

**May 22, 2002**

# **Sex selection**

**A survey of laboratory methods and clinical results**

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## ***Introduction***

The concept of sex selection is likely to be as old as mankind<sup>1</sup>. Myths regarding specific activities of the couple e.g. timing or frequency of intercourse<sup>2 3 4 5</sup>, in order to obtain the desired gender are abound even in literate western populations. There are reports illustrating variations in human sex ratio in large populations within the range of 0.505 to 0.538 males per birth<sup>6 7</sup>. Variations have been seen in comparisons between multiple and singleton pregnancies (range 0.428 to 0.506 cf. singletons 0.513) and hypothetically attributed to possible genetic factors or interactions of previous pregnancies<sup>8</sup>. Possible variations due to extreme situations affecting the whole population e.g. earthquakes have been reported (0.501 males per birth cf. 0.516)<sup>9</sup>. Although statistically significant, the variations from the generally accepted “natural” ratio of just above 0.50 are small. At least when examined in large populations the effects of such sex selection treatment should be possible to discover. A problem is, however, that most reports only deal with small selected populations, where a ratio deviating from 0.50 could be due to random factors, or from bias in the selection of cases.

Several reports suggest a possible link between occupation and gender imbalance among offspring e.g. 58 divers in Australia had 45 sons and 85 daughters<sup>10</sup>. A sample of 863 pilots in the German Air Force implied that helicopter pilots with 1000 to 2000 flying hours (108 children in total) had a significantly different sex ratio (0.38), while other helicopter pilots had not. In the same study, among fighter pilots, those with up to 1000 flying hours had a significantly increased sex ratio (0.57, N=471) and those with 1000 to 2000 flying hours had a decreased ratio (0.42, N=264)<sup>11</sup>. However, the groups with significant differences were relatively small and there is no evidence in these studies to eliminate bias in selection of the cases. In another study<sup>12</sup>, 62 pilots and astronauts subjected to high G-force exposure had a higher frequency of daughters (male sex ratio 0.39; N=166) than pilots with low exposure (ratio 0.51; N=582), as judged from published biographies of an Air War College and military classification and assignment histories.

To interpret the available data correctly it is important to bear in mind that factors influencing the natural sex selection are very poorly understood. In a study of semen

samples from 95 men<sup>13</sup> attending an andrology laboratory for infertility investigation, the proportion of Y-bearing spermatozoa, assessed by quantitative PCR, was on average 50.3% (range 41.9-56.7). Special procedures for sperm selection did not change the proportions in the individual samples. However, the human live birth sex ratio does not seem to be a simple reflection of the X-Y ratio in semen, indicating that other factors, e.g. uterine environment, may play a role in the final sex ratio<sup>14</sup>. Thus, evaluation of sex selection methods cannot only rely on data obtained from populations of “enriched” spermatozoa, but must include the outcome of pregnancies resulting from treatment with these sperm populations.

In general, sex-selection may be performed at three different levels (pre conception, PGD and post implantation). The last level, post-implantation, is the oldest laboratory method and the most invasive. It includes karyotyping of cells obtained from the placenta or amniotic fluid and possible termination of the pregnancy if the foetus is of the undesired sex. These methods will not be covered in this review. The two other levels, selection of spermatozoa carrying X- or Y- spermatozoa (pre-conceptual selection) and pre-implantation genetic diagnosis (PGD) are the scope for this review.

### ***Pre-conceptual selection***

Efficient laboratory methods for pre-conceptual sex selection have primarily been developed in veterinary medicine, where the economical advantages of sex selection in domestic animals has been a major promoting factor. In addition, the evaluation of the efficacy of the methods is supposedly easier in the large-scale insemination programs that are routinely used in the modern cattle breeding industry, compared to the situation in humans. The advances in high technology methods for veterinary medicine now allow the collection of 6 million spermatozoa of each sex per hour or 18 million X-spermatozoa per hour<sup>15</sup>.

