytes), D3 embryo formation (D3 embryos/2PN), blastocyst development (blastocyst/2PN), pregnancy (+hCG/initiated cycle), and live birth rates (live births/initiated cycle) were compared between ICSI and ICSI-AOA cycles. *P*-values <0.05 were considered as statistically significant.

Results

Mouse oocyte calcium analysis reveals normal Ca^{2+} patterns during fertilization in patients with embryo developmental problems

To study whether impaired embryo development was caused by abnormal sperm-related Ca^{2+} patterns during oocyte activation, the MOCA test was performed on the male partner's sperm and compared to Ca^{2+} response patterns observed with sperm from a person with proven normal Ca^{2+} oscillatory behavior and normal embryo development.

The majority of sperm samples, except for that from P13, assessed by MOCA displayed Ca^{2+} oscillatory patterns with high frequency (>10 Ca^{2+} spikes) during 2-h measurement in most of the oocytes injected and showed AxF scores comparable to control sperm (Fig. 1). Moreover, all AxF scores were higher than 9, excluding a sperm–oocyte-activating deficiency regardless of the sperm sample characteristics (Supplementary Table SV) (Vanden-Meerschaut *et al.*, 2013). Of all the couples, only P13 showed a high proportion of oocytes with 0 or 1–2 Ca^{2+} peaks and therefore they had the lowest AxF score with a value of 24.1 AU. This patient suffered from severe asthenozoospermia, although a previous vitality test revealed 59% live sperm cells (Supplementary Table SV). During ICSI and ICSI-AOA, the hypo-osmotic swelling (HOS) test was used to ensure injection of live sperm cells (Ramu and Jeyendran, 2013). However, it was not possible to perform the HOS test during MOCA and immotile sperm cells were injected, of which some could have been dead at the moment of the injection, explaining the decreased total Ca^{2+} release observed.

Preimplantation embryo development and clinical outcomes were not improved after ICSI-AOA treatment in couples with embryo developmental problems

In total, 17 ICSI-AOA cycles were compared to 44 previous ICSI cycles from the same patient cohort (P1–P17). The detailed laboratory and clinical outcomes obtained per couple after ICSI and ICSI-AOA cycles are described in Supplementary Tables SII and SIII.

A

Patient	P1	P2	P4	P5	P6	P7	P8	P9	P10	P11	P13	P14	P15	P16	Control
N oocytes	15	20	24	24	23	23	33	26	24	33	19	15	20	19	81
AxF (AU)	93.3	101.0	76.7	52.0	90.8	50.4	56.2	123.2	107.6	77.0	24.1	46.1	82.8	56.2	70.6

B

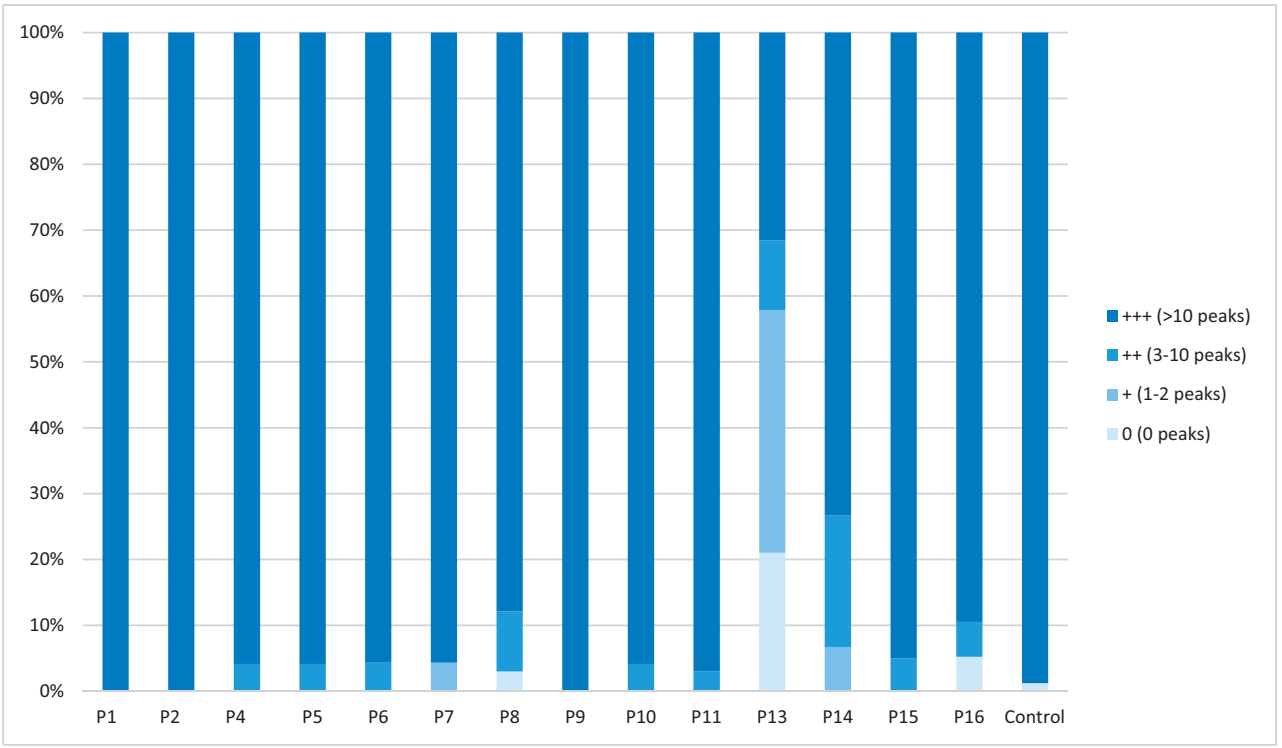


Figure 1. Mouse oocyte calcium analysis outcome in couples with embryo developmental problems (P1–P17). (**A**) The AxF product reveals normal calcium (Ca^{2+}) release (AxF > 9) similar to the control sample. (b) Frequency scoring of Ca^{2+} oscillations. Oocytes were classified according to the number of Ca^{2+} rises observed during the 2-h measurement: +++: >10 peaks; ++: 3–10 peaks; +: 1–2 peaks; 0: 0 peaks; AxF: product of the amplitude and frequency scores; AU: arbitrary units; N: number of oocytes surviving after Ca^{2+} analysis.

From the ICSI-AOA cycles, a total number of 262 cumulus–oocyte complexes (COCs) (mean 15.41 ± 7.60) were collected, from which 190 MII oocytes (mean 11.18 ± 5.41) were used for treatment. From previous ICSI cycles, a total number of 602 COCs (mean 13.37 ± 3.80) were obtained from which 421 (mean 9.12 ± 3.21) were MII oocytes. After ICSI-AOA, a total fertilization rate of 68.95% (131/190) was achieved, which was not significantly different from the fertilization rate of 76.25% (321/421) obtained after ICSI cycles (Table I). Embryo developmental capacity of fertilized oocytes was assessed by evaluating embryo formation on D3 and D5. After ICSI-AOA, a total D3 embryo formation rate of 66.41% (87/131) and blastocyst formation of 13.74% (18/131) were achieved, which were similar to D3 embryo formation of 71.96% (231/321) and blastocyst formation of 9.35% (30/321) obtained after conventional ICSI. No difference was found in the ratio of good quality D3 embryo formation (41.12% vs 33.59%) and good quality blastocyst formation (2.49% vs 3.82%) between ICSI and ICSI-AOA cycles, respectively (Table I). Of note, good quality embryo formation was substantially decreased in our patient population when compared to the ICSI average rates obtained during the study period in our IVF clinic (61.4% for good quality D3 embryo formation and 22.0% for good quality blastocyst formation) (Supplementary Table SVI).

The exact D3 and D5 embryo development obtained after ICSI and ICSI-AOA treatments is shown per patient in Table II. Only patient P2, P13, and P17 showed more than a 20% increase in blastocyst development after AOA, while only P12 showed more than 20% increase in good quality D5 embryo formation. Overall blastocyst formation remained very poor after AOA treatment (13.74%) in comparison to the average blastocyst rate obtained in our IVF clinic after ICSI (57.3%) (Supplementary Table SVI).

Blastocysts were transferred in fresh or after vitrification/warming when at least Blastocyst 1 quality (Supplementary Table SI) was achieved. Exceptionally, four embryos with lower quality than Blastocyst 1 (compacting) were transferred on D5 in some ICSI cycles where the patients did not have a better option. Overall, after ICSI-AOA, a total pregnancy rate of 29.41% (5/17) was achieved, which was similar to the pregnancy rate of 25.0% (11/44) obtained after previous ICSI cycles (Table I). Previous ICSI cycles resulted in three biochemical pregnancies, one miscarriage, and seven live births (15.91%, 7/44), while the ICSI-AOA cycles resulted in one ectopic pregnancy and four live births (23.52%, 4/17) (Table I). None of the new-borns had major or minor congenital malformations. Body weight and Apgar scores were normal, but one live birth after ICSI ended up as preterm delivery (Supplementary Table SVII) due to premature rupture of membranes.

Table I Comparison of preimplantation embryo development and clinical outcomes after previous ICSI and subsequent ICSI-AOA cycles in couples with embryo developmental problems (P1–P17).

Parameter	ICSI	AOA	*P*-value
Cycles (oocyte retrievals)	44	17	
Cumulus–oocyte complexes	602	262	
Fertilization rate (2PN/MII)	76.25% (321/421)	68.95% (131/190)	0.059
D3 embryo formation rate (D3 embryos/2PN)	71.96% (231/321)	66.41% (87/131)	0.257
Good quality D3 embryo formation rate (D3 good quality embryos/2PN)[a]	41.12% (132/321)	33.59% (44/131)	0.167
Blastocyst formation rate (D5 embryos/2PN)	9.35% (30/321)	13.74% (18/131)	0.180
Good quality blastocyst formation rate (D5 good quality embryos/2PN)[a]	2.49% (8/321)	3.82% (5/131)	0.535
Embryos transferred in fresh[b]	22	7	
Cryopreserved embryos	12	11	
Embryos transferred after thawing	7	4	
Oocyte retrievals with fresh or frozen embryo transfer (ET/initiated cycle)[c]	63.63% (28/44)	58.82% (10/17)	0.774
+hCG after fresh ET	8	4	
+hCG after frozen-thawed ET	3	1	
Pregnancy rate (+hCG/initiated cycle)	25.00 (11/44)	29.41% (5/17)	0.752
Biochemical pregnancy	3	0	
Ectopic pregnancy	0	1	
Miscarriage	1	0	
Ongoing pregnancy (>12 weeks)	7	4	
Live birth after fresh ET	6	3	
Live birth after frozen-thawed ET	1	1	
Live birth rate (live births/initiated cycle)	15.91% (7/44)	23.53% (4/17)	0.481

[a]Good quality embryos are embryos with Qualities A and B following criteria described in Supplementary Table S1.
[b]Four embryos in compacting stage were transferred on D5 in some ICSI cycles where the patients did not have a better option.
[c]Embryo transfer is considered as procedure. In one embryo transfer after ICSI and one embryo transfer after ICSI-AOA. Two embryos were transferred simultaneously.
AOA: assisted oocyte activation; 2PN: 2 pronuclei; MII: metaphase II oocytes; D2, D3, D5: Day 2, Day 3, Day 5 of embryo development; ET: embryo transfer procedure; +hCG: positive hCG test (>10 mIU/ml).

Table II D3 and D5 embryo quality after ICSI and ICSI-AOA cycles in couples with embryo developmental problems (P1–P17).

Patient	ICSI cycles, n	D3 embryo formation rate (D3 embryos/2PN)	Good quality D3 embryo formation rate (D3 good quality embryos/2PN)	Blastocyst formation (D5 embryos/ 2PN)	Good quality blastocyst formation (D5 good quality embryos/2PN)	ICSI-AOA cycles. n	D3 embryo formation rate (D3 embryos/2PN)	Good quality D3 embryo formation rate (D3 good quality embryos/2PN)	Blastocyst formation (D5 embryos/ 2PN)	Good quality blastocyst formation (D5 good quality embryos/2PN)
P1	3	42.86% (12/28)	7.14% (2/28)	0.00% (0/28)	0.00% (0/28)	1	52.94% (9/17)	0.00% (0/17)	0.00% (0/17)	0.00% (0/17)
P2	4	96.43% (27/28)	75.00% (21/28)	14.29% (4/28)	7.14% (2/28)	1	100.00% (5/5)	60.00% (3/5)	40.00% (2/5)	20.00% (1/5)
P3	2	90.00% (9/10)	50.00% (5/10)	10.00% (1/10)	10.00% (1/10)	1	62.50% (5/8)	37.50% (3/8)	0.00% (0/8)	0.00% (0/8)
P4	3	65.71% (23/35)	34.29% (12/35)	11.43% (4/35)	2.86% (1/35)	1	59.09% (13/22)	18.18% (4/22)	9.09% (2/22)	0.00% (0/22)
P5	2	80.00% (8/10)	50.00% (5/10)	10.00% (1/10)	0.00% (0/10)	1	75.00% (3/4)	50.00% (2/4)	0.00% (0/4)	0.00% (0/4)
P6	2	42.86% (6/14)	7.14% (1/14)	7.14% (1/14)	0.00% (0/14)	1	80.00% (4/5)	20.00% (1/5)	0.00% (0/5)	0.00% (0/5)
P7	2	66.67% (8/9)	55.56% (6/9)	11.11% (1/9)	11.11% (1/9)	1	80.00% (8/10)	70.00% (7/10)	10.00% (1/10)	0.00% (1/10)
P8	2	41.67% (5/12)	0.00% (0/12)	8.33% (1/12)	0.00% (0/12)	1	16.67% (1/6)	0.00% (0/6)	0.00% (0/6)	0.00% (0/6)
P9	2	55.56% (5/9)	11.11% (1/9)	11.11% (1/9)	0.00% (0/9)	1	42.86% (3/7)	14.29% (1/7)	14.29% (1/7)	14.29% (1/7)
P10	4	89.58% (43/48)	50.00% (24/48)	10.42% (5/48)	2.08% (1/48)	1	100.00% (8/8)	75.00% (6/8)	25.00% (2/8)	12.50% (1/8)
P11	3	95.65% (22/23)	82.61% (19/23)	8.70% (2/23)	0.00% (0/23)	1	100.00% (4/4)	100.00% (4/4)	25.00% (1/4)	0.00% (0/4)
P12	2	60.00% (9/15)	26.67% (4/15)	6.67% (1/15)	0.00% (0/15)	1	40.00% (2/5)	40.00% (2/5)	20.00% (1/5)	20.00% (1/5)
P13	2	53.33% (8/15)	20.00% (3/15)	6.67% (1/15)	0.00% (0/15)	1	50.00% (5/10)	10.00% (1/10)	40.00% (4/10)	0.00% (0/10)
P14	2	50.00% (5/10)	40.00% (4/10)	10.00% (1/10)	0.00% (0/10)	1	83.33% (5/6)	66.67% (4/6)	16.67% (1/6)	16.67% (1/6)
P15	4	60.87% (14/23)	34.78% (8/23)	13.04% (3/23)	0.00% (0/23)	1	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)
P16	2	78.57% (11/14)	21.43% (3/14)	7.14% (1/14)	0.00% (0/14)	1	83.33% (5/6)	16.67% (1/6)	0.00% (0/6)	0.00% (0/6)
P17	3	100.0% (18/18)	83.33% (15/18)	11.11% (2/18)	11.11% (2/18)	1	100.0% (7/7)	71.43% (5/7)	42.86% (3/7)	0.00% (0/7)
TOTAL	44	71.96% (231/321)	41.12% (132/321)	9.35% (30/321)	2.49% (8/321)	17	64.41% (87/131)	33.59% (44/131)	13.74% (18/131)	3.82% (5/131)

AOA: assisted oocyte activation; 2PN: 2 pronuclei; MII: metaphase II oocytes; D2, D3, D5: Day 2, Day 3, Day 5 of embryo development.

Genetic screening does not identify pathogenic variants in *SCMC* genes in couples with embryo developmental problems

To unravel a possible genetic defect causing embryo developmental problems following ICSI, DNA sequence analysis of several recently identified female genes (*NLRP2*, *NLRP5*, *NLRP7*, *TLE6*, *PADI6*, and *KHDC3L*) associated with EDA was conducted. In addition, analysis of the *PLCZ1* gene of the patient's partner DNA was performed to exclude a possible male factor involved in abnormal calcium release. The patient cohort being sequenced consisted of 42 couples, including the 17 couples that underwent ICSI-AOA (P1–P17) and 25 additional couples that met the inclusion criteria (P18–P42), but who did not undergo AOA (Supplementary Table II). Two samples (female P15 and male P33) could not be screened due to poor DNA quality. Moreover, *PLCZ1* was not screened in P3, P17, and P31 as these couples used a sperm donor.

Sequencing of the selected genes revealed several variants, which were categorized according to the guidelines of the American College of Medical Genetics (ACMG) (Richards *et al.*, 2015). No Class 4 (likely pathogenic) or Class 5 (pathogenic) variants were detected, however, some interesting Class 3 (variants of uncertain significance, VUS) were observed. We identified 10 unique heterozygous VUS in SCMC genes (Table III) in 14 female patients. Most of the variants were predicted to be tolerated by at least one *in silico* prediction algorithm, except for the *TLE6* c.1100C>T (p.Pro367Leu) variant encountered in P4, that it is predicted to be damaging by the three *in silico* pathogenicity prediction tools and has a high Grantham score (Table III). P4 did not benefited from ICSI-AOA, as both D3 and D5 embryo development were similar to the rates obtained after ICSI. Specifically, blastocyst development remained very poor (9.09% blastocyst and 0% good quality blastocyst rate) after AOA treatment (Table II). In addition, we identified a synonymous *NLRP2* c.1572T>C (p.Asp524=) variant in four unrelated patients (P3, P12, P17, and P32) and the intronic *NLRP5* c.623-12_623-11insTTC (p.?) in two unrelated patients (P1 and P24) (Table III). The prevalence of both variants in the affected individuals in our cohort (9.5% and 4.8%, respectively) is significantly increased compared to its prevalence in the control population (0.61% and 0.69% gnomAD, respectively). From these patients, P1, P3, P12, and P17 underwent ICSI-AOA treatment. ICSI-AOA treatment was not beneficial for P1 and P3, as both patients remained with 0% blastocyst formation. However, P12 showed a 20% increase in good quality blastocyst formation after AOA, while P17 had a more than 20% increase in blastocyst formation but the quality of the embryos did not improve (Table II). However, the benefit observed after AOA in P17 requires further investigation as this patient used a sperm donor sample.

Interestingly, while *PLCZ1* mutations were not expected in this patient population, we identified a previously reported homozygous *PLCZ1* mutation (c.1499C>T; p.Ser500Leu) in P35, displaying impaired embryonic development, but not showing typical fertilization failure. Although this variant is classified as benign, mostly because its prevalence in the control population is 3%, it is predicted to be damaging by SIFT and PROVEAN *in silico* predictive tools (Table III). The same mutation has been previously reported at a high frequency (9/37, 24.3%) in patients suffering from fertilization failure after ICSI (Yoon *et al.*, 2008; Torra-Massana *et al.*, 2019). In this case, the patient showed 74.1% (20/27) fertilization rate, but 0% (0/20) blastocyst formation after ICSI (Supplementary Table SII). ICSI-AOA treatment has been recommended to the couple, but they have not yet undergone another IVF cycle.

Discussion

In this study, we showed that AOA treatment using ionomycin neither improved preimplantation embryo development nor clinical outcomes in our patient cohort with repetitive embryo developmental problems after ICSI (≤15% blastocyst formation) (Table I). Impaired embryo development not influenced by a poor fertilization or by cycle-to-cycle variability was ensured by our strict inclusion criteria.

At present, there is abundant evidence that ICSI in combination with AOA increases fertilization rates and consequently blastocyst formation in patients with total fertilization failure or low fertilization (<33%) after ICSI (Bonte *et al.*, 2019; Li *et al.*, 2019; Shan *et al.*, 2022). The rationale behind the success of this treatment is that oocyte activation failure (either sperm or oocyte-related) is mostly caused by a deficiency in sperm or oocyte factors participating in the Ca^{2+} releasing machinery, thus the artificial induction of Ca^{2+} oscillations during fertilization helps to overcome this problem. Likewise, when the deficiency occurs in proteins acting downstream of the Ca^{2+} oscillations, AOA is likely to not be beneficial (Sang *et al.*, 2018; Cardona Barberán *et al.*, 2020). In the past years, AOA has also been suggested for couples with other indications, such as embryo developmental problems after ICSI regardless of the patient infertility condition (Ebner *et al.*, 2015; Mateizel *et al.*, 2022; Tsai *et al.*, 2022; Yin *et al.*, 2022), but also for specific infertile populations that generally suffer from IVF failure, such as diminished ovarian reserve (Tsai *et al.*, 2022), PCOS, primary ovarian insufficiency (POI), advanced maternal age, and female unexplained infertility among others (Lv *et al.*, 2020). However, the benefit of AOA to treat embryo developmental problems is still not clear and literature shows contradictory results (Ebner *et al.*, 2015; Lv *et al.*, 2020; Mateizel *et al.*, 2022; Tsai *et al.*, 2022; Yin *et al.*, 2022).

Ebner *et al.* (2015) proposed the use of AOA with calcimycin (GM508 Cult-Active) to treat 57 couples with fertilization rate >30% and EDA (no transfer), complete developmental delay (no morula/blastocyst on D5), or reduced blastocyst formation (≤15%) in at least one ICSI cycle. The authors' hypothesis was based on different reports showing a correlation between cell division and Ca^{2+} availability (Berridge, 1995), and reporting that the Ca^{2+} fluctuations normally preceding mitotic division, disappear in arrested embryos (Sousa *et al.*, 1996). Moreover, some studies performed in animal models also suggest that altering the Ca^{2+} released during oocyte activation burdens both fertilization but also embryo development (Ozil and Huneau, 2001; Ozil *et al.*, 2005, 2006). All these data seem to support the use of AOA for these couples and in accordance, Ebner *et al.* (2015) showed a highly significant increase in blastocyst formation for all patient groups in comparison to their previous ICSI cycle. In addition, an increase in implantation, clinical pregnancy, and live birth rates was also reported (Ebner *et al.*, 2015).

Although Ebner *et al.* (2015) showed promising results, two more recent sibling-oocyte studies could not corroborate the benefit of AOA to treat embryo developmental problems (Mateizel *et al.*, 2022;

Table III Variants of uncertain significance detected in *SCMC* genes and *PLCZ1* in couples with embryo developmental problems (P1–P42).

Patient	Gene	c.Nomenclature	p.Nomenclature	RefSNP	Classification criteria—Pathogenic[a]	Classification criteria—Benign[a]	MAF (gnomAD -all)	Polyphen[b]	SIFT[b]	PROVEAN[b]	Grantham[b]	State
P1	*NLRP5*	c.623-12_623-11insTTC	p.?	rs538671525	/	/	0.006874	NA	NA	NA	NA	HET
P3	*NLRP2*	c.1572T>C	p.Asp524=*	rs61735084	/	BP7	0.006144	NA	NA	N	0	HET
P4	*TLE6*	c.1100C>T	p.Pro367Leu	rs140669738	PM2	/	0.000113	PD	D	D	98	HET
P6	*TLE6*	c.805A>G	p.Lys269Glu	rs147398793	PM2	BP4	0.000071	B	T	N	56	HET
P12	*NLRP2*	c.1572T>C	p.Asp524=*	rs61735084	/	BP7	0.006144	NA	NA	N	0	HET
P13	*NLRP2*	c.2920C>G	p.Leu974Val	rs61735074	PM2	/	0.004792	PD	T	N	32	HET
P16	*NLRP2*	c.1217C>G	p.Thr406Arg	rs139903547	PM2. PP3	/	0.006266	PD	T	D	71	HET
P17	*NLRP2*	c.1572T>C	p.Asp524=*	rs61735084	/	BP7	0.006144	NA	NA	N	0	HET
P18	NLRP7	c.930G>T	p.Gln310His	rs145973556	PM2	/	0.001744	PD	T	N	24	HET
P24	*NLRP5*	c.623-12_623-11insTTC	p.?	rs538671525	/	/	0.006874	NA	NA	NA	NA	HET
P32	*NLRP2*	c.1572T>C	p.Asp524=*	rs61735084	/	BP7	0.006144	NA	NA	N	0	HET
P35	*PLCZ1*	c.1499C>T*	p.Ser500Leu**	rs10505830	PM1. PP2	BA1. BP4. BP6	0.030961	T	D.T	D	145	HOM
P36	*TLE6*	c.893C>T	p.Thr298Met	rs141406529	PM2	BP4	0.000039	PD	T	D	81	HET
P38	*PADI6*	c.286G>A	p.Ala96Thr	rs181841908	PM2	BP4	0.001099	B	T	N	58	HET
P40	*NLRP2*	c.1346C>T	p.Thr449Met	rs144271525	PM2	/	0.000182	PD	T	N	81	HET

Variant classification was checked in Varsome (https://varsome.com) in February 2022.
*Variant of uncertain significance with some benign evidence (synonymous variants).
**Variant classified as benign. Although it has been reported previously in patients with fertilization failure both in heterozygous and homozygous state.
[a]Classification criterion according to guidelines from the American College of Medical Genetics.
[b]Mutation assessment by Polyphen. SIFT. PROVEAN and Grantham.
RefSNP: reference single-nucleotide polymorphism number; MAF: major allele frequency; GnomAD: The Genome Aggregation Database; NA: not available; D: damaging; PD: probably damaging; T: tolerated; N: neutral; B: benign; HET: heterozygous; HOM: homozygous.

Yin *et al.*, 2022). Yin *et al.* (2022) included 140 patients with normal fertilization rate >70%, but D3 good quality embryo rate ≤30% in previous ICSI cycles. After AOA treatment with ionomycin no differences were seen in cleavage, D3 good quality embryos and available blastocyst rates between the non-AOA and AOA groups. Additionally, implantation, miscarriage, clinical pregnancy, and live birth rates were not influenced by AOA (Yin *et al.*, 2022). Mateizel *et al.* (2022) included 19 patients with fertilization rate >30% and D5 poor embryo quality in at least one previous ICSI cycle. In the subsequent cycle, AOA treatment with calcimycin (GM508 Cult-Active) did not increase fertilization, D5 good embryo quality, or utilization rates in comparison to the non-AOA sibling control group (Mateizel *et al.*, 2022). Even though these two studies differed in inclusion criteria and in the Ca^{2+} ionophore selected for AOA treatment, the use of sibling oocytes as control group provides a more unbiased dataset to evaluate the effect of AOA than comparing the AOA results to previous ICSI cycles as performed by Ebner *et al.* (2015) and this study. Nonetheless, we ensured that all patients had at least two ICSI cycles showing embryo developmental problems, while Ebner *et al.* (2015) compared the ICSI-AOA cycle to the immediately preceding ICSI cycle regardless of the patients having more than one ICSI cycle with impaired embryonic development. While 23/57 of the patients included in that study had more than one ICSI cycle with abnormal embryo development, 34 patients couples had only one failed ICSI cycle (Ebner *et al.*, 2015). The latter group may have introduced some bias as there is difference in gamete quality between cycles.

Additionally, we have formerly reported that calcimycin (GM508 Cult-Active) shows less calcium releasing and fertilizing potential than ionomycin in both mouse and human oocytes (Nikiforaki *et al.*, 2016). Noteworthy, the activation rate in both mouse and human oocytes was very low (<15%) after calcimycin treatment (Nikiforaki *et al.*, 2016). Furthermore, it has also been reported that for patients with fertilization failure after ICSI, AOA treatment using ionomycin gives higher activation than when using calcimycin (Jia *et al.*, 2023) and that failed ICSI-AOA cycles using calcimycin can be rescued using ionomycin (Ebner *et al.*, 2021). The later may be explained by the fact that some patients might require more potential stimuli than others (Ebner and Shebl, 2022). Considering this, our study agrees with Yin *et al.* (2022) and Mateizel *et al.* (2022) and reports new valuable data on the inefficacy of ionomycin treatment at fertilization to improve embryo development.

Some articles have also studied the effect of AOA on specific infertility conditions that are likely to cause IVF failure due to poor embryo quality. Tsai *et al.* (2022) reported an increase in cleavage rate and good quality D3 embryos after ICSI-AOA in comparison to ICSI in patients with diminished ovarian reserve ≥40 years that fulfilled POSEIDON Group 4 criteria (Tsai *et al.*, 2022). In addition, Lv *et al.* (2020) treated patients with different infertility indications (male factor infertility caused by oligoastenoteratozoospermia (OAT), female factor infertility caused by advanced age, unexplained cause, PCOS, POI, or a combination of male and female factors) and observed that higher live births were obtained in all groups except POI after AOA treatment, but blastocyst rates were only improved in the OAT group, unexplained female infertility and combined male and female infertility (Lv *et al.*, 2020). Although AOA seems beneficial for some of these patient populations, the total initial cleavage and blastocyst rates obtained after ICSI in both studies were already high (Lv *et al.*, 2020; Tsai *et al.*, 2022), which may question the necessity for AOA treatment in these patient groups.

Recently, there was a case report of a patient with PN arrest, who benefited from a later AOA application (Xi *et al.*, 2020). Calcium release during fertilization in this patient was enough to induce PN formation but not to promote first mitotic cleavage. Later ionophore application at the time of embryo arrest (Day 2) recovered mitotic activity, then good quality blastocysts were produced and a live birth was achieved (Xi *et al.*, 2020). For patients with successful fertilization but later developmental arrest, AOA application at the time of the arrest, making the treatment patient-specific, could be a better strategy to recover embryo development (Sousa *et al.*, 1996; Ebner and Shebl, 2022). Studying whether patients with embryo developmental problems have a calcium deficiency during the first cleavage stages would increase our knowledge on calcium dynamics during embryonic development and will help identify specific patient populations that could benefit of later AOA treatment. As mentioned before, AOA will in theory only benefit patients suffering from a Ca^{2+} deficiency. Therefore, for the first time, we also investigated whether the male partner's sperm in couples with embryo developmental problems were able to induce normal Ca^{2+} oscillatory patterns during oocyte activation. All sperm samples (except that from P13) showed a high frequency of Ca^{2+} spikes and AxF scores similar to the control sample when injected into mouse oocytes (MOCA) (Fig. 1) (Vanden-Meerschaut *et al.*, 2013), meaning that the embryo developmental problems observed in our patient cohort may have been caused by oocyte-related factors that cannot be restored by AOA. All MOCA experiments were performed with frozen-warmed sperm samples for practical reasons, and although fresh sperm was used for all ICSI cycles, previous research performed in our lab confirmed that frozen-warmed human sperm gives comparable calcium oscillation patterns as fresh sperm (Vanden-Meerschaut *et al.*, 2013). In addition, the use of swim-up for sperm processing technique during MOCA was preferred as it is a faster method than density gradient centrifugation. From the literature, it has been reported that there is no difference in fertilization rates and thus, calcium-releasing capacity, when using sperm samples processed with density gradient centrifugation or direct swim-up method (Palini *et al.*, 2017). In addition, PLCζ content has been showed to be reduced after cryopreservation (Moreau *et al.*, 2019), but even though MOCA results could show a slightly reduced calcium release in comparison to the situation in which fresh sperm is used, frozen-warmed patient samples were compared to a frozen-warmed control patient with normal fertilization and embryo development and the results showed no signal of calcium deficiency.

Normal Ca^{2+} release was expected from all patients included in the study as we excluded those with fertilization rates <60%, similar to Yin *et al.* (2022). However, we detected one patient (P13) that, although having normal fertilization (93.75%), showed a high proportion of oocytes with 0 or 1–2 Ca^{2+} peaks and therefore had the lowest AxF score (24.1 AU). This patient suffered from asthenozoospermia, but vitality test by eosin-nigrosin staining revealed 59% live sperm cells. During ICSI and ICSI-AOA treatments, the HOS test was performed to ensure injection of live sperm, as reflected by the high fertilization rates observed in this patient. The HOS test evaluates the sperm membrane integrity in hypo-osmotic conditions. Live sperm cells in a hypo-osmotic media control osmotic pressure by swelling, while dead sperm cells cannot control it and swell until the membrane disrupts

(Ramu and Jeyendran, 2013). The advantage of this test is that live sperm are identified by a swollen tail and can still be used for ICSI. We could not perform this test before MOCA, which explains why some oocytes did not show calcium release. P13 showed a considerable improvement in the blastocyst rate, increasing from 6.67% (1/15) after ICSI to 40.0% (4/10) after ICSI-AOA treatment (Table II). However, this couple did not have good quality blastocysts after either ICSI (0.0%, 0/15) or ICSI-AOA (0.0%, 0/10).

Embryo developmental problems after ICSI can be caused by a broad variety of factors (Mohebi and Ghafouri-Fard, 2019; Paonessa *et al.*, 2021; Sfakianoudis *et al.*, 2021). Recent research has brought to light the important role the SCMC plays during first embryo cleavages (Bebbere *et al.*, 2016), as different patients suffering from embryo developmental problems have been detected to present with variants in SCMC genes (Xu *et al.*, 2016; Wang *et al.*, 2018; Mu *et al.*, 2019; Lin *et al.*, 2020; Zheng *et al.*, 2021). In this article, we also investigated the underlying genetic causes of the embryo developmental problems observed in our patient cohort by screening for genetic variants in female SCMC components (*TLE6*, *PADI6*, *NRLP2*, *NRLP5*, *NRLP7*, and *KDHC3L9*) and in *PLCZ1* to exclude a possible male factor. No major pathogenic variants (Classes 4 and 5) were detected, but we did encounter some VUS (Class 3) in SCMC genes (Table III). Classification of variants was performed using the Varsome platform, a search engine that integrates information from more than 130 genomic databases and combines different types of data (i.e. computational, predictive, functional, population, segregation) to classify human genetic variants according to the ACMG guidelines (Kopanos *et al.*, 2019). Varsome is regularly updated, refined, and assessed, thus variant classification can change depending on the moment of the consult. At the time of consultation, we did identify 10 heterozygous VUS variants. Of note is the *TLE6* variant c.1100C>T (p.Pro367Leu) encountered in P4, which is predicted to be damaging by all *in silico* pathogenicity prediction tools and is found in a very low frequency in the general population (Table II). This variant is present in one of the 7 beta-transducin repeat domains (WD domains) that *TLE6* contains and is required for protein interaction. All disease-causing variants reported in *TLE6* are located in the WD repeat domains of the protein which makes it very likely that this variant is also causal (Alazami *et al.*, 2015; Lin *et al.*, 2020). In addition, AOA treatment was not able to overcome the impaired embryonic development observed after ICSI (Supplementary Table SII) and both D3 embryo (59.1%, 13/22) and blastocyst formation (9.1%, 2/22) rates, as well as good quality D3 embryo (18.2%, 4/22) and good quality blastocyst formation (0.0%, 0/22) rates remained very low (Table II). This result agrees with the fact that the male partner's sperm was able to induce normal Ca^{2+} release (Fig. 1). Yet, functional analysis tests should be performed to assess the causality of variants reported in this article. To assess pathogenicity of SCMC variants, multiple strategies could be applied: immunostaining of the specific protein in unfertilized or arrested embryos from the patient (Xu *et al.*, 2016), comparison of protein levels after transfection of Hela cells with complementary RNA (cRNA) constructs containing the protein of interest with and without the mutation (Mu *et al.*, 2019), as well as production of mouse models carrying the variant (Yu *et al.*, 2014).

All SCMC variants reported in the literature are identified in homozygosis or compound heterozygosis, in contrast to the findings in this article (Alazami *et al.*, 2015; Xu *et al.*, 2016). Since only exonic and flanking intronic regions were sequenced, there may be disease-causing variants in other intronic regions such as promotors or enhancers that were not detected. We cannot exclude the possibility that other heterozygous variants are present in undiscovered genes also participating in early preimplantation embryonic divisions. During recent years, mutations in other female genes involved in oocyte maturation arrest, like *PATL2* and *TUBB8*, have also been detected in patients showing EDA (Chen *et al.*, 2017; Wu *et al.*, 2019), meaning that other proteins participating in crucial pathways occurring prior to embryonic development, like oocyte maturation and fertilization, can have a later effect in embryo development. Finally, the detection rate of SCMC mutations reported in the literature is rather low (Mu *et al.*, 2019; Lin *et al.*, 2020). For instance, Lin *et al.* (2020) only detected *TLE6* mutations in 3/403 individuals screened. Moreover, many mutations in SCMC have been identified in patients from consanguineous families which have an increased risk for recessive genetic disorders (Xu *et al.*, 2016; Wang *et al.*, 2018; Zheng *et al.*, 2021). Consanguinity is more common in north and sub-Saharan Africa, the Middle East, and central and south Asia (Bittles and Black, 2010). This may explain why we did not identify any clear pathogenic variant screening 42 Caucasian patients. Finally, targeted genetic screening seems insufficient to detect pathogenic mutations causing impaired embryonic development as this is a multifactorial condition, with many required molecular factors not discovered yet. A better strategy would be to perform whole-exome or whole-genome sequencing, although these techniques are more expensive and require complex bioinformatic analysis (Capalbo *et al.*, 2021).

Unexpectedly, we did find a homozygous mutation in *PLCZ1* (c.1499C>T; p.Ser500Leu), previously reported to cause fertilization failure after ICSI in 9/37 patients screened within one study (Torra-Massana *et al.*, 2019). Functional analysis revealed no decreased fertilization rate after injecting IVM human oocytes with mutated *PLCZ1* p.Ser500Leu cRNA in comparison to wild-type (WT) *PLCZ1* cRNA (Torra-Massana *et al.*, 2019). This result could partially explain the phenotype seen in P35, as this couple had normal fertilization rate after routine ICSI (74.07%), but all embryos arrested before achieving the blastocyst state (Supplementary Table SIII). Further research should investigate whether sperm with *PLCZ1* p.Ser500Leu cRNA is able to support embryo development until blastocyst stage as does *PLCZ1* WT cRNA in mouse oocytes (Saunders *et al.*, 2002). Moreover, AOA was able to restore fertilization rates in these patients, regardless of the *PLCZ1* c.1499C>T (p.Ser500Leu) mutation being in heterozygous or homozygous state, and live births were achieved (Torra-Massana *et al.*, 2019). Unfortunately, P35 has not undergone an ICSI-AOA cycle yet. AOA will most possibly overcome the impaired embryo development, as it seems to be caused by a PLCζ deficiency. Moreover, to confirm the causality of the *PLCZ1* variant, the Ca^{2+} releasing pattern induced by the patient sperm sample remains to be studied.

In conclusion, our data show that AOA treatment is not indicated for patients with a history of embryo developmental problems after ICSI (≤15% blastocyst formation), as it probably results from oocyte-related factors not linked to disturbed Ca^{2+} patterns. Despite the sample size being low, we decided to stop the study and not include more patients, as no beneficial effect was observed after ICSI-AOA. Besides, there is only scarce evidence in the literature to support the direct benefit of AOA treatment for couples suffering from this condition. Moreover, patient heterogenicity complicates the comparison of the results in these studies and brings to light the necessity for a strict

definition of embryo developmental problems to reduce AOA overtreatment. Although no major abnormalities have been found in children born after ICSI-AOA (Vanden-Meerschaut *et al.*, 2014a; Mateizel *et al.*, 2018; Shan *et al.*, 2022), this technique should be reserved for couples with a clear deficiency in post-fertilization oocyte Ca^{2+} dynamics (Ferrer-Buitrago *et al.*, 2018b; Cardona Barberán *et al.*, 2020). In addition, we also described different heterozygous SCMC VUS variants in patients with embryo developmental problems; yet, causality should be determined by functional assessment. It also remains to be investigated whether *PLCZ1* variants can cause both fertilization failure and EDA infertility phenotypes. Finally, genetic screening using whole-exome sequencing techniques may reveal novel genes and variants involved in embryo development and allow the design of more appropriate and personalized treatment options for these couples. For patients with clear molecular defects, WT cRNA or recombinant protein injection during ICSI may restore the abnormal protein content and allow correct embryonic development. Injection of *WEE2*, *TRIP13*, and *CDC20 WT* cRNA molecules in patients with the corresponding mutation could restore oocyte maturation and fertilization potential (Sang *et al.*, 2018; Zhang *et al.*, 2020; Zhao *et al.*, 2020). For patients with and without clear molecular defects, but still oocyte-suspected cytoplasmic deficiency, nuclear transfer (NT) could represent a more general approach. NT consists of replacing poor-quality oocyte or zygote cytoplasm (containing abnormal proteins required for fertilization and/or embryo development) with good-quality cytoplasm from a healthy oocyte or zygote (Craven *et al.*, 2017). Recently, using an embryo arrest mouse model, NT was shown to significantly increase blastocyst formation rates (Costa-Borges *et al.*, 2020; Tang *et al.*, 2020). Moreover, in humans, NT has already been proposed to overcome EDA (Zhang *et al.*, 2016) as well as fertilization failure after ICSI-AOA (Tang *et al.*, 2022).

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its Supplementary Material.

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Authors' roles

D.B. and B.H. designed the study. A.C.B., D.B., A.B., V.T., R.P., F.M., and I.D.C. performed the experiments, and collected and analyzed the data. A.C.B. wrote the original draft and revised the article. A.B., D.S., P.D.S., P.C., L.L., and F.V.M. revised and edited the article. B.H. revised and approved the final article. All authors read and agreed to the published version of the article.

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Conflict of interest

A.C.B., D.B., A.B., V.T., R.P., F.M., I.D.C., L.L., D.S., P.D.S., P.C., and F.V.M. have nothing to disclose. B.H. reports a research grant from the Flemish Fund for Scientific Research and reports being a board member of the Belgian Society for Reproductive Medicine and the Belgian Ethical Committee on embryo research.

References

Alazami AM, Awad SM, Coskun S, Al-Hassan S, Hijazi H, Abdulwahab FM, Poizat C, Alkuraya FS. TLE6 mutation causes the earliest known human embryonic lethality. *Genome Biol* 2015;**16**:240.

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Hum Reprod* 2011;**26**:1270–1283.

Bebbere D, Masala L, Albertini DF, Ledda S. The subcortical maternal complex: multiple functions for one biological structure? *J Assist Reprod Genet* 2016;**33**:1431–1438.

Berridge MJ. Calcium signalling and cell proliferation. *Bioessays* 1995;**17**:491–500.

Bittles AH, Black ML. Consanguinity, human evolution, and complex diseases. *Proc Natl Acad Sci USA* 2010;**107**:1779–1786.

Bonte D, Ferrer-Buitrago M, Dhaenens L, Popovic M, Thys V, de Croo I, de Gheselle S, Steyaert N, Boel A, Vanden Meerschaut F *et al.* Assisted oocyte activation significantly increases fertilization and pregnancy outcome in patients with low and total failed fertilization after intracytoplasmic sperm injection: a 17-year retrospective study. *Fertil Steril* 2019;**112**:266–274.

Capalbo A, Poli M, Riera-Escamilla A, Shukla V, Kudo Høffding M, Krausz C, Hoffmann ER, Simon C. Preconception genome medicine: current state and future perspectives to improve infertility diagnosis and reproductive and health outcomes based on individual genomic data. *Hum Reprod Update* 2021;**27**:254–279.

Cardona Barberán A, Boel A, Vanden Meerschaut F, Stoop D, Heindryckx B. Diagnosis and treatment of male infertility-related fertilization failure. *J Clin Med* 2020;**9**:3899.

Chen B, Li B, Li D, Yan Z, Mao X, Xu Y, Mu J, Li Q, Jin L, He L et al Novel mutations and structural deletions in TUBB8: expanding mutational and phenotypic spectrum of patients with arrest in oocyte maturation, fertilization or early embryonic development. *Hum Reprod* 2017;**32**:457–464.

Condic ML. The role of maternal-effect genes in mammalian development: are mammalian embryos really an exception? *Stem Cell Rev Rep* 2016;**12**:276–284.

Costa-Borges N, Spath K, Miguel-Escalada I, Mestres E, Balmaseda R, Serafín A, Garcia-Jimenéz M, Vanrell I, González J, Rink K *et al.* Maternal spindle transfer overcomes embryo developmental arrest caused by ooplasmic defects in mice. *Elife* 2020;**9**:e48591.

Craven L, Tang M-X, Gorman GS, De Sutter P, Heindryckx B. Novel reproductive technologies to prevent mitochondrial disease. *Hum Reprod Update* 2017;**23**:501–519.

De Leeneer K, Hellemans J, Steyaert W, Lefever S, Vereecke I, Debals E, Crombez B, Baetens M, Van Heetvelde M, Coppieters F *et al*. Flexible, scalable, and efficient targeted resequencing on a benchtop sequencer for variant detection in clinical practice. *Hum Mutat* 2015;**36**:379–387.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S, Ozil J-P. Egg-to-embryo transition is driven by differential responses to Ca2+ oscillation number. *Dev Biol* 2002;**250**:280–291.

Ebner T, Oppelt P, Wober M, Staples P, Mayer RB, Sonnleitner U, Bulfon-Vogl S, Gruber I, Haid AE, Shebl O. Treatment with Ca2+ ionophore improves embryo development and outcome in cases with previous developmental problems: a prospective multicenter study. *Hum Reprod* 2015;**30**:97–102.

Ebner T, Shebl O. Effect of A23187 ionophore treatment on human blastocyst development—a sibling oocyte study. *J Assist Reprod Genet* 2022;**39**:1233–1234.

Ebner T, Shebl O, Oppelt P. First live births after application of a ready-to-use ionomycin in cases of failed Artificial Oocyte Activation (AOA) using calcimycin. *Fertil Steril* 2021;**116**:e199.

Ferrer-Buitrago M, Bonte D, De Sutter P, Leybaert L, Heindryckx B. Single Ca2+ transients vs oscillatory Ca2+ signaling for assisted oocyte activation: limitations and benefits. *Reproduction* 2018a;**155**: R105–R119.

Ferrer-Buitrago M, Dhaenens L, Lu Y, Bonte D, Vanden Meerschaut F, De Sutter P, Leybaert L, Heindryckx B. Human oocyte calcium analysis predicts the response to assisted oocyte activation in patients experiencing fertilization failure after ICSI. *Hum Reprod* 2018b;**33**:416–425.

Grantham R. Amino acid difference formula to help explain protein evolution. *Science* 1974;**185**:862–864.

Jia L, Chen P, Su W, He S, Guo Y, Zheng L, Fang C, Liang X. Artificial oocyte activation with ionomycin compared with A23187 among patients at risk of failed or impaired fertilization. *Reprod Biomed Online* 2023;**46**:35–45.

Kashir J, Heindryckx B, Jones C, de Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 2010;**16**:690–703.

Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Albarca Aguilera M, Meyer R, Massouras A. VarSome: the human genomic variant search engine. *Bioinformatics* 2019;**35**:1978–1980.

Lefever S, Pattyn F, de Wilde B, Coppieters F, de Keulenaer S, Hellemans J, Vandesompele J. High-throughput PCR assay design for targeted resequencing using primerXL. *BMC Bioinformatics* 2017;**18**:400.

Li J, Zheng X, Lian Y, Li M, Lin S, Zhuang X, Chen L, Liu P, Qiao J. Artificial oocyte activation improves cycles with prospects of ICSI fertilization failure: a sibling oocyte control study. *Reprod Biomed Online* 2019;**39**:199–204.

Lin J, Xu H, Chen B, Wang W, Wang L, Sun X, Sang Q. Expanding the genetic and phenotypic spectrum of female infertility caused by TLE6 mutations. *J Assist Reprod Genet* 2020;**37**:437–442.

Lv M, Zhang D, He X, Chen B, Li Q, Ding D, Hao Y, Xue R, Ji D, Zou W *et al*. Artificial oocyte activation to improve reproductive outcomes in couples with various causes of infertility: a retrospective cohort study. *Reprod Biomed Online* 2020;**40**:501–509.

Mateizel I, Santos-Ribeiro S, Segers I, Wouters K, Mackens S, Verheyen G. Effect of A23187 ionophore treatment on human blastocyst development—a sibling oocyte study. *J Assist Reprod Genet* 2022;**39**:1235.

Mateizel I, Verheyen G, van de Velde H, Tournaye H, Belva F. Obstetric and neonatal outcome following ICSI with assisted oocyte activation by calcium ionophore treatment. *J Assist Reprod Genet* 2018;**35**:1005–1010.

Meng X, Melo P, Jones C, Ross C, Mounce G, Turner K, Child T, Coward K. Use of phospholipase C zeta analysis to identify candidates for artificial oocyte activation: a case series of clinical pregnancies and a proposed algorithm for patient management. *Fertil Steril* 2020;**114**:163–174.

Mohebi M, Ghafouri-Fard S. Embryo developmental arrest: review of genetic factors and pathways. *Gene Rep* 2019;**17**:100479.

Moreau J, Fargeon S, Gatimel N, Parinaud J, Léandri RD. Expression of phospholipase PLC Zeta in human spermatozoa: impact of cryopreservation. *Andrology* 2019;**7**:315–318.

Mu J, Wang W, Chen B, Wu L, Li B, Mao X, Zhang Z, Fu J, Kuang Y, Sun X *et al*. Mutations in NLRP2 and NLRP5 cause female infertility characterised by early embryonic arrest. *J Med Genet* 2019; **56**:471–480.

Mu J, Zhang Z, Wu L, Fu J, Chen B, Yan Z, Li B, Zhou Z, Wang W, Zhao L *et al*. The identification of novel mutations in PLCZ1 responsible for human fertilization failure and a therapeutic intervention by artificial oocyte activation. *Mol Hum Reprod* 2020;**26**:80–87.

Nikiforaki D, Vanden Meerschaut F, de Roo C, Lu Y, Ferrer-Buitrago M, de Sutter P, Heindryckx B. Effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. *Fertil Steril* 2016;**105**: 798–806.e2.

Ozil JP, Banrezes B, Tóth S, Pan H, Schultz RM. Ca2+ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev Biol* 2006;**300**:534–544.

Ozil JP, Huneau D. Activation of rabbit oocytes: the impact of the Ca2+ signal regime on development. *Development* 2001;**128**: 917–928.

Ozil JP, Markoulaki S, Toth S, Matson S, Banrezes B, Knott JG, Schultz RM, Huneau D, Ducibella T. Egg activation events are regulated by the duration of a sustained [Ca2+]cyt signal in the mouse. *Dev Biol* 2005;**282**:39–54.

Palini S, Stefani S, Primiterra M, Benedetti S, Barone S, Carli L, Vaccari E, Murat U, Feichtinger W. Comparison of in vitro fertilization outcomes in ICSI cycles after human sperm preparation by density gradient centrifugation and direct micro swim-up without centrifugation. *JBRA Assist Reprod* 2017;**21**:89–93.

Paonessa M, Borini A, Coticchio G. Genetic causes of preimplantation embryo developmental failure. *Mol Reprod Dev* 2021;**88**:338–348.

Ramu S, Jeyendran RS. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. In: Carrell DT, Aston KI (eds). *Spermatogenesis: Methods and protocols. Methods Mol Biol*, Vol. **927**. Totowa, NJ: Humana Press, 2013, 21–25.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E *et al*. Standards and guidelines for the interpretation of sequence variants: a joint consensus

recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;**17**:405–424.

Rybouchkin AV, Van der Straeten F, Quatacker J, De Sutter P, Dhont M. Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. *Fertil Steril* 1997;**68**:1144–1147.

Sang Q, Li B, Kuang Y, Wang X, Zhang Z, Chen B, Wu L, Lyu Q, Fu Y, Yan Z *et al.* Homozygous mutations in WEE2 cause fertilization failure and female infertility. *Am J Hum Genet* 2018;**102**:649–657.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLCζ: a sperm-specific trigger of Ca2+ oscillations in eggs and embryo development. *Development* 2002;**129**: 3533–3544.

Sfakianoudis K, Maziotis E, Karantzali E, Kokkini G, Grigoriadis S, Pantou A, Giannelou P, Petroutsou K, Markomichali C, Fakiridou M *et al.* Molecular drivers of developmental arrest in the human preimplantation embryo: a systematic review and critical analysis leading to mapping future research. *Int J Mol Sci* 2021;**22**: 8353.

Shan Y, Zhao H, Zhao D, Wang J, Cui Y, Bao H. Assisted oocyte activation with calcium ionophore improves pregnancy outcomes and offspring safety in infertile patients: a systematic review and meta-analysis. *Front Physiol* 2022;**12**:2513.

Sousa M, Barros A, Tesarik J. Developmental changes in calcium dynamics, protein kinase C distribution and endoplasmic reticulum organization in human preimplantation embryos. *Mol Hum Reprod* 1996;**2**:967–977.

Stein P, Savy V, Williams AM, Williams CJ. Modulators of calcium signalling at fertilization. *Open Biol* 2020;**10**:200118.

Tang M, Boel A, Castelluccio N, Barberán C, Christodoulaki A, Bekaert A, Popovic B, Vanden Meerschaut M, de Sutter F, Menten P *et al.* Human germline nuclear transfer to overcome mitochondrial disease and failed fertilization after ICSI. *J Assist Reprod Genet* 2022;**39**:609–618.

Tang M, Popovic M, Stamatiadis P, van der Jeught M, van Coster R, Deforce D, de Sutter P, Coucke P, Menten B, Stoop D *et al.* Germline nuclear transfer in mice may rescue poor embryo development associated with advanced maternal age and early embryo arrest. *Hum Reprod* 2020;**35**:1562–1577.

Torra-Massana M, Cornet-Bartolomé D, Barragán M, Durban M, Ferrer-Vaquer A, Zambelli F, Rodriguez A, Oliva R, Vassena R. Novel phospholipase C zeta 1 mutations associated with fertilization failures after ICSI. *Hum Reprod* 2019;**34**:1494–1504.

Tóth S, Huneau D, Banrezes B, Ozil JP. Egg activation is the result of calcium signal summation in the mouse. *Reproduction* 2006;**131**: 27–34.

Tsai TE, Lin PH, Lian PF, Li CJ, Vitale SG, Mikuš M, Su WP, Tsai HW, Tsui KH, Te Lin L. Artificial oocyte activation may improve embryo quality in older patients with diminished ovarian reserve undergoing IVF-ICSI cycles. *J Ovarian Res* 2022;**15**:1–9.

Vanden-Meerschaut F, D'Haeseleer E, Gysels H, Thienpont Y, Dewitte G, Heindryckx B, Oostra A, Roeyers H, Van Lierde K, De Sutter P. Neonatal and neurodevelopmental outcome of children aged 3-10 years born following assisted oocyte activation. *Reprod Biomed Online* 2014a;**28**:54–63.

Vanden-Meerschaut F, Leybaert L, Nikiforaki D, Qian C, Heindryckx B, de Sutter P. Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. *Hum Reprod* 2013;**28**:87–98.

Vanden-Meerschaut F, Nikiforaki D, Heindryckx B, de Sutter P. Assisted oocyte activation following ICSI fertilization failure. *Reprod Biomed Online* 2014b;**28**:560–571.

Wang X, Song D, Mykytenko D, Kuang Y, Lv Q, Li B, Chen B, Mao X, Xu Y, Zukin V *et al.* Novel mutations in genes encoding subcortical maternal complex proteins may cause human embryonic developmental arrest. *Reprod Biomed Online* 2018;**36**:698–704.

Wu L, Chen H, Li D, Song D, Chen B, Yan Z, Lyu Q, Wang L, Kuang Y, Li B *et al.* Novel mutations in PATL2: expanding the mutational spectrum and corresponding phenotypic variability associated with female infertility. *J Hum Genet* 2019;**64**:379–385.

Xi H, Fu Y, Liu C, Lu X, Sui L, Chen Y, Zhao J. Assisted oocyte activation with calcium ionophore 44 hours after intracytoplasmic sperm injection resulting in successful pregnancy. *Gynecol Endocrinol* 2020;**36**:1035–1037.

Xu Y, Shi Y, Fu J, Yu M, Feng R, Sang Q, Liang B, Chen B, Qu R, Li B *et al.* Mutations in PADI6 cause female infertility characterized by early embryonic arrest. *Am J Hum Genet* 2016;**99**:744–752.

Yeste M, Jones C, Amdani SN, Patel S, Coward K. Oocyte activation deficiency: a role for an oocyte contribution? *Hum Reprod Update* 2016;**22**:23–47.

Yin M, Li M, Li W, Wu L, Yan Z, Zhao J, Ouyang J, Lyu Q, Yan Z, Li B. Efficacy of artificial oocyte activation in patients with embryo developmental problems: a sibling oocyte control study. *Arch Gynecol Obstet* 2022;**305**:1225–1231.

Yoon S-Y, Jellerette T, Salicioni AM, Lee HC, Yoo M, Coward K, Parrington J, Grow D, Cibelli JB, Visconti PE *et al.* Human sperm devoid of PLC, zeta 1 fail to induce Ca2+ release and are unable to initiate the first step of embryo development. *J Clin Invest* 2008; **118**:3671–3681.

Yu XJ, Yi Z, Gao Z, Qin D, Zhai Y, Chen X, Ou-Yang Y, Wang ZB, Zheng P, Zhu MS *et al.* The subcortical maternal complex controls symmetric division of mouse zygotes by regulating F-actin dynamics. *Nat Commun* 2014;**5**:4887.

Zhang Z, Li B, Fu J, Li R, Diao F, Li C, Chen B, Du J, Zhou Z, Mu J *et al.* Bi-allelic missense pathogenic variants in TRIP13 cause female infertility characterized by oocyte maturation arrest. *Am J Hum Genet* 2020;**107**:15–23.

Zhang J, Zhuang G, Zeng Y, Grifo J, Acosta C, Shu Y, Liu H. Pregnancy derived from human zygote pronuclear transfer in a patient who had arrested embryos after IVF. *Reprod Biomed Online* 2016;**33**:529–533.

Zhao L, Xue S, Yao Z, Shi J, Chen B, Wu L, Sun L, Xu Y, Yan Z, Li B *et al.* Biallelic mutations in CDC20 cause female infertility characterized by abnormalities in oocyte maturation and early embryonic development. *Protein Cell* 2020;**11**:921–927.

Zheng W, Hu H, Dai J, Zhang S, Gu Y, Dai C, Guo J, Xu X, Li Y, Zhang S *et al.* Expanding the genetic and phenotypic spectrum of the subcortical maternal complex genes in recurrent preimplantation embryonic arrest. *Clin Genet* 2021;**99**:286–291.

BMC Pregnancy and Childbirth

RESEARCH

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Calcium ionophore improves embryonic development and pregnancy outcomes in patients with previous developmental problems in ICSI cycles

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Abstract

Background: Calcium (Ca^{2+}) ionophores are now mainly considered as efficient treatments for fertilization failure. Recently, its application for rescuing poor embryo development was proposed but still non-routine. This study aimed to explore whether Ca^{2+} ionophore improves embryo development and pregnancy outcomes in patients with poor embryo development in previous intracytoplasmic sperm injection (ICSI) cycles.

Methods: This study included 97 patients undergoing assisted oocyte activation (AOA) with Ca^{2+} ionophore (calcimycin, A23187) treatment. Preimplantation embryonic development and clinical outcomes were compared between ICSI-AOA cycles (AOA group) and previous ICSI cycles of the same patients in which poor embryo developmental potential was present (non-AOA group). Subgroups stratified by maternal age (< 35, 35–40, ≥ 40 years, respectively) were analyzed separately.

Results: A total of 642 MII oocytes were collected in AOA group, and 689 in non-AOA group. Significantly higher day 3 good quality embryo rate ($P = 0.034$), good quality blastocyst formation rate ($P < 0.001$), and utilization rate ($P < 0.001$) were seen in AOA group. Similar results were seen in each subgroup. For pregnancy outcomes, there were significant differences in clinical pregnancy rate ($P = 0.039$) and live birth rate ($P = 0.045$) in total group. In subgroup aged < 35 years, biochemical ($P = 0.038$), clinical ($P = 0.041$), and ongoing pregnancy rate ($P = 0.037$) in AOA group were significantly higher than that in non-AOA group. No significant improvement for clinical outcomes for subgroups aged 35–40 and aged ≥40.

Conclusion: The study suggests that calcimycin could improve preimplantation development and pregnancy outcomes in patients aged < 35 years with embryo developmental problems in previous ICSI cycles.

Keywords: Poor embryo development, Assisted oocyte activation, Calcium ionophore, Age

Introduction

Infertility has become a health problem of global concern affecting about 15% of couples and 80% of them could find a solution with the advancement of assisted reproductive technology (ART) [1]. Part of failed patients undergoing in vitro fertilization (IVF) suffered from poor embryo developmental potential. Embryo quality

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positively correlated to pregnancy outcomes [2, 3], but the cause of poor embryo development is still unclear and there is a lack of effective treatment in clinic [4, 5].

Some studies reported that Calcium (Ca^{2+}) signal deficiency or insufficiency during oocyte activation is associated with embryo arrest, cleavage anomalies, or poor preimplantation development and blastocyst quality [6–9]. During mammalian fertilization, sperm releases sperm-specific phospholipase C zeta (PLCζ) into the ooplasm immediately after the fusion of sperm and oocyte plasma membranes, and then results in Ca^{2+} oscillations which are essential for normal fertilization and the onset of embryogenesis by triggering downstream signaling pathways. This process is known as oocyte activation [10–12]. A sequence of cellular events during oocyte activation, including maternal mRNA recruitment, cytoskeletal rearrangement, embryonic genome activation, and initiation of cleavage [13–16], is the premise of oocyte-to-embryo transition and early embryonic development [11, 17, 18] and dependents on various amount of Ca^{2+} oscillations [13]. In general, increase of Ca^{2+} during activation has later effect on subsequent embryonic development [19, 20] and oppositely, oocyte activation failure resulting from sperm- or oocyte-related calcium insufficiency could lead to poor embryo development.

Assisted oocyte activation (AOA), including mechanical, electrical, and chemical stimuli, has been considered as a feasible approach which can increase Ca^{2+} in the oocyte and induce oocyte activation efficiently [8]. Calcium ionophores including ionomycin and calcimycin (A23187) are the most common approaches used for AOA in ART [21], and have been widely used for rescuing fertilization failure and globozoospermia [22]. Recently, researchers tried to improve embryo development with AOA at intracytoplasmic sperm injection (ICSI) in patients with various early embryo developmental problems. It has been reported that successful cleavage after calcium ionophore treatment could be achieved in cases with previous embryo arrest at the pronuclear stage [23]. A multicentric prospective study reported that calcium ionophore could rescue patients with completely developmental arrest or delay, or reduced blastocyst formation rate [24]. But a study using sibling oocytes as the control group in the same cycle reported that calcium ionophore was not able to improve embryo laboratory outcomes as well as pregnancy outcomes in patients who suffered from poor embryo development but achieved more than 70% normal fertilization rate in previous ICSI cycles [25].

Previous studies were still sparse, based on small sample size, and no consensus has been reached. Of central concern, AOA treatment protocols as well as targeted patients were obviously different among these studies. AOA was sometimes performed immediately after ICSI for 15 minutes and sometimes performed after 60 minutes of ICSI for 10 minutes [24–26]. In fact, Ca^{2+} oscillations begin at 15–30 minutes after ICSI [27, 28], which might be relevant to delayed release of PLCζ due to delayed disintegration of spermatozoa plasma membrane at ICSI [29]. Thus, we applied calcium ionophore at 15–30 minutes after ICSI and aimed to evaluate the effect of calcium ionophore on patients with poor embryo developmental potential in previous ICSI cycles.

Materials and methods

Patients

The study was performed at the Hospital for Reproductive Medicine Affiliated to Shandong University. A total of 97 female patients undergoing calcimycin (A23187) treatment for AOA (AOA group) were collected from March 2020 to December 2021 and met one of the following AOA indications in previous ICSI cycles: (1) no good quality embryo on day 3 and no good quality blastocyst; (2) at least one good quality embryo on day 3 but no good quality blastocyst; (3) or less than 20% good quality blastocyst formation rate [24]. Their preceding conventional ICSI cycles without AOA served as the control group (non-AOA group). Patients with normal fertilization rate < 30% in previous ICSI cycles were excluded because that is conventional indication for AOA [24]. Patients with MII oocyte retrieved < 2 were also ruled out, which was a compromise between minimizing the contingency of poor embryo development and adequate sample size. The patients were divided into three subgroups by their age (< 35, 35–40, ≥ 40 years, respectively) in order to explore the effect of AOA at different age stages.

ICSI and ionophore oocyte activation

Controlled ovarian hyperstimulation (COH) protocols were determined by experienced physicians depending on the patients' baseline data and willing. Follicle growth was initiated by either recombinant follicle stimulating hormone or human menopausal gonadotropin in general. Ovulation was induced by human chorionic gonadotropin (hCG) at a dose of 4000–10,000 IU. Oocyte retrieval was performed 36 hours after hCG administration. The time interval between removing granule cells and performing ICSI in non-AOA cycles was same as that in AOA cycles. Routinely, the time interval ranges from 0.5 h to 2 h, which depends on the maturation rate of the obtained oocytes and the number of ICSI patients on the day of oocyte retrieval. The ICSI procedure was identical in both non-AOA and AOA cycles and performed by experienced operators. Sperm used for ICSI were all fresh sperm and most were ejaculated sperm. During calcium

ionophore oocyte activation cycles, MII oocytes were exposed to A23187 (5 μM, Sigma, St. Louis, MO, USA) for 15 minutes after 15 minutes of ICSI, and then rinsed for three times. The base solution for oocyte activation is cleavage-stage medium, which is G1-plus (Vitrolife) in our center.

Embryo culture and transfer

Embryos were cultured with sequential culture media supplied by Vitrolife (G-IVF, G1 and G2; Scandinavian IVF Science, Sweden). Puissant's criteria [30] was used for embryo scoring on day 3. Embryo transfer was considered on the third day of embryo culture when at least one available embryo was formed, and no more than three fresh cleaved embryos were transferred at once. Alternatively, all embryos were cultured up to blastocyst stage. Blastocyst scores were assessed according to Gardner and Lane's criteria [31]. In general, only blastocysts over 4BC grade were graded as good quality and transferrable in our center. If there were transferrable blastocysts, fresh embryo transfer with one blastocyst was performed. Surplus embryos after fresh embryo transfers were all incubated up to blastocyst stage and frozen. During IVF cycles, if there was a risk of ovarian hyperstimulation syndrome, or there were any conditions that might have impaired endometrial receptivity or patients' specific wishes, embryos were all cultured for blastocyst cryopreservation and fresh embryo transfer would not be performed. Embryos used for frozen embryo transfers were all at blastocysts stage in the study and only one blastocyst was transferred in each frozen embryo transfer cycle. Frozen embryo transfer procedure was reported previously [32, 33].

Outcomes

Primary outcomes included day 3 good quality embryo rate (good quality embryos on day 3/2PN (pronuclear) zygotes), and good quality blastocyst formation rate (good quality blastocysts/2PN zygotes). Secondary outcomes were utilization rate ((day 3 good quality embryos transferred*0.55 + day 3 non-good quality embryos transferred*0.45 + blastocyst transferred or frozen*1.00)/MII oocytes), biochemical pregnancy (a rising hCG concentration > 25 IU/L 12 days after transfer) rate, clinical pregnancy (visualization of gestational sacs through an abdominal ultrasound) rate, miscarriage (spontaneous pregnancy loss before 28 weeks of gestation) rate, and live birth (delivering at least one live-born infant after ≥28 weeks of gestation) rate.

Statistical analysis

Variables were tested for normal distribution using Kolmogorov-Smirnov test or Shapiro-Wilk test based on the sample size. According to the outcome of distribution, either paired t test or Wilcoxon sign rank test was applied for descriptive data and McNemar test or chi-square test for categorical variables.

All statistical analyses were performed using SPSS software, version 26.0. A 2-sided P value less than 0.05 was considered statistically significant.

Results

Participants

A total of 97 female patients were included in this study and each patient was included only once. Patients underwent ICSI were due to unexplained infertility (44.33%), male factor infertility (41.24%), and female factor infertility (14.43%). The mean (± SD) maternal age was 34.84 ± 4.92 years, body mass index (BMI) was 23.87 ± 2.86 kg/m^2, anti-Mullerian hormone (AMH) was 2.91 ± 2.20 ng/ml, and they had undergone 2.24 ± 1.40 previous ICSI cycles. No significant difference was observed between non-AOA and AOA cycles in total group and each subgroup as far as COH protocols and gonadotropin dose were concerned (Table 1 and Table S1 in Additional File 1). There was no significant difference between non-AOA and AOA cycles in terms of growth hormone application in each subgroup (Table S2 in Additional File 1). 7 male patients supplied epididymal or testicular sperm in their both AOA cycles and non-AOA cycles and the rest were all ejaculated sperm (Table S2 in Additional File 1).

Total included patients

When all included infertile patients were analyzed, 642 MII oocytes for ICSI were collected in AOA group and 689 in non-AOA group. After ICSI, significantly lower normal fertilization rate was found in AOA group ($P = 0.006$). There was no significant difference in multi-PN formation rate ($P = 0.112$) and cleavage rate ($P = 0.575$). However, day 3 good quality embryo rate ($P = 0.034$), good quality blastocyst formation rate ($P < 0.001$), and utilization rate ($P < 0.001$) were significantly higher in AOA group than that in non-AOA group (Table 1).

Seventy-one embryos were transferred in 46 transfer cycles in non-AOA group and 86 embryos were transferred in 69 transfer cycles in AOA group. No significant difference in terms of biochemical pregnancy rate ($P = 0.098$), but clinical pregnancy rate ($P = 0.039$), ongoing pregnancy rate ($P = 0.021$), and live birth rate ($P = 0.045$) were significantly higher in AOA group than that in non-AOA group. 4 babies were delivered in non-AOA group and 16 in AOA group. No significant difference in early miscarriage rate ($P = 0.726$) and late miscarriage rate ($P = 0.421$) between AOA group

Table 1 COH characteristics and preimplantation development outcomes in non-AOA and AOA group for total patients

	Non-AOA	AOA	*P* value
Cycles, n	97	97	
Age, year	33.62 ± 5.25	34.84 ± 4.92	
COH protocol, n (%)			0.122
GnRH-agonist protocol	50 (51.55%)	46 (47.42%)	
GnRH-antagonist protocol	38 (39.18%)	36 (37.11%)	
Other unconventional protocol	9 (9.28%)	15 (15.46%)	
Gn total dose, IU	2171.55 ± 932.35	2273.33 ± 1018.15	0.379
No. of MII oocytes, n	689	642	
Normal fertilization rate, n (%)	503 (73.00%)	422 (65.73%)	0.006*
Multi-PN formation rate, n (%)	19 (2.76%)	22 (3.43%)	0.112
Cleavage rate, n (%)	493 (98.01%)	413 (97.87%)	0.575
Day 3 good quality embryo rate, n (%)	145 (28.83%)	163 (38.63%)	0.034*
Good quality blastocyst formation rate, n (%)	18 (3.58%)	117 (27.73%)	< 0.001*
Utilization rate, n (%)	50.60 (7.34%)	144.65 (22.53%)	< 0.001*

Values are presented as mean ± SD or n (%). *$P < 0.05$.

COH, controlled ovarian hyperstimulation; Gn, gonadotropin; MII, metaphase II; PN, pronuclear.

Table 2 Clinical outcomes in non-AOA and AOA group for total patients

	Non-AOA	AOA	*P* value
Embryo transfer cycles, n	46	69	
No. of embryos transferred, n	71	86	
Biochemical pregnancy rate, %	28.26% (13/46)	43.48% (30/69)	0.098
Clinical pregnancy rate, %	19.57% (9/46)	37.68% (26/69)	0.039*
Ongoing pregnancy rate, %	10.87% (5/46)	28.99% (20/69)	0.021*
Ectopic pregnancy rate, %	11.11% (1/9)	0	–
Pregnancy loss rate, %			
Early miscarriage rate	37.50% (3/8)	23.08% (6/26)	0.726
Late miscarriage rate	12.50% (1/8)	3.85% (1/26)	0.421
Live birth rate, %	8.70% (4/46)	23.19% (16/69)	0.045*

Values are presented as % (n/total n). *$P < 0.05$

and non-AOA group (Table 2). One ectopic pregnancy occurred in non-AOA group. No birth defects were found.

Significantly higher proportion of cryo-embryo transfer ($P < 0.001$) and blastocyst transfer ($P < 0.001$) were noted in AOA group (Table S3 in Additional File 1). When pregnancy outcomes of cleavage and blastocyst transfers were analyzed separately, there was no significant difference between AOA and non-AOA group in cleavage-stage embryo transfer cycles in terms of biochemical, clinical and ongoing pregnancy rate as well as live birth rate ($P = 0.441$, $P = 0.645$, $P = 0.109$, $P = 0.722$, $P = 0.722$, respectively) (Table 3). In blastocyst transfer cycles, quality of embryos transferred in AOA group is higher than that in non-AOA group although the difference was not statistically significant (Table S4 in Additional File 1). Significantly higher biochemical pregnancy rate ($P = 0.003$) and clinical pregnancy rate ($P = 0.008$) were observed in AOA group than that in non-AOA group, while no significant difference

Table 3 Pregnancy outcomes in non-AOA and AOA group for cleavage-stage embryo transfer and blastocyst transfer cycles

	Cleavage-stage embryo transfer			Blastocyst transfer		
	Non-AOA	AOA	*P* value	Non-AOA	AOA	*P* value
Biochemical pregnancy rate, %	36.36% (12/33)	26.92% (7/26)	0.441	7.69% (1/13)	53.49% (23/43)	0.003*
Clinical pregnancy rate, %	24.24% (8/33)	19.23% (5/26)	0.645	7.69% (1/13)	48.84% (21/43)	0.008*
Ongoing pregnancy rate, %	12.12% (4/33)	15.38% (4/26)	0.722	7.69% (1/13)	37.21% (16/43)	0.092
Live birth rate, %	12.12% (4/33)	15.38% (4/26)	0.722	0	27.91% (12/43)	–

Values are presented as % (n/total n). *$P < 0.05$

in ongoing pregnancy rate ($P=0.092$) was found in spite of a discrepancy of nearly 20%. No live birth was achieved in non-AOA group and 12 babies were born in AOA group (0 vs. 27.91%). 3 ongoing pregnancies in AOA group were still in the third trimester (Table 3).

Subgroup analysis

Fifty-two patients were included in the subgroup aged <35 years, 27 in the subgroup aged 35–40 years, and 18 in the subgroup aged ≥40 years. Preimplantation embryonic development outcomes for each subgroup are shown in Table S1 (Table S1 in Additional File 1).

In women aged <35 years, 408 MII oocytes were collected in non-AOA group and 399 in AOA group. Normal fertilization rate, multi-PN formation rate and cleavage rate did not differ between AOA and non-AOA group ($P=0.063$, $P=0.701$, $P=0.735$, respectively). However, AOA significantly improved day 3 good quality embryo rate ($P=0.001$), good quality blastocyst formation rate ($P<0.001$), and utilization rate ($P<0.001$).

In women aged 35–40, 175 MII oocytes were retrieved in non-AOA group and 148 in AOA group. There were no significant differences in normal fertilization rate, multi-PN formation rate, cleavage rate, and day 3 good quality embryo rate between AOA group and non-AOA group ($P=0.669$, $P=0.285$, $P=0.109$, $P=0.540$, respectively). Good quality blastocyst formation rate ($P<0.001$) and utilization rate ($P=0.001$) were significantly higher in AOA group than that in non-AOA group.

In women aged ≥40 years, 106 MII oocytes were obtained in non-AOA group and 95 in AOA group. Normal fertilization rate was significantly lower in AOA group ($P=0.007$). There were 3 multi-PN zygotes in AOA group and no multi-PN zygotes were formed in non-AOA group (3.16% vs. 0). No significant difference in cleavage rate and day 3 good quality embryo rate between AOA and non-AOA group was found ($P>0.999$, $P=0.789$, respectively). Nevertheless, AOA improved good quality blastocyst formation rate ($P=0.002$) and utilization rate ($P=0.006$) significantly.

In terms of pregnancy outcomes, significant difference between non-AOA group and AOA group was only observed in subgroup aged <35 years. For this subgroup, 42 embryos were transferred in 26 transfer cycles in non-AOA group and 56 embryos were transferred in 44 transfer cycles in AOA group. More cryo-embryo transfers ($P=0.004$) and blastocyst transfers ($P=0.005$) were carried out in AOA group (Table S3 in Additional File 1). Biochemical pregnancy rate ($P=0.038$), clinical pregnancy rate ($P=0.041$), and ongoing pregnancy rate ($P=0.037$) were statistically higher in AOA group compared with non-AOA group. 3 babies were delivered in non-AOA group and 12 babies in AOA group. No significant difference in live birth rate between non-AOA and AOA group was seen (11.54% vs. 27.27%, $P=0.121$) (Fig. 1). Of note, 3 ongoing pregnancies in AOA cycles (33 weeks, 35 weeks, 39 weeks of gestation, respectively) had not completed clinical follow-up yet when the article was done. There was one early pregnancy loss in non-AOA group and 4 in AOA group, which was not significantly different ($P>0.999$) (Table S5 in Additional File 1). In subgroups aged 35–40 and aged ≥40, no significant difference between non-AOA group and AOA group was observed in terms of biochemical pregnancy rate, clinical pregnancy rate, ongoing pregnancy rate, live birth rate (Fig. 1), as well as pregnancy loss rate (Table S5 in Additional File 1).

Discussion

Calcium ionophore can promote extracellular calcium influx as well as calcium release from intracellular store, which can compensate oocyte- or sperm-borne calcium insufficiency [34]. Improvements in cases of globozoospermia or fertilization failure with the application of calcium ionophores for AOA have been repeatedly demonstrated [22, 35]. However, only few studies explored its effect in cases with embryonic development problems and no consistent conclusions were achieved yet. Clinical use of calcium ionophore in these patients is still not considered as conventional treatment measure. Given the importance of calcium for oocyte activation and embryonic development, we supposed that Ca^{2+} oscillations induced by PLCζ after sperm-ooplasm fusion were far from enough to complete preimplantation embryonic development in patients with previous ICSI failure due to embryo developmental problems. Once intracellular calcium stores have been exhausted, oocytes need exogenous calcium supplement [36].

