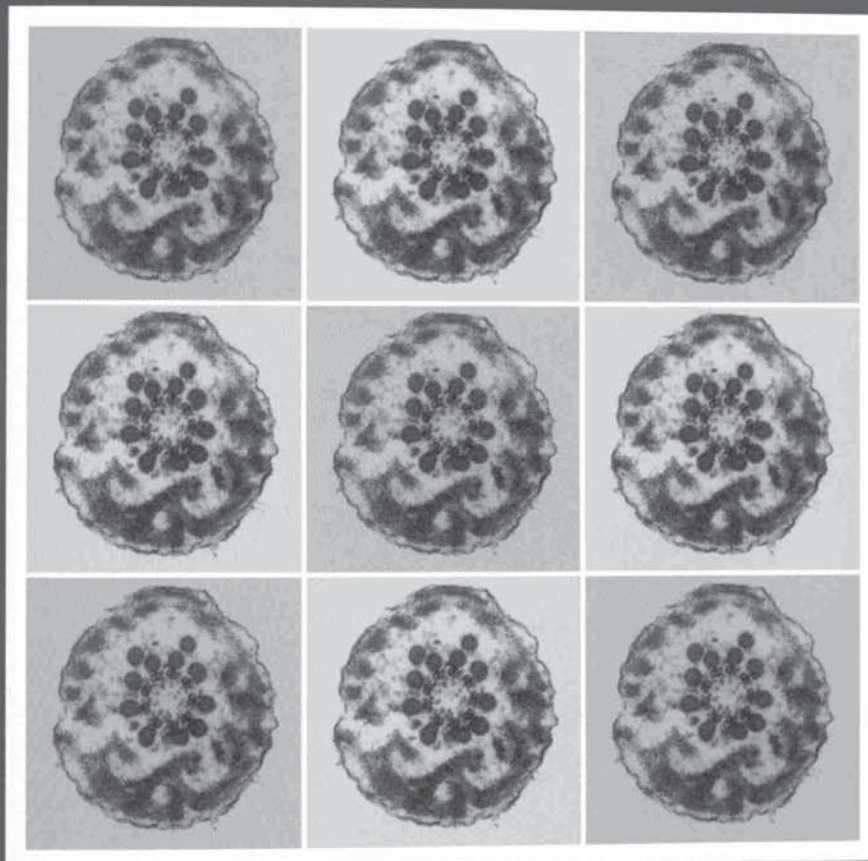
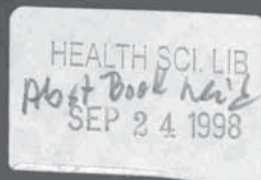


HUMAN REPRODUCTION

Volume 13 number 9 September 1998



Infertility and fibrous sheath dysplasia

An international journal published for ESHRE, covering research and clinical work in
human reproduction and relevant animal studies

ISSN 0268-1161 Coden HUREEE

Oxford University Press

Petitioner ABS Global, Inc. - Ex. 1037, Cover

OUTSTANDING CONTRIBUTION

Births of normal daughters after MicroSort sperm separation and intrauterine insemination, in-vitro fertilization, or intracytoplasmic sperm injection

E.F.Fugger^{1,2,4}, S.H.Black^{1,3}, K.Keyvanfar¹ and J.D.Schulman^{1,3}

¹Genetics & IVF Institute, 3020 Javier Road, Fairfax, VA 22031,

²Department of Obstetrics and Gynecology, and ³Department of Human Genetics, Medical College of Virginia, Richmond, VA, USA

⁴To whom correspondence should be addressed

The world's first deliveries of normal babies after use of flow cytometric separated human sperm cells (MicroSort®) for preconception gender selection are reported. Offspring were of the desired female gender in 92.9% of the pregnancies. Most of these pregnancies and births were achieved after simple intrauterine insemination.

