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Lack of Significant Morphological Differences Between Human X and Y Spermatozoa and Their Precursor Cells (Spermatids) Exposed to Different Prehybridization Treatments

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ABSTRACT: Human X and Y spermatozoa were previously compared by several nonmolecular techniques. Recent studies show that in many of the previous investigations, the methods used to identify the spermatozoa were nonspecific and thus produced contradictory findings. In the present study, the comparison of the 2 germ cell types, X and Y, were performed following fluorescence in situ hybridization (FISH), which is the most reliable genotyping technique currently available. The FISH technique was performed under 3 different treatments: permeabilization with liquid N_2, fixation with Carnoy's, and chromatin decondensation with lithium di-iodosalicylate. Mature and immature germ cells (spermatozoa and spermatids) were compared. Lithium showed higher hybridization efficiency, while liquid N_2 and Carnoy's fixative maintained better morphological integrity of cells with lower hybridization. The sperm exhibiting hybridization signals were not different in any of the morphometric or qualitative comparisons from those that did not exhibit signals. No significant deviation of the sex ratio from 1:1 was seen in either the mature or immature germ cell population. The spatial distribution of X and Y chromosome-specific signals in the sperm head were identical. The hybridization treatments did not have any preferential effect on the cells of specific genotype (X or Y). Neither head parameters (length, HL; width, HW; area, HA) nor tail length (TL) significantly differed between X and Y populations of spermatozoa under any of the treatments. Similarly, the haploid, X-specific round cells did not differ from Y-specific ones by their size (diameter) and shape. These results indicate that neither mature sperm nor their precursors possess significant morphological differences between X and Y genotypes.

Key words: Spermatid, FISH technique, morphological comparison.
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The discovery in the 1920s of the existence of X and Y spermatozoa indicated the potential benefits of separation of these gender-determining gametes (Cui, 1997a; Amann and Seidel, 1982). Since that time, attempts have been made to develop techniques and procedures to isolate and identify sex chromosome-specific sperm. None of the isolation methods developed so far has claimed complete success (Botchan et al, 1997; De Jonge et al, 1997; Hossain et al, 1998). Only the enrichment of 1 genotype over the other (X over Y or vice versa) was achieved to some extent by some isolation methods (Botchan et al, 1997; Hossain et al, 1998). Nevertheless, it is generally agreed that if morphological differences between X and Y spermatozoa could be accurately assessed, this would help to refine the effectiveness of the isolation techniques for X and Y populations (Cui, 1997a). The DNA content difference between X and Y sperm is obvious because of the size difference between X and Y chromosomes, and there is no dispute about this issue (Sumner et al, 1971; Johnson and Schulman, 1994). On the other hand, as reported in the literature, there is contradictory information regarding the morphological differences of mature X and Y spermatozoa, and this has been an issue of scientific debate over the years (Cui and Mathews, 1993; Cui, 1997b; Geraedts, 1997).

Difficulty has also been experienced in accurately identifying the identity (X or Y type) of spermatozoa (Windsor et al, 1993; Flaherty and Mathews, 1996). Most of the identifying methods, including the widely used ones such as ''barr body'' or ''F body'' techniques were found to be nonspecific and unreliable (Windsor et al, 1993). DNA-based methods, which were developed in recent times, produced reliable results in identifying X and Y spermatozoa (Flaherty and Mathews, 1996). Such methods include the polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) techniques (Flaherty and Mathews, 1996; Cui, 1997a). With PCR, lysis of the cell is required,

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whereas with FISH, intact sperm can be visualized along with their genotypic tag (fluorescing X and Y chromosomes). The FISH method of identifying spermatozoa in their reasonably intact state created a potential opportunity for morphological comparisons of X and Y types. Application of FISH to sperm cells requires pretreatment in order to decondense chromatin before the actual procedure can be performed (Chevret et al, 1994; Spriggs et al, 1995). A large variety of such pretreatments have been reported that differ from one another and, therefore, affect the spermatozoa in mechanistically different ways (Spriggs et al, 1995). In the present study we have compared the X and Y populations under some of the commonly used prehybridization treatments (Martin et al, 1991). Furthermore, to complement the comparison of mature X and Y spermatozoa, we performed similar investigations on their precursor cells (spermatids). Inclusion of the precursor cells in the comparison of X and Y sperm populations make our investigation uniquely different from previous investigations that addressed the issue of morphological differences of X and Y.

