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Original Article

Sperm processing affected ratio of X- and Y-bearing sperm

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Abstract: Studies showed that assisted reproductive technology changed the sex ratio at birth, but the effect of semen processing (swim-up technique and density-gradient separation) on enriching either X- or Y-bearing sperm is always controversial. We recruited 30 healthy males and their semen samples were obtained by masturbation after sexual abstinence for 2-7 days. Each sample was divided into 3 aliquots: 1 as control without sperm processing, and the other 2 were handled with density-gradient or swim-up separation. Fluorescent in situ hybridization (FISH) was used to verify the ratio of X- and Y-bearing sperm. We found X/Y sperm ratio was significantly different among the 3 groups, with gradient group vs control group (1.04±0.04 vs 1.0±0.03, P<0.001), swim-up group vs control group (0.48±0.04 vs 1.0±0.03, P<0.001), and gradient group vs swim-up group (P<0.001). We concluded that swim-up technique enriched Y-bearing sperm and density-gradient separation enriched X- bearing sperm, though X/Y sperm ratio before fertilization is not necessarily parallel with human sex ratio at birth.

Keywords: Swim-up sperm processing, density-gradient sperm processing, fluorescent in situ hybridization (FISH), sex ratio at birth

Introduction

Several studies have shown that sex ratio at birth resulting from assisted reproductive technology (ART) was different from that of none male-preferred countries [1, 2]. It has been demonstrated that the artificial technology, IVF/ICSI technology, transfer of blastocysts or cleavage stage embryos, transfer of thawed embryo or fresh embryo, may lead to significant difference in sex ratio at birth [3]. Semen processing is a very important step in ART IUI. Studies about the effect of semen processing on enriching either X- or Y-bearing sperm is always controversial [4-8]. In 1993 it has been demonstrated for the first time X-bearing spermatozoa were statistically larger and longer than Y-bearing spermatozoa [9], and we hypothesized that these factors will affect the semen processing results. Here we design this study using fluorescent in situ hybridization (FISH) to verify whether different sperm preparation method influence the ratio of X- or Y-bearing sperm.

Materials and methods

Semen samples

Normal semen samples were obtained from 30 healthy donors who came for semen analysis for infertile examination at Center for Reproductive Medicine, The Third Affiliated Hospital, Sun Yat-sen University, China. The samples were obtained by masturbation after 2-7 days of sexual abstinence, allowed to liquefy at room temperature for up to 60 minutes and assessed for initial semen parameters according to the World Health Organization guidelines 2010 [10]. Inclusion criteria: volume>2.0 ml; density: (15-40) × 10^6/ml; progressing sperm>32%; pH 7.2-8.0; time of liquefaction<60 min; white blood cells (WBC)<1 × 10^6/ml.

Every semen sample were divided into three aliquots: one remained unprocessed, the other two aliquots went through swim-up or density-gradient separation respectively.

Density-gradient separation group

Gradient medium (spermgrad, Vitrolife) and sperm wash medium (spermRinse, Vitrolife) were used. We used spermgrad gradients of 90% and 40%. All procedures were conducted under sterile conditions. Media was brought to 37°C before use. Semen was centrifuged for 15 minutes at 2000 rpm. The upper and lower layers were carefully aspirated without disturbing the pellet. 10 ml spermRinse was added to re-suspend the pellet and centrifuged for 10 minutes at 1500 rpm. The supernatant was removed, and 0.5 mL PBS was added to re-suspend the pellet, and ready for detection.

Swim-up separation group

Semen samples were kept in an incubator before liquefaction was completed. The processing started by placing the semen sample in a conical tube and adding 1 volume of semen sample to 2 volume of medium (spermRinseTM 10101, Vitrolife). The tube was then placed on a stand and tilted at an angle of 45° and incubated for 1 h at 37°C without changing this angle. Lastly, the upper sperm solution was extracted and washed by using 10 ml PBS centrifuged at 1500 rpm for 2 times. The supernatant was removed, and 0.5 mL PBS was added to re-suspend the pellet, and ready for detection.

Control group

Semen samples were kept in an incubator before liquefaction was completed. Then the semen was washed by using 10 ml PBS centrifuged at 1500 rpm for 2 times. The supernatant was removed, and 0.5 mL PBS was added to re-suspend the pellet, and ready for detection.

Fluorescent in situ hybridization (FISH)

Sperm sample was fixed in 5 ml Carnoy's solution (Sigma) and centrifuged at 1200 g for 5 min, the pellet was re-suspended, and fixation was repeated for three times. Smear 20 μl suspension onto the slides and left it air-dry at room temperature (RT).