In this study, we showed that calcimycin treatment significantly improved day 3 good quality embryo rate, good blastocyst formation rate, as well as utilization rate. Calcimycin seemed be able to help embryos step over the developmental arrest point by making up for calcium deficiency. Moreover, advanced age does not limit the application of calcimycin. Meanwhile, calcimycin significantly improved post-implantation development as well as pregnancy outcomes. Importantly, significant improvement of clinical outcomes only observed in women <35 years old. Higher implantation rate after AOA was reported as long as the oocytes were derived from patients younger than 36 years [37]. It is well known that fertility declines with maternal age because of reduced oocyte quality except for premature follicles recruitment, increasing ovulatory disorders, and impaired luteal phase [38]. Intrinsic defect of oocytes from patients with advanced age, such as oocyte

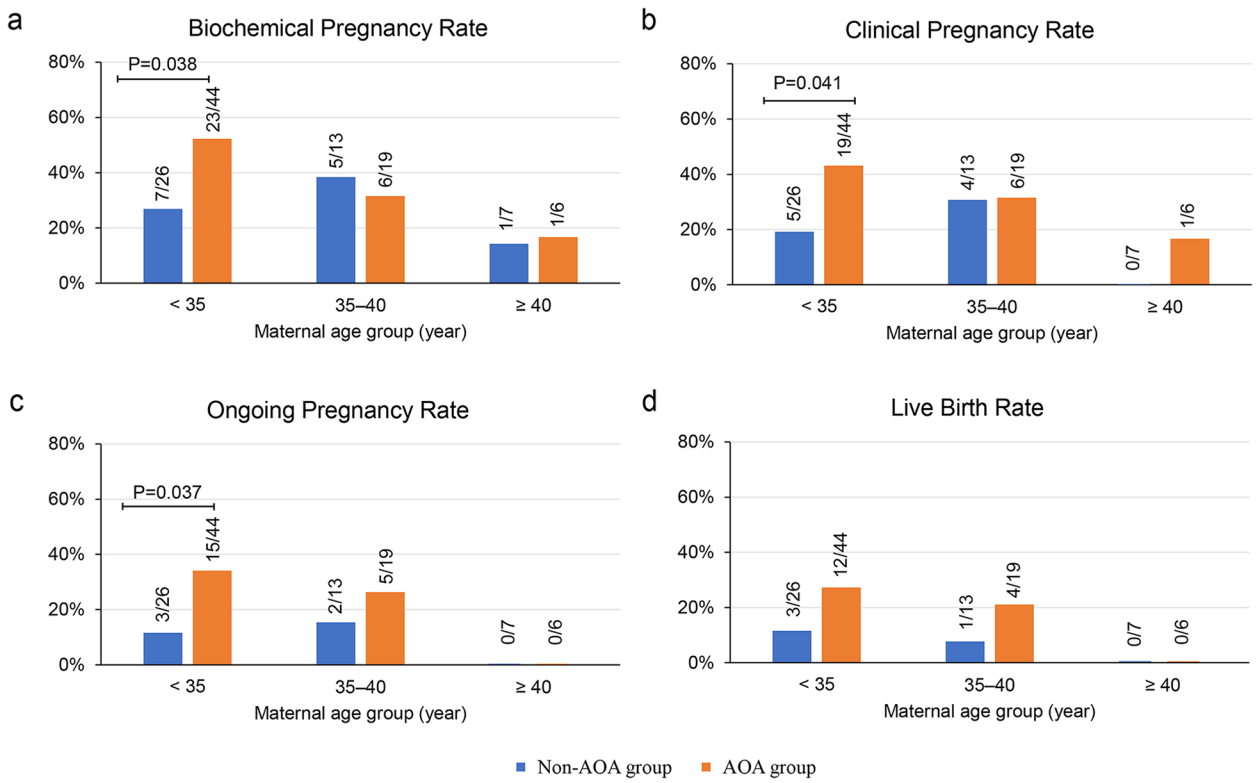


Fig. 1 Comparison of clinical outcomes between non-AOA and AOA group for each subgroup. **a** biochemical pregnancy rate, **b** clinical pregnancy rate, **c** ongoing pregnancy rate, **d** live birth rate. Fractions above bars indicate the proportion of embryo transfers that achieved biochemical pregnancy, clinical pregnancy, ongoing pregnancy or live birth

aneuploidy and mitochondrial dysfunction, instead of oocyte activation failure, is responsible to age-related infertility and may not be able to be rescued by AOA [26]. Besides, current ionophores used in human oocytes can only induce single transient Ca^{2+} shock, unlike Ca^{2+} oscillations in physiologic condition, but their benefits were still significant [21]. Calcium ionophore may activate fully cellular events during oocyte activation which are essential for embryo development by promoting adequate calcium release for oocyte activation. Multiple lines of evidence showed that oocyte activation and subsequent development were results of calcium signal summation [39], and oocytes can be fully activated as long as calcium release reaches a specific threshold [13]. And the molecular effects induced by ionophores are comparable with that in physiologic condition [17]. Oocytes can be tolerant to the change of physiologic calcium pattern to some extent [36]. However, single Ca^{2+} transient induced by AOA is still considered as a limited solution. We suspected that oocytes derived from women of advanced age cannot be tolerant to artificial Ca^{2+} fluctuations as the younger women. Excessive Ca^{2+} after AOA may be even noxious stimuli for aging oocytes.

Interestingly, although AOA significantly improved embryonic development, we noted that normal fertilization rate in AOA group was lower. Subgroup analysis found that the lower normal fertilization rate in AOA group was only present in women aged ≥40 years. The explanation is that female's age grew in AOA cycles compared to their own previous non-AOA cycles, and fertility rapidly declines over 40 years old [40]. Meanwhile, age-related reduction of oocytes retrieved partially contributed to the lower fertilization rate. Some studies reported that less retrieved oocytes are associated to total failed fertilization [41, 42]. However, progressing aging was inevitable in clinical practice when AOA was considered to be used to rescue previous poor embryo quality.

The study on sibling oocytes reported that AOA performed 1 hour after ICSI did not improve embryo and pregnancy outcomes in patients with previous poor embryo development but more than 70% normal fertilization rate [25]. They concluded that patients without fertilization problems might have normal Ca^{2+} signal pattern and normal development. Some studies have emphasized that AOA is not beneficial for all patients

with oocyte activation deficiency [18, 26]. Besides, short of calcium during oocyte activation is not the only reason for poor embryo development. Sperm DNA damage, sperm chromatin abnormalities, oocyte abnormalities in structural proteins and mitochondria, or transcription factors and other regulatory proteins could also lead to poor prognosis of embryo [43, 44], which cannot be rescued by AOA. Maternal age and fertilization rate may act as significant reference indicators for application of AOA in cases with poor embryo development.

The lack of homogeneous AOA protocols impairs the inter-study comparison. We applied calcium ionophore at 15–30 minutes after ICSI in order to mimic natural calcium rise in conventional ICSI mode. Variations in Ca^{2+} response and oocyte activation rate between different AOA protocols have also been mentioned [34]. Double ionophore application were proposed to rescue some patients' developmental problems, which might benefit from simulating a more physiologic calcium signal pattern [36]. Recombinant PLCζ protein can be considered for triggering Ca^{2+} oscillations in oocytes but there are still many limitations on its application, such as low activity, the use of fusion proteins, and unassessed post-implantation development [21]. Determining the scope of ionophore application and standardizing agents, dosage, timing, duration, and times of ionophore exposure are necessary [45].

Our results showed that calcimycin was a safe treatment in terms of pregnancy outcomes and congenital defect, confirming previous safety studies [35, 46]. Follow-up studies of children born following calcium ionophores activation reported reassuring results about neurodevelopmental and language developmental outcomes [47, 48]. But more large-scale prospective studies with long-term follow-up of AOA-born children are still needed.

The study included all day 3 and blastocyst transfers, fresh and frozen embryo transfers to observe the overall condition of clinical outcomes. More blastocysts were formed and frozen in AOA group, which contributed to more frozen embryo transfers. It has been reported that frozen or fresh transfer have no significant effects on clinical outcomes [49], while the effect of embryo stage when transferring is debatable [50–52]. It is commonly accepted that clinical pregnancy rate of blastocyst transfer is significantly higher than that of cleavage-stage embryo transfer [52–54]. There was part of included patients with relative normal day 3 good quality embryo rate in non-AOA cycles but significant lower good quality blastocyst formation rate whose pregnancy outcomes may be improved by early-stage embryo transfer and in vivo early embryo development [55]. Therefore, improved pregnancy outcomes by AOA were only observed in blastocyst transfer cycles in our study. In non-AOA group, embryos marginally developed into blastocyst stage and were transferred or frozen. AOA significantly improved the quality of blastocysts, allowing preferential selection of most optimal grade embryos for transfer that may have higher development potential [56]. In addition, AOA may improve post-implantation development that contributed to more satisfactory pregnancy outcomes. Admittedly, the present study is limited by small sample size and intrinsic deficits of retrospective analysis. We plan to study further with multicenter joint and expanded sample size in the future.

Conclusion

In conclusion, calcimycin treatment is now mainly used for fertilization failure and its application for improving embryonic development is still non-routine. In this study, with a stratified analysis of age, we show for the first time that calcimycin can be applied as a feasible treatment in women aged < 35 years with poor embryo development in previous ICSI cycles.

Abbreviations

ART: assisted reproductive technology; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; PLCζ: phospholipase C zeta; Ca^{2+}: Calcium; AOA: assisted oocyte activation; COH: controlled ovarian hyperstimulation; hCG: human chorionic gonadotropin; PN: pronuclear; BMI: body mass index; AMH: anti-Mullerian hormone..

Supplementary Information

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Additional file 1.

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contribution statements

All authors contributed to the study conception and design. H.Z. and K.W. performed micromanipulations and embryo culture. J.L., M.Z., X.S. and R.C. performed data collection. X.C. and Y.D. analyzed the patient data. X.C. was a major contributor in writing the manuscript. All authors commented on previous versions of the manuscript and approved the final manuscript. B.L. and K.W. had responsibility for the integrity and accuracy of any part of the study, and the final decision to submit the manuscript for publication. The authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

This study complied with the Declaration of Helsinki (World Medical Assembly) and was reviewed and approved by the Ethics Committee of Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University (2021/Ethical Review #01). Informed consent was obtained from all patients before they were included in the retrospective clinical study during ethics review.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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References

1. Saha S, Roy P, Corbitt C, Kakar SS. Application of Stem Cell Therapy for Infertility. Cells. 2021;10(7). https://doi.org/10.3390/cells10071613.
2. Oron G, Son WY, Buckett W, Tulandi T, Holzer H. The association between embryo quality and perinatal outcome of singletons born after single embryo transfers: a pilot study. Hum Reprod. 2014;29(7):1444–51. https://doi.org/10.1093/humrep/deu079.
3. Zhu J, Lian Y, Li M, Chen L, Liu P, Qiao J. Does IVF cleavage stage embryo quality affect pregnancy complications and neonatal outcomes in singleton gestations after double embryo transfers? Journal of assisted reproduction and genetics. 2014;31(12):1635–41. https://doi.org/10.1007/s10815-014-0351-8.
4. Li J, Chen Q, Wang J, Huang G, Ye H. Does growth hormone supplementation improve oocyte competence and IVF outcomes in patients with poor embryonic development? A randomized controlled trial. BMC Pregnancy Childbirth. 2020;20(1):310. https://doi.org/10.1186/s12884-020-03004-9.
5. Liu B, Zhao H, Wu K, Großhans J. Temporal Gradients Controlling Embryonic Cell Cycle. Biology. 2021;10(6). https://doi.org/10.3390/biology10060513.
6. Rogers NT, Halet G, Piao Y, Carroll J, Ko MS, Swann K. The absence of a Ca(2+) signal during mouse egg activation can affect parthenogenetic preimplantation development, gene expression patterns, and blastocyst quality. Reproduction. 2006;132(1):45–57. https://doi.org/10.1530/rep.1.01059.
7. Martinez M, Durban M, Santalo J, Rodriguez A, Vassena R. Assisted oocyte activation effects on the morphokinetic pattern of derived embryos. J of assist rep and gen. 2021;38(2):531–7. https://doi.org/10.1007/s10815-020-02025-9.
8. Ebner T, Montag M. Artificial oocyte activation: evidence for clinical readiness. Rep biomed on. 2016;32(3):271–3. https://doi.org/10.1016/j.rbmo.2015.12.004.
9. Murugesu S, Saso S, Jones BP, Bracewell-Milnes T, Athanasiou T, Mania A, et al. Does the use of calcium ionophore during artificial oocyte activation demonstrate an effect on pregnancy rate? A meta-analysis. Fertil Steril. 2017;108(3):468–82 e3. https://doi.org/10.1016/j.fertnstert.2017.06.029.
10. Williams CJ. Signalling mechanisms of mammalian oocyte activation. Hum Reprod Update. 2002;8(4):313–21. https://doi.org/10.1093/humupd/8.4.313.
11. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Dev (Cambridge, England). 2002;129(15):3533–44. https://doi.org/10.1242/dev.129.15.3533.
12. Nomikos M, Swann K, Lai FA. Starting a new life: sperm PLC-zeta mobilizes the Ca2+ signal that induces egg activation and embryo development: an essential phospholipase C with implications for male infertility. Bioessays. 2012;34(2):126–34. https://doi.org/10.1002/bies.201100127.
13. Ferrer-Buitrago M, Bonte D, De Sutter P, Leybaert L, Heindryckx B. Single Ca(2+) transients vs oscillatory Ca(2+) signaling for assisted oocyte activation: limitations and benefits. Reproduction. 2018;155(2):R105–R19. https://doi.org/10.1530/REP-17-0098.
14. Ben-Yosef D, Shalgi R. Oocyte activation: lessons from human infertility. Trends Mol Med. 2001;7(4):163–9. https://doi.org/10.1016/s1471-4914.
15. Horner VL, Wolfner MF. Transitioning from egg to embryo: triggers and mechanisms of egg activation. Dev Dyn. 2008;237(3):527–44. https://doi.org/10.1002/dvdy.21454.
16. Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. Hum Reprod Update. 2010;16(6):690–703. https://doi.org/10.1093/humupd/dmq018.
17. Ferrer-Buitrago M, Tilleman L, Thys V, Hachem A, Boel A, Van Nieuwerburgh F, et al. Comparative study of preimplantation development following distinct assisted oocyte activation protocols in a PLC-zeta knockout mouse model. Mole Hum Rep. 2020;26(11):801–15. https://doi.org/10.1093/molehr/gaaa060.
18. Vanden Meerschaut F, Nikiforaki D, De Gheselle S, Dullaerts V, Van den Abbeel E, Gerris J, et al. Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. Hum Reprod. 2012;27(7):1977–84. https://doi.org/10.1093/humrep/des097.
19. Bos-Mikich A, Whittingham DG, Jones KT. Meiotic and mitotic Ca2+ oscillations affect cell composition in resulting blastocysts. Dev Biol. 1997;182(1):172–9. https://doi.org/10.1006/dbio.1996.8468.
20. Anifandis G, Michopoulos A, Daponte A, Chatzimeletiou K, Simopoulou M, Messini CI, et al. Artificial oocyte activation: physiological, pathophysiological and ethical aspects. Syst Biol Reprod Med. 2019;65(1):3–11. https://doi.org/10.1080/19396368.2018.1516000.
21. Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. Reprod Biomed On. 2014;28(5):560–71. https://doi.org/10.1016/j.rbmo.2014.01.008.
22. Bonte D, Ferrer-Buitrago M, Dhaenens L, Popovic M, Thys V, De Croo I, et al. Assisted oocyte activation significantly increases fertilization and pregnancy outcome in patients with low and total failed fertilization after intracytoplasmic sperm injection: a 17-year retrospective study. Fertil Steril. 2019;112(2):266–74. https://doi.org/10.1016/j.fertnstert.2019.04.006.
23. Darwish E, Magdi Y. A preliminary report of successful cleavage after calcium ionophore activation at ICSI in cases with previous arrest at the pronuclear stage. Reprod Biomed On. 2015;31(6):799–804. https://doi.org/10.1016/j.rbmo.2015.08.012.
24. Ebner T, Oppelt P, Wober M, Staples P, Mayer RB, Sonnleitner U, et al. Treatment with Ca2+ ionophore improves embryo development and outcome in cases with previous developmental problems: a prospective multicenter study. Hum Reprod. 2015;30(1):97–102. https://doi.org/10.1093/humrep/deu285.
25. Yin M, Li M, Li W, Wu L, Yan Z, Zhao J, et al. Efficacy of artificial oocyte activation in patients with embryo developmental problems: a sibling oocyte control study. Arch Gynecol Obstet. 2021. https://doi.org/10.1007/s00404-021-06329-8.
26. Lv M, Zhang D, He X, Chen B, Li Q, Ding D, et al. Artificial oocyte activation to improve reproductive outcomes in couples with various causes of infertility: a retrospective cohort study. Reprod Biomed On. 2020;40(4):501–9. https://doi.org/10.1016/j.rbmo.2020.01.001.
27. Nakano Y, Shirakawa H, Mitsuhashi N, Kuwabara Y, Miyazaki S. Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. Mole Hum Reprod. 1997;3(12):1087–93. https://doi.org/10.1093/molehr/3.12.1087.
28. Yanagida K, Katayose H, Hirata S, Yazawa H, Hayashi S, Sato A. Influence of sperm immobilization on onset of Ca(2+) oscillations after ICSI. Hum Reprod. 2001;16(1):148–52. https://doi.org/10.1093/humrep/16.1.148.

29. Dozortsev D, Qian C, Ermilov A, Rybouchkin A, De Sutter P, Dhont M. Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm-oocyte interaction. Hum Reprod. 1997;12(12):2792–6. https://doi.org/10.1093/humrep/12.12.2792.
30. Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F. Embryo scoring as a prognostic tool in IVF treatment. Hum Reprod. 1987;2(8):705–8. https://doi.org/10.1093/oxfordjournals.humrep.a136618.
31. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril. 2000;73(6):1155–8. https://doi.org/10.1016/s0015-0282.
32. Yin C, Li LJ, Ma S, Zhao H, Xu L, Li C, et al. Efficiency and safety of laser-assisted hatching on vitrified-warmed blastocyst transfer cycles: a prospective control trial. Lasers Med Sci. 2022;37(3):1931–42. https://doi.org/10.1007/s10103-021-03453-4.
33. Liu H, Zhao H, Yu G, Li M, Ma S, Zhang H, et al. Conventional in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI): which is preferred for advanced age patients with five or fewer oocytes retrieved? Arch Gynecol Obstet. 2018;297(5):1301–6. https://doi.org/10.1007/s00404-018-4696-6.
34. Nikiforaki D, Vanden Meerschaut F, de Roo C, Lu Y, Ferrer-Buitrago M, de Sutter P, et al. Effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. Fertil Steril. 2016;105(3):798–806 e2. https://doi.org/10.1016/j.fertnstert.2015.11.007.
35. Mateizel I, Verheyen G, Van de Velde H, Tournaye H, Belva F. Obstetric and neonatal outcome following ICSI with assisted oocyte activation by calcium ionophore treatment. J of Assist Reprod and Gen. 2018;35(6):1005–10. https://doi.org/10.1007/s10815-018-1124-6.
36. Shebl O, Reiter E, Enengl S, Allerstorfer C, Schappacher-Tilp G, Trautner PS, et al. Double ionophore application in cases with previous failed/low fertilization or poor embryo development. Reprod Biomed On. 2021. https://doi.org/10.1016/j.rbmo.2021.11.008.
37. Borges E Jr, de Almeida Ferreira Braga DP, de Sousa Bonetti TC, Iaconelli A Jr, Franco JG Jr. Artificial oocyte activation using calcium ionophore in ICSI cycles with spermatozoa from different sources. Reprod Biomed On. 2009;18(1):45–52. https://doi.org/10.1016/s1472-6483.
38. Vander Borght M, Wyns C. Fertility and infertility: Definition and epidemiology. Clin Biochem. 2018;62:2–10. https://doi.org/10.1016/j.clinbiochem.2018.03.012.
39. Toth S, Huneau D, Banrezes B, Ozil JP. Egg activation is the result of calcium signal summation in the mouse. Reproduction. 2006;131(1):27–34. https://doi.org/10.1530/rep.1.00764.
40. Female age-related fertility decline. Committee Opinion No. 589. Fertil Steril. 2014;101(3):633–4. https://doi.org/10.1016/j.fertnstert.2013.12.032.
41. Mahutte NG, Arici A. Failed fertilization: is it predictable? Curr Opin in Obst and Gynecol. 2003;15(3):211–8. https://doi.org/10.1097/00001703-200306000-00001.
42. Javed M, Esfandiari N, Casper RF. Failed fertilization after clinical intracytoplasmic sperm injection. Reprod Biomed On. 2010;20(1):56–67. https://doi.org/10.1016/j.rbmo.2009.10.010.
43. Krey LC, Grifo JA. Poor embryo quality: The answer lies (mostly) in the egg. Fertil Steril. 2001;75(3):466–8. https://doi.org/10.1016/s0015-0282(00)01778-7.
44. Bashiri Z, Amidi F, Amiri I, Zandieh Z, Maki CB, Mohammadi F, et al. Male Factors: the Role of Sperm in Preimplantation Embryo Quality. Reprod Sci (Thousand Oaks, Calif). 2021;28(7):1788–811. https://doi.org/10.1007/s43032-020-00334-z.
45. Heytens E, Soleimani R, Lierman S, De Meester S, Gerris J, Dhont M, et al. Effect of ionomycin on oocyte activation and embryo development in mouse. Reprod Biomed On. 2008;17(6):764–71. https://doi.org/10.1016/s1472-6483(10)60403-8.
46. Miller N, Biron-Shental T, Sukenik-Halevy R, Klement AH, Sharony R, Berkovitz A. Oocyte activation by calcium ionophore and congenital birth defects: a retrospective cohort study. Fertil Steril. 2016;106(3):590–6. e2. https://doi.org/10.1016/j.fertnstert.2016.04.025.
47. Vanden Meerschaut F, D'Haeseleer E, Gysels H, Thienpont Y, Dewitte G, Heindryckx B, et al. Neonatal and neurodevelopmental outcome of children aged 3–10 years born following assisted oocyte activation. Reprod Biomed On. 2014;28(1):54–63. https://doi.org/10.1016/j.rbmo.2013.07.013.
48. D'Haeseleer E, Vanden Meerschaut F, Bettens K, Luyten A, Gysels H, Thienpont Y, et al. Language development of children born following intracytoplasmic sperm injection (ICSI) combined with assisted oocyte activation (AOA). Intern J of lang & Commun Dis. 2014;49(6):702–9. https://doi.org/10.1111/1460-6984.12100.
49. Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, et al. Transfer of Fresh versus Frozen Embryos in Ovulatory Women. New Eng J of Med. 2018;378(2):126–36. https://doi.org/10.1056/NEJMoa1705334.
50. Blake DA, Proctor M, Johnson NP. The merits of blastocyst versus cleavage stage embryo transfer: a Cochrane review. Hum Reprod. 2004;19(4):795–807. https://doi.org/10.1093/humrep/deh104.
51. Martins WP, Nastri CO, Rienzi L, van der Poel SZ, Gracia C, Racowsky C. Blastocyst vs cleavage-stage embryo transfer: systematic review and meta-analysis of reproductive outcomes. Ultra in Obst & Gynecol : The offic J of the Intern Soc of Ultrasound in Obst and Gynecol. 2017;49(5):583–91. https://doi.org/10.1002/uog.17327.
52. Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. The Coch Data of System Rev. 2016;6:CD002118. https://doi.org/10.1002/14651858.CD002118.pub5.
53. Clua E, Rodríguez I, Arroyo G, Racca A, Martínez F, Polyzos NP. Blastocyst versus cleavage embryo transfer improves cumulative live birth rates, time and cost in oocyte recipients: a randomized controlled trial. Reprod Biomed On. 2022;44(6):995–1004. https://doi.org/10.1016/j.rbmo.2022.01.001.
54. Eftekhar M, Mohammadi B, Tabibnejad N, Mortazavi LM. Frozen-thawed cleavage stage versus blastocyst stage embryo transfer in high responder patients. Zygote (Cambridge, England). 2020;28(6):511–5. https://doi.org/10.1017/s0967199420000428.
55. Mahdavinezhad F, Kazemi P, Fathalizadeh P, Sarmadi F, Sotoodeh L, Hashemi E, et al. In vitro versus In vivo: Development-, Apoptosis-, and Implantation- Related Gene Expression in Mouse Blastocyst. Iran J of Biotech. 2019;17(1):e2157. https://doi.org/10.21859/ijb.2157.
56. Zhao YY, Yu Y, Zhang XW. Overall Blastocyst Quality, Trophectoderm Grade, and Inner Cell Mass Grade Predict Pregnancy Outcome in Euploid Blastocyst Transfer Cycles. Chi Med J. 2018;131(11):1261–7. https://doi.org/10.4103/0366-6999.232808.

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International Journal of Language & Communication Disorders

Research Report

Language development of children born following intracytoplasmic sperm injection (ICSI) combined with assisted oocyte activation (AOA)

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Abstract

Background

The effect of assisted reproduction technology (ART) on language development is still unclear. Moreover, different techniques are introduced at rapid pace and are not always accompanied by extensive follow-up programmes.

Aims

To investigate the language development of 3–10-year-old children born following ART using intracytoplasmic sperm injection (ICSI) combined with assisted oocyte activation (AOA), which is a highly specialized technique applied in cases with a history of fertilization failure following conventional ICSI. Secondly, a comparison is made between the language development of singletons and twins.

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singletons compared with twins. For the general language, none of the children scored within the clinical zone for language disability corresponding with a percentile lower than 5.

Conclusion & Implications

This study presents the first data concerning language outcome in 3–10-year-old children born following AOA. General language scores of the AOA children in this study are located within the normal ranges. The language development of singletons was significantly better compared with twins. Although the results are reassuring for language development, in future long-term follow-up studies in this population are necessary.

References

Bakker, P., 1987, Autonomous languages of twins. *Acta geneticae medicae et gemellologiae*, **36**(2), 233–238.

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De Graaf-Peters, V. B. and Hadders-Algra, M., 2006, Ontogeny of the human central nervous system: what is happening when?, *Early Human Development*, **82**, 257–266.

View | PubMed | Web of Science® | Google Scholar

Esfandiari, N.,Javed,M. H.,Gotlieb,L. and Casper,R. F., 2005, Complete failed fertilization after intracytoplasmic sperm injection—analysis of 10 years' data. *International Journal of Fertility and Women's Medicine*, **50**, 187–192.

PubMed | Web of Science® | Google Scholar

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Gucuyener, K., Arhan, E., Soysal, A.S., Ergenekon, Z., Turan, O., Onal, E., Koç, E., Turkyilmaz, C., Atalay, Y., 2011, Language assessment of non-handicapped twins at 5 years of age. *Pediatrics International*, **53**(6), 944–949.

View | PubMed | Web of Science® | Google Scholar

Hay, D.A., Prior, M., Collett, S., Williams, M. 1987, Speech and language development in preschool twins. *Acta Genet Med Gemellol (Roma)*, **36**(2), 213–223.

View | CAS | PubMed | Web of Science® | Google Scholar

Heindryckx, B., de Gheselle, S., Gerris, J., Dhont, M. and de Sutter, P., 2008, Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reproductive Biomedicine Online*, **17**, 662–668.

View | PubMed | Web of Science® | Google Scholar

Hendriksen, J. and Hurks, P., 2009, *WPSSI-III-NL Wechsler Preschool and Primary Scale of Intelligence*, 3rd edn (Amsterdam: Pearson Assessment and Information BV).

Google Scholar

Heytens, E., Soleimani, R., Lierman, S., de Meester, S., Gerris, J., Dhont, M., van der Elst, J. and de Sutter, P., 2008, Effect of ionomycin on oocyte activation and embryo development in mouse. *Reproductive Biomedicine Online*, **17**, 764–71.

View | CAS | PubMed | Web of Science® | Google Scholar

Kort, W., Schittekatte, M., Bosmans, M., Compaan, E. L., Dekker, P. H., Vermeir, G. and Verhaeghe, P., 2005, *WISC-III-NL, Wechsler Intelligence Scale for Children. Handleiding en verantwoording*, 3rd edn (Amsterdam: Harcourt Test).

Google Scholar

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Nekkebroeck, J., Bonduelle, M., Desmyttere, S., van den Broeck, W. and Ponjaert-Kristoffersen, I., 2008, Socio-emotional and language development of 2-year-old children born after PGD/PGS and parental well-being. *Human Reproduction*, **23**(8), 1849–1857.

View | PubMed | Web of Science® | Google Scholar

Palermo, G. D., Neri, Q. V., Takeuchi, T and Rosenwaks, Z., 2009, ICSI: where we have been and where we are going? *Seminars Reproductive Medicine*, **27**, 191–201.

View | PubMed | Web of Science® | Google Scholar

Paul, R., 2001, Language disorders from infancy through adolescence. In *Assessment and Intervention* (St. Louis, MO: Mosby).

Google Scholar

Place, I. and Englert, Y., 2003, A prospective longitudinal study of the physical, psychomotor and intellectual development of singleton children up to 5 years who were conceived by intracytoplasmic sperm injection compared with children conceived spontaneously and by *in vitro* fertilization. *Fertility and Sterility*, **80**(6), 1388–1397.

View | PubMed | Web of Science® | Google Scholar

Rutter, M., Thorpe, K., Greenwood, R., Northstone, K. and Golding, J., 2003, Twins as a natural experiment to study the causes of mild language delay: I: design; twin–singleton differences in language and obstetric risks. *Journal of Child Psychology and Psychiatry, and Allied Disciplines*, **44**(3), 326–341.

View | PubMed | Web of Science® | Google Scholar

Sansavini, A., Guarini, A., Justice, L. M., Savini, S., Broccoli, S., Alessandroni, R. and Faldella, G.,

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Journal of Medicine, **346**(10), 731–737.

View | PubMed | Web of Science® | Google Scholar

Semel, E., Wiig, E. H. and Secord, W. A., 2010, *Clinical Evaluation of Language Fundamentals. 4* (Amsterdam: Pearson, Nederlandse versie).

Google Scholar

Squires, J., Carter, A. and Kaplan, P., 2003, Developmental monitoring of children conceived by intracytoplasmic sperm injection and *in vitro* fertilization. *Fertility and Sterility*, **79**(2), 453–454.

View | PubMed | Web of Science® | Google Scholar

Tarlatzis, B. C. and Grimbizis, G., 1999, Pregnancy and child outcome after assisted reproduction techniques. *Human Reproduction*, **14**, 231–243.

View | PubMed | Web of Science® | Google Scholar

Thorpe, K., Rutter, M. and Greenwood, R., 2003, Twins as a natural experiment to study the causes of mild language delay: II: Family interaction risk factors. *Journal of Child Psychology and Psychiatry*, **44**(3), 342–355.

View | PubMed | Web of Science® | Google Scholar

Tomblin, J. B., Records, N. L., Buckwalter, P., Zhang, X., Smith, E. and O'Brien, M., 1997, Prevalence of specific language impairment in kindergarten children. *Journal of Speech Language and Hearing Research*, **40**(6), 1245–1260.

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Wagenaar, K., van Weissenbruch, M. M., Knol, D. L., Cohen-Kettenis, P. T., Delemarre-van de Waal, H. A. and Huisman, J., 2009, Information processing, attention and visual-motor function of adolescents born after *in vitro* fertilization compared with spontaneous conception. *Human Reproduction*, **24**(4), 913–921.

CAS | PubMed | Web of Science® | Google Scholar

Woldringh, G. H., Horvers, M., Janssen, A. J., Reuser, J. J., de Groot, S. A., Steiner, K., d'Hauwers, K. W., Wetzels, A. M. and Kremer, J. A., 2011, Follow-up of children born after ICSI with epididymal spermatozoa. *Human Reproduction*, **26**(7), 1759–1767.

CAS | PubMed | Web of Science® | Google Scholar

Wolke, D. and Meyer, R., 1999, Cognitive status, language attainment, and prereading skills of 6-year-old very preterm children and their peers: the Bavarian Longitudinal Study. *Developmental Medicine Child Neurology*, **41**(2), 94–109.

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COMMENTARY

Artificial oocyte activation: evidence for clinical readiness

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Abstract Artificial oocyte activation using Ca^{2+}ionophores or similar compounds is a widely applied technique in IVF laboratories. This is all the more interesting as most of the agents aiming for intracellular Ca^{2+} increase do not result in physiological Ca^{2+} oscillations but much rather cause a single Ca^{2+} transient. Two observations from mammals may explain why a rather non-physiological single Ca^{2+} peak caused by ionophores is sufficient to rescue cycles showing severe male factor infertility, deficient oocyte maturation, developmental problems in humans, or both. On the one hand, it has been shown that it is mainly the initial Ca^{2+} rise that drives further downstream events, in particular calcium/calmodulin-dependent protein kinase II (CaMKII) action, and on the other, it is possible that this enzyme remains active even in the absence of Ca^{2+}. It therefore seems that mammalian oocytes can respond to a wide range of intracellular Ca^{2+} signals and have a surprisingly high degree of tolerance for changes in cytosolic Ca^{2+}. As epigenetic consequences or differences in gene expression have not been studied to date, artificial oocyte activation has to be considered as experimental and should only be applied with a proper indication.

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KEYWORDS: artificial oocyte activation, ICSI, ionophore

It has been known for decades that periodic changes in internal Ca^{2+} mediate a large range of cell functions involved in oocyte activation and subsequent cell function. The effectiveness of these calcium oscillations depends on their number, frequency, temporal modulation and amplitude.

On the other hand, cytosolic calcium deficiency, regardless of whether the problem is sperm- or oocyte-born, will have a profound effect on cell physiology. Evidence shows that in such cases, fertilization after intracytoplasmic sperm injection (ICSI) (Ebner et al., 2012, 2015a; Montag et al., 2012) and mitotic cleavage (Ebner et al., 2015b) may be severely impaired. After all, it is generally acknowledged that intracellular Ca^{2+} is limited and, once internal stores have been depleted, the oocyte is reliant on influx of extracellular Ca^{2+} to compensate for this loss (Berridge et al., 1998). The AOA technique would facilitate such a mechanism.

As Vanden Meerschaut et al. (2014a) highlighted, AOA, in particular using Ca^{2+}ionophores such as A23187 or ionomycin, is a widely applied technique in human IVF laboratories. The problem with AOA, however, is that all application modes published to date (Vanden Meerschaut et al., 2014a) are all but standardized, which would be a prerequisite for the safe handling of oocytes in clinical use, as well as preventing meaningful comparison between different IVF studies. In general, it is interesting that artificial activation is widely used, as most of the compounds used for AOA do not result in physiological Ca^{2+} oscillations but much rather cause a single Ca^{2+} transient.

Although it is evident that oscillatory Ca^{2+} signalling is the normal stimulus during mammalian fertilization, Ducibella et al. (2003) have highlighted whether periodical changes in Ca^{2+} are indeed necessary for normal development.

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In mammalian fertilization, the spatio–temporal information about internal Ca^{2+} is transduced by downstream factors that mediate oocyte activation and subsequent preimplantation embryonic development. One such effector, CaMKII, has been found to oscillate in temporal synchrony with repetitive Ca^{2+}peaks (Markoulaki et al., 2003). This was demonstrated not only after regular fertilization but also after parthenogenetic activation (Winston and Maro, 1995) and multiple sequential ionophore pulses (Markoulaki et al., 2003). It indicates that artificial Ca^{2+} recruitment results in a proper biological downstream response. Even more interestingly, CaMKII activity obviously rises and falls with the initial Ca^{2+} transient in a directly proportional manner (Markoulaki et al., 2003), thus reflecting the situation commonly found in AOA (presence of a single calcium rise). Although oscillatory Ca^{2+} signalling may be a mechanism to maintain the sensitivity of CaMKII for the duration of Ca^{2+} signalling in regular fertilization (Ducibella et al., 2003), autophosphorylation of CaMKII, once activated, may keep the enzyme active even without the presence of calcium (Johnson et al., 1998); a scenario that would explain how a Ca^{2+} stimulus can be transmitted in the absence of its physiological repetitive pattern.

Moreover, evidence shows that, in humans, any reduction in the frequency of Ca^{2+} peaks can be compensated by both a higher amplitude and a longer total duration of the same, thus increasing the total amount of calcium released (Nikiforaki et al., 2014). This hypothesis is in agreement with mammalian data showing that oocyte activation is tolerant to perturbations in the calcium oscillation pattern as long as the total amount of calcium release is uncompromised and passes a critical threshold (Ozil et al., 2005; Toth et al., 2006). For daily practice, this would mean that an increase in length and number of exposures to ionophore could indeed show a benefit in selected cases (although this contradicts the need for a standardized procedure).

Taken together, it seems that mammalian oocytes can respond to a wide range of intracellular Ca^{2+} signalling parameters (Ducibella et al., 2003) and have a surprisingly high degree of tolerance for prolonged changes in cytosolic Ca^{2+} (Ozil et al., 2005; Toth et al., 2006).

The simple fact, however, that AOA can rescue cycles showing severe male factor infertility, deficient oocyte maturation (Kim et al., 2015), developmental problems, or both, should not mask the non-physiological nature of ionophore treatment. Given the key role of Ca^{2+} oscillations in regular ovum activation, it has been speculated that they are linked to long-term development, possibly via chromatin remodelling and reprogramming of gene expression (Ducibella et al., 2003). The observed failure in the completion of oocyte activation (Vitullo and Ozil, 1992) as well as the impairment in postimplantation viability of parthenogenetically activated mammalian oocytes (Ozil and Huneau, 2001) indicates that defective Ca^{2+} treatment could interfere with epigenetic reprogramming of the genome which, in turn, could result in an altered gene expression pattern (Ozil and Huneau, 2001). This would affect protein synthesis and degradation during the first cell cycle (Kurokawa and Fissore, 2003).

Recently, it became evident that the placenta of offspring born via assisted reproductive technologies has a reduced methylation level at H19 (Nelissen et al., 2013), which might explain the parallel finding that the epigenetic status of children conceived after IVF or ICSI differs from that after spontaneous conception (Whitelaw et al., 2014). The actual extent to which AOA, as one of the upcoming laboratory technologies, might affect DNA methylation is unclear; however, the absence of imprinting disorders in AOA live-births reported so far (Deemeh et al., 2015; Vanden Meerschaut et al., 2014b) suggests that theoretical changes in methylation might not be correlated with the overall transcriptional levels of the associated genes (Rancourt et al., 2012).

Considering the additional fact that most assisted reproduction techniques, such as ICSI, prolonged in-vitro culture or cryopreservation are thought to be related to an altered gene expression (Giritharan et al., 2010; Monzo et al., 2012), it is a realistic scenario that AOA could affect gene expression. It seems, however, to be less than expected. Compared with conventional IVF, the combination of ICSI with a chemical activation step resulted in a gene expression pattern that was found to be closer to IVF than to ICSI alone, suggesting that AOA effectively mimics, at the genetic level, a proportion of the events initiated by sperm entrance (Bridges et al., 2011).

So it is reassuring that the continuous follow-up of children born after AOA revealed that neonatal and neurodevelopmental outcome of children aged between 3 and 10 years is within expected ranges (Vanden Meerschaut et al., 2014b) and so is their language development (D'haeseleer et al., 2014).

Most likely, it is the rather inflationary use of AOA substances that has led some scientists to comment on the imminent scenario of using AOA, particularly Ca^{2+}-ionophores, as a routine procedure (Santella and Dale, 2015; van Blerkom et al., 2015). These authors consider ionophores as still "experimental", which they actually are, and the authors of this comment fully support that notion. A more open-minded commentary dealing with ionophore has stated that "it is defining modifications in treatment strategies that concurrently advances our knowledge of human fertilization and opens new avenues for research into the subcellular complexity of the earliest stages of development" (Albertini, 2015). This reflects the history of IVF, where we have to acknowledge that certain procedures were introduced without full understanding of the underlying mechanism. Moving forward does imply following a line that transfers a procedure from research to experimental to clinical. The history of AOA is probably one of the best examples and is currently in the transition process from experimental to clinical – provided that it is applied with a proper indication. To conclude, there is definitely a need for more prospective studies with a larger number of patients to confirm the observations so far and to further address the safety of AOA.

References

Albertini, D.F., 2015. What we have here is a failure to fertilize: back to basics. J. Assist. Reprod. Genet. doi:10.1007/s10815-015-0514-2.

Berridge, M.J., Bootman, M.D., Lipp, P., 1998. Calcium – a life and death signal. Nature 395, 645–648.

Bridges, P.J., Jeoung, M., Kim, H., Kim, J.H., Lee, D.R., Ko, C., Baker, D.J., 2011. Methodology matters: IVF versus ICSI and embryonic gene expression. Reprod. Biomed. Online 23, 234–244.

Deemeh, M.R., Tavalaee, M., Nasr-Esfahani, M.H., 2015. Health of children born through artificial oocyte activation: a pilot study. Reprod. Sci. 22, 322–328.

D'haeseleer, E., Vanden Meerschaut, F., Bettens, K., Luyten, A., Gysels, H., Thienpont, Y., De Witte, G., Heindryckx, B., Oostra, A., Roeyers, H., Sutter, P.D., van Lierde, K., 2014. Language development of children born following intracytoplasmic sperm injection (ICSI) combined with assisted oocyte activation (AOA). Int. J. Lang. Commun. Disord. 49, 702–709.

Ducibella, T., Schultz, R.M., Ozil, J.P., 2003. Role of calcium signals in early development. Semin. Cell Dev. Biol. 17, 324–332.

Ebner, T., Köster, M., Shebl, O., Moser, M., Van der Ven, H., Tews, G., Montag, M., 2012. Application of a ready-to-use calcium ionophore increases rates of fertilization and pregnancy in severe male factor infertility. Fertil. Steril. 98, 1432–1437.

Ebner, T., Montag, M., Van der Ven, K., Van der Ven, H., Shebl, O., Oppelt, P., Hirchenhain, J., Krüssel, J., Maxrath, B., Gnoth, C., Friol, K., Tigges, J., Wünsch, E., Luckhaus, J., Beerkotte, A., Weiss, D., Grunwald, K., Struller, D., Etien, C., 2015a. Live birth after artificial oocyte activation using a ready-to-use ionophore: a prospective multicentre study. Reprod. Biomed. Online 30, 359–365.

Ebner, T., Oppelt, P., Wöber, M., Staples, P., Mayer, R.B., Sonnleitner, U., Bulfon-Vogl, S., Gruber, I., Haid, A.E., Shebl, O., 2015b. Treatment with Ca2+ ionophore improves embryo development and outcome in cases with previous developmental problems: a prospective multicenter study. Hum. Reprod. 30, 97–102.

Giritharan, G., Li, M.W., Di Sebastiano, F., Esteban, F.J., Horcajadas, J.A., Lloyd, K.C., Donjacour, A., Maltepe, E., Rinaudo, P.F., 2010. Effect of ICSI on gene expression and development of mouse preimplantation embryos. Hum. Reprod. 25, 3012–3024.

Johnson, J., Bierle, B.M., Gallicano, G.I., Capco, D.G., 1998. Calcium/calmodulin-dependent protein kinase II and calmodulin: regulators of the meiotic spindle in mouse eggs. Dev. Biol. 204, 464–477.

Kim, J.W., Yanh, S.H., Yoon, S.H., Kim, S.D., Jung, J.H., Lim, J.H., 2015. Successful pregnancy and delivery after ICSI with artificial oocyte activation by calcium ionophore in in-vitro matured oocytes: a case report. Reprod. Biomed. Online 30, 373–377.

Kurokawa, M., Fissore, R.A., 2003. ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphosphate receptor-1 down-regulation, and embryo development. Mol. Hum. Reprod. 9, 523–533.

Markoulaki, S., Matson, S., Abbott, A.L., Ducibella, T., 2003. Oscillatory CaMKII activity in mouse egg activation. Dev. Biol. 258, 464–474.

Montag, M., Köster, M., van der Ven, K., Bohlen, U., van der Ven, H., 2012. The benefit of artificial oocyte activation is dependent on the fertilization rate in a previous treatment cycle. Reprod. Biomed. Online 24, 521–526.

Monzo, C., Haouzi, D., Roman, K., Assou, S., Dechaud, H., Hamamah, S., 2012. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. Hum. Reprod. 27, 2160–2168.

Nelissen, E.C., Dumoulin, J.C., Daunay, A., Evers, J.L., Tost, J., van Montfoort, A.P., 2013. Placentas from pregnancies conceived by IVF/ICSI have a reduced DNA methylation level at the H19 and MEST differentially methylated regions. Hum. Reprod. 28, 1117–1126.

Nikiforaki, D., Vanden Meerschaut, F., Qian, C., De Croo, I., Lu, Y., Deroo, T., Van den Abbeel, E., Heindryckx, B., De Sutter, P., 2014. Oocyte cryopreservation and in vitro culture affect calcium signalling during human fertilization. Hum. Reprod. 29, 29–40.

Ozil, J.P., Huneau, D., 2001. Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development. Development 128, 917–928.

Ozil, J.P., Markoulaki, S., Toth, S., Matson, S., Banrezes, B., Knott, J.G., Schultz, R.M., Huneau, D., Ducibella, T., 2005. Egg activation events are regulated by the duration of a sustained $[Ca^{2+}]_{cyt}$ signal in the mouse. Dev. Biol. 282, 39–54.

Rancourt, R.C., Harris, H.R., Michels, K.B., 2012. Methylation levels at imprinting control regions are not altered with ovulation induction or in vitro fertilization in a birth cohort. Hum. Reprod. 27, 2208–2216.

Santella, L., Dale, B., 2015. Assisted yes, but where do we draw the line? Reprod. Biomed. Online doi:10.1016/j.rbmo.2015.06.013.

Toth, S., Huneau, D., Banrezes, B., Ozil, J.P., 2006. Egg activation is the result of calcium signal summation in the mouse. Reproduction 131, 1–9.

van Blerkom, J., Cohen, J., Johnson, M., 2015. A plea for caution and more research in the "experimental" use of ionophores in ICSI. Reprod. Biomed. Online 30, 323–324.

Vanden Meerschaut, F., Nikiforaki, D., Heindryckx, B., De Sutter, P., 2014a. Assisted oocyte activation following ICSI fertilization failure. Reprod. Biomed. Online 28, 560–571.

Vanden Meerschaut, F., D'Haeseleer, E., Gysels, H., Thienpont, Y., Dewitte, G., Heindryckx, B., Oostra, A., Roeyers, H., Van Lierde, K., De Sutter, P., 2014b. Neonatal and neurodevelopmental outcome of children aged 3–10 years born following assisted oocyte activation. Reprod. Biomed. Online 28, 54–63.

Vitullo, A.D., Ozil, J.P., 1992. Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. Dev. Biol. 151, 128–136.

Whitelaw, N., Bhattacharya, S., Hoad, G., Horgan, G.W., Hamilton, M., Haggarty, P., 2014. Epigenetic status in the offspring of spontaneous and assisted conception. Hum. Reprod. 29, 1452–1458.

Winston, N., Maro, B., 1995. Calmodulin-dependent protein kinase II is activated transiently in ethanol-stimulated mouse oocytes. Dev. Biol. 170, 350–352.

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and aneuploidy screening using NGS or aCGH (ICSI n=31, average 10.55 embryos per couple and IVF n=100, average 13.02 embryos per couple). Fresh ejaculated sperm samples were used for fertilization in both groups. Patients with poor sperm (<5M/ml and <10% motility) and those with known translocations were excluded from analysis to avoid any influence on ploidy rates. GEE models were used to compare ploidy rates per couple in the IVF and ICSI cohorts, taking into account female age at oocyte retrieval.

RESULTS: ICSI cycles resulted in a significantly higher embryo aneuploidy rate per couple compared to IVF, 79.1% vs. 70.9% respectively (p=0.001) adjusting for female age, which was not significantly different between the two groups (ICSI 36.9 yrs, IVF 38.3 yrs; p=0.13). Aneuploidy rates were not affected by the incidence of banking with no significant difference in the data between those embryos which were frozen-thawed before biopsy and those which were biopsied as part of a fresh cycle. Considering that only euploid embryos were transferred, there was no significant difference in clinical pregnancy rate between each fertilization method (45.7% ICSI vs. 46.1% IVF). However, blastulation rates were significantly higher in euploid IVF embryos (47.7% vs. 23.0%), so utilisation rate improved compared to ICSI which could indicate higher cumulative pregnancy rates in the future.

CONCLUSIONS: Embryo aneuploidy rate (per couple) was significantly higher when oocytes were fertilized by ICSI, compared to those that were fertilized by IVF. ICSI was initially developed to overcome male factor infertility, accounting for 40% of couples seeking ART. However, the proportion of ICSI cycles worldwide is 66%, with some regions almost exclusively doing ICSI. There is currently no evidence that ICSI is beneficial for couples with no underlying male factor. The use of ICSI is increasing, often being utilized even when sperm is suitable for IVF; where embryo selection is based on morphological development alone without PGS, this could unknowingly increase the risk of an aneuploid embryo being transferred, and be a contributing factor to cycle outcome.

Supported by: This study was kindly supported by Westmead Fertility Centre.

P-594 Wednesday, October 10, 2018 6:30 AM

LONG-TERM FOLLOW UP OF THE BABIES BORN FROM ICSI WITH CALCIUM IONOPHORE ACTIVATION.

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OBJECTIVE: Repeated fertilization failures in ICSI are rare but distressful events for patients. Therefore, oocyte activation by calcium (Ca) ionophore following ICSI has been applied to enhance fertilization. However, few follow up studies regarding children born after Ca ionophore have been reported and no statistically significant differences in fetal development at birth were indicated compared to those without artificial oocyte activation. The present study was conducted to clarify whether Ca ionophore activation affected on the babies development until 5 years old.

DESIGN: Retrospective cohort study.

MATERIALS AND METHODS: Development of 24 babies born from ICSI treated with Ca ionophore for repeated fertilization failures from 2004 to 2016 were investigated by sending questionnaires to their parents. Sex retio, gestational age, congenital abnormalities at birth, birth weight and height were compared to the data of national growth survey on preschool children collected by Ministry of Health, Labor and Welfare (MHLW) of Japan. Height, weight and congenital abnormalities diagnosed at the age of 1 and a half, 2, 3 and a half and 5 were also followed up in 6 babies out of 24. Physical development including body weight and height until 5 years old was assessed by comparing to the range between the 97th and 3rd percentile curves of the national growth survey.

RESULTS: The sex ratio (f/m) was 0.6 (15 males and 9 females, respectively). Average gestational age (days) was 272.5±11.9. 21 trisomy was confirmed in one baby. Average birth height (cm) of males and females were 48.35±1.85 and 49.17 ±2.50, respectively. Average birth weight (kg) of males and females were 2.89 ± 0.43 and 2.92 ± 0.42, respectively. Birth height of all babies were plotted between the 97th and 3rd percentile curves of the national growth survey. Birth weight of 20 babies were plotted between the 97th and 3rd percentile curves, but 4 babies were out. Except for one male, height and weight of all 5 babies at the age from 1 to 5 were plotted between the 97th and 3rd percentile curves. There were no significant differences in physical development between the data of babies born from ICSI with Ca ionophore and national average by MHWL. Any abnormalities were not diagnosed until 5 years old.

CONCLUSIONS: The present study demonstrates that oocyte activation with Ca ionophore does not influence physical development of children. However, the safety of Ca ionophore should not be concluded from the present study due to sample size. 21 trisomy was confirmed in one baby. It is known as the result of a chromosome abnormality which occurs in the first meiotic cell cycle and is also known to be caused by chromosome nondisjunction during the early stages of embryo cleavage. Further scrutiny is required to determine the influence of Ca ionophore on the prognosis of babies.

P-595 Wednesday, October 10, 2018 6:30 AM

INVESTIGATION OF FERTILIZATION AND CLINICAL OUTCOMES FOLLOWING ICSI IN PATIENTS WITH PRIMARY CILIARY DYSKINESIA.

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OBJECTIVE: This retrospective study investigated the testicular sperm extraction (TESE)-ICSI outcomes of 9 couples with male Primary Ciliary Dyskinesia (PCD) between September 2013-December 2017 at one fertility clinic. Two pronuclei (2PN) rate, blastocyst development rate, good-quality blastocyst (Grade 3BB and above on day 5 by the Gardner scale) rate, clinical pregnancy rate (CPR), and live birth rate were investigated and compared between embryos derived from motile testicular spermatozoa, immotile testicular spermatozoa and ejaculated sperm.

DESIGN: A retrospective study in a reproductive center.

MATERIALS AND METHODS: TESE was performed on 9 PCD patients following semen analysis. Oocytes for TESE-ICSI were collected over a total of 18 oocyte retrieval cycles. All testicular spermatozoa retrieved were immotile and treated with pentoxifylline. The HOST was conducted on spermatozoa in which stimulating motility was unsuccessful to ensure viable spermatozoa was used for ICSI. All embryos were transferred by single frozen-thawed embryo transfers (FET). All patients involved gave written consent and international review board approval was granted. Statistical significance was calculated by application of the student T-test with a p-value of <0.05.

RESULTS: The mean female and male ages were 32.5±3.8 and 36.2±5.4, respectively. Sperm retrieval rate was 100% (9/9) with 22.2% (2/9) motile sperm retrieval following pentoxifylline treatment. Of the 9 patients who underwent TESE, motile sperm could be confirmed in the ejaculate of 1 patient. No significant differences in TESE-ICSI results using motile testicular spermatozoa, immotile spermatozoa and ejaculated sperm were reported in terms of 2PN rate (71.1%, 65.4%, 73.7%, respectively), blastocyst development rate (37.5%, 23.4%, 53.8%, respectively) and good-quality blastocyst rate (6.3%, 10.9%, 38.5%, respectively). CPR was 27.3% (3/11), 36.4% (4/11) and 50.0% (2/4), respectively, with no significant difference between the groups. Live birth rate using motile sperm, immotile sperm and ejaculated motile sperm were 33.3% (1/3) with 1 couple reporting miscarriage and 1 ongoing pregnancy, 75.0% (3/4), 50.0% (1/2), respectively. There are currently four female infants from testicular spermatozoa and one male infant from ejaculated sperm. No perinatal anomalies were reported.

CONCLUSIONS: Our results indicate sperm derivation does not affect fertilization or clinical outcomes following TESE-ICSI for patients with PCD. We therefore suggest that ejaculated motile sperm is used as first means of treatment, followed by fresh TESE and sequential HOS test following failed attempts.

clinicians, reproductive professionals and researchers." Expert review of molecular diagnostics vol. 19,6 (2019): 443-457. doi:10.1080/14737159.2019.1614916.

Muratori, M et al. "Sperm selection with density gradient centrifugation and swim up: effect on DNA fragmentation in viable spermatozoa." Scientific reports vol. 9,1 7492. 16 May. 2019, doi:10.1038/s41598-019-43981-2.

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7.3

ARTIFICIAL OOCYTE ACTIVATION: A RESCUE APPROACH FOR PATIENTS WITH NO BLASTOCYST FORMATION

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Introduction: Low or absent blastocyst formation remains a major cause of cycle cancellation in IVF, leaving patients without embryos available for transfer. Artificial oocyte activation (AOA) using calcium ionophores can enhance oocyte activation and early embryonic development, yet evidence for its benefit in patients with recurrent poor blastulation remains limited. This study aimed to determine whether AOA improves fertilization, blastocyst formation, and clinical outcomes in patients with a history of low or no blastocyst rate, thereby reducing cycle cancellation and providing new opportunities for embryo transfer.

Method: This prospective paired study analyzed 104 ICSI cycles from 52 patients with <30 % or no blastocyst formation in a previous cycle. Each patient served as her own control, comparing a non-AOA cycle with a subsequent AOA cycle. AOA was performed using Ca^{2+} ionophore (A23187, CultActive, Gynemed) for 15 min post-ICSI. Wilcoxon signed-rank tests compared continuous variables; McNemar test evaluated cancellation rate; Fisher's exact test compared clinical outcomes.

Results: No significant differences were observed between cycles without and with artificial oocyte activation (AOA) regarding the number of retrieved oocytes, mature oocytes, or fertilization rates ($p > 0.05$). In contrast, AOA enhanced blastocyst development, with the blastocyst formation rate increasing from 11.9% to 40.9% ($p < 0.001$). Consequently, the cycle cancellation rate decreased from 65.4% to 11.5% ($p < 0.001$). Importantly, 28 patients who previously had no embryo transfer achieved at least one transfer following AOA. Among embryotransfer cycles, pregnancy (62.1% vs 33.3%, $p = 0.13$), clinical pregnancy (44.8% vs 6.7%; $p = 0.02$), ongoing (37.9% vs 6.7%; $p = 0.04$), and live-birth (37.9% vs 6.7%; $p = 0.04$) rates were all higher after AOA. The miscarriage rate per pregnancy decreased from 80.0% to 38.9% ($p = 0.16$), indicating no increase in early pregnancy loss and suggesting improved embryo developmental competence after AOA.

Conclusion: AOA markedly improved blastocyst formation and reduced cycle cancellation, enabling embryo transfer in most previously non-transferable cases. Post-transfer outcomes, including clinical and ongoing pregnancy and live birth, were significantly higher in AOA cycles, with no increase in miscarriage. AOA may therefore represent a valuable laboratory strategy to enhance developmental competence and clinical outcomes in patients with recurrent poor blastocyst formation.

Disclosures: No external funding or competing interests.

REFERENCES:

Ruan JL, Liang SS, Pan JP, Chen ZQ, Teng XM. Artificial oocyte activation with Ca^{2+} ionophore improves reproductive outcomes in patients with fertilization failure and poor embryo development in previous ICSI cycles. Front Endocrinol (Lausanne). 2023;14:1244507.

Shan Y, Zhao H, Zhao D, Wang J, Cui Y, Bao H. Assisted oocyte activation with calcium ionophore improves pregnancy outcomes and offspring safety in infertile patients: a systematic review and meta-analysis. Front Physiol. 2022;12:751905.

Tsai TE, Lin PH, Lian PF, Li CJ, Vitale SG, Mikuš M, Su WP, Tsai HW, Tsui KH, Lin LT. Artificial oocyte activation may improve embryo quality in older patients with diminished ovarian reserve undergoing IVF-ICSI cycles. J Ovarian Res. 2022;15(1):102.

Karagianni M, Thivaiou L, Oraiopoulou C, Papadopoulou MI, Christoforidis N, Papatheodorou A, Chatziparasidou A. The effect of artificial oocyte activation (AOA) in cases with history of low or no blastocyst rate. Hum Reprod. 2025;40(Suppl 1): deaf097.443.

Sendy F, Hemmings R, Kadoch IJ, Jamal W, Phillips S. The effect of artificial oocyte activation on blastocyst development in patients with low blastocyst rates: a retrospective cohort study. Cureus. 2025;17(8):e90557.

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7.4

EMBRYO ABANDONMENT PATTERNS AND PATIENT DISPOSITION CHOICES WITH AUTOMATED DIGITAL BILLING: A SINGLE-CENTRE STUDY

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Introduction: Advances in assisted reproductive technologies have enabled long-term cryopreservation

	Raw semen	DGC	Zymot	p	Effect size
Sperm concentration (x10^6/mL)	58.37; 12.9 [a] [29.68 – 87.07]	13.26; 3.1 [b] [6.42 – 20.11]	13.70; 3.5 [b] [5.91 – 21.49]	0.002	0.630
Progressive motility (%)	60.73; 3.3 [a] [53.48 – 67.98]	60.18; 8.1 [a] [42.18 – 78.18]	92.46; 1.3 [b] [89.55 – 95.36]	0.001	0.594
Acrosome integrity (%)	83.09; 2.6 [a] [77.40 – 88.80]	55.91; 3.18 [b] [48.83 – 62.99]	68.46; 6.07 [a,b] [54.93 – 81.98]	<0.001	0.555
High mitochondrial activity (%)	68.32; 2.9 [a] [61.93 – 74.70]	77.82; 2.9 [a] [71.43 – 84.21]	93.32; 2.4 [b] [88.11 – 98.52]	<0.001	0.727

CONCLUSIONS: Zymot efficiently selects sperm with intact mitochondria and progressive motility, but highly affects acrosome integrity.

IMPACT STATEMENT: This study adds important information regarding sperm recovered by the Zymot device, showing that embryologists should be aware that the acrosome structure is affected by this method, which could, ultimately, impair oocyte fertilization.

SUPPORT: Sao Paulo Research Foundation (grant # 2019/05879-7).

O-11 11:29 AM Monday, October 16, 2023

SPERM CRYOPRESERVATION IS ASSOCIATED WITH AN INCREASED INCIDENCE OF ABNORMALLY-FERTILIZED EMBRYOS IN *IN VITRO* FERTILIZATION CYCLES (IVF) USING INTRA-CYTOPLASMIC SPERM INJECTION (ICSI).

Alvin To, M.D., M.P.H,[1] Timothy Dunn, M.D.,[2] Anna Claire Reynolds, M.D.,[2] Jean-Claire Dillon, MD,[2] Laurie McKenzie, M.D.,[1] Laura Detti, M.D.[3] [1]Baylor College of Medicine, Dept. of Obstetrics and Gynecology, Division of Reproductive Endocrinology & Infertility, Houston, TX; [2]Houston, TX; [3]Baylor College of Medicine, Houston, TX.

OBJECTIVE: To assess if there is any increased incidence of abnormally-fertilized embryos when using semen cryopreservation for IVF/ICSI cycles.

MATERIALS AND METHODS: A retrospective analysis was performed of all consecutive IVF/ICSI cycles at our academic program from 2018-2021. To evaluate the effect of sperm cryopreservation on the formation of abnormal pronuclei (PN) oocytes, we excluded any cycles where review of the medical record identified a contributing component of male-factor infertility per the doctor of record. We also excluded any cycles performed for oocyte cryopreservation or those that utilized donor oocytes or conventional insemination. In this remaining patient cohort, we evaluated for any relationship of sperm cryopreservation with the occurrence of any abnormally-fertilized embryos; defined as an embryo with either 1 or 3 pronuclei (PN). We did not exclude cycles using cryopreserved donor sperm for the analysis if used in cases without male-factor infertility. Fertilization in all cycles was assessed 18 hours after ICSI by time-lapse imaging to ensure correct pronuclei assessment. For statistical analysis, SPSS software was employed for all analyses. Univariate analyses were performed using *t*-tests for continuous variables, while categorical variables were compared with x^2 and Fisher's exact test where appropriate. For any variables found to be significant on univariate analysis, a generalized linear model was used to control for these variables. For all statistical assessments, $p<0.05$ was considered significant. Odds ratios (OR) were calculated for abnormal PN occurrence, with an adjusted OR developed by controlling for maternal age.

RESULTS: 390 cycles were identified for evaluation, 328 (84.1%) cycles utilizing fresh semen samples and 62 (15.9%) using cryopreserved semen specimens. 43 of the 62 (69.4%) frozen semen samples were donor sperm specimens. 39 of these (90.7%) were utilized by LGBTQ+ couples. 31.7% (104/328) of fresh semen cycles had one or more abnormally-fertilized embryo compared to 45.2% (28/62) of cycles using frozen semen. The OR for any abnormal fertilization event was 1.815, with 95% confidence interval (CI) of (1.048, 3.145; $p<0.05$). After adjusting for maternal age, the OR remained statistically significant at 1.843 (95% CI of 1.056, 3.22; $p<0.05$).

CONCLUSIONS: In the absence of male-factor infertility, utilization of frozen sperm in IVF/ICSI cycles was associated with increased odds of abnormally-fertilized embryos.

IMPACT STATEMENT: Sperm cryopreservation is associated with increased odds of having abnormally-fertilized embryos after ICSI.

SUPPORT: No relevant disclosures

O-12 11:40 AM Monday, October 16, 2023

THE ASSESSMENT OF SPERM RETRIEVAL RATES AND CLINICAL OUTCOMES WITH TESTICULAR SPERM EXTRACTION WITH RESPECT TO THE ETIOLOGY OF AZOOSPERMIA.

Yuka Iwamoto, B.S.,[1] Shimpei Mizuta, M.HS.,[2] Kohei Yamaguchi, M.D., Ph.D.,[2] Takumi Takeuchi, M.D., PH.D.,[3] Hidehiko Matsubayashi, M.D., PH.D.,[2] Tomomoto Ishikawa, M.D., PH.D.[2] [1]Reproduction Clinic Osaka, Osaka-Shi, Osaka, Japan; [2]Reproduction Clinic Osaka, Osaka, Japan; [3]Reproduction Clinic Tokyo, Tokyo, Japan.

OBJECTIVE: Sperm retrieval rates (SRR) and clinical outcomes after intracytoplasmic sperm injection (ICSI) in testicular sperm extraction (TESE) cases in relation to the etiology of azoospermia have not well been investigated yet. Here we report our latest nine-year experience in TESE.

MATERIALS AND METHODS: This study investigated SRR of conventional TESE in obstructive azoospermia (OA) and microdissection TESE in cryptozoospermia and non-obstructive azoospermia (NOA) patients between September 2013 and January 2023 (2217 TESE attempts with 2123 patients). The etiologies of NOA were categorized as unexplained, Klinefelter's syndrome (KS), post chemotherapy, post orchiopexy, and microdeletion of azoospermia factor (AZF) c on the Y chromosome. A total of 944 couples had 2259 TESE-ICSI cycles (257 couples and 610 cycles with OA and 446 couples and 1069 cycles with NOA) were evaluated with respect to fertilization, embryonic development and clinical pregnancy rates (CPR).

RESULTS: SRR of patients with first TESE attempts (47.6%) was significantly higher than that of patients who previously failed sperm retrieval (38.6%) ($P<0.001$). In the first TESE cases, SSRs were 100% (256/256) in OA, 21.4% (160/748) in unexplained NOA, 51.7% (93/180) in KS, 44.1% (37/84) in post chemotherapy, 75.4% (46/61) in post orchiopexy, and 81.8% (45/55) in AZFc microdeletion, respectively. SRR of OA was significantly higher, while that of unexplained NOA was lower than any other groups. Normal fertilization rates in OA (62.6%) and post chemotherapy (59.4%) were significantly higher, but that of AZFc microdelecion (35.7%) was significantly lower than any other groups. Blastocyst development rate and good-quality blastcyst rate in AZFc microdeletion (30.6 and 11.9%) were significantly lower than in OA (52.0% and 23.7%), unexplained NOA (45.7% and 21.0%), post chemotherapy (50.8% and 24.0%), post orchiopexy (45.6% and 17.7%) and KS (42.0% of blastulation).CPRs per embryo transfer were lower in unexplained NOA (33.9%), AZFc microdeletion (31.0%), and post orchiopexy (29.4%) than in OA (41.9%). We have had a total of 719 newborns so far with comparable congenital anomaly rate comparing to those with ejaculated sperm-ICSI.

CONCLUSIONS: The success of sperm recovery, fertilization and pre- and post-implantation development was significantly influenced by the etiology of azoospermia.

IMPACT STATEMENT: Our study emphasizes that the etiology of NOA provides clinically valuable prognostic information to couples considering surgical sperm retrieval.

O-13 11:51 AM Monday, October 16, 2023

USE OF CALCIUM IONOPHORE OOCYTE ACTIVATION POST-ICSI RESULTS IN HIGHER BLASTOCYST FORMATION RATES.

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OBJECTIVE: It is known that sperm-induced calcium oscillations trigger mitochondrial respiration, thus causing adenosine triphosphate release. Decreased or absent calcium oscillations result in arrested embryonic development and impaired cleavage division. To date, the literature has primarily investigated calcium ionophore as a therapeutic for poor fertilization rates, but has not reached consensus regarding improvement in blastocyst formation rates. The purpose of this study is to determine if artificial oocyte activation with calcium ionophore improves blastocyst formation rates in patients with a known history of poor blastocyst formation.

Age Group (years)	Control blast rate	Ca++ blast rate	p-value	Control euploid rate	Ca ++ euploid rate	P-value
<35	81/342 (23%)	144/350 (41%)	<0.05	50/81 (62%)	100/144 (69%)	0.76
35-37	160/651 (24%)	321/822 (39%)	<0.05	70/160 (44%)	182/321 (47%)	<0.05
38-39	135/618 (22%)	333/918 (36%)	<0.05	44/135 (33%)	154/333 (46%)	<0.05
40-42	58/268 (22%)	147/423 (35%)	<0.05	10/58 (17%)	46/147 (31%)	0.06
>42	31/135 (23%)	47/178 (26%)	0.66	5/31 (16%)	6/47 (13%)	0.91

MATERIALS AND METHODS: This was a retrospective cohort study evaluating patients undergoing ICSI at a single private-practice. Patients with <25% blastocyst formation per 2PN rates were offered the option to apply assisted calcium activation in a subsequent cycle. Patients' initial cycles served as controls, and these patients' subsequent cycles using calcium ionophore served as the experimental arm. In cycles using calcium ionophore (Tocris ionomycin, Fisher Scientific), post-ICSI oocytes were exposed to calcium ionophore within an hour of undergoing ICSI. Blastocyst culture was performed as is routine thereafter and blastocyst formation rate was defined as the number of day 5 or 6 blastocysts divided by the number of 2 pronuclei embryos post-fertilization.

RESULTS: 202 patients underwent initial cycles without use of calcium ionophore and then subsequently proceeded with 365 cycles using calcium ionophore. Fertilization was not significantly different between the control and experimental cycles. Total blastocyst formation rates rates were significantly higher in all age groups except for those over 42yo, and euploidy rates were significantly higher in patients aged 35-39.

CONCLUSIONS: Calcium ionophore can improve blastocyst formation rates in patients with a history of poor embryonic development. Further prospective data is needed to evaluate the impact of calcium ionophore on euploidy.

IMPACT STATEMENT: As blastocyst culture has become increasingly the standard, use of calcium ionophore may represent a useful treatment option for patients whose embryos have difficulty progressing to the blastocyst stage.

SUPPORT: No support was received for this study

O-14 12:02 PM Monday, October 16, 2023

BLASTOCYST DIAMETER AND INNER CELL MASS AREA AS EMBRYO PARAMETERSTO PREDICT BLASTOCYST QUALITY AND PREGNANCY RATE.

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OBJECTIVE: To verify whether there is an association between the blastocyst diameter and its inner cell mass (ICM) area with blastocyst embryoscope parameters and pregnancy rates.

MATERIALS AND METHODS: A retrospective observational study was performed in a reproductive medicine center in Brazil. Data was collected from November/2020 to March/2023. A total of 156 single frozen embryo transfers (FET) were evaluated. Inclusion criteria included women less than 40 years old, autologous oocytes only, single pregnancies, no genetic test performed, only blastocyst stage transfers. All embryos went through a time-lapse incubator (EmbryoScope®, Vitrolife®), and received the KidScore. The measurement of the ICM area and the entire embryo diameter were performed just before transfer. The samples were divided into two groups: G1, pregnant versus G2, not pregnant. After that, the entire sample was divided according to Kidscore, with Cutoff in 7. Variables were expressed as mean±standard deviation (SD). Student and chi-square tests were applied; and also Pearson correlation. Statistical significance was defined as $p<0.05$.

RESULTS: The pregnancy rate was 73/156 (46.79%) and the clinical pregnancy rate was 62/156 (39.74%). Comparing clinical pregnancy (G1) versus no pregnancy (G2), the following results were found, respectively: ICM area, μm^2, 2721.55±765.84 vs. 2636.94±829.71, p=0,527; blastocyst diameter, μm, 165.15±19.6 vs. 158.82±23.2, p=0.080 and blastocyst diameter/ICM area, $\mu m/\mu m^2$, 0.06±0.002 vs. 0.066±0.035, p=0.506. When data were compared according to KidScore (<7 versus >7), the following results were found, respectively, pregnancy rate, 43/96 (44,8%) vs. 30/60 (50%) p=0.526; clinical pregnancy rate, 37/96 (38.5%) vs. 25/60 (41.7%), p=0.698; blastocyst diameter, μm, 151.49±19.46 vs. 176.96±16.28, $p<0.001$; ICM area, μm^2, 2688.98±886.50 vs. 2640.88±661.47, p=0.719; blastocyst diameter/ICM area, $\mu m/\mu m^2$, 0.062±0.04 vs. 0.07±0.02, p=0.059. A positive correlation between KidScore and blastocyst diameter was observed ($r=0.567$, $p<0.001$).

CONCLUSIONS: Data obtained by time-lapse has allowed the identification of new variable information about morphokinetics as additional markers for embryo viability and implantation potential. Previous studies reported that blastocyst expansion was a predictor for live birth. In our study, there was no difference regarding blastocyst parameters and pregnancy rate. When the blastocyst diameter was compared between Kidscore values, it was observed to have a higher diameter when Kidscore was >7, also showing a positive correlation between both.

IMPACT STATEMENT: In this report, it was not seen an association between the blastocyst diameter and its ICM area with pregnancy rates. However, a positive correlation was seen between Kidscore and blastocyst diameter. Although there is no statistical difference in the pregnancy rate when the kidscore is higher, or when the blastocyst diameter is larger, our findings can be used in practice as a predictor of good quality embryos. It is believed that a larger sample could increase the pregnancy rate for embryos with higher Kidscore and larger diameter.

ORAL ABSTRACT SESSION: FERTILITY PRESERVATION- INFERTILITY & CANCER

O-15 10:45 AM Monday, October 16, 2023

VARIATIONS IN APPROACHES TO SPERM BANKING IN ADOLESCENT BOYS WITH CANCER: A MIXED METHODS STUDY.

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OBJECTIVE: Despite sperm banking as an established, effective, fertility preservation option, pre-treatment sperm banking rates remain low at many pediatric centers.[1,2,3] This study aimed to identify institutional barriers and facilitators to sperm banking and decision-making for adolescent males with cancer among allied health professionals who had completed Oncofertility-related communication and fertility training.

MATERIALS AND METHODS: This explanatory, sequential, mixed-methods study utilized a survey and two subsequent qualitative focus groups to assess the process of adolescent male sperm banking among former learners of ECHO (Enriching Communication skills for Health professionals in Oncofertility) and ENRICH (Educating Nurses about Reproductive Issues in Cancer Healthcare). The survey examined clinician perspectives and practices surrounding support of sperm banking decision-making. A semi-structured focus group guide included a vignette of an adolescent male newly diagnosed with cancer, followed by questions regarding the sperm banking process, barriers, and facilitators. Descriptive statistics were utilized to report survey data. Focus group transcripts were analyzed using constant comparison.

RESULTS: ECHO/ENRICH alumni (N=108) responded to an online survey (31% social workers; 25% psychologists; 14% nurses; 15% nurse practitioners; 10% other). Most participants (67%) reported they never or rarely used a sperm banking decision tool as they had no access or were not aware of one; 98% reported such tools would be helpful. Focus groups were conducted with 15 participants, who were mostly nurses/nurse practitioners (60%) and social workers (27%). There was great variability between and within sites as

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Neurodevelopmental status of children aged 12 to 60 months conceived with artificial oocyte activation in a Cross-Sectional study

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The potential effect of artificial oocyte activation (AOA) following intracytoplasmic sperm injection (ICSI) on neurodevelopment remains uncertain. Since AOA does not fully replicate the physiological Ca^{2+} oscillations essential for fertilization, concerns persist regarding its safety. This study evaluates neurodevelopmental outcomes in children conceived via ICSI-AOA compared to conventional ICSI. A multicentre, cross-sectional study was conducted at Keio University Hospital and eight affiliated institutions, assessing 158 children (ICSI: *n* = 81, ICSI-AOA: *n* = 77) aged 12–60 months using the Japanese Ages and Stages Questionnaire, 3rd Edition (J-ASQ-3), covering communication, gross motor, fine motor, problem-solving, and personal-social skills. No significant differences were found in J-ASQ-3 subdomain scores across age groups, and fully adjusted models showed no significant differences in children scoring below the monitoring zone. Subgroup analyses compared differences between AOA protocols (A23187: *n* = 59; ionomycin: *n* = 15). AOA protocols were not significantly associated with score variations. Neurodevelopmental outcomes in children conceived via ICSI-AOA were comparable to those conceived via conventional ICSI, suggesting that AOA does not adversely affect early childhood neurodevelopment. These findings support the safety of AOA in assisted reproduction, though further longitudinal research is needed to assess long-term outcomes.

Keywords Intracytoplasmic sperm injection (ICSI), Artificial oocyte activation (AOA), Safety, Ages and stages questionnaires 3rd edition (ASQ-3), Neurodevelopment

Artificial oocyte activation (AOA) supports fertilization by temporarily elevating calcium (Ca^{2+}) concentrations in oocytes and is an effective treatment for oocyte activation deficiency (OAD). The three AOA methods include chemical, electrical, and mechanical stimulations. We previously demonstrated that the live birth rate was significantly higher for couples with fertilization rates < 50% through ICSI-alone in the intracytoplasmic sperm injection (ICSI)-AOA (ICSI-AOA) group (18.0% [57/316]) than in the ICSI-alone group (4.7% [4/85]) across 4,893 oocytes, 198 couples, and 649 oocyte retrieval cycles at 18 Japanese facilities[1]. Recent reports indicate that clinical pregnancy rates were higher for infertile couples with fertilization failure history using ICSI-AOA (41.6%, *n* = 35/84) than using ICSI-alone (8.3%, *n* = 5/60)[2]. Several studies have reported that ICSI-AOA using a

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Ca^{2+} ionophore (A23187 or ionomycin), classified as chemical stimulation, is effective for addressing male factor fertilization failure[3,4].

AOA cannot replicate the normal patterns of calcium oscillation[5]raising concerns about longitudinal neurodevelopment in children[6]. Although a meta-analysis on AOA showed that the risk of adverse obstetric, neonatal, or congenital outcomes in children born via ICSI-AOA is comparable to those born via ICSI-alone (relative risk = 1.27, 95% confidence interval [CI] = 0.70–2.28)[7]aberrant DNA methylation and gene expression changes have been reported in rodent offspring treated with ionomycin[8,9] and in human cleavage-stage embryos exposed to A23187 [10], suggesting that ICSI-AOA poses a potential risk of neurodevelopmental and psychiatric disorders[5,10].

Recent studies have reported aberrant DNA methylation in imprinted genes (e.g., *H19*, *SNRPN)*—associated with Beckwith-Wiedemann, Angelman, and Prader-Willi syndromes—in human embryos following AOA[11]. While these epigenetic changes appear to resolve during later developmental stages, the study included only seven live births after ICSI-AOA, leaving long-term implications uncertain. To date, no phenotypic abnormalities have been documented in children born following AOA; however, existing evidence remains limited by small sample sizes and short follow-up periods. Given the expanding clinical application of AOA, rigorous long-term safety assessments are essential. These should include molecular investigations of neurodevelopment and imprint stability, complemented by extended longitudinal follow-up of children.

A 2020 survey by the Japanese Ministry of Health, Labour, and Welfare reported that 67.3% of assisted reproduction facilities had adopted AOA[12]and it has been covered by insurance since 2022. In the United Kingdom, the Human Fertilization and Embryology Authority (HFEA) removed AOA from its list of treatment add-on interventions lacking sufficient evidence of effectiveness in 2023 due to growing evidence supporting its efficacy and classification as a clinical-grade procedure[13]. The HFEA specified that AOA use should be cautiously limited to cases with specific clinical indications, including OAD, fertilization rates < 30%, or cephalic round globozoospermia[14]. Additionally, the European Society of Human Reproduction and Embryology Special Interest Group has advised caution regarding ICSI-AOA, avoiding the use of the term "recommendation"[14] due to insufficient evidence regarding its safety.