Selection of Y- or X-bearing spermatozoa has been the goal for several methods developed for human and domestic animals. The selected population of spermatozoa are then usually inseminated. In the last decade additional methods have used *in vitro fertilization*. Common for all these methods is that the selection method provide viable spermatozoa, which can fertilize an egg, or at least be made to fertilize an egg by means of Assisted Reproduction Techniques (ART). The pre-conception selection methods

consist mainly of passing sperm through different gradients or flow cytometry. In addition, methods based on immunological techniques and electrophoresis have been tested, but these have yet to be shown to be of clinical value<sup>16 17 18</sup>.

The basis for the selection procedure is the expected differences in nuclear constituency of X- and Y-chromosome bearing spermatozoa (on average larger head, neck and tail<sup>19</sup>, a higher dry mass and 2-3% more DNA<sup>20</sup> in X-chromosome bearing). Theoretically, this should give a bimodal distribution of spermatozoa with respect to density or cell surface characteristics, as charge or protein expression, that could interact with components in the media used. However, particularly for human spermatozoa there are several problems. For example, the pleomorphic appearance of sperm heads creates a variability between cells that is much larger than the small differences in size, mass, and density due to X- and Y-chromosome bearing spermatozoa, making the two populations overlap to a considerable extent.

## **Sperm selection by gradients**

Different methods to select highly motile spermatozoa devoid of seminal plasma have been developed to increase success in various forms of ART. Some have been claimed to change the sex ratio at birth. The more common variants have been centrifugation through gradient layers of albumin, varying density preparations as Percoll™ and Ficoll™, or microbead suspensions like Sephadex™. In addition, methods based on sperm migration through media of different composition (“swim up”) have been described.

### **Albumin gradients**

The relatively simple method using an albumin gradient was introduced by Ericsson<sup>21</sup>. In the initial study, successful separation of X- and Y-bearing spermatozoa was reported. Y-bearing spermatozoa were identified by a bright spot caused by a quinacrine dihydrochloride dye. However, these results have been questioned. It is possible that the quinacrine dye could cause bright spots in the presence of high concentrations of albumin, thus causing a high frequency of stained cells, falsely interpreted as Y-chromosome bearing spermatozoa. Nevertheless, there have been several reports on the sex of conceptions after insemination with “sex separated

spermatozoa". Most of these reports show significant results among the reported pregnancies (see Table I for summary). The major concerns regarding these studies is that there does not appear to be a study which is independent from the clinics performing sex selection on a commercial basis. In addition, in general, only monitored pregnancies are documented while patients subject to withdrawal are not included. Having said this, there are no clinical studies – assessing fetal or sex of the child - that refute that the gender outcome can be altered by the albumin gradient separation method. The method has an apparent 15-30% possibility to result in the “undesired” gender - a risk that is not likely to be acceptable for the prevention of inheritance of severe diseases.

Although the clinical outcome has not been challenged by an independent study, several laboratory studies refute that the albumin gradient method, or in fact other gradient methods, indeed alters the ratio between X- and Y-bearing spermatozoa. Chen *et al.*<sup>22</sup> found a small increase in X-chromosome bearing spermatozoa as revealed by fluorescent chromosome-specific probes (FISH). With similar methods, others have not achieved a clinically significant increase in Y-bearing spermatozoa.<sup>23 24 25</sup> In a study using albumin gradient separation, 14 out of 18 couples wishing to have boys were reported to have had successful sperm separation despite the fact that FISH data could not show any shift in the X-Y ratio in the selected sperm populations.<sup>26</sup>

In conclusion, if the albumin separation method does allow a gender selection, it is far from absolute and it is not due to enrichment of one type of spermatozoa. The effect, as Ericson has claimed, appears to be that the treatment gives functional advantages to one type of spermatozoa, or to the zygote developing after fertilization.

