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The effect of the follicular fluid on sperm chromatin quality in comparison with conventional media

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Abstract. – BACKGROUND: Follicular fluid (FF) is biological fluid rich in nutrients, growth factors, hormones and may affect the sperm quality. Sperm washing has been done using conventional media in laboratory procedure so far.

AIM: This study aimed to investigate the effects of FF on survival and maintenance of chromatin integrity post swim up.

MATERIALS AND METHODS: Each washed semen sample was divided into two parts; the control group was incubated in the media, and the experimental groups incubated in the media containing 10% follicular fluid. Smears were prepared after 20 min, 180 min, 24 hours and no incubation times. Sperm chromatin changes like protamine, histone, DNA denaturation, sperm chromatin stability and motility were evaluated at different times.

RESULTS: Incubation of sperm in the follicular fluid increased sperms with normal histone, normal chromatin protamine and sperm with normal head size ($p < 0.05$).

CONCLUSIONS: Administration of follicular fluid into the culture media of the sperms that had been separated by swim up method could improve the sperm quality. Further studies are recommended for understanding the mechanism of the structural change of the sperm chromatin.

Key Words:
Follicular fluid, Conventional media, Protamine, Sperm chromatin.

Introduction

In normal semen, 10-15% of the sperms show chromatin abnormalities. The existence of more than 30% of sperms with chromatin disintegrity in semen leads to pregnancy loss[1]. The sperms with extra histone or protamine deficiency and DNA denaturation are prone to premature chromatin condensation during fertilization[2,3]. Premature condensation of sperm nucleus takes more time for decondensation and results in pronucleus asynchrony[2]. The fertilizing of these pronuclei, can lead to undesirable blastocyst quality, implantation and pregnancy outcome[4]. The histone replacement by protamine is one of the many important events occurring during spermatogenesis for stability of the sperm chromatin[1].

The protamines are united and condensed by disulphide bond in the testes and epididymis and this stability is reinforced by zinc ions. Furthermore, this structural complexity of sperm chromatin has a critical role in fertility[5].

The sperm chromatin configuration has many advantages including the DNA protection against nuclease, protease, and infection. It also facilitates the sperm transport in the seminiferous tubules. Sperm chromatin condensation protects the sperm DNA against oxidative stress and DNA damages[6]. Disturbances in the nuclear condensation may have a negative impact on the male fertility as this highly organized sperm chromatin is known to have a crucial influence on the fertilizing process[7,8].

Infertile men are reported to have a higher fraction of sperm with chromatin defects and DNA breaks than fertile controls[9]. Moreover, the sperm DNA defects may have a possible negative impact on the outcome of assisted reproductive techniques[10].

Therefore, a successful fertilization requires a sperm with a minimum defect in quality and function[11]. In vitro incubation in a media, which contains the normal body fluid components, may improve the sperm quality. One of these candidates, follicular fluid (FF), is a natural secretion of the female reproductive tract. FF is a biological fluid, which meets the sperm in the uterine tube. Its positive effects on acrosomal reaction and sperm capacitation have already been confirmed[12].

Considering the difficulty and costly procedure of assisted reproductive technique (ART),

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this study was designed to improve the sperm chromatin by adding FF to media. Furthermore, it has been questioned whether or not the FF is able to improve the sperm DNA quality. Therefore, the objectives of this study was to find the effects of FF on survival and maintenance of chromatin integrity post swim up

Material and Methods

Follicular Fluids Collection and Preparation

The sampling process was approved by the Ethical committee of Shiraz University of Medical Sciences. FF was obtained from thirty-six healthy and fertile female donors with at least two previous pregnancies. For FF preparation, the mature follicles were punctured and the blood free FF was collected and centrifuged for 20 minutes at 1200 rpm. It was then filtered, inactivated and stored at –70°C until use. One hour before use, it was placed in an incubator set to 37 °C and 5% CO_2 and mixed with Ham's F10 media containing 5% human serum albumin (Sigma, Steinheim, Germany). 10% FF was prepared and pH and osmolarity were also adjusted[13].