Key words: flow cytometric separation/MicroSort/sex selection/XSort/YSort

Introduction

A reliable method for preconception gender selection of offspring in humans has been sought for many years. Historical approaches, including the search for physical, biochemical, and immunological differences, have produced many methods that attempt X and Y sperm cell separation. Among the more prevalent methods reported are albumin gradients, Percoll® gradients, Sephadex columns, and a modified swim-up, all subsequently resulting in unconfirmed sperm cell separation after DNA analysis and inconsistent gender reports (Flaherty and Matthews, 1996; Reubinoff and Schenker, 1996).

Any method for separation of X and Y chromosome-bearing spermatozoa must rely on an identifiable difference between these living cells. The only currently recognizable distinction between living X- and Y-bearing spermatozoa is their total DNA content, owing to the larger size of the X chromosome (Pinkel *et al.*, 1982). In 1987, Johnson *et al.* reported flow cytometric separation (FCS) of animal X- and Y-bearing spermatozoa into two enriched populations based on total DNA difference (United States Patent #5,135,759). Subsequently, pregnancies and births have been reported in the rabbit (Johnson *et al.*, 1989), swine (Johnson, 1991), ovine (Morrell and Dresser, 1989), and bovine (Cran *et al.*, 1993) species using such flow sorted spermatozoa. Over 400 offspring, all normal, have been born using flow sorting technology, including three successive generations in swine (L.A.Johnson, personal communication) and five successive generations in rabbits (Morrell and Dresser, 1989). The first successful separation of

human X- and Y-bearing sperm cells based on the 2.8% total DNA content difference using FCS was reported by Johnson and ourselves (Johnson *et al.*, 1993), and has been substantiated by fluorescence in-situ hybridization (FISH) analysis (Vidal *et al.*, 1998). The efficacy of any preconception sperm separation procedure can be determined on a sample aliquot by using FISH techniques with X and Y chromosome-specific probes to stain individual spermatozoa. Levinson *et al.* (1995) subsequently reported the first human pregnancy using MicroSort and preimplantation embryo genetic testing for the prevention of X-linked hydrocephalus.

We now describe the first births and clinical trial data from the use of MicroSort followed by intrauterine insemination (IUI), in-vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI). This report presents results with MicroSort FCS designed to achieve enrichment in X chromosome-bearing spermatozoa (XSort®).

Materials and methods

Based on the birth of over 400 normal offspring from four species of animals and negative results of the Ames mutagenicity test (Hazelton Washington, Vienna, VA, USA) for the specific dye and laser, an Institutional Review Board approved trial was established to assess flow cytometric separation of X- and Y-bearing spermatozoa in human subjects.

Subject selection

Individuals entered the trial for prevention of sex-linked disorders in offspring or for family balancing. Family balancing was defined as sorting for the gender found in <50% of the children in that family. Couples were provided with written information, consent documents, and personal counselling regarding the risks and benefits of MicroSort.

Sperm preparation and staining

Fresh or frozen semen specimens were provided for sorting. Fresh semen specimens were allowed to liquefy at 35°C for 30 min. Frozen specimens were thawed at room temperature in a laminar flow hood for 15 min. Specimens were evaluated for volume, count, motility, progression, viability, and percent abnormal cells using WHO standards (World Health Organization, 1992). Specimens to be sorted were extended, centrifuged, resuspended, filtered through glass wool to remove debris and non-motile sperm cells (Johann *et al.*, 1989), and treated with a solution of Hoechst 33342, the vital fluorochrome bisbenzimidazole (Calbiochem-Behring Corporation, La Jolla, CA, USA) using previously described techniques (Johnson *et al.*, 1993).