### **Other gradients**

Using Percoll™, Ficoll™ or Sephadex™ filtration for sperm selection has not been shown to give clinically significant results in clinically controlled studies. Corson *et al.* 1983 reported a successful conception of XX karyotype after Sephadex filtration. Kaneko *et al.* 1983 reported significant separation of X- and Y-bearing spermatozoa using Ficoll™ and Percoll™ filtration, but assessments were only based on quinacrine staining (thus unreliable). Beckett *et al.* 1989<sup>27</sup> used karyotyping of sperm chromosomes, Y-body analysis and DNA analysis, and could not show any consistent

enrichment for X-chromosome bearing spermatozoa after Sephadex gel filtration. Several other studies evaluating the methods with chromosome specific DNA-probes have not demonstrated significant shifts in the X-Y ratio<sup>13 28 29 30</sup>.

Combinations of density gradient centrifugation with sperm migration in a medium (“swim up”) to be used for intrauterine insemination (IUI) or *in vitro fertilization* (IVF) have been reported to give clinically meaningful results<sup>31 32</sup> (Table I). However, only a small number of couples achieving clinical pregnancy were retrospectively reported. In the first study<sup>31</sup> laboratory assessment of Y-bearing spermatozoa was performed with quinacrine showing 84% Y-chromosome bearing spermatozoa in the population prepared by swim up, while the Percoll preparation showed only 49% quinacrine stained spermatozoa. In contrast, other authors have not found meaningful changes in the ratio between X- and Y-bearing spermatozoa when assessing the methods using molecular genetic techniques<sup>33 34</sup>.

## **Flow cytometry**

Flow cytometry for selection of gender is based on variability in chromatin staining by the DNA-binding fluorescent dye Hoechst 33342, detection of fluorescence from individual cells, and sorting of individual cells based on fluorescence. Selected spermatozoa are then used for IUI, IVF or ICSI.

The evaluation of the efficacy of sperm sorting has mainly been performed with polymerase chain reaction (PCR) or FISH on treated spermatozoa, or by biopsy of the developing embryo (Pre-implantation Genetic Diagnosis, PGD).

To date, the difference in the DNA content of X- and Y-bearing spermatozoa in humans is the smallest in the animal kingdom. However, purities of X- and Y-bearing sperm of 70-90%, as judged by PCR and FISH methods have been reported<sup>35 36</sup>. Studies to date suggest there is no change in the frequency of spermatozoa with aneuploidy<sup>37</sup>, however there were no diploid spermatozoa in the populations after MicroSort® processing, suggesting that this process eliminates diploid cells.

The main concerns regarding the safety of the sorting technique are the possible hazards imposed especially on the DNA of the spermatozoa. A chemical compound is used that binds to DNA and emits energy in form of light when hit by a laser beam.

Also the laser beam itself conveys energy to the atoms in and around the DNA. This surplus energy, together with the presence of many chemical compounds, not the least water, may create molecular species that can break other molecular bonds and thereby disrupt and cause damages in the DNA. In the mature sperm head, there are no mechanisms for repair of damaged DNA; this process can only take place in the egg after fertilization. The higher number of disruptions of the DNA, the higher likelihood for errors. However, the likelihood of one major, easily distinguishable defect to appear repeatedly in different individuals is almost zero. In contrast, an increase of variable damage would only be possible to distinguish from the “back ground noise” after several thousands of births using the technique.

Laser is by definition transmission of energy in the form of light. Different levels of laser power have been used in different species in conjunction with DNA-binding Hoechst 33342: laser-effect above 75 mW decreased fertilization rate and embryo growth in rabbits<sup>38</sup>, while in swine laser-effect at 25 mW, but not at 125 mW, had a negative effect on fertilization and embryonic development<sup>39</sup>.

The use of ultraviolet light (to illicit fluorescence) and the DNA-binding fluorescent dye (bisbenzimidazole) can contribute to DNA damages in the spermatozoa which had been sonicated before staining and sorting<sup>40</sup>. However, direct interaction between DNA and bisbenzimidazole is believed to be reversible and does not affect the frequency of endogenous DNA nicks in human sperm DNA, although that does not rule out the occurrence of mutations in embryos arising from sorted spermatozoa<sup>41</sup>.

