Investigation of Time-Dependent Changes in Spermatozoa DNA Condensation and Progressive Motility

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ABSTRACT

This study aimed to investigate time-dependent changes in spermatozoa DNA condensation and progressive motility and the correlation between DNA condensation and progressive motility of spermatozoa. Semen samples collected from 30 male patients were subdivided into two aliquots. The first aliquot was left unprocessed and labelled 'raw'. The second aliquot was processed by a swim-up method and was evaluated at the 0th, 3rd and 24th hours. All samples were scored for routine semen analysis. Spermatozoa DNA condensation was evaluated by using the acridine orange and diff-quick stains. The percentages of the changes in the progressive motility and DNA condensation of spermatozoa were estimated. There were no significant differences between samples of the raw and the 0th, 3rd and 24th hours after processing in the percentage of spermatozoa DNA condensation. In all patients, the percentage of spermatozoa progressive motility was higher at the 0th and 3rd hours after processing than in the raw samples. There was no correlation between the percentages of the changes in spermatozoa DNA condensation and progressive motility. Spermatozoa selection can be performed reliably at the 3rd hour after processing of spermatozoa for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) applications. In addition, it is possible to say that the obtaining and processing of spermatozoa that can be performed on the day before the ovum pick-up (OPU) when it is required would not cause to any change in spermatozoa DNA condensation.

Key Words: *Spermatozoa, DNA condensation, progressive motility*

Spermatozoa DNA Kondensasyon ve Progresif Motilitesinde Zamana Bağlı Değişimlerin Araştırılması

ÖZET

Bu çalışmada, spermatozoaların DNA kondensasyonu ve progresif motilitesinde zamana bağlı olarak meydana gelen değişimlerin ve DNA kondensasyonu ile progresif motilite arasındaki ilişkinin araştırılması amaçlandı. Otuz erkek hastadan toplanan semen örnekleri, 2 aliquota ayrıldı. İlk aliguot herhangi

bir işlemden geçirilmedi ve 'raw' olarak adlandırıldı. İkinci aliquot swim-up yöntemi ile yıkanarak 0., 3. ve 24. saatlerde değerlendirildi. Tüm semen örneklerine rutin semen analizi yapıldı. Spermatozoaların DNA kondensasyonları acridine orange ve diff-quick boyaları ile değerlendirildi. Spermatozoaların DNA kondensasyonları ve progresif motilitelerindeki değişimlerin yüzdeleri hesaplandı. Spermatozoaların DNA kondensasyon oranlarında, raw ile yıkama sonrası 0., 3. ve 24. saat örnekleri arasında istatistiksel olarak anlamlı farklılıklara rastlanmadı. Tüm hastalarda progresif motilite oranı, yıkama sonrası 0. ve 3. saatlerde raw örneklerine göre daha yüksekti. Spermatozoaların DNA kondensasyonları ve progresif motilitelerindeki değişimlerin yüzdeleri arasında herhangi bir ilişki saptanmadı. In vitro fertilizasyon (IVF) ve intrastoplazmik sperm injeksiyonu (ICSI) uygulamalarında, spermatozoa seçimi, yıkama işleminden sonra 3. saatte güvenle yapılabilir. Ayrıca, gerekli olduğu durumlarda spermatozoa eldesi ve hazırlığının, ovum pick-up (OPU) işleminden bir gün önce yapılmasının, spermatozoa DNA kondensasyonunda herhangi bir değişime neden olmayacağını söylemek mümkündür.

Anahtar kelimeler: *Spermatozoa, DNA kondensasyonu, progresif motilite*

INTRODUCTION

Semen analysis is the first step in the evaluation of male infertility, including concentration, motility, progression, morphology and vitality evaluation of spermatozoa. However, semen analysis could not provide exact information on fertility because there is a significant overlap between semen parameters of infertile and fertile men (1).

In recent years, evaluation of DNA integrity and maturation of spermatozoa has gained more importance in the diagnosis of male infertility. Significant differences in the levels of spermatozoa DNA damage among fertile and infertile men have been shown (2). Thus, it is thought that spermatozoa DNA integrity provides more information compared to routine semen parameters for fertility evaluation (3). However, whether there is an association between the semen parameters and the DNA damage is not exactly known. While some studies have shown positive correlation between DNA damage of spermatozoa and abnormal semen parameters, others have found no correlation between DNA damage of spermatozoa and semen parameters.