Semen Samples Preparation

The semen was taken from thirty-six volunteering healthy men. It was collected from the University laboratory for marriage genetic consultation. The samples were matched with WHO criteria including sperm motility, count and normal morphology. After being allowed to liquefy for 30 minutes, the samples were washed twice with Ham's F10 at 2500 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in Ham's F10 containing serum and allowed the sperm to swim up.[14] The swimming up sperms was divided into two parts. The first part was incubated in 10% FF and the second was kept in Ham's F10 media as the control. Smears were prepared at the start of the experiment and the preparation time was mentioned as zero (Time0). At the 20-minute (Time1), 180-minute (Time2) and 24 hour (Time3) intervals, smears were also prepared once again and protamine, histone content of the sperm, DNA denaturation and sperm chromatin stability were evaluated.

Sperm Chromatin Assessments

Aniline blue (Merck, Darmstadt, Germany) staining was used to assess the sperm nucleus histone. The smears were fixed with glutaraldehyde and stained with aniline blue. The sperms with extra histone or protamine deficiency showed different colors after aniline blue staining. Depending on the intensity of the color, the sperms were classified into two categories. Those with light blue and normal histone were recorded as score zero; those with dark blue and extra histone content were graded as score 1. The percentage of the intensity of different sperm colors was recorded for each sample[15].

Chromomycine A3 (CMA3) (Merck, Darmstadt, Germany) is a fluorochrome that competes with protamines for association with DNA. The degree of protamine of the mature sperm is evaluated with staining intensity of CMA3. The smears were fixed with Carnoy and were stained with CMA3 solution in Mc Elvin buffer[16]. The bright immature sperm was indicated as CMA3 positive (CMA3, score1) and non-bright and mature sperm was the CMA3 negative (CMA3, score0) sperm. For each sample, the percentage of CMA3 negative and positive has been recorded.

Sodium Dodecyl Sulfate (SDS) (Merck, Darmstadt, Germany) reaction and Gemsa staining (Merck, Darmstadt, Germany) indicate the sperm chromatin stability. The immature sperm decondensation chromatin resistance is evaluated by this technique. The degree of head swelling was recorded as score0 (Normal head) and the score 1 with complete head swelling. The percentages of the head completely swollen and head with normal size were recorded in each sample. The sperms were incubated in 1% SDS in burate buffer for 60 min. the sperms were fixed with glutaraldehyde and smears were prepared[17].

Acridine Orange (AO) (BDH, Chemical Ltd, Poole, England) tests assessed the DNA integrity or denaturation. The sperms were fixed with acetic alcohol, stained with AO, and examined with a fluorescence microscope (Nikon, E800, Japan). AO intercalated with double-stranded DNA (score 0) emits green fluorescence and AO associated with single-stranded DNA (score 1) emits red fluorescence. The percentage of the sperm nuclei with green and red colors was recorded and DNA denaturation was evaluated[18].

Statistical Analysis

All the data were analyzed by SPSS vers. 15, 2006 (SPSS Inc., Chicago, IL, USA) for windows and presented as the Mean ± SEM. Comparison of the quality of sperm according to their histone and protamine content, DNA stability and chromatine

denaturation of sperm that was incubated in FF and matching media were analyzed using Repeated Measurement ANOVA. A *p*- value of less than 0.05 was considered as significant.

Results

As shown in Figure 1 the percentage of the sperms with normal histones was not significantly changed with time in both control and experimental groups ($p = 0.105$) The percentage of the sperms with intermediate or extra histones that exposed to FF, was also not significant ($p = 0.353$). There were no significant difference in histone content of chromatine between control and experimental groups ($p = 0.134$).

As displayed in Figure 2, the percentage of sperm with normal chromatin protamine (CMA3, Score 0) did not change during different times of incubation in Ham's F10 as a control. The sperm with normal protamine increased after incubation in FF, but it was significant only after 24h of incubation in FF ($p = 0.002$). The percentage of sperms with protamine deficiency (CMA3, Score1) significantly decreased after different times of incubation in FF ($p = 0.002$) but it did not significantly change in control group ($p = 0.185$). There was no significant difference between control and FF group ($p = 0.645$).

