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OUTSTANDING CONTRIBUTION

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

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We report the world's first deliveries of normal babies after use of flow cytometric separated human sperm cells (MicroSort®) for preconception gender selection. 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 and Reubinooff and Schenker, 1996).

Any method for separation of X- and Y-chromosome bearing sperm must rely on an identifiable difference between these living cells. The only currently recognizable distinction between living X- and Y-bearing sperm is their total DNA content, due 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 sperm into two enriched populations based on total DNA difference. Subsequently, pregnancies and births have been reported in the rabbit (Johnson et al., 1989), swine (Johnson et al., 1991), ovine (Morrell and Dresser, 1989), and bovine (Cran et al., 1993) using such flow sorted sperm. Over 400 offspring, all normal, have been born using flow sorting technology including three successive generations in swine (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 sperm. 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 sperm (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, Virginia) for the specific dye and laser, an Institutional Review Board (IRB) approved trial was established to assess flow cytometric separation of X- and Y-bearing sperm 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 less than 50% of the children in that family. Couples were provided written information, consent documents, and personal counsel 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 35C for 30 minutes. Frozen specimens were thawed at room temperature in a laminar flow hood for 15 minutes. 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) 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, sperm were sorted using buffered sheath fluid with either a modified Epics® 753 (Coulter Corporation, Hialeah, FL) or a modified FACS® Vantage (Becton-Dickinson Immunocytometry Systems, San Jose, California). Fluorescence emitted from each sperm cell was detected through a 400nm long pass filter, and the enriched fraction of the sorted sample was collected. The motility and progression of the sorted specimen was evaluated at 35C under paraffin oil using an inverted microscope with Hoffman optics (Leitz, Buntun Instruments, Rockville, MD).

Fluorescence in situ hybridization

Approximately 3,000 to 10,000 sperm 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). Briefly, the sorted cells were washed two times for 6 minutes at 1300g with PBS. The pellet was resuspended in 10 l of PBS and air dried in a 0.5cm² area on a glass slide for 10 minutes at 40C. The dried cells were subsequently fixed three times each with 2, 5, and 10 l of fixative (75% methyl alcohol, 25% acetic acid) respectively. Each slide was washed three times with 40ml of a 2X concentration of SSC (0.3 M NaCl, 30mM sodium citrate) (Vysis, Inc., Downers Grove, IL) at 37C and allowed to air dry. The fixed spermatozoa were treated

with 50mM dithiothreitol (DTT) in 0.1M Tris-HCl (pH 8.0 at room temperature) for 8 to 10 minutes, washed three times with 2X concentration of SSC and air dried at room temperature. The treated slides were denatured simultaneously with 1 l of Vysis Spectrum CEP X orange/Y green probe mixture, 10.5 l Vysis Spectrum CEP Hybridization buffer (Vysis, Inc., Downers Grove, IL) and 3.5 l of distilled water at 75C under a 22 X 22 mm cover glass for 5 minutes. 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 minutes at 42C. The slides were washed with a 0.4X concentration of SSC (pH 7.0) at 75C and counter stained with DAPI (4',6-diamidino-2-phenylindole). The sperm cells were screened using a 60X objective on a Nikon Optiphot-2 (Nikon, Inc., Melville, New York) 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 analyzed by the FITC/Rhodamine filter. Sperm 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 sperm. A total of 200 spermatozoa were 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, California), transferred into 1ml Nunc cryotubes (Nunc, Kamstrup, DK) or into 0.25ml straws (IMV, Minneapolis, Minnesota) and subsequently frozen in a programmable rate freezer (TS Scientific, Perskie, PA) using liquid nitrogen vapor (Mahadevan and Trounson et al., 1984). Frozen sorted specimens were stored at -196C in liquid phase nitrogen.

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

Results

IVF and ICSI

Sperm sorted for the X-bearing chromosome was used for 27 patients in 33 IVF/ ICSI cycles. A total of 7 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 130,000 total motile sperm cells per insemination in 92 patients with an average age of 33.9 years. The average post-MicroSort FISH analysis for XSorts was 84.5% X-bearing sperm. Twenty-two clinical pregnancies occurred in these 208 IUI cycles (10.6% pregnancy rate/cycle). This rate is 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 GIFT 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 were achieved with MicroSort XSorts followed by IUI, IVF, or ICSI. Nine patients have given birth to 11 normal healthy babies, 7 pregnancies resulted in spontaneous miscarriage, there was 1 ectopic pregnancy, and 12 pregnancies are ongoing. Of the 14 pregnancies with known fetal or birth gender, 13 had only female

conceptions (92.9%). 88.2% (15/17 including three sets of twins) of the fetuses were 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 intrauterine insemination. 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 hemophilia, 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 pre-implantation embryo testing represents an even more powerful tool for increasing the number of unaffected female embryos conceived, and reducing or eliminating the transfer to the uterus of male embryos (manuscript in preparation).

Current MicroSort technology provides an average of approximately 85% X-bearing sperm in the enriched sorted sample as determined by FISH analysis. This increase would be expected to make it approximately 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 substantially increase the probability of having a daughter.

Several characteristics specific to human sperm cells present unique challenges for FCS compared to domestic animal sperm. Some of the issues that affect FCS include the percent total DNA difference between X- and Y-bearing sperm, 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 sperm compared to 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 sperm are more oval and highly variable morphologically than the relatively paddle shaped bull sperm, 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 to approximately 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 sperm also present a comparatively high level of heterogeneity within and between individuals.

Pregnancies after intrauterine insemination using less than 1 million motile sperm cells have been obtained in the bovine (Seidel et al., 1996) and the human (Trout et al., 1995 and Centola, 1997). Intrauterine insemination using 200,000 to more than 200 million motile sperm in 1761 cycles have showed no significant differences between the number of sperm cells inseminated and pregnancy rate (Cressman et al., 1996). Current MicroSort technology results in reduced number of sperm cells compared to the quantity usually used for medical

IUIs, but still readily achieve many pregnancies. This is consistent with the observation that men with Kallman's syndrome who have greatly reduced sperm counts but functionally normal sperm 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 enhance the number of normal motile cells prior to FCS. 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 million or more cells generally result in approximately 100,000 to 300,000 motile cells for IUI. Smaller number of sperm can be used, of course, for ICSI or IVF.

Concerns regarding the use of UV and Hoechst 33342 (bisbenzimidazole) for FCS of human sperm cells have been expressed (Ashwood-Smith, 1994 and Munne, 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 is reversible at 37°C. Hoechst 33342 absorbs at 358nm, a substantial distance from the maximum DNA absorption of approximately 260nm, excites at 367nm and emits at 461nm. While some somatic cells have been found to be sensitive to bisbenzimidazole staining (Van Zandt and Fry, 1983; Durand and Olive, 1982), 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 M bisbenzimidazole as assessed by PCR analysis of the B-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 UV exposure during passage through a fluorescence activated cell sorter. Concerns regarding embryo development in the bovine (Cran et al., 1994) and rabbit (McNutt and Johnson, 1996) were not observed in our human experience that resulted in a 92% embryo cleavage rate (unpublished data) and 21.2% pregnancy rate per transfer cycle. Most important, as in the animal data in several species with hundreds of normal births, all offspring born from FCS of human sperm 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-enrichment from 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 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 and Markle and Nam, 1982). Our experience with couples who actually inquired about gender selection indicates that 53.6% (675/1259) of such inquirers 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 Great Britain. The majority of couples (90.5%) in our study were seeking gender preselection for family balancing purposes, were in their mid 30's, had 2-3 children of the same sex, and desired only one more child.

In summary, a significant number of individual normal births in humans have 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.

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