Materials and Methods

Study Design and Experiments

Ejaculated semen specimens (n = 5) and testicular biopsy specimens (n = 3) underwent FISH followed by morphological evaluations. Smears were prepared on glass slides using semen sperm, Percoll-washed sperm, and spermatids recovered from biopsy (Hossain et al, unpublished data). Before performing FISH, the slides were pretreated with one of the following: liquid nitrogen (LN), fixative (FIX), lithium di-iodosalicylate (LIS), or no treatment (NT). The LN treatment involved immersing the slide into liquid N_2 for 1 minute followed by thawing and raising the temperature to 37°C for 5 minutes for 10 consecutive cycles. The FIX method involved a 30-minute exposure of the slide to Carnoy's fixative (Martin et al, 1991; Goldman et al, 1993). In the LIS group, sperm were treated with dithiothreitol for 20 minutes followed by lithium di-iodosalicylate for 20 or 180 minutes at ambient temperature (Spriggs et al, 1995). Three thousand three hundred spermatozoa and 520 round cells, processed by these prehybridization treatments, were evaluated. Along with the qualitative features, 5 morphometric parameters were assessed for comparison of X and Y sperm and their precursor cells (spermatids). Appropriate controls (wet and dry preparations with no prehybridization and hybridization treatments) were maintained.

Fluorescence In Situ Hybridization

Slides that were representative of the prehybridization treatments (LN, FIX, LIS, and NT) were hybridized following a common FISH protocol (Estop et al, 1998). The manufacturer's (Vysis, Inc, Downers Grove, Ill) suggested steps were strictly followed to perform FISH using 2 direct-labeled probes: fluorored-X and fluorogreen-Y for tagging X and Y sperm, respectively. The Spectrum CEP direct chromosome enumeration probes (Vysis) produce distinctive fluorescent ''dots'' within the DNA, and assure fast, efficient results with less background.

Round Cell Preparation for Analysis

Cryopreserved testicular biopsy samples obtained from males with nonobstructive azoospermia who were undergoing in vitro fertilization were used. Biopsy tissues were obtained, processed, and utilized following recent protocols (Mendoza and Tesarik, 1996; Vanderzwalmen et al, 1997). Briefly, centrifugation of the samples was performed at 600 $\times$ *g* through a discontinuous Percoll gradient in 3 layers (50%, 70%, 90%) for 20 minutes. The 50%, 70%, and 90% Percoll fractions were washed independently with human tubal fluid-HEPES medium supplemented with 10% synthetic serum substitute (Irvine Scientific, Santa Ana, Calif) and centrifuged for 5 min at 1000 $\times$ *g*. Cell smears on clean glass slides prepared with the round cells were air-dried and then treated with Carnoy's fixative.

Microscopic Evaluation and Measurement

The FISH slides were analyzed with a fluorescence microscope (Olympus B-MAX 60 fitted with an fluorescein isothiocyanate/rhodamine double bandpass filter set) to assess the hybridization signals and morphological features of the head and the tail. The non-FISH group (control) was evaluated by the same microscope with phase contrast, brightfield view, or both. Spermatozoa were measured for head length (HL), head width (HW), and tail length (TL) under oil immersion ($\times$1000). Considering the shape of the sperm head as an ellipse, head area (HA) was calculated by the formula HA = $\pi \times 0.5$ HL $\times$ 0.5 HW. In round cells, only the cell diameter (D) was measured. All measurements were in ocular micrometer units (omus).

The location of the X and Y chromosomes in the sperm head was approximated following the previously described procedure (Geraedts and Pearson, 1975). Briefly, the well-delineated sperm head was hypothetically divided into 4 quadrants by 2 imaginary lines, one from head tip to the origin of the tail, and the other across the width (Figure 3). The location of the hybridization signals, orange (red) for X and green for Y in reference to these 4 quadrants were scored for 200 spermatozoa of each genotype.

Statistical Analysis

Analysis of variance was performed using multivariate as well as univariate methods. Post-hoc comparisons were made with Tukey's procedure. Because the sample size was large, the normality assumption of the observations was not required; however, as a precautionary measure and, when possible, nonparametric procedures were also performed. The level of statistical significance for all analyses was set at $P < .05$.

