Article

Separation of X- and Y-bearing human spermatozoa by sperm isolation medium gradients evaluated by FISH

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Abstract

An eight-layer discontinuous sperm isolation medium (PureSperm gradient) was evaluated in separation of human spermatozoa according to sex chromosomes by fluorescence in-situ hybridization (FISH). This study was carried out on spare samples from normozoospermic and oligozoospermic patients referred to the Royan Institute for infertility treatment. Semen analysis was assessed according to World Health Organization criteria. X- and Y-bearing spermatozoa were simultaneously identified in the neat semen (control) and sperm isolation medium fractions from the same samples using FISH and chromosome specific DNA probes. At least 1000 spermatozoa were scored for each sample. The proportions of X- and Y-bearing spermatozoa were determined by presence of red or green fluorescent signals. Before separation, there was no significant difference in the percentage of spermatozoa with the specific signals of X and Y chromosomes. After separation, in both normal and oligozoospermic patients, the percentage of X-bearing spermatozoa in the bottom layer slightly exceeded that in the top layer ($P \leq 0.001$). In both the normal and oligozoospermic groups, the difference between the frequencies of Y-bearing spermatozoa in the top and bottom layers was significant ($P \leq 0.001$). It seemed that eight-step discontinuous gradient was not a reliable method for the separation X- and Y-bearing spermatozoa for clinical purposes.

Keywords: *FISH, PureSperm, sex chromosome, spermatozoa*

Introduction

After the discovery of X and Y spermatozoa in the 1920s (Painter, 1921), several techniques have been developed to separate these cells according to their characteristics. Since the 1970s, when Ericsson *et al.* (1973) reported enrichment of Y-bearing human spermatozoa using an albumin gradient, there has been great interest in preconception sex selection.

Methods such as discontinuous albumin gradients (Ericsson *et al.*, 1973), Percoll gradient (Kaneko *et al.*, 1984) and swim-up procedure (Check and Katsoff, 1993) have been reported to separate X-and Y-bearing spermatozoa, but none of these methods was capable of altering the sex ratio. Only flow cytometry (FCM), which is based on the 2.8% total DNA content difference between X- and Y-bearing spermatozoa, has been proven to be effective (Johnson *et al.*, 1993). The first human pregnancy using MicroSort (Genetic and IVF Institute, Fairfax, VA, USA) combined with preimplantation embryo genetic testing for the prevention of X-linked hydrocephalus was reported by Levinson *et al.* (1995), and a clinical trial showed the safety and effectiveness of this method for preconception gender selection (Schulman and Karabinus, 2005).

Since the introduction of sperm-sexing technology, several laboratory validation methods have been developed to verify

the purity of sorted cells. In early studies, the efficiency of sperm separation was evaluated using quinacrine staining, but later a number of investigations showed that this method was not reliable (Van Kooij and Van Oost, 1992). The hamster egg penetration assay was also used to determine the type of sex chromosome in human spermatozoa (Brandriff *et al.*, 1986). However, this method was time consuming and only those spermatozoa that penetrated zona-free hamster eggs could be karyotyped. In recent years, fluorescence in-situ hybridization (FISH) has been the most effective method for evaluation of sorted spermatozoa and studying aneuploidy (Guttenbach and Schmid, 1990; Wyrobek *et al.*, 1990). This technique involves the hybridization of chromosome-specific DNA probes labelled with fluorochromes to complementary DNA sequences on the target chromosome. This highly sensitive and specific method permits the study of much larger numbers of spermatozoa in less time than other techniques.

The need for effective sperm preparation in the assisted reproductive techniques led to the use of density gradient separation protocols for removal of immotile spermatozoa, leukocytes, epithelial cells, particulate debris, microbial contamination (Shyam *et al.*, 2005) and even apoptotic spermatozoa with the aid of additional methods such as magnetic activated cell sorting (Said *et al.*, 2006). Using these density gradients for sorting X- and Y-bearing spermatozoa is an idea that has been investigated by different researchers.

Using the FISH technique, it was demonstrated that albumin gradient (Wang *et al.*, 1994a; Flaherty and Mathews, 1996), Sephadex column (Vidal *et al.*, 1993) and swim-up techniques (Han *et al.*, 1994) did not significantly affect the proportion of X- to Y-bearing spermatozoa. A discontinuous Percoll gradient did appear to alter this ratio, although the degree of enrichment was insufficient for clinical use (Wang *et al.*, 1994b).

In 1995, Percoll was removed from the market for clinical use because of the high concentration of endotoxins (Svalander *et al.*, 1995) and new density gradient media such as PureSperm (Nidacon, Goteborg, Sweden) and Ixaprep (Medicult, Copenhagen, Denmark), which contained lower concentrations of endotoxins, were developed.

In the present study, two-colour FISH was used to determine the X- and Y-bearing human spermatozoa before and after applying the PureSperm discontinuous density gradient system.

Materials and methods

Sperm preparation

Semen samples were obtained from two groups: the first group included three donors with normal parameters and the second group included three donors diagnosed as oligozoospermic with a sperm concentration of 10–15 × 10^6/ml, with almost normal motility and morphology according to World Health Organization criteria (WHO, 1999). All of the donors were selected from patients who attended the clinical laboratory of the Royan Institute and their spare samples were used for this study. After liquefaction, each sample underwent manual semen analysis.

