Separating X-Bearing Human Spermatozoa Through a Discontinuous Percoll Density Gradient Proved to Be Inefficient by Double-Label Fluorescent In Situ Hybridization

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Purpose: Double-label fluorescence in situ hybridization (FISH) was used to evaluate the efficiency of separating X- and Y-chromosome-bearing spermatozoa through 12-step discontinuous Percoll gradients.

Methods: Liquefied normal semen samples from 10 healthy donors were overlaid onto 25% Percoll and centrifuged. Parts of the sperm pellet were saved as control, while the remaining portion was separated by 12-step Percoll gradient. After centrifugation, the spermatozoa in the 80% Percoll layer were collected. The X:Y ratio of the control and separated spermatozoa was verified by double-label FISH (CEP SOX/SGY probes) and scored blindly by one observer. Differences in the X:Y ratios between matched groups were analyzed by paired *t* tests.

Results: The overall average labeling efficiency was 99.2%. A significant enrichment ($P = 0.02$) of X-bearing spermatozoa was obtained in Percoll separated fractions (mean X:Y ratio = 52.2:46.4) compared with the control group (X:Y ratio = 49.5:48.4). Discontinuous Percoll gradients also decreased the proportion of aneuploid spermatozoa (from 1.0 to 0.8%), but the differences were nonsignificant.

Conclusions: Discontinuous Percoll separation did increase the X:Y ratio significantly, but the enrichment of X-bearing spermatozoa is insufficient for clinical use in preconceptional sex selection.

KEY WORDS: fluorescence in situ hybridization; Percoll; X-sperm preselection.

INTRODUCTION

At present, more than 200 X-linked recessive diseases have been reported (1). These disorders, such as hemophilia, certain muscular dystrophies, Hunter's syndrome, etc., are transmitted by heterozygote female carriers and affect half of their male offspring. The separation of Y-bearing from X-bearing spermatozoa could prevent the birth of affected male offspring, if the separation procedures are reliable and reveal that the number, viability and fertilization capacity of the spermatozoa obtained are adequate (see Ref. 2 for review).

Methods using Sephadex columns (3,4), discontinuous Percoll gradients (5,6), and flow cytometry (7,8) have been reported to enrich viable X-bearing sperm to a considerable extent. Among these sex preselection procedures, the 12-step discontinuous Percoll density gradient method described by Iizuka *et al.* (6) gives the most dramatic results: up to 94% of X-bearing sperm populations can be purified from 12-step discontinuous Percoll gradient. Iizuka *et al.* used quinacrine mustard to evaluate separation. Fluorochrome quinacrine stains the F-body at the long arm of the Y chromosome, allowing differentiation of Y- from X-bearing spermatozoa (9,10). Using these separated spermatozoa for artificial insemination was reported to result in the birth of six female infants in six pregnancies (6). Since then, Percoll density gradients have been widely used in attempts to avoid male offspring with X-chromosome linked genetic disorders or for other social purposes.

However, the quinacrine mustard staining method has proved to be unreliable, since it detects a fluorescent spot in only 25 to 50% of male diploid cells (11) even though 100% of these cells contain a Y-chromosome. These underestimates, coupled with the

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false positive and false negative rate reported by Thompson and Niebruhr (12), suggest that the F-body test cannot accurately estimate the number of Y-chromosome-bearing cells. Because doubt is thus cast on the scoring method used by Iizuka *et al.*, their initial results (94% of X-sperm enrichment) require reconfirmation by a more specific method.

Since then, the feasibility of enriching X-bearing sperm with discontinuous Percoll gradient separation has been further investigated by several groups, but with controversial results (13–15). van Kooij and van Oost (13) could not demonstrate any difference by Southern blot analysis in the sex ratio of spermatozoa obtained from Percoll separated fractions. Using indirect double-label fluorescent in situ hybridization (FISH), Wang *et al.* (14) concluded that Percoll centrifugation is of limited use for preconceptional sex selection, while Tohyama *et al.* (15) demonstrated a dramatic enrichment of X-bearing sperm after Percoll centrifugation separation and recommended it for clinical application.