Therefore, we aimed to investigate the impact of ICSI-AOA on paediatric neurodevelopment. A cross-sectional design was employed, involving Keio University and eight affiliated institutions under the Japanese Institute for Standardization of Assisted Reproductive Technology (JISART), supported by Tokyo Medical University and the University of Tokyo. Neurodevelopmental assessment was conducted using the Japanese version of the Ages and Stages Questionnaires, 3rd Edition (J-ASQ-3), which evaluates communication, gross motor, fine motor, problem-solving, and personal-social skills.

Results

Cohort characteristics

Overall, 158 cases were analysed, among which 81 and 77 were in the ICSI-alone and ICSI-AOA groups, respectively, with one twin pregnancy in the ICSI-AOA group. The median maternal age at delivery (ICSI, 35.0 [interquartile range = 32.0–38.0] vs. ICSI-AOA, 36.0 [33.0–39.0] years) and paternal age (ICSI, 38.0 [34.5–41.0] vs. ICSI-AOA, 37.0 [33.0–40.0] years), the proportion of mothers employed as office workers (ICSI, 17.3% vs. ICSI-AOA, 28.9%), and maternal educational background were comparable (Table 1). Household income, which was categorized into five ranges from < 3 to > 10 million yen, did not differ significantly between the groups. Fathers were significantly more likely to be employed as teachers in the ICSI-AOA group (9.2%) than in the ICSI-alone group (1.2%).

We examined the influence of sibling presence and social engagement on neurodevelopment by analysing singleton birth rates, sibling composition, and levels of social engagement between the groups. The singleton birth rates were 98.7% and 100% in the ICSI-AOA and ICSI-alone groups, respectively, showing comparable rates. No significant differences in sibling composition were observed between the groups. The proportion of children participating in social activities was similar between the ICSI-AOA (77.9%) and ICSI-alone (85.2%) groups (Table 1).

J-ASQ-3 evaluation between ages 12 and 60 months

All 81 and 77 children in the ICSI-alone and ICSI-AOA groups, respectively, completed the J-ASQ-3 questionnaire without any missing responses. The participants were categorized into nine groups based on age, each completing the corresponding age-appropriate J-ASQ-3 questionnaire. Specifically, the ICSI-alone and ICSI-AOA groups showed no significant differences in any of the five J-ASQ-3 subdomain scores—communication, gross motor, fine motor, problem-solving, or personal-social skills—across all age groups (Fig. 1). Among the 45 fully adjusted mean differences evaluated, a single *P*-value (the personal-social skills score at 48 months) reached the threshold for statistical significance between the ICSI-AOA and ICSI-alone groups (adjusted mean difference = 7.76; 95% CI = 0.47–15.05; $P = 0.038$) (Table 2). However, Q-Q plot analysis demonstrated that this value was well within the expected null distribution, indicating no evidence of systematic deviation or a true effect (Supplementary Fig. S1). This result underscores the observed data consistency with the expected random variation, reinforcing the findings' overall reliability. Subsequently, the number of cases below the cutoff and within the monitoring zone for the five J-ASQ-3 subdomain scores were compared, revealing no significant developmental differences in the five subdomains between the ICSI-alone and ICSI-AOA groups (Table 3).

J-ASQ-3 evaluation between A23187 and ionophore

Each AOA method poses distinct neurodevelopmental risks. Therefore, we evaluate the J-ASQ-3 scores associated with each reagent used for AOA. The AOA protocol comprised 59, 15, and 3 cases treated with A23187, ionomycin, and electrical stimulation, respectively. No mechanical stimulation, $SrCl_2$, or phospholipase

Variable	ICSI	ICSI-AOA	*P*-value
No.	81	76	
Maternal characteristics			
Age at delivery, median (IQR), y	35.0 (32.0–38.0)	36.0 (33.0–39.0)	0.14
Educational level (%)			
High school dropout	2 (2.5)	0 (0)	0.50
High school graduate or vocational school graduate	29 (35.8)	26 (34.2)	0.87
College dropout	1 (1.2)	1 (1.3)	>0.99
College graduate	43 (53.1)	43 (56.6)	0.87
Graduate school degree holder	3 (3.7)	6 (7.9)	0.32
Declined or unknown	3 (3.7)	0 (0)	0.25
Profession (%)			
Healthcare worker	19 (23.5)	12 (15.8)	0.24
Office worker	14 (17.3)	22 (28.9)	0.09
Teacher	7 (8.6)	8 (10.5)	0.79
Public servant	3 (3.7)	2 (2.6)	>0.99
Manufacturing worker	1 (1.2)	2 (2.6)	0.61
Agriculture	2 (2.5)	0 (0)	0.50
Business/store owner	1 (1.2)	2 (2.6)	0.61
Service provider	12 (14.8)	5 (6.6)	0.13
Housewife	20 (24.7)	23 (30.3)	0.48
Declined or unknown	2 (2.5)	0 (0)	0.50
Paternal characteristics			
Age at delivery, median (IQR), y	38.0 (34.5–41.0)	37.0 (33.0–40.0)	0.55
Educational level (%)			
High school dropout	0 (0)	0 (0)	>0.99
High school graduate or vocational school graduate	30 (37.0)	25 (32.9)	0.62
College dropout	1 (1.2)	3 (3.9)	0.36
College graduate	42 (51.9)	39 (51.3)	>0.99
Graduate school degree holder	6 (7.4)	9 (11.8)	0.42
Declined or unknown	2 (2.5)	0 (0)	0.50
Profession (%)			
Healthcare worker	9 (11.1)	4 (5.3)	0.25
Office worker	31 (38.3)	32 (42.1)	0.63
Teacher	1 (1.2)	7 (9.2)	0.03
Public servant	9 (11.1)	3 (3.9)	0.13
Manufacturing worker	9 (11.1)	9 (11.8)	>0.99
Agriculture	0 (0)	1 (1.2)	0.48
Business/store owner	10 (12.3)	7 (9.2)	0.61
Service provider	10 (12.3)	9 (11.8)	>0.99
Househusband	0 (0)	2 (2.6)	0.23
Declined or unknown	2 (2.5)	2 (2.6)	>0.99
Family income (%)			
<3 million yen/year	1 (1.2)	1 (1.3)	>0.99
3 million yen/year≤, <5 million yen/year	7 (8.6)	11 (14.5)	0.32
5 million yen/year≤, <7 million yen/year	24 (29.6)	17 (22.4)	0.36
7 million yen/year≤, <10 million yen/year	30 (37.0)	25 (32.9)	0.62
10 million yen/year≤	16 (19.8)	22 (28.9)	0.20
Declined or unknown	3 (3.7)	0 (0)	0.25
Children characteristics			
Obstetrical complications (%)			
GDM	3 (3.7)	2 (2.6)	>0.99
HDP	3 (3.7)	4 (5.3)	0.71
Placenta previa	3 (3.7)	1 (1.3)	0.62
Vaginal delivery	48 (59.3)	45 (58.4)	0.92
Gestational age at birth, median (IQR)	39.0 (38.0–40.0)	39.0 (37.0–40.0)	0.79
Preterm ≤ 36 weeks (%)	2 (3.7)	5 (6.5)	
Continued			

Variable	ICSI	ICSI-AOA	*P*-value
Full-term 37–41 weeks (%)	79 (97.5)	72 (93.5)	
Post-term ≥ 42 weeks (%)	0 (0)	0 (0)	
Birth weight, median (IQR)	3120.0 (2928.0-3418.0)	3045.0 (2776.0-3324.0)	0.19
<2,500 g (%)	5 (6.2)	12 (15.6)	
2,501–2,999 g (%)	26 (32.1)	22 (28.6)	
3,000–3,499 g (%)	37 (45.7)	31 (40.3)	
3,500–3,999 g (%)	10 (12.3)	11 (14.3)	
≥4,000 g (%)	3 (3.7)	1 (1.3)	
Sex (%)			
Female	42 (51.9)	38 (49.4)	0.87
Male	39 (48.1)	39 (50.6)	
Number of the offspring (%)			
Singleton	81 (100.0)	75 (98.7)	0.48
Twin	0 (0)	1 (1.3)	
Sibling composition (%)			
Yes	47 (58.0)	41 (53.2)	0.43
No	32 (39.5)	36 (46.8)	
Declined or unknown	2 (2.5)	0 (0)	
Use of group childcare services (e.g., preschools or daycare)			
Yes	69 (85.2)	60 (77.9)	0.12
No	10 (12.3)	17 (22.1)	
Declined or unknown	2 (2.5)	0 (0)	

Table 1. Parental and children Characteristics. *IQR* interquartile range, *ICSI* intracytoplasmic sperm injection, *AOA* artificial oocyte activation, *y* years, *GDM* gestational diabetes mellitus, *HDP* hypertensive disorders of pregnancy.

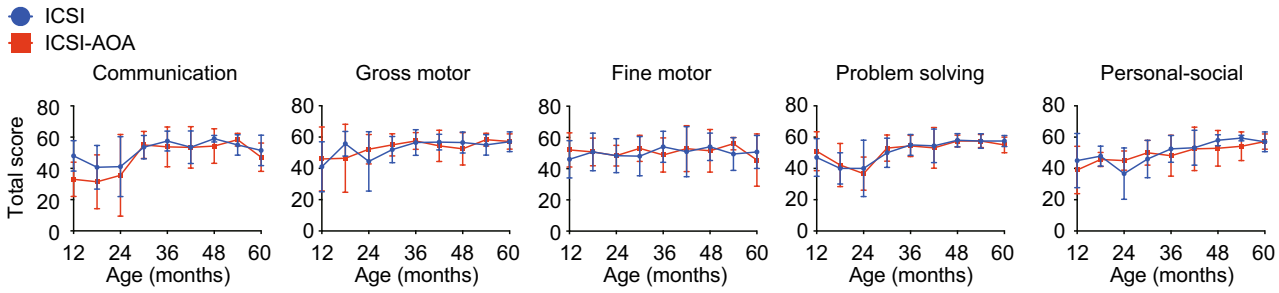


Fig. 1. J-ASQ-3 cores at age 12–60 months | Total raw scores on each J-ASQ-3 domain (communication, gross motor, fine motor, problem-solving, and personal-social) for children born via ICSI alone and ICSI-AOA at 12, 18, 24, 30, 36, 42, 48, 54, and 60 months of age. No significant group differences are found in any of the J-ASQ-3 domains (**A**–**E**). Error bars represent 95% confidence intervals, indicating variation within the sample. *J-ASQ-3* Japanese version of the Ages and Stages Questionnaire 3rd Edition, *ICSI* intracytoplasmic sperm injection, *AOA* artificial oocyte activation.

C zeta (PLCζ) cases were reported (Supplementary Table S1). The analysis showed no significant differences in any of the five J-ASQ-3 subdomain scores across all age categories between the ionomycin and A23187 groups (Fig. 2). Similarly, the number of cases below the cutoff and within the monitoring zone for the five J-ASQ-3 subdomain scores was similar between the A23187 and ionomycin groups.

Validation of risk factors in children scoring below the monitoring zone on the J-ASQ-3

Identifying factors associated with neurodevelopmental delays is essential for recognizing at-risk children and enabling timely interventions. We identified populations at high risk of developmental delay requiring evaluation or follow-up within each subdomain. Children scoring below the monitoring zone on the J-ASQ-3 (Supplementary Fig. S2) were identified, and their backgrounds, including maternal and paternal age, child's sex, presence of siblings, group living status, gestational age at delivery, birth weight, and delivery mode, were compared with those of children scoring within the normal range. Among these factors, maternal age, sibling composition, and child's sex were significantly associated with scores below the monitoring zone: higher

Variable	ICSI-alone	ICSI-AOA	Mean difference (95% CI)	*P*-value	Adjusted[b] mean difference (95% CI)	*P*-value
No.	81	77				
J-ASQ-3 score, mean (SD)[a]						
Age 12 months						
No.	4 (male: 3, female: 1)	5 (male: 4, female: 1)				
Communication	48.8 (11.1)	33.0 (11.0)	6.70 (-11.21 to 24.60)	0.43	10.42 (-7.28 to 28.12)	0.18
Gross motor	40.0 (18.3)	46.0 (20.4)	7.95 (-9.89 to 25.78)	0.35	-4.35 (-51.52 to 42.81)	0.81
Fine motor	47.5 (11.0)	52.0 (11.0)	-5.71 (-23.18 to 11.75)	0.49	-8.01 (-30.81 to 14.80)	0.48
Problem-solving	43.8 (11.1)	51.0 (12.5)	-4.02 (-18.19 to 10.16)	0.55	-11.71 (-35.31 to 11.89)	0.24
Personal-social	45.0 (17.3)	39.0 (15.2)	-1.34 (-11.36 to 8.68)	0.78	-0.17(-25.58 to 25.24)	0.99
Age 18 months						
No.	8 (male: 3, female: 5)	7 (male: 2, female: 5)				
Communication	38.1 (14.9)	31.4 (17.3)	6.70 (-11.21 to 24.60)	0.43	7.74 (-12.47 to 27.94)	0.41
Gross motor	54.4 (8.2)	46.4 (21.7)	7.95 (-9.89 to 25.78)	0.35	8.97 (-11.32 to 29.26)	0.35
Fine motor	45.0 (19.6)	50.7 (8.9)	-5.71 (-23.18 to 11.75)	0.49	-7.00 (-25.23 to 11.22)	0.41
Problem-solving	38.1 (11.6)	42.1 (13.8)	-4.02 (-18.19 to 10.16)	0.55	-4.23. (-19.53 to 11.08)	0.55
Personal-social	44.4 (11.5)	45.7 (4.5)	-1.34 (-11.36 to 8.68)	0.78	-1.81 (-11.60 to 7.99)	0.69
Age 24 months						
No.	8 (male: 3, female: 5)	9 (male: 4, female: 5)				
Communication	46.3 (12.2)	35.6 (26.1)	10.69 (-10.57 to 31.96)	0.29	14.17 (-8.92 to 37.26)	0.21
Gross motor	49.4 (12.7)	52.2 (9.4)	-2.85 (-14.28 to 8.58)	0.60	-0.99 (-12.74 to 10.76)	0.86
Fine motor	50.6 (9.0)	48.3 (6.6)	2.29 (-5.82 to 10.41)	0.56	3.69 (-5.84 to 13.22)	0.42
Problem-solving	44.4 (13.2)	36.7 (10.6)	7.71 (-4.61 to 20.03)	0.20	10.34 (-1.63 to 22.31)	0.084
Personal-social	40.6 (12.1)	45.0 (6.1)	-4.38 (-14.91 to 6.16)	0.38	-2.49 (-12.45 to 7.47)	0.60
Age 30 months						
No.	6 (male: 5, female: 1)	5 (male: 3, female: 2)				
Communication	54.2 (8.0)	55.0 (7.1)	3.75 (-6.00 to 13.49)	0.43	-0.85 (-15.38 to 13.67)	0.89
Gross motor	53.3 (8.2)	55.0 (7.1)	-1.00 (-8.12 to 6.12)	0.77	-2.10 (-15.04 to 10.85)	0.71
Fine motor	50.0 (12.2)	53.0 (8.4)	5.25 (-5.20 to 15.70)	0.30	-1.26 (-17.47 to 14.95)	0.86
Problem-solving	51.7 (9.3)	53.0 (8.4)	0.63 (-6.13 to 7.38)	0.85	-0.45 (-16.35 to 15.44)	0.95
Personal-social	48.3 (12.1)	50.0 (7.9)	4.38 (-6.47 to 15.22)	0.40	-4.24 (-21.94 to 13.45)	0.58
Age 36 months						
No.	10 (male: 6, female: 4)	8 (male: 4, female: 4)				
Communication	57.5 (6.4)	53.8 (12.8)	3.75 (-5.99 to 13.49)	0.43	3.64 (-7.53 to 14.81)	0.49
Gross motor	56.5 (8.2)	57.5 (5.4)	-1.0 (-8.12 to 6.12)	0.77	2.78 (-4.60 to 10.15)	0.43
Fine motor	54.0 (9.9)	48.8 (10.9)	5.25 (-5.20 to 15.70)	0.30	7.59 (-4.33 to 19.51)	0.19
Problem-solving	55.0 (6.7)	54.4 (6.8)	0.63 (-6.13 to 7.38)	0.85	1.65 (-4.44 to 7.74)	0.57
Personal-social	52.5 (8.6)	48.1 (13.1)	4.38 (-6.47 to 15.22)	0.41	2.62 (-8.04 to 13.28)	0.60
Age 42 months						
No.	16 (male: 6, female: 10)	16 (male: 8, female: 8)				
Communication	55.7 (6.0)	53.4 (13.3)	2.19 (-5.25 to 9.62)	0.55	2.01 (-6.06 to 10.08)	0.61
Gross motor	57.5 (4.1)	54.4 (10.0)	3.13 (-2.50 to 8.75)	0.26	2.96 (-3.04 to 8.95)	0.32
Fine motor	53.8 (11.2)	53.1 (13.8)	0.63 (-8.43 to 9.68)	0.89	-0.046 (-9.87 to 9.78)	0.99
Problem-solving	56.6 (6.3)	53.1 (13.0)	3.44 (-4.06 to 10.94)	0.35	3.47 (-4.52 to 11.46)	0.38
Personal-social	55.3 (7.4)	52.5 (13.9)	2.81 (-5.23 to 10.86)	0.48	3.20 (-5.03 to 11.43)	0.43
Age 48 months						
No.	11 (male: 8, female: 3)	14 (male: 7, female: 7)				
Communication	59.1 (2.0)	54.3 (11.1)	4.81 (-1.67 to 11.28)	0.13	5.30 (-2.65 to 13.23)	0.18
Gross motor	56.8 (6.4)	52.5 (10.3)	4.32 (-3.06 to 11.69)	0.24	5.93 (-1.73 to 13.59)	0.12
Fine motor	54.6 (8.5)	51.4 (13.6)	3.12 (-6.63 to 12.86)	0.52	3.21 (-6.40 to 12.83)	0.49
Problem-solving	58.2 (4.0)	57.5 (3.8)	0.68 (-2.58 to 3.94)	0.67	1.26 (-1.73 to 4.25)	0.39
Personal-social	58.2 (3.4)	52.9 (11.4)	5.33 (-1.48 to 12.13)	0.12	7.76 (0.47 to 15.05)	0.038
Age 54 months						
No.	8 (male: 2, female: 6)	6 (male: 3, female: 3)				
Communication	60.0 (0.0)	60.0 (0.0)	NA	NA	NA	NA
Gross motor	55.0 (6.6)	58.3 (4.1)	-3.33 (-9.98 to 3.32)	0.37	-4.59 (-11.11 to 1.94)	0.15
Continued						

Variable	ICSI-alone	ICSI-AOA	Mean difference (95% CI)	*P*-value	Adjusted[b] mean difference (95% CI)	*P*-value
Fine motor	49.4 (10.5)	55.0 (4.5)	-5.65 (-14.85 to 3.60)	0.83	-6.71 (-16.56 to 3.13)	0.16
Problem-solving	57.5 (4.6)	57.5 (4.2)	0.00 (-5.24 to 5.24)	0.24	-1.05 (-6.50 to 4.41)	0.68
Personal-social	59.4 (1.8)	54.2 (9.2)	5.21 (-4.40 to 14.82)	0.96	5.10 (-2.64 to 12.84)	0.17
Age 60 months						
No.	10 (male: 4, female: 6)	7 (male: 4, female: 3)				
Communication	51.5 (9.7)	47.1 (9.1)	4.36 (-5.59 to 14.31)	0.37	3.12 (-10.17 to 16.41)	0.62
Gross motor	50.5 (9.8)	51.4 (6.3)	-0.93 (-9.96 to 8.10)	0.83	-6.79 (-17.57 to 4.00)	0.20
Fine motor	51.5 (9.4)	42.9 (19.5)	8.64 (-6.45 to 23.73)	0.24	1.66 (-18.48 to 21.79)	0.86
Problem-solving	58.0 (3.5)	55.0 (5.0)	3.00 (-1.37 to 7.37)	0.16	0.13 (-5.31 to 5.56)	0.96
Personal-social	57.0 (6.3)	57.1 (4.9)	-0.14 (-6.23 to 5.94)	0.96	0.56 (-6.71 to 7.83)	0.87

Table 2. Comparison of the J-ASQ-3 scores between the ICSI-alone and ICSI-AOA Groups. *J-ASQ-3* Japanese Ages and Stages Questionnaire, 3rd Edition, *ICSI* intracytoplasmic sperm injection, *AOA* artificial oocyte activation, *NA* not applicable, *SD* standard deviation. [a]J-ASQ-3 scores in each subdomain can range from 0 to 60, with lower and higher scores indicating greater delay and competence, respectively. [b]The adjusted models include the use of AOA, gestational age at birth, children sex, and age at assessment.

Variable	ICSI-alone	ICSI-AOA	Odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)	*P*-value
No.	81	77			
Below cutoff, No (%)					
Communication	1 (1.2)	7 (9.1)	8.00 (0.96–66.63)	8.85 (0.96–75.78)	0.054
Gross motor	3 (3.7)	5 (6.5)	1.81 (0.42–7.82)	1.75 (0.40–7.73)	0.46
Fine motor	4 (4.9)	4 (5.2)	1.06 (0.25–4.37)	1.05 (0.25–4.40)	0.94
Problem-solving	2 (2.5)	3 (3.9)	0.61 (0.26–9.85)	1.56 (0.24–9.92)	0.64
Personal-social	3 (3.7)	6 (7.8)	2.20 (0.53–9.12)	2.16 (0.51–9.05)	0.29
Any domain	7 (8.6)	12 (15.6)	1.95 (0.73–5.25)	1.92 (0.70–5.26)	0.20
Below monitoring, No (%)					
Communication	6 (7.4)	14 (18.2)	2.78 (1.01–7.65)	2.82 (1.00–8.00)	0.051
Gross motor	16 (19.8)	16 (20.8)	1.07 (0.49–2.32)	1.02 (0.46–2.29)	0.96
Fine motor	12 (14.8)	8 (10.4)	0.67 (0.26–1.73)	0.64 (0.25–1.69)	0.37
Problem-solving	12 (14.8)	10 (13.0)	0.86 (0.35–2.12)	0.80 (0.31–2.06)	0.65
Personal-social	11 (13.6)	12 (15.6)	1.18 (0.49–2.85)	1.13 (0.45–2.82)	0.8
Any domain	28 (34.6)	28 (36.4)	1.08 (0.56–2.08)	1.04 (0.52–0.21)	0.91

Table 3. Comparison of the numbers below cutoff and in monitoring zones based on the J-ASQ-3 scores between the ICSI-alone and ICSI-AOA Groups. *NA* not applicable, *J-ASQ-3* Japanese Ages and Stages Questionnaire, 3rd Edition, *ICSI* intracytoplasmic sperm injection, *AOA* artificial oocyte activation. [a]The adjusted models include the use of AOA, gestational age at birth, children sex, and children age at assessment.

maternal age (35.0 [32.0–38.0] vs. 37.0 [33.0–39.8] years; OR = 1.10 [1.01–1.20], $P = 0.037$), absence of siblings (OR = 4.00 [0.20–0.78], $P = 0.007$), and male sex (OR = 2.57 [1.31–5.05], $P = 0.006$) (Supplementary Table S2).

Discussion

This is the first multicentre cross-sectional study to evaluate the long-term safety of ICSI, followed by AOA on neurodevelopment in a large cohort of children. Our findings suggest that the neurodevelopmental safety of ICSI-AOA is comparable to that of ICSI-alone in terms of social and motor development in 12–60 months. Analysis of scores below the cutoff and within the monitoring zone revealed no significant differences across all subdomains. No significant differences in the J-ASQ-3 scores were observed between the AOA methods using A23187 and ionomycin, suggesting that variations in the AOA techniques do not adversely affect neurodevelopmental outcomes.

Previous studies evaluating the language abilities of children aged 3–10 years conceived via ICSI-AOA, using the Reynell Developmental Language Scales, found that 4 of the 20 children scored below the 10th percentile in specific subdomains[15]. The analysis of children scoring below the cutoff or requiring monitoring revealed no significant differences, suggesting that AOA does not negatively impact communication. Key methodological differences likely account for the discrepancies in these findings. A previous study assessed a population with a high proportion of twins, conducted evaluations over an extended single-day period, and tested children in an unfamiliar environment with unfamiliar individuals present[15]. However, our study used parent-reported

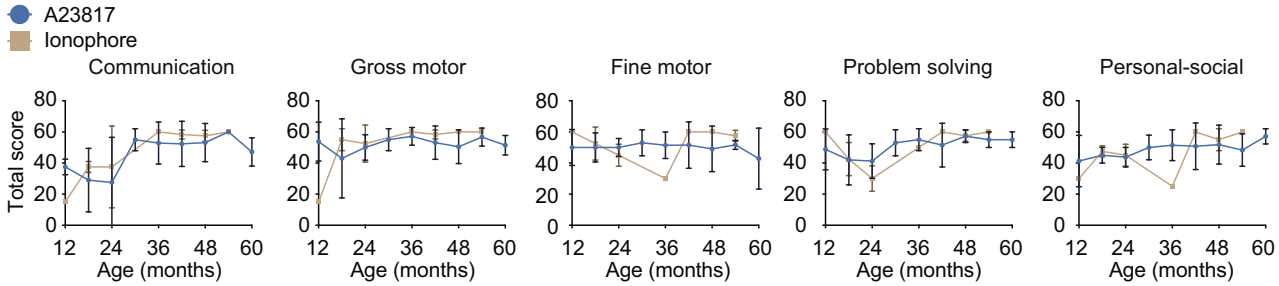


Fig. 2. J-ASQ-3 scores for comparing neurodevelopment in children conceived with ICSI-AOA using A23817 or ionophores | Total raw scores on each J-ASQ-3 domain (communication, gross motor, fine motor, problem-solving, and personal-social) for children born with ICSI-AOA using A23817 or ionophores at 12, 18, 24, 30, 36, 42, 48, 54, and 60 months of age. Both groups have no significant differences at all age points and in all domains (**A**–**E**). Data points represent mean scores; the bars represent 95% confidence intervals and indicate within-sample variation. *J-ASQ-3* Japanese version of the Ages and Stages Questionnaire 3rd Edition; *ICSI* intracytoplasmic sperm injection, *AOA* artificial oocyte activation.

assessments conducted in approximately 15 min within the comfort of their homes, likely providing a more accurate reflection of the children's true abilities. Given experimental evidence linking altered calcium signalling during fertilization to changes in gene expression and early neurodevelopment, larger cohort studies with long-term follow-up are needed to clarify the potential implications of AOA.

This study did not include children born after AOA with $SrCl_2$ or PLCζ. Precision medicine approaches have been explored to detect OAD in advance, targeting sperm-related (including PLCζ and PLCζ I mutations) and oocyte-related (including wee1-like protein kinase 2, PAT1 homolog 2, tubulin beta-8 chain, and transducin-like enhancer protein 6 mutations) factors[16]. However, an effective method for selecting the appropriate AOA protocol is yet to be established. $SrCl_2$ and PLCζ are considered potent agents for oocyte activation; however, their clinical application remains limited. The activation mechanism of $SrCl_2$ is well characterized in murine oocytes but remains poorly understood in human oocytes[17]. Similarly, PLCζ shows significant variability in expression levels and functional capacity among individuals, complicating the development of standardized therapeutic dosing regimens[18]. These limitations contribute to insufficient data supporting the efficacy and safety of $SrCl_2$ and PLCζ in human applications, hindering their broader clinical adoption. Ca^{2+} ionophores have well-defined activation mechanisms, and substantial clinical evidence supports their efficacy and early postnatal safety. They have become the preferred agents for AOA in routine clinical practice[12] leading to a larger proportion of infants being born following their use.

Recent literature highlights concerns about neurodevelopmental delays in children born during the coronavirus disease 2019 (COVID-19) pandemic, particularly regarding language and communication skills[13,19–21]. These delays are attributed to multifactorial influences, including social restrictions (e.g., lockdowns), economic instability, and increased parental stress. Maternal COVID-19 is associated with cytokine storms, which can impair foetal development and induce DNA damage[21]. Since the pandemic was declared in March 2020, assuming that all children included in this study were exposed to its social consequences is reasonable. Many participants experienced significant early developmental stages during the pandemic. Reduced social interactions, limited access to developmental resources, and heightened family stress may have exerted similar developmental effects on both groups. This shared context may explain the lack of significant differences in communication scores between the ICSI-AOA and ICSI-alone groups in our study and could influence outcomes in future studies using the ASQ-3.

Early intervention is a well-established method of improving developmental outcomes in children with developmental delays. A study on the Early Start Denver Model, conducted over 2 years involving children diagnosed with autism spectrum disorder (ASD) at 18–30 months, showed improvements in Intelligence Quotient, adaptive behaviour, and electroencephalograph patterns that were similar to those of typically developing children[22,23]. The American Academy of Pediatrics recommends combining standardized screening tests for ASD in children aged 9–30 months to support early identification of developmental disorders[24–26]. Although the J-ASQ-3 used in this study is a screening tool rather than a diagnostic instrument, reevaluating children below the monitoring zone and initiating early intervention may promote developmental improvements. We compared confounding variables associated with the J-ASQ-3 scores in children who scored below the cutoff, those in the monitoring zone, and typically developing children to explore the factors contributing to developmental risk beyond AOA. While no significant differences were found in the below-cutoff group, an analysis of children in the monitoring zone revealed that having siblings was associated with a lower adjusted OR for being in the monitoring zone (adjusted OR = 0.40, 95% CI = 0.20–0.78). Male children had a higher adjusted OR than female children (adjusted OR = 2.57, 95% CI = 1.31–5.05). These results support the known higher risk of developmental disorders in males[27] and suggest that having siblings promotes developmental progress[28]. Overall, this analysis indicates that AOA is possibly a minimal risk factor for developmental disorders in this cohort.

In our cohort, paternal age did not differ significantly between the conventional ICSI and ICSI-AOA groups (median 37.0 vs. 38.5 years; $P = 0.91$). Moreover, paternal age was not predictive of scoring below the ASQ-

3 monitoring threshold. Although recent evidence suggests that advanced paternal age may subtly influence offspring neurodevelopment[29]these data indicate that it is unlikely to have confounded the association between AOA and neurodevelopmental outcomes.

Epigenetic mechanisms govern gene expression, cellular function, and developmental processes, and DNA methylation plays a central role in normal embryonic development. Aberrant DNA methylation patterns in infants have been linked to an increased risk of ASD[9]. A comparative analysis of 962 assisted reproduction technology (ART)-conceived and 983 naturally conceived singleton newborns revealed a global trend of hypomethylation in ART-conceived individuals, with 607 differentially methylated CpG sites identified, affecting genes associated with growth and neurodevelopment[30]. Although these findings highlight the potential epigenetic risks associated with ART, whether specific ART procedures contribute to these alterations remains unclear. Our analysis showed that children conceived with AOA exhibited no neurodevelopmental differences compared to those conceived without AOA. These differences suggest that AOA, despite its mechanistic role in overcoming incomplete oocyte activation, a process known to disrupt epigenetic reprogramming during zygotic genome activation[31]does not introduce additional neurodevelopmental risks.

To further explore the observed trend, it is noteworthy that in our cohort, the ICSI-AOA group showed numerically low J-ASQ-3 communication scores at 12, 18, and 24 months of age. However, this difference was no longer apparent by 30 months, suggesting any early developmental lag may be transient. This pattern, combined with the absence of significant between-group differences in the proportion of children falling below the threshold or requiring monitoring, suggests that AOA is unlikely to have lasting adverse effects on communication development. Nevertheless, these findings warrant cautious interpretation, and ongoing developmental surveillance remains important.

This study demonstrated AOA's safety for neurodevelopment in children up to 60 months, reinforcing the recent decision by the HFEA[13] and the fact-finding survey's results in Japan, which recommended upgrading AOA to clinical grade based on accumulating evidence[32]. As evidence of AOA's efficacy and safety continues to increase, its evaluation in clinical practice is expected to improve, potentially expanding its indications. However, adhering to the current indications for AOA use is crucial, particularly in clinical settings, given the limited understanding of its long-term effects.

Limitations

This study employed a cross-sectional design, assessing children aged 12–60 months using the J-ASQ-3, with no longitudinal follow-up. While heterogeneity and potential biases from environmental and genetic factors could not be eliminated, minimal bias was suggested, as no significant differences in social or environmental backgrounds were observed, except for a higher number of fathers with teaching professions in the ICSI-AOA group than in the ICSI-alone group. The COVID-19 pandemic may have affected developmental outcomes, given its impact on early childhood environments. Finally, this study lacked adequate data to compare various AOA methods, underscoring the necessity of longitudinal multicentre studies to assess the long-term safety and effectiveness of alternative techniques[29].

Conclusions

This is the first study to demonstrate the long-term safety of ICSI, followed by AOA on neurodevelopment in a large cohort of children. Based on the findings, no significant concerns regarding any adverse effects of AOA on childhood neurodevelopment have been identified. These data add to the body of evidence available to physicians when considering approaches for couples experiencing fertilization failure. However, more detailed studies on the long-term childhood development born after all AOA types are needed before any definitive conclusions can be drawn.

Participants and methods

Study design and participants

We collected data on mothers who gave birth via ICSI-alone or ICSI-AOA at Keio University Hospital and eight JISART facilities between August 2018 and June 2023. Based on responses from the eight participating facilities, A23187-mediated AOA was performed using either a HEPES-buffered calcium ionophore (CIM-10, Kitazato Corporation, Japan) or A23187 obtained from SIGMA (C7522, USA), Gynemed (GM508 CultActive, Germany), or FujiFilm (019-20111, Japan). GM508 CultActive was supplied as a ready-to-use A23187 ionophore solution, with its concentration undisclosed. When preparing other A23187 solutions, stock concentrations were diluted in HEPES-buffered medium with DMSO to achieve final working concentrations of either 5 μM or 10 μM. In all cases, AOA was initiated within 30 min after ICSI and consisted of a single exposure at 37 °C for 5–15 min. Ionomycin (I-0634, Sigma-Aldrich, Belgium) was also used as a calcium ionophore. In this protocol, oocytes were exposed to a 5 μM ionomycin solution for 10 min, starting 20 min after ICSI.

The neurodevelopmental survey focused on children aged 12–60 months who were conceived via ICSI-alone or ICSI-AOA between August 2018 and January 2023. The exclusion criteria included cases involving testicular sperm extraction, mothers aged ≥ 43 years at the time of embryo transfer, and double embryo transfers using ICSI-alone and ICSI-AOA. No age restrictions were applied for fathers. Prior to completing the J-ASQ-3 and an additional questionnaire, written informed consent was obtained from all participants. To specifically assess the impact of ICSI-AOA on neurodevelopmental outcomes, we established a control group matched by offspring age. For each child born following ICSI-AOA, one age-matched control child was selected from the IVF database of the corresponding facility. These children were conceived and delivered during the same time period to ensure comparable durations of postnatal follow-up. All procedures were conducted in accordance with the relevant guidelines and regulations.

On December 8, 2023, all facilities were instructed to collect relevant cases and complete the case report forms. Among the 375 identified women, 326 children aged 12–60 months were selected. Questionnaires were distributed equally to both groups (163 ICSI-AOA and 163 ICSI) totalling 326 children on January 5, 2024, with February 18, 2024, as the submission deadline. However, participants were not informed about the survey in advance; therefore, a 1,000-yen gift card was included with the questionnaire.

Valid responses were obtained from 158 children (158/266, 59.4%), comprising 77 of 128 (60.2%) in the ICSI-AOA group and 81 of 138 (58.7%) in the ICSI group. Among the remaining cases: 60 questionnaires were undelivered, 104 were not returned, 3 were declined, and 1 was excluded for not meeting inclusion criteria. Parents were also asked to complete a paper-based questionnaire on factors influencing child development, including sibling composition and social activities (e.g., daycare or kindergarten), parental educational background, occupation, and household income.

Assessment of neurodevelopment

The J-ASQ-3 is a level 1 screening tool that evaluates five developmental domains. This questionnaire, completed by parents or caregivers, is known for its high reliability and validity. It has been translated into several languages and is widely used in clinical and research settings internationally[33–36] .The J-ASQ-3 is a Japanese translation of the ASQ-3, with items adapted to Japanese culture and developmental standards for children in Japan[37]. The cutoff is defined as scores ≤-2.0 standard deviations (SD) below the age-specific mean, indicating a strong suspicion of developmental delay, whereas the monitoring zone (-1.0 to -2.0 SD) suggests the need for evaluation or follow-up. In this study, we defined scores ≤ − 1.0 SD as "below monitoring" to include both the monitoring zone and below-cutoff categories, due to the limited number of children scoring below the cutoff. A binomial logistic regression analysis was performed using AOA presence, child's sex, gestational age, and J-ASQ-3 age as independent variables, with children below the cutoff or in the monitoring zone as the dependent variable.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA), with statistical significance considered at $P<0.05$. Power analyses, based on the primary outcome, were used to investigate the mean differences in the J-ASQ-3 subdomain scores between children conceived with and without AOA. Covariance analysis was used to determine the mean differences in the J-ASQ-3 subdomains between the ICSI-alone and ICSI-AOA groups. We used quantile-quantile plots (Q-Q plots) analysis to assess the overall distribution of P-values derived from the analysis of 45 adjusted mean differences in J-ASQ-3 scores between ICSI-alone and ICSI-AOA. This method was selected to rigorously evaluate whether the observed P-value distribution aligns with the expected null distribution, enabling the identification of potential deviations due to systematic biases or random variation. The Q-Q plot analysis strengthens our multiple comparison framework robustness by providing a visual and statistical assessment of conformity.

The percentages of children identified as being below the monitoring threshold and cutoff levels indicative of delays, as defined by the ASQ-3 developer, were computed for each subdomain and the overall J-ASQ-3 outcome. Additionally, the odds ratios (ORs) were calculated using a binary logistic regression model. The independent variables included AOA presence, sex, gestational age at birth, and age in months during the J-ASQ-3 assessment. All results were reported with 95% confidence intervals (CIs).

Data availability

The data supporting the findings of this study will be made available from the corresponding authors upon reasonable request.

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References

1. Akashi, K. et al. Artificial oocyte activation using Ca(2+) ionophores following intracytoplasmic sperm injection for low fertilization rate. *Front. Endocrinol. (Lausanne)*. **14**, 1131808. https://doi.org/10.3389/fendo.2023.1131808 (2023).
2. Ruan, J. L., Liang, S. S., Pan, J. P., Chen, Z. Q. & Teng, X. M. Artificial oocyte activation with Ca(2+) ionophore improves reproductive outcomes in patients with fertilization failure and poor embryo development in previous ICSI cycles. *Front. Endocrinol. (Lausanne)*. **14**, 1244507. https://doi.org/10.3389/fendo.2023.1244507 (2023).
3. Bonte, D. et al. Assisted oocyte activation significantly increases fertilization and pregnancy outcome in patients with low and total failed fertilization after intracytoplasmic sperm injection: a 17-year retrospective study. *Fertil. Steril.* **112**, 266–274. https://doi.org/10.1016/j.fertnstert.2019.04.006 (2019).
4. Murugesu, S. et al. Does the use of calcium ionophore during artificial oocyte activation demonstrate an effect on pregnancy rate? A meta-analysis. *Fertil Steril* **108**, 468–482 e463. https://doi.org/10.1016/j.fertnstert.2017.06.029 (2017).
5. Vanden Meerschaut, F., Nikiforaki, D., Heindryckx, B. & De Sutter, P. Assisted oocyte activation following ICSI fertilization failure. *Reprod. Biomed. Online*. **28**, 560–571. https://doi.org/10.1016/j.rbmo.2014.01.008 (2014).
6. Ducibella, T. & Fissore, R. The roles of Ca2+, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Dev. Biol.* **315**, 257–279. https://doi.org/10.1016/j.ydbio.2007.12.012 (2008).
7. Long, R. et al. Risk of birth defects in children conceived by artificial oocyte activation and intracytoplasmic sperm injection: a meta-analysis. *Reprod. Biol. Endocrinol.* **18** https://doi.org/10.1186/s12958-020-00680-2 (2020).
8. Yin, M. et al. DNA methylation and gene expression changes in mouse pre- and post-implantation embryos generated by intracytoplasmic sperm injection with artificial oocyte activation. *Reprod. Biol. Endocrinol.* **19**, 163. https://doi.org/10.1186/s12958-021-00845-7 (2021).
9. Mouat, J. S. & LaSalle, J. M. The promise of DNA methylation in Understanding multigenerational factors in autism spectrum disorders. *Front. Genet.* **13**, 831221. https://doi.org/10.3389/fgene.2022.831221 (2022).

10. Deemeh, M. R., Tavalaee, M. & Nasr-Esfahani, M. H. Health of children born through artificial oocyte activation: a pilot study. *Reprod. Sci.* **22**, 322–328. https://doi.org/10.1177/1933719114542017 (2015).
11. Liang, R. et al. Is there any effect on imprinted genes H19, PEG3, and SNRPN during AOA? *Open. Med. (Wars).* **17**, 174–184. https://doi.org/10.1515/med-2022-0410 (2022).
12. Harada, S. et al. Fact-finding survey on assisted reproductive technology in Japan. *J. Obstet. Gynaecol. Res.* **49**, 2593–2601. https://doi.org/10.1111/jog.15780 (2023).
13. UK fertility regulator. Launches improved ratings for fertility treatment 'add-ons. *HFEA*. https://www.hfea.gov.uk/treatments/treatment-add-ons/ (2023).
14. Lundin, K. et al. Good practice recommendations on add-ons in reproductive medicine†. *Hum. Reprod.* **38**, 2062–2104. https://doi.org/10.1093/humrep/dead184 (2023).
15. Vanden Meerschaut, F. et al. Neonatal and neurodevelopmental outcome of children aged 3–10 years born following assisted oocyte activation. *Reprod. Biomed. Online.* **28**, 54–63. https://doi.org/10.1016/j.rbmo.2013.07.013 (2014).
16. Campos, G., Sciorio, R. & Esteves, S. C. Total fertilization failure after ICSI: insights into pathophysiology, diagnosis, and management through artificial oocyte activation. *Hum. Reprod. Update.* **29**, 369–394. https://doi.org/10.1093/humupd/dmad007 (2023).
17. Lu, Y. et al. Strontium fails to induce Ca(2+) release and activation in human oocytes despite the presence of functional TRPV3 channels. *Hum Reprod Open* **2018**, hoy005. https://doi.org/10.1093/hropen/hoy005 (2018).
18. Kashir, J. et al. Variance in total levels of phospholipase C zeta (PLC-ζ) in human sperm May limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertil. Steril.* **99**, 107–117e103. https://doi.org/10.1016/j.fertnstert.2012.09.001 (2013).
19. Hessami, K. et al. COVID-19 pandemic and infant neurodevelopmental impairment: A systematic review and Meta-analysis. *JAMA Netw. Open.* **5**, e2238941. https://doi.org/10.1001/jamanetworkopen.2022.38941 (2022).
20. Sato, K., Fukai, T., Fujisawa, K. K. & Nakamuro, M. Association between the COVID-19 pandemic and early childhood development. *JAMA Pediatr.* **177**, 930–938. https://doi.org/10.1001/jamapediatrics.2023.2096 (2023).
21. Fajardo-Martinez, V. et al. Neurodevelopmental delay in children exposed to maternal SARS-CoV-2 in-utero. *Sci. Rep.* **14**, 11851. https://doi.org/10.1038/s41598-024-61918-2 (2024).
22. Dawson, G. et al. Randomized, controlled trial of an intervention for toddlers with autism: the early start Denver model. *Pediatrics* **125**, e17–23. https://doi.org/10.1542/peds.2009-0958 (2010).
23. Dawson, G. et al. Early behavioral intervention is associated with normalized brain activity in young children with autism. *J. Am. Acad. Child. Adolesc. Psychiatry.* **51**, 1150–1159. https://doi.org/10.1016/j.jaac.2012.08.018 (2012).
24. Services, C. f. M. M. *CMS proposals to implement certain disclosure provisions of the Affordable Care Act*. http://www.cms.gov/apps/media/press/factsheet.asp?Counter=4221 (2012).
25. Lipkin, P. H. & Macias, M. M. Promoting optimal development: identifying infants and young children with developmental disorders through developmental surveillance and screening. *Pediatrics* **145** https://doi.org/10.1542/peds.2019-3449 (2020).
26. Hyman, S. L., Levy, S. E. & Myers, S. M. Identification, evaluation, and management of children with autism spectrum disorder. *Pediatrics* **145** https://doi.org/10.1542/peds.2019-3447 (2020).
27. Polyak, A., Rosenfeld, J. A. & Girirajan, S. An assessment of sex bias in neurodevelopmental disorders. *Genome Med.* **7**, 94. https://doi.org/10.1186/s13073-015-0216-5 (2015).
28. Tsinivits, D. & Unsworth, S. The impact of older siblings on the Language environment and Language development of bilingual toddlers. *Appl. Psycholinguist.* **42**, 325–344. https://doi.org/10.1017/S0142716420000570 (2021).
29. Potabattula, R. et al. Effects of paternal and chronological age on BEGAIN methylation and its possible role in autism. *Aging (Albany NY).* **15**, 12763–12779. https://doi.org/10.18632/aging.205275 (2023).
30. Håberg, S. E. et al. DNA methylation in newborns conceived by assisted reproductive technology. *Nat. Commun.* **13**, 1896. https://doi.org/10.1038/s41467-022-29540-w (2022).
31. Shafqat, A. et al. Oocyte activation, calcium release and epigenetic remodelling: lessons from Cancer models. *Front. Cell. Dev. Biol.* **10**, 781953. https://doi.org/10.3389/fcell.2022.781953 (2022).
32. Shionoya, N. et al. Survey of in vitro fertilization add-ons in Japan (Izanami project). *Front. Endocrinol. (Lausanne).* **15**, 1404601. https://doi.org/10.3389/fendo.2024.1404601 (2024).
33. Bernard, J. Y. et al. Breastfeeding duration and cognitive development at 2 and 3 years of age in the EDEN mother-child cohort. *J. Pediatr.* **163**, 36–42e31. https://doi.org/10.1016/j.jpeds.2012.11.090 (2013).
34. Richter, J. & Janson, H. A validation study of the Norwegian version of the ages and stages questionnaires. *Acta Paediatr.* **96**, 748–752. https://doi.org/10.1111/j.1651-2227.2007.00246.x (2007).
35. Lopes, S., Graça, P., Teixeira, S., Serrano, A. M. & Squires, J. Psychometric properties and validation of Portuguese version of Ages & Stages Questionnaires (3rd edition): 9, 18 and 30 Questionnaires. *Early Hum. Dev.* **91**, 527–533. https://doi.org/10.1016/j.earlhumdev.2015.06.006 (2015).
36. Small, J. W., Hix-Small, H., Vargas-Baron, E. & Marks, K. P. Comparative use of the ages and stages questionnaires in low- and middle-income countries. *Dev. Med. Child. Neurol.* **61**, 431–443. https://doi.org/10.1111/dmcn.13938 (2019).
37. Mezawa, H. et al. Psychometric profile of the ages and stages questionnaires, Japanese translation. *Pediatr. Int.* **61**, 1086–1095. https://doi.org/10.1111/ped.13990 (2019).

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Author contributions

K.M. conceptualized the research question and study design, conducted investigation, data curation, and formal analysis, and prepared the original draft. M.Y. contributed to conceptualization, methodology, and validation, supervised the study, acquired funding, and critically revised the manuscript. K.A., S.K., T.A., and M.H. contributed to methodology and critically revised the manuscript. S.C.J. performed formal analysis and validation. H.U. conducted data curation and investigation. Y.S. contributed to methodology and formal analysis. Y.H. and M.T. contributed to supervision and manuscript revision. S.N. contributed to formal analysis and manuscript revision. Y.O. contributed to funding acquisition and manuscript revision. N.K. coordinated project administration and revised the manuscript. All authors performed manuscript revision and approved the final version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
This study was approved by the Institutional Research Ethics Board of Keio University School of Medicine (approval number: 20231127). Collaborating institutions obtained approval from their respective ethics committees to participate in the study. This study was conducted in accordance with the Declaration of Helsinki.

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Artificial oocyte activation with Ca^{2+} ionophore improves reproductive outcomes in patients with fertilization failure and poor embryo development in previous ICSI cycles

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Research question: Does artificial oocyte activation (AOA) by a calcium ionophore (ionomycin) improve the previous fertilization failure or poor embryo development of intracytoplasmic sperm injection (ICSI) account for male factor infertility or other infertility causes?

Design: This retrospective study involved 114 patients receiving ICSI-AOA in Shanghai First Maternity and Infant Hospital with previous ICSI fertilization failure or poor embryo development. The previous ICSI cycles of the same patients without AOA served as the control group. The fertilization rates, cleavage rates, transferable embryo rates and blastocyst formation rates of the two groups were compared. Additionally, the clinical pregnancy, implantation rate and live birth rates were also compared to assess the efficiency and safety of AOA. Furthermore, two subgroup analyses were performed in this study based on the cause of infertility and the reason for AOA. The fertilization rate, embryonic development potential and clinical outcome were compared among groups.

Results: Among 114 ICSI-AOA cycles, the fertilization rate, top-quality embryo rate, implantation rate, clinical pregnancy per patient and live birth rate per patient were improved significantly compared with previous ICSI cycles ($p<0.05$ to $P< 0.001$), and the miscarriage rate in the AOA group was significantly lower than that of the control group ($p<0.001$). In the AOA subgroups based on the cause of infertility, the fertilization rates of each subgroup were significantly improved compared with previous control cycles except for the mixed factor infertility subgroup ($p<0.05$ to $p<0.001$). In the AOA subgroups based on the reason for AOA, the fertilization rates of each subgroup were significantly increased compared with those in their previous ICSI cycle without AOA ($p<0.001$); however, there was no significant difference in the top-quality embryo rate. No significant improvement was found in the implantation rates and the clinical pregnancy rate in each subgroup except for the poor embryo

development subgroup. In the 114 AOA cycles, 35 healthy infants (21 singletons and 7 twins) were delivered without major congenital birth defects or malformations.

Conclusion: This study showed that AOA with the calcium ionophore ionomycin can improve the reproductive outcomes of patients with previous fertilization failure and poor embryo development after ICSI.

KEYWORDS

artificial oocyte activation, ionomycin, intracytoplasmic sperm injection, fertilization failure, embryo development

Introduction

Intracytoplasmic sperm injection (ICSI) is mainly used for male factor infertility. The average fertilization rate after ICSI is approximately 70% to 80% (1). However, 1%~5% of ICSI cycles occurring still result in total fertilization failure (TFF) or almost complete fertilization failure, defined as a fertilization rate of less than 30% (2). Oocyte activation deficiency (OAD) is the main cause of ICSI fertilization failure, which may be related to sperm or oocyte factors leading to responsiveness of gamete interaction (3, 4).

In the past decade, an increasing number of studies have shown that sperm-specific phospholipase Cζ (phospholipase C zeta, PLCζ) is strongly associated with sperm-specific oocyte activation failure (OAF) (5). The low expression of PLCζ is one of the main reasons for fertilization failure (6–8). ICSI combined with artificial oocyte activation could mimic physiological calcium changes that occur during fertilization (9). During normal fertilization, sperm-specific PLCζ is released into the oocyte cytoplasm, triggering calcium shock and promoting the release of Ca^{2+} in the endoplasmic reticulum. The temporary increase of Ca^{2+} concentration in oocytes generates Ca^{2+} oscillations to activate oocytes and induces a cortical granular response. Oocytes recover and complete meiosis, extrude the second polar body and form two pronuclei (8). The endoplasmic reticulum is the main source of intracellular Ca^{2+} increase, which is mainly triggered by the successful induction of IP3 pathway by sperm-specific phospholipase C-ζ (PLC-ζ) around the sperm nucleus. The failure of oocyte activation leading to non-fertilization may be mainly caused by the failure of PLC-induced IP3 pathway (10). However, the molecular mechanism of OAD induced by oocyte-borne oocyte activation factors is unclear. According to previous reports, human oocytes with ICSI-TFF often carry with specific problems, such as abnormal cell structure, abnormal gene expression, abnormal chromosomes, immature oocytes and abnormal spindles (11). All of these problems may cause oocyte activation failure after ICSI.

AOA is the most commonly used technology to solve sperm-related OAF at present, and healthy live births have been delivered after using various activation methods. Abnormalities in the oocyte activating signal pathway may lead to the occurrence of OAF (12). To date, AOA is the most effective method to solve ICSI-TFF, low fertilization and OAF caused by sperm factors (13). However, large-scale clinical studies are still lacking, and there are also disputes regarding the efficiency of AOA and the safety of offspring. Therefore, this study analysed the clinical pregnancy outcomes and neonatal health of AOA patients who received the Ca^{2+} ionophore ionomycin during ICSI and evaluated the efficacy and safety of ionomycin on the fertilization rate and embryo development.

Materials and methods

Patients

This retrospective analysis was performed on the clinical data of couples receiving ICSI-AOA due to poor fertilization or poor embryo development after a previous ICSI cycle from January 2019 to December 2020 in Shanghai First Maternity and Infant Hospital affiliated with Tongji University. The indications for ICSI-AOA are as follows: previous ICSI fertilization failure (TFF or fertilization rate ≤ 30%) and poor embryo development (complete embryo development arrest, reduced blastocyst formation on day 5 (≤15%)). Controlled ovarian stimulation and oocyte retrieval were carried out according to standard protocols (14). A total of 114 couples were enrolled in this retrospective analysis (Figure 1). Most patients started ICSI with ovarian stimulation using either the long agonist or antagonist protocols. Ovulation was triggered by intradermal injection of 5000~10,000 IU HCG. Patients with AOA cycles in the previous cycle and half-AOA cycles were excluded from this study. The previous cycles without AOA were considered as a control group. The interval between the two stimulation cycles was no more than six months.

In addition, two subgroup analyses were performed in this study. According to the cause of infertility, patients were further divided into six subgroups: (i) oligoasthenoteratozoospermia (OAT), including oligozoospermia (low number of sperm), asthenozoospermia (poor sperm movement), and teratozoospermia (abnormal sperm shape); (ii) advanced age (age>35 years); (iii) primary ovarian insufficiency (POI, patients with primary ovarian insufficiency); (iv) unexplained

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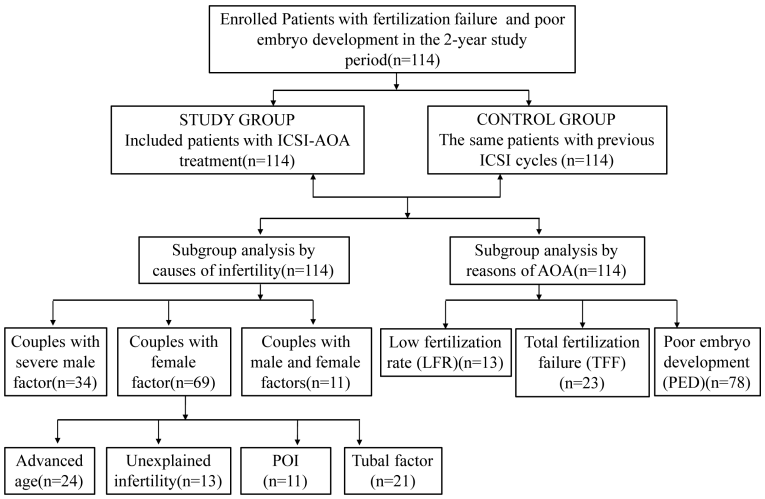


FIGURE 1
Flowchart of patients. ICSI, intracytoplasmic sperm injection; AOA, artificial oocyte activation; POI, patients with primary ovarian insufficiency.

infertility; (v) tubal factor; and (vi) mixed factor infertility (M+F). Based on the reason for AOA, patients were divided into three subgroups for analysis: total fertilization failure (TFF); low fertilization rate (LFR, fertilization rate <30%); and poor embryo development (PED). Similarly, the oocyte activation cycle was compared with the previous ICSI cycle of the same patients. The fertilization rate, embryonic development potential and clinical outcome were compared among groups.

This study was approved by the institutional review board of Shanghai First Maternity and Infant Hospital affiliated with Tongji University. All the enrolled patients in this study signed written informed consent and were informed of the potential risks of using AOA technology.

ICSI and calcium ionophore AOA procedure

The oocytes collected from each cycle were cultured at 37°C in a humidified triple-gas incubator with 6% CO_2 and 5% oxygen (Thermo Fisher Scientific, USA) for at least 2 h before removing cumulus cells. Subsequently, all metaphase II oocytes were treated according to the standard ICSI procedure (15). Oocyte activation with ionomycin was performed according to previous reports (16, 17). Briefly, thirty minutes after ICSI, the oocytes were immediately exposed to activated liquid droplets (G-mops, Vitrolife, Sweden) containing 10 μmol/l ionomycin (Sigma Aldrich, USA) for 10 minutes, rinsed with medium (G-mops, Vitrolife, Sweden) 3 times, and transferred to embryo culture medium (G1, Vitrolife, Sweden).

Fertilization check, embryo grading and embryo transfer

Fertilization observations were carried out 18-19 h after ICSI under an inverted microscope. Only oocytes with two clear pronuclei (2PN) and two polar bodies (2PB) were considered to be fertilized normally. Quality assessment of embryos was carried out according to routine guidelines described previously (18). Embryos were cultured *in vitro* to the third day (D3), and only embryos with 7-9 cells from normal fertilized oocytes and a fragment ratio <10% were defined as top-quality embryos. Then, top-quality embryos were transferred under transvaginal ultrasound guidance. The remaining top-quality embryos were cryopreserved for the next cycle. Additionally, all the remaining cleavage-stage embryos were cultured up to the blastocyst stage, the blastocysts were scored morphologically according to the Gardner scale (2), and the available blastocysts were cryopreserved for later transfer. According to provincial guidelines, up to two embryos can be transferred in any transfer cycle.

Study outcome assessment and clinical pregnancy

The following parameters were used to compare previous routine ICSI cycles with ICSI-AOA cycles of the same patient. The primary outcome measure was the 2PN fertilization rate, defined as the number of normal fertilized oocytes divided by the number of injected MII oocytes. Secondary outcome measures included the cleavage rate (number of cleaved zygotes/number of fertilized oocytes), top-quality embryo rate (number of high-quality embryos/number of cleaved zygotes), blastocyst formation rate (number of blastocysts formed/number of blastocysts cultured), and implantation rate (number of gestational sacs seen on scanning divided by the number of embryos replaced). Clinical pregnancy was defined as the presence of at least one gestational sac on ultrasound at 6 weeks. A baby born alive after 20 gestation weeks was classified as a live birth. The miscarriage rate was defined as the number of miscarriages before 20 weeks divided by the number of women with a positive pregnancy test. Additionally, information on the patient's pregnancy outcomes was followed up until March 2021 by telephone survey, including gestation weeks, gender of babies,

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birth weight and body length, as well as major birth defects. There were still 40 and 103 frozen embryos remaining in the previous ICSI cycles and the AOA cycles, respectively, by the end of the study.

Statistical analysis

The one-sample Kolmogorov–Smirnov test was used to test the normal distribution of continuous variables. Continuous variables were given as the mean ± SD if normally distributed. Comparisons between groups were performed with paired t tests for continuous variables and chi-square tests for categorical variables, where appropriate. Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS Inc., Version 14.0, Chicago, IL, USA). A two-tailed value of $P < 0.05$ was considered statistically significant.

Results

Patients' baseline clinical characteristics

Between January 2019 and December 2020, 114 patients who underwent ICSI-AOA were enrolled in this study with a history of poor fertilization or embryo development in the first ICSI cycles. The flow chart of the subjects is shown in Figure 1.

The average age of the women was 33.86 ± 4.981 years, and they had suffered infertility for 4.09 ± 2.991 years before ART treatment (Table 1). The other baseline characteristics of the patients are shown in Table 1, including infertility types, infertility years, infertility factors, female body mass index (BMI), antral follicle counts (AFC), and basal FSH. All couples received routine ICSI and AOA (Table 1). The stimulation protocol used in each group was similar between the two groups (P >0.05) (Table 2).

TABLE 1 General characteristics of the enrolled patients (mean ± SD).

Item	Value
No. of patients	114
Female age (y), mean ± SD	33.86 ± 4.981
Male age (y), mean ± SD	35.71 ± 5.799
Type of infertility, n (%)	
Primary infertility	72 (63.16%)
Secondary infertility	42 (36.84%)
Duration of infertility (y)	4.09 ± 2.991
Indication, n (%)	
Female factors	68(59.65%)
Male factors	35(30.70%)
Mixed	11(9.65%)
BMI for women (kg/m^2)	22.29 ± 3.291
AFC	9.99 ± 7.378
Basal FSH(IU/L)	7.24 ± 3.923

Values are shown as the mean ± SD or number. BMI, female body mass index; AFC, antral follicle count.

Embryological characteristics in previous standard ICSI cycles and AOA cycles

The stimulation protocols adopted in both groups were comparable. No significant differences were found in the number of oocytes obtained between the two groups. However, the numbers of oocytes fertilized, cleavage embryos, top-quality embryos, transferred embryos and frozen embryos were significantly higher in the AOA group than in the control group. Furthermore, the cycle cancellation rate, overall fertilization rate and top-quality embryo rate in the AOA group were significantly higher than those in the control group (P< 0.001). However, the cleavage rate, the transferable embryo rate, and blastocyst formation rate were comparable between the two groups (P>0.05) (Table 2).

Pregnancy outcomes in previous standard ICSI cycles and AOA cycles

During the study period, women in the AOA group and women in the control group had undergone 84 and 60 embryo transfer cycles, respectively. The proportions of fresh transfer and FET were similar between the two groups (p>0.05). The clinical pregnancy rate per transfer (41.67% versus 8.33%, P < 0.001), clinical pregnancy rate per patient (30.7% versus 4.39%, P < 0.001) and implantation rate (34.59% versus 6.02%, P < 0.001) were significantly higher in the AOA group than in the control group, whereas the miscarriage rate in the AOA group was significantly lower than that in the control group (17.14% versus 100%, p< 0.001). Furthermore, the live birth rate per transfer (30.95%) and live birth rate per patient (28.95%) were greatly improved after AOA treatment compared to those in the control group (Table 3).

Subgroup analysis based on the cause of infertility

Subgroup analysis was performed into six categories according to the cause of infertility. The fertilization rates of each subgroup were significantly increased compared with those in their corresponding previous ICSI cycles without AOA except for patients in the M+F group. However, there was no significant difference in the top-quality embryo rate among all the subgroups except for the M+F group. The implantation rates were significantly increased in the OAT and unexplained infertility subgroups. The pregnancy rate was significantly improved in the OAT patients. For the OAT patients, the fertilization rate, implantation rate and clinical pregnancy rate were significantly increased, but no improvement was found in the top-quality embryo rate. In the unexplained infertility group, the fertilization rate and implantation rate were significantly increased after AOA, but no significant difference was found in the top-quality embryo rate and clinical pregnancy rate. For the M+F patients, the top-

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TABLE 2 Comparison of clinical outcomes previous standard ICSI cycles and AOA cycles.

	Control group (before)	AOA group (after)	$t/\chi^2/Z$ value	*P* value
No. of patients	114	114		
COS protocols				0.648
long protocol (%)	59(51.75)	66(57.89)	0.868	
Antagonist protocol (%)	40(35.09)	35(30.70)	0.497	
Other protocol (%)	15(13.16)	13(11.40)	0.163	
No. of oocytes obtained	6.89 ± 4.450	7.75 ± 5.048	1.830	0.070
No. of MII oocytes	5.33 ± 3.571	6.16 ± 4.153	-2.079	0.040
No. of oocytes fertilized	2.81 ± 2.844	4.78 ± 3.643	-5.317	0.000
Fertilization rate, %	52.63(320/608)	77.64(545/702)	90.813	0.000
No. of cleavage embryos	2.55 ± 2.677	4.50 ± 3.605	-5.625	0.000
Cleavage rate, %	90.94(291/320)	94.13(513/545)	3.312	0.077
No. of top-quality embryos	0.48 ± 0.790	1.24 ± 1.626	-4.984	0.000
Top-quality embryos rate, %	18.90(55/291)	27.49(141/513)	7.423	0.006
No. of transferable embryos	1.08 ± 1.390	2.07 ± 2.315	-5.13	0.000
Transferable embryos rate, %	42.27(123/291)	46.00(236/513)	1.049	0.306
No. of embryos frozen	0.76 ± 1.046	1.54 ± 1.838	-5.114	0.000
Blastocyst formation rate, %	23.53(28/119)	23.87(53/222)	0.005	0.943
Cancellation rate per cycle, %	45.61(52/114)	28.95(33/114)	6.772	0.009

COS, controlled ovarian stimulation; MII, metaphase II; ICSI, intracytoplasmic sperm injection; 2PN, two pronuclei; AOA, artificial oocyte activation; positive number/total number in brackets.

quality embryo rate was significantly increased after AOA, but no significant improvement was found in the top-quality embryo rate, implantation rate or clinical pregnancy rate. For the advanced age, POI and tubal factor patients, the fertilization rate was also significantly increased, but no significant increases were found in the top-quality embryo rate, implantation rate, and clinical pregnancy rate per patient after AOA treatment compared with their previous ICSI cycle (Figure 2).

Subgroup analysis based on the reason for AOA

Subgroup analysis was performed in three groups based on the reason for AOA. The fertilization rates of each subgroup were significantly increased compared with those in their previous ICSI cycle without AOA; however, there was no significant difference in the top-quality embryo rate. In the PED group, the fertilization rate,

TABLE 3 Comparison of pregnancy outcomes between previous standard ICSI cycles and AOA cycles.

	Control group	AOA group	$t/\chi^2/Z$ value	*P* value
Total number of transfer cycles	60	84		
Fresh transfer (%)	25(15/60)	26.19(22/84)	0.026	0.872
FET (%)	75(45/60)	73.81(62/84)	0.026	0.872
No. of embryos transferred	0.73 ± 1.071	1.18 ± 1.151	-3.538	0.001
Clinical pregnancy rate per transfer (%)	8.33(5/60)	41.67(35/84)	19.385	0.000
Clinical pregnancy rate per patients (%)	4.39(5/114)	30.70(35/114)	27.287	0.000
Implantation rate (%)	6.02(5/83)	34.59(46/133)	32.985	0.000
Miscarriage rate (%)	100(5/5)	17.14(6/35)	15.065	0.000
Live birth rate per transfer (%)	0	34.52(29/84)		
Live birth rate per patients (%)	0	25.44(29/114)		

FET, frozen-thawed embryo transfer; positive number/total number in brackets.

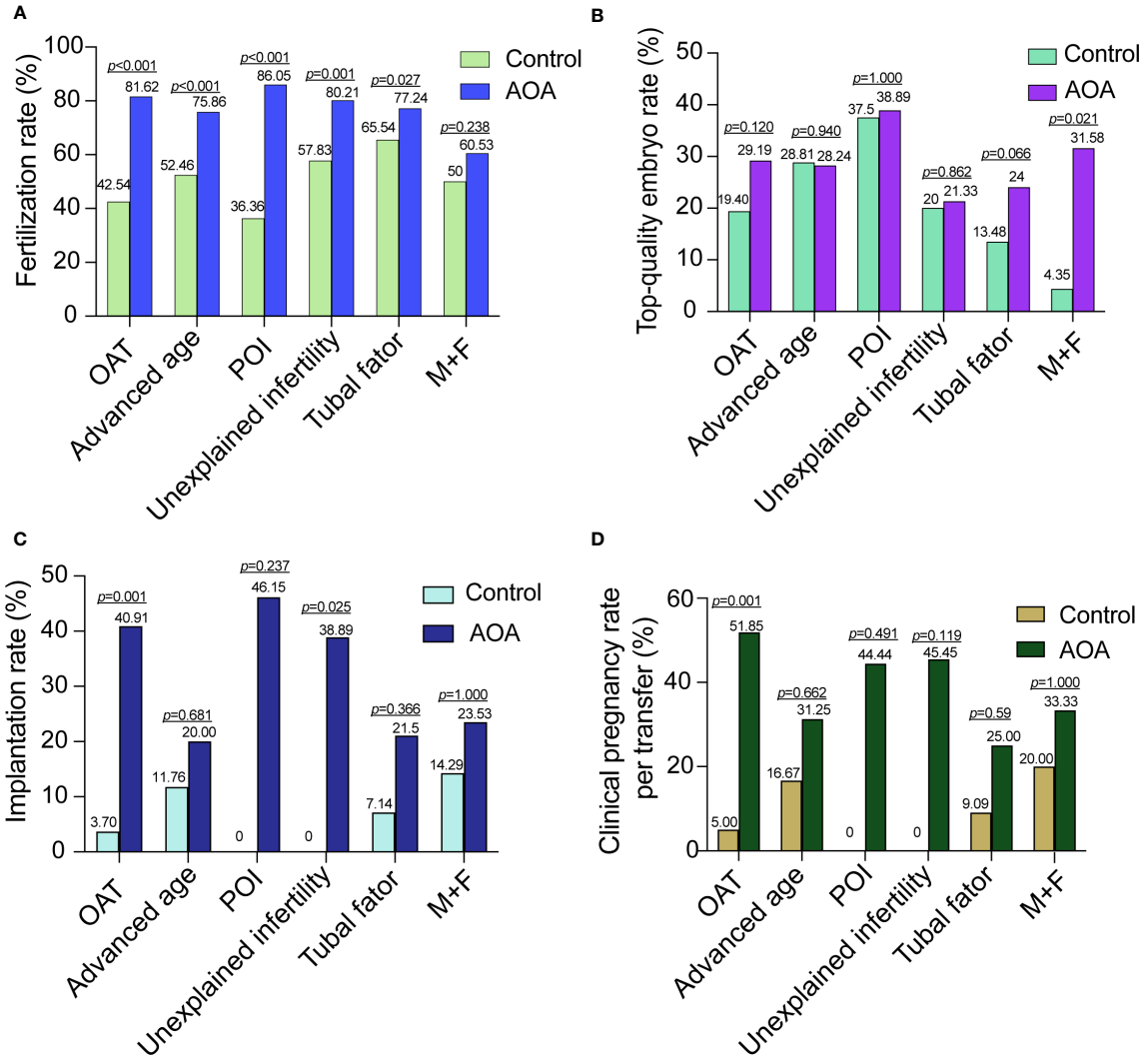


FIGURE 2
Comparison of activation effects in AOA subgroups based on the cause of infertility. **(A)** Comparison of fertilization rates between AOA subgroups and control groups. **(B)** Comparison of the top-quality embryo rate between AOA subgroups and control groups. **(C)** Comparison of the implantation rate between AOA subgroups and control groups. **(D)** Comparison of the clinical pregnancy rate between AOA subgroups and control groups. Control, previous ICSI cycles; *AOA*, artificial oocyte activation; *OAT*, oligoasthenoteratozoospermia; *POI* patients with primary ovarian insufficiency; *M+F* patients with male and female factors.

implantation rate and clinical pregnancy rate were highly increased after AOA, and the top-quality embryo rate was increased slightly after AOA treatment compared with their previous ICSI cycle without AOA but did not reach statistical significance. In the TFF and LFR groups, the fertilization rate was greatly improved, but no data on implantation rates and the clinical pregnancy rate were shown in the control group because no embryos could be transferred in these two groups (Figure 3).

The neonatal outcomes in the AOA group

In the AOA group, 21 singletons and 7 twins among 35 babies were born. All newborns, including 15 boys and 20 girls, had no major birth defects or congenital malformations. Only one baby was born with an atrial septal defect in preterm with gestational weeks of 28 w+4 d. The other neonatal characteristics in the AOA group are shown in Table 4 in terms of gestational weeks, body length, birth weight, low birth weight and preterm birth rate.

Discussion

In this study, we demonstrated that AOA with ionomycin can improve the reproductive outcome of patients with previous total ICSI fertilization failure and poor embryo development.

Our results indicated that AOA treatment significantly improved the fertilization rate, implantation rate, clinical pregnancy and live birth rates per patient compared with previous ICSI cycles, which is in accordance with previous reports (19–21). The evidence suggested that AOA can improve the fertilization rate and the developmental ability of embryos and

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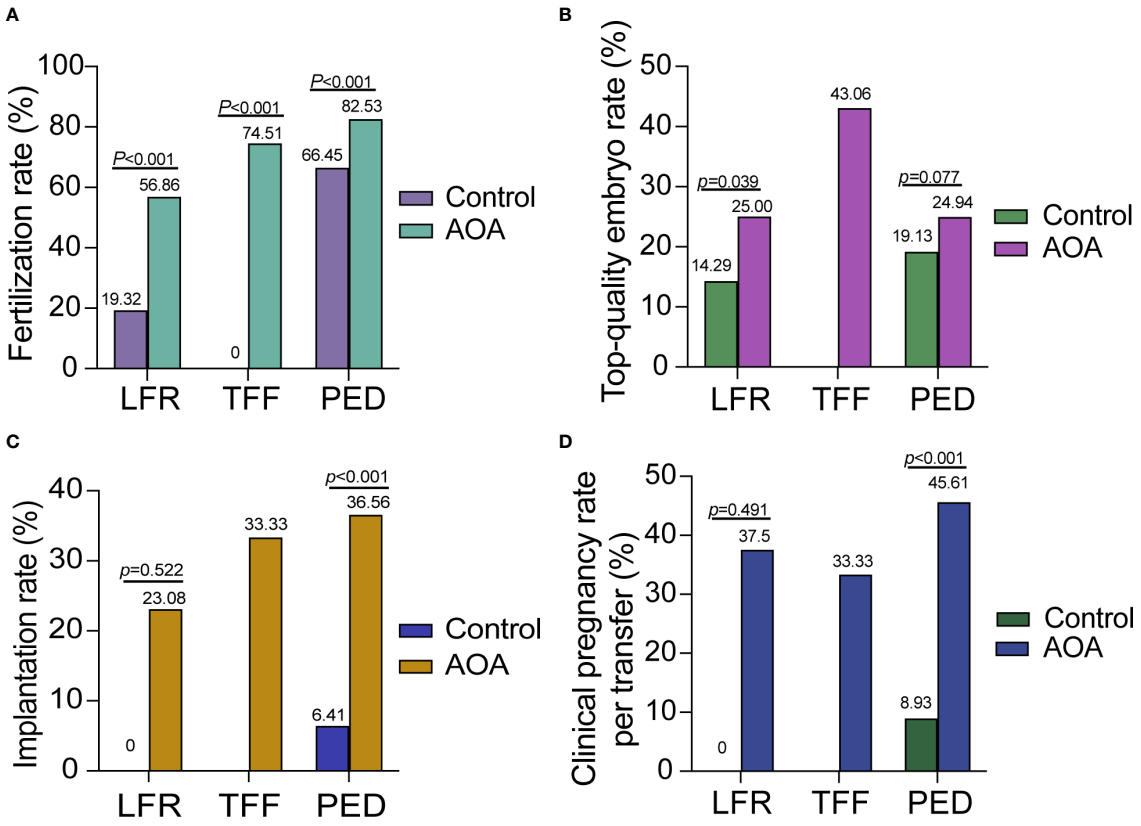


FIGURE 3
Comparison of activation effects in AOA subgroups based on the reason for AOA. **(A)** Comparison of fertilization rates between AOA subgroups and control groups. **(B)** Comparison of the top-quality embryo rate between AOA subgroups and control groups. **(C)** Comparison of the implantation rate between AOA subgroups and control groups. **(D)** Comparison of the clinical pregnancy rate between AOA subgroups and control groups. *Control* previous ICSI cycles; *AOA* artificial oocyte activation; *LFR* low fertilization; *TFF* total fertilization failure; *PED* poor embryo development.

potentially provide more good-quality embryos. Additionally, some studies have also demonstrated that AOA leads to better cumulative pregnancy and live birth rates. However, one study (22) indicated that although AOA can improve the fertilization rate, it might not improve the developmental ability of embryos and pregnancy outcomes. This discrepancy might be derived from the different activation methods used. In that study, double ionophores were applied in cases with previous failed/low fertilization or poor embryo development, and patients with a previous single ionophore were used as the control group.

TABLE 4 Neonatal characteristics in the AOA group.

Item	Value
Birth babies	35
Baby's gender	
Male, n	15
Female, n	20
Singletons, n	21
Twins, n	7
Gestational weeks (wk)	37.42 ± 3.200
Body length (cm)	48.26 ± 3.883
Birth weight (g)	2894.57 ± 773.143
Low birth weight rate (%) (<2,500 g)	28.57(10/35)
Preterm birth rate (%) (<37 wk)	25.71(9/35)
Birth defect rate (%)	2.86(1/35)

ICSI is the most effective method to treat male infertility, but complete fertilization failure will still occur occasionally in a few patients. Abnormal calcium oscillations after fertilization may be the principal mechanism for poor prognosis in patients with TFF. In this study, we found that the fertilization rate, implantation rate and clinical pregnancy rate were significantly increased in patients with TFF and LFR compared with previous ICSI cycles. AOA treatment with ionomycin can change the calcium oscillation pattern and improve the fertilization rate and clinical outcome of these patients. In the past 20 years, the application of AOA has successfully solved many infertility cases due to OAF caused by sperm factors, and Ca^{2+} ionophores have been successfully applied in cases of complete globozoospermia (23) or severe male factor infertility (24). Those results are consistent with our study, couples with OAT can benefit from ICSI-AOA in terms of higher fertilization rate, which in turn increases the number of available embryos and may improve the implantation and pregnancy rates. Recent researches found novel sperm-related oocyte activators, which showed that the ACTL7A/PLCZ1 mutation and DPY19

deletion decreased the expression of functional PLCζ, resulting in acrosomal detachment and oocyte activation failure associated with male infertility, and ICSI-AOA could rescue TFF in individuals with the ACTL7A/PLCZ1/DPY19 pathogenic variants (9, 25, 26). However, ICSI-AOA is not always beneficial for patients with previous LFR and a suspected oocyte-related activation deficiency (27), except Vanden Meerschaut F et al. reported that ICSI-AOA was very efficient in patients with a suspected oocyte-related activation deficiency and previous TFF after conventional ICSI. Our data demonstrated that the implantation rate, top-quality embryo rate and clinical pregnancy rate were not significantly different in the LFR group compared with previous ICSI cycles. AOA can cause calcium oscillation in oocytes, thereby improving fertilization rates in patients with LFR but may not improve embryo development and pregnancy outcomes in these patients.

Based on our study, we found that AOA treatment can improve the embryo development and reproductive outcome of patients with poor embryo development. This result is consistent with other studies, which demonstrated that AOA treatment can overcome the developmental incompetence of embryos and is an additional indication for ionophore treatment (14, 28). It was reported that the different frequencies and patterns of calcium oscillations could affect the induction of oocyte activation and embryo development (29). The calcium-binding proteins and intracellular calcium channels in the human endoplasmic reticulum that regulate inositol 1,4,5-trisphosphate receptors (IP3Rs) play an important role in the regulation of calcium signalling during oocyte maturation, fertilization, and early embryo development, and calcium signalling is a key factor in early embryo development (30). Therefore, AOA with calcium ionophores enhances calcium oscillations and may have beneficial effects on embryo development. However, one study on sibling oocytes showed that AOA using ionomycin 1 h after ICSI did not benefit the early or late development of embryos derived from patients with a history of embryo developmental problems (31). Admittedly, oocyte activation deficiencies are not the only reason for poor embryo development. Sperm DNA damage, oocyte abnormalities in structural proteins and mitochondria, or transcription factors may also contribute to poor prognosis of embryos (32), and none of them can be rescued by AOA.