Hoechst 33342 and flow sort procedures decreases motility, vitality, capacitation status, and fertilization rate *in vitro* of boar spermatozoa<sup>42</sup>. Furthermore, in human spermatozoa Hoechst 33342 in concentrations above 90  $\mu\text{M}$  impaired motility (beat cross frequency), and in concentrations above 900  $\mu\text{M}$  point mutations in the  $\beta$ -globin gene in sperm DNA could be detected<sup>43</sup>. The typical concentration used for sorting human spermatozoa is 9  $\mu\text{M}$ <sup>37</sup>.

Studies in domestic animals<sup>44 45 46 47 48 49 50 51 52 53</sup> have resulted in apparently normal offspring. There is data in cattle, swine and rabbit where at least three (rabbit nine) successive generations have been followed up with respect to malformation and reproductive function after flow cytometry sorting of spermatozoa<sup>45 47 54</sup>. Although,

several generations have been generated after sorting procedures, the total number of offspring is only a few hundred. Furthermore, the embryo survival has been reported to be lower after sperm sorting, although no in depth explanation has been given (rabbits<sup>47</sup>, swine<sup>55</sup>, cattle<sup>46</sup>). In comparison with conditions for human sperm sorting, spermatozoa from these species were exposed to higher stain concentrations and longer incubations times. However, it must be considered that the lower exposure used for human sperm may reflect a relatively easily accessible sperm chromatin, meaning that the molecular exposure of human sperm DNA to possibly damaging compounds may even be greater.

To date there are no reports indicating an increase in major malformations after flow sorting of human spermatozoa. A large-scale FDA evaluation is under way. Specific procedures e.g. rigorous cleaning of the machines between samples effectively eliminates the possibility of contamination between sorts (either between patients or from micro organisms e.g. viruses).

## **Clinical results**

Among animals the birth of live offspring has been reported for rabbit, pig<sup>44 45</sup>, sheep<sup>48 52</sup>, cattle<sup>46 50 51</sup>, and horse<sup>53</sup>.

In a case report<sup>56</sup>, flow cytometry was used to select X-bearing spermatozoa in order to prevent an X-linked disease. The procedure resulted in 80-86% X-bearing spermatozoa which in turn resulted in 24 embryos which could be diagnosed with regard to sex: 22 out of 23 were of female gender.

In 1998 Fugger *et al.*<sup>57</sup> reported births of normal female children after sperm sorting procedures and subsequent IUI, IVF or ICSI. Indications were sex-linked disorders or family balancing. A total of 27 patients were treated in 33 cycles with X-sorted spermatozoa, resulting in seven pregnancies after IVF or ICSI treatment. IUI was performed in 208 cycles in 92 patients. There were 22 clinical pregnancies, of which seven resulted in spontaneous miscarriage, one was ectopic, and 12 were still ongoing when the report was published. Nine pregnancies resulted in eleven healthy children. In 17 cases where the sex was known at the time of publication 15 were female. In a subsequent report on 332 patients, 96 pregnancies were achieved in 663 cycles; the

desired gender was obtained in 94% (37/39) of cases for parents desiring females and in 73% (11/15) of cases for those desiring males. At publication, 47 pregnancies were ongoing.<sup>58</sup>

MicroSort® have kindly provided us with a confidential report<sup>59</sup>, which was submitted as part of an FDA IDE (Investigational Device Exemption) clinical trial. MicroSort® has been developed under a patent licence from the USDA at the Genetics & IVF Institute in Fairfax, Virginia (USA). The evaluation includes a 1-year follow up of babies born. As of December 2001, a total of 1088 patients have participated; 1596 X-sort cycles have been performed (87% X sperm by FISH) and 403 Y-sort cycles (72% Y sperm by FISH). A total of 354 clinical pregnancies had been achieved, 252 children had been born and 78 pregnancies are still ongoing. Out of 288 pregnancies aimed at female gender: 91% (194/214) have resulted in the desired gender. Out of 66 pregnancies aimed at male gender: 70% (26 of 37) have resulted in the desired gender. The clinical success was a 14% pregnancy rate after IUI (224/1 547), 17% after sorting frozen spermatozoa (5/30), 28% after IVF (13/47), 30% after ICSI (104/348), 24% after frozen embryo transfer (11/46). A total of 16% of clinical pregnancies have ended in miscarriage (57/354). Regarding data obtained from PGD to assess the flowcytometry method, in approximately 90% of the cases the sex could be determined. Of these, X-sort gave 90% female embryos (273/305) and Y-sort 61% (245/403) male embryos. Interestingly sperm sorting before PGD is likely to increase the number of embryos of the desired sex available for biopsy and subsequently increase the chances of success of the procedure.