Flow cytometric separation

The specimens were sorted by FCS as previously described (Johnson *et al.*, 1993). Briefly, spermatozoa were sorted using buffered sheath

fluid with either a modified Epics® 753 (Coulter Corporation, Hialeah, FL, USA) or a modified fluorescence-activated cell sorter (FACS® Vantage; Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). Fluorescence emitted from each sperm cell was detected through a 400-nm long pass filter, and the enriched fraction of the sorted sample was collected. The motility and progression of the sorted specimen were evaluated at 35°C under paraffin oil using an inverted microscope with Hoffman optics (Leitz; Bunton Instruments, Rockville, MD, USA).

Fluorescence in-situ hybridization

Approximately 3000 to 10 000 spermatozoa from the enriched sorted fraction were used to determine the proportion of X- and Y-bearing cells by FISH using alpha-satellite DNA probes specific for the X and Y chromosomes. The FISH procedure was a modification (Pieters *et al.*, 1990) of one DNA probe standard protocol (Vysis Inc., Downers Grove, IL, USA). Briefly, the sorted cells were washed two times for 6 min at 1300 g with phosphate-buffered saline (PBS). The pellet was resuspended in 10 µl of PBS and air dried in a 0.5-cm² area on a glass slide for 10 min at 40°C. The dried cells were subsequently fixed three times each with 2, 5, and 10 µl of fixative (75% methyl alcohol, 25% acetic acid), respectively. Each slide was washed three times with 40 ml of a 2× concentration of SSC (0.3 M NaCl, 30 mM sodium citrate) (Vysis Inc.) at 37°C and allowed to air dry. The fixed spermatozoa were treated with 50 mM dithiothreitol in 0.1 M Tris-HCl (pH 8.0 at room temperature) for 8 to 10 min, washed three times with 2×SSC and air dried at room temperature. The treated slides were denatured simultaneously with 1 µl of Vysis Spectrum CEP X orange/Y green probe mixture, 10.5 µl Vysis Spectrum CEP hybridization buffer (Vysis) and 3.5 µl of distilled water at 75°C under a 22×22-mm cover glass for 5 min. Similar analyses could also be performed with X and Y probes from other sources. The sperm DNA and the X and Y probe mixture were hybridized for 30 to 45 min at 42°C. The slides were washed with a 0.4×SSC (pH 7.0) at 75°C and counter-stained with DAPI (4',6-diamidino-2-phenylindole). The sperm cells were screened using a 60× objective on a Nikon Optiphot-2 (Nikon Inc., Melville, New York, USA) equipped with a dual band pass fluorescein isothiocyanate (FITC)/rhodamine cube and DAPI filter. The sorted sperm cells were identified first using the DAPI filter then analysed by the FITC/rhodamine filter. Spermatozoa identified with one distinct red spot were classified as haploid X chromosome-bearing cells and those with one distinct green spot as haploid Y chromosome-bearing spermatozoa. A total of 200 spermatozoa was counted for each patient.

Post-sort specimen cryopreservation

Sorted specimens to be frozen were extended with TEST yolk buffer freezing medium (Irvine Scientific, Santa Ana, CA, USA), transferred into 1-ml Nunc cryotubes (Nunc, Kamstrup, Denmark) or into 0.25-ml straws (IMV, Minneapolis, MN, USA) and subsequently frozen in a programmable rate freezer (TS Scientific, Perskie, PA, USA) using liquid nitrogen vapour (Mahadevan and Trounson, 1984). Frozen sorted specimens were stored at -196°C in liquid phase nitrogen.

Frozen specimens used for IVF or ICSI were thawed at room temperature, centrifuged, and resuspended in Ménézo's B2 (Fertility Technologies Inc., Natick, MA, USA) or Whittingham's T6 (Quinn *et al.*, 1984) medium prior to use.

Results

IVF and ICSI

Spermatozoa sorted for the X-bearing chromosome were used for 27 patients in 33 IVF/ICSI cycles. A total of seven clinical

pregnancies resulted in a 21.2% (7/33) clinical pregnancy rate per initiated cycle.