We also observed that there was no significant difference in the top-quality embryo rate, embryo implantation rate or clinical pregnancy rate per patient in the advanced age and POI groups compared with the control group. These findings indicated that OAF might not be the main factor that affects infertility in advanced age and POI women. Problems with oocyte-derived and deficiencies in downstream signalling pathways may be the main cause of infertility in women of advanced age and POI (33). Furthermore, age-related defects may lead to mitochondrial DNA alignment, accompanied by mitochondrial dysfunction and changes in the gene expression profile (34). However, these studies suggested that AOA may not improve embryo development with advanced age and POI patients but may improve fertilization rates and thus potentially provide more transferable embryos (20). Vanden Meerschaut et al. also reported some cases of oocyte-related OAD, which could be overcome by AOA (27). In our study, the implantation rate was improved in patients with unexplained infertility, and the top-quality rate was also increased in patients with M+F.

We also found that the miscarriage rate in the AOA group was significantly lower than that in the control group. A prospective study demonstrated that the miscarriage rate was decreased in OAT, PCOS and unexplained infertility patients, which was in line with our study (35). Calcium oscillation is a landmark initial event in oocyte activation, and AOA can generate a single transient calcium oscillation, which may alter the activities of specific proteins and enzymes downstream of the Ca^{2+}-regulated pathway and may also have a profound impact on gene expression and development at the later stage of embryos.

Although AOA can improve the treatment outcome of patients with fertilization failure, as a new technology in the assisted reproduction field, its safety still needs more in-depth study. At present, most reports believe that AOA technology will not increase the probability of embryo chromosome abnormalities or the risk of foetal birth defects. Miller et al. compared 83 offspring born with AOA and 595 offspring born after ICSI and demonstrated that there was no significant difference between the two groups in the rate and type of birth defects, birth weight, gestational age, and foetal sex of the offspring (36). A meta-analysis also confirmed that there was no significant difference in congenital defects or types of congenital defects between ICSI-AOA and traditional ICSI (37). These reports were consistent with our study, which showed no statistically significant differences in the birth defect rate, birth weight, gestational age, preterm birth rate, early-neonatal death rate, and foetal sex ratio between the activation and control groups. However, most current studies all have small sample sizes, and we only followed up on early foetal health. Moreover, another study revealed that the application of high concentrations of ionomycin after mouse sperm ICSI increased the frequency and amplitude of Ca^{2+} release, affecting mitochondrial energy metabolism, increasing reactive oxygen species (ROS) and decreasing ATP, and blastocyst formation (38). Furthermore, the impact of AOA on the gene expression and epigenetics of early human embryos is still lacking large amounts of research, and thus, further studies are needed to evaluate the long-term effect of AOA on offspring.

There are still some limitations in our study. First, we performed a retrospective self-controlled study. The previous ICSI cycles of patients served as the control group, and ICSI-AOA was performed in the second cycle based on the previous condition of patients. Patients may not use the same regimen twice. The health status of patients may be improved by medications in the second cycles, and the number and quality of oocytes retrieved may be improved by adjusting the ovulation stimulation protocol. Undoubtedly, the best control group would comprise patients undergoing ICSI-AOA versus patients undergoing ICSI only, and the reproductive outcomes can be compared between the two groups. The reason why we did not undergo a second cycle without AOA was that once the patients had already failed in the first ICSI cycle and exhibited fertilization failure or poor embryo development, they usually asked for something to change in the

second cycle, and the physicians may probably recommend undergoing ICSI-AOA; thus, we lack a control group who are in a second cycle without AOA. Second, there may be some potential bias and confounders that cannot be excluded, and the POI, unexplained infertility and mixed factor infertility subgroups only had a small number of patients in each group; thus, the results should be interpreted with caution. Although the existing evidence was probably at a low level, this study might shed light on further research on AOA treatment, and another prospective, multicentre study with a larger sample size and parallel control would be needed in the future to provide a clearer answer.

In conclusion, our study demonstrated that AOA treated with ionomycin can obtain better reproductive outcomes in infertile couples with previous fertilization failure and poor embryo development. Subgroup analysis showed that couples with male/female factors and unexplained infertility may also benefit from AOA treatment in terms of improving fertilization rate and embryo development potential. However, before such procedures can be widely used in clinical practice, it is necessary to determine more physical agents and protocols that better mimic physiological Ca^{2+} release and conduct further research to evaluate the efficiency and safety of such treatments. At present, AOA should still be limited to application in patients with appropriate indications.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of Shanghai First Maternal and Infant hospital (KS23210). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JR was involved in study design, execution, data analysis, project administration, roles/writing-original manuscript. JP and SL were involved in data collection and management ZC was involved in methodology, review and editing, critical discussion. XT was involved in review and editing, supervision critical discussion, and final approval of the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Sun B, Yeh J. Calcium oscillatory patterns and oocyte activation during fertilization: a possible mechanism for total fertilization failure (TFF) in human. *In Vitro Fertilization? Reprod Sci* (2021) 28:639–48. doi: 10.1007/s43032-020-00293-5

2. ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine. Electronic address: coticchio.biogenesi@grupposandonato.it. The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reprod BioMed Online* (2017) 35:494–510. doi: 10.1016/j.rbmo.2017.06.015

3. Montag M, Köster M, van der Ven K, Bohlen U, van der Ven H. The benefit of artificial oocyte activation is dependent on the fertilization rate in a previous treatment cycle. *Reprod BioMed Online* (2012) 24:521–6. doi: 10.1016/j.rbmo.2012.02.002

4. Jones C, Meng X, Coward K. SPERM FACTORS AND EGG ACTIVATION: Phospholipase C zeta (PLCZ1) and the clinical diagnosis of oocyte activation deficiency. *Reproduction* (2022) 164:F53–66. doi: 10.1530/REP-21-0458

5. Dai J, Dai C, Guo J, Zheng W, Zhang T, Li Y, et al. Novel homozygous variations in *PLCZ1* lead to poor or failed fertilization characterized by abnormal localization patterns of PLCζ in sperm. *Clin Genet* (2020) 97:347–51. doi: 10.1111/cge.13636

6. Nomikos M, Yu Y, Elgmati K, Theodoridou M, Campbell K, Vassilakopoulou V, et al. Phospholipase Cζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril* (2013) 99:76–85. doi: 10.1016/j.fertnstert.2012.08.035

7. Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* (2010) 16:690–703. doi: 10.1093/humupd/dmq018

8. Meng X, Melo P, Jones C, Ross C, Mounce G, Turner K, et al. Use of phospholipase C zeta analysis to identify candidates for artificial oocyte activation: a case series of clinical pregnancies and a proposed algorithm for patient management. *Fertility Sterility* (2020) 114:163–74. doi: 10.1016/j.fertnstert.2020.02.113

9. Tavalaee M, Nasr-Esfahani MH. Expression profile of *PLC ζ*, *PAWP*, and *TR-KIT* in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. *Andrology* (2016) 4:850–6. doi: 10.1111/andr.12179

10. Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. *Reprod BioMedicine Online* (2014) 28:560–71. doi: 10.1016/j.rbmo.2014.01.008

11. Kilani S, Chapman MG. Meiotic spindle norMality predicts live birth in patients with recurrent in *vitro* fertilization failure. *Fertil Steril* (2014) 101:403–6. doi: 10.1016/j.fertnstert.2013.10.045

12. Yu Y, Nomikos M, Theodoridou M, Nounesis G, Lai FA, Swann K. PLCζ causes Ca(2+) oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P(2). *Mol Biol Cell* (2012) 23:371–80. doi: 10.1091/mbc.E11-08-0687

13. Yan Z, Fan Y, Wang F, Yan Z, Li M, Ouyang J, et al. Novel mutations in PLCZ1 cause male infertility due to fertilization failure or poor fertilization. *Hum Reprod* (2020) 35:472–81. doi: 10.1093/humrep/dez282

14. Chen ZQ, Wang Y, Ng EHY, Zhao M, Pan JP, Wu HX, et al. A randomized triple blind controlled trial comparing the live birth rate of IVF following brief inCubation versus standard inCubation of gametes. *Hum Reprod* (2019) 34:100–8. doi: 10.1093/humrep/dey333

15. Kong P, Liu Y, Zhu Q, Yin M, Teng X. Effect of male age on pregnancy and neonatal outcomes in the first frozen-thawed embryo transfer cycles of IVF/ICSI treatment. *Andrology* (2021) 9:1540–8. doi: 10.1111/andr.13031

16. Nasr-Esfahani MH, Razavi S, Javdan Z, Tavalaee M. Artificial oocyte activation in severe teratozoospermia undergoing intracytoplasmic sperm injection. *Fertil Steril* (2008) 90:2231–7. doi: 10.1016/j.fertnstert.2007.10.047

17. Jia L, Chen P, Su W, He S, Guo Y, Zheng L, et al. Artificial oocyte activation with ionomycin compared with A23187 among patients at risk of failed or impaired fertilization. *Reprod BioMed Online* (2023) 46:35–45. doi: 10.1016/j.rbmo.2022.08.105

18. Jia L, Chen P-Y, Guo Y-C, Zhang Z-Q, Gong X, Chen J-B, et al. Prediction of cumulative live birth rate in women aged 40 years and over undergoing *in vitro* fertilization/intracytoplasmic sperm injection. *Reprod Dev Med* (2020) 4:233–8. doi: 10.4103/2096-2924.305931

19. Ebner T, Montag MOocyte Activation Study Group, , Montag M, van der Ven K, van der Ven H, et al. Live birth after artificial oocyte activation using a ready-to-use ionophore: a prospective multicentre study. *Reprod BioMed Online* (2015) 30:359–65. doi: 10.1016/j.rbmo.2014.11.012

20. Li J, Zheng X, Lian Y, Li M, Lin S, Zhuang X, et al. Artificial oocyte activation improves cycles with prospects of ICSI fertilization failure: a sibling oocyte control study. *Reprod BioMed Online* (2019) 39:199–204. doi: 10.1016/j.rbmo.2019.03.216

21. Shebl O, Trautner PS, Enengl S, Reiter E, Allerstorfer C, Rechberger T, et al. Ionophore application for artificial oocyte activation and its potential effect on morphokinetics: a sibling oocyte study. *J Assist Reprod Genet* (2021) 38:3125–33. doi: 10.1007/s10815-021-02338-3

22. Shebl O, Reiter E, Enengl S, Allerstorfer C, Schappacher-Tilp G, Trautner PS, et al. Double ionophore application in cases with previous failed/low fertilization or poor embryo development. *Reprod BioMed Online* (2022) 44:829–37. doi: 10.1016/j.rbmo.2021.11.008

23. Taylor SL, Yoon SY, Morshedi MS, Lacey DR, Jellerette T, Fissore RA, et al. Complete globozoospermia associated with PLCζ deficiency treated with calcium ionophore and ICSI results in pregnancy. *Reprod BioMed Online* (2010) 20:559–64. doi: 10.1016/j.rbmo.2009.12.024

24. Ebner T, Köster M, Shebl O, Moser M, van der Ven H, Tews G, et al. Application of a ready-to-use calcium ionophore increases rates of fertilization and pregnancy in severe male factor infertility. *Fertil Steril* (2012) 98:1432–7. doi: 10.1016/j.fertnstert.2012.07.1134

25. Zhao S, Cui Y, Guo S, Liu B, Bian Y, Zhao S, et al. Novel variants in *ACTL7A* AND *PLCZ1* are associated with male infertility and total fertilization failure. *Clin Genet* (2023) 103:603–8. doi: 10.1111/cge.14293

26. Tavalaee M, Nomikos M, Lai FA, Nasr-Esfahani MH. Expression of sperm PLCζ and clinical outcomes of ICSI-AOA in men affected by globozoospermia due to DPY19L2 deletion. *Reprod BioMedicine Online* (2018) 36:348–55. doi: 10.1016/j.rbmo.2017.12.013

27. Vanden Meerschaut F, Nikiforaki D, De Gheselle S, Dullaerts V, Van den Abbeel E, Gerris J, et al. Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. *Hum Reprod* (2012) 27:1977–84. doi: 10.1093/humrep/des097

28. Tsai T-E, Lin P-H, Lian P-F, Li C-J, Vitale SG, Mikuš M, et al. Artificial oocyte activation may improve embryo quality in older patients with diminished ovarian reserve undergoing IVF-ICSI cycles. *J Ovarian Res* (2022) 15:102. doi: 10.1186/s13048-022-01036-7

29. Cooney MA, Malcuit C, Cheon B, Holland MK, Fissore RA, D'Cruz NT. Species-specific differences in the activity and nuclear localization of murine and bovine phospholipase C zeta 1. *Biol Reprod* (2010) 83:92–101. doi: 10.1095/biolreprod.109.079814

30. Yang YR, Follo MY, Cocco L, Suh P-G. The physiological roles of primary phospholipase C. *Adv Biol Regul* (2013) 53:232–41. doi: 10.1016/j.jbior.2013.08.003

31. Yin M, Li M, Li W, Wu L, Yan Z, Zhao J, et al. Efficacy of artificial oocyte activation in patients with embryo developmental problems: a sibling oocyte control study. *Arch Gynecol Obstet* (2022) 305:1225–31. doi: 10.1007/s00404-021-06329-8

32. Bashiri Z, Amidi F, Amiri I, Zandieh Z, Maki CB, Mohammadi F, et al. Male factors: the role of sperm in preimplantation embryo quality. *Reprod Sci* (2021) 28:1788–811. doi: 10.1007/s43032-020-00334-z

33. Jones KT, Lane SIR. Molecular causes of aneuploidy in mamMalian eggs. *Development* (2013) 140:3719–30. doi: 10.1242/dev.090589

34. Yin M, Yu W, Li W, Zhu Q, Long H, Kong P, et al. DNA methylation and gene expression changes in mouse pre- and post-implantation embryos generated by intracytoplasmic sperm injection with artificial oocyte activation. *Reprod Biol Endocrinol* (2021) 19:163. doi: 10.1186/s12958-021-00845-7

35. Lv M, Zhang D, He X, Chen B, Li Q, Ding D, et al. Artificial oocyte activation to improve reproductive outcomes in couples with various causes of infertility: a retrospective cohort study. *Reprod BioMed Online* (2020) 40:501–9. doi: 10.1016/j.rbmo.2020.01.001

36. Miller N, Biron-Shental T, Sukenik-Halevy R, Klement AH, Sharony R, Berkovitz A. Oocyte activation by calcium ionophore and congenital birth defects: a retrospective cohort study. *Fertil Steril* (2016) 106:590–6. doi: 10.1016/j.fertnstert.2016.04.025

37. Long R, Wang M, Yang QY, Hu SQ, Zhu LX, Jin L. Risk of birth defects in children conceived by artificial oocyte activation and intracytoplasmic sperm injection: a meta-analysis. *Reprod Biol Endocrinol* (2020) 18:123. doi: 10.1186/s12958-020-00680-2

38. Chen C, Sun T, Yin M, Yan Z, Yu W, Long H, et al. Ionomycin-induced mouse oocyte activation can disrupt preimplantation embryo development through increased reactive oxygen species reaction and DNA damage. *Mol Hum Reprod* (2020) 26:773–83. doi: 10.1093/molehr/gaaa056

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Assisted Oocyte Activation With Calcium Ionophore Improves Pregnancy Outcomes and Offspring Safety in Infertile Patients: A Systematic Review and Meta-Analysis

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This study aimed to evaluate the efficacy and safety of calcium ionophore during assisted oocyte activation (AOA). This meta-analysis contained randomized controlled trials and prospective observational and retrospective trials. The summary odds ratio (OR) with 95% confidence intervals (CIs) was calculated for clinical pregnancy rate and live birth rate. Both fixed and random effects models were applied. A total of 22 studies were included into this meta-analysis. Seventeen of the included studies showed that calcium ionophore increased the clinical pregnancy rate (OR, 2.14; 95% CI, 1.38–3.31). Similarly, 14 studies indicated that AOA with calcium ionophore during intracytoplasmic sperm injection (ICSI) improved the live birth rate considerably (OR, 2.65; 95% CI, 1.53–4.60). Moreover, fertilization, blastocyst formation, and implantation rate were higher after using AOA with calcium ionophore combined with ICSI. In addition, calcium ionophore did not increase top-quality embryo rate, cleavage rate, miscarriage rate, congenital birth defects, and neonatal sex ratio. Therefore, calcium ionophore followed by ICSI not only significantly improved live birth and overall pregnancy, but also did not affect the incidence of miscarriage, congenital birth defects, and neonatal sex ratio. This meta-analysis indicated that using calcium ionophore to activate oocytes was beneficial for couples with poor fertilization rates following ICSI.

Keywords: calcium ionophore, ICSI, miscarriage, congenital birth defects, pregnancy, assisted oocyte activation

INTRODUCTION

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are common techniques in the field of assisted reproduction (Hamberger et al., 1998; Kissin et al., 2014). The number of ICSI cycles has increased notably as the number of infertile individuals has increased, with average fertilization rates estimated to reach 70%, but fertilization failure still occurs in 1–5% of ICSI cycles (Bhattacharya et al., 2013; Johnson et al., 2013; ESHRE/Alpha, 2017). ICSI enables male factor infertility to be treated in fertilization. The failure of standard ICSI to fertilize patients with a sufficient number of oocytes (usually >3) is primarily due to oocyte activation failure, which may be interrelated with oocyte or sperm factors (Vanden Meerschaut et al., 2014; Bonte et al., 2019).

Oocyte activation is a spatial-temporal, regulated process, and caused by a series of intracellular Ca^{2+} oscillations from sperm entry into the ooplasm during sperm-oocyte fusion (Stricker, 1999; Swain and Pool, 2008). This process is triggered by sperm-borne phospholipase C zeta (PLC zeta) which is transported to the oocyte at sperm entry into the ooplasm (Saunders et al., 2002). Ca^{2+} oscillations are recognized as indispensable for successful oocyte activation, fertilization, and embryogenesis (Ramadan et al., 2012). Insufficient calcium may enable fertilization failure and cleavage anomalies. Thus, increasing appropriate calcium artificially during the ICSI period could avoid the above situation.

Currently, assisted oocyte activation (AOA) in conjunction with ICSI has been developed and broadly applied in improving clinical outcomes of male factor infertility, embryo developmental block, and patients who remain infertile after ICSI (Borges et al., 2009a; Kashir et al., 2010; Montag et al., 2012; Kang et al., 2015; Ferrer-Buitrago et al., 2018). AOA methods include electrical, mechanical, and chemical activation, of which calcium ionophore belonging to chemical AOA is the most universally used and most effective technique, and it activates oocytes not only by enhancing intracellular Ca^{2+} but also with the same recruiting method from the culture medium outside the oocyte (Kashir et al., 2010; Amdani et al., 2013; Nomikos et al., 2013; Vanden Meerschaut et al., 2014; Yeste et al., 2016). Calcimycin (Yoon et al., 2013; Ebner et al., 2014) and ionomycin (Deemeh et al., 2014) are two commonly used calcium ionophores. Calcimycin (also referred to as A23187) can be either home-made or a ready-to-use solution called GM508 CultActive (Gynemed).

Recently, calcium ionophore has been widely used in various centers and obtained satisfactory results. Yet, considering that calcium oscillation is consistent with the active and rapid demethylation of the paternal genome and passive DNA demethylation of the maternal genome, it appears reasonable that an altered calcium signal, as reported following calcium ionophore treatment, might be related to modifications in genomic imprinting (Nikiforaki et al., 2016). Moreover, several meta-analysis studies assessed the value of calcium ionophore during AOA in improving reproductive outcomes, and researchers found that ICSI-AOA played a positive role in enhancing fertilization rate and clinical pregnancy outcome (Sfontouris et al., 2015; Murugesu et al., 2017). However, the small number of research samples reduces the credibility of AOA effectiveness. Identifying and evaluating more cases would contribute to more reliable conclusions.

The effect of ICSI-AOA on post-implantation embryo development and pregnancy effectiveness must be taken into consideration deeply. With the widespread application of chemical activators, their safety in offspring has attracted more and more attention. In this meta-analysis, we aim to identify, appraise, and assess the efficacy and safety of calcium ionophore as a method of AOA in promoting pregnancy outcomes and reducing miscarriage and congenital birth defects.

MATERIALS AND METHODS

This meta-analysis and systematic review was conducted and reported according to PRISMA guidelines.

Search Strategy

PubMed, Embase, Scopus, and Cochrane Library databases were searched up to February 2020. The language and publication date were not limited. The following Medical Subject Headings (MeSH) terms and free-text terms were employed in searching the databases. MeSH terms included infertility and calcium ionophores. Free-text terms consisted of calcium ionophore, ionophores, calcium, A23187, calcimycin, ionmycin, AOA, oocyte activation, eggs activation, artificial eggs activation, assisted eggs activation, sterility reproductive, sterility, reproductive, subfertility, sub-fertility, infertility, ICSI, IVF, embryo transfer, blastocyst, miscarriage, pregnancy, and birth and congenital birth defects. The terms were combined with "AND" or "OR" Boolean operators. We expanded the search by using the "related article" function. Furthermore, we manually searched the reference list to identify any articles not captured by electronic database searches. Any search filters or limitations were not used in this study. In addition, full-text articles were authorized for inclusion. Using the aforementioned strategies, two groups of ICSI-AOA with calcium ionophore and ICSI-only (non-AOA) were compared and identified, and related data were extracted.

Eligibility Criteria

All randomized controlled trials (RCTs) and observational studies involving comparison between "ICSI-AOA with calcium ionophore" with "ICSI-only (non-AOA)" patient groups and also those containing outcome indicators were included in this study. We excluded non-controlled studies that only reported outcome incidence and did not report outcome indicators.

Data Extraction

Data collection of each study was completed by two independent reviewers (YS and HZ). Then a standardized Excel spreadsheet was used for performing quantitative data entry and processing. The accuracy of the collected and extracted data was acknowledged by a third reviewer (DZ). The content of data extraction was as follows: characteristics (author, publication year, country, study type, study period, study population, and oocyte activation); study groups (ICSI-AOA infertility patients with calcium ionophore vs. ICSI-only (non-AOA) control group); and outcome indicators (fertilization, cleavage, top-quality embryo, blastocyst, implantation, miscarriage, clinical pregnancy, live births, congenital birth defects, and neonatal sex ratio). The detailed information is summarized in **Table 1**. When multiple publications were involved in the same patient cohort, we chose the largest sample study.

Risk of Bias and Quality Assessment

Five RCTs and 17 observational studies that evaluated the association between AOA with calcium ionophore and clinical outcome were included in this study. The quality assessment of RCTs was performed through the Cochrane risk of bias

TABLE 1 | Study characteristics.

No.	References	Country	Study type	Study period	Study population	Oocyte activation protocol	Control	Cases number	Outcome indicators
1	Li J. et al. (2019)	China	Sibling oocytes control study-prospective cohort study	Oct 2015–Dec 2017	Previous ICSI cycles of no or low fertilization or were diagnosed with severe teratozoospermia	Calcium ionophore (ionomycin) exposure twice for 5 min with a 30 min interval	Sibling oocytes	50 patients Survival MII oocytes after ICSI: 513 oocytes AOA: 262 oocytes; control: 251 oocytes	Fertilization rate, Cleavage rate, blastocyst rate, Implantation rate, Pregnancy rate, Live-birth rate, Abortion rate, Fetal defect
2	Bonte et al. (2019)	Belgium	Retrospective cohort study	Apr 2001–Apr 2018	Previous ICSI cycles of no or low fertilization (≤33.3%)	$CaCl_2$ injection and Calcium ionophore (ionomycin) exposure twice for 10 min with a 30 min interval	Before-after self contrast	122 couples AOA: 191 cycles, 2,476 oocytes; control: 243 cycles, 2351 oocytes	Fertilization rate, Pregnancy rate, Live-birth rate, Abortion rate, Fetal defect
3	Li B. et al. (2019)	China	Retrospective cohort study	Jan 2011–Dec 2016	(1) ICSI fertilization rate ≤50; (2) good quality embryo rate ≤30%; (3) severe oligoasthenoteratozoospermia (OAT); (4) TESA or PESA	Calcium ionophore (ionomycin) exposure for 10 min	Conventional ICSI	AOA:169 patients control:507 patients	Implantation rate, Pregnancy rate, Live-birth rate, Abortion rate, Fetal defect
4	Fawzy et al. (2018)	Egypt	RCT	Apr 2015–Jan 2016	Two previous ICSI cycles of low fertilization (0–30%) or with male-factor infertility undergoing their first ICSI cycle	Group I ($SrCl_2$ for 60 min post-ICSI) group II (GM508 CultActive for 20 min post-ICSI)	Randomized controlled	343 participants (SrCl2 AOA:115, calcimycinAOA:113 and ICSI:115)	Fertilization rate, Cleavage rate, top Embryo rate, Blastocyst rate, Implantation rate, Pregnancy rate, Live-birth rate, Abortion rate
5	Mateizel et al. (2018)	Belgium	Single-center observational study- prospective cohort study	2004–2015	Low ($n = 14$) or no ($n = 16$) fertilization in previous cycles, globozoospermia ($n = 6$), small acrosome ($n = 2$), and poor embryo development ($n = 1$)	$CaCl_2$ injection and Calcium ionophore (ionomycin) exposure twice for 7 min with a 30 min interval or calcium ionophore (GM508 CultActive) exposure for 15 min	Conventional ICSI	237 cycles, 74 pregnancies with ICSI+AOA, 47 newborns.	Fetal defect
6	Miller et al. (2016)	Israel	Retrospective cohort study	2006–2014	Failed fertilization after one ICSI procedure in the presence of at least five mature oocytes without oocyte abnormality or had <10% fertilization rate	Calcium ionophore (A23187) exposure for 10 min	Conventional ICSI	AOA: 83 pregnancies; ICSI: 595 pregnancies.	Abortion rate, fetal defect
7	Aydinuraz et al. (2016)	Turkey	Prospective, randomized and single blind study	Dec 2013–Feb 2014	Teratozoospermia (<4% according to WHO 2010 reference limits) and a low fertilization rate (<50%) in the previous cycle	Calcium ionophore (GM508 CultActive) exposure for 15 min	Sibling oocytes	21 couples AOA: 97 MII oocytes; Control: 97 MII oocytes	Fertilization rate, cleavage rate, top embryo rate, implantation rate, pregnancy rate, live-birth rate, abortion rate
8	Dayong Hao et al. (2016)	China	Sibling oocytes control study	Jan 2015–Dec 2015	Previous ICSI cycles of low fertilization ≤30%, globozoospermia, or poor embryo development	Calcium ionophore (A23187) exposure for 15 min	Sibling oocytes	AOA: 12 patients; ICSI: 12 patients	Fertilization rate, top embryo rate, fetal defect
9	Ebner et al. (2015)	Germany/ Austria	Prospective multicentre study	Duration almost 2-year	Previous ICSI cycles of low fertilization <50%, at least three cumulus–oocyte complexes	Calcium ionophore (GM508 CultActive) exposure for 15 min	Before-after self contrast	AOA: 101 cycles; Control: 101 cycles	Fertilization rate, blastocyst rate
10	Caglar Aytac et al. (2015)	Turkey	Prospective, randomized controlled study	Jan 2014–Aug 2014	DOR and partners with normal sperm parameters	Calcium ionophore (GM508 CultActive) exposure for 15 min	Randomized controlled	AOA: 148 patients; Control: 148 patients	Fertilization rate, cleavage rate, pregnancy rate, live-birth rate
11	Kang et al. (2015)	Korea	Retrospective study	Jan 2006–Jun 2013	Previous ICSI cycles of no or low fertilization (<45%)	Calcium ionophore (A23187) exposure for 5 min	Conventional TESE	AOA: 29 cycles; Control: 480 cycles	Pregnancy rate, live-birth rate

(Continued)

TABLE 1 | Continued

No.	References	Country	Study type	Study period	Study population	Oocyte activation protocol	Control	Cases number	Outcome indicators
12	Ebner et al. (2014)	Austrian	Prospective, multicenter, uncontrolled intervention study	Duration 1 year	Complete developmental arrest (no transfer), or complete developmental delay (no morula/blastocyst on Day 5), or significantly reduced blastocyst formation (≤15%) in a previous cycle	Calcium ionophore (GM508 CultActive) exposure for 15 min	Before-after self contrast	AOA: 57 cycles; Control: 57 cycles	Fertilization rate, cleavage rate, top embryo rate, blastocyst rate, implantation rate, pregnancy rate, live-birth rate, abortion rate, fetal defect
13	Deemeh et al. (2014)	Iran	Historical cohort study	Feb 2008–May 2010	(1) Testicular sperm extraction (TESE); (2) severe teratozoospermia (sperm with abnormal morphology ≥98%)	Calcium ionophore (ionomycin) exposure for 10 min	Conventional ICSI	AOA-ICSI: 275 cycle (TESE: 150, severe teratozoospermia: 125); Control: 406 cycle	Pregnancy rate, live-birth rate, abortion rate, fetal defect
14	Yoon et al. (2013)	America	Retrospective study	2007–2011	Previous ICSI cycles of no or low fertilization (≤50%)	Calcium ionophore (A23187) exposure for 30 min	Before-after self contrast	AOA: 185 cycles; Control: 185 cycles	Implantation rate, pregnancy rate, live-birth rate, abortion rate, fetal defect
15	Eftekhar et al. (2013)	Iran	Prospective, randomized, unblinded, clinical trial	Apr 2012–Dec 2012	Teratoospermic partner (normal morphology <14%) undergoing to ICSI cycles	Calcium ionophore(A23187) exposure for 5 min	Randomized controlled	AOA: 19 patients; ICSI:19 patients	Pregnancy rate, live-birth rate, abortion rate
16	Ebner et al. (2012)	Germany/ Austria	Prospective multicenter study	Aug 2009–Mar 2011	Patients with azoospermia (44%) or cryptozoospermia (56%) (e.g., showing <0.1 spermatozoa/mL)	Calcium ionophore (GM508 CultActive) exposure for 15 min	Before-after self contrast	AOA: 75 cycles; Control: 88 cycles	Fertilization rate, blastocyst rate, implantation rate, pregnancy rate, live-birth rate, fetal defect
17	Montag et al. (2012)	Germany	Retrospective cohort study	2003–2009	Patients with 0, 1–29, or 30–50% fertilization in a previous ICSI cycle	Calcium ionophore (A23187) exposure for 15 min	Before-after self contrast	AOA: 129 cycles; Control: 103 cycles	Fertilization rate, implantation rate, pregnancy rate, live-birth rate, abortion rate, fetal defect
18	Vanden Meerschaut et al. (2012)	Belgium	Prospective case series	Jan 2006–Dec 2011	Previous ICSI cycles of no or low fertilization in whom the MOAT >84%	$CaCl_2$ injection and Calcium ionophore (ionomycin) exposure twice for 10 min with a 30 min interval	Sibling oocytes	AOA: 14patients Control: 14 patients	Fertilization rate
19	Borges et al. (2009a)	Brazil	RCT	Jan 2006–Jul 2007	(1) Testicular sperm aspiration in non-obstructive-azoospermic patients (TESA-NOA group, $n = 58$); (2) TESA in obstructive-azoospermic patients (TESA-OA group, $n = 48$); (3) and percutaneous epididymal sperm aspiration in obstructive-azoospermic patients (PESA-OA, $n = 98$)	Calcium ionophore (A23187) exposure for 30 min	Randomized controlled	TESA-NOA AOA: 29 cycles; Control: 29 cycles; TESA-OA control: 24 cycles; AOA: 24 cycles; PESA-OA control: 49 cycles; AOA: 49cycles	Fertilization rate, top embryo rate, implantation rate, pregnancy rate
20	Borges et al. (2009b)	Brazil	RCT	Jan 2006–Jul 2007	The ejaculated group ($n = 92$), the epididymal group ($n = 82$), and the testicular group ($n = 140$)	Calcium ionophore (A23187) exposure for 30 min	Randomized controlled	Ejaculated AOA: 46 cycles; Control: 46 cycles Epididymal; AOA: 41 cycles; Control: 41 cycles; Testicular AOA: 70 cycles; Control:70 cycles	Fertilization rate, top embryo rate, implantation rate, pregnancy rate, abortion rate

(Continued)

TABLE 1 | Continued

No.	References	Country	Study type	Study period	Study population	Oocyte activation protocol	Control	Cases number	Outcome indicators
21	Heindryckx et al. (2008)	Belgium	Prospective cohort study	None	Failed or low fertilization in previous ICSI cycles or who had well-known sperm-borne activation deficiencies such as globozoospermia	$CaCl_2$ injection and Calcium ionophore (ionomycin) exposure twice for 10 min with a 30 min interval	Before-after self contrast	AOA: 30 patients; Control: 30 patients	Fertilization rate, pregnancy rate, live-birth rate, fetal defect
22	Kyono et al. (2012)	Japan	Retrospective study	1 April 2004 and 31 October 2010	Previous ICSI cycles of low fertilization (≤30%)	Group I (SrCl2 for 60–120 min post-ICSI) group II (A23187 5–10 min post-ICSI)	Fetal defect: conventional ICSI Fertilization rate/Pregnancy rate/Implantation rate/abortion rate: before-after self contrast	SrCl2: 35 patients A23187: 50 patients; Conventional ICSI: 530 patients	Fertilization rate, pregnancy rate, implantation rate, abortion rate, fetal defect

assessment tool (Higgins, 2011). Seven domains were considered in the risk of bias assessment: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias. The risk evaluation result was categorized as "low risk (with less than two high-risk components)," "unclear risk (with more than three unclear-risk components)," or "high risk (with more than four high-risk components)" of bias.

The risk of observational studies was evaluated using the Newcastle-Ottawa Quality Assessment Scale (NOS), which used a score system based on three categories: selection of subjects, comparability of study groups, and the assessment of exposure. According to the NOS score, we graded the studies as low quality (0–3 points), medium quality (4–6 points), and high quality (7–9 points). The maximum score is 9 points. The above quality assessment was undertaken by two reviewers (JW and YC) independently, and further confirmed by a third reviewer (YS). A funnel plot was used to assess publication bias.

Outcomes of Interest

The main purpose of this meta-analysis was to appraise the efficacy and safety of AOA with calcium ionophore in combination with ICSI compared with the regular ICSI process, including clinical pregnancy rate and live birth rate. The secondary outcomes of interest were: fertilization rate, cleavage rate, top-quality embryo rate, blastocyst formation rate, implantation rate, miscarriage rate, congenital birth defects, and neonatal sex ratio. The explanation of each noun is shown as follows (Zegers-Hochschild et al., 2009; ESHRE/Alpha, 2017): Clinical pregnancy means a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs to define clinical signs of pregnancy. Clinical pregnancy rate is the number of clinical pregnancies expressed per 100 embryo transfer cycles. Fertilization rate is 2PN zygotes divided by the number of injected mature oocytes. Cleavage rate is defined as the proportion of zygotes which cleave into embryos on day 2 post-insemination. Top-quality embryo rate is defined as the proportion of day 3 embryos with a high score or grade. Blastocyst formation rate is defined as the proportion of 2PN zygotes which are at the blastocyst stage at day 5 or day 6. Implantation rate is the number of gestational sacs observed divided by the number of embryos transferred. Live birth rate is defined as the number of deliveries that resulted in at least one live born baby, expressed per 100 embryo transfer cycles. Congenital birth defects refer to all structural, functional, and genetic anomalies diagnosed in the neonatal period.

Statistical Analysis

A direct comparison meta-analysis was reported via odds ratio (OR) with 95% confidence intervals (CIs). We measured pooled ORs through a fixed effects model and calculated heterogeneity by Q-test and I^2 statistics. A random effects model was employed instead when significant heterogeneity appeared with $P < 0.1$ and $I^{2>}$ 50%. Both fixed and random effects models were applied in this study. We conducted subgroup analyses based on clinical

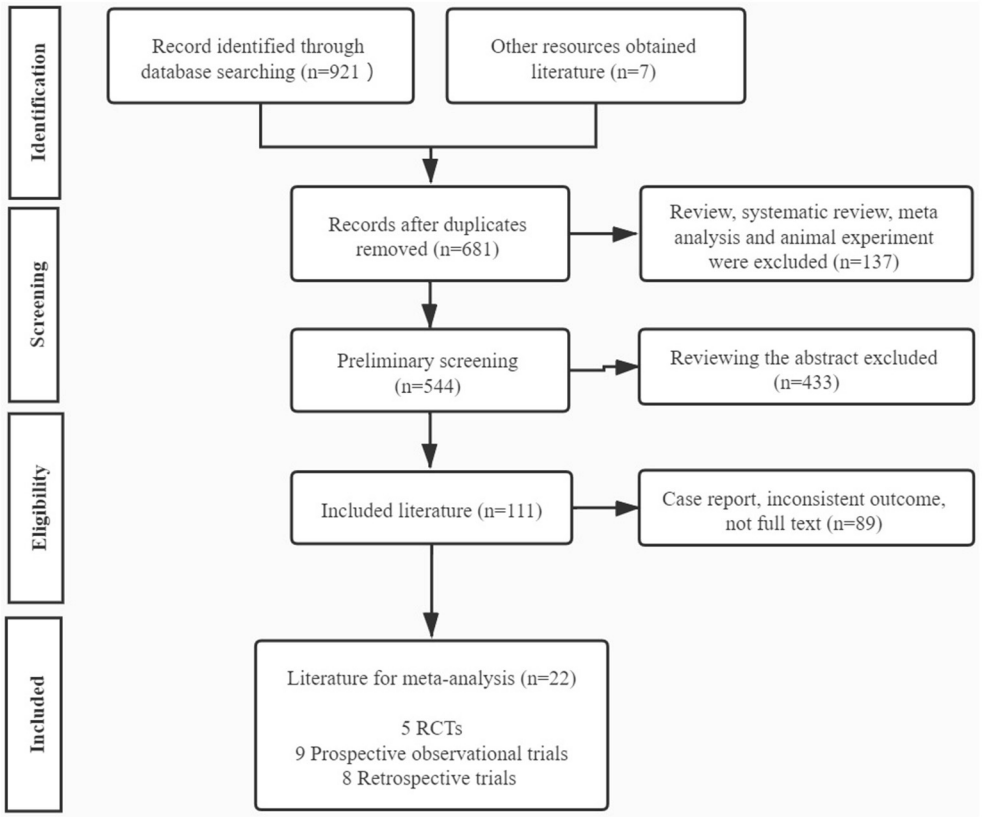


FIGURE 1 | Flowchart of the search method and selection.

diversity and performed two subgroup analyses. Subgroup analyses were stratified into two categories: "previous fertilization failure or low fertilization rate" and "embryo developmental problems [embryonic development block or sperm factor or diminished ovarian reserve (DOR)]." All statistical analysis was accomplished using Review Manager Version 5.3 statistical software and STATA 13.0.

RESULTS

Study Selection and Characteristics

Our study initially yielded 921 articles via the database search and 7 articles through other resources, of which 681 studies were reserved after removing the duplicates. Then 137 articles including reviews, meta-analysis, and animal experiments were excluded. A total of 544 articles were initially screened out, and 433 articles were excluded by reading abstracts. Subsequently, 111 papers conformed to our eligibility criteria, of which 89 studies were precluded for the following reasons: case report, inconsistent outcome, or no full text. Eventually, 22 full-text articles were included in our meta-analysis, consisting of 5 RCTs (Borges et al., 2009a,b; Eftekhar et al., 2013; Caglar Aytac et al., 2015; Fawzy et al., 2018), 9 prospective observational trials (Heindryckx et al., 2008; Ebner et al., 2012, 2014, 2015; Vanden Meerschaut et al., 2012; Aydinuraz et al., 2016; Dayong Hao et al., 2016; Mateizel et al., 2018; Li J. et al., 2019), and 8 retrospective trials (Kyono et al., 2012; Montag et al., 2012; Yoon et al., 2013; Deemeh et al., 2014; Kang et al., 2015; Miller et al., 2016; Bonte et al., 2019; Li B. et al., 2019; **Table 1**). The flowchart of literature identification and selection is summarized in **Figure 1**.

Methodological Quality Assessment

The quality of these five RCTs was evaluated using the Cochrane Risk of Bias tool. Only one study was considered to have high quality, and the remaining four articles all reached a moderate risk of bias (**Supplementary Figure 1**). The RCTs were regularly graded as high risk with complete blinding. To assess the quality of the included observational studies, the NOS was adopted to divide the studies into good, fair, and poor. The great majority of articles indicated good quality at a low risk of bias (six studies scored 9 points, three studies scored 8 points, and five studies scored 7 points), and the other three studies were recognized as medium quality at moderate risk (two studies scored 6 points and one study scored 5 points). The overall quality of the observational studies was high (**Supplementary Table 1**). Sensitivity analyses and publication bias assessments were executed for remarkable heterogeneity among studies.

Primary Outcome

Pregnancy Rate

In this analysis, 17 studies investigated the effect of AOA with calcium ionophore on the pregnancy rate especially (Heindryckx et al., 2008; Borges et al., 2009a,b; Ebner et al., 2012, 2014; Kyono et al., 2012; Montag et al., 2012; Eftekhar et al., 2013; Yoon et al., 2013; Deemeh et al., 2014; Caglar Aytac et al., 2015; Kang et al., 2015; Aydinuraz et al., 2016; Fawzy et al., 2018; Bonte et al., 2019; Li B. et al., 2019; Li J. et al., 2019). It was 34.87% (649/1,861) in the ICSI-AOA with calcium ionophore group and 33.68% (799/2,372) in the non-AOA group. Patients undergoing AOA with calcium ionophore had a higher pregnancy rate compared with the control (OR 2.14; 95% CI, 1.38–3.31; $P = 0.0006$; $I^2 = 82\%$; **Figure 2A**). The funnel plot of pregnancy rate was not asymmetric and Egger's test showed no statistically significance ($P = 0.064$), which suggested no publication bias (**Supplementary Figure 2A**). Considering the heterogeneity was statistically significant ($P < 0.00001$; $I^2 = 82\%$), we conducted subgroup analyses. In the "previous fertilization failure or low fertilization rate" subgroup (OR 3.79; 95% CI, 1.90–7.59; $P = 0.0002$; $I^2 = 67\%$) and "embryo developmental problems (embryonic development block or sperm factor or DOR)" subgroup (OR 2.28; 95% CI, 1.21–4.29; $P = 0.01$; $I^2 = 73\%$), the results illustrated that AOA with calcium ionophore uncommonly enhanced the pregnancy rate (**Figure 2B**) in both subgroups, which indicated that the included study populations were not the main sources of variance in the pregnancy rate. The funnel plot showed no publication bias (**Supplementary Figure 2B**).

Live Birth Rate

A total of 14 studies reported the live birth rate (Heindryckx et al., 2008; Ebner et al., 2012, 2015; Montag et al., 2012; Eftekhar et al., 2013; Yoon et al., 2013; Deemeh et al., 2014; Caglar Aytac et al., 2015; Kang et al., 2015; Aydinuraz et al., 2016; Fawzy et al., 2018; Bonte et al., 2019; Li B. et al., 2019; Li J. et al., 2019). The analysis showed that AOA with calcium ionophore heightened the offspring live birth rate. The funnel plots showed obvious publication bias among these articles (**Supplementary Figure 3A**). Owing to the I^2 value (80%) representing high heterogeneity among the included studies, the pooled OR was performed through a random effects model (OR 2.65; 95% CI, 1.53–4.60; $P = 0.0005$; $I^2 = 80\%$; **Figure 3A**), and further subgroup analyses were executed. Moreover, the heterogeneity reduced (OR 3.23; 95% CI, 2.31–4.52; $P < 0.00001$; $I^2 = 29\%$) and publication bias disappeared when we excluded the five low-quality studies (Ebner et al., 2012; Kang et al., 2015; Mateizel et al., 2018; Bonte et al., 2019; Li B. et al., 2019; **Figure 3B**), and the results were consistent with before the elimination. In subgroup analyses, consistent results revealed that AOA with calcium ionophore significantly increased the birth rate of re-assisted pregnancy in the "previous fertilization failure or low fertilization rate" subgroup (OR 4.76; 95% CI, 2.01–11.25; $P = 0.0004$; $I^2 = 65\%$) and "embryo developmental problems (embryonic development block or sperm factor or DOR)" subgroup (OR 4.59; 95% CI, 1.35–15.65; $P = 0.01$; $I^2 = 72\%$; **Figure 3C**), and no publication bias appeared (**Supplementary Figure 3B**). Hence, the high heterogeneity in this part was caused by the design and quality of studies but not the fact that included studies recruited diverse study populations.

Secondary Outcomes

Fertilization Rate

Fifteen articles investigated the effect of AOA with calcium ionophore on fertilization rate (Heindryckx et al., 2008; Borges et al., 2009a,b; Ebner et al., 2012, 2014, 2015; Kyono et al., 2012; Montag et al., 2012; Vanden Meerschaut et al., 2012; Caglar Aytac et al., 2015; Aydinuraz et al., 2016; Dayong Hao et al., 2016; Fawzy et al., 2018; Bonte et al., 2019; Li J. et al., 2019). It was 62.92% (6,851/10,888) in the ICSI-AOA group and 38.93% (3,772/9,690) in the non-AOA group. AOA with calcium ionophore significantly raised the fertilization rate of patients re-undergoing ICSI (OR 2.54; 95% CI, 1.52–4.24; $P = 0.0004$; $I^2 = 98\%$; **Figure 4A**). The funnel plot showed obvious publication bias among the included studies (**Supplementary Figure 4A**). Considering the extremely high heterogeneity, we conducted subgroup analyses. They illustrated that for the "previous fertilization failure or low fertilization rate" subgroup, AOA with calcium ionophore significantly enhanced the fertilization rate (OR 3.77; 95% CI, 2.28–6.23; $P < 0.00001$; $I^2 = 97\%$); whereas, for the "embryo developmental problems (embryonic development block or sperm factor or DOR)" subgroup, AOA with calcium ionophore did not influence the fertilization rate (OR 1.18; 95% CI, 0.64–2.14; $P = 0.60$; $I^2 = 96\%$; **Figure 4B**, **Supplementary Figure 4B**). These results demonstrated a significant association between fertilization rate and types of people included.

Cleavage Rate

Five studies reported the cleavage rate (Ebner et al., 2014; Caglar Aytac et al., 2015; Aydinuraz et al., 2016; Fawzy et al., 2018; Li J. et al., 2019). It was 93.72% (2,702/2,883) in the AOA treatment group and 85.92% (1,300/1,513) in the control group. Employing the random effects model, this difference was not discovered to be of statistical significance: OR 1.68; 95% CI, 0.90–3.13; $P = 0.10$; $I^2 = 74\%$ (**Figure 5**, **Supplementary Figure 5**). The results indicated that there was no association between cleavage rate and AOA with calcium ionophore treatment.

Top-Quality Embryo Rate

Six studies explored the effect of AOA with calcium ionophore on top-quality embryos (Borges et al., 2009a,b; Ebner et al., 2014; Aydinuraz et al., 2016; Dayong Hao et al., 2016; Fawzy et al., 2018). The top-quality embryo rate was 62.70% (2,264/3,611) in the AOA treatment group and 49.78% (1,222/2,455) in the control group. Using the random effects model, we found AOA with calcium ionophore could not improve the top-quality embryo rate (OR 1.15; 95% CI, 0.72–1.82; $P = 0.55$; $I^2 = 92\%$; **Figure 6**, **Supplementary Figure 6**).

Blastocyst Formation Rate

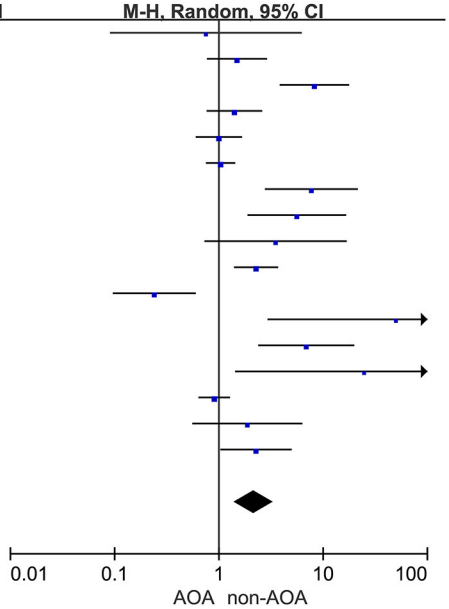
Five studies represented the related information about blastocyst formation (Ebner et al., 2012, 2014, 2015; Fawzy et al., 2018;

A

Study or Subgroup	AOA Events	AOA Total	non-AOA Events	non-AOA Total	Weight	Odds Ratio M-H, Random, 95% CI
Aydinuraz 2016	2	6	4	10	2.9%	0.75 [0.09, 6.23]
Aytac 2015	31	101	19	83	7.3%	1.49 [0.77, 2.90]
Bonte 2019	82	225	8	123	6.9%	8.24 [3.83, 17.74]
Borges1 2009	32	102	25	102	7.4%	1.41 [0.76, 2.60]
Borges2 2009	39	157	39	157	7.8%	1.00 [0.60, 1.67]
Deemeh 2014	93	275	134	406	8.3%	1.04 [0.75, 1.43]
Ebner 2012	25	73	5	79	5.9%	7.71 [2.76, 21.52]
Ebner 2014	23	51	5	39	5.6%	5.59 [1.88, 16.59]
Eftekhar 2013	7	17	3	18	4.1%	3.50 [0.73, 16.85]
Fawzy 2018	104	228	31	115	7.9%	2.27 [1.40, 3.70]
Hee JK 2015	6	29	250	480	6.3%	0.24 [0.10, 0.60]
Heindryckx 2008	20	61	0	50	1.9%	49.89 [2.93, 849.93]
Hye JY 2013	49	170	4	72	5.7%	6.88 [2.38, 19.90]
Koichi Kyono 2012	15	59	0	35	1.8%	24.73 [1.43, 427.74]
Li B 2019	81	169	256	507	8.3%	0.90 [0.64, 1.28]
Li JS 2019	10	26	6	24	5.2%	1.88 [0.56, 6.32]
Montag 2012	30	112	10	72	6.8%	2.27 [1.03, 4.99]
Total (95% CI)		**1861**		**2372**	**100.0%**	**2.14 [1.38, 3.31]**
Total events	649		799			

Heterogeneity: Tau² = 0.57; Chi² = 90.12, df = 16 (P < 0.00001); I² = 82%

Test for overall effect: Z = 3.42 (P = 0.0006)



B

Study or Subgroup	AOA Events	AOA Total	non-AOA Events	non-AOA Total	Weight	Odds Ratio M-H, Random, 95% CI
1.7.1 previous ICSI cycles of no or low fertilization						
Aydinuraz 2016	1	6	4	10	2.9%	0.30 [0.02, 3.63]
Bonte 2019	82	225	8	123	9.0%	8.24 [3.83, 17.74]
Fawzy 2018	104	228	31	115	10.4%	2.27 [1.40, 3.70]
Heindryckx 2008	20	61	0	50	2.3%	49.89 [2.93, 849.93]
Hye JY 2013	49	170	4	72	7.5%	6.88 [2.38, 19.90]
Koichi Kyono 2012	15	59	0	35	2.3%	24.73 [1.43, 427.74]
Li JS 2019	10	26	6	24	6.7%	1.88 [0.56, 6.32]
Montag 2012	30	112	10	72	8.9%	2.27 [1.03, 4.99]
Subtotal (95% CI)		**887**		**501**	**50.1%**	**3.79 [1.90, 7.59]**
Total events	311		63			

Heterogeneity: Tau² = 0.54; Chi² = 21.43, df = 7 (P = 0.003); I² = 67%

Test for overall effect: Z = 3.77 (P = 0.0002)

1.7.2 embryo developmental problems						
Aytac 2015	31	101	19	83	9.6%	1.49 [0.77, 2.90]
Borges1 2009	32	102	25	102	9.8%	1.41 [0.76, 2.60]
Borges2 2009	39	157	39	157	10.3%	1.00 [0.60, 1.67]
Ebner 2012	25	73	5	79	7.7%	7.71 [2.76, 21.52]
Ebner 2014	23	51	5	39	7.3%	5.59 [1.88, 16.59]
Eftekhar 2013	7	17	3	18	5.2%	3.50 [0.73, 16.85]
Subtotal (95% CI)		**501**		**478**	**49.9%**	**2.28 [1.21, 4.29]**
Total events	157		96			

Heterogeneity: Tau² = 0.42; Chi² = 18.49, df = 5 (P = 0.002); I² = 73%

Test for overall effect: Z = 2.54 (P = 0.01)

Total (95% CI)		**1388**		**979**	**100.0%**	**2.96 [1.83, 4.81]**
Total events	468		159			

Heterogeneity: Tau² = 0.52; Chi² = 48.77, df = 13 (P < 0.00001); I² = 73%

Test for overall effect: Z = 4.40 (P < 0.0001)

Test for subgroup differences: Chi² = 1.14, df = 1 (P = 0.29), I² = 12.1%

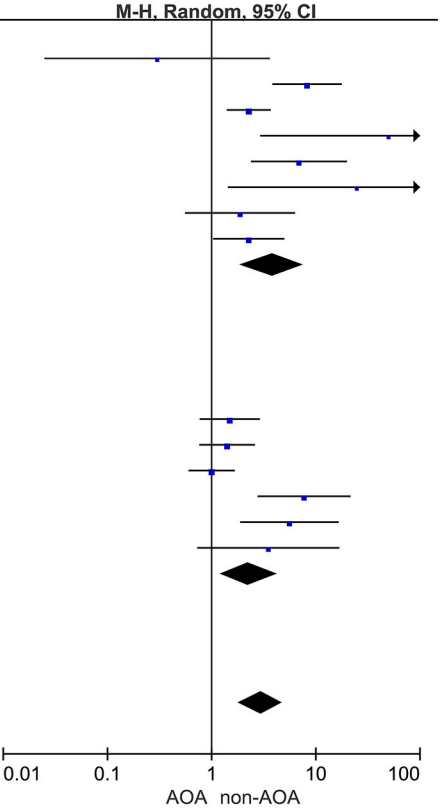


FIGURE 2 | (A) Forest plot of pregnancy rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore. **(B)** The subgroup analysis of pregnancy rate between AOA treatment and non-AOA treatment patient groups.

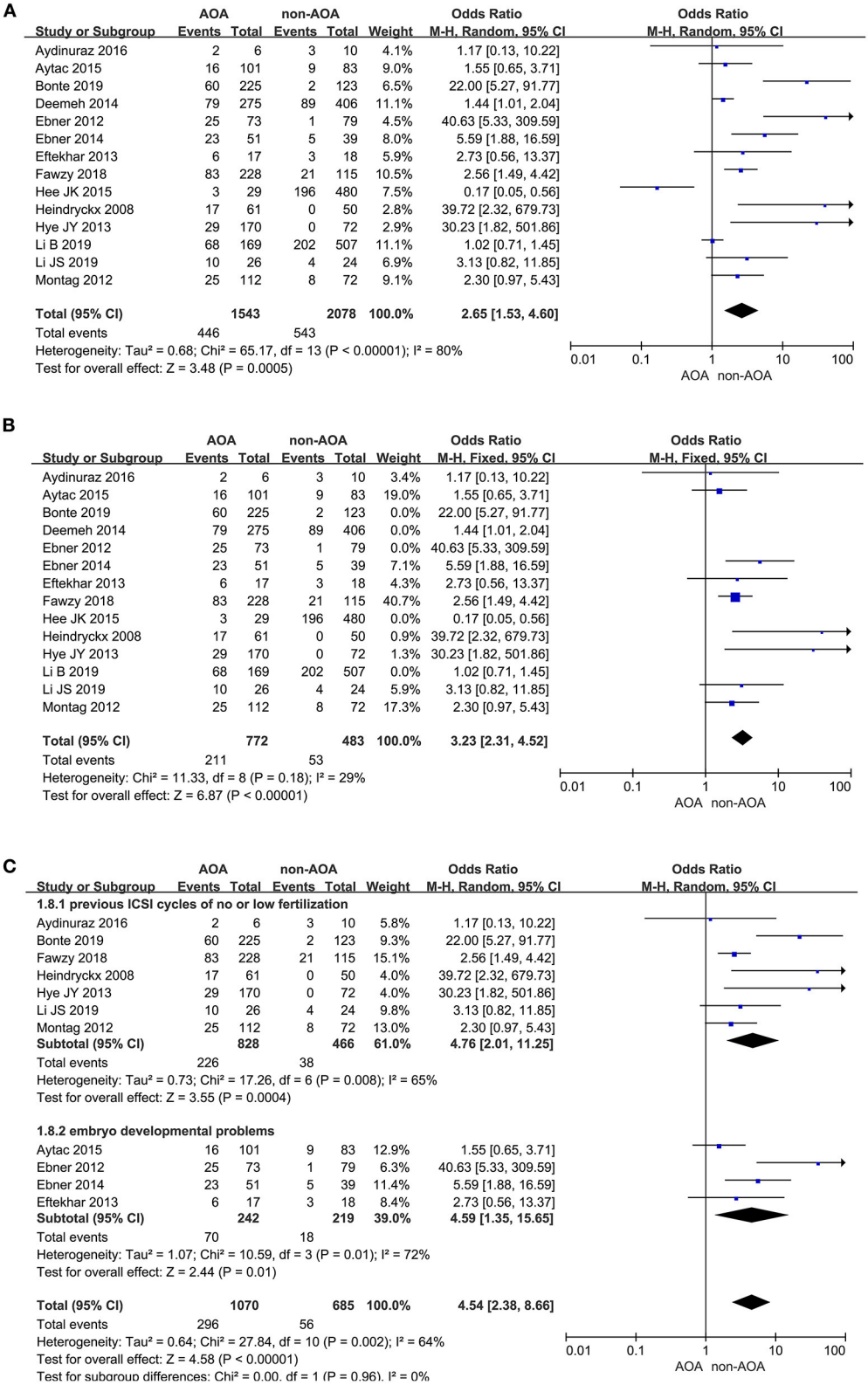


FIGURE 3 | (A) Forest plot of live birth rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore. **(B)** Forest plot of live birth rate between AOA treatment and non-AOA treatment patient groups after reducing the heterogeneity. **(C)** The subgroup analysis of live birth rate between AOA treatment and non-AOA treatment patient groups.

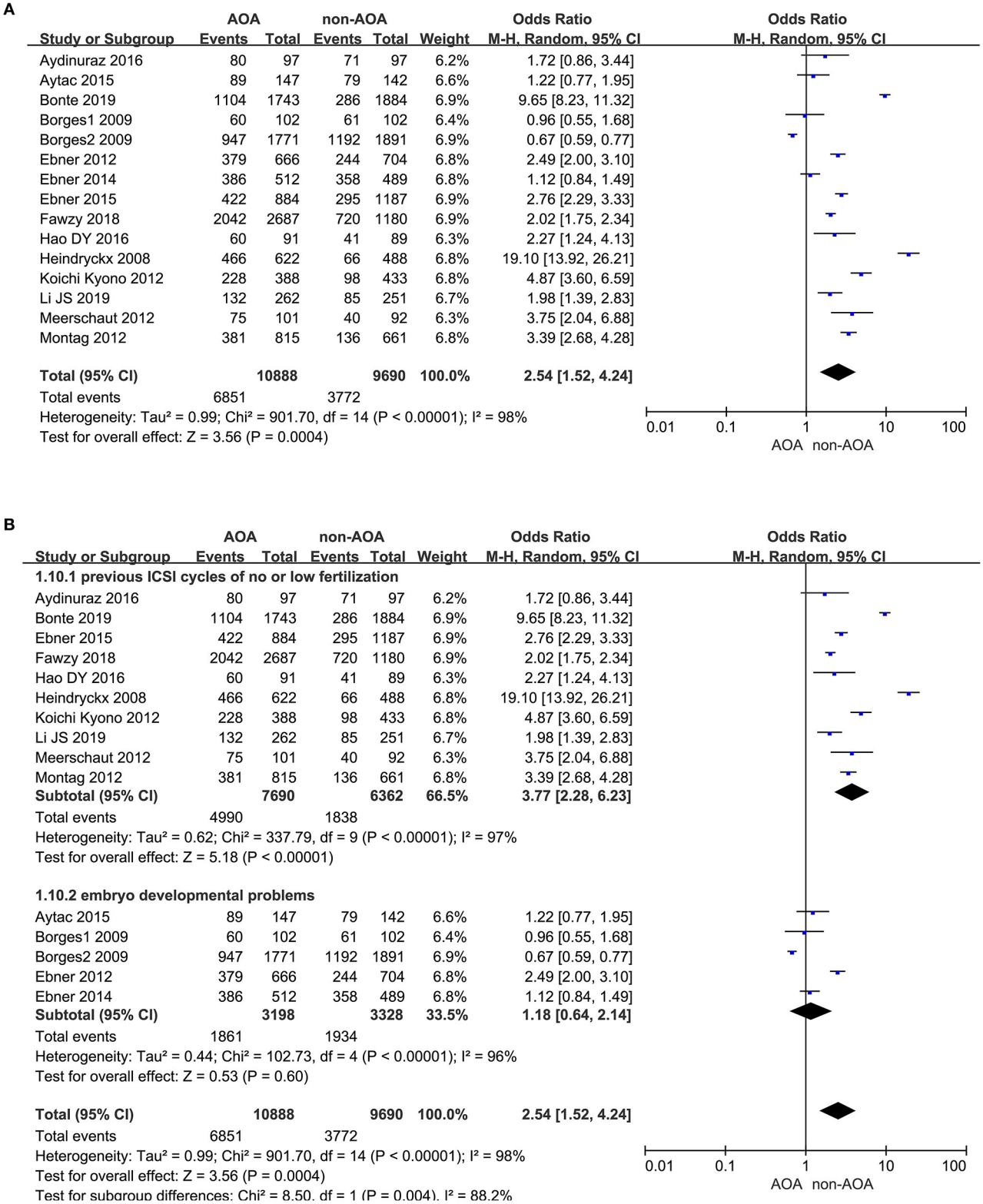


FIGURE 4 | (A) Forest plot of fertilization rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore. **(B)** The subgroup analysis of fertilization rate between AOA treatment and non-AOA treatment patient groups.

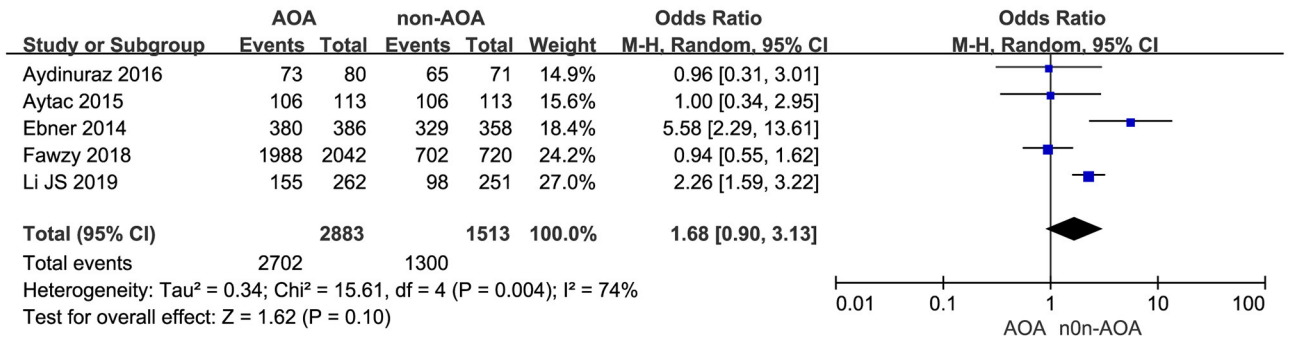


FIGURE 5 | Forest plot of cleavage rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore.

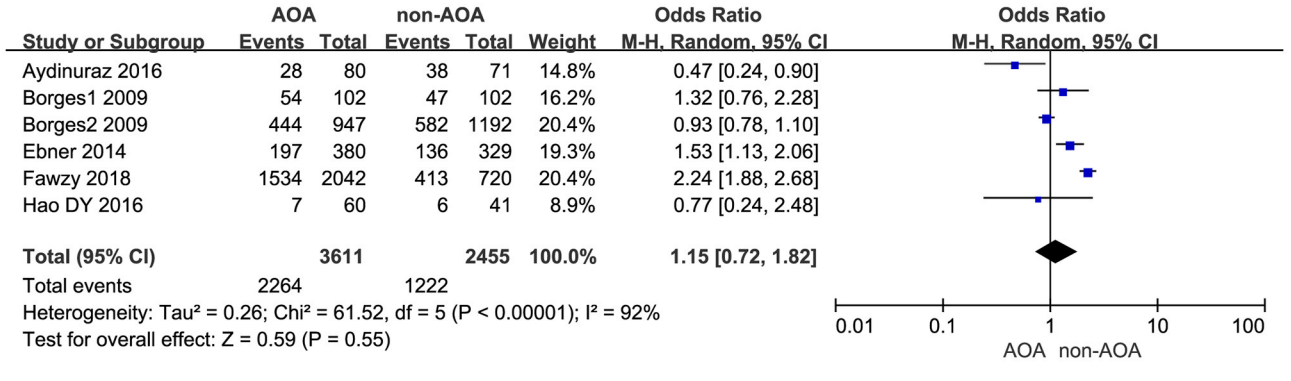


FIGURE 6 | Forest plot of top-quality embryo rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore.

Li J. et al., 2019). It was 55.55% (1,481/2,666) in the ICSI-AOA group and 32.77% (386/1,178) in the non-AOA group. Applying a random effects model to analyze the blastocyst formation rate, a statistically significant difference was observed: OR 3.59; 95% CI, 1.34–9.60; $P = 0.01$; $I^2 = 94\%$ (**Figure 7**, **Supplementary Figure 7**). The pooled results suggested AOA with calcium ionophore improved the blastocyst formation rate.

Implantation Rate

Eleven articles presented the implantation rate, of which there were 488 (488/2,208) implanted embryos in the AOA with calcium ionophore group and 473 (473/2,218) implanted embryos in the non-AOA group (Borges et al., 2009a,b; Ebner et al., 2012, 2014; Kyono et al., 2012; Montag et al., 2012; Yoon et al., 2013; Aydinuraz et al., 2016; Fawzy et al., 2018; Li B. et al., 2019; Li J. et al., 2019). A statistically significant difference of implantation rate was observed between ICSI-AOA and non-AOA groups. In view of the high heterogeneity ($P < 0.00001$; $I^2 = 85\%$), we derived the pooled OR with a random effects model (OR 2.38; 95% CI, 1.38–4.11; $P = 0.002$; $I^2 = 85\%$; **Figure 8A**). The funnel plot remained skewed which suggested publication bias (**Supplementary Figure 8A**). Given the remarkable heterogeneity, subgroup analyses were conducted. For the "previous fertilization failure or low fertilization rate" subgroup, AOA with calcium ionophore obviously increased its implantation rate (OR 2.68; 95% CI, 1.42–5.06; $P = 0.002$; $I^2 = 56\%$); however, for the "embryo developmental problems (embryonic development block or sperm factor or DOR)" subgroup, AOA with calcium ionophore could not improve the implantation rate (OR 2.85; 95% CI, 0.84–9.72; $P = 0.09$; $I^2 = 91\%$; **Figure 8B**, **Supplementary Figure 8B**). For the "previous fertilization failure or low fertilization rate" subgroup, AOA in combination with ICSI could improve the implantation rate significantly, but for the "embryo developmental problems (embryonic development block or sperm factor or DOR)" subgroup, AOA could not increase the implantation rate. These results suggested that the high heterogeneity between studies might be due to the different populations included in the study.

Miscarriage Rate

Thirteen of the included studies investigated the effect of AOA with calcium ionophore on pregnancy outcome (Borges et al., 2009b; Kyono et al., 2012; Montag et al., 2012; Eftekhar et al., 2013; Yoon et al., 2013; Deemeh et al., 2014; Ebner et al., 2014; Aydinuraz et al., 2016; Miller et al., 2016; Fawzy et al., 2018; Bonte et al., 2019; Li B. et al., 2019; Li J. et al., 2019). Among the included infertility patients, 20.23% (124 of 613) of patients in the ICSI-AOA group and 19.53% (214 of 1,096) of patients in control group experienced miscarriage. Using a fixed effects

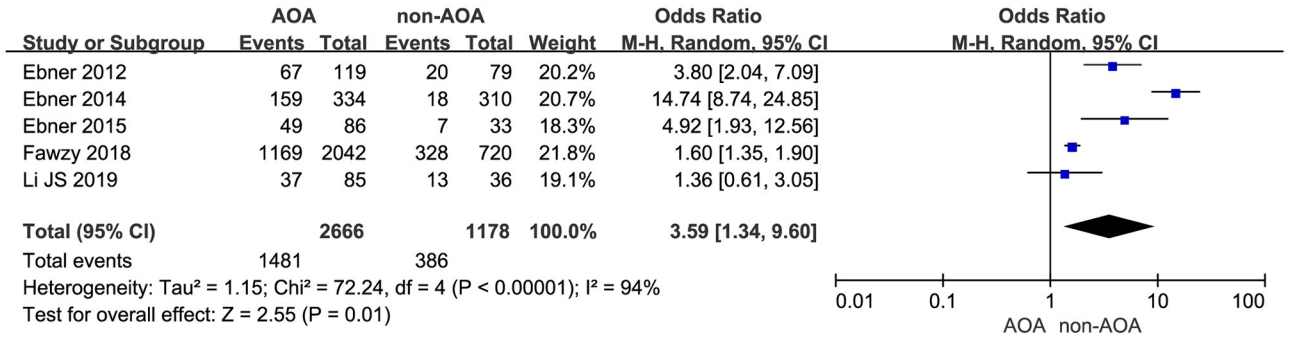


FIGURE 7 | Forest plot of blastocyst formation rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore.

model, we did not discover a statistically significant difference between these two groups (OR 0.78; 95% CI, 0.57–1.07; $P = 0.12$; $I^2 = 0$; **Figure 9**). Hence, AOA with calcium ionophore did not increase the miscarriage rate. Based on the Begg's test, the *P*-value equaled 0.945 > 0.05, which implied no indication of asymmetry among the included 13 studies (**Supplementary Figure 9**).

Congenital Birth Defects

Thirteen out of 22 studies reported the rate of congenital birth defects (Heindryckx et al., 2008; Ebner et al., 2012, 2014; Kyono et al., 2012; Montag et al., 2012; Yoon et al., 2013; Deemeh et al., 2014; Dayong Hao et al., 2016; Miller et al., 2016; Mateizel et al., 2018; Bonte et al., 2019; Li B. et al., 2019; Li J. et al., 2019). The incidence of birth defects was 2.15% (11/512) in the ICSI-AOA treatment group and 3.97% (151/3,808) in the non-AOA control group. According to this meta-analysis, no significant difference was found between the ICSI-AOA group and only ICSI group in congenital anomalies (OR 1.33; 95% CI, 0.70–2.53; $P = 0.38$; $I^2 = 0$; **Figure 10A**). The result suggested that AOA with calcium ionophore did not affect the safety of offspring. Additionally, funnel plots (symmetric) and Egger's test illustrated no obvious publication bias (**Supplementary Figure 10A**). Furthermore, subgroup comparison of different activators between ionomycin and calcimycin were conducted. In the "ionomycin" subgroup (Heindryckx et al., 2008; Deemeh et al., 2014; Bonte et al., 2019; Li B. et al., 2019; Li J. et al., 2019), congenital birth defects occurred in 0.78% (2/256) of patients in the ICSI-AOA group and 1.6% (5/312) in the non-AOA group, respectively. Also in this meta-analysis, no significant difference was found in the ionomycin subgroup (OR 0.85; 95% CI, 0.20–3.71; $P = 0.85$; $I^2 = 28\%$; **Figure 10B**). In the "calcimycin" subgroup (Ebner et al., 2012, 2015; Kyono et al., 2012; Montag et al., 2012; Yoon et al., 2013; Dayong Hao et al., 2016; Miller et al., 2016), no significant difference was found between the ICSI-AOA group (3.05%, 6/197) and non-AOA group (4.33%, 42/971) (OR 1.63; 95% CI, 0.67-3.96; $P = 0.28$; $I^2 = 0$; **Figure 10B**). These results suggested that using different types of calcium ionophore (ionomycin and calcimycin) for AOA would not increase the incidence of birth defects in the offspring of infertile patients. The study of Mateizel et al. (2018) was excluded due to four newborns resulting from mixed transplantation of calcimycin and ionomycin activation cycles. Another research study was excluded, in which only 10 newborns after calcimycin treatment were included, and no birth defects occurred (Kyono et al., 2012). After $SrCl_2$ activation treatment, 12 babies were born without birth defects, so they were not included in the subgroup analysis. The funnel plot is shown in **Supplementary Figure 10B**.

Neonatal Sex Ratio

Seven studies reported the neonatal sex and were analyzed by one-arm analysis (Heindryckx et al., 2008; Yoon et al., 2013; Miller et al., 2016; Mateizel et al., 2018; Bonte et al., 2019; Li B. et al., 2019; Li J. et al., 2019). After undergoing AOA with calcium ionophore treatment, 167 boys and 149 girls were born. Using a fixed effects model, no significant difference was discovered: OR 0.53; 95% CI, 0.47–0.59; $P = 0.71$; $I^2 = 0$ (**Figure 11**). There was no evidence of publication bias in the neonatal sex ratio (**Supplementary Figure 11**). These above results revealed that AOA with calcium ionophore had no effect on the sex ratio of newborns.

DISCUSSION

Summary of Findings

Calcium ionophore treatment during AOA is a common chemical activation method and is always used in cases with low fertilization rates or developmental block. Several studies showed that the use of AOA with calcium ionophore treatment improved pregnancy and live birth rate to a certain degree. As the number of studies increase, it is necessary to find more reliable evidence of calcium ionophore in improving pregnancy outcome. In this study, a meta-analysis was performed to explore the efficacy and safety of calcium ionophore treatment during AOA in improving pregnancy outcome and decreasing the incidence of congenital birth defects.

This meta-analysis was conducted based on five RCTs and 17 observational studies. Two publications (Kyono et al., 2012; Fawzy et al., 2018) dealt with $SrCl_2$, which is not a regular Ca-ionophore but is thought to use calcium channels since Ca^{2+} and Sr^{2+} are very similar molecules. Recently, Lu et al. (2018) found that $SrCl_2$ activation cannot stimulate calcium oscillation or parthenogenic division in matured oocytes *in vitro*

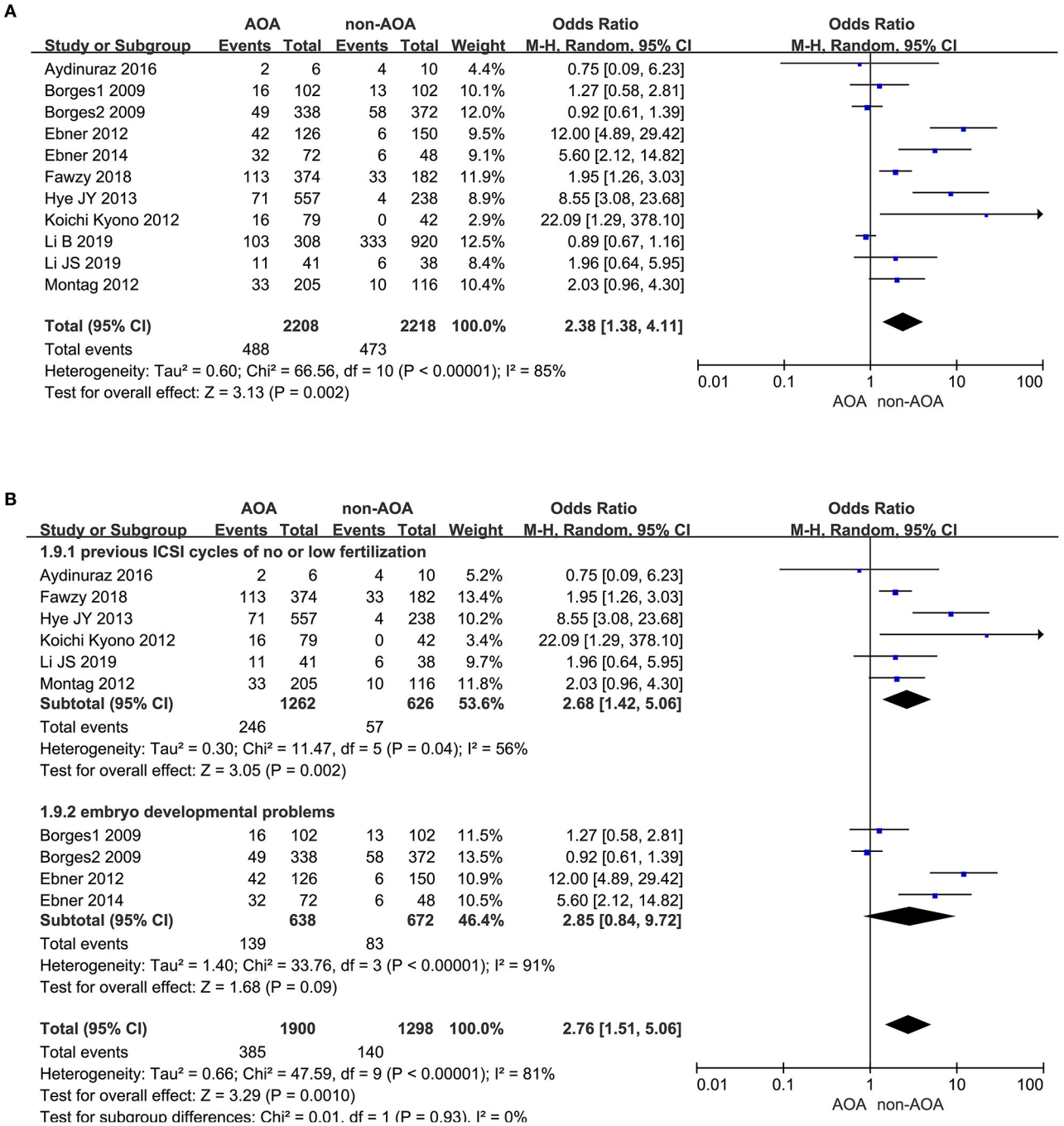


FIGURE 8 | (A) Forest plot of implantation rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore. **(B)** The subgroup analysis of implantation rate between AOA treatment and non-AOA treatment patient groups.

or those with smooth endoplasmic reticulum. Therefore, these two publications referring to $SrCl_2$ were also included. One study (Ebner et al., 2014) reported that Ca^{2+}-ionophore was meant to activate mitosis at the cleavage stage and not activate the oocyte, which was controversial but we still included the study. The only fact is that both classical AOA and stimulation of mitosis have the same underlying causative effect which is a reduced/depleted intracellular calcium level. Luckily, Ebner et al. did apply the Ca^{2+}-ionophore stimulus exactly at the same time for both indications (classical AOA and stimulation of mitosis) according to their ethical vote, so that the application mode was exactly as all other studies mentioned. The pregnancy rate in 17 studies

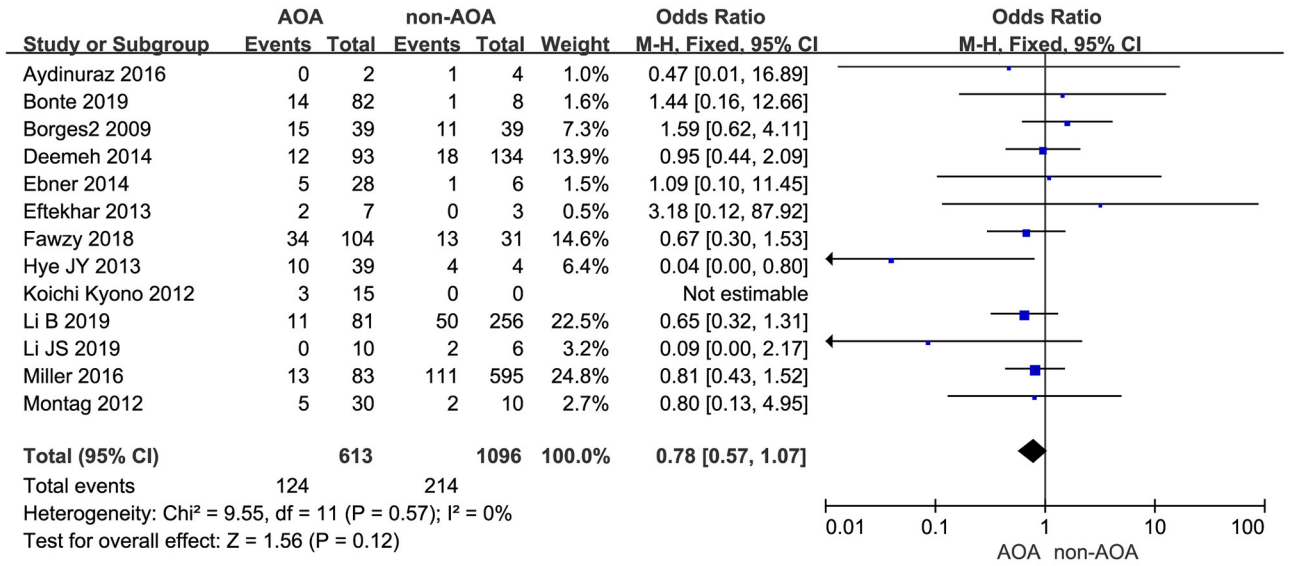


FIGURE 9 | Forest plot of miscarriage rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore.

showed it was also higher in the ICSI-AOA group (34.87%, 649/1,861) than in the non-AOA group (33.68%, 799/2,372). Only 14 studies reported the effect of AOA with calcium ionophore on live birth rate, and it was higher in the ICSI-AOA group (28.90%, 446/1,543) than in the non-AOA group (26.13%, 543/2,078). Focusing on the primary outcomes, calcium ionophore in combination with ICSI significantly increased the pregnancy rate and live birth rate. The secondary outcomes reported that AOA with calcium ionophore played a positive role in improving fertilization, blastulation, and implantation rate, which suggested that AOA with calcium ionophore was effective for oocyte activation. The results of subgroup analysis of the fertilization rate suggested that for patients in the "previous fertilization failure or low fertilization rate" subgroup, in general, AOA significantly increased the fertilization rate of patients, but could not improve the fertilization rate of patients with embryo developmental problems. Similarly, the implantation rate had the same results as the fertilization rate. And AOA did not enhance the cleavage and top-quality embryo rate. Moreover, calcium ionophore in combination with ICSI did not improve the incidence of miscarriage, congenital birth defects, and neonatal sex ratio, which suggested that AOA with calcium ionophore is safe for oocyte activation.