No significant increase in abnormalities is reported; however, at least 750 children must be evaluated to confirm that there is no significant risk of major malformations linked to the MicroSort® technique. Considering the method for prevention of, for instance x-linked disorders, the risk for abnormalities is considerably decreased.

## **Conclusions**

Although the flowcytometry sorting method is generally accepted as a reliable method for pre-conceptual sex selection, there are several issues:

- The equipment, expertise and technology involved in sorting are expensive. The method for sperm selection (MicroSort®) is only available under a commercial licence. All sorts are performed on the premises of GIVF in the USA. However, pregnancy rates are good thus allowing the use of sperm in IUI. This widens the availability of the technology and does reduce costs.
- Although relatively successful with regard to sorting, the risk for “error” can be 10-30%, which may be unacceptable for a couple where the aim is to avoid a severe sex-linked disease.

### ***Pre-implantation Genetic Diagnosis (PGD)***

Pre-implantation diagnosis (PGD) has the advantage of investigating the chromosome content of the embryo. To achieve the correct chromosomal diagnose, the DNA content of the biopsied cell is examined, either with PCR<sup>60 61 62 63 64 65</sup>, or, now more commonly, with FISH<sup>66</sup>.

#### **Advantages and problems with PGD**

When sex determination is based on two blastomeres of each embryo, the diagnosis is likely to be highly reliable and reflect the desired gender at birth<sup>67</sup>. To date studies on biopsied embryos suggest that removal of single cells does not significantly damage the embryo. For example implantation rates remains relatively high. In contrast to the situation in sperm sorting, only the biopsied cells are exposed to dyes or chemicals, while the remaining cells, which will develop into a child, are not exposed. One disadvantage with PGD is that there are in general only a few embryos to examine and thus few, or in some cases no, embryos with the desired gender are available for transfer to the uterus.

#### **Clinical Results**

Vandervorst *et al.* have published 5 years' experience of PGD for the diagnosis of severe inherited disorders<sup>67</sup>. From October 1993 to October 1998, 183 PGD cycles were done in 92 couples. PCR was used for specific diagnosis and FISH for sexing (n=64). The result of 24 (of 29) PGD were confirmed by prenatal diagnosis or after birth. One pregnancy was terminated after misdiagnose. As a result of one misdiagnosis,

replacement of embryos now only occurs if the results of two blastomeres are identical. The implantation rate was 12% and the pregnancy rate per embryo transfer was 26.3 % for X-linked disorders (47 cycles).

The Farah Hospital, Amann, Jordan has the most extensive experience in performing PGD for family balancing and family size. They have been performing this technique for several years and implantation rates are above the normal range. Out of 66 children born from PGD, all had the desired gender and were normal at 1 year follow up<sup>68</sup>. In addition to its use in sex selection, sex chromosome aneuploidy is detected by FISH PGD.

A very large collation of data has been made on behalf of the European Society for Human Reproduction and Embryology (ESHRE) by its Preimplantation Genetic Diagnosis Consortium<sup>69 70</sup>. The latest publication, accumulating data up to May 2001, includes results from 25 centres, totalling 1560 couples, mainly referred for inherited diseases (only 30 couples referred for sex selection due to social reasons)<sup>71</sup>. Data on success in obtaining the desired gender is not included, but a clinical pregnancy rate of 35% was achieved for couples seeking sex selection for social reasons, which was better than for any other PGD group