The formation of highly packaged chromatin (chromatin condensation) is a complex process, and it results in creation of mature spermatozoa during spermatogenesis (4). Arginine-rich protamins in DNA of spermatozoa are replaced with lysine-rich somatic histones during forming of nuclear condensation. The strong interaction between positive loaded protamine and negative loaded DNA skeleton leads to chromatin condensation by facilitating connections between DNA and basic protamine. Chromatin condensation increases stabilization of spermatozoa DNA making it stronger thus motility of spermatozoa around oocytes increases and being protected from mutagens. Condensed chromatin becomes decondensed rapidly by dispersing in cytoplasm of spermatozoa during pronucleus development. Spermatozoa cannot complete chromatin condensation always; this developmental anomaly affects fertilization ability of spermatozoa negatively. Hence, the stability of spermatozoa nucleus is suggested as a significant factor which affects fertilization rates in assisted reproductive techniques.

Defective chromatin packaging leads to spermatozoa DNA damage (5). Increase of DNA strand breaks in spermatozoa indicates incomplete maturation of spermatozoa. There is an association between DNA fragmentation and defective chromatin packaging in spermatozoa (6,7). It has been found that DNA strand breaks increase in men have abnormal semen parameters (8,9). In addition, it has been reported that increase of spermatozoa with DNA fragmentation causes decrease in the rates of natural conception (10).

There are multiple assays to evaluate chromatin integrity, including TdT-mediated-dUTP nick end labeling (TUNEL), single gel electrophoresis assay (COMET), sperm chromatin structure assay (SCSA), the in situ nick translation assay, acridine orange test and colorimetric tests as sperm chromatin dispersion test (SCD) and dyes such as Toluidine Blue or Aniline Blue. Colorimetric tests measure the packaging quality of spermatozoa chromatin. Mature or immature spermatozoa chromatin displays different capacity in binding to dyes used for colorimetric tests. This difference is linked anomalies in the quality of chromatin packaging because of modification in nucleoprotein components occurs during spermiogenesis (11). A fixative (methanol), an acidic dye (eosin) and a cationic dye (methylene blue and derivatives) compose diff-quick stain. While the nucleus with normal DNA is stained lightly, the nucleus with fragmented DNA is stained darkly with diff-quick stain. In the acridine orange test, acridine orange dye is used; it is an intercalating dye appears red

when bound to fragmented DNA and green when bound to double-stranded DNA under fluorescent microscopy (12).

In this study, we aimed to investigate the changes in spermatozoa DNA condensation and progressive motility at the 0th, 3rd and 24th hours after semen processing and the correlation between DNA condensation and progressive motility of spermatozoa. This study also would provide possibility to make a comparison of the outcomes of acridine orange and diff-quick stains.

MATERIALS AND METHODS

Thirty semen samples were collected from men undergoing routine semen analysis in the Andrology Laboratory of Education and Research Hospital; all participating individuals gave informed consent. All samples were collected after a 3-5 day abstinence period and were allowed to liquefy for half an hour before analysis. Routine semen parameters were evaluated according to World Health Organization's (WHO) semen analysis guidelines (13). The specimens were subdivided into two aliquots. The first aliquot was left unprocessed and labelled 'raw.' The second aliquot was processed by a swim-up method and was evaluated at the 0th, 3rd and 24th hours for spermatozoa progressive motility. The percentage of the change in the progressive motility of spermatozoa was estimated.

DNA condensation of spermatozoa was evaluated by using the acridine orange and diff-quick stains. Therefore, the slides were prepared by smearing 5 µl of semen samples for each group, and were fixed in methanol at 4 °C for at least 30 minutes. Then slides were stained with diff-quick and acridine orange stains and they were examined respectively under the light and fluorescent microscopy. While sperm with normal light staining heads show condensed sperm (with intact DNA); sperm with abnormal dark staining heads show decondensed sperm (with DNA damaged) by the diff-quick stain. In the acridine orange staining, green fluorescing sperm are condensed sperm (with intact DNA); red fluorescing cells are decondensed sperm (with DNA damaged). In the evaluation, at least 200 sperm cells were counted for each slide, DNA condensed and decondensed sperm were recorded as a percentage of the total number of the cells counted. The percentage of the change in sperm DNA condensation was estimated.