Biological and Clinical Significance

Conventional IVF resulted in low fertilization rates or even no fertilization. The most likely cause is oocyte activation failure, followed by few or poor quality of sperm or oocyte characteristics. ICSI is widely used to treat infertility caused by male factors, but low fertilization and complete fertilization failure still arise in a few special infertility populations due to abnormal cell mitosis and embryo cleavage. Calcium has a major role in cell mitosis and embryo cleavage (Swanson et al., 1997; Goud et al., 1999; Tosti, 2006). Inadequate physiological or artificial calcium causes cleavage anomalies or embryo arrest. Multiple methods were proved to solve activation/fertilization failure problems after ICSI, among which calcium ionophore is advantageous and has no evidence of toxicity or harmful outcome (Miller et al., 2016). In addition, ICSI in combination with AOA by calcium ionophore has mitigated the above infertility problem. Multiple studies reported that oocyte fertilization that failed after ICSI was subsequently activated by calcium ionophore, and the activated oocytes developed normally (Kashir et al., 2010). However, the safety and efficacy of AOA with calcium ionophore are still a routine trepidation. Hence, large-sample clinical studies and meta-analyses or reviews need to be investigated. Recently, two related meta-analyses that primarily concentrated on the efficacy of AOA were reported (Sfontouris et al., 2015; Murugesu et al., 2017). The results demonstrated that fertilization, blastulation, implantation, and clinical pregnancy and live birth rate increased with statistical significance in infertility patients treated with calcium ionophore. Calcium ionophore appears to be the most popular method in oocyte activation. With the increase of AOA-related articles, a new meta-analysis was needed to discuss the safety and efficacy of AOA with calcium ionophore. In this study, we included 22 studies and mainly explored the efficiency and safety of AOA with calcium ionophore in the assisted reproductive field. Our results indicated that AOA with calcium ionophore not only significantly increased the fertilization, blastulation, implantation, and clinical pregnancy and live birth rate, but also did not affect the incidence of miscarriage, congenital birth defects, and neonatal sex ratio.

Inconsistent types of included studies may limit the possibility in obtaining a more reliable conclusion and cause high heterogeneity. There were five RCTs, nine prospective observational trials, and eight retrospective trials in this study.

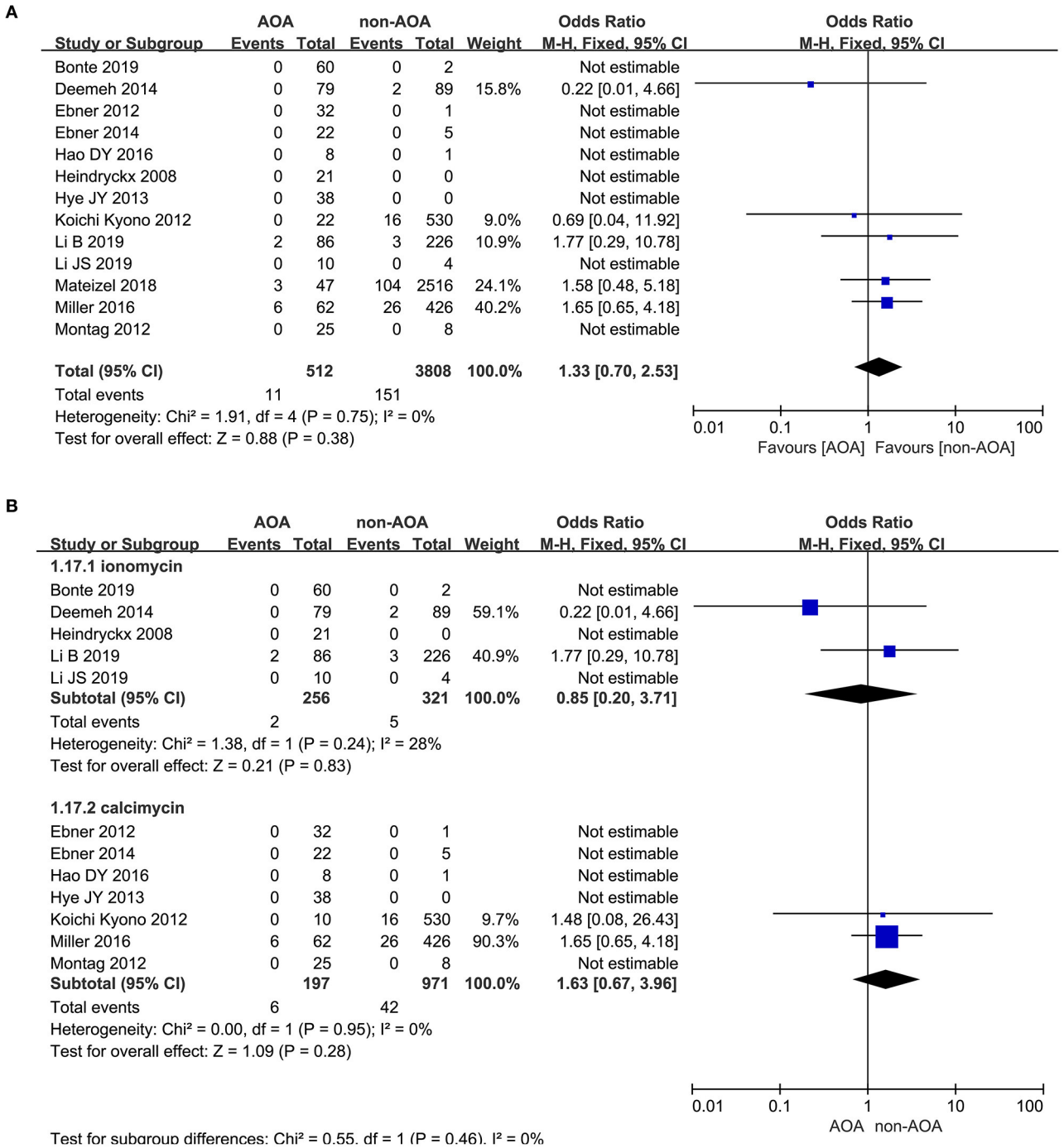


FIGURE 10 | (A) Forest plot of congenital birth defect rate between AOA treatment and non-AOA treatment patient groups using calcium ionophore. **(B)** The subgroup analysis of birth defect rate between AOA treatment and non-AOA treatment patient groups.

More RCT studies promoted a creditable outcome. Fertilization failure after ICSI was uncommon, leading to difficultly in designing and conducting a methodologically precise RCT, so a small number of RCT studies were included in this study. Our results tended to support the notion that calcium ionophore treatment was safe and effective. The analysis results indicated that AOA with calcium ionophore resulted in a statistically significant improvement in live birth rate. The degree of heterogeneity was high and publication bias appeared. Subsequently, we performed a sensitivity analysis to discover the

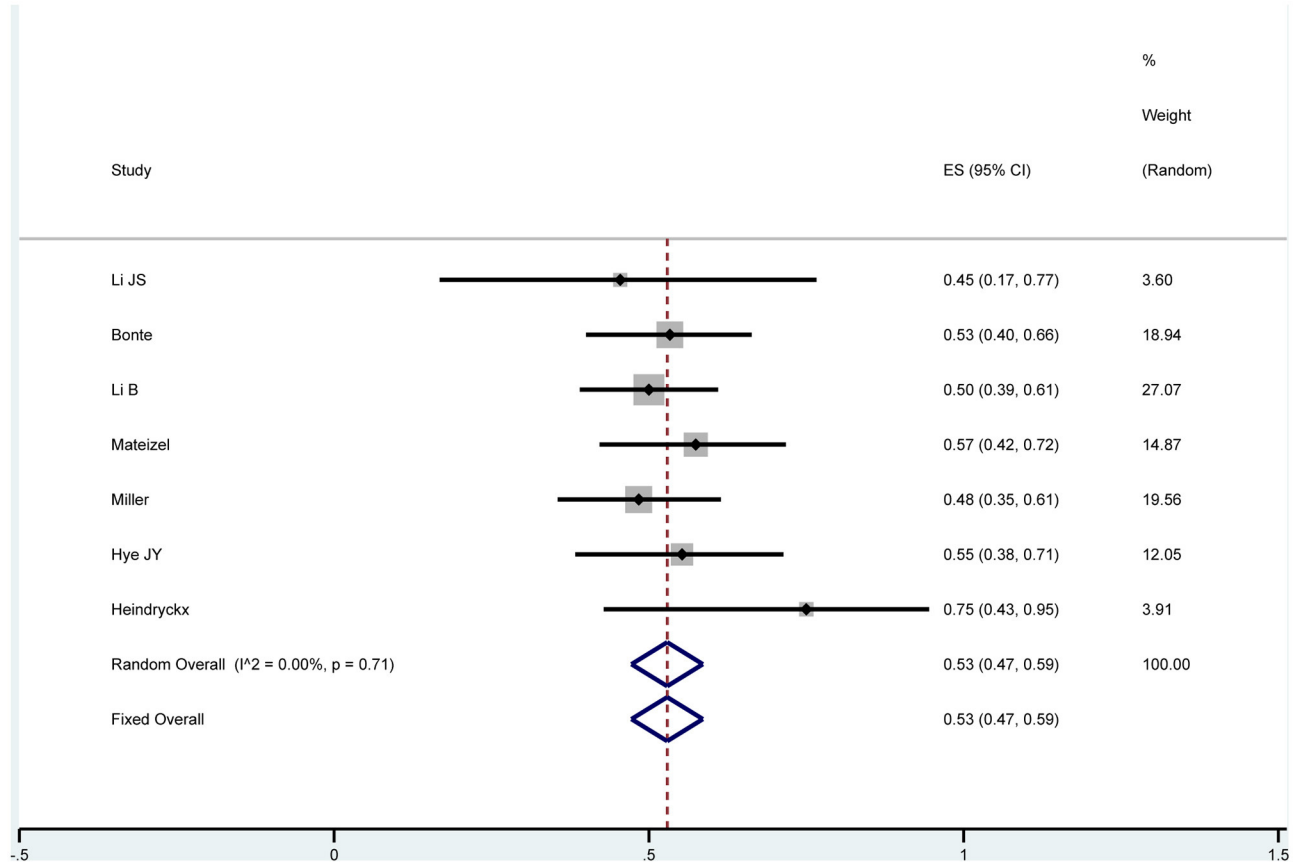


FIGURE 11 | Forest plots of the included studies reporting data on sex ratio.

cause of heterogeneity in the 14 studies. The results indicated that five low-quality studies (Ebner et al., 2012; Deemeh et al., 2014; Kang et al., 2015; Bonte et al., 2019; Li B. et al., 2019) had a great influence on heterogeneity. After excluding these five low-quality studies, the heterogeneity and publication bias disappeared among those remaining nine studies. The meta analysis of the remaining nine studies also suggested AOA with calcium ionophore significantly increased the live birth rate. Through comprehensively analyzing the causes of bias, we found that two studies (Ebner et al., 2012; Bonte et al., 2019) covered pre- and post-control of their own case involving patients with fertilization failure or low fertilization rate in the previous cycle, and the live birth rate with the treatment of calcium ionophore in the next cycle was significantly higher than those without calcium ionophore in the previous cycle. Another two studies (Deemeh et al., 2014; Li B. et al., 2019) showed that AOA with calcium ionophore could improve the birth rate of patients with previous fertilization failure or low fertilization rate, as well as patients with testicular sperm extraction (TESE) or severe teratozoospermia. However, Kang et al. (2015) thought that patients with previous fertilization failure or low fertilization rate could not achieve the same birth rate as those who received TESE when they were treated with TESE again. Therefore, there is heterogeneity in the inclusion and evaluation for live birth rate in these five studies.

Furthermore, in subgroup analyses, consistent results where AOA with calcium ionophore significantly increased the birth rate of re-assisted pregnancy were found in the "previous fertilization failure or low fertilization rate" subgroup (OR 4.76; 95% CI, 2.01–11.25; $P = 0.0004$; $I^2 = 65\%$) and "embryo developmental problems (embryonic development block or sperm factor or DOR)" subgroup (OR 4.59; 95% CI, 1.35–15.65; $P = 0.01$; $I^2 = 72\%$). Hence, the high heterogeneity in this part was caused by the design and quality of studies but not the fact that the included studies recruited diverse study populations. High clinical heterogeneity could be explained by patient characteristics, fertility treatment plans of different centers, and distinct inclusion and exclusion criteria. After eliminating specific studies by sensitivity analysis, our findings were collated by a mixture of random and fixed models. Moreover, the statistical heterogeneity could be handled by subgroup analysis. And subgroup analysis did not change our results. It increased the credibility of the findings in AOA with calcium ionophore promoting live birth rate. The overall pregnancy rate was significantly increased in the

ICSI-AOA group. And no publication bias appeared. After eliminating specific studies by sensitivity analysis, we found high heterogeneity appearing among these 17 included studies. Aiming to analyze the reasons for high heterogeneity, subgroup analysis was performed. The results demonstrated that AOA with calcium ionophore uncommonly enhanced the pregnancy rate in both subgroups. It ensured more credible results in AOA with calcium ionophore promoting overall pregnancy rate.

For the secondary results, in view of the fact that we were more interested in the safety of AOA for offspring, the analysis results indicated that AOA with calcium ionophore not only reduced the incidence of congenital birth defects, but also a decrease was seen in the ICSI-AOA group. Furthermore, the degree of heterogeneity was low. However, although 13 studies were included, 8 studies had few newborns (Heindryckx et al., 2008; Ebner et al., 2012, 2015; Montag et al., 2012; Yoon et al., 2013; Dayong Hao et al., 2016; Bonte et al., 2019; Li J. et al., 2019). Therefore, increasing the number of newborns contributed to a more reliable conclusion in assessing the safety of AOA with calcium ionophore. Similar results were found in miscarriage rate. AOA with calcium ionophore did not affect the pregnancy outcome and a similar miscarriage rate was discovered in the ICSI-AOA group and non-AOA group. Thirteen articles mentioned miscarriage rate, among which one study reported that no clinical pregnancy occurred in the only-ICSI group. The number of research articles and cases was acceptable, and low heterogeneity and no publication bias occurred. Therefore, the conclusion that AOA with calcium ionophore did not promote miscarriage rate was evidence-based. Moreover, the fertilization, blastocyst formation, and implantation rate all improved with a statistical difference. However, in the subgroup analyses, ICSI-AOA could obviously improve the fertilization and implantation rate of patients with "previous fertilization failure or low fertilization rate," but not the patients with "embryo developmental problems." It reminded us that it was of little significance to use calcium ionophore during AOA to activate oocytes for patients with "embryo developmental problems." Compared with the non-AOA group, no evidence in improving cleavage and top-quality embryo rate was found, but a trend of promotion appeared. The gender of the fetus at birth was also of concern to us. The results revealed that AOA with calcium ionophore had no effect on the sex ratio of newborns using by one-arm analysis. This study did not pay attention to the baby's development in the future, and ignored whether gene expression was affected by treatment by calcium ionophore.

CONCLUSION

These existing studies proved that AOA by calcium ionophore following ICSI not only significantly improved live birth, overall pregnancy, implantation, fertilization, and blastocyst formation rates, but also did not affect the incidence of miscarriage and congenital birth defects. Strong evidence for the efficacy and safety of calcium ionophore application in improving oocyte activation has been discovered. This meta-analysis indicated that using calcium ionophore to activate oocytes was beneficial for couples with poor fertilization rates following ICSI. We would suggest using AOA with calcium ionophore in routine practice for failed fertilization following ICSI.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YS designed the paper. HZ wrote the manuscript. YS and DZ drew the figures. JW, DZ, and YC revised and supervised the project. HB contributed to supervision and reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.751905/full#supplementary-material

Supplementary Table 1 | NOS for the risk of bias and the quality assessment of included studies.

Supplementary Figure 1 | Risk of bias of the included RCTs.

Supplementary Figure 2 | (A) Funnel plots of publication bias in pregnancy rate. **(B)** Funnel plots of publication bias in pregnancy rate subgroup analysis.

Supplementary Figure 3 | (A) Funnel plots of publication bias in live birth rate. **(B)** Funnel plots of publication bias in live birth rate subgroup analysis.

Supplementary Figure 4 | (A) Funnel plots of publication bias in fertilization rate. **(B)** Funnel plots of publication bias in fertilization rate subgroup analysis.

Supplementary Figure 5 | Funnel plots of publication bias in cleavage rate.

Supplementary Figure 6 | Funnel plots of publication bias in top-quality blastocyst rate.

Supplementary Figure 7 | Funnel plots of publication bias in blastocyst formation rate.

Supplementary Figure 8 | (A) Funnel plots of publication bias in implantation rate. **(B)** Funnel plots of publication bias in implantation rate subgroup analysis.

Supplementary Figure 9 | Funnel plots of publication bias in miscarriage rate.

Supplementary Figure 10 | (A) Funnel plots of publication bias in congenital birth defect rate. **(B)** Funnel plots of publication bias in congenital birth defect rate subgroup analysis.

Supplementary Figure 11 | Funnel plots of publication bias in sex ratio.

REFERENCES

Amdani, S. N., Jones, C., and Coward, K. (2013). Phospholipase C zeta (PLCzeta): oocyte activation and clinical links to male factor infertility. *Adv. Biol. Regul.* 53, 292–308. doi: 10.1016/j.jbior.2013.07.005

Aydinuraz, B., Dirican, E. K., Olgan, S., Aksunger, O., and Erturk, O. K. (2016). Artificial oocyte activation after intracytoplasmic morphologically selected sperm injection: a prospective randomized sibling oocyte study. *Hum. Fertil.* 19, 282–288. doi: 10.1080/14647273.2016.1240374

Bhattacharya, S., Maheshwari, A., and Mollison, J. (2013). Factors associated with failed treatment: an analysis of 121,744 women embarking on their first IVF cycles. *PLoS ONE* 8:e82249. doi: 10.1371/journal.pone.0082249

Bonte, D., Ferrer-Buitrago, M., Dhaenens, L., Popovic, M., Thys, V., De Croo, I., et al. (2019). Assisted oocyte activation significantly increases fertilization and pregnancy outcome in patients with low and total failed fertilization after intracytoplasmic sperm injection: a 17-year retrospective study. *Fertil. Steril.* 112, 266–274. doi: 10.1016/j.fertnstert.2019.04.006

Borges, E. Jr, de Almeida Ferreira Braga, D. P., de Sousa Bonetti, T. C., Iaconelli, A.,Jr., and Franco, J. G. Jr. (2009a). Artificial oocyte activation using calcium ionophore in ICSI cycles with spermatozoa from different sources. *Reprod. Biomed. Online* 18, 45–52. doi: 10.1016/S1472-6483(10)60423-3

Borges, E. Jr, de Almeida Ferreira Braga, D. P., de Sousa Bonetti, T. C., Iaconelli, A. Jr., and Franco, J. G. Jr. (2009b). Artificial oocyte activation with calcium ionophore A23187 in intracytoplasmic sperm injection cycles using surgically retrieved spermatozoa. *Fertil. Steril.* 92, 131–136. doi: 10.1016/j.fertnstert.2008.04.046

Caglar Aytac, P., Kilicdag, E. B., Haydardedeoglu, B., Simsek, E., Cok, T., and Parlakgumus, H. A. (2015). Can calcium ionophore "use" in patients with diminished ovarian reserve increase fertilization and pregnancy rates? A randomized, controlled study. *Fertil. Steril.* 104, 1168–1174. doi: 10.1016/j.fertnstert.2015.07.1163

Dayong Hao, R. Z., Zhang, X., Shen, C., Yang, Y., Wu, W., Liu, Y., et al. (2016). A control study of artificial assisted activation of oocytes in the same cycle. *Natl. Med. J.* 96, 3489–3493. doi: 10.3760/cma.j-issn.0376-2491.2016.43.010

Deemeh, M. R., Tavalaee, M., and Nasr-Esfahani, M. H. (2014). Health of children born through artificial oocyte activation: a pilot study. *Reprod. Sci.* 22, 322–328. doi: 10.1177/1933719114542017

Ebner, T., Koster, M., Shebl, O., Moser, M., Van der Ven, H., Tews, G., et al. (2012). Application of a ready-to-use calcium ionophore increases rates of fertilization and pregnancy in severe male factor infertility. *Fertil. Steril.* 98, 1432–1437. doi: 10.1016/j.fertnstert.2012.07.1134

Ebner, T., Montag, M., Oocyte, G., Montag, M., Van der Ven, K., Van der Ven, H., et al. (2015). Live birth after artificial oocyte activation using a ready-to-use ionophore: a prospective multicentre study. *Reprod. Biomed. Online* 30, 359–365. doi: 10.1016/j.rbmo.2014.11.012

Ebner, T., Oppelt, P., Wober, M., Staples, P., Mayer, R. B., Sonnleitner, U., et al. (2014). Treatment with Ca2+ ionophore improves embryo development and outcome in cases with previous developmental problems: a prospective multicenter study. *Hum. Reprod.* 30, 97–102. doi: 10.1093/humrep/deu285

Eftekhar, M., Janati, S., Rahsepar, M., and Aflatoonian, A. (2013). Effect of oocyte activation with calcium ionophore on ICSI outcomes in teratospermia: a randomized clinical trial. *Iran. J. Reprod. Med.* 11, 875–882.

ESHRE/Alpha (2017). The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reprod. Biomed. Online* 35, 494–510. doi: 10.1016/j.rbmo.2017.06.015

Fawzy, M., Emad, M., Mahran, A., Sabry, M., Fetih, A. N., Abdelghafar, H., et al. (2018). Artificial oocyte activation with SrCl2 or calcimycin after ICSI improves clinical and embryological outcomes compared with ICSI alone: results of a randomized clinical trial. *Hum. Reprod.* 33, 1636–1644. doi: 10.1093/humrep/dey258

Ferrer-Buitrago, M., Dhaenens, L., Lu, Y., Bonte, D., Vanden Meerschaut, F., De Sutter, P., et al. (2018). Human oocyte calcium analysis predicts the response to assisted oocyte activation in patients experiencing fertilization failure after ICSI. *Hum. Reprod.* 33, 416–425. doi: 10.1093/humrep/dex376

Goud, P. T., Goud, A. P., Van Oostveldt, P., and Dhont, M. (1999). Presence and dynamic redistribution of type I inositol 1,4,5-trisphosphate receptors in human oocytes and embryos during *in-vitro* maturation, fertilization and early cleavage divisions. *Mol. Hum. Reprod.* 5, 441–451. doi: 10.1093/molehr/5.5.441

Hamberger, L., Lundin, K., Sjogren, A., and Soderlund, B. (1998). Indications for intracytoplasmic sperm injection. *Hum. Reprod.* (13 Suppl.) 1, 128–133. doi: 10.1093/humrep/13.suppl_1.128

Heindryckx, B., De Gheselle, S., Gerris, J., Dhont, M., and De Sutter, P. (2008). Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod. Biomed. Online* 17, 662–668. doi: 10.1016/S1472-6483(10)60313-6

Higgins, J. P. T. (2011). "Chapter 8: Assessing risk of bias in included studies," in *Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0,* ed J. A. C. Stern (Cochrane Collaboration). Available online at: www.handbook.cochrane.org

Johnson, L. N., Sasson, I. E., Sammel, M. D., and Dokras, A. (2013). Does intracytoplasmic sperm injection improve the fertilization rate and decrease the total fertilization failure rate in couples with well-defined unexplained infertility? A systematic review and meta-analysis. *Fertil. Steril.* 100, 704–711. doi: 10.1016/j.fertnstert.2013.04.038

Kang, H. J., Lee, S. H., Park, Y. S., Lim, C. K., Ko, D. S., Yang, K. M., et al. (2015). Artificial oocyte activation in intracytoplasmic sperm injection cycles using testicular sperm in human *in vitro* fertilization. *Clin. Exp. Reprod. Med.* 42, 45–50. doi: 10.5653/cerm.2015.42.2.45

Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J., and Coward, K. (2010). Oocyte activation, phospholipase C zeta and human infertility. *Hum. Reprod. Update* 16, 690–703. doi: 10.1093/humupd/dmq018

Kissin, D. M., Jamieson, D. J., and Barfield, W. D. (2014). Monitoring health outcomes of assisted reproductive technology. *N. Engl. J. Med.* 371, 91–93. doi: 10.1056/NEJMc1404371

Kyono, K., Kumagai, S., Nishinaka, C., Nakajo, Y., Uto, H., Toya, M., et al. (2012). Birth and follow-up of babies born following ICSI with oocyte activation using strontium chloride or calcium ionophore A23187. *J. Mamm. Ova Res.* 29, 35–40. doi: 10.1274/jmor.29.35

Li, B., Zhou, Y., Yan, Z., Li, M., Xue, S., Cai, R., et al. (2019). Pregnancy and neonatal outcomes of artificial oocyte activation in patients undergoing frozen-thawed embryo transfer: a 6-year population-based retrospective study. *Arch. Gynecol. Obstet.* 300, 1083–1092. doi: 10.1007/s00404-019-05298-3

Li, J., Zheng, X., Lian, Y., Li, M., Lin, S., Zhuang, X., et al. (2019). Artificial oocyte activation improves cycles with prospects of ICSI fertilization failure: a sibling oocyte control study. *Reprod. Biomed. Online* 39, 199–204. doi: 10.1016/j.rbmo.2019.03.216

Lu, Y., Reddy, R., Ferrer Buitrago, M., Vander Jeught, M., Neupane, J., and De Vos, W. H. (2018). Strontium fails to induce Ca(2+) release and activation in human oocytes despite the presence of functional TRPV3 channels. *Hum. Reprod. Open* 2018:hoy005. doi: 10.1093/hropen/hoy005

Mateizel, I., Verheyen, G., Van de Velde, H., Tournaye, H., and Belva, F. (2018). Obstetric and neonatal outcome following ICSI with assisted oocyte activation by calcium ionophore treatment. *J. Assist. Reprod. Genet.* 35, 1005–1010. doi: 10.1007/s10815-018-1124-6

Miller, N., Biron-Shental, T., Sukenik-Halevy, R., Klement, A. H., Sharony, R., and Berkovitz, A. (2016). Oocyte activation by calcium ionophore and congenital birth defects: a retrospective cohort study. *Fertil. Steril.* 106, 590–596.e592. doi: 10.1016/j.fertnstert.2016.04.025

Montag, M., Koster, M., van der Ven, K., Bohlen, U., and van der Ven, H. (2012). The benefit of artificial oocyte activation is dependent on the fertilization rate in a previous treatment cycle. *Reprod. Biomed. Online* 24, 521–526. doi: 10.1016/j.rbmo.2012.02.002

Murugesu, S., Saso, S., Jones, B. P., Bracewell-Milnes, T., Athanasiou, T., Mania, A., et al. (2017). Does the use of calcium ionophore during artificial oocyte activation demonstrate an effect on pregnancy rate? A meta-analysis. *Fertil. Steril.* 108, 468–482. e463. doi: 10.1016/j.fertnstert.2017.06.029

Nikiforaki, D., Vanden Meerschaut, F., de Roo, C., Lu, Y., Ferrer-Buitrago, M., de Sutter, P., et al. (2016). Effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. *Fertil. Steril.* 105, 798–806.e792. doi: 10.1016/j.fertnstert.2015.11.007

Nomikos, M., Kashir, J., Swann, K., and Lai, F. A. (2013). Sperm PLCzeta: from structure to Ca2+ oscillations, egg activation and therapeutic potential. *FEBS Lett.* 587, 3609–3616. doi: 10.1016/j.febslet.2013.10.008

Ramadan, W. M., Kashir, J., Jones, C., and Coward, K. (2012). Oocyte activation and phospholipase C zeta (PLCzeta): diagnostic and therapeutic implications for assisted reproductive technology. *Cell Commun. Signal.* 10:12. doi: 10.1186/1478-811X-10-12

Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., et al. (2002). PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 129, 3533–3544. doi: 10.1242/dev.129.15.3533

Sfontouris, I. A., Nastri, C. O., Lima, M. L., Tahmasbpourmarzouni, E., Raine-Fenning, N., and Martins, W. P. (2015). Artificial oocyte activation to improve reproductive outcomes in women with previous fertilization failure: a systematic review and meta-analysis of RCTs. *Hum. Reprod.* 30, 1831–1841. doi: 10.1093/humrep/dev136

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* 211, 157–176. doi: 10.1006/dbio.1999.9340

Swain, J. E., and Pool, T. B. (2008). ART failure: oocyte contributions to unsuccessful fertilization. *Hum. Reprod. Update* 14, 431–446. doi: 10.1093/humupd/dmn025

Swanson, C. A., Arkin, A. P., and Ross, J. (1997). An endogenous calcium oscillator may control early embryonic division. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1194–1199. doi: 10.1073/pnas.94.4.1194

Tosti, E. (2006). Calcium ion currents mediating oocyte maturation events. *Reprod. Biol. Endocrinol.* 4:26. doi: 10.1186/1477-7827-4-26

Vanden Meerschaut, F., Nikiforaki, D., De Gheselle, S., Dullaerts, V., Van den Abbeel, E., Gerris, J., et al. (2012). Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. *Hum. Reprod.* 27, 1977–1984. doi: 10.1093/humrep/des097

Vanden Meerschaut, F., Nikiforaki, D., Heindryckx, B., and De Sutter, P. (2014). Assisted oocyte activation following ICSI fertilization failure. *Reprod. Biomed. Online* 28, 560–571. doi: 10.1016/j.rbmo.2014.01.008

Yeste, M., Jones, C., Amdani, S. N., Patel, S., and Coward, K. (2016). Oocyte activation deficiency: a role for an oocyte contribution? *Hum. Reprod. Update* 22, 23–47. doi: 10.1093/humupd/dmv040

Yoon, H. J., Bae, I. H., Kim, H. J., Jang, J. M., Hur, Y. S., Kim, H. K., et al. (2013). Analysis of clinical outcomes with respect to spermatozoan origin after artificial oocyte activation with a calcium ionophore. *J. Assist. Reprod. Genet.* 30, 1569–1575. doi: 10.1007/s10815-013-0110-2

Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., et al. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, (2009). *Fertil. Steril.* 92, 1520–1524. doi: 10.1016/j.fertnstert.2009.09.009

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POLICY AND PRACTICE

The use of ICSI in ART: Evidence for practice

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ABSTRACT

This article reviews the evidence regarding the safety and efficacy of intra-cytoplasmic sperm injection (ICSI) in order to provide evidence-based clinical and laboratory guidelines and recommendations for use of ICSI within an assisted reproductive technology (ART) service. The guidelines will address the evidence for the use of ICSI rather than conventional IVF insemination; the use of ART techniques supplementary to ICSI; and risks associated with ICSI. This article is not intended to be the only approved standard of practice or to dictate an exclusive course of treatment. Other plans of management may be appropriate, taking into account the needs and medical history of the patient, available resources, and institutional or clinical practice limitations. The Executive Committees of the British Fertility Society (BFS) and Association of Reproductive and Clinical Scientists (ARCS) have approved this report. It was reviewed by members of BFS and ARCS and their input was considered in the preparation of the final document. This article will not consider specific details concerning technical aspects of the ICSI procedure.

Keywords: ICSI, IVF, male factor infertility, unexplained infertility, fertilisation

Introduction

Subfertility affects 15% of heterosexual couples globally, but estimates of the contribution of male factor subfertility to this overall figure vary widely, from 7.5% to 70% of couples (Agarwal et al., 2015). Differences between countries, in the definition of male factor subfertility, and the scarcity of population-based reports make it difficult to establish an accurate estimate, but it is generally accepted that a male factor is implicated in around 50% of subfertility, with 20-30% of cases being exclusively due to male factor(s) (Agarwal et al., 2015).

Intracytoplasmic sperm injection (ICSI) is a laboratory technique for the management of male factor subfertility that aims to assist fertilisation using micromanipulation. A single spermatozoon is selected, immobilised and injected into a mature oocyte from which the surrounding cumulus cells have been removed. Successful pregnancy following ICSI was first described in 1992 (Palermo et al., 1992) and the technique was rapidly incorporated into routine ART practice worldwide, offering hope to a sub-group of men who would otherwise have little or no chance of achieving a pregnancy using their own gametes. The degree to which ICSI has revolutionised the treatment of severe male factor infertility cannot be overstated, and more than 5 million children have been born globally as a result of the technique (Adamson et al., 2018).

ICSI is now the accepted management for men with oligoasthenoteratozoospermia (OAT), a condition that includes oligozoospermia (low number of sperm), asthenozoospermia (poor sperm movement) and teratozoospermia (abnormal sperm shape), and with azoospermia, whether obstructive or non-obstructive. ICSI using surgically retrieved spermatozoa is currently the only available intervention for azoospermic men hoping to father their own

genetic offspring; in this sub-group, provided live spermatozoa can be retrieved from either the epididymis or the testes, ICSI provides the possibility of achieving fertilisation *in vit*ro, and of pregnancy following transfer of resulting embryo(s). However, while ICSI was developed specifically for the management of male factor subfertility, there are numerous reports of its use where a male factor has not been identified. The potential risks associated with an invasive technique that requires *in vitro* manipulation of gametes (Retzloff & Hornstein, 2003), together with both the additional expense and concern that ICSI may be associated with an increase in the incidence of congenital malformations in resulting offspring (Lacamara et al., 2017; Esteves et al., 2018; Luke et al., 2021) make it essential to establish whether or not the use of ICSI, rather than conventional IVF, carries any benefit in cases where there is no identified male factor that is likely to impair fertilisation (Bodri et al., 2015).

As an ART procedure, ICSI is more labour-intensive and technically challenging than conventional IVF and its cost is reported to be 8.3 % higher in four Dutch centres (Bouwmans et al., 2008). The additional cost is frequently borne by fertility patients. A decision to offer ICSI rather than conventional IVF in a given treatment cycle must be evidence-based, and ultimately rests with appropriately qualified Clinical Scientists (clinical embryologists/andrologists).

Current Guidelines

In the UK, the recognised indications for ICSI as listed in the National Institute for Health and Care Excellence (NICE) Guidelines are:

- Severe deficits in semen quality
- Obstructive azoospermia
- Non-obstructive azoospermia

In addition, NICE guidance recognises that treatment using ICSI should be considered for patients in whom a previous IVF treatment cycle has resulted in failed or very poor fertilisation, and recommends that "*couples should be informed that ICSI improves fertilisation rates compared to IVF alone, but once fertilisation is achieved the pregnancy rate is no better than with IVF*" (NICE, 2004).

The fertility sector in the UK is regulated by the Human Fertilisation and Embryology Authority (HFEA), whose 9th Code of Practice (2019; updated in 2021) states that before treatment using ICSI is offered, centres should provide specific information about its risks including the risks of:

- *a reduced number of eggs being available for treatment (compared to IVF), due to eggs being immature or damaged by the process of ICSI*
- *children conceived having inherited genetic, epigenetic or chromosomal abnormalities (including cystic fibrosis gene mutations, imprinting disorders, sex chromosome defects and heritable sub-fertility).*

While the HFEA makes no specific recommendations with respect to clinical criteria for selecting ICSI rather than conventional IVF in ART treatment, it does require that the reason(s) for performing ICSI should be recorded in the patient's medical notes.

In 2020, the American Society for Reproductive Medicine (ASRM) advised that:

- *ICSI without male factor subfertility may be of benefit for selected patients undergoing IVF with preimplantation genetic testing for monogenic disease and previously cryopreserved oocytes.*

- *The additional cost burden of ICSI for non–male factor indications, where data on improved live-birth outcomes over conventional insemination are limited or absent, must be considered.*

The guidelines presented here have been drafted according to levels of evidence as outlined by NICE (2004) (Table 1).

Trends in the use of ICSI

Despite an absence of conclusive evidence to support its use in ART treatment other than for the management of severe male factor subfertility, or where there has been previous fertilisation failure, ICSI is currently used globally more frequently than conventional IVF; in some countries it is used exclusively in place of IVF for all treatment indications (Dyer et al., 2016). The observed rise in the use of ICSI is neither consistent internationally, nor reflects the reported rise in the incidence of male factor subfertility (Andersen et al., 2008; Povey & Stocks, 2010). For example, in the USA, the use of ICSI between 1996 and 2012 almost doubled, from 36.4% to 76.2%, with the greatest increase found in cycles where no male factor had been identified, in which the use of ICSI increased from 15.4% to 66.9% of cycles (Boulet et al., 2015).

It is clear that the use of ICSI in couples with non-male factor subfertility may be beneficial where a previous IVF attempt resulted in low fertilisation or total fertilisation failure (Van der Westerlaken et al., 2005). However, its use in the management of couples with unexplained infertility, advanced female age, low ovarian reserve or low oocyte yield, presumably in attempts to increase fertilisation rates in these groups, has contributed to the reported progressive overall rise in the use of ICSI compared with conventional IVF (Andersen et al., 2008). This increase is taking place despite evidence that while ICSI may indeed increase fertilisation rates compared with conventional IVF in these patient groups, implantation and

pregnancy rates, and overall reproductive outcomes are not improved (Sfontouris et al., 2015; Boulet et al., 2015; Haas et al., 2021). The incidence of total fertilisation failure with ICSI remains 2% per cycle, compared with 5% after conventional IVF (Bhattacharya et al., 2001).

Numerous studies indicate that the use of ICSI in non-male factor subfertility does not yield better clinical outcomes than IVF; rather, it functions as a normaliser of fertilisation in reducing the number of cases of failed or low fertilisation (Palermo et al., 2015). Based on available evidence, ICSI is not indicated for universal use in ART (Babayev et al., 2014; Bosch et al., 2020), and the observed increase in the use of ICSI over conventional IVF is likely to be largely due to concern over the perceived risk of fertilisation failure (Abbas et al., 2020). In a substantial RCT, ICSI was found to offer no advantage in non-male factor cases (Drakopoulos et al., 2019).

Risks for offspring

Early concerns regarding risks to the foetus (Wennerholm et al., 2000; Hansen et al., 2002) and the long-term health impact on children born after ICSI (te Velde et al., 1998; Van Steirteghem et al., 2002; Tournaye, 2002; Retzloff et al., 2003; Karpman et al., 2005) persist, and questions and controversies remain concerning its use (Fauser et al., 2014; Lacamara et al., 2017; Catford et al., 2018; Luke et al., 2021). These concerns do only reflect slight increases in risk above natural or IVF conception, but until such concerns are resolved fully, use of ICSI should remain restricted to patients where evidence indicates that they are outweighed by the benefit of this intervention.

Indications for ICSI

Male factor

Male factor subfertility is defined as abnormality in one or more semen parameters or in sperm function (Zegers-Hochschild et al., 2017). The minimum levels for sperm concentration,

motility and morphology in normal semen were last standardised in the 6th edition of the World Health Organization Laboratory Manual for Examination and Processing of Human Semen (WHO, 2021), using strict criteria for the evaluation of sperm morphology. Based on the 5th centile from data collected using standardised methodologies for semen analysis (Campbell et al., 2021), parameters have been established by the WHO 6th edition that define oligozoospermia (sperm concentration $<16x10^6$/ml; total number of sperm in the ejaculate $<39x10^6$), asthenozoospermia (<42% total motility; <30% progressive motility) and teratozoospermia (<4% normal forms). An international consensus meeting agreed that ICSI should not be used in normozoospermic men (Cairo Consensus Workshop Group, 2020).

Abnormal semen parameters

Although historically, evidence suggests that ICSI results in increased fertilisation rates compared with conventional IVF in the management of "mild" to "moderate" semen abnormality, these terms are often ill-defined, and there is a lack of contemporary evidence comparing outcomes of ICSI and conventional IVF in such cases. Moreover, early studies reported findings from small samples, and the semen parameters used to define abnormality were inconsistent and not easily comparable with those defined by the WHO (2021). Indeed, as pointed out by the Vienna Consensus group (2017), "*...the current visual evaluation of 200 or 400 spermatozoa used in the vast majority of laboratories to assess '% normal forms' has such a large uncertainty of measurement that it cannot be considered a reliable predictor for IVF success/failure for individual men. Unless determined using a more robust methodology, sperm normal forms should not be used to direct ART treatment options*."

A meta-analysis of 8 published studies concluded that the probability of fertilisation is improved significantly with the use of ICSI compared with IVF in couples suffering from "moderate" male factor subfertility, defined as any combination of oligozoospermia,

asthenozoospermia, and/or teratozoospermia (Tournaye et al., 2002). A later study reported that fertilisation rates are improved, and the risk of total failed fertilisation is decreased when ICSI is used rather than conventional IVF in the treatment of patients with "mild" oligoasthenoteratozoospermia, defined as a sperm concentration of 5–20 × 10^6/ml and/or motility of 5%–15%, and/or 2%–8% morphologically normal forms according to Kruger strict criteria (Elizur et al., 2004).

Of the semen characteristics assessed during routine diagnostic semen analysis (WHO, 2021), studies have not tended to examine the effect of each parameter in isolation. However, a high percentage of abnormal spermatozoa has long been associated with reduced fertility, although even in cases of severe teratozoospermia, fertilisation *in vitro* with conventional IVF insemination may be achieved (reviewed by Ombelet et al., 1995). A number of early studies examined the outcome of conventional IVF in cases of isolated teratozoospermia in semen samples with normal sperm concentration and motility, and reported lower limits for the percentage normal forms that could be used to indicate the need to use ICSI rather than conventional IVF insemination ranging from <4% (Kruger et al., 1988; Robinson et al., 1992) to <8% (Zollner et al., 1996). In a retrospective analysis of 2144 cycles, 68.6% of those where semen analysis showed <4% normal forms achieved the normalised median fertilisation rate for the authors' clinic, provided sperm concentration and motility were within the normal range (Robinson et al., 1992). A further study reported that fertilisation rates of >50% were achieved in 36% of cycles using conventional IVF insemination with semen samples where <5% normal forms were present (Lundin et al., 1997). In this later study, the pregnancy rate per embryo transfer in IVF cycles where the percentage of normal forms was <5% was at least equivalent to that in cycles using conventional IVF with normal semen, confirming that fertilising capacity not necessarily impaired in this poor prognosis group (Lundin et al., 1997). A later meta-analysis concluded that isolated teratozoospermia is not associated with decreased clinical

pregnancy rates with IVF with or without the use of ICSI (Hotaling et al., 2011). However, intra- and interlaboratory variation in sperm morphology assessment makes it difficult to prescribe a universal, definitive cut-off for percentage normal forms where ICSI is indicated rather than conventional IVF (Kohn et al., 2018).

For some cases of "moderate" or "mild" male factor subfertility, it may well be that a higher fertilisation rate is achieved using ICSI compared with conventional IVF, but in the absence of a clear consensus regarding the definitions of "mild", "moderate" and "severe" male factor, centres electing to use ICSI rather than IVF should define and justify their criteria for doing so, such as through pre-treatment assessment of the quality of "trial semen preparations" (Vienna Consensus group, 2017).

Guidance and recommendations for the use of ICSI where semen parameters are abnormal
ART laboratories should define validated centre-specific criteria for semen parameters that determine when to use ICSI rather than conventional IVF to ensure the rate of total failed fertilisation rates remains below 5% per IVF cycle. **GPP** In deriving centre-specific criteria for ICSI, pre-treatment assessment of the quality of semen preparations may be used, with defined parameters in prepared samples to indicate use of ICSI. **B** ICSI should be used in cases of severe male factor subfertility. B ICSI may be considered in cases of moderate male factor subfertility. **B**

Azoospermia

It is estimated that azoospermia accounts for up to 10% of male factor subfertility. ICSI is currently the only available treatment option for azoospermia other than the use of donor semen (Practice Committee of the ASRM in collaboration with the Society for Male Reproduction and Urology, 2019). The aetiology of obstructive azoospermia (OA) may be congenital, iatrogenic, the result of surgery (vasectomy), inflammation or injury, the presence of cysts in ejaculatory ducts and prostate, or Young's syndrome (Esteves & Agarwal, 2013; Nieschlag et al., 2010). Non-obstructive azoospermia (NOA) has its origins in spermatogenic failure without obstruction (Cerván-Martín et al., 2020).

For OA and NOA, both NICE Guidelines in the UK and best practice guidelines in the US include ICSI after testicular or epididymal sperm extraction by surgical sperm retrieval (SSR) for fertility treatment and management (NICE, 2013; Schlegel et al., 2021).

Round spermatids

Following the first report of fertilisation after ICSI using round spermatids in the management of azoospermia (Fishel et al., 1997), the ASRM stated that the technique should be considered experimental (ASRM, 2008) and in several countries, including the UK, treatment with ICSI using round spermatids is currently banned. However, using sophisticated microscopy and artificial oocyte activation (AOA) for a carefully selected patient group, 14 live births have been reported following ICSI using round spermatids (Tanaka et al., 2015). The same group have reported favourable findings concerning the health and cognitive development at 2 years of age of 90 babies born following ICSI using round spermatids, concluding that there were no significant differences between this small sample of ICSI infants conceived by round spermatid injection compared to those conceived naturally (Tanaka et al., 2018). From the limited number of cases reported to date, the success rate with ICSI and round spermatid injection appears to be low (3% live births from 2657 treatment cycles; Tanaka et al., 2018).

Guidance and recommendations for the use of ICSI in azoospermia
ICSI should be used as the method of insemination, rather than conventional IVF, in all ART treatment cycles where surgically retrieved sperm are used. **A** The use of round spermatids for ICSI is considered experimental and not permitted in the UK. **GPP**

Globozoospermia

Globozoospermia is a rare cause of male subfertility characterised by the presence of round-headed, acrosomeless spermatozoa which are incapable of sperm-oocyte fusion or binding the human zona pellucida (Aitken et al., 1990). It accounts for fewer than 0.1% of cases of male subfertility (Dam et al., 2007). In men with partial globozoospermia, the fertilisation and live birth rates following ICSI do not differ from those of the general ICSI population (Dam et al., 2012).

In men where all the sperm in the ejaculate are globozoospermic, ICSI is necessary to achieve fertilisation *in vitro*. In the absence of large studies, it is difficult to evaluate the success rate of ICSI in cases of total globozoospermia, but it is known that while fertilisation can be achieved and successful live births can result, the rates of fertilisation and successful pregnancy outcome are variable and unpredictable. In general, fertilisation, pregnancy and live birth rates are lower with ICSI in globozoospermia than in other groups of subfertile men (reviewed by Dam et al., 2007), the reasons for which are not clear. The condition can present with a wide spectrum of effects (Coutton et al., 2015a,b), and the success rates following treatment may vary even between members of the same family (Kilani et al., 2004). While it is unclear which patients with total globozoospermia will succeed following ICSI, even with the limited data

available it is clear that ICSI should be offered to all globozoospermic patients. Currently, although there are known genetic causes for this disorder (DPY19L2, SPARA16, ZPBP, and PICK1; Fesahat et al., 2020), the prognostic value of genetic assessment with regard to outcome prior to treatment using ICSI has yet to be established (Coutton et al., 2015b).

The negative correlation between globozoospermia and ICSI outcomes highlights the need for appropriate management of these patients (Fesahat et al., 2020). A number of reports suggest artificial oocyte activation (AOA) following ICSI may improve fertilisation potential in men with total globozoospermia, bypassing the requirement of the acrosome and sperm function factors responsible for oocyte activation. Reduced expression of candidate genes in sperm responsible for oocyte activation, such as PLCζ, has been reported in men with globozoospermia compared with fertile men and may explain reduced fertilisation rates with ICSI using globozoospermic sperm in the absence of AOA (Taylor et al, 2010; Kamali-Dolat Abadi et al., 2016; Tavalaee & Nasr-Esfahani, 2016). Although live births following ICSI have been achieved in globozoospermia both with and without AOA (Kilani et al., 1998; Stone et al., 2000; Shang et al., 2019; Niu et al 2020), importantly, higher fertilisation rates have been reported following ICSI with AOA, than following conventional ICSI alone (Chansel-Debordeaux et al., 2015). Therefore, it has been proposed that AOA maybe used to enhance fertilisation in globozoospermic cases (Yanagida et al., 2008). ICSI with AOA should be considered in treatment of total globozoospermia (see *artificial oocyte activation (AOA)* below).

Guidance and recommendations for the use of ICSI in globozoospermia
ICSI should be used as the method of insemination, rather than conventional IVF, in all cases of total globozoospermia **A**

Genetic counselling should be offered to globozoospermic men prior to treatment. **C** AOA may be considered in cases of total globozoospermia. **C**

Sperm DNA fragmentation

Tests of sperm DNA fragmentation use different techniques and caution should be exercised in interpreting and comparing results obtained from the use of different tests. Results must be interpreted according to validated data obtained from the specific test used (WHO 2021). There is increasing evidence to suggest that sperm DNA fragmentation (SDF) significantly affects reproductive outcomes, with reported associations between elevated sperm DNA fragmentation in semen and an increased incidence of miscarriage (Robinson et al., 2012), and reduced fertilisation rates, embryo quality, and pregnancy rates with ART (Simon et al., 2010; 2011), although a systematic review found significant limitations of the evidence due to weakness of methodologies and study designs (Cissen et al., 2016). As a primary intervention, lifestyle changes that reduce the generation of oxidative stress in spermatozoa have been suggested for patients with high sperm DNA fragmentation, with the aim of reducing SDF levels prior to ART (Esteves et al., 2021; Aitken & Bakos, 2021). While acknowledging that adherence to a healthy diet could improve semen quality and fecundity, the authors of a systematic review of observational studies concluded that such studies "*may prove associations but not causation*", and that any observed associations must be confirmed with large prospective cohort studies and well-designed RCTs (Salas-Huetos et al., 2017).

In the management of patients where SDF levels remain high, it has been suggested that ICSI should be used, with the specific selection of progressively motile and morphologically normal spermatozoa for injection, since these characteristics may be

associated with lower levels of DNA fragmentation (Belloc et al., 2014). However, others have reported no such association (Le et al., 2019), and this remains controversial.

Several meta-analyses have reported conflicting results regarding the relationship between elevated SDF and ART outcomes including miscarriage and clinical pregnancy rates (Li et al., 2006; Zhao et al., 2014; Osman et al., 2015; Zhang et al., 2015; Deng et al., 2019; Ribas-Maynou et al., 2021). While findings remain inconclusive, there is a suggestion that the negative impact of DNA fragmentation on ART outcomes may be less with ICSI compared with IVF (Simon et al., 2017; Malic Voncina et al., 2021), although ICSI does not appear to result in a reduction in miscarriage rates compared with IVF in patients with elevated SDF (Zhao et al., 2014). There is a need for well-designed RCTs, where matched patients with similarly elevated SDF levels, as assessed using the same assay, are allocated to IVF and ICSI treatment groups, to provide conclusive evidence for any benefit from the use of ICSI in the management of elevated SDF.

Since DNA fragmentation may be associated with oxidative stress in the male reproductive tract, with levels of DNA fragmentation reported to be significantly higher in ejaculated compared with testicular spermatozoa (Moskovtsev et al., 2010; Bradley et al., 2016), it has been suggested that use of testicular sperm for ICSI may benefit patients with elevated SDF (Esteves et al., 2017). While the findings of a few small-scale studies support this practice, these have largely reported outcomes in couples with previous failed ICSI cycles using ejaculated spermatozoa (Pabuccu et al., 2017; Arafa et al., 2018).

Guidance and recommendations for the use of ICSI in elevated sperm DNA fragmentation levels

Further studies are needed to establish whether or not the use of ICSI in normospermic patients with elevated sperm DNA fragmentation levels leads to improved outcomes compared with IVF. **GPP**

Further studies are needed to establish whether or not the use of testicular sperm should be considered in patients with elevated sperm DNA fragmentation levels if previous ART cycle(s) with ejaculated sperm have been unsuccessful. **GPP**

Other indications

Unexplained infertility

Use of ICSI as first line treatment. Unexplained infertility, thought to represent 30% of subfertility, is defined as a failure to conceive despite normal genito-urinary anatomy, patent fallopian tubes, a healthy cervix and uterus, normal ovarian and testicular function with normal semen, and normal coital frequency (O'Flynn, 2014; Zegers-Hochschild et al., 2017). In patients with well-defined unexplained infertility, the rate of total failed fertilisation is approximately 5% per cycle with conventional IVF (Bhattacharya et al., 2001), and this has prompted investigations into the use of ICSI in such patients. However, many of the studies that have examined the efficacy of ICSI in non-male factor subfertility have included female factor such as tubal disease as well as unexplained infertility.

Initially, in the absence of evidence for improved pregnancy outcomes compared with conventional IVF, ICSI was not recommended for the management of patients with unexplained infertility (ASRM, 2012).

A small RCT comparing pregnancy rates in 60 couples with unexplained infertility following treatment either with IVF or ICSI found no significant differences in implantation, clinical pregnancy or live-birth rates between the two treatment groups (Foong et al., 2006).

One prospective cohort study where sibling oocytes were inseminated either using ICSI or conventional IVF found that for 42 couples with unexplained infertility and confirmed >4% normal sperm morphology, there was no statistical difference in fertilisation rate between ICSI and IVF (Younes et al., 2019).

The majority of studies that have examined the efficacy of ICSI in the treatment of non-male factor subfertility, including female factors and unexplained infertility, suggest that ICSI offers no advantage over conventional IVF in terms of either fertilisation or clinical pregnancy rates (reviewed by Abbas et al., 2020). An early Cochrane review (van Rumste et al., 2000), updated by the same authors in 2003, attempted to compare live birth rates following ICSI or IVF in couples with non-male factor subfertility (unexplained infertility or subfertility due to tubal disease). Two studies that reported live birth rates, but that used alternation rather than randomisation to allocate treatment, reported that for men with borderline semen parameters (10-20 million spermatozoa per ml, 30-50% motility, 4-14% normal forms), fertilisation rates were significantly higher per oocyte retrieved with IVF (477/736; 64.8%) than with ICSI (400/748; 53.5%), but there were no differences between the IVF and ICSI groups in terms of pregnancy, miscarriage or live birth rates (van Rumste et al., 2000, 2003). The authors were able to identify only one RCT (415 patients, 435 treatment cycles) that reported pregnancy rate, but not live birth or miscarriage rates or other adverse outcomes such as congenital malformations (Bhattacharya et al., 2001). They concluded that taking into consideration the additional cost, complexity, possible adverse effects and long term implications of ICSI compared with IVF, for couples with non-male factor subfertility IVF, and not ICSI, should be the treatment of choice. They acknowledged that this remained an open question at that time, in the absence of research focusing on live-birth rates and adverse events.

More recently, a retrospective population-based cohort analysis of 14,693 women who underwent their first cycle of IVF or ICSI between July 2009 and June 2014 in Victoria,

Australia found no difference in the cumulative live birth rate between ICSI and conventional IVF in couples with non-male factor (unexplained and female factor) subfertility (Li et al., 2018). Similarly, an analysis of clinical pregnancy and live birth rates in all fresh stimulated IVF and ICSI treatment cycles recorded on the UK HFEA registry database between 1998 and 2016 found no evidence that ICSI is more effective than conventional IVF in the treatment of couples with subfertility where semen parameters were normal (569,605 cycles; Supramaniam et al., 2020).

Evidence that in cases of unexplained infertility there is no difference between clinical pregnancy or live birth rates following either conventional IVF or ICSI (ASRM, 2020) is strongly supported by the findings of the only RCT to examine this directly (Dang et al., 2021). A further multi-centre RCT to investigate whether ICSI is more effective than conventional IVF in patients without severe male factor subfertility is currently being undertaken in Denmark, with patient enrolment between November 2019 and December 2023 (Berntsen et al., 2021).

Use of split IVF-ICSI as first line treatment. As a form of insurance against total failed fertilisation in patients with unexplained infertility, sibling oocytes may be "split" between IVF and ICSI insemination in the first ART treatment cycle. A systematic review of 11 studies and a total of 901 couples with well-defined unexplained infertility, where sibling oocytes were randomly allocated to insemination using conventional IVF or ICSI, found marginally higher fertilisation rates, and a significantly lower risk of total failed fertilisation with ICSI than with IVF (Johnson et al., 2013). The authors concluded that in the management of well-defined unexplained infertility, it may be appropriate to use ICSI, or split IVF-ICSI inseminations in order to avoid total fertilisation failure and potentially to increase cumulative pregnancy rates. However, variations between the data sets made it impossible to undertake a meta-analysis of pregnancy outcomes (Johnson et al., 2013).

Several retrospective studies have suggested that the parameters of prepared sperm samples may be indicative of fertilisation outcomes using conventional IVF (Rhemrev et al., 2001; Repping et al., 2002; Wiser et al., 2012). This has led to the suggestion that for couples with unexplained infertility where prepared sperm samples do not meet defined criteria for IVF, it is appropriate to use ICSI, or split IVF-ICSI insemination (Wiser et al., 2012). In a study of 259 cycles of split IVF-ICSI in couples with unexplained infertility, only 8 (3%) cycles failed to achieve fertilisation in sibling oocytes inseminated with conventional IVF (Lee et al., 2017). Thus, while split IVF-ICSI insemination for unexplained infertility may reduce the risk of total fertilisation failure, evidence suggests that this strategy will "rescue" only around 3% of cycles from total fertilisation failure.

Guidance and recommendations ART treatment in couples with unexplained infertility
ICSI should not be considered as first-line therapy for unexplained infertility. **A** Split IVF-ICSI inseminations should not be used as first-line therapy for couples with unexplained infertility. **A** Further research is needed to establish whether ICSI results in better clinical outcomes than IVF in couples with unexplained infertility. **GPP**

Use of ICSI following total failed fertilisation with IVF

Rescue ICSI in the same ART cycle. Rescue ICSI is the process where metaphase II oocytes that have failed to fertilise following IVF insemination are injected with sperm using ICSI. One study reported a fertilisation rate of 74.6 % with rescue ICSI of 1831 unfertilised oocytes from 313 cycles in patients with complete failed fertilisation following IVF (Cao et al., 2016).

Historically, concerns that some oocytes that fail to develop pronuclei following conventional IVF insemination may have been penetrated by spermatozoa, and that rescue ICSI using these oocytes would increase the likelihood of polyploid embryos developing led to the antecedent of ARCS (ACE) and subsequently the HFEA in the UK to stipulate that oocytes which have failed to show signs of normal fertilisation after IVF or ICSI insemination must not be re-inseminated, whether by IVF or ICSI (21.4(c); HFEA Code of Practice, 9th Edition). There is a paucity of data either to support or refute this concern, although a single report found 22.6% of 509 rescue ICSI cycles undertaken between 2014 and 2019 resulted in one or more embryos with 3 pronuclei (Chen et al., 2021).

A systematic review of rescue ICSI which included 1,863 patients found an overall pregnancy rate of 14.4%, but contained insufficient data to enable an evaluation of any effect on malformation rates (Beck-Fruchter et al., 2014). A further systematic review which included 22 original studies found that implantation and clinical pregnancy rates following fresh embryo transfer after rescue ICSI were relatively low (5% and 10% respectively; Paffoni et al., 2021). However, the transfer of cryopreserved rescue ICSI embryos yielded significantly higher implantation and pregnancy rates (18% and 36% respectively), leading the authors to conclude that rescue ICSI in combination with frozen embryo transfer to overcome technical and biological issues associated with delayed fresh transfer after rescue ICSI may be beneficial (Paffoni et al., 2021).

With regard to risks to offspring, a single study which compared 233 children born after rescue ICSI with 906 children born following conventional ICSI found no differences in neonatal outcomes (Chen et al., 2014). Among the studies examined for their systematic review (Paffoni et al., 2021), fewer than 180 births deriving from fresh and frozen cycles using rescue ICSI were described, and other than 2 miscarriages due to malformations and 1 case of microtia, no relevant health problems in newborns were reported. Therefore, information to

date from the small number of births that have resulted from rescue ICSI does not suggest an increase in adverse outcomes, including malformation rates, following its application.

ICSI in the subsequent ART cycle. Total fertilisation failure after conventional IVF insemination is considered a reliable predictor of failed fertilisation in subsequent IVF cycles, with a reported recurrence rate ranging from 29% (Barlow et al., 1990) to between 45% and 70% (Roest et al., 1998). Numerous studies suggest that ICSI is beneficial in overcoming previous fertilisation failure using conventional IVF (Palermo et al., 1996; Benadiva et al., 1999), and this is reflected in the NICE guidelines which state that ICSI should be considered for couples in whom a previous IVF treatment cycle has resulted in failed or very poor fertilisation (NICE, 2004). However, it has been reported that fertilisation may be achieved in between 94% and 97% of subsequent repeat IVF insemination in such patients (Lipitz et al., 1994; Kinzer et al., 2008). Moreover, conventional IVF insemination after a cycle of fertilisation failure cycle has been shown to result in pregnancy and live birth rates equivalent to those achieved in other patients following a routine second cycle of IVF (22% compared with 24%) and with patients who had ICSI in their second cycle (24%; Kinzer et al., 2008). In many cases, total fertilisation failure is related more to a suboptimal response to ovarian stimulation than to semen parameters, and improved stimulation in a subsequent cycle may increase the chance of fertilisation with conventional IVF (Kinzer et al., 2008).

Split IVF-ICSI in the subsequent ART cycle. Following a cycle of unexplained total failed fertilisation with conventional IVF, a number of small studies have reported outcomes after splitting mature sibling oocytes between ICSI and conventional IVF insemination in the subsequent cycle (Elizur et al., 2004; van der Westerlaken et al., 2005). The findings suggest that performing ICSI on at least some of the oocytes will avoid repeated total fertilisation failure. However, the possibility should be considered that, in some instances, failed fertilisation reflects poor oocyte quality, or other sub-optimal clinical or laboratory factors,

rather than poor sperm function. Each case of unexplained total failed fertilisation following conventional IVF should be assessed as to whether or not alternative management, such as modification of ovarian stimulation in a subsequent cycle, may be more appropriate than changing the method of insemination from IVF to ICSI (Kinzer et al., 2008).

Use of ICSI following poor fertilisation with IVF. There is no consistent definition of a "poor" fertilisation rate in the literature. The Vienna Consensus on KPIs in the ART laboratory established a competency reference value of ≥60% normal fertilisation as the accepted minimum rate after conventional IVF, with poor fertilisation defined as <25% normal fertilisation of inseminated oocytes (ESHRE SIG/Alpha, 2017).

ICSI in the subsequent ART cycle. There is evidence from a small randomised study to support the use of ICSI as a secondary measure to avoid a repetition of a poor (<25%) fertilisation rate (Van der Westerlaken et al., 2005), but there are no data available comparing live birth outcomes after conventional IVF or ICSI treatment specifically in patients with previous poor fertilisation following IVF.

In the UK, the use of ICSI for patients with a history of poor fertilisation with IVF is endorsed by NICE guideline CG156 (NICE 2013) and by the HFEA (2019), although 'poor' is not defined.

Split IVF-ICSI in the subsequent cycle. An alternative strategy in cycles following poor fertilisation with IVF is to split oocytes between insemination using conventional IVF and ICSI (Elizur et al., 2004; Van der Westerlaken et al., 2005; Kinzer et al., 2008). In one retrospective study, ICSI did not significantly improve fertilisation rates in 27 couples with normal semen characteristics and low fertilisation rates (≤30%) in previous IVF treatments, nor were differences observed in embryo cleavage rates after ICSI or conventional IVF (Elizur et al.,

2004). It can be concluded that there may be no benefit in performing split IVF-ICSI in couples with normal sperm characteristics following an IVF cycle with poor fertilisation.

Guidance and recommendations for the ART cycle following an IVF treatment cycle with total failed fertilisation or poor fertilisation
ICSI can be used as the method of insemination for patients with unexplained infertility where a previous IVF treatment cycle has resulted in total failed fertilisation. **B** ICSI may be used as the method of insemination when a previous IVF treatment cycle has resulted in poor fertilisation, with "poor" defined as <25% of mature (MII) oocytes inseminated oocytes achieving normal fertilisation. **B** Despite its prohibition in the UK, evidence to date does not support concerns that rescue ICSI may increase the incidence of abnormalities. Where permitted by law, rescue ICSI may be considered. **B**

Cryopreserved oocytes

Oocyte vitrification currently requires the removal of cumulus cells prior to cryopreservation which, together with vitrification itself, may alter the structure of the zona pellucida, possibly through premature cortical granule discharge, potentially resulting in inhibition of sperm penetration after conventional IVF insemination. Although there is a paucity of direct data to indicate that ICSI is required to fertilise human cryopreserved oocytes, it is known that cumulus cells serve multiple functions including mechanical entrapment of sperm and sperm capacitation (Van Soom et al., 2002), release of chemotactic factors to aid fertilisation (Oren-Benaroya et al., 2008) and mediation or promotion of fertilisation (Tanii et al., 2011). Therefore, ICSI has been adopted as the method of insemination for vitrified-warmed oocytes

(reviewed by Gook & Edgar, 2007). The ASRM have advised that ICSI without male factor subfertility may be of benefit for select patients undergoing ART with previously cryopreserved oocytes (ASRM, 2020).

Guidance and recommendations for the use of ICSI with cryopreserved oocytes
ICSI should be used as the method of insemination of thawed/warmed cryopreserved oocytes. **C**

In vitro *matured oocytes*

ICSI is sometimes used for insemination of in *vitro* matured oocytes, on the assumption that structural alterations to the zona pellucida may occur during in *vitro* maturation (IVM) thereby preventing fertilisation using conventional IVF. It has been reported that the fertilisation rate is higher using ICSI compared with IVF, for both cumulus-intact (84.1% and 56.3% respectively; n=289; $p<0.01$) and denuded IVM oocytes (84.5% and 39.5% respectively; n=152; $p<0.01$; Hwang et al., 2000). The cleavage rates for both sets of fertilised oocytes were similar.

These findings contrast with those of a second RCT carried out in patients with polycystic ovary syndrome (PCOS), where 113 sibling IVM oocytes were assigned randomly for insemination either using ICSI or IVF. No significant difference was found between the IVF or ICSI groups of IVM oocytes in terms of fertilisation, blastocyst development, or clinical pregnancy rates for fresh and cryopreserved embryo transfers between the two groups (Walls et al., 2012).

Further studies are needed to establish the recommended method for insemination of IVM oocytes.

Guidance and recommendations for the use of ICSI after *in vitro* maturation
Conventional IVF may be used as the method of insemination for cumulus intact *in vitro* matured oocytes, unless ICSI is indicated by poor semen parameters. **C** Further studies are required comparing outcomes following IVF and ICSI with *in vitro* matured oocytes. **GPP**

Preimplantation genetic testing (PGT)

The ASRM have advised that ICSI without male factor subfertility may be of benefit for select patients undergoing ART with PGT for monogenic disease (PGT-M; ASRM, 2020). Historically, ICSI has been used for PGT-M cycles using fresh oocytes in order to ensure monospermic fertilisation and to eliminate the risk of introducing contaminating DNA from sperm that may remain attached to the zona pellucida following conventional IVF insemination. However, a recent cohort study looking at 927 patients undergoing PGT-M where IVF or ICSI was used to achieve fertilisation found no difference between the two groups in the incidence of PCR failure and/or incomplete diagnosis, and both groups showed negligible contamination with paternal DNA (Feldman et al., 2017).

In PGT for aneuploidy (PGT-A), conventional insemination (IVF) may be used where semen parameters are normal. However, in a small retrospective study to evaluate rates of euploidy, aneuploidy, and mosaicism in trophectoderm biopsy samples following IVF (n=75) or ICSI (n=227) in PGT-A cycles using next generation sequencing (NGS), although similar rates of euploid, aneuploid, and “no result embryos” were found in both groups, higher rates of mosaicism were found in IVF embryos The authors concluded that ICSI may be preferable

to conventional IVF insemination to minimise the rate of mosaic results in NGS PGT-A cycles (Palmerola et al., 2019).

Guidance and recommendations for the use of ICSI prior to genetic testing of embryos
Conventional IVF may be used as the method of insemination for PGT-M cycles, unless ICSI is indicated by poor semen parameters. **B** Further studies are necessary to establish whether or not ICSI is necessary to minimise the rate of mosaic results in NGS PGT-A cycles. **GPP**

Advanced maternal age

There is currently no conclusive evidence that ICSI offers any advantage over conventional IVF in women of advanced age, with the majority of studies failing to identify a difference in fertilisation or pregnancy rates in patients with normal semen parameters (Luna et al., 1998; Tannus et al., 2017; ASRM, 2012).

A retrospective study of 54 couples with non-male factor subfertility, where sibling oocytes were inseminated randomly using either ICSI (n=245) or IVF (n=259), reported an increase in the fertilisation rate and percentage of good quality embryos following ICSI compared with IVF in women aged 35-39 years; the same increases were not seen in women aged 40-45 years (Farhi et al., 2019). In a separate retrospective study of IVF and ICSI outcomes in 685 women aged ≥40 years with unexplained infertility, the pregnancy and live birth rates were the same in both groups, irrespective of the method of insemination (Gennarelli et al., 2019).

Guidance and recommendations for the use of ICSI for advanced maternal age
Conventional IVF should be used rather than ICSI as the method of insemination for oocytes from women of all ages, unless ICSI is indicated by poor semen parameters. **A**

Low oocyte yield

Current evidence does not support the use of ICSI rather than conventional IVF for low oocyte yield (ASRM, 2012). An RCT of 104 treatment cycles in 96 couples with non-male factor subfertility where ≤6 oocytes were retrieved reported no significant difference in fertilisation rate, embryo number or pregnancy rates whether IVF or ICSI was used for insemination (Moreno et al., 1998). These findings are supported by a recent large European multicentre retrospective analysis of outcomes in 4891 patients with non-male factor subfertility following treatment using either ICSI (n=4227) or conventional IVF (n=664) (Drakopoulos et al., 2019). In this series, fresh and cumulative live birth rates did not differ significantly for IVF and ICSI in women showing poor (1-3 oocytes), suboptimal (4-9 oocytes), normal (10-15 oocytes), and high (>15 oocytes) response to ovarian stimulation.

Guidance and recommendations for the use of ICSI for low oocyte yield
Conventional IVF should be used rather than ICSI as the method of insemination for oocytes from women with low oocyte yield, unless ICSI is indicated by poor semen parameters. **A**

HIV/Hepatitis B/Hepatitis C positive patients

In the treatment of patients with blood-borne viruses (BBV), perceived risks are seroconversion after treatment of the uninfected female partner where the male partner is BBV-positive,

transmission of different strains of HIV to the female partner in sero-concordant couples, vertical transmission to the baby, and infection of clinical staff (Devaux et al., 2003; Ethics Committee of the ASRM, 2015).

Although there is currently insufficient evidence to support the use of ICSI rather than conventional IVF in the treatment of patients who test positive for HIV, Hepatitis B or Hepatitis C, it has been proposed that ICSI, where each oocyte is exposed to a single spermatozoon, may pose less risk of viral transmission than conventional IVF (Wu et al., 2015). Semen washing and preparation procedures to reduce or eradicate viral load are common practice in treating sero-discordant couples where the male partner is BBV-positive (Garrido et al., 2004, 2009; Inoue et al., 2017).

A study of 741 sero-discordant couples where the male partner was HIV-positive reported no seroconversion in female partners after intra-uterine insemination (IUI; 2400 cycles), or IVF/ICSI (total 283 cycles; no details were provided concerning the number of IVF or ICSI cycles) when semen was washed prior to use in treatment. Moreover, there was no vertical transmission in the newborns in any of the treatment groups (Savasi et al., 2007). A similar, larger multi-centre retrospective analysis of outcomes in 1036 sero-discordant couples was carried out by the European CREAThE Network, which found no seroconversion in the female partners when washed semen was used for IUI (2840 cycles), IVF (107 cycles) or ICSI (394 cycles), although approximately 7% of cases were lost to follow up and no results were available for evaluation of potential vertical transmission (Bujan et al., 2007). The most recent systematic review and meta-analysis concluded that semen washing provides a safe and effective treatment prior to ART for HIV sero-discordant couples. In the studies reviewed, with a total of 11,585 ART cycles in 3994 women, there were no reported instances of seroconversion in HIV-negative women after IUI, IVF or ICSI treatment with washed sperm

from their HIV-positive partners. Among those studies that measured HIV transmission to infants, there were no reports of cases of vertical transmission (Zafer et al., 2016).

The 2021 ESHRE guideline on medically assisted reproduction (MAR) in patients with a viral infection/disease supports the use of IUI, IVF or ICSI in sero-discordant couples and when both partners test positive for hepatitis B virus (HBV), hepatitis C virus (HCV) or human immunodeficiency virus (HIV), and recommends specific semen preparation procedures to reduce or eradicate viral load (ESHRE, 2021).

Guidance and recommendations for treating viral positive patients
There is insufficient evidence to indicate that ICSI is necessary rather than conventional IVF in the treatment of BBV-positive patients and sero-discordant couples, unless ICSI is indicated by poor semen parameters. **B**

Prevention of polyspermy

The reported incidence of zygotes with three pronuclei ranges between 4% and 8% following conventional IVF (Staessen & Van Steirteghem, 1997; Macas et al., 1996; Plachot et al., 1992; Porter et al., 2003; cited in Joergensen et al., 2015), and of 6% following ICSI (Staessen & Van Steirteghem, 1997). Triploidy may arise either through polyspermic fertilisation of IVF-inseminated oocytes, or through oocyte-derived meiotic failure, which will manifest as triploidy in both IVF- and ICSI-inseminated oocytes (Porter et al., 2003).

It is not possible to establish the origin of triploidy without further investigations that are not routinely available in the clinical setting, so for couples in whom conventional IVF results in a high proportion of triploid zygotes, the use of ICSI in subsequent cycles is likely to minimise the risk of a recurrence if the origin of triploidy is polyspermy (Egozcue et al., 2002;

Jun et al., 2006). However, if triploidy following IVF originates through oocyte-derived meiotic failure, ICSI will not resolve the problem, which may recur in subsequent cycles.

Guidance and recommendations for the prevention of polyspermy
ICSI may be considered when a previous IVF cycle resulted in a high percentage of zygotes developed 3 or more pronuclei in a previous IVF cycle. **GPP** Reduction in sperm insemination concentration at IVF may be considered when a previous IVF cycle resulted in a high percentage of zygotes developed 3 or more pronuclei in a previous IVF cycle. **GPP**

Use of frozen semen

In mouse models it has been shown that when cryopreserved sperm are used for insemination, higher fertilisation rates are achieved with ICSI than with conventional IVF (Szczygiel et al., 2002). However, there have been several retrospective studies in humans comparing outcomes following conventional IVF using cryopreserved donor sperm with those using fresh semen samples showing no significant difference in pregnancy rates (Cohen et al., 1985; Robinson et al., 1993; Le Lannou et al., 1995; Clarke et al., 1997). A retrospective study of 69 cycles of conventional IVF using cryopreserved donor sperm reported an association between poor outcomes and progressive motility of <88% in the sperm preparation used for treatment (Wang et al., 2009). Further studies are needed to establish the recommended method of fertilisation when using frozen sperm, and to define a reference value for percentage sperm motility in the prepared sample that indicates the use of ICSI rather than IVF when using cryopreserved semen.