Intrauterine insemination

Intrauterine insemination was performed after appropriate clinical monitoring in 208 cycles using an average of 130×10^3 total motile sperm cells per insemination in 92 patients with an average age of 33.9 years. The average post-MicroSort FISH analysis for XSort samples was 84.5% X-bearing spermatozoa. Twenty-two clinical pregnancies occurred in these 208 IUI cycles (10.6% pregnancy rate/cycle). This rate was rather similar to that reported for medical donor insemination in 10 796 cycles (8.9% pregnancy rate/cycle) (Johnston *et al.*, 1994) and for donor IUI and gamete intra-Fallopian transfer in 21 760 cycles (11.0% pregnancy rate/cycle) (Human Fertilization and Embryology Authority, 1997) without MicroSort.

Pregnancies and births

A total of 29 clinical pregnancies was achieved with MicroSort XSort followed by IUI, IVF, or ICSI. Nine patients have given birth to 11 normal healthy babies, seven pregnancies resulted in spontaneous miscarriage, there was one ectopic pregnancy, and 12 pregnancies are ongoing. Of the 14 pregnancies with known fetal or birth gender, 13 had only female conceptions (92.9%). A total of 88.2% (15/17 including three sets of twins) of the fetuses was of the female gender.

Discussion

We demonstrate the achievement of a substantial number of pregnancies and deliveries of normal babies after use of MicroSort in humans. Importantly, most of these pregnancies were achieved after simple IUI. In 92.9% of the pregnancies, offspring were solely of the desired female gender. All children born so far have been normal and healthy.

The ability to separate X- and Y-bearing sperm cells provides new opportunities for women who are carriers of X-linked disorders. There are over 350 X-linked diseases in humans, including haemophilia, Duchenne muscular dystrophy, and X-linked hydrocephalus. In most cases, the X-linked diseases are only expressed in the male offspring of carrier mothers. The use of MicroSort for the enrichment of the X chromosome-bearing sperm cells can now allow for the preferential conception of unaffected female offspring. Combining MicroSort with IVF or ICSI and subsequent preimplantation embryo testing represents an even more powerful tool for increasing the number of unaffected female embryos conceived, and reducing or eliminating the transfer of male embryos to the uterus.

Current MicroSort technology provides an average of ~85% X-bearing spermatozoa in the enriched sorted sample as determined by FISH analysis. This increase would be expected to make it ~ five to six times more likely to have a female than a male child ($85/15 = 5.7$) after an XSort. MicroSort technology now provides couples who desire family balancing with a preconception method to increase substantially the probability of having a daughter.

Several characteristics specific to human sperm cells present unique challenges for FCS compared with domestic animal spermatozoa. Some of the issues that affect FCS include the percentage total DNA difference between X- and Y-bearing spermatozoa, physical shape and morphology, variation in the size of the Y chromosome, heterogeneity of the sperm cells, and the range of individual variability. Human sperm cells present a relatively small average total DNA difference (2.8%) between X- and Y-bearing spermatozoa compared with that of some domestic animals (3.6 to 4.1%). In addition, variation in the amount of human Y heterochromatin can result in the Y chromosome being as much as 50% larger in one individual than another. Human spermatozoa are more oval and have a more highly variable morphology than the relatively paddle-shaped bull spermatozoa, and these factors may affect cell orientation in the FCS system. It is not uncommon for the average human sperm ejaculate to contain 30 to 50% abnormal cells by WHO standards, and 85% abnormal cells by Kruger strict criteria evaluation, compared with ~ 5 to 10% abnormal cells in bull semen. Sperm cell morphology and, more specifically, acrosome and membrane integrity may affect dye uniformity and light refraction issues relevant to fluorescence detection. Human spermatozoa also present a comparatively high level of heterogeneity within and between individuals.

