

The effects of follicular fluid on human X, Y bearing sperm ratio by Fluorescent in Situ Hybridization (FISH)

Soghra Bahmanpour¹ Ph.D., Mohammad Reza Namavar¹ Ph.D., Tahereh Talaei¹ Ph.D., Zohreh Mazaheri¹ M.Sc., Ahmad Monabati² Ph.D.

1 Department of Anatomy, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

2 Department of Pathology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

Received: 11 April 2009; accepted: 18 August 2009

Abstract

Background: Some evidences showed that the secretion of uterine tube, vagina and follicular fluid (FF) affects X and Y-chromosome populations. Sperm selection with X or Y chromosome can added to oocyte for gender desired. The isolation of X, Y sperm have done and all efforts in this field are done to make culture media similar to in vivo condition. The objective of this study was to find if the FF can influence the ratio of X or Y chromosomes, therefore we added human FF to culture media to separate X and Y sperms.

Materials and Methods: Normal semen sample from 36 healthy men were selected. Then the samples were divided into control and experimental groups: control group sperms have been incubated with conventional culture media (Ham's F-10) and experimental group with conventional culture media + 10% human FF. For sperm isolation, swim up technique was used. After 24 hours of incubation, slides smear were prepared. Then, we used the Fluorescent in Situ Hybridization (FISH) method to evaluate the effect of follicular fluid on the population ratio of X and Y containing sperms.

Results: Although the incubation of sperm in FF and Ham's F-10 increased Y sperm (59.44 % in control and 61.42% in experimental groups) in comparison with X sperm (40.56% in control and 38.5 in experimental groups) significantly ($p < 0.05$), but the Y (or X) bearing sperm did not significantly change in experimental group in comparison with Y (or X) bearing sperm in control group

Conclusion: This study showed that using the swim up method for collecting sperms and adding FF to culture media can improve some sperm parameters, but did not has significant effects on population of X and Y sperm.

Key words: *FISH technique, X, Y sperm, Follicular fluid, Conventional media, Swim up.*

Introduction

Preselection of the gender of offspring is a subject that has held man's attention since the beginning of recorded history. Although scientific

studies on genes have been conducted recently, sex selection and gender preference have been considered since ancient time. Anaxagoras, a Greek scientist was the first person who related the sex of fetus to testis (1). In ancient times the Greek believed that the semen from the right testis leads to male and the left testis will lead to female borne children. Sometimes people, who would like to have a desired gender, in particularly male gender, go under surgery to have their left testis removed (2).

Corresponding Author:

Soghra Bahmanpour, Department of Anatomical Sciences, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

E-mail: bahmans@sums.ac.ir

Most scientific hypotheses for producing the desired sex of offspring address separation of X- and Y-bearing sperm, and most have had limited, if any success. One of the hypotheses is based on physical characteristics of sperm, on supposed differences in size and shape. X and Y chromosome-bearing sperms are different morphologically and structurally. The X chromosome-bearing sperms are 2 or 3 folds greater in size than Y chromosome-bearing sperm and in mammalian placental it is similar to autosomal chromosome and it makes pairs with its homologues. The X-bearing sperm has 2.8% more DNA than the Y-bearing sperm. It swims more slowly and has a longer life span (3). X chromosome is protected phylogenetically and evolutionary and more than half of its genes are involved in sex determination (4). Y chromosome, in contrast, differs from autosomal chromosome and most of its nucleus content belongs to heterochromatin and it is not involved in protein coding. In spite of its genetic condones, Y chromosome is necessary for male development and fertility (5). There has been no experimental verification of differences based on size and shape, and the results from attempts to verify separation of X- and Y-bearing sperm based on density have not been completely proved (6).