To clarify these conflicts and to evaluate the actual effectiveness of 12-step discontinuous Percoll gradients in separating X-chromosome-bearing sperm cells for clinical sex preselection, we used a sensitive and highly accurate direct double-label FISH technique to investigate the ratio of X- and Y-bearing spermatozoa before and after Percoll separation.

MATERIALS AND METHODS

Semen Samples

Semen samples used in this experiment were obtained from 10 healthy donors undergoing routine semen analysis in an in vitro fertilization (IVF) program. The parameters of all semen samples were normal according to World Health Organization criteria (1993). The mean $\pm$ standard deviation values for the 10 samples were as follows: sperm volume, 3.08 ± 1.2 ml; concentration, $116.8 \pm 73 \times 10^6$ /ml; and motility, $69.2 \pm 13.2\%$.

Discontinuous Percoll Gradient Centrifugation

The procedure described by Iizuka *et al.* (6) was followed in this study. Briefly, Percoll (Pharmacia, Sweden) was made isotonic (100%) by adding 1 vol of 0.1 M HEPES-buffered 1.5 M NaCl, pH 7.4, to 9 vol of Percoll. Various concentrations of Percoll (25 to 80% in 5% stepwise concentrations) were prepared

by diluting the resulting isotonic Percoll solution with 0.01 M HEPES-buffered human tubal fluid (HTF) medium (HEPES-HTF). Discontinuous 12-step Percoll gradients were prepared in round-bottomed nitrocellulose tubes by consecutively layering 0.7 ml each of 80 to 30% Percoll solutions.

Less than 2 ml of each liquefied semen sample was first washed through 4 ml of 25% Percoll and centrifuged in a swing-out rotor at 250g for 20 minutes. Parts of the precipitated sperm pellets (control) were immediately prepared for double-label FISH analysis. The remainder were then resuspended in 0.7 ml of 25% Percoll and placed as the topmost layer of the discontinuous density gradient prepared as described. After centrifugation at 250g for 30 min, 0.3–0.5 ml of the 80% Percoll layer was collected through a pin hole in the bottom of the tube.

Pretreatment of Spermatozoa

The DNA of spermatozoa is tightly compacted, but FISH requires denaturation of the target cell's chromosome DNA before hybridization with specific nucleic acid probes. Therefore, an accurate FISH result in spermatozoa is highly dependent on proper decondensation of the sperm chromatin before hybridization.

Both the Percoll-treated and the control spermatozoa were treated by a modified Martini and co-workers' method (16) to decondense chromatin as follows. The sperm samples were diluted to 3 ml with HEPES-HTF medium and centrifuged at 175g for 10 min. The concentrated spermatozoa were spread on clean slides and allowed to air-dry, followed by incubation for 10 min in fresh, cold fixative (ethanol:methanol, 3:1). After being washed with $2\times$ standard saline citrate (SSC) to remove the remaining fixative, the slides were incubated in decondensation solution [1 M Tris-HCl buffer, pH 9.5, containing 25 mM dithiothreitol (DTT)] for 30 min at room temperature. When the decondensation step was completed, the slides were washed once in $2\times$ SSC, once in phosphate-buffered saline, and finally dehydrated through an ethanol series (70 to 85 to 95%) and air-dried.

Fluorescent In Situ Hybridization

A pair of sex chromosome-specific DNA probes (CEPSOX/SGY; Vysis, USA) with directly labeled fluorescent dye was used to determine the ratio of X- and Y-bearing spermatozoa. A standard overnight hybridization procedure was performed according to the manufacturer's specifications. After five stringent

washing steps, all slides were dehydrated through an ethanol series, air-dried, and mounted with a glycerol: phosphate-buffered saline mixture containing DAPI II (4,6-diamidino-2-phenylindole; Vysis) as a nuclear counterstain reagent. Five hundred spermatozoa of the control and Percoll-separated specimens from each donor were scored blindly by one observer using an Olympus microscope with epifluorescence and DAPI/fluorescein isothiocyanate/propidium iodide triple filter blocks. Only spermatozoa with unique tails and without any overlapping were counted. Nuclei with more than one separate signal were scored as aneuploid cells.