Pregnancies after IUI using $<1 \times 10^6$ motile sperm cells have been obtained in bovine (Seidel *et al.*, 1996) and human (Trout *et al.*, 1995; Centola, 1997) species. Intrauterine insemination using 200 000 to more than 200×10^6 motile spermatozoa in 1761 cycles has shown no significant differences between the number of sperm cells inseminated and pregnancy rate (Cressman *et al.*, 1996). Current MicroSort technology results in a reduced number of sperm cells compared with the quantity usually used for medical IUI, but still readily achieves many pregnancies. This is consistent with the observation that men with Kallman's syndrome, who have greatly reduced sperm counts but functionally normal spermatozoa, readily impregnate their wives through intercourse (Burris *et al.*, 1988). In the human, pre-sort processing of the semen sample is required to remove undesirable cells and to enhance the number of normal motile cells prior to FACS. Subsequently, a substantial number of cells passing through the flow cytometer are eliminated due to lack of orientation, undetermined fluorescence difference, and other factors. The retrieval rate currently can vary between 0.6% and 1.2% of the sperm cells passing through the cytometer. Sperm samples starting with 50×10^6 or more cells generally result in ~100 000 to 300 000 motile cells for IUI. Smaller numbers of spermatozoa can be used, of course, for ICSI or IVF.

Concerns regarding the use of ultraviolet light and Hoechst 33342 (bisbenzimidazole) for FCS of human sperm cells have been expressed (Ashwood-Smith, 1994; Munné, 1994) and clarified (Johnson and Schulman, 1994). Bisbenzimidazole is a non-intercalating non-cytotoxic DNA stain that preferentially binds to triplet adenine and thymine base pairs in the minor groove outside of the double helix, and the binding is reversible at 37°C. Hoechst 33342 absorbs at 358 nm, a substantial distance from the maximum DNA absorption of ~ 260 nm, excites at 367 nm and emits at 461 nm. While some somatic

cells have been found to be sensitive to bisbenzimidazole staining (Durand and Olive, 1982; Van Zandt and Fry, 1983), sperm nuclear DNA has undergone changes including compaction and stabilization that is uniquely different from other cell types. Watkins *et al.* (1996) found no evidence of mutagenicity of human sperm cells stained with $<900 \mu\text{M}$ bisbenzimidazole as assessed by polymerase chain reaction analysis of the beta-globin gene. Catt *et al.* (1997) also reported no increase in the incidence of endogenous DNA nicks in human sperm cells after bisbenzimidazole staining and ultraviolet exposure during passage through a fluorescence-activated cell sorter. Concerns regarding embryo development in bovine (Cran *et al.*, 1994) and rabbit (McNutt and Johnson, 1996) species were not observed in our experience with humans, which resulted in a 92% embryo cleavage rate (unpublished data) and 21.2% pregnancy rate per transfer cycle. Most importantly, as in the animal data from several species with hundreds of normal births, all offspring born from FCS of human spermatozoa have been normal and healthy.

MicroSort provides a significantly high degree of X-bearing sperm enrichment and represents the only validated method for preferential conception of daughters. MicroSort also achieves Y-chromosome enrichment of sperm populations (YSort®), but to a lesser extent than the X-chromosome enrichment from the XSort results reported here; our ongoing trial with YSorts will be subsequently reported.

Fundamental principles of liberty affirm that couples should be free to choose their own reproductive options. The preferential conception of a daughter in couples at risk for transmitting an X-linked disease provides the couple with a higher likelihood of an unaffected child. Furthermore, several surveys have indicated that a majority of couples approve in principle of preconception gender selection for purposes of family balancing (Rosenzweig and Adelman, 1976; Markle and Nam, 1982). Our experience with couples who actually enquired about gender selection indicates that 53.6% (675/1259) of such enquirers desired female offspring. This slight preference for girls is consistent with other reports by Batzofin (1987) for American couples and Liu and Rose (1995) in the UK. The majority of couples (90.5%) in our study were seeking gender preselection for family balancing purposes, were in their mid thirties, had two or three children of the same sex, and desired only one more child.