According to the chromosomal characteristic of X and Y bearing sperms, they can separate by different methods (7, 8). Most recently, it is reported that the combination of FISH and flowcytometry can separate X and Y bearing sperms. The outcome of pregnancy revealed that 92% of the offspring were females when X bearing sperms were used and 81.5% of the offspring were males when Y bearing sperms were used in ART (9). It is suggested that the environmental factor like reproductive secretions including the follicular, tubal and vaginal fluid can affect the sex ratio and X and Y chromosome population (10-12). Follicular fluid is a biological and inexpensive fluid, which its chemical component can affect the sperm, oocyte and embryo development (13). This fluid contains some compound like cytokine, progesterone and antioxidant material (14, 15). Studies have already reported that the follicular fluid affected the sperm motility and acrosomal reaction. The probable influence of the follicular fluid ingredients on X or Y chromosomes may change the ratio of those incubated in follicular fluid (14).

Due to costliness, difficult preparation and production and the chemical nature of ART drugs, the present study evaluated the effect of one of the

natural female reproductive tracts secretion on X and Y sperm chromosomes. Because the previous data for sex selection methods confirmed that Fluorescent in Situ Hybridization (FISH) are properly evaluated reliable method and the double-label FISH is one of the best methods for the chromosome selection and detection (7). Therefore we designed this study to evaluate the follicular fluid effects on X and Y sperm chromosomes ratio by FISH.

Materials and methods

Follicular fluid preparation

The follicular fluids were prepared from 36 egg donor healthy women and the ethics rule was used as well. The follicular fluid was clear and without blood mixture. Follicular fluid was centrifuged at 1000 rpm at 20 minutes, and then the supernatant was removed. The fluid was passed through 0.45 micrometer Millipore (Whatman, Germany) and inactivated in 56°C water bath. The processed follicular fluid was aliquot and stored at -80°C until used (14, 16).

Semen preparation

Normal sperm samples were obtained from 36 volunteer men. The semen samples were accepted healthy according to WHO standard criteria. The ethic committee of the University approved the use of the volunteer's semen for the present study. The semen mixed and washed two times with Ham's F10 medium (Sigma, Germany) and centrifuged at 2500 rpm for 10 minutes. The supernatant was removed carefully and gently and after second wash, the pellet was overlaid with 1 ml of Ham's F10 medium taking care not to disturb it and allow the sperm to swim-up into medium for 30 minute. The supernatant contained swim up sperm was separated and then it divided into two groups. In the control group the sperm was overlaid by the Ham's F10 media only and was incubated in it for 24 hours and let the sperm to swim up during this time through media. In the experimental groups the sperm were overlaid by Ham's F10 mixed with 10% follicular fluid and was incubated in it for 24 hours and let the sperm to swim up during incubation. After 24 hours, the control and experimental groups were washed with PBS and then it centrifuged at 2500 rpm for 10 minutes and the supernatant was removed and discarded. Then the Carnoy's fixative was added (3:1, Methanol: acetic acid) (Merck, Germany), and the sample were fixed and smear was prepared and stored at -20°C until used.

FISH technique

Before the starting FISH technique, the slides were removed from -20 °C and left on the room temperature. The sperm nucleus decondensation was done either with NaOH (3M) or dithiothreitol (DTT) and lithium diiodosalicylate (LIS) (Sigma, Aldrich, Germany). After decondensation, the smear was dehydrated through Ethanol series and then air-dried. Then the hybridization was done. Briefly, ten micro litter of probe fluorescent labeled was placed on the semen smear within a marked area of a slide. X centromeric probe labeled with a green fluorophore (FITC spectrum) and X centromeric probe with a red fluorophore (Texas Red spectrum). The probe was prepared from Cytocell, England Company. Then each slide was mounted with a clear cover slip and sealed with rubber cement. Denaturation was done at 73 °C hotplate (Bibby, England) and then the slide was hybridized at least 6 hours or overnight at 37 °C in a humid incubator (Napco, USA). After hybridization, the cover slip and rubber cement were removed and the slides were dipped in the 2× SSC container (saline-sodium citrate) at 73 °C water bath (Memmert, Germany) and followed by washing in 0.05% Tween 20 in 0.4 ×SSC (Merck, Germany), and air-dried in dark room (17,18). Thereafter, the slides were counter stained with 4', 6-diamino-2-phenylindole (DAPI) along with antifade (Cytocell, England) at the final stage of hybridization for counter staining and prevent of fading of fluorescent probes during studying under microscope. The slides were studied under the Epifluorescent microscope (Nikon, E800, Japan), the DAPI/Texas red was emitted the red color of the Y chromosome sperm which it observed as a red spot and the DAPI/FITC was emitted the green color which was the sign of the X chromosome (Figure1). One hundred sperms were counted in each field and the data were analyzed by ANOVA and differences with $p < 0.05$ were significant statistically.