Guidance and recommendations for the use of frozen semen
ICSI should be considered if the percentage motility of cryopreserved sperm after preparation does not meet the laboratory's criteria for IVF. **B** ART laboratories should define validated centre-specific criteria for prepared cryopreserved sperm to determine when to use ICSI rather than conventional IVF to minimise the risk of poor or failed fertilisation. **GPP** Further studies are required comparing outcomes of IVF and ICSI using frozen ejaculated sperm. **GPP**

ICSI-associated techniques

A number of techniques have been developed, with the aim of improving ICSI outcomes by selecting sperm with specific morphological or physiological characteristics, by visualising the meiotic spindle in the oocyte at the time of ICSI, or by attempting to activate the oocyte after sperm injection.

Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

In routine ICSI, sperm are selected for injection using microscopy with 200x – 400x magnification. For IMSI, higher magnification (>6000x) is used, enabling the selection of motile sperm according to detailed morphological assessment (Bartoov et al., 2002).

Two Cochrane reviews have concluded that current evidence from RCTs neither supports nor refutes the clinical use of IMSI rather than routine ICSI, when comparing outcomes in terms of live birth, risk of miscarriage and clinical pregnancy rate (He et al., 2018; Teixeira et al., 2020).

The significant increase in time required for IMSI procedures in comparison to routine ICSI as well as the additional expense in terms of both practitioners' time and the cost of essential microscopic equipment should be considered before selecting IMSI for routine use (Berkovitz et al., 2005).

Guidance and recommendations for the use of IMSI
There is no evidence that the use of IMSI as a method of sperm selection and insemination results in an increase in clinical pregnancy or live birth rates compared with routine ICSI. **A**

Physiological Intra-Cytoplasmic Sperm injection (PICSI)

PICSI enables the selection of spermatozoa according to their ability to bind to hyaluronic acid or hyaluronan, a major component of the cumulus complex surrounding the oocyte (Jakab et al., 2005). The head of a mature spermatozoon has a specific receptor that allows it to bind to hyaluronan, while immature sperm lack this hyaluronan-binding capacity (Huszar et al., 2006). Hyaluronan-selected sperm are reported to have reduced levels of DNA damage and aneuploidy (Pregl Breznik et al., 2013).

A Cochrane review comparing outcomes following conventional ICSI and PICSI found insufficient evidence to determine whether the use of sperm selected by hyaluronan binding improves live birth or pregnancy outcomes compared with the use of conventionally selected sperm (McDowell et al., 2014). A later review found that the rate of live birth, clinical pregnancy, implantation, embryo quality, fertilisation and miscarriage were similar for both ICSI and PICSI (Avalos-Duran, 2018).

A large multi-centre RCT comparing outcomes following conventional ICSI and PICSI found no significant difference in terms of live birth rates (Miller et al., 2019), although a

reduced risk of miscarriage was identified (Kirkman-Brown et al., 2019), and the age-related increase in miscarriage rate (after clinical pregnancy) was mitigated by PICSI (West et al., 2022).

As with IMSI, PICSI takes longer than routine ICSI, together with the need to use relatively expensive, commercially prepared PICSI dishes for sperm selection means that the cost of the procedure is significantly higher than that of routine ICSI.

Guidance and recommendations for the use of PICSI
There is no evidence that PICSI results in an increase in live birth rates compared with routine ICSI. **A** Further studies are required to establish whether PICSI could be recommended where there is an increased miscarriage risk. **C**

Computer-assisted polarised light microscopy

The integrity of the oocyte's spindle can be assessed according to its birefringence using computer-assisted polarisation microscopy (Rienzi et al., 2005). In a study where sibling oocytes were randomly allocated to selection for ICSI according to the alignment of the meiotic spindle or to ICSI without selection, fertilisation and embryo morphology was improved in the spindle-aligned group (Asa et al., 2017), but a similar study found no significant difference between the selected and unselected oocyte groups in terms of clinical pregnancy rates (Swiatecka et al., 2014). Other groups have reported increased fertilisation potential, embryo development and implantation and clinical pregnancy rates in embryos derived from selected oocytes where the meiotic spindle had been visualized at the time of ICSI (Wang et al., 2001; Madaschi et al., 2008, 2009).

A meta-analysis which included 10 controlled trials concluded that while fertilisation rates were higher when oocytes were selected for injection using meiotic spindle visualisation than with routine ICSI using unselected oocytes, the clinical pregnancy and implantation rates were not statistically different (Petersen et al., 2009).

As with PICSI and IMSI, there are considerable additional risks associated with meiotic spindle visualisation and additional costs in terms of equipment and practitioners' time.

Guidance and recommendations for the use of ICSI with spindle visualisation
There is no evidence to indicate that ICSI with oocyte selection by meiotic spindle visualisation yields an increase in clinical pregnancy and implantation rates compared with routine ICSI using unselected oocytes. **A**

Artificial oocyte activation (AOA)

The failure of some mature oocytes to fertilise following ICSI may be associated with sperm-associated oocyte activation deficiency. On penetration of the oocyte, phospholipase C-zeta (PLCζ) from functional sperm triggers the periodic release of Ca^{2+} from intracellular reserves resulting in the cytosolic calcium (Ca^{2+}) oscillations associated with oocyte activation. PLCζ, and other stimulants such as calcium ionophore A23187, ionomycin, strontium chloride or even electric pulses have been used to produce cytosolic Ca^{2+} influx and activate oocytes artificially. There are concerns that AOA may lead to an increased risk of malformations, and further studies are required to establish whether or not AOA is an effective and safe method to use in attempts to overcome ICSI failure (Anifandis et al., 2019).

A meta-analysis of published reports of the use of calcium ionophore in AOA concluded that rates of fertilisation, cleavage, blastulation, and implantation, as well as overall pregnancy and live-birth rates, were significantly higher with the use of calcium ionophore for AOA after ICSI compared with no activation (Murugesu et al., 2017). However, until studies have been carried out that examine any potential epigenetic consequences or differences in gene expression associated with AOA, its use remains experimental (Ebner & Montag, 2016).

An RCT has compared outcomes following ICSI with or without AOA in 343 couples with primary infertility and 2 previous ICSI treatment cycles with low (<30%) or no fertilisation and/or with male factor subfertility. The findings indicated that AOA using either SrCl2 (n=115) or calcimycin (n=113) resulted in improved clinical pregnancy and live birth rates compared with ICSI without AOA (n=115; Fawzy et al., 2018).

Guidance and recommendations for the use of ICSI with artificial oocyte activation
AOA should not be used routinely with ICSI as its safety, in terms of the potential developmental consequences and birth outcomes, has yet to be established. **A** ICSI with AOA may be used where two previous routine ICSI cycle(s) have resulted in <30% or no fertilisation. **A** Where AOA is used, patients should be advised that safety, in terms of the potential developmental consequences and birth outcomes, has not been established. **GPP** Patients should be provided with safety data relating to the specific AOA technique used. **GPP**

Methods for selecting viable sperm from immotile samples

For samples where all the spermatozoa are immotile, whether ejaculated (total asthenozoospermia), testicular or epididymal, identifying live sperm for use in ICSI is

challenging. Reviews of the functional tests that are available and may be used for the identification and selection of viable sperm for ICSI from an immotile population agree that for most, larger series are needed to evaluate fully each method and their indications before considering their wider application (Herbemont & Sifer, 2015; Simopoulou et al., 2016; Vaughan & Sakkas, 2019).

Modified Hypo Osmotic Swelling Test (HOST)

The hypo osmotic sperm swelling test (HOST) was introduced as a functional assay for infertility diagnosis (Jeyendran et al., 1984). It distinguishes between live and dead immotile spermatozoa through exposure to hypo-osmotic conditions. Water influx into the cytoplasm of live sperm through the functioning plasma membrane results in differing tail swelling patterns, distinguishing them from dead sperm with no tail swelling. HOST may be used for the identification and selection of live sperm for use in ICSI from a population of immotile forms.

In an RCT comparing fertilisation and clinical pregnancy rates when immotile testicular sperm were selected for use in ICSI according to either HOST or morphology showed that rates of both were significantly higher using the HOST compared with morphological assessment for sperm selection (Sallam et al., 2005). According to the WHO (2021), HOST can be used in cases of total asthenozoospermia in order to select viable spermatozoa for ICSI. To date, no studies have evaluated the effect on the children born following ICSI and sperm selection using HOST.

Motility stimulants

Early comparisons of different chemicals for their ability to enhance sperm motility (Hammitt et al., 1989) led to the first clinical use of pentoxyphylline (PF) as an initiator of motility in severe male factor subfertility (Yovich et al., 1988) and in immotile testicular sperm prior to ICSI (Tasdemir et al., 1998). PF and its fellow methylxanthine derivative theophylline are

phophodiesterase inhibitors and increase levels of intracellular cyclic AMP which plays a role in sperm motility. Both are now commonly used with sperm samples with no or only occasional motile sperm to reduce significantly the length of time spent performing ICSI with such samples. A retrospective comparison of outcomes following ICSI in 77 cases where all the testicular sperm retrieved after SSR were immotile found that for the 47 cycles where supplementation with PF was used, some sperm became motile allowing easier identification of vital sperm, shortening the length of time taken for ICSI, yielding improved fertilisation rates and an increase in the number of embryos available for use in treatment compared with those without use of PF (Kovacic et al., 2006).

Despite concerns regarding the safety of use of these chemical compounds for sperm motility enhancement and ICSI there is, as yet, no evidence of an increase in adverse outcomes for offspring. No anomalies were found in the offspring following PF treatment in two case reports where live births were reported (Hattori et al., 2011; Ebner et al., 2014). A review of 122 babies born after ICSI with sperm selection following motility enhancement with PF found no evidence of an increase in adverse outcomes, including malformation rates, following the use of PF (Navas et al., 2017). However, to date there have been no large follow-up studies of pregnancy and neonatal outcomes after use of methylxanthine motility enhancers reported.

Laser assisted immotile sperm selection (LAISS)

Laser assisted immotile sperm selection (LAISS) targets the tips of immotile sperm with a laser beam of approximately 200 μJ and an irradiation time of about 2 ms (Aktan et al., 2004). The thermal effect of the laser causes a localised increase in temperature resulting in changes to the sperm cell membrane proteins, and spermatozoa whose tails curl after the laser shot are regarded as viable and can be selected for use in ICSI. LAISS has the advantages that it can be performed with normal culture media, does not require the use of chemical reagents, is easy to

perform, the selected spermatozoa can be used directly for ICSI, and the laser energy level can be adjusted as needed. The percentage of immotile spermatozoa classified as viable by the laser test has been shown to be similar to that detected by the HOST (Aktan et al, 2004).

A small number of studies have reported the use of LAISS for the selection of viable but immotile spermatozoa for ICSI (Nordhoff, 2015; Simopoulou et al., 2016; Chen et al., 2019), and it appears that similar fertilisation and embryo cleavage rates can be achieved using LAISS to that with use of motile testicular spermatozoa (Chen et al., 2016). A single study of ICSI using testicular sperm found no negative effect on pregnancy rates, perinatal or neonatal outcomes after using LAISS for selection of viable immotile sperm in 33 cycles compared with 99 cycles where motile sperm were used (Chen et al., 2021).

While LAISS is an effective and rapid method of identifying viable sperm for ICSI, the requirement for expensive, specialised equipment and the lack of studies comparing its effectiveness with alternative methods for selection of viable immotile sperm mean its use has not been adopted widely.

Guidance and recommendations for the use of methods for the selection of viable sperm from a population of immotile forms
HOST, pentoxifylline and LAISS can be used in the absence of motile sperm for the selection of viable immotile sperm for use in ICSI. **B** Further research is required to investigate the long-term effects on children born as a result of the use of HOST, pentoxifylline or LAISS for viable immotile sperm selection. **GPP**

Risks of ICSI

Damage to oocytes

Degeneration of oocytes after injection is a recognised risk associated with ICSI (Rubino et al., 2016), and it has been suggested that the rate of oocyte damage following ICSI should be no greater than 10% (ESHRE SIG/Alpha, 2017; Hughes, 2012). While it is possible that oocyte damage may be a consequence of the procedure itself, equally it may be a reflection of poor oocyte quality, where non-viable oocytes are more vulnerable than good quality oocytes to microinjection (Rosen et al., 2006).

Inherited male subfertility

For the 20-25% of couples where the male partner has severe male factor subfertility, there is a risk that associated genetic factors will be inherited by offspring that are conceived after ICSI (reviewed by Krausz & Riera-Escamilla, 2018). Genetic defects detected in the sperm of men with severe male factor subfertility include deletion of heterochromatin, and an increase in DNA fragmentation, telomere length and apoptosis (Loke & Craig, 2016), and genetic testing is recommended in the evaluation of the severely oligozoospermic (total sperm count $<5 \times 10^6$) or azoospermic male patient (Krausz & Riera-Escamilla, 2018). Given the increased risk of chromosomal abnormalities in these men, it is recommended that karyotyping and a Y chromosome microdeletion assay is performed (ASRM, 2015).

Couples who are contemplating ICSI treatment where Y chromosome microdeletion(s) have been identified in the male partner (Colaco & Modi, 2018) should receive appropriate genetic counselling, since a male child has 100% risk of inheriting this disorder and will require fertility treatment with ICSI if he wishes to father children in later life.

Preliminary data suggest that young men conceived following ICSI may have impaired spermatogenesis (Rumbold et al., 2019), although the mechanisms underlying this remain unclear and require confirmation through larger studies. Semen analyses in 54 young male adults conceived following ICSI found that median sperm concentration, total sperm count and

total motile sperm count were significantly lower compared to the same parameters in semen from 57 spontaneously-conceived peers (Belva et al., 2016). In a later study, the group reported that in the same ICSI 54 male offspring, the risk of sperm concentration and total sperm count below the reference values was increased, and semen parameters did not correlate in paired father-son semen analyses (Belva et al., 2019). There is a need for large studies that compare spermatogenesis and sperm quality between offspring conceived after ICSI for the treatment of male factor and non-male factor subfertility in order to clarify the nature and extent of any intergenerational impact.

Guidance and recommendations for the management of
Genetic counselling should be offered to couples who are contemplating ICSI treatment where Y chromosome microdeletion(s) have been identified in the male partner. **C**

Birth defects and child development

While some of the adverse outcomes that have been reported in ART offspring may be due to the increased risk of health problems in the offspring of couples pursuing ART, rather than a consequence of the ART itself (Sanchez-Calabuig, 2014), it remains difficult to distinguish between the two. Early follow-up studies of ICSI children provided preliminary reassurance that the procedure is safe, in that any slight increase identified in the incidence of neonatal malformations or de novo abnormalities observed in ICSI offspring may be related to the genetics of the subfertile couples rather than to ICSI itself. A comparison of 3372 children conceived by ICSI and 9016 children conceived naturally found a relative risk for congenital malformation of 1.44 (95% CI: 1.25–1.6) for ICSI (Loke & Craig, 2016). An analysis of 6163 IVF and ICSI births in South Australia among a total of 308,974 births in the same period concluded that the risk of birth defects associated with ICSI remained increased, although even

after multivariate adjustment the possibility of further confounding variables could not be ruled out (Davies et al., 2012).

A population-based cohort study compared ART cycles undertaken between 2004 and 2015 reported to the US Society for Assisted Reproductive Technology Clinic Outcome Reporting System (SART CORS) with a 10:1 sample of non-ART births within the same study period and concluded that ART in general is associated with increased risks of birth defects compared with natural conception (Luke et al., 2021). Risks of a major non-chromosomal birth defect, cardiovascular defect and any defect were increased in singleton children, and of chromosomal defects were increased in twins, and the use of ICSI was found to increase this risk further, with the greatest increase where a male factor was identified. Thus, compared to naturally-conceived singletons the study reported a 30% increase in the risk of a major non-chromosomal birth defect with the use of ICSI in the absence of male factor, increasing to 42% in the presence of a male factor diagnosis.

In contrast, a recent systematic review and meta-analysis found no support for any robust conclusions concerning the relative risk of chromosomal abnormalities in pregnancies and children following conventional IVF, ICSI or natural conception (Berntsen et al., 2021). No RCTs examining the risk of chromosome abnormalities in ICSI offspring were identified, all studies had a critical risk of bias, and only five studies were eligible for pooled analyses on adjusted data. These showed no evidence of an increased risk of overall chromosome abnormalities when comparing ICSI to either conventional IVF or natural conception. In contrast, meta-analyses on unadjusted data did show an increased risk of overall chromosome abnormalities in ICSI compared to both conventional IVF and natural conception, and an increased risk of *de novo* abnormalities in ICSI compared to natural conception. Despite this, based on a very low certainty of evidence, the authors concluded that no indication of an

increased risk of chromosome abnormalities in ICSI offspring could be found, and that if it does exist, the absolute risk is small (Berntsen et al., 2021).

Regarding neurodevelopmental outcomes, a national cohort study of 18-month-old naturally-conceived infants of fertile and subfertile couples found no differences in their psychomotor development, although slight delays in some cognitive/language milestones were noted in the subfertile group (Zhu et al., 2009). Among children born to subfertile couples, either naturally or following fertility treatment, those conceived following ICSI displayed the highest relative risk of delay for most milestones investigated. An earlier, smaller study of 97 ICSI, 80 IVF and 110 naturally-conceived children at 5 years of age had found no significant differences in IQ between ICSI offspring and other groups, concluding that any differences observed were more likely due to parental cognitive ability than to the mode of conception (Leslie et al., 2003). Psychological evaluation of 300 singletons conceived after ICSI found that although a higher proportion of ICSI children obtained scores below the cut-off on some of the visual-spatial tests that merited further follow-up, ICSI did not appear to be associated with effects on psychological well-being or cognitive development at age 5 (Ponjaert-Kristoffersen et al., 2004). These findings were supported tentatively by a later systematic review of 80 studies published worldwide (Bay et al., 2013), and a more recent systematic review of 24 studies concluded that cognitive and motor performance are comparable between ICSI and naturally-conceived offspring, explaining any differences that had been reported as being due to limitations in methodology (Catford et al., 2018). Although an association has been reported between ICSI and autism (Sandin et al., 2013; Kissin et al., 2015), causation has not been established, and when confounding factors such as multiple gestation are taken into account the reported association is not supported (Barad et al., 2015).

Many of the reports to date have attempted to draw comparisons between ICSI outcomes with those of spontaneous conceptions in fertile women, rather than couples who

have experienced subfertility and either conceived spontaneously or following ART treatment without the use of ICSI (Esteves et al., 2018).

Imprinting disorders. Some male subfertility may be associated with epigenetic defects arising during of spermatogenesis, and include failure to complete de-methylation of maternal imprints, failure to establish fully paternal methylation imprints, variation in the protamine levels and increased histone retention in sperm (reviewed by McSwiggin & O'Doherty, 2018). A comparison of DNA methylation between 174 naturally-conceived and 20 ICSI-conceived twins revealed significant differences in DNA methylation at the H19/IGF2 ICR (Loke et al., 2015). In contrast, a study comparing X-inactivation in newborn placentas from 60 ICSI-conceived, 72 IVF-conceived and 52 naturally-conceived females, found no difference in DNA methylation patterns between the three groups (Wu et al., 2014).

Despite the undoubted links between some male subfertility and abnormal DNA methylation, and the limited evidence suggesting that epigenetic defects may be more severe in ICSI offspring than in those conceived after conventional IVF, whether the differences in DNA methylations seen in ICSI offspring are driven specifically by ICSI, or are simply associated with elements of male subfertility remains unclear (Loke & Craig, 2016).

Long term health outcomes

Few studies have investigated the long-term health outcomes of ICSI offspring and a careful consideration of confounding variables is needed. Significant confounders include parental age, socio-economic status, and educational level. A contributing factor to early reports of increased pregnancy complications is likely to have been the transfer of multiple embryos resulting in multiple pregnancies (Fauser et al., 2014). Attempts to assess the general physical health of children born from ICSI have reported issues as diverse as significantly increased

incidences of surgical interventions (Bonduelle et al., 2004), cryptorchidism, hospital admissions and urogenital surgeries (Ludwig et al., 2009; Bonduelle et al., 2005) and in blood pressure (Belva et al., 2007) compared with spontaneously conceived children. Conversely, another study found no difference (Knoester et al., 2007). The current published expert consensus suggests no difference in the medical health of ICSI children when matched to age and gender matched controls in the fertile population (Fauser et al., 2014).

The suggestions that, compared to spontaneously conceived children, ICSI-conceived children may be prone to adiposity (an investigation of 217 ICSI-conceived singletons; Belva et al, 2012), and exhibit altered insulin resistance and differences in cortisol levels (an investigation of 42 ICSI-conceived children; Gkourogianni et al, 2014) require substantiation.

Further research into health outcomes in adolescence and adulthood is required before conclusions can be drawn about the long-term safety of ICSI compared to IVF.

Provision of information and informed consent

It is the responsibility of ART clinics to obtain informed consent from patients prior to the commencement of treatment to ensure that patients fully understand its nature, purpose and implications. Clinic staff should establish that patients understand the choices available for their treatment, including the choice of method of insemination of the eggs collected, as well as their consequences, risks and, where applicable costs (HFEA Code of Practice). The laboratory criteria for the insemination method recommended should be discussed with patients prior to consent.

The recommendation to undertake ICSI rather than conventional IVF during ART treatment rests primarily with Clinical Scientist specialists in clinical embryology, and should be based on semen parameters assessed according to methods described in the most recent World Health Organisation laboratory manual (WHO, 2021). Each ART clinic should establish

its own criteria, set out in the clinic's protocols, to stipulate when IVF or ICSI insemination should be used, and the rationale for using ICSI in individual cases should be detailed in the patients' medical records. Any decision to change the proposed and consented insemination method at any point during treatment should be made with patients' informed consent following discussion with appropriately qualified and trained personnel.

Guidance and recommendations for counselling and taking consent for patients embarking on ICSI treatment
Clinics should provide patients with appropriate information to enable them to provide informed consent regarding the use of ICSI in their fertility treatment. **GPP** Appropriate patient information should include any cost implications of the use of ICSI. **GPP**

Guidance and recommendations

ART laboratories should define validated centre-specific criteria for semen parameters that determine when to use ICSI rather than conventional IVF to ensure the rate of total failed fertilisation rates remains below 5% per IVF cycle. ***GPP***

In deriving centre-specific criteria for ICSI, pre-treatment assessment of the quality of semen preparations may be used, with <90% progressive motility in prepared samples as a threshold for considering use of ICSI. ***B***

ICSI should be used in cases of severe male factor subfertility. ***A***

ICSI may be considered in cases of moderate male factor subfertility. ***B***

ICSI should be used as the method of insemination, rather than conventional IVF, in all ART treatment cycles where surgically retrieved sperm are used. ***A***

The use of round spermatids for ICSI is considered experimental and not permitted in the UK. ***GPP***

ICSI should be used as the method of insemination, rather than conventional IVF, in all cases of total globozoospermia. ***A***

Genetic counselling should be offered to globozoospermic men prior to treatment. ***C***

AOA may be considered in cases of total globozoospermia. ***C***

Further studies are needed to establish whether or not the use of ICSI in normospermic patients with elevated sperm DNA fragmentation levels leads to improved outcomes compared with IVF. ***GPP***

Further studies are needed to establish whether or not the use of testicular sperm should be considered in patients with elevated sperm DNA fragmentation levels if previous ART cycle(s) with ejaculated sperm have been unsuccessful. ***GPP***

ICSI should not be considered as first-line therapy for unexplained infertility. ***A***

Split IVF-ICSI inseminations should not be used as first-line therapy for couples with unexplained infertility. ***A***

Further research is needed to establish whether ICSI results in better clinical outcomes than IVF in couples with unexplained infertility. ***GPP***

ICSI can be used as the method of insemination for patients with unexplained infertility where a previous IVF treatment cycle has resulted in total failed fertilisation. ***B***

ICSI may be used as the method of insemination when a previous IVF treatment cycle has resulted in poor fertilisation, with "poor" defined as <25% of inseminated oocytes achieving normal fertilisation. ***B***

Despite its prohibition in the UK, evidence to date does not support concerns that rescue ICSI may increase the incidence of abnormalities. Where permitted by law, rescue ICSI may be considered. ***B***

ICSI should be used as the method of insemination of thawed/warmed cryopreserved oocytes. ***C***

Conventional IVF may be used as the method of insemination for cumulus intact in vitro *matured oocytes, unless ICSI is indicated by poor semen parameters.* ***C***

Further studies are required comparing outcomes following IVF and ICSI with in vitro *matured oocytes.* ***GPP***

Conventional IVF may be used as the method of insemination for PGT-M cycles, unless ICSI is indicated by poor semen parameters. ***B***

Further studies are necessary to establish whether or not ICSI is necessary to minimise the rate of mosaic results in NGS PGT-A cycles. ***GPP***

Conventional IVF should be used rather than ICSI as the method of insemination for oocytes from women of all ages, unless ICSI is indicated by poor semen parameters. ***A***

Conventional IVF should be used rather than ICSI as the method of insemination for oocytes from women with low oocyte yield, unless ICSI is indicated by poor semen parameters. ***A***

There is insufficient evidence to indicate that ICSI is necessary rather than conventional IVF in the treatment of BBV-positive patients and sero-discordant couples, unless ICSI is indicated by poor semen parameters. ***B***

ICSI may be considered when a previous IVF cycle resulted in a high percentage of zygotes developed 3 or more pronuclei in a previous IVF cycle. ***GPP***

Reduction in sperm insemination concentration at IVF may be considered when a previous IVF cycle resulted in a high percentage of zygotes developed 3 or more pronuclei in a previous IVF cycle. ***GPP***

ICSI should be considered if the percentage motility of cryopreserved sperm after preparation does not meet the laboratory's criteria for IVF. ***B***

ART laboratories should define validated centre-specific criteria for prepared cryopreserved sperm to determine when to use ICSI rather than conventional IVF to minimise the risk of poor or failed fertilisation. ***GPP***

Further studies are required comparing outcomes of IVF and ICSI using frozen ejaculated sperm. ***GPP***

There is no evidence that the use of IMSI as a method of sperm selection and insemination results in an increase in clinical pregnancy or live birth rates compared with routine ICSI. ***A***

There is no evidence that PICSI results in an increase in live birth rates compared with routine ICSI. ***A***

Further studies are required to establish whether PICSI could be recommended where there is an increased miscarriage risk. ***C***

There is no evidence to indicate that ICSI with oocyte selection by meiotic spindle visualisation yields an increase in clinical pregnancy and implantation rates compared with routine ICSI using unselected oocytes. ***A***

AOA should not be used routinely with ICSI as its safety, in terms of the potential developmental consequences and birth outcomes, has yet to be established. ***A***

ICSI with AOA may be used where two previous routine ICSI cycle(s) have resulted in <30% or no fertilisation. ***A***

Where AOA is used, patients should be advised that safety, in terms of the potential developmental consequences and birth outcomes, has not been established. ***GPP***

Patients should be provided with safety data relating to the specific AOA technique used. ***GPP***

HOST, pentoxifylline and LAISS can be used in the absence of motile sperm for the selection of viable immotile sperm selection for use in ICSI. ***B***

Further research is required to investigate the long-term effects on children born as a result of the use of HOST, pentoxifylline or LAISS for viable immotile sperm selection. ***GPP***

Genetic counselling should be offered to couples who are contemplating ICSI treatment where Y chromosome microdeletion(s) have been identified in the male partner. ***C***

Clinics should provide patients with appropriate information to enable them to provide informed consent regarding the use of ICSI in their fertility treatment. ***GPP***

Appropriate patient information should include any cost implications of the use of ICSI. ***GPP***

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article. The scope of the article was prepared and authors identified by MJP and GH. Evidence was critically appraised and graded by authors for each section. The overall paper was compiled by VNB and MJP with GH and JK providing critical input. The final paper including the evidence grading was approved by all the authors with any disagreements resolved through discussion with final decision made by VNB, MJP, GH and JK.

References

Abbas, A. M., Hussein, R. S., Elsenity, M. A., Samaha, I. I., El Etriby, K. A., Abd El-Ghany, M. F., Khalifa, M. A., Abdelrheem, S. S., Ahmed, A. A., & Khodry, M. M. (2020). Higher clinical pregnancy rate with in-vitro fertilization versus intracytoplasmic sperm injection in treatment of non-male factor infertility: Systematic review and meta-analysis. *Journal of Gynecology Obstetrics and Human Reproduction 49*(6), 101706. https://doi.org/10.1016/j.jogoh.2020.10170

Adamson, G. D., de Mouzon, J., Chambers, G. M., Zegers-Hochschild, F., Mansour, R., Ishihara, O., Banker, M., & Dyer, S. (2018). International Committee for Monitoring Assisted Reproductive Technology: world report on assisted reproductive technology, 2011. *Fertility and Sterility 110*(6), 1067-1080. https://doi.org/10.1016/j.fertnstert.2018.06.039

Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology 13*(37). https://doi.org/10.1186/s12958-015-0032-1

Aitken, R.J., Kerr, L., Bolton, V., & Hargreave, T. (1990). Analysis of sperm function in globozoospermia: implications for the mechanism of sperm-zona interaction. *Fertility and Steriity 54*(4), 701-707. https://doi.org/10.1016/s0015-0282(16)53833-3

Aitken, R. J., & Bakos, H. W. (2021). Should we be measuring DNA damage in human spermatozoa? New light on an old question. *Human Reproduction 36*(5), 1175-1185. https://doi.org/10.1093/humrep/deab004

Aktan, T. M., Montag, M., Duman, S., Gorkemli, H., & Yurdakul, T. (2004). Use of a laser to detect viable but immotile spermatozoa. *Andrologia 36*(6), 366–369.

https://doi.org/10.1111/j.1439-0272.2004.00636.x

American Society for Reproductive Medicine (2008). Intracytoplasmic sperm injection (ICSI). *Fertil Steril* 90 (5 Suppl), S187. https://doi.org/10.1016/j.fertnstert.2008.08.045

American Society of Reproductive Medicine and Society of Assisted Reproductive Technology (2012). Intracytoplasmic sperm injection (ICSI) for non male factor infertility: a committee opinion. *Fertility and Sterility 98*(6), 1395-1399. https://doi.org/10.1016/j.fertnstert.2012.08.026

American Society of Reproductive Medicine and Society of Assisted Reproductive Technology (2020). Intracytoplasmic sperm injection (ICSI) for non-male factor infertility: a committee opinion. *Fertility and Sterility 114*(2), 239-245. https://10.1016/j.fertnstert.2020.05.032

American Society for Reproductive Medicine (2015) Diagnostic evaluation of the infertile male: a committee opinion. *Fertility and Sterility 103*(3), e18-25. https://doi.org/10.1016/j.fertnstert.2014.12.103

American Society for Reproductive Medicine in collaboration with the Society for Male Reproduction and Urology. (2019). The management of obstructive azoospermia: a committee opinion. *Fertility and Sterility 111*(5), 873-880. https://doi.org/10.1016/j.fertnstert.2019.02.013

American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: a guideline. *Fertility and Sterility 99*(1), 37-43. https://doi.org/10.1016/j.fertnstert.2012.09.028

Andersen, A. N., Carlsen, E., & Loft, A. (2008). Trends in the use of intracytoplasmic sperm injection marked variability between countries. *Human Reproduction Update 14*(6), 593-604. https://doi.org/10.1093/humupd/dmn032

Anifandis, G., Michopoulos, A., Daponte, A., Chatzimeletiou, K., Simopoulou, M., Messin,i

C. I., Polyzos, N. P., Vassiou, K., Dafopoulos, K., & Goulis, D. G. (2019). Artificial oocyte activation: physiological, pathophysiological and ethical aspects. *Systems Biology in Reproductive Medicine 65*(1), 3-22. https://doi.org/10.1080/19396368.2018.1516000

Arafa. M., AlMalki, A. AlBadr, M., Burjaq, H., Majzoub, A., AlSaid, S., & Elbardisi, H. (2018). ICSI outcome in patients with high DNA fragmentation: Testicular versus ejaculated spermatozoa. *Andrologia 50*(1). https://doi.org/10.1111/and.12835

Asa, I., Tabatabaee, R., Farrokhi, A., & Nejatbakhsh, R. (2017). Relationship between meiotic spindles visualization and intracytoplasmic sperm injection outcomes in human oocytes. *Anatomy and Cell Biology 50*(1), 26–32. https://doi.org/10.5115/acb.2017.50.1.26

Avalos-Durán, G., Cañedo-Del Ángel, A. M. E., Rivero-Murillo, J., Zambrano-Guerrero, J. E., Carballo-Mondragón, E,, & Checa-Vizcaíno, M. Á. (2018). Physiological ICSI (PICSI) vs. Conventional ICSI in Couples with Male Factor: A Systematic Review. *JBRA Assisted Reproduction 22*(2), 139-147. https://doi.org/10.5935/1518-0557.20180027

Aydin, T., Yücel, B., Sofikerim, M., Karadag, M., & Tokat, F. (2016). Azoospermia Type Does Not Affect Intracytoplasmic Sperm Injection Results When Microdissection Testicular Sperm Extraction Is Performed. *Journal of Reproductive Medicine 61*(11-12), 581–584.

Babayev, S. N., Park, C. W., & Bukulmez, O. (2014). Intracytoplasmic sperm injection indications: how rigorous? *Seminars in Reproductive Medicine 32*(4), 283-290. https://doi.org/10.1055/s-0034-1375180

Barad, D. H., Kushnir, V. A., Albertini, D., & Gleicher, N. (2015). CDC analysis of ICSI/autism: association is not causation. *Human Reproduction 30*(7), 1745-1746. https://doi.org/10.1093/humrep/dev104

Barlow, P., Englert, Y., Puissant, F., Lejeune, B., Delvigne, A., Van Rysselberge, M., & Leroy, F. (1990). Fertilization failure in IVF: why and what next? *Human Reproduction*

5(4), 451-456. https://doi.org/10.1093/oxfordjournals.humrep.a137122

Barratt, C. L. R., Björndahl, L., De Jonge, C. J., Lamb, D. J., Martini, F. O., McLachlan, R., Oates, R. D., van der Poel, S., John, B. S., Sigman, M., Sokol, R., & Tournaye, H. (2017). The diagnosis of male infertility: An analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Human Reproduction Update 23*(6), 660–680. https://doi.org/10.1093/humupd/dmx021

Bartoov, B., Berkovitz, A., Eltes, F., Kogosowski, A., Menezo, Y., & Barak, Y. (2002). Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *Journal of Andrology 23*(1), 1-8. https://doi.org/10.1002/j.1939-4640.2002.tb02595.x

Bay, B., Mortensen, E. L., & Kesmodel, U. S. (2013). Assisted reproduction and child neurodevelopmental outcomes: a systematic review. *Fertility and Sterility 100*(3), 844-853. https://doi.org/10.1016/j.fertnstert.2013.05.034

Beck-Fruchter, R., Lavee, M., Weiss, A., Geslevich, Y., & Shalev, E. (2014). Rescue intracytoplasmic sperm injection: a systematic review. *Fertility and Sterility 101*(3), 690-698. https://doi.org/10.1016/j.fertnstert.2013.12.004

Belloc, S., Benkhalifa, M., Cohen-Bacrie, M., Dalleac, A., Chahine, H., Amar, E., & Zini, A. (2014). Which isolated sperm abnormality is most related to sperm DNA damage in men presenting for infertility evaluation. *Journal of Assisted Reproduction and Genetics 31*(5), 527-532. https://doi.org/10.1007/s10815-014-0194-3

Belva, F., Bonduelle, M., Roelants, M., Michielsen, D., Van Steirteghem, A., Verheyen, G., & Tournaye, H. (2016). Semen quality of young adult ICSI offspring: the first results. *Human Reproduction 31*(12), 2811-2820. https://doi.org/10.1093/humrep/dew245. Epub 2016 Oct 5

Belva, F., Bonduelle, M., & Tournaye, H. (2019). Endocrine and reproductive profile of boys and young adults conceived after ICSI. *Current Opinions in Obstetrics and Gynecology*

31(3), 163-169. https://doi.org/10.1097/GCO.0000000000000538

Belva, F., Henriet, S., Liebaers, I., Van Steirteghem, A., Celestin-Westreich, S., & Bonduelle, M. (2007). Medical outcome of 8-year-old singleton ICSI children (born >or=32 weeks' gestation) and a spontaneously conceived comparison group. *Human Reproduction 22*(2), 506–515. https://doi.org/10.1093/humrep/del372

Belva, F., Painter, R.C., Schiettecatte, J., Bonduelle, M., Roelants, M., Roseboom, T. J., Tournaye, H., & De Schepper, J. (2013). Gender-specific alterations in salivary cortisol levels in pubertal intracytoplasmic sperm injection offspring. *Hormone Research in Paediatrics 80*(5), 350-355. https://doi.org/10.1159/000355515

Benadiva, C. A., Nulsen, J., Siano, L., Jennings, J., Bet Givargis, H., & Maier D. (1999). Intracytoplasmic sperm injection overcomes previous fertilization failure with conventional in vitro fertilization. *Fertility and Sterility 72*(6), 1041-1044. https://doi.org/10.1016/s0015-0282(99)00403-3

Berkovitz, A., Eltes, F., Yaari, S., Katz, N., Barr, I., Fishman, A., & Bartoov, B. (2005). The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Human Reproduction 20*(1), 185-190. https://doi.org/10.1093/humrep/deh545

Berntsen, S., Nøhr, B., Grøndahl, M. L., Petersen, M. R., Andersen, L. F., Englund, A. L., Knudsen, U. B., Prætorius, L., Zedeler, A., Nielsen, H. S., Pinborg, A., & Freiesleben, N. C. (2021). In vitro fertilisation (IVF) versus intracytoplasmic sperm injection (ICSI) in patients without severe male factor infertility: study protocol for the randomised, controlled, multicentre trial INVICSI. *British Medical Journal Open 11*(6), e051058. https://doi.org/10.1136/bmjopen-2021-051058

Berntsen, S., Laivuori, H., la Cour Freiesleben, N., Loft, A, Söderström-Anttila, V., Oldereid N. B., Romundstad, L. B., Magnusson, Å., Petzold, M., Bergh, C., & Pinborg, A. (2021).

A systematic review and meta-analysis on the association between ICSI and chromosome abnormalities. *Human Reproduction Update 27*(5), 801-847. https://doi.org/10.1093/humupd/dmab005

Bhattacharya, S., Hamilton, M., Shaaban, M., Khalaf, Y., Seddler, M., Ghobara, T., Braude, P., Kennedy, R., Rutherford, A., Hartshorne, G., & Templeton, A. (2001). Conventional invitro fertilization versus intracytoplasmic sperm injection for the treatment of non-male-factor infertility: a randomized controlled trial. *Lancet 357*(9274), 2075-2079. https://doi.org/10.1016/s0140-6736(00)05179-5B

Bodri, D., Sugimoto, T., Yao Serna, J., Kondo, M., Kato, R., Kawachiva, S., & Matsumoto, T. (2015). Influence of different oocyte insemination techniques on early and late morphokinetic parameters: retrospective analysis of 500 time-lapse monitored blastocysts. *Fertility and Sterility 104*(5), 1175-1181.e1-2. https://doi.org/10.1016/j.fertnstert.2015.07.1164

Bonduelle, M., Bergh, C., Niklasson, A., Palermo, G.D., & Wennerholm, U. B., Collaborative Study Group of Brussels, Gothenburg and New York (2004). Medical follow-up study of 5-year-old ICSI children. *Reproductive BioMedicine Online 9*(1), 91–101. https://doi.org/10.1016/s1472-6483(10)62116-5

Bonduelle, M., Wennerholm, U. B., Loft, A., Tarlatzis, B. C., Peters, C., Henriet, S., Mau, C., Victorin-Cederquist, A., Van Steirteghem, A., Balaska, A., Emberson, J. R., & Sutcliffe, A. G (2005). A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. *Human Reproduction 20*(2), 413–419. https://doi.org/10.1093/humrep/deh592

Bosch, E., Espinós, J. J., Fabregues, F., Fontes, J., García-Velasco, J., Llácer, J., Requena, A., Checa, M. A., & Bellver, J., Spanish Infertility SWOT Group (SISG) (2020). ALWAYS ICSI? A SWOT analysis. *Journal of Assisted Reproduction and Genetics 37*(9), 2081-

2092. https://doi.org/10.1007/s10815-020-01836-0

Boulet, S. L., Mehta, A., Kissin, D. M., Warner, L., Kawwass, J. F., & Jamieson, D. J. (2015). Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection. *JAMA 313*(3), 255-263. https://doi.org/10.1001/jama.2014.17985

Bouwmans, C. A., Lintsen, B. M., Eijkemans, M. J., Habbema, J. D., Braat, D. D., & Hakkaart, L. (2008). A detailed cost analysis of in vitro fertilization and intracytoplasmic sperm injection treatment. *Fertility and Sterility 89*(2), 331-341. https://doi.org/10.1016/j.fertnstert.2007.03.003

Bradley, C. K., McArthur, S. J., Gee, A. J., Weiss, K. A., Schmidt, U., & Toogood, L. (2016). Intervention improves assisted conception intracytoplasmic sperm injection outcomes for patients with high levels of sperm DNA fragmentation: a retrospective analysis. *Andrology 4*(5), 903-910. https://doi.org/10.1111/andr.12215

Bujan, L., Hollander, L., Coudert, M., Gilling-Smith, C., Vucetich, A., Guibert, J., Vernazza, P., Ohl, J., Weigel, M., Englert, Y., & Semprini, A.E. (2007). Safety and efficacy of sperm washing in HIV-1-serodiscordant couples where the male is infected: Results from the European CREAThE network. *AIDS 21*(14), 1909–1914. https://doi.org/10.1097/QAD.0b013e3282703879

Bungum, L., Bungum, M., Humaidan, P., & Andersen, C. (2004). A strategy for treatment of couples with unexplained infertility who failed to conceive after intrauterine insemination. *Reprod Biomed Online* 8: 584–589. https://doi.org/10.1016/s1472-6483(10)61107-8

Cairo Consensus and Workshop Group (2020). The current status and future of andrology: A consensus report from the Cairo workshop group. *Andrology 8*(5), 27-52. https://doi.org/10.1111/andr.12720

Campbell, M. J., Lotti, F., Baldi, E., Schlatt, S., Festin, M. P., Bjorndahl, L., Toskin, I., & Barratt C. L. R. (2021). Distribution of Semen Examination Results 2020 - a follow up of

data collated for the WHO semen analysis manual 2010. *Andrology 9*(3), 817-822. https://doi.org/10.1111/andr.12983

Cao, S., Wu, X., Zhao, C., Zhou, L., Zhang, J., & Ling, X. (2016). Determining the need for rescue intracytoplasmic sperm injection in partial fertilisation failure during a conventional IVF cycle. *Andrologia* 48(10), 1138-1144. https://doi.org/10.1111/and.12551

Carlsen, E., Giwercman, A., Keiding, N., & Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *British Medical Journal 305*(6854), 609–613. https://doi.org/10.1097/00006254-199303000-00023

Catford, S. R., McLachlan, R. I., O'Bryan, M. K., & Halliday, J. L. (2018). Long-term follow-up of ICSI-conceived offspring compared with spontaneously conceived offspring: a systematic review of health outcomes beyond the neonatal period. *Andrology 6*(5), 635-653. https://doi.org/10.1111/andr.12526

Cerván-Martín, M., Castilla, J. A., Palomino-Morales, R. J., & Carmona, F. D. (2020). Genetic Landscape of Nonobstructive Azoospermia and New Perspectives for the Clinic. *Journal of Clinical Medicine 9*(2), 300. https://doi.org/10.3390/jcm9020300

Chansel-Debordeaux, L., Dandieu, S., Bechoua, S., & Jimenez, C. (2015). Reproductive outcome in globozoospermic men: update and prospects. *Andrology 3*(6), 1022-1034. https://doi.org/10.1111/andr.12081

Chen, H. H., Feng, G. X., Zhang, B., Zhou, H., Wang, C. Z., Shu, J. H., Gan, X., Lin, R., Huang, D., & Huang, Y. (2017). A new insight into male fertility preservation for patients with completely immotile spermatozoa. *Reproductive Biology and Endocrinology 15*(1), 74. https://doi.org/10.1186/s12958-017-0294-x

Chen, H. H., Zhou, H., Shu, J. H., Gan, X. Y., Wang, C. Z., Lin, R. Y. (2019). A point of confusion for embryologists in the identification of viable spermatozoa by the eosin-nigrosin test. *Clinical and Experimental Reproductive Medicine 46*(1), 36–40.

https://doi.org/10.5653/cerm.2019.46.1.36

Chen, H. H., Wang, C., Zhou, H., Shu, J., Gan, X., Xu, K., Wu, Z., Deng, X., Huang, G., & Lin, R. (2021). Laser-assisted selection of immotile spermatozoa has no effect on obstetric and neonatal outcomes of TESA-ICSI pregnancies. *Reproductive Biology and Endocrinology 19*(1), 159. https://doi.org/10.1186/s12958-021-00835-9

Chen, L., Xu, Z., Zhang, N., Wang, B., Chen, H., Wang, S., & Sun, H. (2014). Neonatal outcome of early rescue ICSI and ICSI with ejaculated sperm. *Journal of Assisted Reproduction and Genetics 31*(7), 823-828. https://doi.org/10.1007/s10815-014-0245-9

Chen, W., Bai, H., Li, M., Xue, X., & Shi, J. (2021). Effects of three pro-nuclei (3PN) incidence on laboratory and clinical outcomes after early rescue intracytoplasmic sperm injection (rescue-ICSI): an analysis of a 5-year period. *Gynecological Endocrinology 37*(2), 137-140. https://doi.org/10.1080/09513590.2020.1757640

Cissen, M., van Wely, M., Scholten, I., Mansell, S., de Bruin, J. P., Mol, B. W., Braat, D., Repping, S., & Hamer, G. (2016). Measuring sperm DNA fragmentation and clinical outcomesof medically assisted reproduction: a systematic review and meta-analysis. P*LoS One 11*(11), e0165125. https://doi.org/10.1371/journal.pone.0165125

Clarke. G. N., Bourne, H., Hill, P., Johnston, W. I., Speirs, A., McBain, J. C. & Baker, H. W. (1997). Artificial insemination and in-vitro fertilization using donor spermatozoa: a report on 15 years of experience. *Human Reproduction 12*(4), 722-726. https://doi.org/10.1093/humrep/12.4.722

Cohen, J., Edwards, R. G., Fehilly, C. B., Fishel, S. B., Hewitt, J., Rowland, G. F., Steptoe, P. C., Walters, D. E., & Webster, J. (1985). In vitro fertilization using cryopreserved donor semen in cases where both partners are infertile. *Fertility and Sterility 43*(4), 570-574. https://doi.org/10.1016/s0015-0282(16)48499-2

Colaco, S., & Modi, D. (2018). Genetics of the human Y chromosome and its association

with male infertility. *Reproductive Biology and Endocrinology 16*(1), 14. https://doi.org/10.1186/s12958-018-0330-5

Coutton, C., Arnoult, C., & Ray, P. F. (2015). Comprehensive investigation in patients affected by globozoospermia. *Andrology* 3(6), 1183. https://doi.org/10.1111/andr.12096

Coutton, C., Escoffier, J., Martinez, G., Arnoult, C., & Ray, P. F. (2015). Teratozoospermia: spotlight on the main genetic actors in the human. *Human Reproduction Update 21*(4), 455-485. https://doi.org/10.1093/humupd/dmv020

Dam, A. H., Feenstra, I., Westphal, J. R., Ramos, L., van Golde, R. J., & Kremer, J. A. (2007). Globozoospermia revisited. *Human Reproduction Update 13*(1), 63-75. https://doi.org/10.1093/humupd/dml047

Dam, A. H. D. M., Pijnenburg, A. J. E., Hendriks, J. C. M., Westphal, H., Ramos, L., & Kremer, J. A. M. (2012). Intracytoplasmic sperm injection in partial globozoospermia. *Fertility and Sterility 97*(1), 60-66. https://doi.org/10.1016/j.fertnstert.2011.10.003

Dang, V. Q., Vuong, L. N., Luu, T. M., Pham, T., Ho, T. M., Ha, A. N., Truong, B. T., Phan, A. K., Nguven, D. P., Pham, T. N., Pham, Q. T., Wang, R., Norman, R. J., & Mol, B. W. (2021). Intracytoplasmic sperm injection versus conventional in-vitro fertilisation in couples with infertility in whom the male partner has normal total sperm count and motility: an open-label, randomised controlled trial. *Lancet 397*(10284), 1554-1563. https://doi.org/10.1016/s0140-6736(21)00535-3

Davies, M. J., Moore, V. M., Wilson, K. J., Van Essen, P., Priest, K., Scott, H., Haan, E. A., & Chan, A. (2012). Reproductive technologies and the risk of birth defects. *New England Journal of Medicine 366*(19), 1803-1813. https://doi.org/10.1056/NEJMoa1008095

Deng, C., Li, T., Xie, Y., Guo, Y., Yang, Q. Y., Liang, X., Deng, C. H., & Liu, G. H. (2019). Sperm DNA fragmentation index influences assisted reproductive technology outcome: A systematic review and meta-analysis combined with a retrospective cohort study.

Andrologia 51(6), e13263. https://doi.org/10.1111/and.13263

Drakopoulos, P., Garcia-Velasco, J., Bosch, E., Blockeel, C., de Vos, M., Santos-Ribeiro, S., Makrigiannakis, A., Tournaye, H., & Polyzos, N. P. (2019). ICSI does not offer any benefit over conventional IVF across different ovarian response categories in non-male factor infertility: a European multicenter analysis. *Journal of Assisted Reproduction and Genetics 36*(10), 2067-2076. https://doi.org/10.1007/s10815-019-01563-1

Dyer, S., Chambers, G. M., de Mouzon, J., Nygren, K. G., Zegers-Hochschild, F., Mansour, R., Ishihara, O., Banker, M., & Adamson, G.D. (2016). International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted Reproductive Technology 2008, 2009 and 2010. *Human Reproduction 31*(7), 1588-1609. https://doi.org/10.1093/humrep/dew082

Ebner, T., Shebl, O., Mayer, R. B., Moser, M., Costamoling, W., & Oppelt, P. (2014). Healthy live birth using theophylline in a case of retrograde ejaculation and absolute asthenozoospermia. *Fertility and Sterility 101*(2), 340–3. https://doi.org/10.1016/j.fertnstert.2013.10.006

Ebner, T., & Montag., M. (2016). Artificial oocyte activation: evidence for clinical readiness. *Reproductive BioMedicine Online 32*(3), 271-273. https://doi.org/10.1016/j.rbmo.2015.12.004

Egozcue, S., Blanco, J., Vidal, F., & Egozcue, J. (2002). Diploid sperm and the origin of triploidy. *Human Reproduction 17*(1), 5-7. https://doi.org/10.1093/humrep/17.1.5

Elizur, S. E., Levron, J., Seidman, D. S., Kees, S., Levran, D., & Dor, J. (2004). Conventional in vitro fertilization versus intracytoplasmic sperm injection for sibling oocytes in couples with mild oligoteratoasthenozoospermia and couples with normal sperm. *Fertility and Sterility 82*(1), 241-243. https://doi.org/10.1016/j.fertnstert.2003.11.053

ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive

Medicine (2017). The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reproductive BioMedicine Online 35*(5), 494-510. https://doi.org/10.1016/j.rbmo.2017.06.015

ESHRE (2021). Guideline for Medically assisted reproduction in patients with a viral infection or disease. *Human Reproduction Open*. https://doi.org/10.1093/hropen/hoab037

Esteves, S. C., & Agarwal, A. (2013). Reproductive outcomes, including neonatal data, following sperm injection in men with obstructive and nonobstructive azoospermia: case series and systematic review. *Clinics (Sao Paulo) 68*(Suppl 1), 141–150. https://doi.org/10.6061/clinics/2013(sup01)16

Esteves, S. C., Roque, M., & Agarwal, A. (2016). Outcome of assisted reproductive technology in men with treated and untreated varicocele: Systematic review and meta-analysis. *Asian Journal of Andrology 18*(2), 254–258. https://doi.org/10.4103/1008-682X.163269

Esteves, S. C., Roque, M., Bradley, C. K., & Garrido, N. (2017). Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis. *Fertility and Sterility 108*(3), 456-467. https://doi.org/10.1016/j.fertnstert.2017.06.018

Esteves, S. C., Roque, M., Bedoschi, G., Haahr, T., & Humaidan, P. (2018). Intracytoplasmic sperm injection for male infertility and consequences for offspring. *Nature Reviews Urology 15*(9), 535-562. https://doi.org/10.1038/s41585-018-0051-8

Esteves, S. C., Zini, A., Coward, R. M., Evenson, D. P., Gosálvez, J., Lewis, S. E. M., Sharma, R., & Humaidan, P. (2021). Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations. *Andrologia 53*(2), e13874. https://doi.org/10.1111/and.13874

Ethics committee of the American Society for Reproductive Medicine (2015). Human

immunodeficiency virus (HIV) and infertility treatment: A committee opinion. *Fertility and Sterility 104*(1), e1–e8. https://doi.org/10.1016/j.fertnstert.2015.04.004

Fesahat, F., Henkel, R., & Agarwal, A. (2020). Globozoospermia syndrome: An update. *Andrologia*. *52*(2), e13459. https://doi.org/10.1111/and.13459

Farhi, J., Cohen, K., Mizrachi, Y., Weissman, A., Raziel, A., & Orvieto, R. (2019). Should ICSI be implemented during IVF to all advanced-age patients with non-male factor subfertility? *Reproductive Biology and Endocrinology 17*(1), 30. https://doi.org/10.1186/s12958-019-0474-y

Fauser, B. C., Devroey, P., Diedrich, K., Balaban, B., Bonduelle, M., Delemarre-van de Waal, H. A., Estella, C., Ezcurra, D., Geraedts, J. P., Howles, C. M., Lerner-Geva, L., Serna, J., & Wells, D., Evian Annual Reproduction (EVAR) Workshop Group 2011. (2014). Health outcomes of children born out after IVF/ICSI: A review of current expert opinion and literature. *Reproductive BioMedicine Online 28*(2), 162-182. https://doi.org/10.1016/j.rbmo.2013.10.013

Fawzy, M., Emad, M., Mahran, A., Sabry, M., Fetih, A. N., Abdelghafar, H., & Rasheed, S. (2018). Artificial oocyte activation with SrCl2 or calcimycin after ICSI improves clinical and embryological outcomes compared with ICSI alone: results of a randomized clinical trial. *Human Reproduction 33*(9), 1636-1644. https://doi.org/10.1093/humrep/dey258

Feldman, B., Aizer, A., Brengauze, M., Dotan, K., Levron, J., Schiff, E., & Orvieto, R. (2017). Pre-implantation genetic diagnosis- should we use ICSI for all? *Journal of Assisted Reproduction and Genetics 34*(9), 1179-1183. https://doi.org/10.1007/s10815-017-0966-7

Fesahat, F., Henkel, R., & Agarwal, A. (2020). Globozoospermia syndrome: An update. *Andrologia 52*(2): e13459. https://doi.org/10.1111/and.13459

Fishel, S., Green, S., Hunter, A., Lisi, F., Rinaldi, L., Lisi, R., & McDermott, H. (1997).

Human fertilization with round and elongated spermatids. *Human Reproduction 12*(2), 336-340. https://doi.org/10.1093/humrep/12.2.336

Foong, S. C., Fleetham, J. A., O'Keane,. J. A., Scott, S. G., Tough, S. C., & Greene, C. A. (2006). A prospective randomized trial of conventional in vitro fertilization versus intracytoplasmic sperm injection in unexplained infertility. *Journal of Assisted Reproduction and Genetics 23*(3), 137–140. https://doi.org/10.1007/s10815-005-9008-y

Gennarelli, G., Carosso, A., Canosa, S., Filippini, C., Cesarano, S., Scarafia, C., Brunod, N., Revelli, A., & Benedetto, C. (2019). ICSI Versus Conventional IVF in Women Aged 40 Years or More and Unexplained Infertility: A Retrospective Evaluation of 685 Cycles with Propensity Score Model. *Journal of Clinical Medicine 8*(10), 1694. https://doi.org/10.3390/jcm8101694

Garrido, N., Gil-Salom, M., Martínez-Jabaloyas, J. M., & Meseguer, M. (2009). First report of the absence of viral load in testicular sperm samples obtained from men with hepatitis C and HIV after washing and their subsequent use. *Fertility and Sterility 92*(3), 1012–1015. https://doi.org/10.1016/j.fertnstert.2009.01.159

Garrido, N., Meseguer, M., Bellver, J., Remohí, J., Simón, C., & Pellicer, A. (2004). Report of the results of a 2 year programme of sperm wash and ICSI treatment for human immunodeficiency virus and hepatitis C virus serodiscordant couples. *Human Reproduction 19*(11), 2581–2586. https://doi.org/10.1093/humrep/deh460

Ghanem, M., Bakr, N. I., Elgayaar, M. A., El Mongy, S., Fathy, H., & Ibrahim, A. H. (2005). Comparison of the outcome of intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia in the first cycle: a report of case series and meta-analysis. *International Journal of Andrology 28*(1), 16–21. https://doi.org/10.1111/j.1365-2605.2005.00491.x

Gkourogianni, A., Kosteria, I., Telonis, A. G., Margeli, A., Mantzou, E., Konsta, M.,

Loutradis, D., Mastorakos, G., Papassotiriou, I., Klapa, M. I., Kanaka-Gantenbein, C., & Chrousos, G. P. (2014). Plasma metabolomic profiling suggests early indications for predisposition to latent insulin resistance in children conceived by ICSI. *PloS One 9*(4), e94001. https://doi.org/10.1371/journal.pone.0094001

Goldman, M. B., Thornton, K. L., Ryley, D., Alper, M. M., Fung, J. L., Hornstein, M. D., & Reindollar, R. H. (2014). A randomized clinical trial to determine optimal infertility treatment in older couples: the Forty and Over Treatment Trial (FORT-T). *Fertility and Sterility 101*(6), 1572–1574. https://doi.org/10.1016/j.fertnstert.2014.03.012

Gook, D. A., Edgar, D. H. (2007). Human oocyte cryopreservation. *Human Reproduction Update 13*(6), 591-605. https://doi.org/10.1093/humupd/dmm028

Guzick, D. S., Overstreet, J. W., Factor-Litvak, P., Brazil, C.K., Nakajima, S. T., Coutifaris, C., Carson, S. A., Cisneros, P., Steinkampf, M. P., Hill, J. A., Xu, D., & Vogel, D. L. (2001). Sperm morphology, motility, and concentration in fertile and infertile men. *New England Journal of Medicine 345*(19), 1388–1393. https://doi.org/10.1056/NEJMoa003005

Guzick, D.S., Sullivan, M.W., Adamson, G.D., Cedars, M.I., Falk, R.J., Peterson, E.P., Steinkampf, M.P. (1998). Efficacy of treatment for unexplained infertility. *Fertility and Sterility 70*(2), 207–213. https://doi.org/10.1016/s0015-0282(98)00177-0

Hammitt, D. G., Bedia, E., Rogers, P. R., Syrop, C. H., Donovan, J. F., & Williamson, R. A. (1989). Comparison of motility stimulants for cryopreserved human semen. *Fertility and Sterility 52*(3), 495-502. https://doi.org/10.1016/s0015-0282(16)60925-1

Haas, J., Miller, T. E., Nahum, R., Aizer, A., Kirshenbaum, M., Zilberberg, E., Lebovitz, O., & Orvieto, R. (2021). The role of ICSI vs. conventional IVF for patients with advanced maternal age – a randomised controlled trial. *Journal of Assisted Reproduction and Genetics 38*(1), 95-100. https://doi.org/10.1007/s10815-020-01990-5

Hansen, M., Kurinczuk, J. J., Bower, C., & Webb, S. (2002). The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *New England Journal of Medicine* *346*(10), 725-730. https://doi.org/10.1056/nejmoa010035

Hattori, H., Nakajo, Y., Ito, C., Toyama, Y., Toshimori, K., & Kyono, K. (2011). Birth of a healthy infant after intracytoplasmic sperm injection using pentoxifylline-activated sperm from a patient with Kartagener's syndrome. *Fertility and Sterility 95*(7), 2431.e2439–2411. https://doi.org/10.1016/j.fertnstert.2011.03.074

He, F., Wang, M. J., Li, S. L., Zhang, C. Y., & Hu, L. N. (2018). IMSI versus ICSI for male factor infertility: A meta-analysis. *Zhonghua Nan Ke Xue* 24(3), 254-262. (Article in Chinese)

Hershlag, A., Paine, T., Kvapil, G., Feng, H., & Napolitano, B. (2002). In vitro fertilization intracytopasmic sperm injection split: an insemination method to prevent fertilization failure. *Fertility and Sterility 77*(2), 229-232. https://doi.org/10.1016/s0015-0282(01)02978-8

HFEA (2019). Commissioning Guidance for fertility Treatment. https://www.hfea.gov.uk/media/2920/commissioning-guidance-may-2019-final-version.pdf

HFEA (2021). Code of Practice 10th Edition. https://portal.hfea.gov.uk/media/1527/2019-12-16-code-of-practice-9th-edition-december-2019.pdf

Hotaling, J. M., Smith, J. F., Rosen, M., Muller, C. H., & Walsh, T.J. (2011). The relationship between isolated teratozoospermia and clinical pregnancy after in vitro fertilization with or without intracytoplasmic sperm injection: a systematic review and meta-analysis. *Fertility and Sterility 95*(3), 1141-1145. https://doi.org/10.1016/j.fertnstert.2010.09.029

Hughes, C. on behalf of the Association of Clinical Embryologists (2012). Association of

clinical embryologists - guidelines on good practice in clinical embryology laboratories 2012. *Human Fertility (Camb) 15*(4), 174-189. https://doi.org/10.3109/14647273.2012.747891

Huszar, G., Ozkavukcu, S., Jakab, A., Celik-Ozenci, C., Sati, G.L., & Cayli, S. (2006). Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. *Current Opinions in Obstetrics and Gynecology 18*(3), 260-267. https://doi.org/10.1097/01.gco.0000193018.98061.2f

Hwang, J. L., Lin, Y. H., & Tsai, Y. L. (2000). In vitro maturation and fertilization of immature oocytes: A comparison study of fertilization techniques. *Journal of Assisted Reproduction and Genetics 17*(1), 39-43. https://doi.org/10.1023/a:1009450014064

Ibrahim, A. A., Awad, H. A., El-Haggar, S., & Mitawi, B. A. (1977). Bilateral testicular biopsy in men with varicocele. *Fertility and Sterility 28*(6), 663–667. https://doi.org/10.1016/s0015-0282(16)42620-8

Inoue, O., Kuji, N., Ito, H., Yamada, M., Hamatani, T., Oyadomari, A., Kato, S., Hanabusa, H., Isaka, K., & Tanaka, M. (2017). Clinical efficacy of a combination of Percoll continuous density gradient and swim-up techniques for semen processing in HIV-1 serodiscordant couples. *Asian Journal of Andrology 19*(2), 208–213. https://doi.org/10.4103/1008-682X.173442

Jakab, A., Sakkas, D., Delpiano, E., Cayli, S., Kovanci, E., Ward, D., Revelli, A., & Huszar, G. (2005). Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertility and Sterility 84*(6), 1665-1673. https://doi.org/10.1016/j.fertnstert.2005.05.068

Jeyendran, R. S., Van der Ven, H. H., Perez-Pelaez, M., Crabo, B. G., & Zaneveld, L. J. (1984). Development of an assay to assess the functional integrity of the human sperm

membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility 70*(1), 219–228. https://doi.org/10.1530/jrf.0.0700219

Joergensen, M. W., Labouriau, R., Hindkjaer, J., Stougaard, M., Kolevraa, S., Bolund, L., Agerholm, I. E., & Sunde, L. (2015). The parental origin correlates with the karyotype of human embryos developing from tripronuclear zygotes. *Clinical and Experimental Reproductive Medicine 42*(1), 14-21. https://doi.org/10.5653/cerm.2015.42.1.14

Johnson, L. N. C., Sasson, I. E., Sammel, M. D., & Dokras, A. (2013). Does intracytoplasmic sperm injection improve the fertilization rate and decrease the total fertilization failure rate in couples with well-defined unexplained infertility? A systematic review and meta-analysis. *Fertility and Sterility 100*(3), 704–711. https://doi.org/10.1016/j.fertnstert.2013.04.038

Jun, S. H., O'Leary, T., Jackson, K. V., & Racowsky, C. (2006). Benefit of intracytoplasmic sperm injection in patients with a high incidence of triploidy in a prior in vitro fertilization cycle. *Fertility and Sterility 86*(4), 825-829. https://doi.org/10.1016/j.fertnstert.2006.03.043

Kamali-Dolat Abadi, M., Tavalaee, M., Shahverdi, A., & Nasr-Esfahani, M. H. (2016). Evaluation of PLCζ and PAWP expression in globozoospermic individuals. *Cell Journal 18*(3), 438-445. https://doi.org/10.22074/cellj.2016.4572

Kanto, S., Sugawara, J., Masuda, H., Sasano, H., Arai, Y., & Kyono, K. (2008). Fresh motile testicular sperm retrieved from nonobstructive azoospermic patients has the same potential to achieve fertilization and pregnancy via ICSI as sperm retrieved from obstructive azoospermic patients. *Fertility and Sterility 90*(5), 2010.e5-2010.e7. https://doi.org/10.1016/j.fertnstert.2008.01.049

Karpman, E., Williams, D. H., & Lipshultz, L. I. (2005). IVF and ICSI in male infertility: update on outcomes, risks, and costs. *The Scientific World Journal 16*(5), 922-932.

https://doi.org/10.1100/tsw.2005.117

Kilani, Z. M., Shaban, M. A., Ghunaim, S. D., Keilani, S. S., & Dakkak, A. I (1998). Triplet pregnancy and delivery after intracytoplasmic injection of round-headed spermatozoa. *Human Reproduction 13*(8), 2177-2179. https://doi.org/10.1093/humrep/13.8.2177

Kilani, Z., Ismail, R., Ghunaim, S., Mohamed, H., Hughes, D., Brewis, I., & Barratt, C.L. (2004). Evaluation and treatment of familial globozoospermia in five brothers. *Fertility and Sterility 82*(5), 1436-1439. https://doi.org/10.1016/j.fertnstert.2004.03.064

Kim, H. J., Yoon, H. J., Jang, J. M., Lee, W. D., Yoon, S. H., & Lim, J. H. (2017). Evaluation of human embryo development in in vitro fertilization- and intracytoplasmic sperm injection- fertilized oocytes: A time – lapse study. *Clinical and Experimental Reproductive Medicine 44*(2), 90-95. https://doi.org/10.5653/cerm.2017.44.2.90

Kinzer, D. R., Barrett, C. B., & Powers, R. D. (2008). Prognosis for clinical pregnancy and delivery after total fertilization failure during conventional in vitro fertilization or intracytoplasmic sperm injection. *Fertility and Sterility 90*(2), 284-288. https://doi.org/10.1016/j.fertnstert.2007.06.025

Kirby, E. W., Wiener, L. E., Rajanahally, S., Crowell, K., & Coward, R. M. (2016). Undergoing varicocele repair before assisted reproduction improves pregnancy rate and live birth rate in azoospermic and oligospermic men with a varicocele: a systematic review and meta-analysis. *Fertility and Sterility 106*(6), 1338–1343. https://doi.org/10.1016/j.fertnstert.2016.07.1093

Kirkman-Brown, J., Pavitt, S., Khalaf, Y., Lewis, S., Hooper, R., Bhattacharya, S., Coomarasamy, A., Sharma, V., Brison, D., Forbes, G., West, R., Pacey, A., Brian, K., Cutting, R., Bolton, V., & Miller, D. (2019). Sperm selection for assisted reproduction by prior hyaluronan binding: the HABSelect RCT. *NIHR Journals Library* Feb 2019. https://doi.org/10.3310/eme06010

Kissin, D. M., Zhang, Y., Boulet, S. L., Fountain, C., Bearman, P., Schieve, L., Yeargin-Allsopp, M., & Jamieson, D. J. (2015). Association of assisted reproductive technology (ART) treatment and parental infertility diagnosis with autism in ART-conceived children. *Human Reproduction 30*(2), 454-465. https://doi.org/10.1093/humrep/deu338

Knoester, M., Vandenbroucke, J. P., Helmerhorst, F. M., van der Westerlaken, L. A., Walther, F. J., & Veen, S. (2007). Matched follow-up study of 5–8 year old ICSI-singletons: comparison of their neuromotor development to IVF and naturally conceived singletons. *Human Reproduction 22*(6), 1638-1646. https://doi.org/10.1093/humrep/dem040

Kohn, T. P., Kohn, J. R., & Lamb, D. J. (2018). Role of sperm morphology in deciding between various assisted reproduction technologies. *European Urology Focus 4*(3), 311-313. https://doi.org/10.1016/j.euf.2018.07.029

Kovacic, B., Vlaisavljevic, V., & Reljic, M. (2006). Clinical use of pentoxifylline for activation of immotile testicular sperm before ICSI in patients with azoospermia. *Journal of Andrology 27*(1), 45-52. https://doi.org/10.2164/jandrol.05079

Krausz, C., & Riera-Escamilla, A. (2018). Genetics of male infertility. *Nature Reviews Urology 15*(6), 369-384. https://doi.org/10.1038/s41585-018-0003-3

Kruger, T. F., Acosta, A. A., Simmons, K. F., Swanson, R. J., Matta, J. F., & Oehninger, S. (1988). Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertility and Sterility 49*(1), 112-117. https://doi.org/10.1016/s0015-0282(16)59660-5

Lacamara, C., Ortega, C., Villa, S., Pommer, R., & Schwarze, J. E. (2017). Are children born from singleton pregnancies conceived by ICSI at increased risk for congenital malformations when compared to children conceived naturally? A systematic review and meta-analysis. *JBRA Assisted Reproduction 21*(3), 251-259. https://doi.org/10.5935/1518-0557.20170047

Le, M. T., Nguyen, T. A. T., Nguyen, H. T. T., Nguyen, T. T. T., Nguyen, V. T., Le, D. D., Nguyen, V. Q. H., & Cao, N. T. (2019). Does sperm DNA fragmentation correlate with semen parameters? *Reprod Med Biol* 18(4), 390-396. https://doi.org/10.1002/rmb2.12297

Le Lannou, D., Gastard, E., Guivarch, A., Laurent, M. C., & Poulain, P. (1995). Strategies in frozen donor semen procreation. *Human Reproduction 10*(7), 1765-1774. https://doi.org/10.1093/oxfordjournals.humrep.a136171

Lee, S. H., Lee, J. H., Park, Y.-S., Yang, K., M. & Lim, C. K. (2017). Comparison of clinical outcomes between *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in IVF-ICSI split insemination cycles. *Clinical and Experimental Reproductive Medicine 44*(2), 96-104. https://doi.org/10.5653/cerm.2017.44.2.96

Leslie, G. I., Gibson, F. L., McMahon, C., Cohen, J., Saunders, D. M., & Tennant, C. (2003). Children conceived using ICSI do not have an increased risk of delayed mental development at 5 years of age. *Human Reproduction 18*(10), 2067-2072. https://doi.org/10.1093/humrep/deg408

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. H. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update 23*(6), 646–659. https://doi.org/10.1093/humupd/dmx022

Li, Z., Wang, L., Cai, J., & Huang, H. (2006). Correlation of sperm DNA damage with IVF and ICSI outcomes: a systematic review and meta-analysis. *Journal of Assisted Reproduction and Genetics 23*(9-10), 367-376. https://doi.org/10.1007/s10815-006-9066-9

Li, Z., Wang, A. Y., Bowman, M., Hammarberg, K., Farquhar, C., Johnson, L., Safi, N. & Sullivan, E. A. (2018). ICSI does not increase the cumulative live birth rate in non-male factor infertility. *Human Reproduction 33*(7), 1322-1330. https://doi.org10.1093/humrep/dey118

Lipitz, S., Rabinovici, J., Goldenberg, M., Bider, D., Dor, J., & Mashiach, S. (1994). Complete failure of fertilization in couples with mechanical infertility: implications for subsequent in vitro fertilization cycles. *Fertility and Sterility 61*(5), 863-866. https://doi.org/10.1016/s0015-0282(16)56697-7

L0ke, Y. J., Galati, J. C., Saffery, R. & Craig J. M. (2015). Association of in vitro fertilization with global and IGF2/H19 methylation variation in newborn twins. *Journal of Developmental Origins of Health and Disease 6*(2), 115-24. https://doi.org/10.1017/S2040174415000161

Loke, Y. J., & Craig, J. M. (2016). Are the effects of IVF on DNA methylation driven by intracytoplasmic sperm injection and male infertility? *Epigenomics 8*(7), 881-884. https://doi.org/10.2217/epi-2016-0041

Ludwig, A. K., Katalinic, A., Thyen, U., Sutcliffe, A. G., Diedrich, K., & Ludwig, M. (2009b). Physical health at 5.5 years of age of term-born singletons after intracytoplasmic sperm injection: results of a prospective, controlled, single-blinded study. *Fertility and Sterility 91*(1), 115–124. https://doi.org/10.1016/j.fertnstert.2007.11.037

Luke, B., Brown, M. B., Wantman, E., Forestieri, N. E., Browne, M. L., Fisher, S. C., Yazdy, M. M., Ethen, M. K., Canfield, M. A., Watkins, S., Nichols, H. B., Farland, L. V,, Oehninger, S., Doody, K. J., Eisenberg, M. L., & Baker, V. L. (2021). The risk of birth defects with conception by ART. *Human Reproduction 36*(1), 116-129. https://doi.org/10.1093/humrep/deaa272

Luna, M., Bigelow, C., Duke, M., Ruman, J., Sandler, B., Grunfeld, L., & Copperman, A. B. (1998). Should ICSI be recommended routinely in patients with four or fewer oocytes retrieved? *Journal of Assisted Reproduction and Genetics 28*(10), 911-915. https://doi.org/10.1007/s10815-011-9614-9

Lundin, K., Söderlund, B., & Hamberger, L. (1997). The relationship between sperm

morphology and rates of fertilization, pregnancy and spontaneous abortion in an in-vitro fertilization/intracytoplasmic sperm injection programme. *Human Reproduction 12*(12), 2676-2681. https://doi.org/10.1093/humrep/12.12.2676

Macas, E., Imthurn, B., Rosselli, M., & Keller, P. J. (1996). The chromosomal complements of multipronuclear human zygotes resulting from intracytoplasmic sperm injection. *Human Reproduction 11*(11), 2496–2501. https://doi.org/10.1093/oxfordjournals.humrep.a019147

Madaschi, C., de Souza Bonetti, T. C., de Almeida Ferreira Braga, D. P., Pasqualotto, F. F., Iaconelli, A. Jr, & Borges, E. Jr (2008). Spindle imaging: a marker for embryo development and implantation. *Fertility and Sterility 90*(1), 194-198. https://doi.org/10.1016/j.fertnstert.2007.05.071

Madaschi, C., Aoki, T., de Almeida Ferreira Braga, D. P., de Cássia Sávio Figueira, R., Semião Francisco, L., Iaconelli, A. Jr, & Borges, E. Jr. (2009). Zona pellucida birefringence score and meiotic spindle visualization in relation to embryo development and ICSI outcomes. *Reproductive BioMedicine Online 18*(5), 681-686. https://doi.org/10.1016/s1472-6483(10)60014-4

Maheshwari, A., Muneer, A., Lucky, M., Mathur, R., & McEleny, K. (2020). A review of varicocele treatment and fertility outcomes. *Human Fertility (Camb)* July7, 1–8. https://doi.org/10.1080/14647273.2020.1785117

Malic Voncina, S., Stendqvist, A., Bungum, M., Schyman, T., & Giwercman, A. (2021). Sperm DNA fragmentation index and cumulative live birth rate in a cohort of 2,713 couples undergoing assisted reproduction treatment. *Fertility and Sterility 16*(6), 1483-1490. https://doi.org/10.1016/j.fertnstert.2021.06.049

Mangoli, E., Khalili, M. A., Talebi, A. R., Agha-Rahimi, A., Soleimani, M., Faramarzi, A., & Pourentezari, M. (2019). IMSI procedure improves clinical outcomes and embryo

morphokinetics in patients with different aetiologies of male infertility. *Andrologia 51*(8), e13340. https://doi.org/10.1111/and.13340. Epub 2019 Jun 13

McDowell, S., Kroon, B., Ford, E., Hook, Y., Glujovsky, D., & Yazdani, A. (2014). Advanced sperm selection techniques for assisted reproduction. *Cochrane Database of Systematic Reviews* Oct 28(10):CD010461. https://doi.org/10.1002/14651858.CD010461.pub2

McSwiggin, H. M., & O'Doherty, A. M. (2018). Epigenetic reprogramming during spermatogenesis and male factor infertility. *Reproduction 156*(2), R9–R21. https://doi.org/10.1530/REP-18-0009

Miller, D., Pavitt, S., Sharma, V., Forbes, G., Hooper, R., Bhattacharya, S., Kirkman-Brown, J., Coomarasamy, A., Lewis, S., Cutting, R., Brison, D., Pacey, A., West, R., Brian, K., Griffin, D., & Khalaf, Y. (2019). Physiological, hyaluronan-selected intracytoplasmic sperm injection for infertility treatment (HABSelect): a parallel, two-group, randomised trial. *Lancet 393*(10170), 416-422. https://doi.org/10.1016/S0140-6736(18)32989-1

Moreno, C., Ruiz, A., Simón, C., Pellicer, A., & Remohí, J. (1998). Intracytoplasmic sperm injection as a routine indication in low responder patients. *Human Reproduction 13*(8), 2126-2129. https://doi.org/10.1093/humrep/13.8.2126

Moskovtsev, S. I., Jarvi, K., Mullen, J. B., Cadesky, K. I., Hannam, T., & Lo, K. C. (2010). Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. *Fertility and Sterility 93*(4), 1142-1146. https://doi.org/10.1016/j.fertnstert.2008.11.005

Murugesu, S., Saso, S., Jones, B. P., Bracewell-Milnes, T., Athanasiou, T., Mania, A., Serhal, P., & Ben-Nagi, J. (2017). Does the use of calcium ionophore during artificial oocyte activation demonstrate an effect on pregnancy rate? A meta-analysis. *Fertility and Sterility 108*(3), 468-482. https://doi.org/10.1016/j.fertnstert.2017.06.029

National Institute for Health and Care Excellence. (2004). Fertility problems: assessment and treatment (NICE guideline [CG 156; para 1.13]). https://www.nice.org.uk/guidance/cg156/chapter/Recommendations#intracytoplasmic-sperm-injection