Statistical Analysis

Differences in the X:Y ratios and the frequencies of aneuploid spermatozoa between matched control and Percoll fractions were analyzed by paired *t* tests. The level of significance was defined as *P* less than 0.05.

RESULTS

As shown in Table I, 12-step discontinuous Percoll centrifugation selected highly motile and morphologically normal spermatozoa. The results of double-label FISH analysis from both groups are summarized in Table II. The average labeling efficiencies were 99.1% in controls (range, 98.2 to 100%) and 99.3% in Percoll fractions (range, 97.4 to 100%).

Based on these high labeling efficiencies, we demonstrated a significant enrichment of X-bearing spermatozoa in discontinuous Percoll-separated fractions (*P* = 0.02; paired *t* test). The X- to Y-bearing sperm ratio varied from 0.74 to 1.21 (mean, 1.03 ± 0.15) in control fractions. After 12-step discontinuous Percoll centrifugation, the average ratio of X- to Y-bearing spermatozoa increased to 1.13 ± 0.1 (range 0.99–1.28). The absolute values of the enrichment were quite

small, and two semen samples even had a slightly decreased ratio of X- to Y-sperm after Percoll separation compared with the control group (from 1.21 in controls to 1.19 and 1.18 in the separation groups, respectively).

We also found a decrease in aneuploid spermatozoa (from 1.0% in controls to 0.8%) in the Percoll-treated samples, but this did not reach statistical significance.

DISCUSSION

After Iizuka and co-workers' (6) first report that centrifugation of spermatozoa through 12-step Percoll gradients yielded enriched (94%) populations of X-bearing spermatozoa, as determined by the inadequate quinacrine scoring method, several other studies were performed to determine accurately by a more specific molecular-based evaluation method, the ratio of X- to Y-bearing spermatozoa resulting from Percoll separation (13–15). Using Southern blot analysis, a 1:1 ratio of X- to Y-bearing spermatozoa was obtained after centrifuging semen through 12-step Percoll gradients, despite 86% of the spermatozoa being scored as X-bearing after quinacrine staining. This suggests that Percoll might interfere with quinacrine staining and lead to falsely elevated frequencies of X-bearing spermatozoa (13).

Recently, Wang *et al.* (14) demonstrated a significant increase in X- to Y-bearing spermatozoa, from 49.0:48.2 in controls to 55.1:44.1 in Percoll separation fractions (*P* < 0.0001), evaluated by indirect double-label FISH and multistep immunofluorescent visualization. Because the percentage of X-bearing sperm in the Percoll fractions was only 55.1%, they concluded that this method is of limited use for preconceptional sex selection. Using a similar evaluation method, however, Tohyama and colleagues (15) reported a dramatic enrichment of the X-bearing sperm ratio obtained from Percoll fractions ($75.1 \pm 13.7\%$; range, 63.9–93.4%) compared with neat semen controls ($49.8 \pm 1.7\%$). They suggest that, for clinical application, human X-bearing sperm can be adequately separated by 12-step Percoll density gradient centrifugation.

In the present study, a highly sensitive and accurate direct double-label FISH method was used to determine X- and Y-bearing sperm before and after 12-step Percoll separation. The overall labeling efficiencies were as high as 99.2% in our FISH analysis. Based on such a highly reliable method, we were unable to demonstrate a dramatic enrichment of X-bearing sperm after 12-step Percoll separation, in contrast to the result

Table I. Motile and Morphologically Abnormal Sperm (%) Before and After X and Y Spermatozoa Separation by Discontinuous Percoll Gradients

	Motile sperm (%)		Atypical sperm (%)	
	Control	Percoll	Control	Percoll
Mean (<i>n</i> = 10)	70.5	89.1	15.3	7.6
SD	11.0	8.1	3.1	2.3
<i>P</i> value	0.0001		0.006	