In summary, a significant number of individual normal births in humans has been achieved using MicroSort, IUI, and IVF and ICSI. Pregnancies have also been attained with the use of frozen sorted sperm cells. MicroSort technology should find broad application, and continued refinement is expected to provide improvements in both sperm purity and recovery.

Acknowledgements

The authors wish to acknowledge Keith Blauer, Daniel Deresh, Brent Hazelrigg, Janelle Jones, Shirley Jones, Barbara Lustig, Gene Levinson, Rekha Matken, Lesa McGahan, Celeste Miller, Mike Norton, Chrispo Opanga, Mike Opsahl, Susanne Stockard, and Loan Thanh for their substantial contributions to the laboratory, clinical, and administrative functions that made this manuscript possible.

References

- Ashwood-Smith, M.J. (1994) Safety of human sperm selection by flow cytometry. *Hum. Reprod.*, **9**, 757.
- Batzofin, J.H. (1987) XY sperm separation for sex selection. *Urol. Clin. North. Am.*, **14**, 609–618.
- Burris, A.S., Clark, R.V., Vantman, D. *et al.* (1988) A low sperm concentration does not preclude fertility in men with isolated hypogonadotropic hypogonadism after gonadotropin therapy. *Fert. Steril.*, **50**, 343–347.
- Catt, S.L., Sakkas, D., Bizzaro *et al.* (1997) Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa. *Mol. Hum. Reprod.*, **3**, 821–825.
- Centola, G.M. (1997) Successful treatment of severe oligozoospermia with sperm washing and intrauterine insemination. *J. Androl.*, **18**, 448–453.
- Cran, D.G., Johnson, L.A., Miller, N.G.A. *et al.* (1993) Production of bovine calves following separation of X- and Y-chromosome bearing sperm and *in vitro* fertilization. *Vet. Rec.*, **132**, 40–41.
- Cran, D.G., Cochrane, D.J., Hohnson, L.A. *et al.* (1994) Separation of X- and Y-chromosome bearing bovine sperm by flow cytometry for use in IVF. *Theriogenology*, **41**, 183.
- Cressman, B.E., Pace-Owens, S., Pliego *et al.* (1996) Effect of sperm dose on pregnancy rate from intrauterine insemination: a retrospective analysis. *Tex. Med.*, **92**, 74–79.
- Durand, R.E. and Olive, P.L. (1982) Cytotoxicity, mutagenicity and DNA damage by Hoechst 33342. *J. Histochem. Cytochem.*, **30**, 111–116.
- Flaherty, S.P. and Matthews, C.D. (1996) Application of modern molecular techniques to evaluate sperm sex selection methods. *Mol. Hum. Reprod.*, **2**, 937–942.
- Human Fertilisation and Embryology Authority (1997) Sixth Annual Report. Donor insemination data, page 27.
- Johann R., Jeyendran R., Vermeiden, J. *et al.* (1989) Human sperm selection by glass wool filtration and two-layer, discontinuous Percoll gradient centrifugation. *Fertil. Steril.*, **51**, 685–690.
- Johnson, L.A. (1991) Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. *Reprod. Domest. Anim.*, **26**, 309–314.
- Johnson, L.A. and Schulman, J.D. (1994) The safety of sperm selection by flow cytometry. *Hum. Reprod.*, **9**, 758–759.
- Johnson, L.A., Flook, J.P., Look, M.V. *et al.* (1987) Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. *Gamet. Res.*, **16**, 1–9.
- Johnson, L.A., Flook, J.P. and Hawk, H.W. (1989) Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. *Biol. Reprod.*, **41**, 199–203.
- Johnson, L.A., Welch, G.R., Keyvanfar, K. *et al.* (1993) Gender preselection in humans? Flow cytometric separation of X and Y spermatozoa for the prevention of X-linked diseases. *Hum. Reprod.*, **8**, 1733–1739.