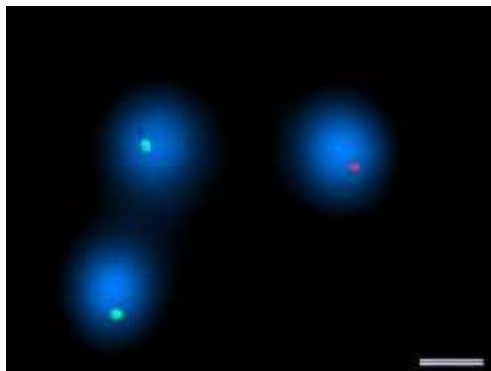


Figure 1. Sperm head with X chromosomes (Green) and Y chromosomes (Red). Scale bar = 10 μ m.

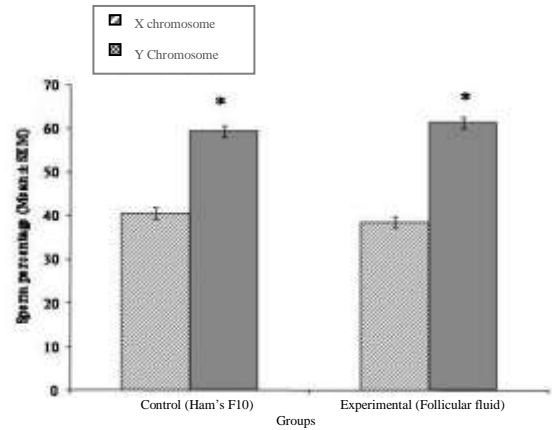


Figure 2. The effect of follicular fluid and Ham's F10 on the X, Y sperm Chromosome ratio.

*Different significantly from X chromosomes ($p < 0.05$).

Results

The results of this study showed that the percentage of Y sperm chromosomes in control groups (60.44%) was higher than the X sperm chromosomes (40.56%) after 24 hours of incubation in Hams'F10 ($p < 0.05$). In experimental groups, the percentage of Y-chromosomes (61.42%) increased in comparison with X chromosomes (38.5%) after 24 hours of incubation in follicular fluid ($p < 0.05$). As these results shows the differences between Y and X sperm chromosomes were significant within groups in both groups (control and experimental). Although the percentage of Y bearing chromosomes sperm in the experimental groups (61.42%) were more than the control group (60.44%) but the differences between two groups were not significant (Figure2). Indeed the significant differences were seen in the Y-chromosomes population of both groups after 24 hours in comparison with X sperm within groups. Although the sperms Y-chromosomes were increased in both groups but the differences between those were not significant. The X bearing chromosomes were reduced in experimental group in comparison with control but it was not significant ($p = 0.29$). These results revealed that the follicular fluid could not change the percentage of the population of X or Y sperm chromosomes compare to Ham's F10 media.

Discussion

X or Y chromosome sperm ratio can differ by environmental conditions such as temperature, nutrition and female reproduction tract secretion. Some evidences showed that the secretion of uterine tube, vagina and follicular fluid affects X and Y-chromosome populations (11, 19). FF can

affect the oocyte maturation and embryo development. The positive effects of FF on acrosomal reaction and capacitation were confirmed (20). One of the specific protocols for the study of X and Y chromosome is FISH. In this procedure the X or Y-chromosomes is labeled with fluorescent probes. We used two color FISH for evaluation of both X and Y chromosome (21). These probes are designed in accordance with different two sperm nucleus structure (21, 22).