Each sample was divided into two aliquots. One aliquot (control) was immediately prepared for double-colour FISH, whereas the other aliquot was applied to an eight-step discontinuous sperm isolation gradient. After evaluating the quality and quantity of the spermatozoa retrieved from the top and bottom layers, they were subsequently prepared for double-colour FISH.

Discontinuous gradients

Different concentrations of discontinuous sperm isolation medium with an osmolality of 300–310 mOsm/kg H_2O were prepared by diluting the medium with Ham's F10: human serum albumin (HSA) 9:1 (pH = 7.2). The eight-step discontinuous gradient was prepared in a conical centrifuge tube (Falcon, USA) by consecutively layering 1 ml of each concentration (35–84%). In order to prepare a 1 ml layer of 84% density, 840 µl of medium was diluted with 160 µl of Ham's F10:HSA. The aliquots of semen (1 ml) were overlaid on the 8 ml of eight-layer density gradient and centrifuged at 200 *g* for 8 min. By using a Pasteur pipette, the upper and lower layers were carefully aspirated, then centrifuged at 800 *g* for 5 min to separate the medium from the sperm pellet. The sperm pellets were disaggregated in phosphate-buffered saline (PBS; pH = 7.4), washed three times and centrifuged at 800 *g* for 5 min. The control samples without any treatment were also washed three times with PBS. The pretreated and control samples were fixed in methanol–acetic acid (3:1) and stored at –20ºC for 20 min. The sperm suspensions were placed on microscope slides for smear preparation, air dried and stored at –20ºC until used for FISH.

Pretreatment of sperm nuclei

The sperm nuclei were decondensed by incubating the slides at room temperature in 0.01 mol/l dithiothreitol (DTT, Sigma, USA) in 0.1 mol/l Tris–HCL (pH = 8) for 5 min and then in 0.01 mol/l LIS (3,5-diiodosalicylic acid, lithium salt) for 10 min. In the final step, the slides were incubated for 10 min in 0.001 mol/l DTT solution in 0.1M Tris (pH:8). After washing in PBS, the slides were placed in 2× saline sodium citrate (SSC) for 10 min at 37ºC, rinsed with PBS and dehydrated in an ethanol series.

FISH

A mixture of directly labelled probes consisting of X(DXZ1) red and Y(DYZ3) green (Qbiogene Inc., USA) were used for this study. The probe mixtures (10 µl) was added to the slide under a cover slip (20 × 20 mm) and sealed with rubber cement. Spermatozoa and probe DNA were co-denatured simultaneously for 5 min at 75ºC. The slides were then incubated in a moist chamber at 37ºC for 4 h to allow hybridization of the DNA probes.

The post-hybridization wash was performed in 0.4× SSC/0.3% NP40 at 73ºC for 2 min, followed by washing in 2× SSC/0.1% NP40 at room temperature for 1 min. Slides were counterstained with 10 ml of 4(,6-diamidino-2-phenylindole) (DAPI) mixed with antifade (Cytocell Technologies Ltd, UK).

Nuclei and signals were examined using a fluorescence microscope (Nikon E800, Japan) equipped with a triple-band pass filter for DAPI/spectrum green/spectrum orange.

Signal and data analysis

Only intact, undamaged and non-overlapped nuclei were scored. X and Y chromosomes in sperm nuclei were recognized by a green or orange fluorescent spot respectively. At least 1000 spermatozoa were scored from each sample.

All statistical analyses were performed using the Statistics Package Social Sciences SPSS.13 (SPSS, USA). Proportions of X- to Y-bearing spermatozoa in each group were compared using the chi-squared test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Hybridization efficiencies of the X and Y probes in all cases were more than 98%. The frequencies of X and Y signals in the control and top/bottom layers of the discontinuous sperm isolation medium were compared in the normal and oligozoospermic groups (**Tables 1** and **2**). There were no inter-individual variations in either group.

Processing of the spermatozoa with discontinuous sperm isolation medium changed the semen characteristics. In the normal group, the sperm concentration in the bottom layer (84%) reduced approximately 35–47% and the percentage motility increased by a factor of 1.4–1.7. In the oligozoospermic group, the sperm concentration in the bottom layer (84%) showed 42–60% reduction and the percentage of motility increased by a factor of 1.2–1.5. The percentage motility in the top layer (35%) in both groups showed a significant reduction compared with the bottom layer ($P = 0.027$) and the control group (($P = 0.013$; data not shown).

In the normal group, a total of 9916 spermatozoa in the control samples and 14,533 spermatozoa in the top ($n = 7822$) and bottom ($n = 6711$) layers of the gradient fractions were examined for the presence of X- and Y-bearing spermatozoa. In this group the ratio of X:Y in the control group (51.2:48.8) compared with the top layer (49.1:50.9) and also between the top and bottom layer (51.9:48.1) was significantly different ($P = 0.006$ and 0.001 respectively). The ratios between the control and bottom layer were not significantly different.