Then the slides were put into 5% NaOH solution (Sigma) for 1 minute, then hypotonic solution for 5 min at RT. The slides were then rinsed with 2 × SSC (Sigma), dehydrated through an ethanol series (70%, 85%, 100%) and air-dried. The probe mix of 7 ul of X/Y probe (cytocell) was then applied to the slides, covered with a coverslip, sealed with rubber cement and denaturized at 78°C for 5 min, then hybridised overnight in a moist chamber at 37°C. The slides were washed for 2 min in 0.4 × SSC at 73°C and 1 min in 2 × SSC/0.1% Nonidet P-40. The sample was counterstained with 10 ml of DAPI in 125 ng/ml antifade solution (Vysis), covered with a glass coverslip, and assessed at 1000 magnification with a fluorescence microscope (Leica) equipped with a three-color filter for fluorescein isothiocyanate (FITC) coupled with the software. Only morphologically intact sperm were assessed. Overlapping sperm nuclei, disrupted nuclei or large nuclei with diffuse signals were not considered. On the images, sperm with a single green or two green signals were classified as X sperm, sperm with a single red or two red signals were classified as Y sperm. At least 10 visions and 1000 X- or Y-bearing sperm were calculated.

Ethics

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Reproductive Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University. Written informed-consent forms were obtained from the anticipatants.

Statistical analysis

The results were analyzed using paired sample t-test and pearson chi-square test (SPSS 19.0).

Results

Semen sample

Totally 30 males provided 30 semen samples. All of the males were primary infertility, and their average age was 26.3±3.1 years. Average volume was 3.50±0.5 ml, average density 60.5±15.1 million/ml, progressing sperm 45%±11%; pH 7.2-8.0; time of liquefaction <60 min; white blood cells (WBC)<1 × 10^6/ml.

FISH result

Sperm with a single green or two green signals were classified as X sperm, sperm with a single red or two red signals were classified as Y sperm while sperm with both green and red sig-

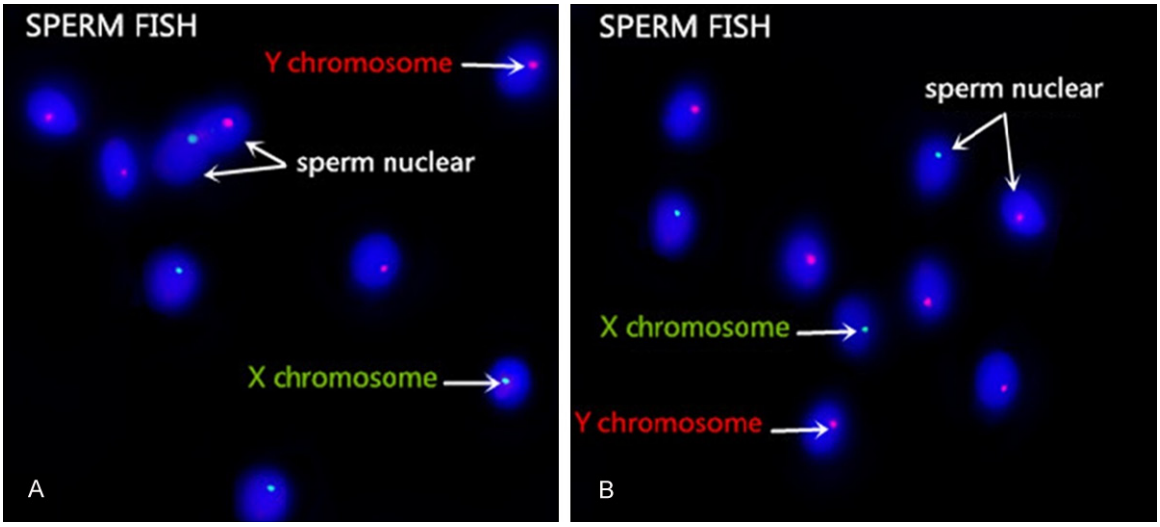
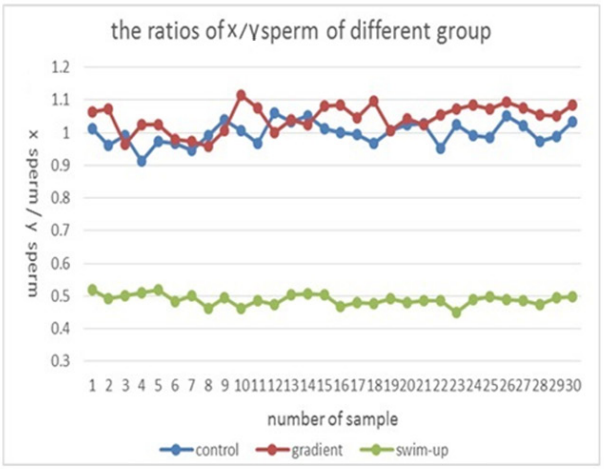
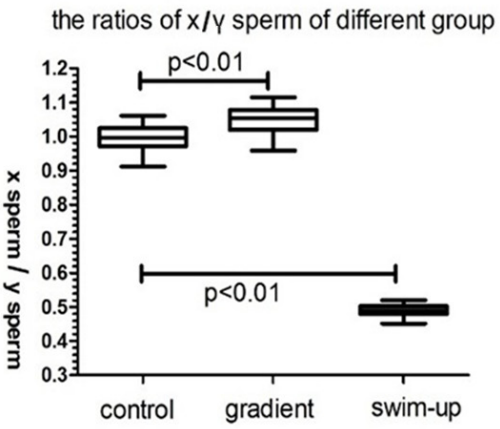