Although numerous methods have been developed as having influence on the gender of the offspring, most lack valid scientific evidence of effectiveness. Preconception gender selection has an important application in reducing the risk of having children with X-linked diseases or unwanted child (23). A preconception, flow cytometric sperm sorting method of gender selection is based upon the detection of differential fluorescence emitted by fluorescently stained X and Y chromosome-bearing sperm (24). Current results indicate that the method is a safe and effective process and is a valuable tool for families wishing to balance the gender distribution of their children or to reduce the likelihood of having children with X-linked disease. The observed major congenital abnormality rate of 2.05% for babies born after the sperm sorting method was the same with that occurring in normal population (8). The previous study showed that the X: Y ratio is almost 50:50 but the some separation methods were changed this ratio in the limited range (25). They reported that some kind of separation likes percoll and albumin gradient could separate these kinds of sperm but there were some controversial in their results (26). The results of present study showed that although the follicular fluid changes the Y percentage population but it is not significant. Indeed the sperm incubation in follicular fluid was associated with imbalance of X: Y ratio but not between the Y-chromosomes of the two groups. This result was the same in both group after incubation of sperm in the media only or mixed with follicular fluid which it is in accordance with other finding (27). Our results were in accordance with the Yan *et al* (2006), (23), which reported that swim up could not separate the X and Y bearing sperm. Although some isolation methods could separate the high purity of X or Y bearing sperm (27), but the other reported no positive results in separation (28). We can conclude that these differences that were not statically significant may be partially dependent on media condition and also belong to follicular fluid nature. As the Y and X sperm have different

feature and entity, extension of time beyond the 24 hours could give another results. Using the different concentration of follicular fluid or a modified swim up procedure may lead to better results. Although the follicular fluid doesn't change the percentage of X or Y bearing sperm chromosomes in comparison of the media, but it probably has improved the quality of sperm by swim up methods.

Acknowledgment

Research Deputy of Shiraz University of Medical Sciences and Health services grant No 84-2766 supported this project financially. The Author wish to thank the Rostami IVF Center, Zeinabieh Hospital in Shiraz and staff of Anatomy and Pathology Department especially Mrs Tabibi and Aleahmad.

References

1. Mittwoch U. Sex in mythology and history. *Arq Bras Endocrinol Metab* 2005; 49:7-13.
2. Scott Sills E. Sex selection of human sperm: Evolution of current techniques and applications. *Arch. Gynecol. Obstet* 1998; 261: 109-115.
3. Paasch U, Grunewald S, Glander HJ. Sperm selection in assisted reproductive techniques. *Soc Reprod Fertil Suppl* 2007; 65: 515-525.
4. Graves JA, Delbridge ML. The X- a sexy chromosome. *Bioassays* 2001; 23: 1091-1094.
5. Jain T, Missmer SA, Gupta RS, Hornstein MD, Preimplantation sex selection demand and preferences in an infertility population. *Fert Steril* 2005; 83: 649-658.
6. Gledhill BL. Selection and separation of X- and Y-chromosome-bearing mammalian sperm. *Gamete Res* 1988; 20: 377-395.
7. Johnson LA, Welch GR, Keyvanfar K, Dorfmann A, Fugger EF, Schulman JD. Gender preselection in humans? Flow cytometric separation of X and Y spermatozoa for the prevention of X-linked diseases. *Hum Reprod* 1993; 8:1733.
8. Levinson G, Keyvanfar K, Wu JC, Fugger EF, Fields RA, Harton GL, et al. DNA-based X-enriched sperm separation as an adjunct to preimplantation genetic testing for the prevention of X-linked disease. *Hum Reprod* 1995; 10: 979-982.
9. Karabinus DS. Flow cytometric sorting of human sperm: MicroSort clinical trial update. *Theriogenology* 2009; 71: 74-79.
10. Schulman JD, Karabinus DS. Scientific aspects of preconception gender selection. *Reprod Biomed Online*. 2005; 10: 125-128.
11. Grant V.J. Sex determination and the maternal dominance hypothesis. *Hum Reprod* 1996; 11: 2371-2375.
12. Seli E, Sakkas D. Sperm nuclear determinants of reproductive outcome: implications for ART. *Hum Reprod* 2005; 11: 337-349.
13. Burrello N, Vicari E, D'Amico L, Satta A, D'Agata R, Calogero AE. Human follicular fluid stimulates the acrosomal reaction by interacting with γ -aminobutyric acid receptor. *Fertility and sterility* 2004; 82: 1086-1090