The results of SDS – Giemsa staining showed that after the sperm incubation in Ham's F10 (as a control) in different time, the percentage of sperms with normal head (SDS, score0) did not significantly change ($p = 0.871$). This criterion significantly increased after 24h incubation in FF ($p < 0.001$). (Figure 3). The percentage of sperms with abnormal head (score 1) significantly decreased after incubation in FF (P=0.003) but not changed in control group ($p = 0.583$). The differences between the control and experimental groups were not significant ($p = 0.443$). After incubation in FF, it seems that the FF could improve the stability of the sperm chromatin.

The results obtained after the sperms were stained with acridine orange showed that percentage of sperms with normal chromatin (double-stranded DNA) decreased with time in the control and increased in the experimental groups but the differences between the control and experimental group were not significant ($p = 0.167$). The percentage of sperms with abnormal chromatin (single-stranded DNA) increased with time in the control and decreased in the experimental groups. but the differences between the control and experimental groups were not significant ($p = 0.219$).

Discussion

A previous investigation showed that defect in the sperm chromatin has negative effects on pregnancy rate both *in vitro* and *in vivo*. It was reported that normal pregnancy would not oc-

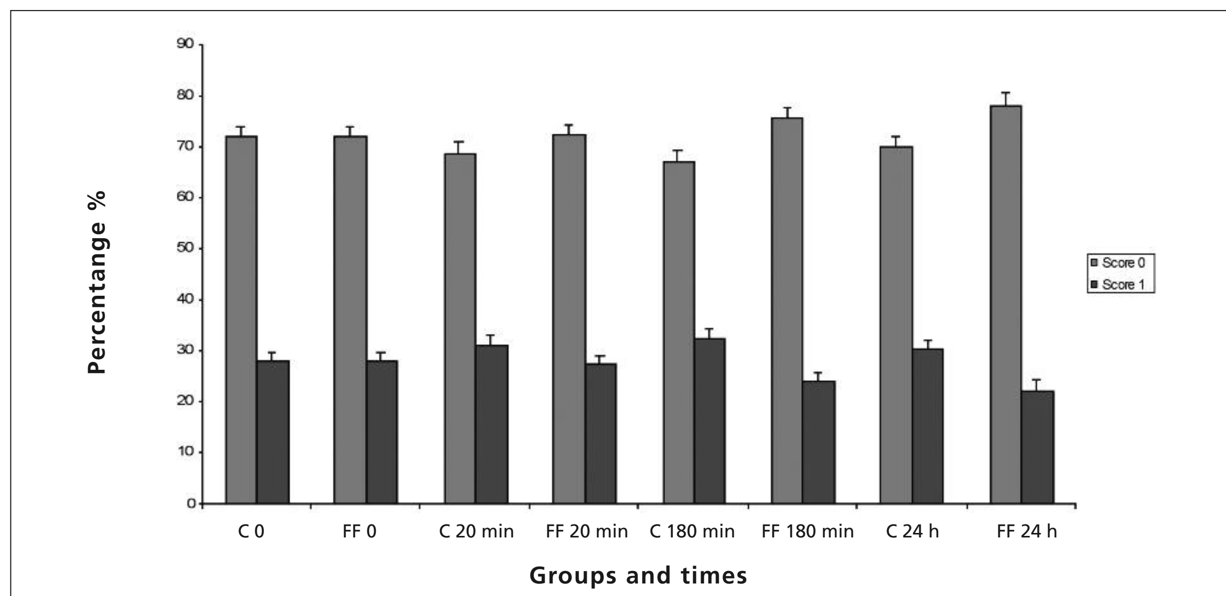

Figure 1. Effect of follicular fluid and conventional media (cotnrol) on sperm histone content: aniline blue.