Navas, P., Paffoni, A., Intra, G., González-Utor, A., Clavero, A., Gonzalvo, M. C., Díaz, R., Peña, R., Restelli, L., Somigliana, E., Papaleo, E., Castilla, J. A., & Viganò, P. (2017). Obstetric and neo-natal outcomes of ICSI cycles using pentoxifylline to identify viable spermatozoa in patients with immotile spermatozoa. *Reproductive BioMedicine Online 34*(4), 414-421. https://doi.org/10.1016/j.rbmo.2017.01.009

Nieschlag, E., Behre, H. M., & Nieschlag, S. (Eds.). (2010). *Andrology: Male Reproductive Health and Dysfunction* (3rd ed.). Springer-Verlag Berlin Heidelberg. https://doi.org/10.1007/978-3-540-78355-8

Niu, X., Ruan, Q., Witz, C. A., & Wang, W. (2020). Comparison of Human Oocyte Activation Between Round-Headed Sperm Injection Followed by Calcium Ionophore Treatment and Normal Sperm Injection in a Patient With Globozoospermia. *Frontiers in Endocrinology (Lausanne) 11*, 183. https://doi.org/10.3389/fendo.2020.00183

Nordhoff, V. (2015). How to select immotile but viable spermatozoa on the day of intracytoplasmic sperm injection? An embryologist's view. *Andrology 3*(2), 156–162. https://doi.org/10.1111/andr.286

O'Flynn, N. (2014). Assessment and treatment for people with fertility problems: NICE guideline. *British Journal of General Practice 64*(618), 50–51. https://doi.org/10.3399/bjgp14X676609

Ohlander, S., Hotaling, J., Kirshenbaum, E., Niederberger, C., & Eisenberg, M. L. (2014). Impact of fresh versus cryopreserved testicular sperm upon intracytoplasmic sperm injection pregnancy outcomes in men with azoospermia due to spermatogenic dysfunction:

a meta-analysis. *Fertility and Sterility 101*(2), 344–349. https://doi.org/10.1016/j.fertnstert.2013.10.012

Ombelet, W., Menkveld, R., Kruger, T. F., & Steeno, O. (1995). Sperm morphology assessment: historical review in relation to fertility. *Human Reproduction Update 1*(6), 543-557. https://doi.org/10.1093/humupd/1.6.543

Oren-Benaroya, R., Orvieto, R., Gakamsky, A., Pinchasov, M., & Eisenbach, M. (2008). The sperm chemoattractant secreted from human cumulus cells is progesterone. *Human Reproduction 23*(10), 2339-2345. https://doi.org/10.1093/humrep/den265

Osman, A., Alsomait, H., Seshadri, S., El-Toukhy, T., & Khalaf, Y. (2015). The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. *Reproductive BioMedicine Online 30*(2), 120-127. https://doi.org/10.1016/j.rbmo.2014.10.018

Pabuccu EG, Caglar GS, Tangal S, Haliloglu AH, Pabuccu R. (2017). Testicular versus ejaculated spermatozoa in ICSI cycles of normozoospermic men with high sperm DNA fragmentation and previous ART failures. *Andrologia 49*(2). https://doi.org/10.1111/and.12609

Paffoni, A., Reschini, M., Pisaturo, V., Guarneri, C., Palini, S., & Viganò, P. (2021). Should rescue ICSI be re-evaluated considering the deferred transfer of cryopreserved embryos in in-vitro fertilization cycles? A systematic review and meta-analysis. *Reproductive Biology and Endocrinology 19*(1), 121. https://doi.org/10.1186/s12958-021-00784-3

Palermo, G., Joris, H, Devroey, P., & Van Steirteghem, A. P. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340(8810), 17-18. https://doi.org/10.1016/0140-6736(92)92425-f

Palermo, G. D., Cohen, J., & Rozenwaks, Z. (1996). Intracytoplasmic sperm injection: a powerful tool to overcome fertilization failure. *Fertility and Sterility 65*(5), 899-908.

https://doi.org/10.1016/s0015-0282(16)58257-0

Palermo, G. D., Neri, Q. V., & Rosenwaks, Z. (2015). To ICSI or Not to ICSI. *Seminars in Reproductive Medicine 33*(2), 92-102. https://doi.org/10.1055/s-0035-1546825

Palmerola, K.L., Vitez, S. F., Amrane, S., Fischer, C. P., & Forman, E. J. (2019). Minimizing mosaicism: assessing the impact of fertilization method on rate of mosaicism after next-generation sequencing (NGS) preimplantation genetic testing for aneuploidy (PGT-A). *Journal of Assisted Reproduction and Genetics 36*(1), 153-157. https://doi.org/10.1007/s10815-018-1347-6

Plachot, M. (2000). Fertilization. *Human Reproduction 15* Suppl 4: 19-30. https://doi.org/10.1093/humrep/15.suppl_4.19

Plachot, M., & Crozet, N. (1992). Fertilization abnormalities in human in-vitro fertilization. *Human Reproduction 7*(Suppl 1): 89–94. https://doi.org/10.1093/humrep/7.suppl_1.89

Ponjaert-Kristoffersen, I., Tjus, T., Nekkebroeck, J., Squires, J., Verté, D., Heimann, M., Bonduelle, M., Palermo, G., & Wennerholm, U. B. Collaborative study of Brussels, Göteborg and New York. (2004). Psychological follow-up study of 5-year-old ICSI children. *Human Reproduction 19*(12), 2791-2797. https://doi.org/10.1093/humrep/deh511

Porter, R., Han, T., Tucker, M. J., Graham, J., Liebermann, J., & Scott Sills, E. (2003). Estimation of second polar body retention rate after conventional insemination and intracytoplasmic sperm injection: in vitro observations from more than 5000 human oocytes. *Journal of Assisted Reproduction and Genetics 20*(9), 371-376. https://doi.org/10.1023/a:1025481011680

Povey, A. C., & Stocks, S. J. (2010) Epidemiology and trends in male subfertility. *Human Fertility (Camb) 13*(4), 182-188. https://doi.org/10.3109/14647273.2010.515700

Petersen, C. G., Oliveira, J. B., Mauri, A. L., Massaro, F. C., Baruffi, R. L., Pontes, A., &

Franco, J.G. Jr. (2009). Relationship between visualization of meiotic spindle in human oocytes and ICSI outcomes: a meta-analysis. *Reproductive BioMedicine Online 18*(2), 235-243. https://doi.org/10.1016/s1472-6483(10)60261-1

Pregl Breznik, B., Kovacic, B., & Vlaisavljvic, V. (2013). Are sperm DNA fragmentation, hyperactivation, and hyaluronan-binding ability predictive for fertilization and embryo development in in vitro fertilization and intracytoplasmic sperm injection? *Fertility and Sterility 99*(5), 1233-1241 https://doi.org/10.1016/j.fertnstert.2012.11.048

Proctor, M., Johnson, N., Van Peperstraten, A., & Philipson, G. (2008). Techniques for surgical retrieval of sperm prior to intracytoplasmic sperm injection (ICSI) for azoospermia. *Cochrane Database of Systematic Reviews* 2008(2), CD002807. https://doi.org/10.1002/14651858.CD002807.pub3

Repping, S., van Weert, J. M., Mol, B. W., de Vries, J. W., & van der Veen, F. (2002). Use of the total motile sperm count to predict total fertilization failure in in vitro fertilization. *Fertility and Sterility 78*(1), 22-28. https://doi.org/10.1016/s0015-0282(02)03178-3

Retzloff, M. G., & Hornstein, M. D. (2003). Is intracytoplasmic sperm injection safe? *Fertility and Sterility 80*(4), 851-859. https://doi.org/10.1016/s0015-0282(03)01014-8

Rhemrev, J. P., Lens, J. W., McDonnell. J., Schoemaker. J., & Vermeiden, J. P. (2001). The postwash total progressively motile sperm cell count is a reliable predictor of total fertilization failure during in vitro fertilization treatment. *Fertility and Sterility 76*(5), 884-891. https://doi.org/10.1016/s0015-0282(01)02826-6

Ribas-Maynou, J., Yeste, M., Becerra-Tomás, N., Aston, K. I., James, E. R., & Salas-Huetos, A. (2021). Clinical implications of sperm DNA damage in IVF and ICSI: updated systematic review and meta-analysis. *Biological Reiewsv Cambridge Philosophical Society 96*(4), 1284-1300. https://doi.org/10.1111/brv.12700

Rienzi, L., Ubaldi, F., Iacobelli, M., Minasi, M. G., Romano, S., & Greco, E. (2005). Meiotic

spindle visualization in living human oocytes. *Reproductive BioMedicine Online 10*(2), 192-198. https://doi.org/10.1016/s1472-6483(10)60940-6

Robinson, J. N., Lockwood, G. M., Dokras, A., Egan, D. M., Ross, C., & Barlow, D. H. (1993). A controlled study to assess the use of in vitro fertilization with donor semen after failed therapeutic donor insemination. *Fertility and Sterility 59*(2), 353-358. https://doi.org/10.1016/s0015-0282(16)55673-8

Robinson, J. N., Lockwood, G. M., Dokras, A., Egan, D. M., Nicholson, S. C., Ross, C., & Barlow, D. H. (1992). Does isolated teratozoospermia affect performance in in-vitro fertilization and embryo transfer? *Human Reproduction 9*(5), 870-874. https://doi.org/10.1093/oxfordjournals.humrep.a138608

Robinson, L., Gallos, I. D., Conner, S. J., Rajkhowa, M., Miller, D., Lewis, S., Kirkman-Brown, J., & Coomarasamy, A. (2012). The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Human Reproduction 27*(10), 2908-17. https://doi.org/10.1093/humrep/des261

Roest, J., Van Heusden, A. M., Zeilmaker, G. H., & Verhoeff, A. (1998). Treatment policy after poor fertilization in the first IVF cycle. *Journal of Assisted Reproduction and Genetics 15*(1), 18-21. https://doi.org/10.1023/a:1022570120207

Rosen, M. P., Shen, S., Dobson, A. T., Fujimoto, V. Y., McCulloch, C. E., & Cedars, M. I. (2006). Oocyte degeneration after intracytoplasmic sperm injection: a multivariate analysis to assess its importance as a laboratory or clinical marker. *Fertility and Sterility 85*(6), 1736-1743. https://doi.org/10.1016/j.fertnstert.2005.12.017

Rubino, P., Vigano, P., Luddi, A., & Piomboni, P. (2016). The ICSI procedure from past to future: a systematic review of the more controversial aspects. *Human Reproduction Update 22*(2), 194-227. https://doi.org/10.1093/humupd/dmv050

Rubio, I., Kuhlmann, R., Agerholm, I. Kirk J., Herrero, J., Escribá, M.J., Bellver, J., &

Meseguer, M. (2012). Limited implantation success of direct-cleaved human zygotes: a time lapse study. *Fertility and Sterility 98*(6), 1458-1463. https://doi.org/10.1016/j.fertnstert.2012.07.1135

Ruiz, A., Remohí, J., Minguez, Y., Guanes, P. P., Simón, C., & Pellicer, A. (1997). The role of in vitro fertilization and intracytoplasmic sperm injection in couples with unexplained infertility after failed intrauterine insemination. *Fertility and Sterility 68*(1), 171–173. https://doi.org/10.1016/s0015-0282(97)81497-5

Rumbold, A. R., Sevoyan, A., Oswald, T. K., Fernandez, R. C., Davies, M. J., & Moore, V. M. (2019). Impact of male factor infertility on offspring health and development. *Fertility and Sterility 111*(6), 104-1053. https://doi.org/10.1016/j.fertnstert.2019.05.006

Salas-Huetos, A., Bullo, M., & Salas-Salvado, J. (2017). Dietary patterns, foods and nutrients in male fertility parameters and fecundability: a systematic review of observational studies. *Human Reproduction Update 23*(4), 371-389. https://doi.org/10.1093/humupd/dmx006

Sallam, H. N., Farrag, A., Agameya, A. F., El-Garem, Y., & Ezzeldin, F. (2005). The use of the modified hypo-osmotic swelling test for the selection of immotile testicular spermatozoa in patients treated with ICSI: a randomized controlled study. *Human Reproduction 20*(12), 3435-3440. https://doi.org/10.1093/humrep/dei249

Salman Yazdi, R., Bakhshi, S., Jannat Alipoor, F., Akhoond, M. R., Borhani, S., Farrahi, F., Panah, M. L., & Gilani, M. A. S. (2014). Effect of 830-nm diode laser irradiation on human sperm motility. *Lasers in Medical Science 29*(1), 97–104. https://doi.org/10.1007/s10103-013-1276-7

Sánchez-Calabuig, M. J., Lopez-Cardona, A. P., Fernández-González, R., Ramos-Ibeas, P., Fonseca Balvís, N., Laguna-Barraza, R., Pericuesta, E., Gutiérrez-Adán, A., & Bermejo-Álvarez, P. (2014). Potential health risks associated to ICSI: insights from animal models

and strategies for a safe procedure. *Frontiers in Public Health 2*, 241. https://doi.org/10.3389/fpubh.2014.00241

Sandin, S., Nygren, K. G., Iliadou, A., Hultman, C. M., & Reichenberg, A. (2013). Autism and mental retardation among offspring born after in vitro fertilization. JAMA *310*(1), 75-84. https://doi.org/10.1001/jama.2013.7222

Savasi, V., Ferrazzi, E., Lanzani, C., Oneta, M., Parrilla, B., & Persico, T. (2007). Safety of sperm washing and ART outcome in 741 HIV-1-serodiscordant couples. *Human Reproduction 22*(3), 772–777. https://doi.org/10.1093/humrep/del422

Schlegel, P. N., Sigman, M., Collura, B., De Jonge, C. J., Eisenberg, M. L., Lamb, D. J., Mulhall, J. P., Niederberger, C., Sandlow, J. I., Sokol, R. Z., Spandorfer, S. D., Tanrikut, C., Treadwell, J. R., Oristaglio, J. T., & Zini, A. (2021). Diagnosis and Treatment of Infertility in Men: AUA/ASRM Guideline PART II. *Journal of Urology 205*(1), 44-51. https://doi.org/10.1097/JU.0000000000001520

Sfontouris, I. A., Kolibianakis, E. M., Lainas, G. T., Navaratnarajah, R., Tarlatzis, B. C., & Lainas, T.G. (2015). Live birth rates using conventional in vitro fertilization compared to intracytoplasmic sperm injection in Bologna poor responders with a single oocyte retrieved. *Journal of Assisted Reproduction and Genetics 32*(5), 691-697. https://doi.org/10.1007/s10815-015-0459-5

Shang, Y. L., Zhu, F. X., Yan, J., Chen, L., Tang, W. H., Xiao, S., Mo, W. K., Zhang, Z. G., He, X. J., Qiao, J., Cao, Y. X., & Li, W. (2019). Novel DPY19L2 variants in globozoospermic patients and the overcoming this male infertility. *Asian Journal of Andrology 21*(2), 183-189. https://doi.org/10.4103/aja.aja_79_18

Shih, K. W., Shen, P. Y., Wu, C. C., & Kang, Y. N. (2019). Testicular versus percutaneous epididymal sperm aspiration for patients with obstructive azoospermia: a systematic review and meta-analysis. *Translational Andrology and Urology 8*(6), 631–640.

https://doi.org/10.21037/tau.2019.11.20

Simon, L., Brunborg, G., Stevenson, M., Lutton, D., McManus, J., & Lewis, S. E. (2010). Clinical significance of sperm DNA damage in assisted reproduction outcome. *Human Reproduction 25*(7), 1594-1608. https://doi.org/10.1093/humrep/deq103

Simon, L., Castillo, J., Oliva, R., & Lewis, S. E. (2011). Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reproductive BioMedicine Online 23*(6), 724-734. https://doi.org/10.1016/j.rbmo.2011.08.010

Simon, L., Zini, A., Dyachenko, A., Ciampi, A., & Carrell, D. T. (2017). A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. *Asian Journal of Andrology 19*(1), 80-90. https://doi.org/10.4103/1008-682x.182822

Simopoulou, M., Gkoles, L., Bakas, P., Giannelou, P., Kalampokas, T., Pantos, K., & Koutsilieris, M. (2016). Improving ICSI: A review from the spermatozoon perspective. *Systems Biology in Reproductive Medicine 62*(6), 359-371. https://doi.org/10.1080/19396368.2016.1229365

Skakkebaek, N. E., Rajpert-De Meyts, E., Buck Louis, G. M., Toppari, J., Andersson, A. M., Eisenberg, M. L., Jensen, T. K., Jørgensen, N., Swan, S. H., Sapra, K. J., Ziebe, S., Priskorn, L., & Juul, A. (2016). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews 96*(1), 55–97. https://doi.org/10.1152/physrev.00017.2015

Staessen, C., & Van Steirteghem, A. C. (1997). The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in-vitro fertilization. *Human Reproduction 12*(2), 321–327. https://doi.org/10.1093/humrep/12.2.321

Stone, S., O'Mahony, F., Khalaf, Y., Taylor, A., & Braude, P. (2000). A normal livebirth

after intracytoplasmic sperm injection for globozoospermia without assisted oocyte activation: case report. *Human Reproduction 15*(1), 139-141. https://doi.org/10.1093/humrep/15.1.139

Sullivan, E. A., Zegers-Hochschild, F., Mansour, R., Ishihara, O., de Mouzon, J., Nygren, K. G., & Adamson, G. D. (2013) International committee for monitoring Assisted Reproductive Technologies (ICMART) world report: assisted reproductive technology report 2004. *Human Reproduction 28*(5), 1375-1390. https://doi.org/10.1093/humrep/det036

Supramaniam, P. R., Granne, I., Ohuma, E. O., Lim, L. N., McVeigh, E., Venkatakrishnan, R., Becker, C. M., & Mittal, M. (2020). ICSI does not improve reproductive outcomes in autologous ovarian response cycles with non-male factor subfertility, *Human Reproduction 35*(3), 583-594 https://doi.org/10.1093/humrep/dez301

Swan, S. H., Elkin, E. F., & Fenster, L. (1997). Have sperm densities declined? A reanalysis of global trend data. *Environmental Health Perspectives 105*(11), 1228–1232. https://doi.org/10.1289/ehp.971051228

Swiatecka, J., Bielawski, T., Anchim, T., Leśniewska, M., Milewski, R., & Wołczyński, S. (2014.). Oocyte zona pellucida and meiotic spindle birefringence as a biomarker of pregnancy rate outcome in IVF-ICSI treatment. *Ginekologia Polska 85*(4), 264-271. https://doi.org/10.17772/gp/1722

Szczygiel, M. A., Kusakabe, H., Yanagimachi, R., & Whittingham, D. G. (2002). Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. *Biology of Reproduction 67*(4), 1278-1284. https://doi.org/10.1095/biolreprod67.4.1278

Tanaka, A., Nagayoshi, M., Takemoto, Y., Tanaka, I., Kusunoki, H., Watanabe, S., Kuroda, K., Takeda, S., Ito. M., & Yanagimachi, R. (2015). Fourteen babies born after round

spermatid injection into human oocytes. *Proceedings of the National Academy of Science USA 112*(47), 14629-14634. https://doi.org/10.1073/pnas.1517466112

Tanaka, A., Suzuki, K., Nagayoshi, M., Tanaka, A., Takemoto, Y., Watanabe, S., Takeda, S., Irahara, M., Kuji, N., Yamagata, Z., & Yanagimachi, R. (2018). Ninety babies born after round spermatid injection into oocytes: survey of their development from fertilization to 2 years of age. *Fertility and Sterility 110*(3), 443-451. https://doi.org/10.1016/j.fertnstert.2018.04.033

Tanii, I., Aradate, T., Matsuda, K., Komiya, A., & Fuse, H. (2011). PACAP-mediated sperm-cumulus cell interaction promotes fertilization. *Reproduction 141*(2), 163-171. https://doi.org/10.1530/rep-10-0201

Tannus, S., Son, W. Y., Gilman, A., Younes, G., Shavit, T., & Dahan, M. H. (2017). The role of Intracytoplasmic sperm injection in non-male factor infertility in advanced maternal age. *Human Reproduction 32*(1), 119-124. https://doi.org/10.1093/humrep/dew298

Taşdemir, I., Taşdemir, M., & Tavukçuoğlu, S. (1998). Effect of pentoxifylline on immotile testicular spermatozoa. *Journal of Assisted Reproduction and Genetics 15*(2), 90-92. https://doi.org/10.1007/bf02766832

Tavalaee, M., & Nasr-Esfahani, M. H. (2016). Expression profile of PLCζ, PAWP, and TR-KIT in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. *Andrology 4*(5), 850-856. https://doi.org/10.1111/andr.12179

Taylor, S. L., Yoon, S. Y., Morshedi, M. S., Lacey, D. R., Jellerette, T., Fissore, R. A., & Oehninger, S. (2010). Complete globozoospermia associated with PLCζ deficiency treated with calcium ionophore and ICSI results in pregnancy. *Reproductive BioMedicine Online 20*(4), 559-564. https://doi.org/10.1016/j.rbmo.2009.12.024

Teixeira, D. M., Hadyme Miyague, A., Barbosa, M. A., Navarro, P. A., Raine-Fenning, N.,

Nastri, C. O., & Martins, W. P. (2020). Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. *Cochrane Database of Systematic Reviews* 2(2), CD010167. https://doi.org/10.1002/14651858.CD010167.pub3

te Velde, E., van Baar, A., & van Kooije, R. (1998). Concerns about assisted reproduction. *Lancet 351*(9115), 1524-1525. https://doi.org/10.1016/s0140-6736(98)22021-6

Tournaye, H. (2003). ICSI: a technique too far? *International Journal of Andrology 26*(2), 63-69. https://doi.org/10.1046/j.1365-2605.2003.00380.x

Tournaye, H., Verheyen, G., Albano, C., Camus, M., Van Landuyt, L., Devroey, P., & Van Steirteghem, A. (2002.) Intracytoplasmic sperm injection versus in vitro fertilization: a randomized controlled trial and a meta-analysis of the literature. *Fertility and Sterility 78*(5), 1030-1037. https://doi.org/10.1016/s0015-0282(02)03377-0

Tunç, L., Gürocak, S., Sözen, S., Tan, O., Alkibay, T., & Bozkirli, I. (2005). Is varicocele a prognostic factor for determining sperm retrieval rate before testicular sperm extraction? *Archives of Andrology 51*(2), 159–164. https://doi.org/10.1080/014850190884318

Tüttelmann, F., Ruckert, C., & Röpke, A. (2018). Disorders of spermatogenesis: Perspectives for novel genetic diagnostics after 20 years of unchanged routine. *Medical Genetics 30*(1), 12–20. https://doi.org/10.1007/s11825-018-0181-7

Van der Borght, M., & Wyns, C. (2018). Fertility and infertility: Definition and epidemiology. *Clinical Biochemistry 62*, 2-10. https://doi.org/10.1016/j.clinbiochem.2018.03.012

Van der Westerlaken, L., Helmerhorst, F., Dieben, S., & Naaktgeboren, N. (2005). Intracytoplasmic sperm injection as a treatment for unexplained total fertilization failure or low fertilization after conventional in vitro fertilization. *Fertility and Sterility 83*(3), 612-617. https://doi.org/10.1016/j.fertnstert.2004.08.029

Van Rumste, M. M., Evers, J. L., Farquhar, C. M., & Blake, D. A. (2000). Intra-cytoplasmic

sperm injection versus partial zona dissection, subzonal insemination and conventional techniques for oocyte insemination during in vitro fertilisation. *Cochrane Database of Systematic Reviews* 2000(2): CD001301. https://doi.org/10.1002/14651858.cd001301

Van Rumste, M. M. E., Evers, J. L. H., & Farquhar, C. (2003). Intra-cytoplasmic sperm injection versus conventional techniques for oocyte insemination during in vitro fertilisation in couples with non-male subfertility. *Cochrane Database of Systematic Reviews 2003*(2): CD001301.

Van Soom, A., Tanghe, S., De Pauw, I., Maes, D., & de Kruif, A. (2002). Function of the cumulus oophorus before and during mammalian fertilization. *Reproduction in Domestic Animals 37*(3), 144-151. https://doi.org/10.1046/j.1439-0531.2002.00345.x

Van Steirteghem, A., Bonduelle, M., Devroey, P., & Liebaers, I. (2002). Follow-up of children born after ICSI. *Human Reproduction Update 8*(2), 111-116. https://doi.org/10.1002/14651858.CD001301

Vaughan, D.A., & Sakkas, D. (2019). Sperm selection methods in the 21st century. *Biology of Reproduction 101*(6), 1076-1082. https://doi.org/10.1093/biolre/ioz032

Walls, M., Junk, S., Ryan, J. P., & Hart, R. (2012). IVF versus ICSI for the fertilization of in-vitro matured human oocytes. *Reproductive BioMedicine Online 25*(6), 603-607. https://doi.org/10.1016/j.rbmo.2012.08.001

Wang, J. G., Douglas, N. C., Prosser, R., Kort, D., Choi, J. M., & Sauer, M. V. (2009). Optimization of IVF pregnancy outcomes with donor spermatozoa. *Journal of Assisted Reproduction and Genetics 26*(2-3), 83-91. https://doi.org/10.1007/s10815-008-9291-5

Wang, R., Danhof, N. A., Tjon-Kon-Fat, R. I., Eijkemans, M. J., Bossuyt, P. M., Mochtar, M. H., van der Veen, F., Bhattacharya, S., Mol, B. W. J., & van Wely, M. (2019). Interventions for unexplained infertility: a systematic review and network meta-analysis. *Cochrane Database of Systematic Reviews 9*(9), CD012692.

https://doi.org/10.1002/14651858.CD012692.pub2

Wang, W. H., Meng, L., Hackett, R. J., & Keefe, D. L. (2001). Developmental ability of human oocytes with or without birefringent spindles imaged by Polscope before insemination. *Human Reproduction 16*(7), 1464-1468. https://doi.org/10.1093/humrep/16.7.1464

Wennerholm, U. B., Bergh, C., Hamberger, L., Lundin, K., Nilsson, L., Wikland, M., & Kallen, B. (2000). Incidence of congenital malformations in children born after ICSI. *Human Reproduction 15*(4), 944-948. https://doi.org/10.1093/humrep/15.4.944

West, R., Coomarasamy, A., Frew, L., Hutton, R., Kirkman-Brown, J., Lawlor, M., Lewis, S., Partanen, R., Payne-Dwyer, A., Roman-Montanana, C., Torabi, F., Tsagdi, S., & Miller, D. (2022). Sperm selection with hyaluronic acid improved live birth outcomes among older couples and was connected to sperm DNA quality, potentially affecting all treatment outcomes. *Human Reproduction 37*(6), 1106-1125. https://doi.org/10.1093/humrep/deac058

Wiser, A., Ghetler, Y., Gonen, O., Piura, E., Berkovits, A., Itskovich, A., Rom, T., & Shulman, A. (2012). Re-evaluation of post-wash sperm is a helpful tool in the decision to perform in vitro fertilisation or intracytoplasmic sperm injection. *Andrologia 44*(2), 73-77. https://doi.org/10.1111/j.1439-0272.2010.01107.x

World Health Organization (2021). WHO laboratory Manual for the Examination and Processing of Human Semen (6th ed). World Health Organization Press, Geneva.

Wu, E.X., Stanar, P., & Ma, S. (2014). X-chromosome inactivation in female newborns conceived by assisted reproductive technologies. *Fertility and Sterility 101*(6), 718-723. https://doi.org/10.1016/j.fertnstert.2014.03.010

Yanagida, K., Fujikura, Y., & Katayose, H. (2008). The present status of artificial oocyte activation in assisted reproductive technology. *Reproductive Medicine and Biology 7*(3),

133-142. https://doi.org/10.1111/j.1447-0578.2008.00210.x

Younes, G., Tannus, S., Son, W. Y., & Dahan, M. H. (2019). When to do intracytoplasmic sperm injection: a prospective comparison. *Archives in Gynecology and Obstetrics 300*(5), 1461–1471. https://doi.org/10.1007/s00404-019-05324-4

Yovich, J. M., Edirisinghe, W. R., Cummins, J. M., & Yovich, J. L. (1988). Preliminary results using pentoxifylline in a pronuclear stage tubal transfer (PROST) program for severe male factor infertility. *Fertility and Sterility 50*(1), 179–81. https://doi.org/10.1016/s0015-0282(16)60030-4

Yu, X. W., Wei, Z. T., Jiang, Y. T., & Zhang, S. L. (2015). Y chromosome azoospermia factor region microdeletions and transmission characteristics in azoospermic and severe oligozoospermic patients. *International Journal of Clinical and Experimental Medicine 8*(9), 14634–14646. http://www.ncbi.nlm.nih.gov/pmc/articles/pmc4658835/

Yu, Z., Wei, Z., Yang, J., Wang, T., Jiang, H., Li, H., Tang, Z., Wang, S., & Liu, J. (2018). Comparison of intracytoplasmic sperm injection outcome with fresh versus frozen-thawed testicular sperm in men with nonobstructive azoospermia: a systematic review and meta-analysis. *Journal of Assisted Reproduction and Genetics 35*(7), 1247–1257. https://doi.org/10.1007/s10815-018-1206-5

Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., de Mouzon, J., Sokol, R., Rienzi, L., Sunde, A., Schmidt, L., Cooke, I. D., Simpson, J. L., & van der Poel, S. (2017). The International Glossary on Infertility and Fertility Care. *Fertility and Sterility 108*(3), 393–406. https://doi.org/10.1016/j.fertnstert.2017.06.005

Zhang, Z., Zhu, L., Jiang, H., Chen, H., Chen, Y., & Dai, Y. (2015). Sperm DNA fragmentation index and pregnancy outcome after IVF or ICSI: a meta-analysis. *Journal of Assisted Reproduction and Genetics 32*(1), 17-26. https://doi.org/10.1007/s10815-014-0374-1

Zhao, J., Zhang, Q., Wang, Y., & Li, Y. (2014). Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. *Fertility and Sterility 102*(4), 998-1005. https://doi.org/10.1016/j.fertnstert.2014.06.033

Zhu, J. L., Basso, O., Obel, C., Hvidtjørn, D., & Olsen, J. (2009). Infertility, infertility treatment and psychomotor development: the Danish National Birth Cohort. *Paediatric and Perinatal Epidemiology 23*(2), 98–106. https://doi.org/10.1111/j.1365-3016.2008.00989.x

Zollner, U., Schleyer, M., & Steck, T. (1996). Evaluation of a cut-off value for normal sperm morphology using strict criteria to predict fertilization after conventional in-vitro fertilization and embryo transfer in asthenozoospermia. *Human Reproduction 11*(10), 2155-2161. https://doi.org/10.1093/oxfordjournals.humrep.a019068

Table 1. Levels of evidence (NICE, 2004)

Grade strength of evidence

A. Requires at least one RCT as part of a body of literature of overall good quality and consistency addressing the specific recommendation. (evidence levels 1a, 1b).

B. Requires the availability of well-controlled clinical studies but has no randomised clinical trials on the topics of recommendations. (evidence levels 2a, 2b, 3).

C. Requires evidence obtained from expert committee reports of opinions and/or clinical experiences of respected authorities. Indicates an absence of directly applicable clinical studies of good quality. (evidence level 4).

GPP. Good practice point

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ARTICLE

Neonatal and neurodevelopmental outcome of children aged 3–10 years born following assisted oocyte activation

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Abstract Assisted oocyte activation (AOA) using a calcium ionophore has been used for more than a decade following intracytoplasmic sperm injection (ICSI) fertilization failure. However, since AOA does not mimic precisely the physiological fertilization process, concerns exist about its use in human assisted reproduction. This study assessed the neonatal and neurodevelopmental outcome of children aged ≥3 years who had been born following AOA in our centre. Twenty-one children participated in the study (81% response rate; mean age 63.6 ± 21.07 months). Neonatal data were collected via questionnaires. Neurodevelopmental outcome was tested using the Reynell Developmental Language Scales or Clinical Evaluation of Language Fundamentals, Wechsler Preschool and Primary Scale of Intelligence or Wechsler Intelligence Scale for Children, and the Movement Assessment Battery for Children III. Behaviour was scored by the Social Communication Questionnaire, the Child Behaviour Checklist and the Teachers Report Form. For all tests and questionnaires, the mean outcomes lay within the expected ranges. These are first data on the developmental outcome of AOA children. The high response rate and the robustness of the tests support the data, which are reassuring although still considered preliminary. Therefore, AOA should still be performed only in selected couples.

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KEYWORDS: assisted oocyte activation, child follow up, failed fertilization, ICSI, ionomycine

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Introduction

Intracytoplasmic sperm injection (ICSI) is typically used in cases of severe semen dysfunction or following failure of conventional IVF. In general, fertilization rates of 70—75% are obtained (Palermo et al., 2009). In contrast, failed fertilization still occurs in 1—3% of ICSI cycles, even when a sufficient number of mature oocytes and motile spermatozoa are available (Esfandiari et al., 2005; Flaherty et al., 1995). The principal reason for fertilization failure following ICSI is defective oocyte activation (Flaherty et al., 1998; Liu et al., 1995; Rawe et al., 2000). The primary cellular event leading to mammalian oocyte activation is the initiation of intracellular calcium oscillations in the oocyte's cytosol. Although doubts have risen recently, substantial evidence suggests that the spermatozoon supplies an activating protein, phospholipase C zeta, into the oocyte's cytosol which triggers calcium oscillations via an inositol-3-phosphate-mediated pathway (Aarabi et al., 2012; Saunders et al., 2002). The oocyte responds to this by the calcium-dependent activation of several down-stream pathways, which are necessary for successful oocyte activation, pre- and post-implantation embryo development (Ducibella et al., 2002; Kashir et al., 2010; Parrington et al., 2007). Therefore, both the spermatozoon and the oocyte are considered to be responsible for successful oocyte activation and embryo development. In this study centre, the mouse oocyte activation test (MOAT), a heterologous ICSI model, is performed to diagnose the activation capacity of a patient's spermatozoa (Rybouchkin et al., 1996).

Several reports show that most, but not all, of the couples suffering from ICSI failure benefit from the application of ICSI combined with assisted oocyte activation (AOA) (Heindryckx et al., 2005, 2008; Montag et al., 2012; Vanden Meerschaut et al., 2012). Different activating agents have been used successfully in human assisted reproduction, e.g. calcium ionophore, electrical pulses and strontium chloride (Baltaci et al., 2010; Kyono et al., 2008; Rybouchkin et al., 1997; Yanagida et al., 2006). However, until adequate scientific evidence is provided regarding its safety and efficacy, ICSI combined with AOA cannot yet be considered an established treatment. The current study centre has been performing ICSI combined with AOA during more than a decade, using the injection of calcium chloride ($CaCl_2$) along with the spermatozoon into the oocyte, followed by a 2-fold calcium ionophore exposure of the injected oocyte (Rybouchkin et al., 1997) and has built up a large experience in the treatment of this rare but challenging group of patients. Rewarding results have been obtained regarding fertilization and pregnancy rates (Heindryckx et al., 2008). Nevertheless, the artificially induced calcium rises do not mimic precisely the physiologically sperm-induced calcium oscillations and little is known yet about the possible adverse effects of ionophores on post-implantation embryo development. Therefore, concerns exist about the use of a calcium ionophore as oocyte-activating agent in human assisted reproduction. However, the influence of ionomycin exposure during the zygote stage on pre- and post-implantation development has been evaluated in the mouse (Heytens et al., 2008). Zygotes treated with ionomycin showed normal pre- and post-implantation development, and normally developing and fertile pups were born originating from ionomycin-treated zygotes. Nevertheless, since these results were obtained from highly fertile animals, results should not be extrapolated without caution to a subfertile human population (Harper et al., 2012). Even though the exposure to the calcium ionophore for the purpose of AOA is short and limited to the zygote stage, it is appropriate to examine possible adverse effects of ionomycin in children born following AOA.

Since the main aim of a IVF-clinic is to ensure the birth of healthy babies, the goal of this study was to report on the neonatal outcome of children born following AOA and on their cognitive, language and motor development and behaviour from the age of 3 years onwards and to compare their outcomes with age-matched norms.

Materials and methods

Participants

All children born following AOA at University Hospital Ghent from October 2001 until September 2008 were eligible for this study. Inclusion criteria for the neurodevelopmental and behavioural assessment were: (i) age $\geq$3 years; (ii) the ability to visit the outpatient clinic; and (iii) Dutch as the mother tongue. Written informed consent was given by the parents and the study was approved by the Ethical Committee of the Ghent University Hospital (EC number: 2011/188; Belgian registry number: B670201110978, approved 19 May 2011).

Assisted oocyte activation

ICSI combined with AOA was performed as previously described (Heindryckx et al., 2008). Briefly, a spermatozoon was injected into the oocyte together with a small amount of 0.1 mol/l $CaCl_2$ (corresponding to the diameter of the oocyte). Thereafter, the oocytes were incubated for 30 min at 37°C in a 6% CO_2 air atmosphere. Next, the oocytes were exposed to a calcium ionophore (10 μmol/l ionomycin, I9657; Sigma-Aldrich, Bornem, Belgium) for 10 min. Following ionophore exposure, the oocytes were washed with Cook Cleavage medium (Cook Ireland, Limerick, Ireland) and were incubated again. After another 30 min, the calcium ionophore treatment was repeated for 10 min. Finally, the oocytes were washed and incubated overnight under 5% O_2.

Neonatal data

A questionnaire concerning obstetric and neonatal parameters was filled in by the parents shortly after the birth of their child. This questionnaire was retrieved from the patients' file at the moment of this study and informed consent was given by the parents to use this questionnaire for data analysis. Additionally, some parents were consulted on the day of the neurodevelopmental assessment if data were missing or unclear.

Follow up by assessment

All children aged $\geq$3 years were invited to the study centre for neurodevelopmental follow up using a set of three

standardized tests. For each child, all tests took place on one single day and were performed with a period of rest in between. The order of testing could be different due to planning. The cognitive, language and motor development tests were performed by two clinical psychologists, one speech therapist and one physiotherapist, respectively.

Cognitive development

To assess cognitive development, the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III-NL, $\leq$7 years) or the Wechsler Intelligence Scale for Children (WISC-III-NL) was used (Hendriksen and Hurks, 2009; Kort et al., 2005). Both tests measure verbal IQ (VIQ), performance IQ (PIQ) and full-scale IQ (FSIQ) and can be completed without reading or writing. Dutch/Flemish norms were used for both tests.

Language development

Language skills were assessed by the Reynell Developmental Language Scales (*Reynell Taalontwikkelingsschalen*, RTOS) or by the Clinical Evaluation of Language Fundamentals (CELF-IV-NL) (Kort et al., 2008; Schaerlaekens et al., 2003). The RTOS is used for children <5 years and is a Dutch/Flemish norm-based practical tool for everyday clinical use. The CELF-IV-NL is used for children aged $\geq$5 years. For both tests, the outcomes are percentile scores and include two scales next to a total percentile score: the comprehension and the expressive scale.

Motor development

The Movement Assessment Battery for Children (Movement ABC-II-NL) was used to identify and evaluate movement problems (Henderson et al., 2010). It provides a comprehensive assessment of movement competence on age-appropriate tasks within three subtests: manual dexterity, ball skills and static and dynamic balance. Outcome score is a percentile score based on Flemish/Dutch norms. Additionally, a questionnaire regarding motor skills in everyday life was given to the parents (*Vragenlijst voor Motorische Vaardigheden van Kleuters* (VMVK, <5 years) or *Coördinatievragenlijst Voor Ouders* (CVO, $\geq$5 years)). The CVO was officially translated from Developmental Coordination Disorder Questionnaire (Wilson et al., 2000).

Follow up by questionnaires

The behaviour of the AOA children aged $\geq$3 years was assessed by three standardized questionnaires. The Social Communication Questionnaire (SCQ) is a worldwide-used screening tool for autism spectrum disorders (ASD) (Rutter et al., 2003a; Warreyn et al., 2004). Furthermore, the Child Behaviour Checklist (CBCL) and the Teacher's Report Form (TRF) were used. These questionnaires are identical, filled in by the parent and teacher, respectively, on which the child is rated on various behavioural and emotional aspects. The CBCL is one of the most widely used standardized measures in child psychology for evaluating maladaptive behavioural and emotional problems in children (Achenbach and Rescorla, 2001). For the CBCL, the usefulness of existing US norms within a Flemish community sample were explored before and the reliability was excellent (Braet et al., 2011). In addition to the total problem score, the CBCL and TRF give two broad syndrome scores: internalizing and externalizing behaviour. All the above mentioned questionnaires were in Dutch and filled in by one or both parents, except for the TRF, which was filled in by the teacher of the child. The TRF was a non-mandatory questionnaire.

Statistical analysis

To compare birthweight and gestational age between the singletons and twins, the Mann–Whitney *U*-test was used. The Fisher's Exact test was used to compare the amount of birth defects in the study population to the amount of birth defects present in a large ICSI population (Davies et al., 2012). The Mann–Whitney *U*-test was used to compare mean results on the developmental tests between study groups. The Wilson's confidence interval for a single proportion method was used to calculate the 95% confidence intervals around the proportion of children with test scores within pathological thresholds (Altman et al., 2008). The Statistical Package for the Social Sciences version 20 (SPSS Statistics, IBM Corp, NY, USA) and R version 2.15.1 (The R Foundation for Statistical Computing) were used for statistical analysis. A P-value < 0.05 was considered statistically significant.

Results

Study population

From 2001 onwards, 53 pregnancies were achieved following AOA in this study centre, of which 41 ended in childbirth (77.4% ongoing pregnancy rate). Six out of those 41 pregnancies were twin pregnancies (14.6%). Eight spontaneous miscarriages (15.1%) and three biochemical pregnancies (5.7%) occurred. One pregnancy was interrupted because of the fetus carrying trisomy 21. From those 47 children born following AOA, 26 were aged 3 years or older at the time of this study and therefore eligible for this study. Twenty-one out of those 26 children aged 3–11 years could be included in the study (81% response rate). The mean ± standard deviation and median (range) age of the included children was 63.6 ± 21.07 and 62.0 (38–124) months, respectively. The mean maternal and paternal age at childbirth was 32 ± 3.0 years and 34 ± 3.6 years, respectively. Two singleton sisters had a mother with Asian descent. All other parents were Caucasians. Two-thirds of the parents had a higher level of education (beyond 18 years of age). All children lived together with both their biological parents.

The fertility history and indications for AOA are shown in Table 1. In 11 couples, the indication for AOA was low or zero fertilization in previous conventional ICSI cycles, whereas for the remaining three couples, AOA had been advised because of total globozoospermia, a condition in which the sperm acrosome is lacking and typically leading to very low or zero fertilization rates using conventional ICSI. Without AOA the mean fertilization rate in this group of 14 couples was 19.9%, whereas following AOA treatment the fertilization rate was restored to 74.1%. A mean of

Table 1 Fertility history and indications for AOA.

Characteristic	*Study population (n = 14 patients)*
Fertility history[a]	
Conventional ICSI without pregnancy	
Cycles	2.1 ± 1.75
Fertilization rate (%)	19.9 ± 19.30
ICSI + AOA	
Cycles	2.5 ± 1.16
Fertilization rate (%)	74.1 ± 20.81
Indication to perform AOA[a]	
Zero fertilization following conventional ICSI	3 (21.4)
Low fertilization (<33.3%) following conventional ICSI	8 (57.1)
Globozoospermia	3 (21.4)
MOAT results[b]	
0—20% activation: certain sperm deficiency	5 (41.7)
21—84% activation: probable sperm deficiency	4 (33.3)
85—100% activation: no sperm deficiency	3 (25.0)

Values are mean ± standard deviation or *n* (%).
AOA = assisted oocyte activation; ICSI = intracytoplasmic sperm injection; MOAT = mouse oocyte activation test.
[a]Data for all 14 parent couples.
[b]Data for 12 couples.

2.1 ± 1.75 conventional ICSI cycles were performed in these couples before they were referred to this study centre. Pregnancies following AOA treatment were achieved after a mean of 2.5 ± 1.16 fresh AOA cycles. For 12 out of 14 couples, a diagnostic MOAT was performed before proceeding to AOA treatment. Nine of the couples were classified into MOAT group 1 or 2, which strongly suggests a sperm deficiency (Vanden Meerschaut et al., 2013).

Neonatal outcome and birth defects

Eleven singletons and 10 twins born following ICSI combined with AOA were included in this study. Neonatal data were retrieved for all of the included children (Table 2). The birthweight of the singletons was significantly higher compared with the twins ($P < 0.01$). Seven out of 21 children (33.3%) were born following a Caesarean section, four of whom were twins. Mean gestational age at birth was 39.4 ± 0.92 weeks and 36.8 ± 2.53 weeks for the singletons and twins, respectively ($P < 0.001$). Five children (solely twins) were admitted to the neonatal intensive care unit (NICU) (mean duration of stay 35 ± 18.8 days, range 1—44 days). Reasons for admission to the NICU were: prematurity ($n = 2$), poor condition of the mother ($n = 2$) and low temperature ($n = 1$). Birth defects were diagnosed in three children (14.3%, 95% CI 5.0—34.6%) at toddler age. In one child, a small arachnoid cyst was found by chance at the age of three. The condition is considered to be benign and did not require any intervention. In another child, a pectus excavatum and genu varum were found. In a third child, vesico-ureteral reflux was diagnosed at the age of two.

Non-respondent analysis

The five children who did not participate were excluded because of: (i) a mother tongue different from Dutch ($n = 3$); and (ii) parents were not able to visit the outpatient clinic ($n = 2$).

All five excluded children were singletons (three girls and two boys), and two of them were sisters. Indications for AOA were globozoospermia ($n = 4$) and failed fertilization following conventional ICSI ($n = 1$). Their mean ± standard deviation and median (range) age at the moment of recruitment for the study was 73.8 ± 33.66 and 77.0 (40—125) months, respectively. The maternal age at childbirth was 28 ± 4.0 years. Mean gestational age of these children was 39.0 ± 1.32 weeks, with none of them born before week 37 of pregnancy. Mean birthweight was 3107 ± 217.8 g.

Neurodevelopmental outcome

The majority of the children were younger than 7 years at the moment of testing. Seven of the 11 singletons were single child. Two singletons were sisters; another pair of singletons comprised a brother and a sister. The results are presented in Table 3.

Cognitive development

Cognitive development was assessed for all included children. Mean FSIQ, PIQ and VIQ scores on the WPPSI-III-NL were 104.2, 105.0 and 102.8, respectively (20 children: 10 singletons, 10 twins). One girl and one boy (not genetically related to each other) scored 77 and 74 for VIQ and PIQ, respectively. However, both their FSIQ scores were considered normal: 93 and 85, respectively. The oldest child was tested using the WISC-III-NL: FSIQ, PIQ and VIQ scores were 131, 130 and 123, respectively. Considering the pathological threshold for FSIQ scoring being <70 (cognitive disability), the proportion of children in this cohort falling below this threshold is 0% (95% CI 0—15.5%).

Language development

One child was excluded because of an incomplete test result due to a lack of co-operation. Eight children aged <5 years were tested using the RTOS. Their total percentile score for language development was 78.2, while their percentile scores on receptive and expressive language skills were 82.0 and 73.3. The remaining 12 children aged ≥5 years were tested using the CELF-IV-NL. Their total, receptive and expressive language percentile scores were 40.1, 32.5 and 40.9, respectively. If both groups were compared, the younger group scored significantly better than the older group of children ($P < 0.05$). Importantly, the older group consisted of eight twins (8/12, 67%), whereas in the younger group only two twins were present (2/8, 25%). Considering the pathological thresholds for language percentile scoring being <5th percentile (language disability), the proportion

Table 2 Neonatal data.

Outcome	*Singletons (n = 11)*	*Twins (n = 10)*	*Total (n = 21)*
Obstetric			
Delivery <37 weeks	0 (0)	2 (20.0)	2 (9.5)
Vaginal delivery	8 (72.7)	6 (60.0)	14 (66.7)
Caesarean section	3 (27.3)	4 (40.0)	7 (33.3)
Neonatal			
Birthweight (g)	3623 ± 324.5[a] (3085–4140)	2696 ± 676.7[a] (1640–3450)	3181 ± 695.5 (1640–4140)
Birthweight <2500 g	0 (0)	3 (30)	3 (14.3)
APGAR <7 at 5 min	0 (0)	0 (0)	0 (0)
Neonatal intensive care	0 (0)	5 (50.0)	5 (23.8)
Duration (days)			
≤7	0 (0)	1 (10.0)	1 (4.8)
>7	0 (0)	4 (40.0)[b]	4 (19.0)
Perinatal mortality	0 (0)	0 (0)	0 (0)
Gender			
Female	8 (72.7)	6 (60.0)	14 (66.7)
Male	3 (27.3)	4 (40.0)	7 (33.3)

Values are *n* (%) or mean ± standard deviation (range).
[a]$P < 0.01$ (Student's t-test). [b]Two of these children were admitted to NICU because of severe illness of their mother (aorta dissection).

of children in this cohort falling below this threshold for the expressive and total language score was 0% (95% CI 0–16.1%), whereas this was 5% (95% CI 0.01–23.6%) for the receptive language score.

Motor development

In the two youngest children, the test was cancelled because of fatigue. Seventeen children were tested using the Movement ABC-II-NL for children aged 3–6 years. The mean total percentile score was 37.1. The remaining two older children (both singletons) were tested using the Movement ABC-II-NL test for children aged 7–10 years. Their mean percentile score was 50.0. Subtest percentile scores are shown in Table 3. Six children (31.6%) scored below the 15th percentile for motor skill development. Among them, 5 (83.3%) were twins, of which two were born before week 37 of gestation. No correlation was found between the birthweight of the included children and the total or subtest scores on motor skill development. Considering the pathological thresholds for motor skill percentile scoring being <5th percentile (Developmental Coordination Disorder), the proportion of children in this cohort falling below this threshold is 10.5% (95% CI 2.9–31.4%) for manual dexterity, 5.3% (95% CI 0.01–24.6%) for aiming and catching and the total motor score, and 0% (95% CI 0–16.8%) for balance. Additionally, the parents were asked to fill out a questionnaire regarding motor skills in everyday life (VMVK or CVO questionnaire). All children ($n = 21$) tested within the normal ranges on these questionnaires (data not shown), meaning that according to the questionnaires no notable co-ordination disorders with influence on everyday life activities are present in these children.

Behaviour

The behaviour of all 21 AOA children was assessed using three validated questionnaires. The SCQ questionnaire was filled out and returned valid for 16 children. The mean score was 6.4 (range 0–13). No correlation was found between paternal and maternal age at birth, APGAR score at 5 min, birthweight and the SCQ score. The CBCL and TRF (non-mandatory) were returned for 19 and eight children, respectively. The mean scores for the CBCL and TRF were 43.8 and 44.0, respectively. Again, these scores fell within the normal range for children of similar age, being <70. Also, for both questionnaires, the mean scores for internalizing and externalizing behaviour lied within the normal range. One girl scored above the norm for internalizing behaviour (CBCL score: 74). Nevertheless, this was not confirmed by her teacher (TRF score: 53).

Discussion

This is the first report on the neurodevelopmental and behavioural outcome of children born following AOA, which is a rarely required, nonetheless highly specialized fertilization technique. The high response rate and the robustness of the performed tests and questionnaires support the validity of the results found in this study.

Others described a group of 22 babies born following AOA (Takisawa et al., 2011a,b). Of these babies, 10 were born following AOA by the calcium ionophore A23187 and 12 following $SrCl_2$ treatment. Growth and health of these babies at birth were not different between both AOA protocols. In a similar communication, weight and height of 21 AOA children was assessed up to the age of six (Sato et al., 2011).

Table 3 Scores of the follow up by assessment.

Test and age group	*No. of children*	*Norm*	*Score*			
Cognition	21		Verbal IQ	Performal IQ	Full scale IQ	
WPPSI-III-NL (≤7 years)	20	100	102.8 (96.4–109.2) (77–129)	105.0 (97.8–112.2) (74–142)	104.2 (97.5–110.8) (85–144)	
WISC-III-NL (>7 years)	1	100	123	130	131	
Language	20		Receptive scale	Expressive scale	Total percentile score	
RTOS (<5 years)	8	50	82.0 (63.4–100.7)[a] (35.5–99)	73.3 (49.4–97.2)[b] (30–99)	78.2 (57.2–99.2)[c] (33.3–99.0)	
CELF-IV-NL (≥5 years)	12	50	32.5 (7.3–57.7)[a] (3.6–94.5)	40.9 (16.6–65.2)[b] (7.1–88.5)	40.1 (16.8–63.4)[c] (9.1–89.7)	
Motor skills	19		Manual dexterity	Aiming and catching	Balance	Total percentile score
Movement ABC-II-NL (3–6 years)	17	50	42.0 (24.4–58.7) (1–95)	43.8 (29.5–58.0) (2–95)	41.5 (25.1–57.9) (5–91)	37.1 (22.3–52.0) (2–84)
Movement ABC-II-NL (7–10 years)	2	50	56.5 (NA) (50–63)	21.0 (NA) (5–37)	77.0 (NA) (63–91)	50.0 (NA) (37–63)
Autism screening	16		Score			
SCQ (≥2 years)	16	<15	6.4 (4.5–8.3) (0–13)			
Maladaptive behaviour screening	19		Internalizing broadband	Externalizing broadband	Total score	
CBCL (1.5–5 years)	11	<70	50.5 (42.7–58.2) (33–74)	47.6 (42.1–53.0) (35–60)	48.6 (42.5–54.8) (30–63)	
CBCL (6–18 years)	8	<70	38.4 (33.6–43.2) (33–50)	39.1 (35.0–43.2) (34–48)	37.3 (32.0–42.5) (29–45)	
TRF (1.5–5 years)	4	<70	47.5 (41.3–53.7) (44–53)	44.8 (36.8–52.7) (38–49)	45.5 (41.7–49.3) (44–49)	
TRF (6–18 years)	4	<70	43.5 (31.7–55.7) (37–52)	42.7 (40.9–44.1) (41–43)	42.5 (32.1–52.9) (33–48)	

Values are mean (95% CI) (min–max) or *n*. Norm: (i) the 50th percentile of the general population (for cognition, language and motor skill assessment); or (ii) the cut-off below which the behaviour is considered normal (for autism and maladaptive behaviour screening).
CBCL = Child Behaviour Checklist; CELF = Clinical Evaluation of Language Fundamentals; Movement ABC-II-NL = Movement Assessment Battery for Children III; NA = not applicable (*n* = 2); RTOS = Reynell Developmental Language Scales; SCQ = Social Communication Questionnaire; TRF = Teachers Report Form; WISC = Wechsler Intelligence Scale for Children; WPPSI = Wechsler Preschool and Primary Scale of Intelligence.
[a]$P < 0.01$. [b,c]$P < 0.05$ comparing RTOS and CELF scores (Mann–Whitney *U*-test), respectively.

For the singletons, the physical growth was within the 10–90% percentile. In the latter report, it was not mentioned whether the children were born following calcium ionophore or strontium treatment.

The neonatal data of this group of children were reassuring. No birth defects were reported at birth, whereas at toddler age birth defects were reported in 14.3% of the children followed up by assessment. However, the occurrence of birth defects in this cohort was not different from a recent reported birth defect rate of 9.7% in 939 ICSI children (Davies et al., 2012). Nonetheless, the sample size of the current study group was not adequate to allow for a meaningful conclusion, as it led to a wide confidence interval for the observed birth defect rate, which indicated that the observed birth defect rate is statistically compatible not only with the rate of the reference cohort (9.7%), but also with a rate that is 3-times higher, which should be interpreted with caution. One should also bear in mind that children born following assisted reproduction technology stay more often in neonatal care units compared with naturally conceived children and are checked very carefully, which might lead to overestimation of anomalies that create an observational bias.

For the assessment of neurodevelopmental health, only children aged 3 years or older were included. It is known that tests of cognitive ability performed on very young children (≤2 years) are not necessarily reliable predictors of long-term intelligence (Bowen et al., 1998; Leslie et al.,

2003). In several studies, no delay of cognitive development was found in ICSI children compared with IVF or naturally conceived controls (Cederblad et al., 1996; Leunens et al., 2008; Place and Englert, 2003; Ponjaert-Kristoffersen et al., 2005). However, one study suggested that IQ scores were significantly lower in ICSI children (Knoester et al., 2008). The latter could not be confirmed by the present study with normal IQ scores. One girl, who scored rather low for VIQ (<85), was known with distinct shyness, especially in new situations, which could have led to a false low score on VIQ. No gross problems were known for this girl at school or in relationship with classroom peers. One boy also scored relatively low for PIQ (<85). This boy was at the lower age limit to perform this test and since this test was performed at the end of the day, tiredness could have been the cause for the lower PIQ score (Murphy and Davidshofer, 1998). Both children had normal FSIQ scores.

At the group level, the mean scores for language development fell within the normal ranges. The younger children tested by the RTOS scored significantly higher on language development compared with the older group tested by the CELF-IV-NL. This difference might be attributed to the fact that the majority of the children in the older group were twins and several reports claim that twins on average score lower on language development tests compared with singletons (Rutter et al., 2003b; Thorpe, 2006).

It has been shown that motor skill development in ICSI children can lag behind (Knoester et al., 2007a). Although the overall results of the present cohort was reassuring, the twins tended to lag behind compared with the included singletons, although the difference was not significant. This might be explained by the significantly lower gestational age at birth for the twins compared with the singletons.

Extensive research regarding the influence of IVF and ICSI on children behaviour and the incidence of attention-deficit hyperactivity disorder and disorders in the autism spectrum (ASD) has been performed. It was shown that the prevalence of ASD was higher among ICSI children (3.4%) compared with the general population (0.6–0.7%) (Knoester et al., 2007b). Older paternal as well as maternal age are considered to be risk factors for ASD (Hultman et al., 2011; Sandin et al., 2012). On the contrary, another group could not confirm a relationship between IVF/ICSI and ASD when results were adjusted according to maternal age and educational level, parity, smoking, birthweight and multiplicity (Hvidtjørn et al., 2011). In this study, the SCQ questionnaire was used to assess the risk for ASD. The mean score for the AOA children fell within the normal range. Nevertheless, taking into account the research of Snow and Lecavalier (2008), one younger child (aged 37 months) scored just above the cut-off value. The latter researchers decreased the cut-off score from 15 to 13 for younger children. Importantly, one should keep in mind that a single questionnaire is not sufficient to diagnose ASD. No significant correlations were found in this study between the SCQ score and paternal and maternal age at birth, APGAR score at 5 min or birthweight. According to the CBCL questionnaire, this group of AOA children did not show any maladaptive behaviour. However, one girl scored above the norm for internalizing behaviour. This could be explained by the fact that ICSI girls tend to score higher on the externalizing and internalizing broadband as well as on the total CBCL score in comparison with IVF girls (Knoester et al., 2007b). Nevertheless, this girl's CBCL score was not confirmed by her teacher, since her TRF score was normal.

This cohort consists of almost 50% twins, which is essentially higher compared with the overall twinning rate following assisted reproduction technology, which was 11.2% in Belgium in 2010 (Belrap, 2012). Since 1 July 2003, assisted reproduction centres in Belgium are legally obliged to follow a strict regulation regarding embryo transfer. In women aged <36 years, only one embryo can be transferred in a first treatment cycle. From the second cycle on, two embryos might be transferred if only moderate embryos are available for transfer. From the third cycle onwards, a maximum of two embryos may be transferred. Since most of the couples undergoing AOA carry a history of several treatment cycles, most of these couples legally applied for the transfer of two embryos, which led to the high twinning rate in the current cohort of children. Since the high success rates of both the study centre's AOA procedure and the current cryopreservation techniques, the centre now encourages the transfer of only one embryo in AOA couples. Consequently, the twinning rate in this AOA population has gone down significantly in the last 5 years.

Four singletons of this cohort are siblings, which is one-third of the singleton cohort. Kramer and Conger (2009) pointed out the important role of brothers and sisters in a child's development. Sibling relations provide an important context for the development of children's understanding of their social, emotional, moral and cognitive worlds. This should be kept in mind when interpreting the results of the current study.

The strengths of this case series are both the high response rate and the use of a range of different validated age-adjusted tests and questionnaires. Although AOA is rarely necessary in modern assisted reproduction, its possible impact on offspring development should not be neglected. However, this follow-up study is also characterized by certain limitations that must be taken into consideration. First of all, since AOA is a rarely used technique, the absolute number of included children is very low which severely limits conclusion making. Also, the fact that all tests were pooled on one day could have influenced the results, since some children showed fatigue and a lack of interest during the course of the day. Unfortunately, due to practical considerations (mostly related to the parents' professional activities), all tests had to be performed on one day. Another concern which needs to be addressed is the fact that two-thirds of the parents of the children that were followed up by assessment had a higher level of education (beyond 18 years of age). Parental education, together with family income, occupational status and place of residence describes the socioeconomic status (SES) of an individual. The influence of SES on the development of children born at term is well established (Ardila et al., 2005). Research shows that SES is associated with a wide range of health, cognitive and socioemotional outcomes in children, with effects beginning prior to birth and continuing into adulthood (Bradley and Corwyn, 2002; Turkheimer et al., 2003). As a matter of fact, parental education is a major component of the SES variables. It is generally accepted that IVF/ICSI children comprises a selected group of children, born to parents with on average higher educa-

tional and socioeconomic levels. Therefore, appropriate reference values for IQ and percentile scores may be higher, so that the apparent lack of departure from the norm, or advantage over it, may be less reassuring than it looks. Finally, this analysis did not include a group of control children, since for all used tests and questionnaires, validated norm values were available. Individual performance was compared with established norms for Dutch-speaking children. Also, since the current cohort of AOA children is very small due to the fact that AOA is a rarely required technique, the prior aim was to describe the neurodevelopmental performance of those children specifically. In future, the optimal design would be a multicentre-based child age-matched control study.

The overall results of this case series of children born following ICSI combined with AOA showed that neonatal, developmental as well as behavioural outcome is reassuring, although the results are still to be considered preliminary. No serious adverse effects were observed; however, the size of this study is too small to reach a definite conclusion. Therefore, AOA should still not be considered routine practice yet. The sample size of this cohort was too small to detect chromosomal aberrations and malformations following AOA.

Couples undergoing AOA (or any other assisted reproduction procedure) should be counselled in advance regarding increased risks of pregnancy complications and higher rates of obstetric interventions. Although conflicting data exist, studies of children born following IVF and ICSI suggest increased rates of congenital malformations, imprinting disorders and a marginally increased risk of cancer (Basatemur and Sutcliffe, 2008). However, the current study and evidence is inadequate to draw definite conclusions about long-term outcomes after assisted reproduction technology and AOA specifically. Prospective multicentre long-term studies are needed to eliminate the effect of confounders. The long-term outcomes after AOA are difficult to evaluate because of the variability in AOA methods and data reporting. Therefore, clinicians should aim for standardization of the AOA procedure as well as the methodology for follow up of children born following AOA. ICSI combined with AOA should still be performed only in cases where a clear indication is present.

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References

Aarabi, M., Yu, Y., Xu, W., Tse, M.Y., Pang, S.C., Yi, Y.J., Sutovsky, P., Oko, R., 2012. The testicular and epididymal expression profile of PLCζ in mouse and human does not support its role as a sperm-borne oocyte activating factor. PLoS One 7, e33496.

Achenbach, T.M., Rescorla, L.A., 2001. Manual for the ASEBA School-age Forms and Profiles. Research Centre for Children, Youth and Families. University of Vermont, Burlington.

Altman, D.S., Machin, D., Bryant, T., Gardner, S., 2008. Statistics with Confidence: Confidence Intervals and Statistical Guidelines. John Wiley and Sons, Bristol, UK.

Ardila, A., Rosselli, M., Matute, E., Guajardo, S., 2005. The influence of the parents' educational level on the development of executive functions 1. Dev. Neuropsychol. 28, 539—560.

Baltaci, V., Ayvaz, O.U., Unsal, E., Aktas, Y., Baltaci, A., Turhan, F., Ozcan, S., Sönmezer, M., 2010. The effectiveness of intracytoplasmic sperm injection combined with piezoelectric stimulation in infertile couples with total fertilization failure. Fertil. Steril. 94, 900—904.

Basatemur, E., Sutcliffe, A., 2008. Follow-up of children born after ART. Placenta 29, S135—S140.

Belrap, 2012. Report 2010 of the College of Physicians in Reproductive Medicine. Available from: <http://www.belrap.be/Documents/Reports/Global/Report_IVF10_14DEC12.pdf>.

Bowen, J., Gibson, F., Leslie, G., Arnold, J., Ma, P., Starte, D., 1998. Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. Lancet 351, 1529—1534.

Bradley, R.H., Corwyn, R.F., 2002. Socioeconomic status and child development. Annu. Rev. Psychol. 53, 371—399.

Braet, C., Callens, J., Schittekatte, M., Soyez, V., Druart, C., Roeyers, H., 2011. Assessing emotional and behavioural problems with the child behaviour checklist: exploring the relevance of adjusting the norms for the Flemisch community. Psychol. Belg. 51, 213—235.

Cederblad, M., Friberg, B., Ploman, F., Sjöberg, N.O., Stjernqvist, K., Zackrisson, E., 1996. Intelligence and behaviour in children born after in-vitro fertilization treatment. Hum. Reprod. 11, 2052—2057.

Davies, M.J., Moore, V.M., Willson, K.J., Van Essen, P., Priest, K., Scott, H., Haan, E.A., Chan, A., 2012. Reproductive technologies and the risk of birth defects. N. Engl. J. Med. 366, 1803—1813.

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R.M., Kopf, G.S., Fissore, R., Madoux, S., Ozil, J.P., 2002. Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. Dev. Biol. 250, 280—291.

Esfandiari, N., Javed, M.H., Gotlieb, L., Casper, R.F., 2005. Complete failed fertilization after intracytoplasmic sperm injection—analysis of 10 years' data. Int. J. Fertil. Womens Med. 50, 187—192.

Flaherty, S.P., Payne, D., Swann, N.J., Mattews, C.D., 1995. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. Hum. Reprod. 10, 2623—2629.

Flaherty, S.P., Payne, D., Matthews, C.D., 1998. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. Hum. Reprod. 13, 155—164.

Harper, J., Magli, M.C., Lundin, K., Barratt, C.L.R., Brison, D., 2012. When and how should new technology be introduced into the IVF laboratory? Hum. Reprod. 27, 303—313.

Heindryckx, B., Van der Elst, J., De Sutter, P., Dhont, M., 2005. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. Hum. Reprod. 20, 2237—2241.

Heindryckx, B., De Gheselle, S., Gerris, J., Dhont, M., De Sutter, P., 2008. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. Reprod. Biomed. Online 17, 662—668.

Henderson, S.E., Sugden, D.A., Barnet, A.L., 2010. Movement Assessment Battery for Children-II-NL. The Psychological Corporation, London.

Hendriksen, J., Hurks, P., 2009. WPSSI-III-NL Wechsler Preschool and Primary Scale of Intelligence, third ed. Pearson Assessment and Information B.V., Amsterdam.

Heytens, E., Soleimani, R., Lierman, S., De Meester, S., Gerris, J., Dhont, M., Van der Elst, J., De Sutter, P., 2008. Effect of

ionomycin on oocyte activation and embryo development in mouse. Reprod. Biomed. Online 17, 764—771.
Hultman, C.M., Sandin, S., Levine, S.Z., Lichtenstein, P., Reichenberg, A., 2011. Advancing paternal age and risk of autism: new evidence from a population-based study and a meta-analysis of epidemiological studies. Mol. Psychiatry 16, 1203—1212.
Hvidtjørn, D., Grove, J., Schendel, D., Schieve, L.A., Sværke, C., Ernst, E., Thorsen, P., 2011. Risk of autism spectrum disorders in children born after assisted conception: a population-based follow-up study. J. Epidemiol. Commun. Health 65, 497—502.
Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J., Coward, K., 2010. Oocyte activation, phospholipase C zeta and human infertility. Hum. Reprod. Update 16, 690—703.
Knoester, M., Vandenbroucke, J.P., Helmerhorst, F.M., van der Westerlaken, L.A., Walther, F.J., Veen, S., 2007a. Matched follow-up study of 5—8 year old ICSI-singletons: comparison of their neuromotor development to IVF and naturally conceived singletons. Hum. Reprod. 22, 1638—1646.
Knoester, M., Helmerhorst, F.M., van der Westerlaken, L.A., Walther, F.J., Veen, S., 2007b. Matched follow-up study of 5—8 year old ICSI singletons: child behaviour, parenting stress and child (health-related) quality of life. Hum. Reprod. 22, 3098—3107.
Knoester, M., Helmerhorst, F.M., Vandenbroucke, J.P., van der Westerlaken, L.A., Walther, F.J., Veen, S., 2008. Cognitive development of singletons born after intracytoplasmic sperm injection compared with in vitro fertilization and natural conception. Fertil. Steril. 90, 289—296.
Kort, W., Schittekatte, M., Compaan, E., 2008. Clinical Evaluation of Language Fundamentals, fourth ed. Pearson assessment and information B.V., Amsterdam.
Kort, W., Schittekatte, M., Bosmans, M., Compaan, E.L., Dekker, P.H., Vermeir, G., Verhaeghe, P., 2005. WISC-III-NL, Wechsler Intelligence Scale for Children. Handleiding en verantwoording, third ed. Harcourt Test Publishers, Amsterdam.
Kramer, L., Conger, K.J., 2009. What we learn from our sisters and brothers: for better or for worse. New Dir. Child Adolesc. Dev. 126, 1—12.
Kyono, K., Kumagai, S., Nishinaka, C., Nakajo, Y., Uto, H., Toya, M., Sugawara, J., Araki, Y., 2008. Birth and follow-up of babies born following ICSI using $SrCl_2$ oocyte activation. Reprod. Biomed. Online 17, 53—58.
Leslie, G.I., Gibson, F.L., McMahon, C., Cohen, J., Saunders, D.M., Tennant, C., 2003. Children conceived using ICSI do not have an increased risk of delayed mental development at 5 years of age. Hum. Reprod. 18, 2067—2072.
Leunens, L., Celestin-Westreich, S., Bonduelle, M., Liebaers, I., Ponjaert-Kristoffersen, I., 2008. Follow-up of cognitive and motor development of 10-year-old singleton children born after ICSI compared with spontaneously conceived children. Hum. Reprod. 23, 105—111.
Liu, J., Nagy, Z., Joris, H., Tournaye, H., Smitz, J., Camus, M., Devroey, P., Van Steirteghem, A., 1995. Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. Hum. Reprod. 10, 2630—2636.
Montag, M., Köster, M., van der Ven, K., Bohlen, U., van der Ven, H., 2012. The benefit of artificial oocyte activation is dependent on the fertilization rate in a previous treatment cycle. Reprod. Biomed. Online 24, 521—526.
Murphy, K.R., Davidshofer, C.O., 1998. Psychological Testing. Prentice Hall, New Jersey.
Palermo, G.D., Neri, Q.V., Takeuchi, T., Rosenwaks, Z., 2009. ICSI: where we have been and where we are going. Semin. Reprod. Med. 27, 191—201.
Parrington, J., Davis, L.C., Galione, A., Wessel, G., 2007. Flipping the switch: how a sperm activates the egg at fertilization. Dev. Dyn. 236, 2027—2038.
Place, I., Englert, Y., 2003. A prospective longitudinal study of the physical, psychomotor, and intellectual development of singleton children up to 5 years who were conceived by intracytoplasmic sperm injection compared with children conceived spontaneously and by in vitro fertilization. Fertil. Steril. 80, 1388—1397.
Ponjaert-Kristoffersen, I., Bonduelle, M., Barnes, J., Nekkebroeck, J., Loft, A., Wennerholm, U.B., Tarlatzis, B.C., Peters, C., Hagberg, B.S., Berner, A., Sutcliffe, A.G., 2005. International collaborative study of intracytoplasmic sperm injection-conceived, in vitro fertilization-conceived, and naturally conceived 5-year-old child outcomes: cognitive and motor assessments. Pediatrics 115, e283—e289.
Rawe, V.Y., Olmedo, S.B., Nodar, F.N., Doncel, G.D., Acosta, A.A., Vitullo, A.D., 2000. Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. Mol. Hum. Reprod. 6, 510—516.
Rutter, M., Bailey, A., Lord, C., 2003a. Social Communication Questionnaire (SCQ). Western Psychological Services, Los Angeles.
Rutter, M., Thorpe, K., Greenwood, R., Northstone, K., Golding, J., 2003b. Twins as a natural experiment to study the causes of mild language delay: I: design; twin-singleton differences in language, and obstetric risks. J. Child Psychol. Psychiatry 44, 326—341.
Rybouchkin, A., Dozortsev, D., Pelinck, M.J., De Sutter, P., Dhont, M., 1996. Analysis of the oocyte activation capacity and chromosomal complement of roundheaded human spermatozoa by their injection into mouse oocytes. Human. Reprod. 11, 2170—2175.
Rybouchkin, A., Van der Straeten, F., Quatacker, J., De Sutter, P., Dhont, M., 1997. Fertilization and pregnancy after assisted oocyte activation and intra-cytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil. Steril. 68, 1144—1147.
Sandin, S., Hultman, C.M., Kolevzon, A., Gross, R., Maccabe, J.H., Reichenberg, A., 2012. Advancing maternal age is associated with increasing risk for autism: a review and meta-analysis. J. Am. Acad. Child Adolesc. Psychiatry 51, e1.
Sato, Y., Nakamura, Y., Sakamoto, E., Tasaka, A., Usui, K., Hattori, H., Ito, Y., Nakajo, Y., Doshida, M., Kyono, K., 2011. Follow up of children following new technology: TESE, IVM, and oocyte activation. Hum. Reprod. 26, I274.
Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development 129, 3533—3544.
Schaerlaekens, A., Zink, I., Van Ommeslaeghe, K., 2003. Reynell Taalontwikkelingsschalen RTOS: Handleiding. Swets and Zeitlinger Publishers, Lisse.
Snow, A.V., Lecavalier, L., 2008. Sensitivity and specificity of the Modified Checklist for Autism in Toddlers and the Social Communication Questionnaire in preschoolers suspected of having pervasive developmental disorders. Autism 12, 627—644.
Takisawa, T., Doshida, M., Hattori, H., Nakamura, Y., Kyoya, T., Shibuya, Y., Nakajo, Y., Tasaka, A., Toya, M., Kyono, K., 2011a. Effect of oocyte activation by calcium ionophore A23187 or strontium chloride in patients with low fertilization rates and follow-up of babies. Hum. Reprod. 26, I178—I179.
Takisawa, T., Sato, Y., Tasaka, A., Ito, Y., Nakamura, Y., Hattori, H., 2011b. Effect of oocyte activation by calcium ionophore A23187 or strontium chloride in patients with low fertilization rates and follow-up of babies. Fertil. Steril. 96, S162.
Thorpe, K., 2006. Twin children's language development. Early Hum. Dev. 82, 387—395.
Turkheimer, E., Haley, A., Waldron, M., D'Onofrio, B., Gottesman, I.I., 2003. Socioeconomic status modifies heritability of IQ in young children. Psychol. Sci. 14, 623—628.
Vanden Meerschaut, F., Nikiforaki, D., De Gheselle, S., Dullaerts, V., Van den Abbeel, E., Gerris, J., Heindryckx, B., De Sutter, P.,

2012. Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. Hum. Reprod. 27, 1977—1984.

Vanden Meerschaut, F., Leybaert, L., Nikiforaki, D., Qian, C., Heindryckx, B., De Sutter, P., 2013. Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. Hum. Reprod. 28, 87—98.

Warreyn, P., Raymaekers, R., Roeyers, H., 2004. Handleiding vragenlijst sociale communicatie. SIG VZW, Destelbergen.

Wilson, B.N., Kaplan, B.J., Crawford, S.G., Campbell, A., Dewey, D., 2000. Reliability and validity of a parent questionnaire on childhood motor skills. Am. J. Occup. Ther. 54, 484—493.

Yanagida, K., Morozumi, K., Katayose, H., Hayashi, S., Sato, A., 2006. Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization. Reprod. Biomed. Online 13, 801—806.

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ASSISTED REPRODUCTION TECHNOLOGIES

Assessing the impact of calcium ionophore on pregnancy outcomes in artificial oocyte activation cycles: a 10-year update of systematic review and meta-analysis

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Abstract

Purpose The objective is to evaluate the effectiveness and safety of calcium ionophore as an artificial oocyte activation (AOA) method on pregnancy outcomes in different groups of intracytoplasmic sperm injection (ICSI) patients, providing potential evidence to establish consensus on the indications of AOA.

Methods A systematic comprehensive search was performed in Medline, Embase, the Cochrane Library, and Google Scholar databases. Studies published from January 2014 to June 2024 were searched for analysis. All studies that compared ICSI with AOA-ICSI in routine indications composing impaired fertilization or embryo developmental arrest in previous cycles, or male-factor infertility were included.

Results Twelve studies were included in the meta-analysis. AOA-ICSI was associated with the increase in the overall fertilization rate (OR 1.99, 95% CI 1.16–3.41) and live birth rate (OR 4.58, 95% CI 1.52–13.80). All secondary outcomes including cleavage, blastocyst, high-quality embryo, implantation, biochemical pregnancy, clinical pregnancy presented superiority or equivalence in AOA-ICSI. And the use of calcium ionophore did not increase the miscarriage rate (OR 0.43, 95% CI 0.08–2.43). In subgroup analysis, AOA-ICSI exhibited a more significant effect on patients with indications of no or low fertilization. However, in patients with non-fertilization factors, no statistically significant improvements were observed in all outcomes.

Conclusion Calcium ionophore is an effective artificial oocyte activation approach to improving pregnancy outcomes after ICSI, particularly in cases with indications of fertilization factors, providing further support for the application of AOA in specific populations. Further validation is needed to comprehensively establish the safety of AOA.

Trial registration PROSPERO registration number CRD42024551481.

Keywords Intracytoplasmic sperm injection · Artificial oocyte activation · Calcium ionophore · Live birth

Introduction

Intracytoplasmic sperm injection (ICSI) has emerged as a significant approach in the field of assisted reproduction with reported fertilization rates ranging from 70 to 80% [1]. It involves the direct injection of single sperm into the oocyte cytoplasm and was initially utilized to treat infertility with severe male factor and other factors after developed in 1992 [2]. Although ICSI can solve most of the non-fertilization problems in previous cycles, there are still cases suffering from recurrent ICSI failure for various reasons [3]. Failed oocyte activation is considered to be the major cause of fertilization failure in ICSI, accounting for approximately 70% of fertilization failure after conventional ICSI [4, 5].

Phospholipase C zeta (PLCζ) is the dominant sperm-specific factor responsible for triggering oocyte activation in mammals. Oocyte activation deficiency(OAD) has been linked to numerous PLCζ abnormalities in patients experiencing failed ICSI. During normal fertilization, sperm-specific PLCζ is released into the oocyte when the sperm enters the oocyte [6]. PLCζ triggers calcium shock and promotes the hydrolyzation of phosphatidylinositol biphosphate (PIP_2) into inositol trisphosphate (IP_3). IP_3 stimulates the release

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of Ca^{2+} through the IP_3 receptors that are located in the endoplasmic reticulum of the oocyte [7–9]. The temporary increase of Ca^{2+} concentration in oocytes generates Ca^{2+} oscillations to activate several oocyte kinases and induces events including cortical granular response and oocyte maturation [10], leading to successful oocyte activation and the onset of embryogenesis [11]. The specific pattern of calcium oscillation during the activation period is thought to have long-term effects on subsequent pre- and post-implantation events [12]. Insufficient or abnormal Ca^{2+} release could cause low fertilization or total fertilization failure after ICSI [13, 14].