In the oligozoospermic group, in total 6265 spermatozoa in the control samples and 2632 and 4034 spermatozoa respectively for the top and bottom layers of the gradient were examined for the presence of X- and Y-bearing spermatozoa. In this group, the ratios of X:Y between the control (48.4:51.6) and top (41.3:58.7)/bottom (50.7:49.3) layers as well as between the top and bottom layers were significantly different ($P \leq 0.001$, $P = 0.027$ and $P \leq 0.001$ respectively).

In both groups there were significantly more X-bearing than Y-bearing spermatozoa in the bottom layer. The number of Y-bearing spermatozoa in the top layer compared with the bottom layer was significantly higher in both the normal and oligozoospermic groups ($P \leq 0.001$), and this difference appeared to be greater in the oligozoospermic group (9.0 versus 2.8%).

Discussion

Female carriers of X-linked disease transmit the genetic disorder to half of their male offspring. Preconception gender selection plays one important role in reducing the risk of having male children afflicted with a serious or fatal X-linked genetic abnormality. Although flow cytometric sperm sorting is the most reliable preconception method of gender selection (Schulman and Karabinus, 2005), the need for expensive instruments prevents its widespread use in reproductive clinics. On the other hand, preimplantation genetic diagnosis for gender selection may result in a lack of embryos with the desired sex. Using a density gradient separation method is a cheap alternative to flow cytometric sperm sorting and can be implemented by reproductive clinics. Most of the published papers in this field involved Percoll and albumin gradient, Sephadex columns and the swim-up method with a diversity of results that is mainly due to using different and sometimes unreliable methods of sexing spermatozoa. For example, Iizuka *et al.* (1987) reported an enriched (94%) population of X-bearing spermatozoa in the 80% Percoll layer, whereas Wang *et al.* (1994a) reported just 57% enrichment. Apparently the main reason for this great difference was the use of quinacrine staining by Iizuka *et al.* (1987). In addition, early studies suggested that albumin gradients enriched Y-bearing spermatozoa (Ericsson *et al.*, 1973; Quinlivan *et al.*, 1982), but

Table 1. Frequencies of X- and Y-bearing spermatozoa in the control (neat semen) and bottom/top layers of an eight-step discontinuous PureSperm gradient in the normospermic group (determined by fluorescence in-situ hybridization).

Sample	*Total no. of spermatozoa examined*	*No. with Y chromosome (%)*	*No. with X chromosome (%)*
Control	9916	4843 (48.8)	5073 (51.2)
Bottom	6711	3226 (48.1)	3485 (51.9)[a]
Top	7822	3983 (50.9)[b]	3839 (49.1)

[a]Significantly higher in bottom layer compared with top layer ($P = 0.001$).
[b]Significantly higher in top layer compared with bottom layer and control ($P = 0.001$ and $P = 0.006$ respectively).

Table 2. Frequencies of X- and Y-bearing spermatozoa in the control (neat semen) and bottom/top layers of eight-step discontinuous PureSperm gradient in the oligozoospermic group (determined by fluorescence in-situ hybridization).

Sample	*Total no. of spermatozoa examined*	*No. with Y chromosome (%)*	*No. with X chromosome (%)*
Control	6265	3230 (51.6)	3035 (48.4)
Bottom	4034	1990 (49.3)	2044 (50.7)[a]
Top	2632	1544 (58.7)[b]	1088 (41.3)

[a]Significantly higher in bottom layer compared with top layer and control ($P \leq 0.001$ and $P = 0.027$ respectively).
[b]Significantly higher in top layer compared with bottom layer and control ($P \leq 0.001$).

single (Vidal *et al.,* 1993) and double (Wang *et al.*, 1994b) label FISH showed no enrichment for Y-bearing spermatozoa.

So far as is known, there are no reports for the efficacy and efficiency of the discontinuous sperm isolation medium method in sperm sorting of sex chromosomes in addition to removal of debris and immotile spermatozoa. The present hypothesis was based on the retrieval of an enriched population of X-bearing spermatozoa from the 84% gradient layer. Indeed, a significant increase in X-bearing spermatozoa was found in 84% gradient fractions in both normospermia and oligozoospermia groups, although the percentages of X-bearing spermatozoa never exceeded 53.6% in any of the samples. In the oligozoospermic group, the difference between the frequencies of Y-bearing spermatozoa in the top and bottom layers appeared to be greater than that in the normal group. It seemed that the discontinuous sperm isolation method, like other density gradients, did not enrich the X-/Y-bearing spermatozoa to a concentration that could be used for preconception sex selection.

It is probable that those theories based on faster swimming of small and lighter male spermatozoa, as well as migration in different density gradients *in vitro*, do not apply to density gradients (Ericsson *et al.*, 1973; Kaneko *et al.*, 1986). Further studies are needed on the biology of X- and Y-bearing spermatozoa, such as surface charges on spermatozoa and different DNA content, in order to find suitable gradients for sperm sorting in clinical situations.

In conclusion, the results indicate that the PureSperm 8-step gradient enriches X-bearing spermatozoa in the 84% fraction, but is of limited use for preconceptional sex selection.

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