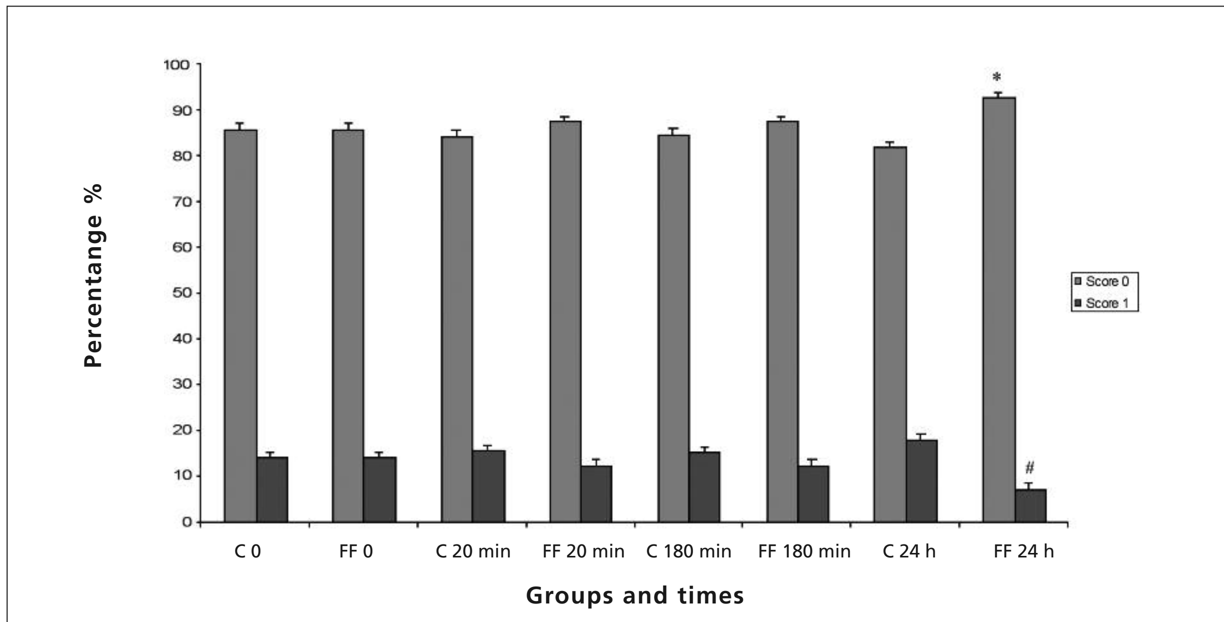

Figure 2. Effect of follicular fluid and conventional media (cotnrol) on sperm nucleus protamine: chromomycine A staining. *Significantly difference with FF0 ($p = 0.002$); #Significant difference with FF0 ($p = 0.002$).

cur, if more than 30% of the sperms showed DNA damage[1]. Therefore, the sperm chromatin stability is necessary for normal chromatin function. Many studies have confirmed that the chromatin integrity and stability affects ART outcomes[6-8]. The present study was designed to investigate the way to improve the sperm chromatin or decrease the damage to the sperm genetic materials during *in vitro* culture. The positive effects of FF on the sperm motility has been shown in the previous studies may be related to the quality of sperm[19-21].