AOA, modified techniques mainly aiming to initiate artificial Ca^{2+} rises, has been applied employing chemical, mechanical or electrical stimulation [15–17]. Chemical agents based on calcium or strontium are most widely used, in clinical practice due to their technical simplicity. The effectiveness and mechanism of strontium chloride remain controversial as it cannot induce the characteristic calcium oscillations in human oocytes [18, 19]. Calcium stimulation is considered to be one of the most efficient methods to compensate for physiological calcium release [20], and is expected to cause relatively small damage to oocytes [21, 22]. Therefore, it has been widely used in clinical practice even though its effect has not been fully confirmed [23, 24]. Besides, indications of using AOA are diverse throughout facilities [25] and patients are commonly treated without a clear consensus of indications. AOA is not only used in fertilization failure cases owing to impaired Ca^{2+} oscillations but also being blindly applied to broader cases of oocyte activation deficiency, meiosis resumption failure, embryo development arrest related to other signaling where its effects are uncertain [26–29]. Additionally, Over the past decade, there has been more high-quality evidence in clinical research that should be considered and an update is urgently needed.

Therefore, we conducted a comprehensive and updated meta-analysis to further elucidate the clinical effectiveness of AOA in improving pregnancy outcomes in every stage. By conducting subgroup analyses based on patient indications, we aim to provide a refined understanding of the potential benefits of AOA in different groups of patients, which may offer new insights for establishing standard therapeutic indications of AOA.

Materials and methods

Search strategy

This systematic review was reported according to the PRISMA 2020 guidelines [30]. The study protocol was registered in PROSPERO (registration number CRD42024551481). The literature search was performed in Medline, Embase, the Cochrane Library, and Google Scholar databases for studies published from January 2014 to June 2024 (extended by 6 months compared to the registered protocol). The search terms applied for the title search was (Intracytoplasmic Sperm Injection OR ICSI OR in vitro fertilization OR IVF OR infertility OR sterility) AND ((oocyte AND activ*) OR (Ca^{2+} OR calcium OR calcimycin)). Search terms were adjusted for each database if necessary. The language of publication was restricted to English. The references of included studies were also screened for additional eligible articles.

Study selection and eligibility criteria

After duplicates had been removed, articles were screened by titles, abstracts, and full texts. Studies were independently selected by two reviewers (JZ and JF), and differences between reviewers were reconciled with a third reviewer (XS). Inclusion criteria for studies were as follows: (i) All studies that focus on the effectiveness of AOA and compared ICSI with AOA-ICSI in routine indications composing impaired fertilization or embryo developmental arrest in previous cycles, or male-factor infertility; (ii) reported at least one of the primary outcomes (fertilization rate or pregnancy rate) and listed detailed number of oocytes in each stage; (iii) randomized controlled and non-randomized controlled trials; (iv) using calcium ionophores (A23187 or ionomycin) as oocyte activation method. Exclusion criteria were: (i) studies that used AOA as rescue; (ii) based on species other than human; (iii) focused on patients with specific conditions, for example, advanced age, PCOS, or certain forms of teratozoospermia.

Data extraction and bias assessment

Data extraction and risk of bias assessment were performed independently by two reviewers (JZ and JF). Disagreements were resolved by consultation with a third reviewer (XS). Study information consisting of the first author, year of publication, country, study period and study design, and patient characteristics including mean age, type of infertility, sperm source, and embryo type were extracted. Fertility-related outcomes including primary outcomes and secondary outcomes were also extracted. Data that did not available was denoted as NA. The primary outcomes for effectiveness were fertilization rate and live birth rate. The fertilization rate is reported as the number of fertilized 2 pronuclei (2PN) oocytes per mature oocyte. The live birth rate is defined as the number of live births per transfer cycle. The secondary outcomes included cleavage, blastocyst, high-quality embryo, implantation, biochemical pregnancy, clinical pregnancy, and miscarriage rate. The cleavage, blastocyst, and high-quality embryo rates are calculated by dividing

the number of oocytes by the number of fertilized oocytes. Implantation, biochemical pregnancy, clinical pregnancy, and live birth rates are measured based on the transfer cycles. The miscarriage rate is defined as the number of miscarriages per number of clinical pregnancies.

The quality of randomized controlled trials was assessed by risk of bias tool 2.0 (RoB 2.0) provided by the Cochrane library [31]. Non-randomized studies were assessed by risk of bias in non-randomized studies of Interventions tool (ROBINS-I) [32]. Publication bias was assessed by funnel plot.

Statistical analysis

Analysis was conducted using the statistical software Review Manager Version 5.0. The effects of AOA-ICSI compared to ICSI were measured using odds ratios with 95% confidence intervals (CI). We assessed heterogeneity using I^2 along with a visual inspection of forest plots. Studies showing tolerable levels of heterogeneity (I^2 values < 30%) were analyzed using a fixed-effect model, and studies showing greater degree of heterogeneity were analyzed using a random-effect model. Subgroup analyses were performed based on different indications. Fertilization factors subgroup include ICSI failures and the use of AOA on account of impaired fertilization, including no or low fertilization rates (< 50%). Non-fertilization factors subgroups include those have normal fertilization rate but have embryo developmental arrest in previous cycles or male-factor infertility. Sensitivity analysis was conducted by removing each article one at a time from the overall effect estimates with high heterogeneity.

Results

Study selection

The electronic and manual search yielded a total of 123 records in the period of the last decade. Full texts of 16 studies were further reviewed for eligibility. Two studies were excluded because their indicators were compared on a cycle basis [33, 34]. Two studies were excluded because of no detailed oocyte number available [35, 36]. Twelve studies met the inclusion criteria and were included in this meta-analysis [15, 24, 26, 27, 37–44]. Figure 1 illustrates the breakdown of the selected studies according to type of study.

Study characteristics

Patient characteristics and study information were extracted and listed in Table 1. Seven studies evaluated the use of AOA in patients with fertilization issues following conventional ICSI. Two studies focused on AOA after ICSI failure resulting from total fertilization failure or a low fertilization rate [43, 44]. Four studies evaluated AOA in patients with no or low fertilization rate or teratozoospermia [15, 38, 39, 41]. One study evaluated AOA in patients with no or low fertilization rate or embryo development deficiency [24]. The remaining five studies assessed AOA for entirely non-fertilization factors. Four studies evaluated AOA solely for cases of embryo development deficiency [26, 27, 37, 40], while one study exclusively considered AOA for male factors [42].

Primary outcomes

For the primary outcomes, AOA was associated with the statistically significant increase of fertilization rate (OR 1.99, 95% CI 1.16–3.41, Fig. 2) and live birth rate (OR 4.58, 95% CI 1.52–13.80, Fig. 3) in ICSI failure patient with all reasons.

Subgroup analysis of primary outcomes

In subgroup analysis, AOA exhibited a significant improve on patients with indications of no or low fertilization in both primary outcomes with fertilization rate (OR 3.19, 95% CI 1.62–6.28, Fig. 4) and live birth rate (OR 11.15, 95% CI 2.96–41.97, Fig. 5). By contrast, in the non-fertilization factors group, no statistical significance was observed in both primary outcomes with including fertilization rate (OR 0.97, 95% CI 0.71–1.33, Fig. 4) and live birth rate (OR 2.17, 95% CI 0.68–6.97, Fig. 5). No enough data was retrieved for subgroup analysis on the age of the female partner, source of sperm, type of embryo, or protocol of AOA. Detailed data for primary outcomes were shown in *Supplemental* Table 1.

Secondary outcomes

Regarding the secondary outcomes, the improvement was not statistically significant in cleavage rate (OR 1.23, 95% CI 0.70–2.15), blastocyst rate (OR 2.08, 95% CI 0.98–4.39), high-quality embryo rate (OR 1.21, 95% CI 0.91–1.61), and implantation rate (OR 2.51, 95% CI 0.91–6.88) (Fig. 6). The results showed AOA-ICSI significantly improved the secondary outcomes including biochemical pregnancy (OR 4.38, 95% CI 1.97–9.75) and clinical pregnancy (OR 2.94, 95% CI 1.38–6.23) (Fig. 7). In the subgroup analysis, AOA exhibited a positive effect on patients with indications of no or low fertilization at stages such as blastocyst rate (OR 1.35, 95% CI 1.04–1.75), biochemical pregnancy rate (OR 8.43, 95% CI 4.41–16.11), and clinical pregnancy rate (OR 3.75, 95% CI 1.63–8.65). By contrast, in the non-fertilization factors group, no statistical significance was observed in most secondary outcomes including cleavage rate

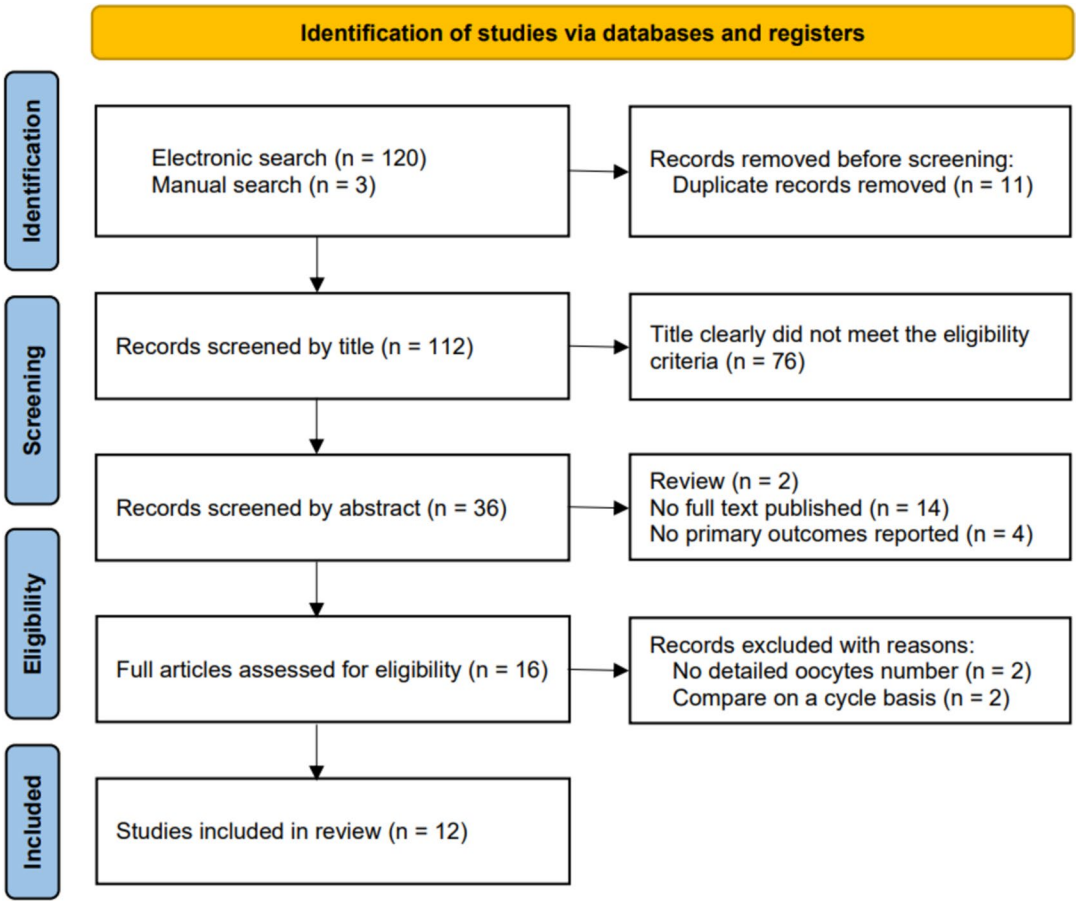


Fig. 1 PRISMA 2020 flow diagram of studies included in the meta-analysis

(OR 0.96, 95% CI 0.28–3.35), blastocyst rate (OR 4.80, 95% CI 0.52–44.68), high-quality embryo rate (OR 1.07, 95% CI 0.65–1.77), implantation rate (OR 2.26, 95% CI 0.43–11.91), biochemical pregnancy rate (OR 2.78, 95% CI 0.95–8.15), and clinical pregnancy rate (OR 2.39, 95% CI 0.76–7.47) (Supplemental Figs. 1–6).

The overall results of each stage were summarized in Table 2. AOA was associated with the statistically significant increase of fertilization rate and live birth rate in ICSI failure patient with all reasons. All secondary outcomes presented superiority or equivalence in the AOA group. The subgroup analysis results of each stage were summarized in Table 3. AOA exhibited a positive effect on patients with indications of no or low fertilization, presenting the same trends but more significant benefits compared to the results in the overall analysis. On the contrary, no statistical significance was observed in both primary outcomes and all secondary outcomes in the non-fertilization factors group. Detailed data for secondary outcomes were shown in *Supplemental* Table 1.

Safety

Limited data are available regarding the association between AOA and other potential adverse outcomes. Our meta-analysis showed a result that the use of calcium ionophore did not increase the possibility of miscarriage rate (OR 0.43, 95% CI 0.08–2.43, Fig. 8). For other adverse outcomes, one study showed no significant difference in rates of oocyte degeneration, miscarriage, preterm delivery, and fatal congenital anomalies between AOA and non-AOA groups [43]. One study analyzed the gestational age and birth weight of infants between the ICSI and AOA-ICSI groups and found no significant difference [42]. One study reported no congenital abnormality in the ten live birth events [41]. One study reported no major birth defects among the newborn babies in both AOA and non-AOA groups [39]. One study showed that there was no significant difference in one early pregnancy loss between the control group and the AOA group [40].

Table 1 Characteristics of included studies

Author, year	Country	Study period	Study type	AOA protocol	Study group	Mean age	Indications	Sperm source	Embryo type
[43]	Japan	January 2015 to December 2019	Large multi-center retrospective study	Ca2 + ionophores (A23187 or ionomycin) within 30 min after ICSI	AOA: 722 MII oocytes sibling oocyte control: 1036 MII oocytes	AOA: 37.7 ± 3.7 control:36.7 ± 3.9 only younger group (≤ 35 years) was included in the meta-analysis	ICSI cycles with no or low fertilization rate (< 50%) with unknown causes	Ejaculated sperm	Frozen embryo
[38]	Turkey	December 2013 to February 2014	RCT	15 min in the 30 µl of pre-equilibrated drops of medium containing Ca2 + ionophore (GM508 Cult-Active	AOA: 97 MII oocytes randomized control: 97 MII oocytes	30.2	Teratozoospermia (< 4% according to WHO 2010 reference limits) and low fertilization rate (< 50%)	Ejaculated sperm	NA
[27]	Belgium	January 2018 to January 2021	Single-center prospective cohort study	Exposed twice to 10 mmol/l Ca2 + ionophore ionomycin (I9657, Sigma-Aldrich, Belgium) for 10 min with a 30-min time interval	AOA: 190 MII oocytes historical control: 421 MII oocytes	33.8 ± 3.6	High fertilization (≥ 60% 2PN) with a minimum of 10 zygotes but low blastocyst rates on D5 (≤ 15%)	Both ejaculated sperm and testicular sperm	Both frozen embryo and fresh embryo
[44]	Belgium	A from April 2001 to April 2018	Retrospective cohort study	Ionomycin (10 mmol/L, I9657; Sigma-Aldrich) for 10 min with a 30-min interval	AOA: 1752 MII oocytes historical control: 1884 MII oocytes	31.2 years, 33.9 years, and 33.7 years for MOAT groups 1, 2, and 3	TFF (fertilization rate of 0–10%) or LF (fertilization rate of 33.3%, based on the 5th percentile of mean fertilization rates)	Ejaculated sperm	Fresh embryo

Table 1 (continued)

Author, year	Country	Study period	Study type	AOA protocol	Study group	Mean age	Indications	Sperm source	Embryo type
[40]	China	March 2020 to December 2021	Prospective cohort study	A23187 (5 µM, Sigma, St. Louis, MO, USA) for 15 min after 15 min of ICSI	AOA: 642 MII oocytes historical control: 689 MII oocytes	AOA: 34.84 ± 4.92 control: 33.62 ± 5.25	(1) No good quality embryo on day 3 and no good quality blastocyst; (2) at least one good quality embryo on day 3 but no good quality blastocyst; (3) or less than 20% good quality blastocyst formation rate	Most were ejaculated sperm	Both frozen embryo and fresh embryo
[26]	Egypt	June 2012 to September 2013	Case report	200 µL of incubated A23187 Ca2 + ionophore solution for 15 min	AOA: 40 MII oocytes historical control: 25 MII oocytes	27	History of incomplete ICSI cycles owing to 2PN arrest	Ejaculated sperm	NA
[37]	Austria	NA	Multi-center prospective study	Ionophore (GM508 Cult-Active; Gynemed, Lensahn, Germany) for 15 min	AOA: 512 MII oocytes historical control: 489 MII oocytes	32.7 ± 4.5	Either (i) complete developmental arrest (no transfer); (ii) complete developmental delay (no morula/blastocyst on day 5); or (iii) significantly reduced blastocyst formation (≤ 15%) in a previous cycle	NA	Frozen embryo
[15]	Egypt	April 2015 to January 2016	RCT	20 min in a commercial calcimycin (Cult-active Ca-Ionophore; Gynemed, Lensahn, Germany)	AOA: 113 MII oocytes randomized control: 115 MII oocytes	AOA: 32.4 ± 5.2 control: 32 ± 5.2	Two previous ICSI cycles of no or low fertilization (0–30%) or with male-factor infertility	Both ejaculated sperm and testicular sperm	Both frozen embryo and fresh embryo

Table 1 (continued)

Author, year	Country	Study period	Study type	AOA protocol	Study group	Mean age	Indications	Sperm source	Embryo type
[41]	China	2013 to 2016	Retrospective cohort study	GM508 cultactive activation medium (Gynemed) or the homemade A23187 medium (10 mM; Calbiochem, San Diego, CA) for 15 min	AOA: 265 MII oocytes historical control: 188 MII oocytes	AOA: 36.4 ± 3.3 control: 36.1 ± 3.7	Severe male infertility, poor fertilization (< 30%) or total fertilization failure in previous ICSI cycles	NA	NA
[39]	China	October 2015 to December 2017	Prospective cohort study	10 µmol/l ionomycin (I9657; Sigma-Aldrich, St. Louis, MO, USA) dissolved in G-IVF PLUS™, which were then cultured twice for 5 min each, with a 30-min interval	AOA: 262 MII oocytes control: 251 MII oocytes	31.28 ± 3.67	Patients who had experienced fertilization failure (fertilization rate < 20%) in their previous ICSI cycles or were diagnosed with severe teratozoospermia	NA	Both frozen embryo and fresh embryo
[24]	China	January 2019 to December 2020	Retrospective cohort study	10 mmol/l ionomycin (Sigma-Aldrich, USA) for 10 min	AOA: 702 MII oocytes historical control: 608 MII oocytes	33.86 ± 4.981	Previous ICSI fertilization failure (TFF or previous ICSI fertilization failure (TFF or fertilization rate ≤ 30%) and poor embryo development (complete embryo development arrest, reduced blastocyst formation on day 5 (≤ 15%))	NA	Both frozen embryo and fresh embryo

Table 1 (continued)

Author, year	Country	Study period	Study type	AOA protocol	Study group	Mean age	Indications	Sperm source	Embryo type
[42]	China	NA	Retrospective cohort study	10 mM calcium ionophore (A23187) (Sigma, MO, USA) prepared in advance and incubated for 10 min	AOA: 883 MII oocytes control: 2671 MII oocytes	AOA: 29.26 ± 3.78 control: 30.13 ± 2.61	Failed ICSI or sperm malformation	Both ejaculated sperm and testicular sperm	Both frozen embryo and fresh embryo

Sensitivity analysis

No obvious effects in results after removing each individual study one by one in fertilization, cleavage, high-quality embryo, biochemical pregnancy, clinical pregnancy, live birth, and miscarriage rate, indicating low sensitivity and stable results. After removing Ebner et al. [37], the heterogeneity for blastocyst rate decreased significantly, and the OR of blastocyst rate became 1.36 [1.10, 1.66] with a significant effect [37]. After removing Zhang et al. the heterogeneity for implantation rate decreased significantly, and the OR of it became 3.67 [1.52, 8.85] with a significant effect [42]. Both the beneficial impact of AOA on blastocyst rate and implantation rate became more pronounced in the remaining studies after removing the outliers.

Risk of bias

All observational studies ware rated as the low or moderate category according to the ROBINS-I bias risk. Randomized control studies were rated as some concerns or no information according to the RoB2.0 (Fig. 9) [45]. The funnel plot of all studies included showed no clear evidence of publication bias (Fig. 10).

Discussion

Main findings

To assess the effectiveness of calcium ionophore treatment for AOA and establish a standard treatment indication, a comprehensive meta-analysis the control group and the AOA group is conducted. This analysis incorporated data from 12 studies, encompassing a total of 16,804 ICSI oocytes. The results of our analysis revealed notable improvements in the overall fertilization rate per mature oocyte. An increase in pregnancy rate and live birth rate per ET and treatment cycle was also observed when AOA was administered following ICSI treatment.

In order to further refine the indications for AOA, a subgroup analysis was performed. The results of our subgroup analysis demonstrate that the use of AOA can improve outcomes more notably in ICSI cases of no or low fertilization while AOA may not be effective in addressing the failure caused by embryo developmental problems.

Interpretation

In clinical practice, AOA may be used as the final option for couples experiencing fertilization failure. Despite the results of many studies, the effectiveness and safety of AOA have not yet been established. The ultimate goal of therapy

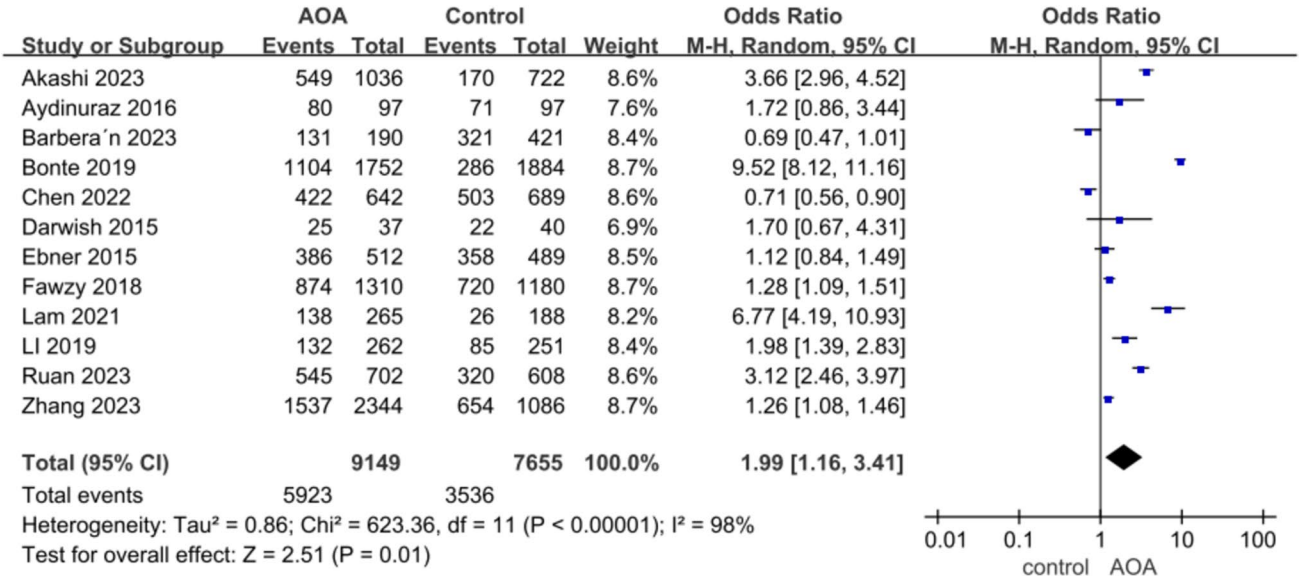


Fig. 2 Forest plot for overall fertilization rate

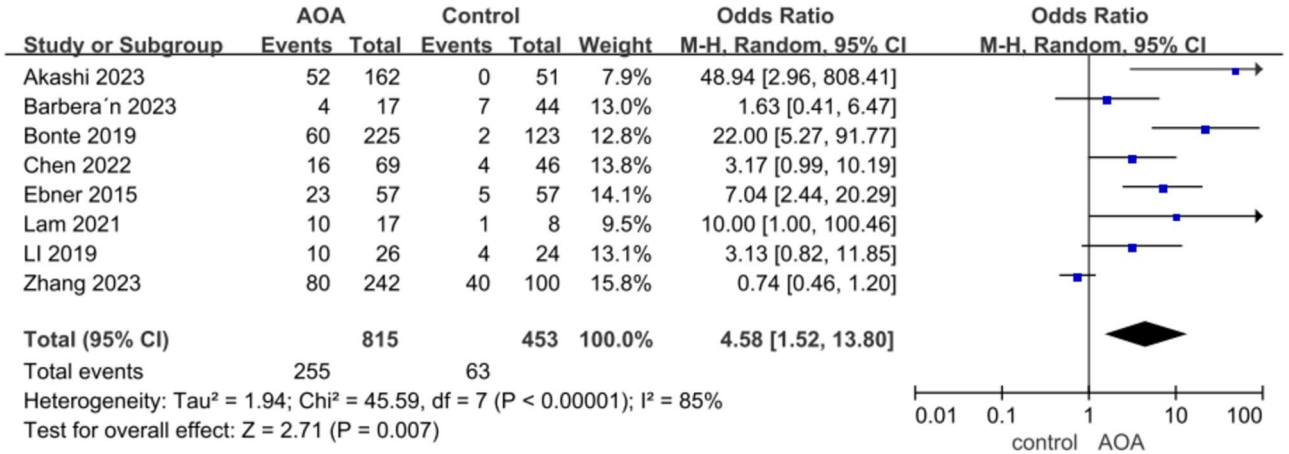


Fig. 3 Forest plot for overall live birth rate

for infertility couples is to improve pregnancy or live birth rates. Our analysis on primary results strongly supported the beneficial of calcium ionophore. AOA therapy can provide infertility couples with more fertilized oocytes and eventually improve their probability to be expecting and owning a child [38]. While some studies have suggested that calcium ions may improve the developmental potential of embryos by regulating the cell cycle in theory [37], our results regarding the stage of cleavage, blastocyst formation, implantation, and high-quality embryo rate do not support this hypothesis. It implied that calcium ionophore can overcome fertilization failures caused by defective Ca^{2+} oscillations but cannot overcome these relevant to oocyte activation and meiosis resumption disorders caused by downstream signaling cascades or defective proteins caused by gene mutations. The lack of positive effects of AOA on embryo development may be also related to the quality or aging of the oocytes. Even though the improvements were not statistically significant in every stage, it is notable that outcomes at each stage following AOA treatment presented superiority or at least equivalence to the control group, indicating that AOA need to be diagnose correctly and cautiously recommended for eligible patients.

In terms of safety, several previous studies have reported that there were no adverse effects of calcium ionophore on in vitro or in vivo embryo development [35, 46–48]. Our meta-analysis showed a result that the use of calcium ionophore did not increase the possibility of miscarriage rate, which is consistent with these previous studies [49, 50]. One study included also conducted a comparison of PGT

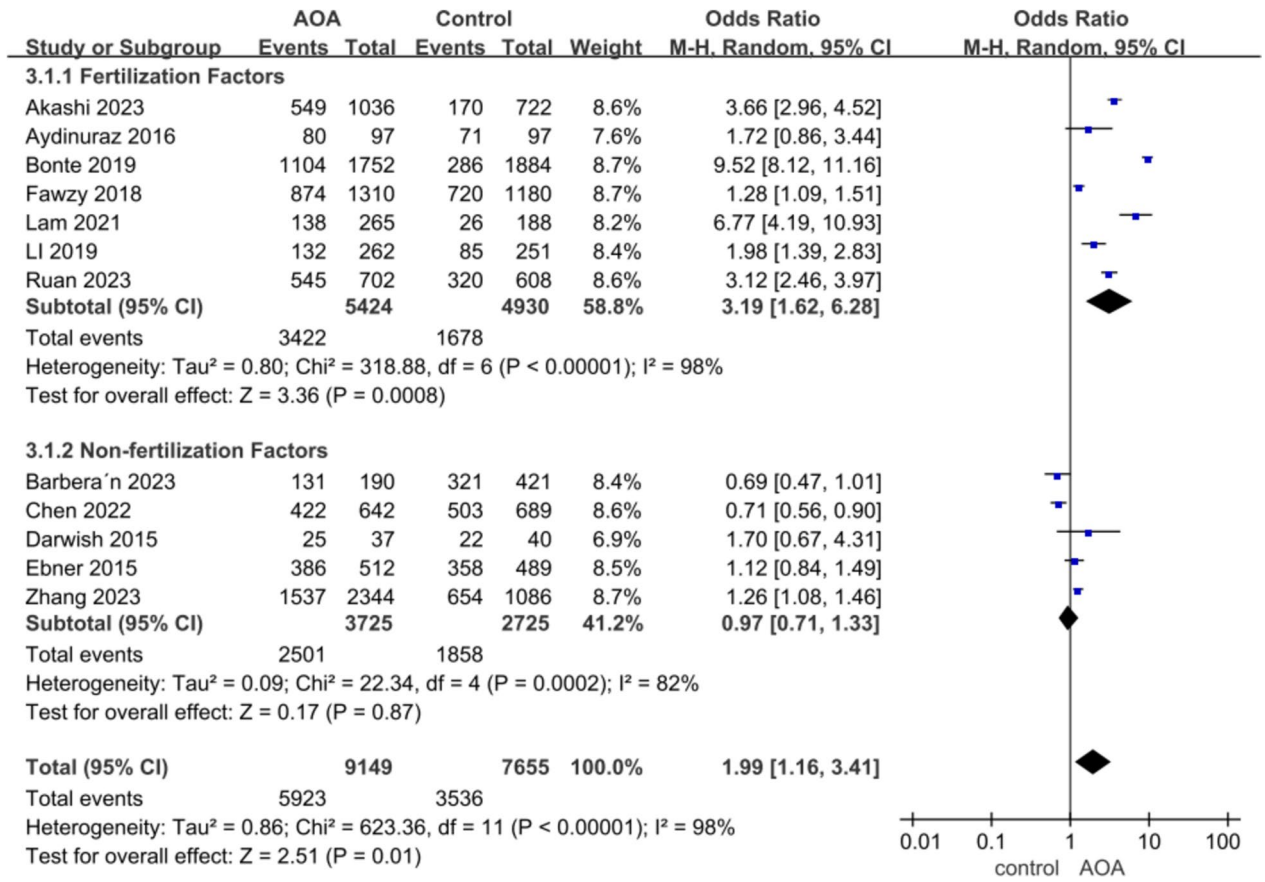


Fig. 4 Forest plot for subgroup analysis of fertilization rate

and AOA-PGT showing that calcium ionophore activation did not affect the proportion of blastocyst aneuploidy, gestational age, and weight at birth [42], which is confirmed by study that indicated the use of high concentrations of A23187 does not increase the risk of chromosome segregation aberrations [51]. However, the evidence for the safety of AOA at the mechanism level is too limited.

The prolonged Ca^{2+} exposure is considered to disrupt the cytoplasmic ion composition and affect cell homeostasis [52]. And non-physiological increases in Ca^{2+} levels might unexpectedly activate proteins in signal transduction pathways, leading to abnormal gene expressions and epigenetic modifications [53]. AOA treatment has been shown to increase the methylation of *DMR2* maternal allele and affect the expression of the imprinted gene *Igf2r*, leading to lower birth weight in mice [54]. AOA is also found to change the imprinting methylation rates at *H19* and *SNRPN* at cleavage stage, inducing epimutations in human embryos [55]. Additionally, the artificial calcium signaling induced by AOA is suspected to have unintended downstream effects. These potential consequences may include an effect on mitochondrial function or other critical developmental pathways. This raises concerns about the long-term impacts of AOA on embryo viability and health [56]. These studies elucidate the mechanisms through which AOA may influence embryo development, but the clinical experience is too limited to conclude whether it will cause clinically significant consequences on embryos and offspring with certainty. More mechanism researched is still urgently needed.

Nevertheless, it has been shown that differences in Ca^{2+} signaling patterns cannot only have effects on implantation and post-implantation development but also influence long-term consequences including birthweight and postnatal growth rate in offspring [56]. Some adverse genetic effects may only appear in the later stages of a child's growth [53]. Only several follow-up studies of children born following calcium ionophores activation have reported reassuring results about neurodevelopmental and language developmental outcomes [49, 58]. Most reports at present support the view that AOA technology will not increase the probability of congenital malformations [35].

There are some studies that showed impaired fertilization rate after AOA due to operation damage or reasons mentioned above [40, 59]. More prospective and mechanism studies are required to confirm the observations and assess the safety of AOA and make aware of the potential risks.

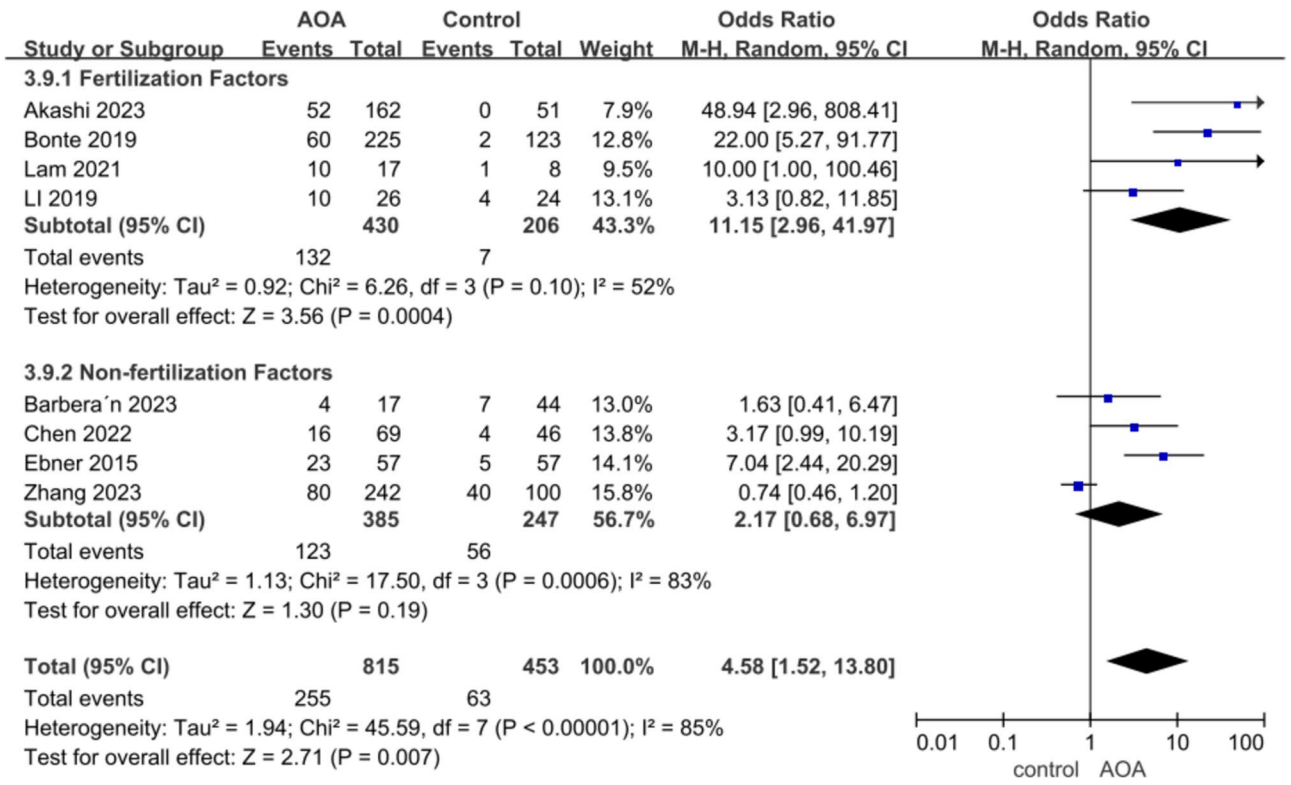


Fig. 5 Forest plot for subgroup analysis of live birth rate

Before making a conclusion, the application of AOA should be strictly limited to patients with appropriate indications, and the health of the children born through this experimental technique should be tracked very closely [56].

Employing AOA as a routine therapy for all ICSI failure patients would be risky given the uncertain benefits and potential safety issues. Therefore, effort should be made to identify the specific patient populations that are most likely to have determinate benefits from AOA and avoid unnecessary exposure for other patients. Currently, there is no authoritative consensus regarding application criteria for AOA treatment for ICSI failure. It has been suggested that AOA may not be universally beneficial for all ICSI patients [60]. Previous studies reported that cases with reasons such as oocyte maturation disorders, severe forms of teratozoospermia, aberrant pronuclear development, and technical problems cannot be improved by AOA [41]. Besides, whether AOA is a possible treatment to overcome embryo developmental problems is still controversial [37].

Our subgroup analysis showed that AOA can improve outcomes more notably in ICSI cases of no or low fertilization while not in the failure caused by embryo developmental problems. In addition, the only study that exclusively included male factor infertility patients (such as sperm malformation) reported a decrease in live birth rates following AOA, suggesting a lack of evidence for this patient population to receive AOA intervention [42]. Studies on sperm malformations of bent neck and sperm flagella abnormalities caused by a *DNAH1* mutation confirmed our point that male factor infertility at least some types of sperm malformation patients may not be suitable candidates for AOA [61, 62]. The current indications for using AOA appear to be broad to some degree [42].

Several diagnostic tests can be used to define the couples who will benefit from AOA in subsequent cycles and assist the clinical management. Previous studies have utilized the mouse oocyte activation test (MOAT) to categorize the reasons for oocyte activation defects [20]. Oocytes with activation rate lower than 20% were diagnosed as sperm-related OAD and showed the most obvious improvement [44]. More sensitive diagnostic tests, such as human oocyte calcium analysis (HOCA), have been employed to predict the effectiveness of AOA on certain patients. Cases with abnormal sperm activation capacity in HOCA are associated with favorable responses to AOA [14]. However, the use of specialized Ca^{2+} imaging equipment limits the application of HOCA [63]. Furthermore, our previous research has shown that patients diagnosed with fertilization failure who are carrying different homozygous or compound heterozygous mutations in *PLCZ1* can achieve successful fertilization and obtain more useable embryos through oocyte activation

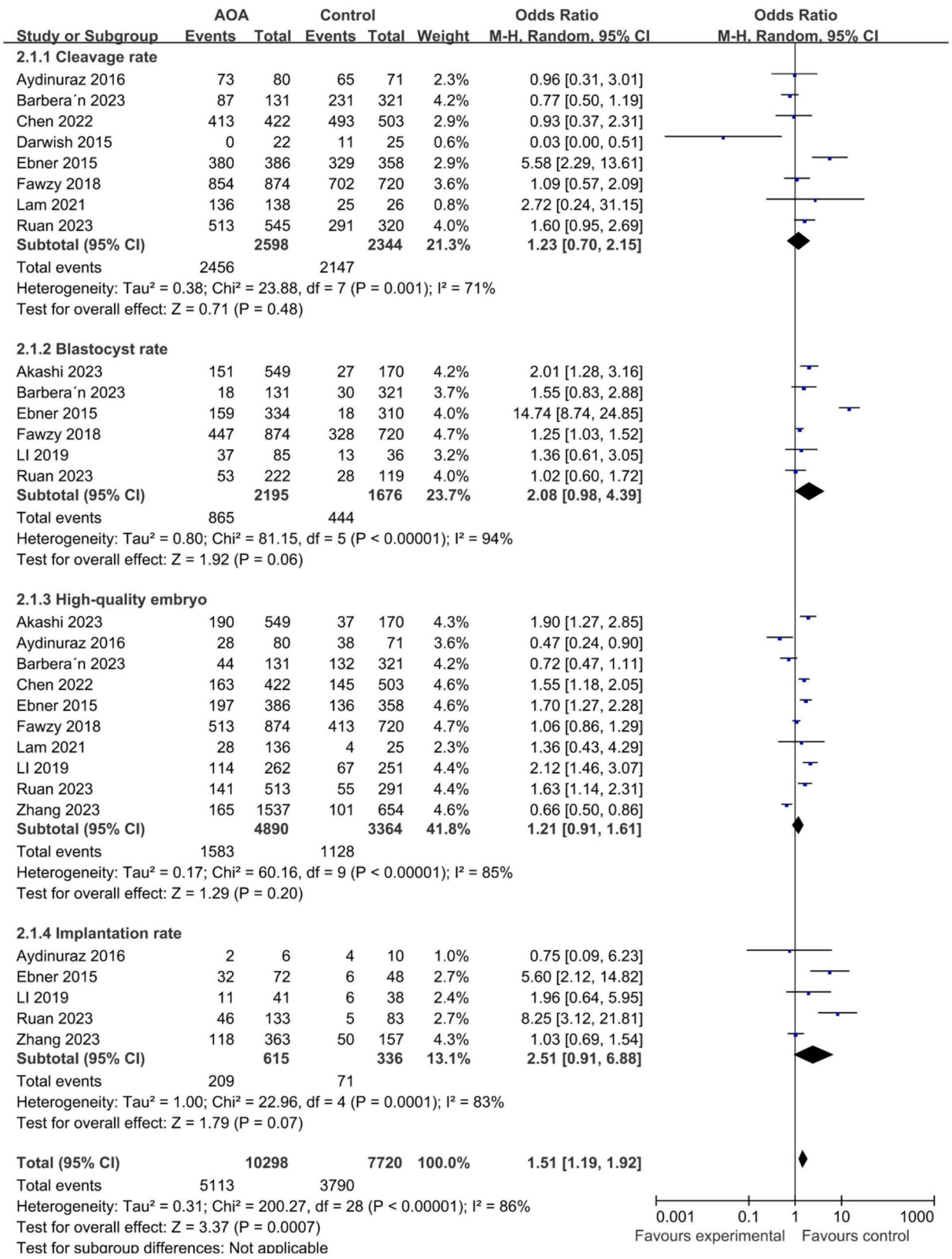


Fig. 6 Forest plot for overall cleavage, blastocyst, high-quality embryo and implantation rate

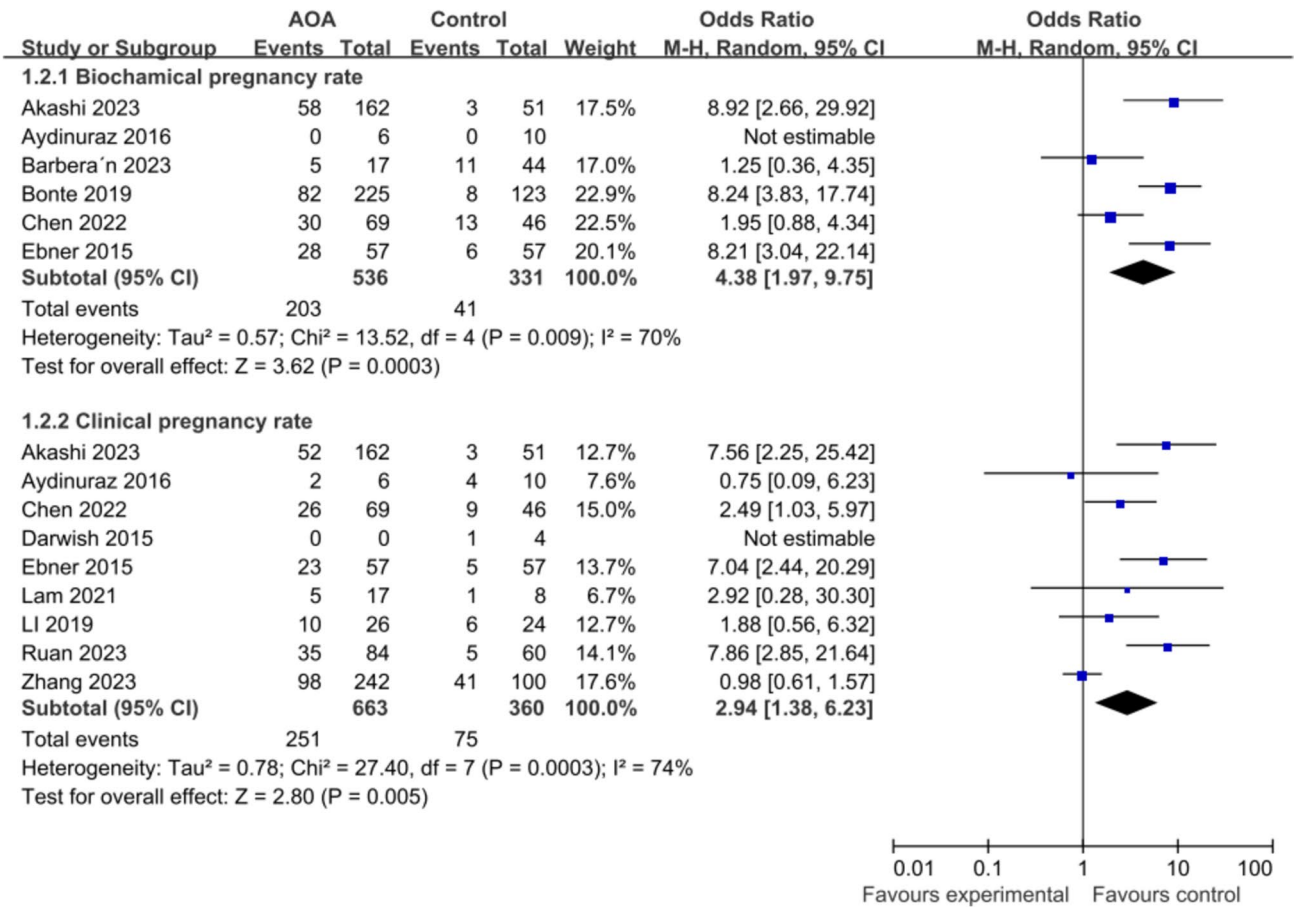


Fig. 7 Forest plot for overall biochemical pregnancy and clinical pregnancy rate

[64], presenting a potential indicator for AOA treatment. Additionally, ICSI with AOA can successfully overcome sperm-related OAD in couples with *ACTL9*, *ACTL7A*, and *IQCN* mutations [65–68]. It may indicate that sequencing of these genes can be used as screen criteria to select appropriate individuals for activation interventions [69].

For AOA protocols, the protocols employed across the included studies vary significantly in type of the calcium

Table 2 Comparison of effectiveness outcomes between AOA-ICSI and ICSI patients

Outcome	Studies	Participants	AOA group	Control group	Odds ratio	95% CI
Fertilization rate[a]	12	16,804	64.74%	46.19%	1.99	[1.16, 3.41]
Cleavage rate[b]	8	4942	94.53%	91.60%	1.23	[0.70, 2.15]
Blastocyst rate[b]	6	3871	37.94%	31.19%	2.08	[0.98, 4.39]
High-quality embryo rate[b]	10	8254	32.37%	33.53%	1.21	[0.91, 1.61]
Implantation rate[c]	5	951	33.98%	21.13%	2.51	[0.91, 6.88]
Biochemical pregnancy rate[c]	6	867	37.87%	12.39%	4.38	[1.97, 9.75]
Clinical pregnancy rate[c]	9	1023	36.34%	13.08%	2.94	[1.38, 6.23]
Live birth rate[c]	8	1268	31.29%	13.91%	4.58	[1.52, 13.80]
Miscarriage rate[d]	6	302	24.64%	56.00%	0.43	[0.08, 2.43]

[a]Per mature oocytes

[b]Per fertilized oocytes cultured

[c]Per transfer cycles

[d]Per clinical pregnancies

Table 3 Subgroup analyses of effectiveness outcomes between AOA-ICSI and ICSI patients

Outcome or Subgroup	With fertilization factors				With non-fertilization factors			
	AOA group	**Control group**	**Odds ratio**	**95% CI**	**AOA group**	**Control group**	**Odds ratio**	**95% CI**
Fertilization rate[a]	63.09%	34.04%	3.19	[1.62, 6.28]	67.14%	68.18%	0.97	[0.71, 1.33]
Cleavage rate[b]	96.27%	95.25%	1.34	[0.92, 1.96]	91.57%	88.15%	0.96	[0.28, 3.35]
Blastocyst rate[b]	39.77%	37.89%	1.35	[1.04, 1.75]	38.06%	7.61%	4.80	[0.52, 44.68]
High-quality embryo rate[b]	42.00%	40.18%	1.33	[0.92, 1.94]	22.98%	28.00%	1.07	[0.65, 1.77]
Implantation rate[c]	32.78%	11.45%	2.82	[0.75, 10.60]	34.48%	27.32%	2.26	[0.43, 11.91]
Biochemical pregnancy rate[c]	35.62%	5.98%	8.43	[4.41, 16.11]	44.06%	20.41%	2.78	[0.95, 8.15]
Clinical pregnancy rate[c]	35.25%	12.42%	3.75	[1.63, 8.65]	39.95%	27.05%	2.39	[0.76, 7.47]
Live birth rate[c]	30.70%	3.40%	11.15	[2.96, 41.97]	31.95%	22.67%	2.17	[0.68, 6.97]
Miscarriage rate[d]	24.72%	75.00%	0.07	[0.01, 0.44]	20.41%	11.11%	1.47	[0.19, 11.59]

[a]Per mature oocytes

[b]Per fertilized oocytes cultured

[c]Per transfer cycles

[d]Per clinical pregnancies

reagents, and the duration, concentration, and timing of calcium treatment (Table 1); however, the available data was insufficient to allow the comparison. In patients with no or low fertilization after ICSI, some study indicated fertilization rates with the use of calcimycin were less efficient compared to ionomycin, causing significantly different Ca^{2+} responses [70, 71]. Ca^{2+} concentration in culture media during AOA can have a negative effect on the efficiency in both mice and humans. No improvement in either the pattern of Ca^{2+} release or mouse embryonic developmental potential after ionomycin exposure in the presence of threefold Ca^{2+} concentrations in the culture medium [72]. A study suggests that different AOA protocols do not have a significant impact on pregnancy outcomes [43]. Further meta-analyses should be conducted in the future to develop more standardized AOA protocols.

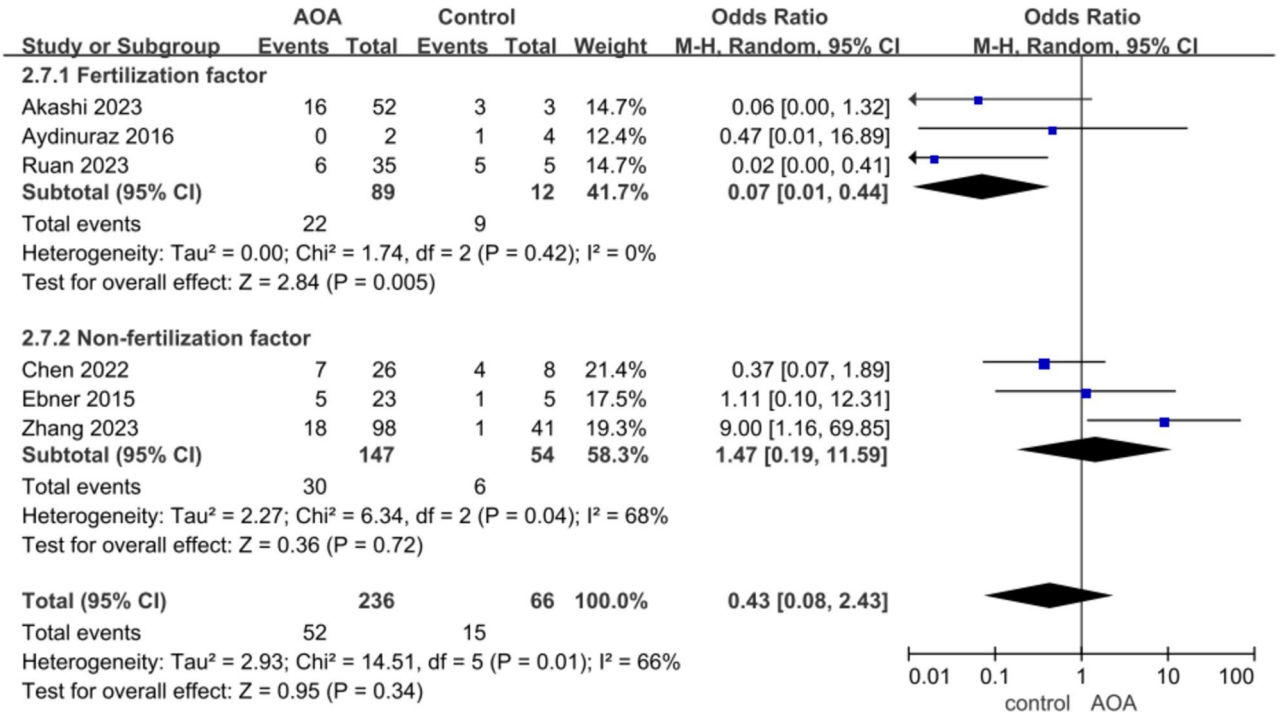


Fig. 8 Forest plot for subgroup analysis of miscarriage rate

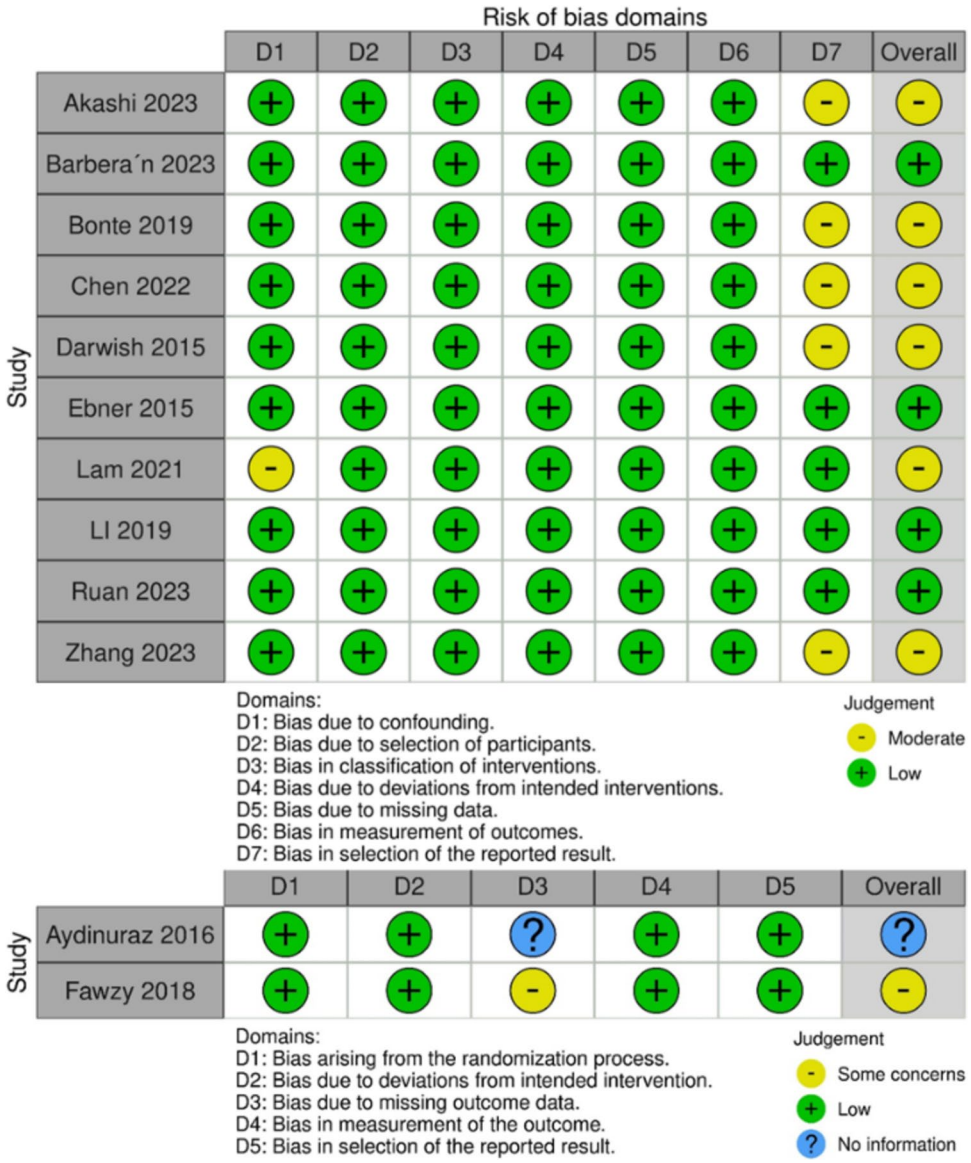


Fig. 9 Judgements about risk of bias of included studies

Strengths and limitations

This study is the first systematic review to include the researches on calcium ionophore as AOA method to treat ICSI failure published after 2017 [73]. With the increasing recognition of AOA technology, the application of AOA has been expanded these years that many centers have started to use AOA to treat recurrent IVF failure cycles due to non-fertilization factors. However, the clinical effectiveness in different indications remains uncertain, and there is still a lack of consensus on the use of AOA for such patients. Only searching for the latest studies also ensure the overall high quality of these included studies. A diversity of findings underpins the strength of our conclusions. The design of our study incorporates several key strengths. Firstly, we comprehensively analyzed indicators at as many stages as possible. By analyzing the effects across different stages, we found that AOA is effective for fertilization and pregnancy and subsequent processes, but does not make a particularly significant contribution to the embryo developmental stages. Secondly, our subgroup analysis provided clear evidence that ICSI failure caused by non-fertilization factors such as embryo developmental problems and teratozoospermia did not benefit from AOA. It also found the heterogeneity of fertilization factor subgroup decreased remarkably compared to the overall group, suggesting different indications were an important source of heterogeneity and enhanced the robustness of the estimation in each subgroup.

This study inevitably entails the subsequent limitations. First, due to the limited number and quality of the articles, we had to perform a mixed analysis of randomized controlled studies and observational studies. More RCTs can

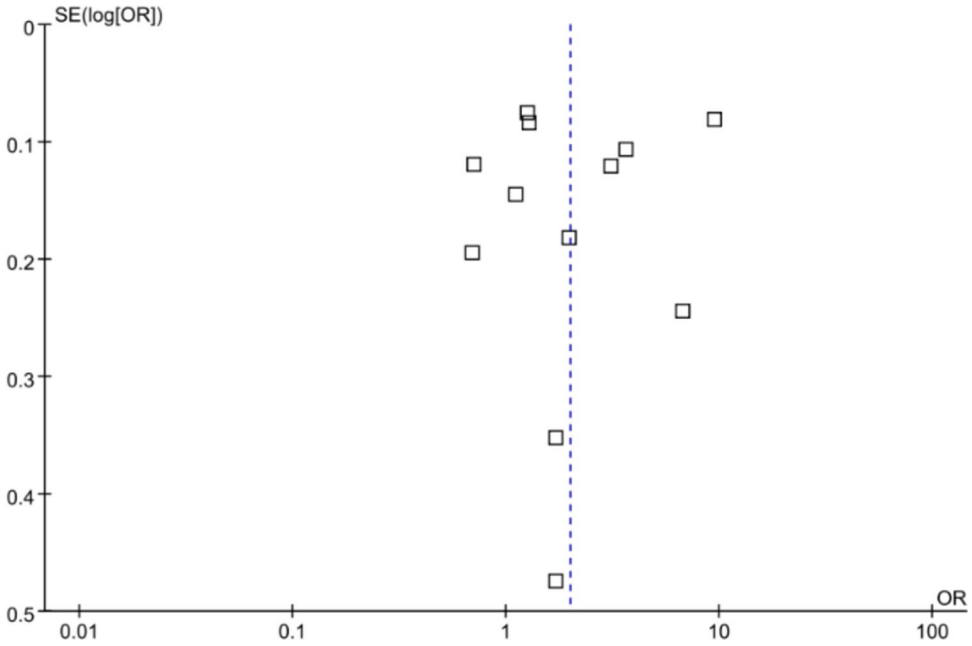


Fig. 10 Funnel plot of included studies

provide more reliable and robust evidence regarding the evaluation of the intervention. However, fertilization failure after ICSI is of low incidence, and once failed in the first ICSI cycle, patients usually ask for something to change in the second cycle, therefore, making it very difficult to design and conduct a methodologically precise RCT. Second, the heterogeneity at some stages was still huge after removing the outliers. In addition to different indications, we have already differentiated to the greatest extent possible, variations in inclusion and exclusion criteria, patient characteristics, and fertility treatment protocols across studies also contributed to unavoidable heterogeneity. Third, it is difficult to further distinguish different indications respectively, for most of studies included couples with several indications for ICSI, making it difficult to differentiate the specific effects of AOA in subgroups. Studies with inclusion criteria of single indication are necessary to confirm the observations in a more extensive population.

Conclusions

The utilization of calcium ionophore treatment for AOA subsequent to ICSI demonstrates a statistically improvement in rates of fertilization, pregnancy, and live birth. The available evidence and our analysis of the miscarriage rate suggest that AOA does not increase the risk of adverse health outcomes in children born. However, there is still limited RCT evidence to judge the effectiveness and safety of ICSI-AOA. Thus, AOA should still be limited to application in patients with appropriate indications. Our subgroup analysis showed that couples with no or low fertilization rate rather than embryo development deficiency benefit more from AOA treatment in terms of improving final live birth rate.

In conclusion, our study demonstrated that AOA using calcium ionophore has the potential to improve fertilization failure and pregnancy outcomes. The result of the subgroup analysis suggested that AOA can be recommended after detailed counseling as a potential treatment for patients with failed ICSI cycles caused by impaired fertilization. Its application on male factors such as sperm abnormalities still lack evidence regarding its effectiveness. For cases of embryo developmental arrest and implantation failure caused by factors other than fertilization, the application of AOA should be cautiously evaluated, taking the patients characteristics into consideration. Further scientific research and follow-up studies should be carried out to provide definitive evidence of the influence of this treatment and obtain more detailed data to guide subsequent research and clinical work.

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Author contribution All authors contributed to the study conception and design. All authors contributed to the study conception and design. The idea for the article was put forward by Jing Fu. Literature search and data analysis were performed by Jingqi Zhang. Visualization was improved by Yilun Sui and Min Xiao. Supervision was performed by Xiaoxi Sun. The first draft of the manuscript was written by Jingqi Zhang and the manuscript was critically revised by Jing Fu. All authors commented on previous versions of the manuscript and approved the final manuscript.

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Data availability The data underlying this article will be shared on reasonable request to the corresponding author. Data regarding any of the subjects in the study has not been previously published unless specified. The detailed protocol is available in PROSPERO (registration number CRD42024551481).

Declarations

Ethics approval This article is a meta-analysis that no ethical approval is required.

Consent of publication This article is a meta-analysis that no consent is required. Standards of Reporting: This systematic review was reported according to the PRISMA 2020 guidelines and PRISMA checklist was fulfilled.

Competing interests The authors declare no competing interests.

References

1. Palermo GD, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: where we have been and where we are going. Semin Reprod Med. 2009;27:191–201.
2. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340:17–8.
3. Yanagida K. Complete fertilization failure in ICSI. Hum Cell. 2004;17:187–93.
4. Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. Hum Reprod. 1998;13(Suppl 1):155–64.
5. Flaherty SP, Payne D, Swann NJ, Mattews CD. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. Hum Reprod. 1995;10:2623–9.
6. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development. 2002;129:3533–44.
7. Swann K, Saunders CM, Rogers NT, Lai FA. PLCzeta(zeta): a sperm protein that triggers Ca2+ oscillations and egg activation in mammals. Semin Cell Dev Biol. 2006;17:264–73.
8. Fukami K, Inanobe S, Kanemaru K, Nakamura Y. Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. Prog Lipid Res. 2010;49:429–37.
9. Yu Y, Nomikos M, Theodoridou M, Nounesis G, Lai FA, Swann K. PLCζ causes Ca(2+) oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P(2). Mol Biol Cell. 2012;23:371–80.
10. Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. Hum Reprod Update. 2010;16:690–703.
11. Ramadan WM, Kashir J, Jones C, Coward K. Oocyte activation and phospholipase C zeta (PLCζ): diagnostic and therapeutic implications for assisted reproductive technology. Cell Commun Signal. 2012;10:12.
12. Ducibella T, Fissore R. The roles of Ca2+, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. Dev Biol. 2008;315:257–79.
13. Yoon SY, Eum JH, Lee JE, Lee HC, Kim YS, Han JE, et al. Recombinant human phospholipase C zeta 1 induces intracellular calcium oscillations and oocyte activation in mouse and human oocytes. Hum Reprod. 2012;27:1768–80.
14. Ferrer-Buitrago M, Dhaenens L, Lu Y, Bonte D, Vanden Meerschaut F, De Sutter P, et al. Human oocyte calcium analysis predicts the response to assisted oocyte activation in patients experiencing fertilization failure after ICSI. Hum Reprod. 2018;33:416–25.
15. Fawzy M, Emad M, Mahran A, Sabry M, Fetih AN, Abdelghafar H, et al. Artificial oocyte activation with SrCl2 or calcimycin after ICSI improves clinical and embryological outcomes compared with ICSI alone: results of a randomized clinical trial. Hum Reprod. 2018;33:1636–44.
16. Mansour R, Fahmy I, Tawab NA, Kamal A, El-Demery Y, Aboulghar M, et al. Electrical activation of oocytes after intracytoplasmic sperm injection: a controlled randomized study. Fertil Steril. 2009;91:133–9.
17. van der Merwe E, Slippers B, Dittrich-Schröder G. Mechanical egg activation and rearing of first instar larvae of *Sirex noctilio* (Hymenoptera: Siricidae). Insects 2023;14.
18. Rogers NT, Hobson E, Pickering S, Lai FA, Braude P, Swann K. Phospholipase Cζ causes Ca2+ oscillations and parthenogenetic activation of human oocytes. Reproduction. 2004;128:697–702.
19. Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. Reprod Biomed Online. 2014;28:560–71.
20. Heindryckx B, Van der Elst J, De Sutter P, Dhont M. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. Hum Reprod. 2005;20:2237–41.
21. Norozi-Hafshejani M, Tavalaee M, Azadi L, Bahadorani M, Nasr-Esfahani MH. Effects of assisted oocyte activation with calcium-ionophore and strontium chloride on in vitro ICSI outcomes. Iran J Basic Med Sci. 2018;21:1109–17.
22. Yamada M, Egli D. Genome transfer prevents fragmentation and restores developmental potential of developmentally compromised postovulatory aged mouse oocytes. Stem Cell Reports. 2017;8:576–88.
23. Jia L, Chen P, Su W, He S, Guo Y, Zheng L, et al. Artificial oocyte activation with ionomycin compared with A23187 among patients at risk of failed or impaired fertilization. Reprod Biomed Online. 2023;46:35–45.
24. Ruan JL, Liang SS, Pan JP, Chen ZQ, Teng XM. Artificial oocyte activation with Ca(2+) ionophore improves reproductive outcomes in patients with fertilization failure and poor embryo development in previous ICSI cycles. Front Endocrinol (Lausanne). 2023;14:1244507.
25. Kashir J, Ganesh D, Jones C, Coward K. Oocyte activation deficiency and assisted oocyte activation: mechanisms, obstacles and prospects for clinical application. Hum Reprod Open 2022;2022:hoac003.
26. Darwish E, Magdi Y. A preliminary report of successful cleavage after calcium ionophore activation at ICSI in cases with previous arrest at the pronuclear stage. Reprod Biomed Online. 2015;31:799–804.
27. Cardona Barberán A, Bonte D, Boel A, Thys V, Paredis R, Machtelinckx F, et al. Assisted oocyte activation does not overcome recurrent embryo developmental problems. Hum Reprod. 2023;38:872–85.
28. Mateizel I, Santos-Ribeiro S, Segers I, Wouters K, Mackens S, Verheyen G. Effect of A23187 ionophore treatment on human blastocyst development-a sibling oocyte study. J Assist Reprod Genet. 2022;39:1225–32.
29. Yin M, Li M, Li W, Wu L, Yan Z, Zhao J, et al. Efficacy of artificial oocyte activation in patients with embryo developmental problems: a sibling oocyte control study. Arch Gynecol Obstet. 2022;305:1225–31.

30. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 2021;372: n71.
31. Sterne JAC, Savović J, Page MJ, Elbers RG, Blencowe NS, Boutron I, et al. RoB 2: a revised tool for assessing risk of bias in randomised trials. BMJ. 2019;366: l4898.
32. Sterne JA, Hernán MA, Reeves BC, Savović J, Berkman ND, Viswanathan M, et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. BMJ. 2016;355: i4919.
33. Lv M, Zhang D, He X, Chen B, Li Q, Ding D, et al. Artificial oocyte activation to improve reproductive outcomes in couples with various causes of infertility: a retrospective cohort study. Reprod Biomed Online. 2020;40:501–9.
34. El-Helaly A, Moussa K, Morsi H, Abu-Sharia D, Hamer J, Farag A. Ionomycin oocyte activation with teratozoospermic male partners in couples undergoing intracytoplasmic sperm injection: a comparative prospective study. International journal of infertility and fetal medicine. 2023;14:12–7.
35. Mateizel I, Verheyen G, Van de Velde H, Tournaye H, Belva F. Obstetric and neonatal outcome following ICSI with assisted oocyte activation by calcium ionophore treatment. J Assist Reprod Genet. 2018;35:1005–10.
36. Nazarian H, Azad N, Nazari L, Piryaei A, Heidari MH, Masteri-Farahani R, et al. Effect of artificial oocyte activation on intracytoplasmic sperm injection outcomes in patients with lower percentage of sperm containing phospholipase Cζ: a randomized clinical trial. Journal of reproduction & infertility. 2019;20:3–9.
37. Ebner T, Oppelt P, Wöber M, Staples P, Mayer RB, Sonnleitner U, et al. Treatment with Ca2+ ionophore improves embryo development and outcome in cases with previous developmental problems: a prospective multicenter study. Hum Reprod. 2015;30:97–102.
38. Aydinuraz B, Dirican EK, Olgan S, Aksunger O, Erturk OK. Artificial oocyte activation after intracytoplasmic morphologically selected sperm injection: a prospective randomized sibling oocyte study. Human fertility (Cambridge). 2016;19:282–8.
39. Li J, Zheng X, Lian Y, Li M, Lin S, Zhuang X, et al. Artificial oocyte activation improves cycles with prospects of ICSI fertilization failure: a sibling oocyte control study. Reprod Biomed Online. 2019;39:199–204.
40. Chen X, Zhao H, Lv J, Dong Y, Zhao M, Sui X, et al. Calcium ionophore improves embryonic development and pregnancy outcomes in patients with previous developmental problems in ICSI cycles. BMC Pregnancy Childbirth. 2022;22:894.
41. Lam KKW, Wong JYY, Cheung TM, Li RHW, Ng EHY, Yeung WSB. A retrospective analysis of artificial oocyte activation in patients with low or no fertilisation in intracytoplasmic sperm injection cycles. J Obstet Gynaecol. 2022;42:648–53.
42. Zhang J, Yao G, Zhang T, Hu J, Yang G, He J, et al. Effect of calcium ionophore (A23187) on embryo development and its safety in PGT cycles. Front Endocrinol (Lausanne). 2022;13: 979248.
43. Akashi K, Yamada M, Jwa SC, Utsuno H, Kamijo S, Hirota Y, et al. Artificial oocyte activation using Ca(2+) ionophores following intracytoplasmic sperm injection for low fertilization rate. Front Endocrinol (Lausanne). 2023;14:1131808.
44. Bonte D, Ferrer-Buitrago M, Dhaenens L, Popovic M, Thys V, De Croo I, et al. Assisted oocyte activation significantly increases fertilization and pregnancy outcome in patients with low and total failed fertilization after intracytoplasmic sperm injection: a 17-year retrospective study. Fertil Steril. 2019;112:266–74.
45. McGuinness LA, Higgins JPT. Risk-of-bias VISualization (robvis): an R package and Shiny web app for visualizing risk-of-bias assessments. Research Synthesis Methods 2020;n/a.
46. Ebner T, Montag M. Artificial oocyte activation: evidence for clinical readiness. Reprod Biomed Online. 2016;32:271–3.
47. Miller N, Biron-Shental T, Sukenik-Halevy R, Klement AH, Sharony R, Berkovitz A. Oocyte activation by calcium ionophore and congenital birth defects: a retrospective cohort study. Fertil Steril. 2016;106:590-6.e2.
48. Vanden Meerschaut F, D'Haeseleer E, Gysels H, Thienpont Y, Dewitte G, Heindryckx B, et al. Neonatal and neurodevelopmental outcome of children aged 3–10years born following assisted oocyte activation. Reprod Biomed Online. 2014;28:54–63.
49. Long R, Wang M, Yang QY, Hu SQ, Zhu LX, Jin L. Risk of birth defects in children conceived by artificial oocyte activation and intracytoplasmic sperm injection: a meta-analysis. Reprod Biol Endocrinol. 2020;18:123.
50. Heindryckx B, De Gheselle S, Gerris J, Dhont M, De Sutter P. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. Reprod Biomed Online. 2008;17:662–8.
51. Capalbo A, Ottolini CS, Griffin DK, Ubaldi FM, Handyside AH, Rienzi L. Artificial oocyte activation with calcium ionophore does not cause a widespread increase in chromosome segregation errors in the second meiotic division of the oocyte. Fertil Steril. 2016;105:807-14.e2.
52. Steinhardt RA, Epel D, Carroll EJ Jr, Yanagimachi R. Is calcium ionophore a universal activator for unfertilised eggs? Nature. 1974;252:41–3.
53. Santella L, Dale B. Assisted yes, but where do we draw the line? Reprod Biomed Online. 2015;31:476–8.
54. Yin M, Yu W, Li W, Zhu Q, Long H, Kong P, Lyu Q. DNA methylation and gene expression changes in mouse pre- and post-implantation embryos generated by intracytoplasmic sperm injection with artificial oocyte activation. Reprod Biol Endocrinol. 2021;19:163.
55. Liang R, Fang F, Li S, Chen X, Zhang X, Lu Q. Is there any effect on imprinted genes H19, PEG3, and SNRPN during AOA? Open Med (Wars). 2022;17:174–84.
56. van Blerkom J, Cohen J, Johnson M. A plea for caution and more research in the 'experimental' use of ionophores in ICSI. Reprod Biomed Online. 2015;30:323–4.
57. Ducibella T, Schultz RM, Ozil J-P. Role of calcium signals in early development. Semin Cell Dev Biol. 2006;17:324–32.
58. D'Haeseleer E, Vanden Meerschaut F, Bettens K, Luyten A, Gysels H, Thienpont Y, et al. Language development of children born following intracytoplasmic sperm injection (ICSI) combined with assisted oocyte activation (AOA). Int J Lang Commun Disord. 2014;49:702–9.
59. Borges E, Jr., de Almeida Ferreira Braga DP, de Sousa Bonetti TC, Iaconelli A, Jr., Franco JG, Jr. Artificial oocyte activation using calcium ionophore in ICSI cycles with spermatozoa from different sources. Reprod Biomed Online 2009;18:45–52.
60. Vanden Meerschaut F, Nikiforaki D, De Gheselle S, Dullaerts V, Van den Abbeel E, Gerris J, et al. Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. Hum Reprod. 2012;27:1977–84.
61. Moaz MN, Khattab S, Foutouh IA, Mohsen EA. Chemical activation of oocytes in different types of sperm abnormalities in cases of low or failed fertilization after ICSI: a prospective pilot study. Reprod Biomed Online. 2006;13:791–4.
62. Liu Z, Guo Y, Chen X, Lin C, Guo X, Jiang M, et al. The effect of ionomycin-induced oocyte activation on multiple morphological abnormalities of the sperm flagella. Systems Biology in Reproductive Medicine. 2023;69:245–54.
63. Nomikos M, Yu Y, Elgmati K, Theodoridou M, Campbell K, Vassilakopoulou V, et al. Phospholipase Cζ rescues failed oocyte activation in a prototype of male factor infertility. Fertil Steril. 2013;99:76–85.
64. Mu J, Zhang Z, Wu L, Fu J, Chen B, Yan Z, et al. The identification of novel mutations in PLCZ1 responsible for human

fertilization failure and a therapeutic intervention by artificial oocyte activation. Mol Hum Reprod. 2020;26:80–7.
65. Zhao S, Cui Y, Guo S, Liu B, Bian Y, Zhao S, et al. Novel variants in ACTL7A and PLCZ1 are associated with male infertility and total fertilization failure. Clin Genet. 2023;103:603–8.
66. Dai J, Zhang T, Guo J, Zhou Q, Gu Y, Zhang J, et al. Homozygous pathogenic variants in ACTL9 cause fertilization failure and male infertility in humans and mice. Am J Hum Genet. 2021;108:469–81.
67. Wei Y, Wang J, Qu R, Zhang W, Tan Y, Sha Y, et al. Genetic mechanisms of fertilization failure and early embryonic arrest: a comprehensive review. Hum Reprod Update. 2024;30:48–80.
68. Wang Y, Chen G, Tang Z, Mei X, Lin C, Kang J et al. Loss-of-function mutations in IQCN cause male infertility in humans and mice owing to total fertilization failure. Molecular Human Reproduction 2023;29:gaad018.
69. Jones C, Meng X, Coward K. Sperm factors and egg activation: phospholipase C zeta (PLCZ1) and the clinical diagnosis of oocyte activation deficiency. Reproduction. 2022;164:F53-f66.
70. Quintana-Vehí A, Martínez M, Zamora MJ, Rodríguez A, Vassena R, Miguel-Escalada I, et al. Significant differences in efficiency between two commonly used ionophore solutions for assisted oocyte activation (AOA): a prospective comparison of ionomycin and A23187. J Assist Reprod Genet. 2023;40:1661–8.
71. Nikiforaki D, Vanden Meerschaut F, de Roo C, Lu Y, Ferrer-Buitrago M, de Sutter P, et al. Effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. Fertil Steril. 2016;105:798-806.e2.
72. Lu Y, Bonte D, Ferrer-Buitrago M, Popovic M, Neupane J, Van der Jeught M, et al. Culture conditions affect Ca(2+) release in artificially activated mouse and human oocytes. Reprod Fertil Dev. 2018;30:991–1001.
73. Murugesu S, Saso S, Jones BP, Bracewell-Milnes T, Athanasiou T, Mania A, Serhal P, Ben-Nagi J. Does the use of calcium ionophore during artificial oocyte activation demonstrate an effect on pregnancy rate? A meta-analysis Fertil Steril. 2017Sep;108:468-482.e3.

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