14. Mendoza C, Tesarik J. Effects of follicular fluid on sperm movement characteristics. *Fertil Steril* 1991; 56: 588-589.
15. Schweigert FJ, Steinhagen B, Raila J, Siemann A, Peet D, Buscher U. Concentrations of carotenoids, retinol and alpha-tocopherol in plasma and follicular fluid of women undergoing IVF. *Hum Reprod* 2003; 18: 1259-1264.
16. Jeon BG, Moon JS, Kim KC, Lee HJ, Choe SY, Rho GJ. Follicular fluid enhances sperm attraction and its motility in human. *J of Assist Reprod and genet* 2000; 18: 407-412.
17. Chen Z, Yan J, Feng HL. Aneuploid analysis of trippronuclear zygotes derived from in vitro fertilization and intracytoplasmic sperm injection in humans. *Fert Steril* 2005, 6: 1845-1848.
18. Flaherty SP, Matthews CD. Application of modern molecular techniques to evaluate sperm sex selection methods. *Mol Hum Reprod* 1996; 2:937-942.
19. Ericsson RJ. X, Y sperm. *Contracept Fert Sex* 1976; 8: 655-668.
20. Cheng FP, Fazeli AR, Voorhout WF, Tremoleda JL, Bevers MM, Colenbrander B. Progesterone in mare follicular fluid induces the acrosome reaction in stallion sperm and enhances in vitro binding to zona pellucida. *International journal of andrology* 1998; 21: 57-66.
21. Kobayashi J, Oguro H, Uchida H, Kohsaka T, Sasada H, Sato E. Assessment of bovine X- and Y-bearing sperm in fractions by discontinuous percoll gradients with rapid fluorescence in situ hybridization. *J Reprod Dev* 2004; 4: 463-469.
22. Johnson LA, Flook J, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol Reprod* 1989; 41: 199-203.
23. Yan J, Feng HL, Chen ZJ, Hu J, Gao X, Qin Y. Influence of swim-up time on the ratio of X- and Y-bearing spermatozoa. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2006; 129: 150-154.
24. Gawicka-Szczgiel M, Kurpisz M. X, Y chromosome-bearing sperm selection and detection methods. A review. *Folia Histochem cytobiol* 1995; 33: 219-127.
25. Van Dyk Q, Mahony M C, Hodgen G D. Differential binding of X- and Y-chromosome-bearing human spermatozoa to zona pellucida *in vitro*. *Andrologia* 2001; 33: 199-205.
26. Flaherty SP, Michalowska J, Swann NJ, Dmowski WP, Mathews CT. Albumin gradient do not enrich Y bearing spermatozoa. *Hum Reprod* 1997; 12: 938-942.
27. Zhou Z, Su L, Tang G. Isolation of X- and Y-bearing spermatozoa. *J Chin Eugen Genet* 1995; 5: 95-97.
28. Han TL, Flaherty SP, Ford JH, Matthews CD. Detection of X and Y bearing human sperm after motile sperm isolation by swim-up. *Fertil Steril* 1993; 60: 1046-1051.