Results

Spermatozoa

Spermatozoa underwent one of the pretreatments (LN, FIX, or LIS) or no treatment (NT) before FISH. The hybridization efficiency in LN, FIX, LIS, and NT groups were $<$20%, $<$10%, $<$70%, and $<$5%, respectively. In the

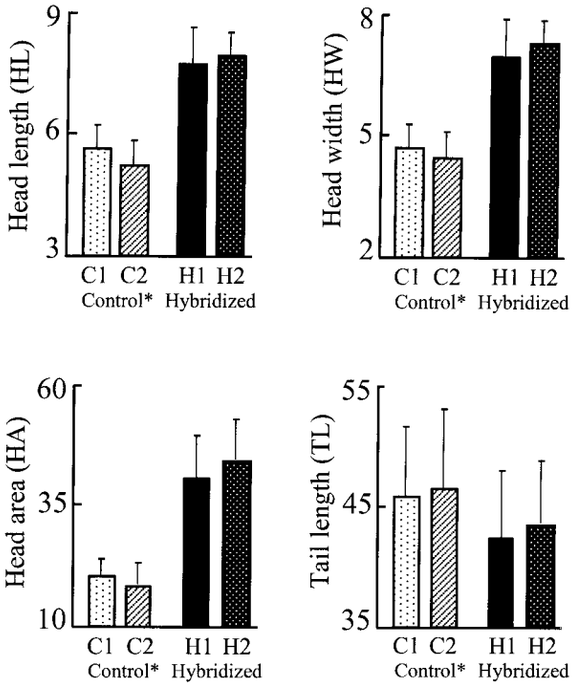

Figure 1. Head (length, width, and area) and tail (length) size comparisons between control and hybridized spermatozoa. Control sperm included measurements of raw semen and Percoll-washed sperm (pooled) in wet (C1) and dry (C2) preparations without any treatment. Hybridized sperm included measurements from raw semen sperm (H1) and Percoll-washed sperm (H2) after FISH. *Indicates control is significantly different ($P < .05$) from the hybridized group.

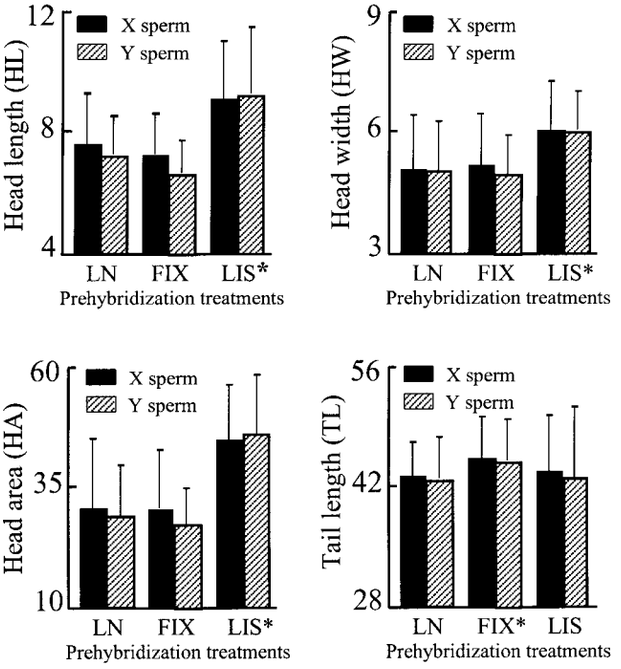

Figure 2. Comparison of head (length, width, and area) and tail (length) measurements between X and Y chromosome-bearing spermatozoa. Heads and tails were measured in sperm undergoing one of the 3 prehybridization treatments (LN, FIX, LIS) followed by a common hybridization procedure. *Indicates significant difference ($P < .05$) between that group and the other groups.

LIS group, <75% and <95% sperm exhibited hybridization signals by 20 and 180 minutes of LIS incubation, respectively. Among the 3 pretreatments, the head and tail morphology of sperm in the LN and FIX groups showed closer resemblance to that of sperm with no pretreatment. Only LIS, especially the prolonged LIS incubation (180 minutes), caused a distortion of the head morphology but produced brighter and larger hybridization signals. As expected, the majority of the hybridization-positive spermatozoa (>98%) produced a single signal in red or green, revealing X and Y genotypes, respectively. No significant deviations of sex ratio from 1:1 were seen in sperm populations in any of the semen donor or treatment groups.