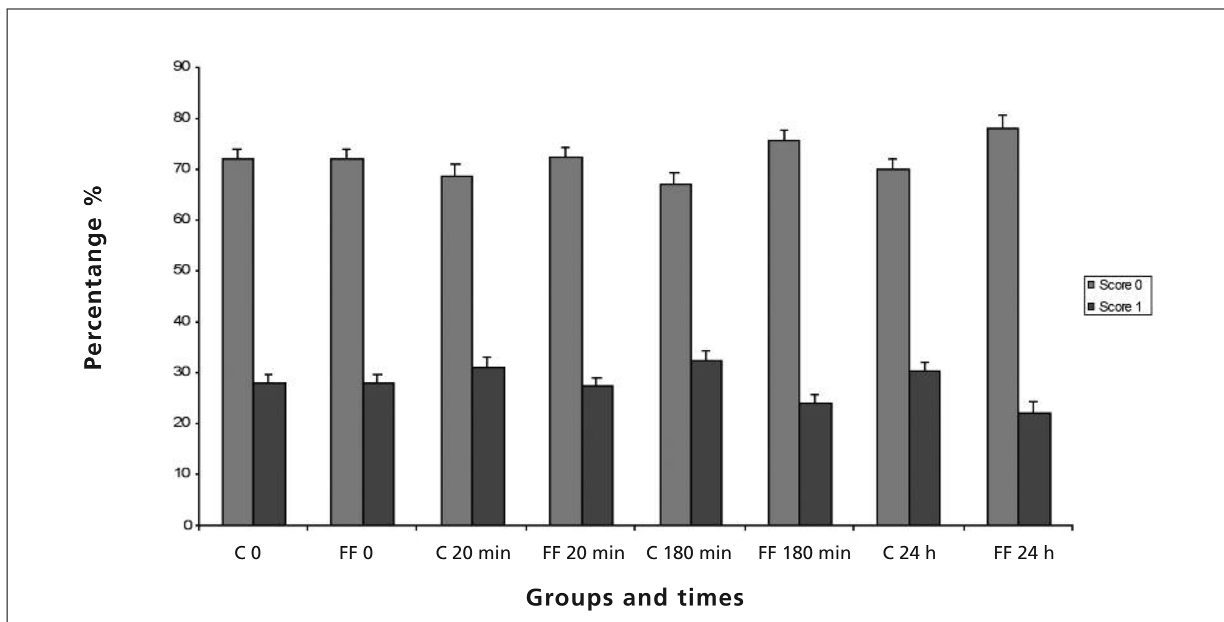

Figure 3. Effect of follicular fluid and conventional media (cotnrol) on sperm chromatine stability: SDS. *Significant difference with FF0 ($p < 0.001$); #Significant difference with FF0 ($p = 0.003$).

FF contains the antioxidant substances like glutathione peroxidase and superoxide dismutase whose ability in inhibition of sperm damage and activating the sperm function was already confirmed[22,23].

Staining smear with aniline blue and CMA3 revealed the positive effects of FF on the sperm chromatin quality just after 24 hours incubation in FF. It showed that as the time of incubation in FF was prolonged, histone replacement by protamine increased and this event was accompanied with extra histone and abnormal chromatin reduction of the sperm. This difference in the percentage of the sperms with normal histone content was not significant. The abnormal sperm with extra histone increased in the control group, but it was not significant in different times.

The increase in the protamination of sperm chromatin by FF in comparison with media was statistically significant only after 24h and the decrease in the sperm with abnormal or protamine deficiency was also significant. When the histone displacement by protamine occurred, histones were first disassembled, then replaced by transitional proteins and finally by protamines[6]. Histone hyperacetylation (*in vitro* and *in vivo*) facilitates nucleosome disassembly and histone replacement by protamine[6,24]. It has been shown that transitional proteins (probably protamine precursors) and protamines are present in cytoplasm (and nucleus) from spermatocyte stages[6,24,25]. Most of the synthetized proprotamine is converted into protamines and intermediate maturation product persists in the sperm nuclei in men[24]. It can be concluded that the sperm histone replacement by protamines may improve the sperm quality and prevent the sperm from DNA damage.

One of the FF components is inorganic phosphate and the previous reports showed that ATP concentration during ovulation would be increased in female genital tract secretion as well as in F.[26,27] and one of the other components of FF is ATPase. This enzyme can dissociate ATP and release inorganic phosphate and energy for sperm motility[27]. Studies have previously reported that hyperacetylation and phosphorylation of histone could lead to histones reduction in sperm chromatin[26,27]. Regarding the results of the present study, we can conclude that the incubation of the sperms in FF component may facilitate sperm chromatin improvement.