Each parameter was summarized as mean ± standard deviation. The differences between the values recorded at different times of parameters were tested with Analysis of Variance. Bonferroni corrected Paired T Test was applied to the parameters in which significant differences were determined. The association between the percentages of changes in spermatozoa DNA condensation and progressive motility was tested with Analysis of Pearson's Correlation. The results were considered as significant if $p < 0.05$.

RESULTS

The percentages of spermatozoa DNA condensation and progressive motility recorded at different times were summarized in the table. There were no significant differences between the samples of the raw and the 0th, 3rd, 24th hours after semen processing in the percentage of spermatozoa DNA condensation. No differences were detected between the outcomes of acridine orange and diff-quick staining ($p > 0.05$, figure 1). There was a positive correlation between the outcomes of both stains. DNA condensed and decondensed spermatozoa stained with acridine orange and diff-quick stains were illustrated in the figure 2.

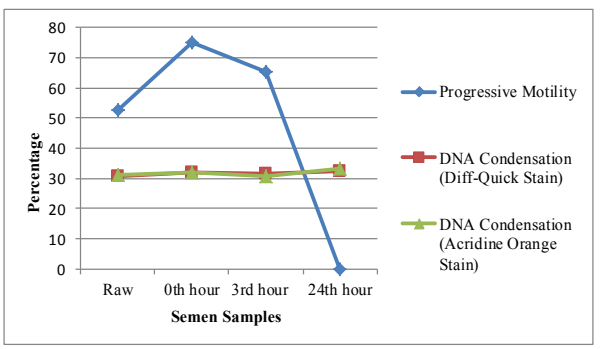

Figure 1. *Time-dependent changes in the percentages of spermatozoa DNA condensation and progressive motility. There are no significant differences in the percentage of DNA condensation among all semen samples ($p > 0.05$). The outcomes of acridine orange and diff-quick staining are not different ($p > 0.05$). There are significant differences in the percentage of progressive motility among all semen samples ($p < 0.05$).*

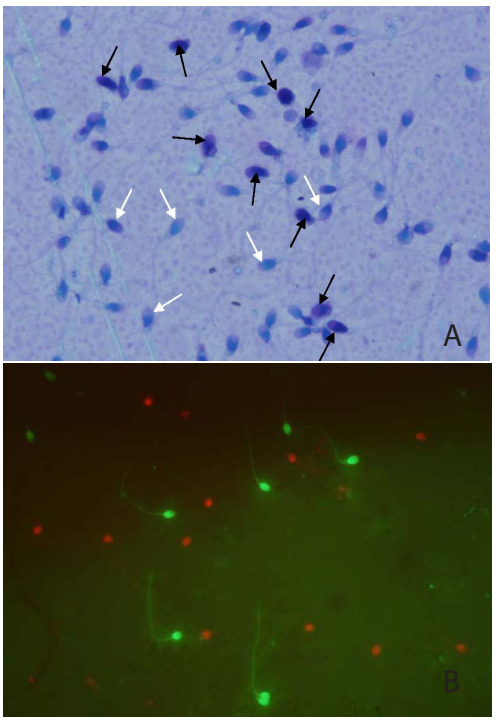

***Figure 2**. Evaluation of spermatozoa DNA condensation.*
A: Diff-Quick staining under light microscopy. Light staining heads (white arrows) show condensed (intact) DNA; dark staining heads (black arrows) show decondensed (damaged) DNA. (X100 magnification)
B: Acridine Orange staining under fluorescent microscopy. Green sperm heads show intact DNA, red sperm

The percentage of spermatozoa progressive motility was lower in the raw samples (52.77% ± 13.73), compared to the samples of the 0th and 3rd hours after semen processing. The percentage of spermatozoa progressive motility was the highest at the 0th hour after processing (75.03% ± 17.08) in all patients. The percentage of spermatozoa progressive motility decreased at the 3rd hour after processing, but it was still higher than in the raw samples (65.43% ± 19.62). There were not progressive motile spermatozoa at the 24th hour after processing. All outcome variables showed significant differences between the samples of the raw and 0th, 3rd, 24th hours after semen processing ($p < 0.05$) (table, figure 1).