The HL, HW, HA, and TL measurements of hybridized sperm (FISH) were different from that of control sperm (non-FISH; Figure 1). Such differences between non-FISH sperm and FISH sperm of both sex types (X and Y) were statistically significant ($P < .05$). It is interesting that hybridization-induced changes in head and tail were of opposite natures; swelling (enlargement) of the head but shrinkage (reduction) of the tail (bottom panels, Figure 1). In all hybridized sperm slides analyzed, the sperm exhibiting hybridization signals were not different in any of the morphometric comparisons from those that did not exhibit signals (data not shown).

None of the head and tail parameters (HL, HW, HA, and TL) significantly differed between X and Y populations of spermatozoa in any of the donors ($P < .123$). Similarly, none of the prehybridization treatments caused any preferential effect on spermatozoa of either type (Figure 2). However, head measurements (HL, HW, HA) in the LIS group were higher ($P < .05$) compared with those of the other 2 treatments (LN and FIX). The relative location of X and Y chromosomes in the head is shown in Figure 3. As seen in the figure, both green and red signals were more prevalent ($P < .05$) in the anterior region of the head (left and right quadrants in the front).

Round Cells

Testicular biopsy samples contained cells of various morphology, size, and shape. In wet preparations of testicular cells, it was possible to confirm the presence of Golgi-phase, acrosome-phase, elongating, and elongated spermatids based on morphological criteria used by others (Mendoza and Tesarik, 1996; Vanderzwalmen et al, 1997). The hybridization efficiency of round cells was higher compared with that of mature spermatozoa (<30% vs <10%) under identical prehybridization/hybridization

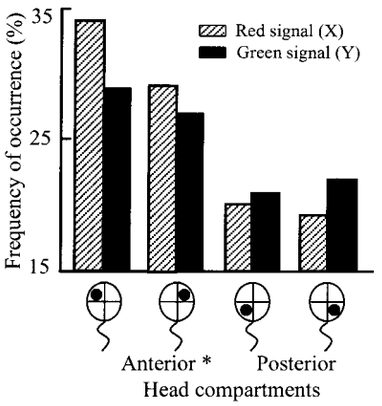

Figure 3. The distribution pattern of X and Y chromosome specific hybridization signals in the head of spermatozoa. The sperm head was hypothetically divided into 4 quadrants and the hybridization signals in these regions were recorded. *Indicates that the group is significantly different ($P < .05$) from the other group.

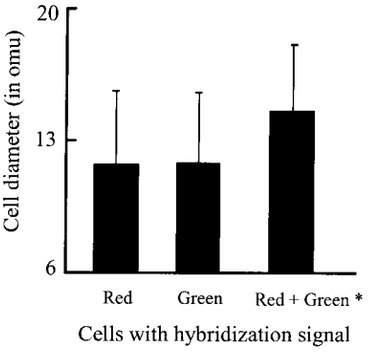

Figure 4. Size comparison of round cells in testicular biopsy samples. Diameter of cells, exhibiting single (haploid) or double (diploid/disomy) hybridization signals, were measured. Cells showing red, green, and combination of red and green signals were identified as carrying X, Y, and XY chromosomes, respectively. *Indicates significant difference ($P < .05$) between that group and the other groups.

conditions (FIX). The size of cells bearing a red signal were not distinguishable from those bearing a green signal (Figure 4). The diameter of cells with green and red signals scored 11.7 ± 3.6 omu and 11.8 ± 3.8 omu, respectively (Figure 4). Round cells bearing 2 signals exhibited a larger diameter (14.6 ± 3.7 omu). The cells showing a single signal (red or green) and diameter of 7–8 μm were considered to be haploid spermatids, whereas those with 2 signals represented disomy or diploidy. Due to the unavailability of a third probe for an autosome, the round cells exhibiting both signals (red and green) remained uncharacterized.