Table II. Labeling Efficiency, Haploid X:Y Spermatozoa Ratios, and Percentage of Aneuploid Spermatozoon (XX, YY, or XY) in Controls and in 80% Percoll Fractions

Semen sample ^a	Control fraction				12-step discontinuous Percoll separation			
	% labeled	X:Y sperm (%)	X/Y	Aneuploid (%)	% labeled	X:Y sperm (%)	X/Y	Aneuploid (%)
1	99.6	52.6:46.6	1.13	0.4	99.6	55.6:43.4	1.28	0.6
2	99.0	41.4:56.0	0.74	1.6	97.4	48.4:48.8	0.99	0.2
3	99.6	51.8:46.8	1.11	1.0	99.8	52.4:46.8	1.12	0.4
4	98.6	53.2:43.8	1.21	1.6	99.0	53.6:45.2	1.19	1.4
5	99.6	49.4:50.2	0.98	0	99.8	50.2:48.2	1.04	1.4
6	98.4	46.2:49.0	0.94	2.2	99.4	53.8:44.6	1.21	1.2
7	100.0	51.4:48.0	1.07	0.6	100.0	53.6:45.0	1.19	1.2
8	98.4	48.8:47.4	1.02	0.6	98.2	49.6:48.2	1.03	0.4
9	98.2	53.6:44.2	1.21	0.4	99.6	53.8:45.6	1.18	0.2
10	99.8	46.8:51.8	0.90	1.2	100.0	51.0:48.4	1.05	0.6
Mean	99.1	49.5:48.4	1.03 ^b	1.0	99.3	52.2:46.4	1.13 ^b	0.8
SD	±0.7		±0.15	±0.7	±0.9		±0.10	0.5

^a Five hundred spermatozoa were calculated per sample.

^b $P = 0.02$ (paired t test) in comparison with the control semen value.

of Tohyama *et al.* (15). Because Tohyama's full paper is not yet available, we cannot evaluate the reason for our contrasting results. Because our data are compatible with Wang and co-workers' (14), we agree with their conclusion that Percoll gradient centrifugation technique cannot enrich X-bearing sperm to a medically significant degree.

Furthermore, to investigate the exact efficiency of X-bearing sperm enrichment after 12-step discontinuous Percoll centrifugation (rather than the effect of a 25% Percoll wash plus 12-step Percoll separation), we used spermatozoa washed in 25% Percoll as a control instead of neat semen samples as used in the Wang *et al.* and Tohyama *et al.* studies (14,15). Therefore, our lower degree of enrichment might imply that the 25% Percoll wash step may produce some preliminary separation. We did confirm that the 12-step discontinuous Percoll centrifugation process can significantly enrich X-bearing sperm, but the degree of enrichment was slight. Since an X- to Y-bearing sperm ratio of at least 80:20 or 20:80 is required to ensure a significant alteration in sex ratio at birth (17), the 52.2% of X-bearing spermatozoa resulting from 12-step Percoll separation seems insufficient to use in preselecting female offsprings.

There are several possible reasons for the favorable female offsprings achieved in Iizuka and co-workers' study (6), using Percoll-separated spermatozoa for intrauterine insemination. First, in their study, the Percoll centrifugation may in fact have significantly altered the ratio of X- and Y-bearing spermatozoa. However, based on our data, we do not believe this is likely. Second, X- and Y-bearing sperm may acquire differing fertilization abilities after Percoll separation.

That is, Percoll separation may reduce the fertilization ability of Y-bearing sperm. Finally, fertilized eggs inseminated by Percoll-separated X- and Y-bearing sperm may have different developmental and implantation potential. Although we cannot monitor the implantation rate of male and female human embryos, the sex ratio of fertilized egg inseminated by Percoll-separated sperm and the cleavage rates of male and female embryos could still be determined. These questions are now being investigated in our laboratory by inseminating donated human oocytes with discontinuous Percoll gradient-separated spermatozoa and analyzing the sex ratio of fertilized eggs and early embryos.

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