There was no correlation between the percentages of the changes in spermatozoa DNA condensation and progressive motility ($p > 0.05$).

DISCUSSION

Naturally, chromatin condensation defects of spermatozoa have negative effects on male fertility because of spermatozoa chromatin's important role in the fertilization. Previous studies have shown that infertile men have more spermatozoa with chromatin condensation defects than fertile men (2,14). Hence, the nuclear integrity of spermatozoa could be regarded as a predictor of the fertility potential. Nevertheless, the biological significance of spermatozoa DNA integrity is well unknown (15). Although fertilization may occur after ICSI with DNA damaged spermatozoa, the first cleavage and later development potential of the male pronucleus remain unclear (16). It has been reported that increase of spermatozoa DNA damage affects embryonic development, increases risk of recurrent miscarriages, and decreases chances of a successful implantation in several previous studies (14,16-18). Another study has detected the decrease in the rate of implantation after ICSI with spermatozoa containing high DNA fragmentation (19). In contrast, some studies have reported no association between spermatozoa DNA damage and the outcomes of IVF and ICSI cycles (20). Yılmaz et al. have shown that there is no significant association between sperm DNA fragmentation and the outcomes of ICSI include fertilization, embryo development, embryo quality, cleavage rate and pregnancy rates (21).

The association between spermatozoa DNA damage and semen parameters is controversial. Some studies have reported either a weak or no correlation between DNA damage and semen parameters (2,22). In a study performed to determine the value of spermatozoa chromatin condensation in the assessment of male fertility, chromatin condensation has been found lower in infertile men compared to fertile men. However, in that study, no correlation has been found between chromatin condensation and concentration, motility, morphology of spermatozoa and it has suggested that chromatin condensation is a value parameter in the assessment of male fertility independent of conventional semen parameters, and the inclusion of chromatin condensation to routine semen analysis (23). On the other hand, there are also some studies, which reported that spermatozoa from patients with abnormal semen parameters have high DNA damage (24-27). It has been reported that chromatin condensation defects and DNA strand breaks increase in morphologically abnormal spermatozoa compared to normal counterpart (28,29). Increased production of re-

***Table 1**. Time-dependent changes in the percentages of spermatozoa DNA condensation and progressive motility.*

	Semen Samples			
	Raw	**0th hour**	**3rd hour**	**24th hour**
*Progressive Motility%	52.77±13.73	75.03±17.08	65.43±19.62	0±0
**DNA Condensation% (Diff-Quick Stain)	30.70±8.34	31.93±8.55	31.67±8.22	32.63±9.29
**DNA Condensation% (Acridine Orange Stain)	31.0±8.79	32.03±8.38	30.90±8.02	33.33±9.32

Values are means ± standard deviation.
* P < 0.05 among all semen samples. ** P > 0.05 among all semen samples.

active oxygen species by immotile or morphologically abnormal spermatozoa may be responsible for DNA damage (27,30). Moskovtsev et al. have significantly correlated spermatozoa DNA damage to concentration, motility, and morphology and they have indicated that the degree of spermatozoa DNA damage increases with the number of abnormal semen parameters (31). Our study showed that there was no any association between spermatozoa DNA condensation and progressive motility.

Degree of spermatozoa DNA damage is expressed with DNA fragmentation index (DFI). There is an association between DNA fragmentation and defective chromatin packaging in spermatozoa. Increased percentage of spermatozoa with DNA fragmentation has decreased the rates of natural conception (10). In two independent studies, it has been found that DFI levels more than 30-40% are incompatible with fertility in vivo, independently of concentration, motility and morphology of spermatozoa (14,32). Bungum et al. have aimed to determine the association between spermatozoa chromatin defects and consequences of IVF, ICSI and IUI by measuring spermatozoa DNA damage with SCSA in total 306 patients. Their results have shown that DFI higher than 27% reduces in vivo pregnancy chance, DFI lower than 27% is compatible with the rates of pregnancy and birth after IVF and ICSI (16). Whereas, Speyer et al. have investigated the rate of implantation after ICSI and IVF cycles with spermatozoa containing high DNA fragmentation and they have detected that there is a negative correlation between the rates of implantation and pregnancy, and DFI for ICSI cycles, but the rates of implantation and pregnancy do not decrease despite the levels of high DFI (≥ 30%) for IVF cycles (19).