- Johnston, R.C., Kovacs, G.T., Lording, D.H. *et al.* (1994) Correlation of semen variables and pregnancy rates for donor insemination: a 15-year retrospective. *Fertil. Steril.*, **61**, 355–359.
- Levinson, G., Keyvanfar, K., Wu, J.C. *et al.* (1995) DNA based X-enriched sperm separation as an adjunct to preimplantation genetic testing for the prevention of X-linked disease. *Mol. Hum. Reprod.* **1**; see *Hum. Reprod.*, **10**, 979–982.
- Liu, P. and Rose, G.A. (1995) Social aspects of over 800 couples coming forward for gender selection of their children. *Hum. Reprod.*, **10**, 968–971.
- Mahadevan M. and Trounson A.O. (1984) Effect of cooling, freezing and thawing rates and storage conditions on preserving of human spermatozoa. *Andrologia*, **16**, 52–60.
- Markle, G.E. and Nam, C.B. (1982) Sex predetermination: its impact on fertility. *Soc. Biol.*, **29**, 168–179.
- McNutt, T.L. and Johnson, L.A. (1996) Flow cytometric sorting of sperm: influence of fertilization and embryo/fetal development in the rabbit. *Mol. Reprod. Dev.*, **43**, 261–267.
- Morrell, J.M. and Dresser, D.W. (1989) Offspring from inseminations with mammalian sperm stained with Hoechst 33342, either with or without flow cytometry. *Mutation Res.*, **224**, 177–183.
- Munné, S. (1994) Flow cytometry separation of X and Y spermatozoa could be detrimental for human embryos. *Hum. Reprod.*, **9**, 758.
- Pieters, M.H.E.C., Geraedts, J.P.M., Meyer, H. *et al.* (1990) Human gametes and zygotes studied by nonradioactive *in situ* hybridization. *Cytogenet. Cell Genet.*, **53**, 15–19.
- Pinkel, D., Glendhill, B.L., Van Dilla, M.A. *et al.* (1982) High resolution DNA measurements of mammalian sperm. *Cytometry*, **3**, 1–9.
- Quinn, P., Warnes, G.M., Kerin, J.F. and Kirby, C. (1984) Culture factors in relation to the success of human *in vitro* fertilization and embryo transfer. *Fertil. Steril.*, **41**, 202–209.
- Reubinoff, B.E. and Schenker, J.G. (1996) New advances in sex preselection. *Fertil. Steril.*, **66**, 343–350.
- Rosenzweig, S. and Adelman, S. (1976) Parental predetermination of the sex of offspring: the attitudes of young married couples with university education. *J. Biosoc. Sci.*, **8**, 335–346.
- Seidel, G.E., Johnson, L.A., Allen, C.A. *et al.* (1996) Artificial insemination with X- and Y-bearing bovine sperm. *Theriogenology*, **1**, 309.
- Trout, S., Bohrer, M., Corsan, G. *et al.* (1995) Is it worthwhile to perform intrauterine insemination of less than one million motile sperm? *American Society of Reproductive Medicine Program Supplement*, Abstract P-082, S132.
- Van Zandt, G. and Fry, C.G. (1983) H33342 staining of mouse bone cells: effects on colony-forming cells. *Cytometry*, **4**, 40–46.
- Vidal, F., Fugger, E.F., Blanco, J. *et al.* (1998) Efficiency of MicroSort flow cytometry for producing sperm populations enriched in X- or Y-chromosome haplotypes: a blind trial assessed by double and triple colour fluorescent *in-situ* hybridization. *Hum. Reprod.*, **13**, 308–312.
- Watkins, A.M., Chan, P.J., Kalugdan, T.H. *et al.* (1996) Analysis of flow cytometer stain Hoechst 33342 on human spermatozoa. *Mol. Hum. Reprod.*, **2**, 709–712.
- World Health Organization (1992) *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. Third Edition. Cambridge University Press, Cambridge, UK.

Received on May 18, 1998; accepted on June 12, 1998