The results of the SDS – Giemsa staining revealed that after 24 hours of incubation in FF, the percentage of the sperms with normal disulphide bond (Normal head) significantly increased ($p < 0.001$), while this kind of sperm was decreased in the control group but it was not significant. The percentage of the sperms with swelling heads significantly decreased after 24h

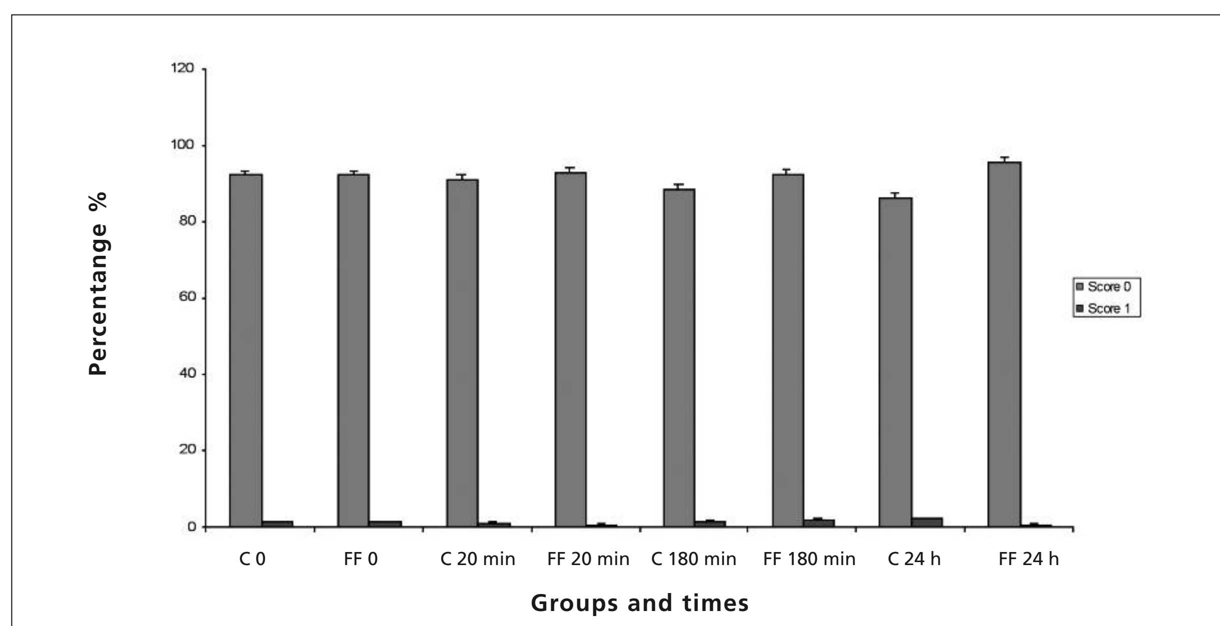

Figure 4. Effect of follicular fluid and conventional media (cotnrol) on sperm chromatine denaturation: acridine orange staining.

incubation in FF ($p = 0.003$) whereas it increased in the control group. The differences between experimental and control group were not significant. Therefore, the abnormal sperm contained lower protamine S-S stability in comparison with the normal sperm[28]. It can be concluded that FF enhances the sperm DNA stability along with displacement of normal protamine with stable S-S bond, leading to a decrease in DNA denaturation. A recent study[29] showed that the sperm thiol content was correlated positively with sperm chromatin integrity. It seemed that the thiol content in oligospermic or infertile samples, which were found to have a higher SH content (fewer disulphide bonds) compared with normal samples.

As the AO staining revealed, the sperms with double-stranded DNA or normal sperms increased in FF compared with the control group, whereas the denaturated single-stranded DNA decreased in FF and increased in the control group. Although these data did not show any significance, it revealed the positive efficacy of FF on the sperm quality. It can be concluded that the antioxidant content of FF may prevent the DNA breakdown and denaturation during *in vitro* incubation in comparison with the control groups. Many studies confirmed that the presence of DNA breaks leads to infertility or failure of assisted reproduction outcome[30,31].

Conclusions

The comparison between the sperm incubating in conventional media and FF did not show significant difference in most of the times. The reasons for such results may be due to low concentration of FF, incubation time and using sperms from normal male with low percent of abnormal sperms. If our samples have been taken from abnormal persons, the results would be changed.

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