DNA damage in spermatozoa has been detected by using a variety of assays however; most of these assays are difficult and take a long time. Diff-quick stain is used routinely to evaluation sperm morphology and it has a very simple and fast protocol. In a previous study, a significant correlation has been found between the results of TUNEL assay and diff-quick stain, also it has shown an association between the percentage of sperm nuclei darkly stained with diff-quick and semen parameters. That study has suggested that diff-quick stain can be adapted as an indicator in evaluation of spermatozoa chromatin (33). Therefore, in our study, we used diff-quick stain to evaluation of spermatozoa DNA damage and compared it to acridine orange stain. Acridine orange test has been used by some laboratories in an attempt to improve male fertility evaluations (34,35). This test is used to measure the susceptibility of spermatozoa DNA to acid-induced denaturation (34). According to our study results, there was a positive correlation between the outcomes of acridine orange and diff-quick stains suggesting that both tests in the evaluation of male fertility are reliable.

Various sperm processing methods are used to ready sperm in IVF applications. The effect of sperm processing methods on DNA quality is exactly unknown. Swim-up and density gradient centrifugation are the two commonly used methods for sperm processing. Ghumman et al. have shown in their study which they investigated the efficiency of single and combined sperm processing methods for isolation of sperm with better DNA integrity that using combination of swim-up and density gradient centrifugation is the most efficient method to eliminate DNA damaged sperm (36). Marchesi et al. also have reported that sperm DNA integrity and motility are improved by semen processing and the modified swim-up method provides the best results (37).

The goal of surgical sperm retrieval is to obtain the best quality sperm and sperm retrieval may be performed either on the day before or on the same day as the OPU. Sperm retrieval performed on the day before the OPU provides practical advantages. It has found that the rates of fertilization and clinical pregnancy, which occurred

with sperm obtained on the day before or on the same day as the OPU, are similar to each other (38). In addition, motility of prepared testicular sperm has increased after 24 hours of culture despite the low initial sperm motility (39). However, it could be considered that preservation of spermatozoa during 24 hours can affect DNA fragmentation. In a previous study, DNA fragmentation of spermatozoa in semen samples preserved in room temperature during 4-24 hours has been evaluated. It has been detected that there is a significant increase in DNA fragmentation of sperm preserved in room temperature during 24 hours, whereas DNA fragmentation is not affected in semen samples preserved in room temperature at periods shorter than 24 hours (40). We used swim-up method as a spermatozoa processing method in our study. The percentage of spermatozoa progressive motility increased after swim-up method compared to raw, but there were no significant differences between the processing and raw samples in the percentage of spermatozoa DNA condensation. In our study, spermatozoa DNA condensation did not decrease even at 24th hour. However, it should be noted that evaluation of the effects on the rates of fertilization, embryo development, pregnancy and live births of waiting time between the obtaining and processing of spermatozoa and the method used for the treatment of infertility is as significant as evaluation of the effects on the semen parameters and spermatozoa DNA condensation of waiting time. In the present study, these data was not investigated, that is the limitations of our study.

CONCLUSION

The results of our study show that the percentage of spermatozoa with progressive motility is higher in the samples of the 0th and 3rd hours after swim-up than in the raw samples. Also, there is no significant difference among the samples of the raw and the 0th, 3rd, 24th hours after processing in the percentage of spermatozoa DNA condensation. Therefore, spermatozoa selection can be performed reliably at the 3rd hour after spermatozoa processing for IVF and ICSI applications. In addition, it is possible to say that the obtaining and processing of spermatozoa that can be performed on the day before the OPU when it is required as testicular spermatozoa retrieval would not cause to any change in spermatozoa DNA condensation.

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