Figure 1. Sperm signals of fluorescent in situ hybridization (FISH). A: Signals of gradient group; B: Signals of swim up group. Sperm with a single green or two green signals were classified as X-bearing sperm, sperm with a single red or two red signals were classified as Y sperm. Sperm with 1 green and 1 red signals or sperm that did not display any signal were disregarded.





Comparison between	Paired Differences					T	Sig
	Mean	Std. Deviation	Std.Error Mean	95% Confidence Interval of the Difference			
				Lower	Upper		
Gradient-swim up	.5562255	.0473422	.0086435	.5385476	.5739033	64.352	0.000
Control- gradient	.0460985	.0517771	0094532	.0654324	-.0267647	-4.877	0.000
Control-swim up	.5101269	.0405377	.0074011	.4949899	.5252640	68.952	0.000

Figure 2. Comparison of X/Y sperm after sperm processing. The swim-up technique selectively enriched Y-bearing sperm and density-gradient separation enriched X-bearing sperm (P<0.001).

nals or sperm that did not display a signal were disregarded (see **Figure 1**).

The X/Y sperm ratio was significantly different among the 3 groups, with gradient group vs control group (1.04±0.04 vs 1.0±0.03, P< 0.001), the 95% confidence interval of the difference is (-0.0654324, -0.0267647); swim-up group vs control group (0.48±0.04 vs 1.0± 0.03, P<0.001), the 95% confidence interval of the difference is (0.4949899, 0.5252640), and gradient group vs swim-up group (P<

0.001), the 95% confidence interval of the difference is (0.538476, 0.5739033) (see **Figure 2**).

Disscussion

The results of our work showed that the swim-up technique selectively enriched Y-bearing sperm and density-gradient separation enriched X-bearing sperm. For control group, the X/Y sperm ratio was nearly 1:1. There are several mechanisms that may account for the changed X/Y sperm ratio after semen processing [11, 12]. 1) The motion capability is different between the X and Y sperm, less X sperm harvested in swim-up processing because they move more slowly than Y sperm; 2) The density and size should be different between the X and Y sperm.

X/Y sperm ratio before fertilization is not necessarily parallel with human sex ratio at birth. We found the male to female ratio at birth was about 1.08-1.10 in our IUI annual reports (not published), no matter for swim-up technique or density-gradient separation. Studied showed that human sperm sorted by flow cytometry and determined by fluorescence in situ hybridization for the use of IVF/ICSI or IUI fertilization, resulted in 93.5% females and 85.3% males for babies born, but not purely resulted in females or males [13]. Studies also showed that short-term storage and swim-up selection did not affect the X/Y sperm ratio in equine spermatozoa [14], and no direct evidence was found that the swim-up procedure for separating motile spermatozoa to use for either intrauterine insemination (IUI) or in vitro fertilization (IVF) would lead to an imbalance of boys and girls [1]. In our study, although the X/Y sperm ratio after swim-up processing was less than 0.5, we did not found sex ratio at birth has been that obvious difference after IUI in clinic. We could not found the similar results in literatures, usually a slight enrichment of X-bearing or Y-bearing spermatozoa after semen processing. More tests should be done to tell the reasons, should it due to our protocol, the semen quality, or the operation, the temperature, the time or medium?

ART changed sex ratio at birth from many aspects besides sperm processing. Many factors during ART may affect the sex ratio of offspring, for example, ovulation pattern, type of artificial reproductive technologies and even the embryo's stage [1, 2, 15]. Study showed that difference between genders observed after IUI in bulls is more likely due to the events occurring after fertilization, but not the thawing or swim-up method of semen preparation [16]. The impact of human fertilization and embryo development in vivo on sex ratio at birth is still a mystery field.

Our conclusion was that swim-up technique enriched Y-bearing sperm and density-gradient separation enriched X- bearing sperm, though X/Y sperm ratio before fertilization is not necessarily parallel with human sex ratio at birth.

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Disclosure of conflict of interest

None.

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