Discussion

In this study human spermatozoa and their precursors (spermatids) were genotyped by FISH and compared by measuring length, width, area, and diameter whenever it was appropriate. The FISH procedure was preceded by 1 of the 3 prehybridization treatments, permeabilization with liquid N_2, fixation with Carnoy's, and chromatin permeabilization with lithium. Drastic differences in hybridization efficiency between sperm with and without pretreatment (10%–100% vs <5%) reconfirmed the necessity of prehybridization treatments in obtaining optimum hybridization results. The resistance of mature sperm cells to hybridization is due to the fact that the spermatozoa-specific chromosomal protein, protamine, traps the DNA in such a way that fluorescent probes are unable to bind the DNA unless they are decondensed by some means (Martin et al, 1991; Goldman et al, 1993; Spriggs et al, 1995). Considerable variation in the hybridization efficiency (10%–100%) among the LN, FIX, and LIS groups is convincing enough to conclude that these 3 prehybridization treatments were not equally effective in making sperm DNA accessible to hybridization. As it appeared, the LN and FIX probably caused only cellular leakage, nuclear membrane leakage, or both, without producing much effect on the chromatin structure, otherwise the hybridization would not be so low in these 2 groups. Therefore, we are of the opinion that when a high hybridization efficiency is a major goal, LIS pretreatment would be the best choice. In the present study, because maintaining the morphological integrity of the sperm cell was a primary concern, the LN and FIX treatments were better suited than the LIS treatment.

The morphometric analyses in this study were based on the measurements of well-delineated sperm; namely, hybridized spermatozoa exhibiting apparently intact shape such as oval head and straight tail. In the LN and FIX groups, it was relatively easy to obtain such sperm. LIS treatment, on the contrary, produced morphological deformities in the head; thus, shorter LIS treatment time (20 minutes) was chosen to avoid such an effect. The hybridized sperm, whether they exhibited a hybridization signal or not, provided identical size measurements. We therefore argue that the hybridization signals perhaps did not represent a selected group of sperm; rather, they represented the entire sperm population. Evaluating the morphology (size and shape) of hybridized spermatozoa, we found that X sperm do not differ from Y sperm in any of the treatment conditions. The objective of our study was not to derive the actual size of X and Y sperm but to compare them. We have been able to demonstrate that with identical treatment, the X and Y exhibited similar shape and size features. It is highly unlikely that all the prehybridization treatments (LN, FIX, and LIS) utilized in this study would work to the same extent in the same direction to eliminate the pre-existing differences of X and Y, if any existed. Furthermore, X and Y sperm having

no prehybridization treatment (NT group) also showed no differences. Thus, our finding cannot be an artifact of the methodology. The finding that the size range of X sperm overlaps with that of Y is further supported by our investigation of spermatids (Figure 4). Recently, to expand the treatment of male factor infertility, the focus has been extended to spermatids (Tesarik, 1997), although most investigations have assessed only the clinical use of them (Tesarik et al, 1996). To our knowledge, this is the first report in which a size comparison of round spermatids of 2 genotypes (X and Y) has been performed. As in mature spermatozoa, we found no size and shape differences between haploid X- and Y-specific round cells.

Contradictory information on X and Y size differences exist in the literature; some supporting the concept, others opposing it (Hendriksen et al, 1996; Cui, 1997a; Geraedts, 1997). Our finding that there is no significant size and shape differences between X and Y, therefore, does not create a new debate, rather it provides further information for using a new approach (Cui, 1997b; Geraedts, 1997). We believe that the majority of the previous studies comparing X and Y size suffered from inaccurate techniques (such as ''barr body'', ''F body,'' etc) to identify X and Y sperm, thus making the conclusions less reliable (Windsor et al, 1993; Flaherty and Mathews, 1996; Cui, 1997a). In contrast, size comparison in our study was based on a large number of samples and involved direct microscopic measurement of sperm identified by FISH, which is the most reliable genotyping technique available. In addition, we performed measurements under several treatment conditions, which allowed us to assess sperm morphology at different states. The presence of 2 different sex chromosomes in X and Y spermatozoa has led to the stipulation of other differences between them (Windsor et al, 1993; Johnson and Schulman, 1994). We argue that even if subtle differences between X and Y spermatozoa exist, these differences should be dismissed for at least 2 reasons: 1) genetic variations evolved from meiotic reshuffling of the chromosomes and 2) morphological variations produced by spermiogenesis (Hiyoshi et al, 1991; Cui, 1997a). In other words, individual variations introduced by meiosis and spermiogenesis should override any small differences of the 2 sperm types (X and Y) caused by their sex chromosomes.

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