## ***Conclusions***

The efficacy of “natural methods” and gradient procedures has not been scientifically proven as effective. Flowcytometry (Microsort®) as a basis for sperm selection does give significant enrichment of both X- and Y-bearing spermatozoa, but the preparations are far from pure. Interestingly the X-sort appears to be more successful. The great advantage of PGD is the certainty of the gender of the embryo, but the advantage is limited to the number of diagnosed embryos that will result in a pregnancy and normal birth. A combined approach, where eggs are fertilized *in vitro* by spermatozoa obtained from a population enriched in one type of spermatozoa through flow cytometry has been developed<sup>56</sup>. This increases the likelihood for embryos of the desired gender, and thereby the availability of embryos for transfer. The disadvantage of this approach is of course the costs of using both flow cytometry and PGD.

The full extent of the risk for inducing inheritable damages to the sperm DNA by the flow cytometry sorting procedures is not completely covered by present scientific investigations. There are well grounded theoretical implications that irradiation energy and radicals generated may cause damages in the DNA. The available numbers of individuals and generations born, and the level of evaluation of possible increases in DNA damage in these individuals, cannot rule out that other, less obvious, damages have occurred.

## ***Acknowledgements***

We are grateful to MicroSort® (USA) for providing us with unpublished information and for their permission for this information to be included in the published version of this document. Prof Ulrik Kvist, Andrology Centre of Karolinska Hospital, Sweden, is acknowledged for valuable suggestions regarding DNA damage and genetic risks.

## Tables

Table I: Summary of published, peer reviewed reports (found in searches at MEDLINE) on clinical outcome (pregnancies) after albumin or other gradient separation.

	Desired gender <sup>1</sup>	Method <sup>2</sup>	Initial group <sup>3</sup>	No of treated couples <sup>4</sup>	leaving study without conception	Couples remaining	Not conceiving	Number of conceptions	Pre-term edning, gender unknown <sup>5</sup>	Pre-term, Male gender <sup>6</sup>	Preterm, Female gener <sup>7</sup> r	molar pregnancy	Normal pregnancies	MALES <sup>8</sup>	FEMALES <sup>8</sup>	Still pregnant <sup>9</sup>	not counted or unknown
Dmowski <i>et al</i> 1979 <sup>72</sup>	Y	A	37	37		37	27	10		1				6	2	2	
Beernink & Ericsson 1982 <sup>73</sup>	Y	A		66	22	44	14	30	3				15	12	4		
	Y	AC											5	1	5	5	
	Y	A											46	38	9		
	Y	AC											7	1	7		
	Y	A												19	7		
Corson <i>et al</i> 1983 <sup>74</sup>	X	S	3					1					2		1	1	
Corson <i>et al</i> 1984 <sup>75</sup>	Y	A		79				40	3		1	1	30	28	7	1	
	Y	A											26	19	7		
	X	S		19				12	1				11	2	8	1	
Jaffe <i>et al</i> 1991 <sup>76</sup>	Y	A	162	112									23	13	10		
	X	AC	87	50									14	3	11		
	CY		107	107									23	14	9		
	CX												17	11	6		
Beernink <i>et al</i> 1993 <sup>77</sup>	Y	A												749	285		
	X	AX												60	133		
Check & Katsoff 1993 <sup>31</sup>	Y	M												25	5		
	Y	P												19	17		
	C													13	13		
Rose & Wong 1998 <sup>26</sup>	Y	A	184	112				31	6					15	4		3
Khatamee <i>et al</i> 1999 <sup>32</sup>	X	M											15	2	13		
	Y	M											37	33	4		

<sup>1</sup> X = Female, Y = Male; C = Control group

<sup>2</sup> A = Albumin gradient; AX = Albumin gradient for X-sort; AC = Albumin gradient + Clomid treatment; S = Sephadex gradient; M = modified swim-up; P = Percoll filtration; C = Control

<sup>3</sup> Number of patients coming for initial consultation for gender selection

<sup>4</sup> Number of patients that were treated

<sup>5</sup> Abortions, ectopic pregnancy

<sup>6</sup> Spontaneous abortions

<sup>7</sup> Miscarriage

<sup>8</sup> Including pre-term ended pregnancies with known gender

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