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Research Article

Does Ambıent Temperature and Sperm Preparatıon Method Affect Sperm DNA Fragmentatıon and Maturatıon

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Abstract

The importance of sperm DNA damage in infertility is known. In addition, sperm maturation defects have been shown to be one of the DNA damage mechanisms and their level is determinant in IUI pregnancies. Especially in varicocele cases, the negative effects of rising testicular temperature are known. Therefore, in our study, we aimed to investigate the effect of temperature used in sperm incubation and also the effects of two sperm selection methods on sperm DNA integrity and chromatin maturation.

This prospective controlled study was conducted in the normospermia (n:40), oligoastenoteratospermia (n:40) groups that applied to Biruni University Hospital between January-June 2018 for semen analysis. Male patients between the ages of 27-45 were included in the study and patients without a diagnosis of endocrinological and / or metabolic disorder and varicocele and / or urinary tract infection were selected. In normospermic and oligoasthenoteratospermic (OAT) cases, swim-up and density gradient methods were used for sperm selection. After sperm selection, samples were kept at 22 and 37 °C, sperm DNA fragmentation was assessed with acridine orange stain, sperm maturation was assessed by acidic aniline blue test.

In both selection methods, sperm motility increased and DNA fragmentation decreased. Especially in cases of OAT, the negative effects of incubation at 37 °C in terms of DNA fragmentation appeared compared to normospermia. In our study, it was found successful in selecting sperms with DNA damage and maturation defect by both gradient and swim-up method in normospermia or OAT cases ($p < 0.05$). In both sperm selection methods, a temperature of 37 °C was observed to cause an increase in sperm DNA fragmentation ($p < 0.05$). There was no difference in results in terms of sperm maturation.

In IVF laboratories, incubation temperature is generally 37 °C in sperm preparation. It has been understood that the use of room temperature in long-term incubations may reduce sperm DNA damage due to its damaging effects. The study should be evaluated with IVF results.

INTRODUCTION

The effects of environmental factors on sperm DNA have been a very interesting subject in recent years. High DNA fragmentation seen in cases of varicocele with high testicular temperature indicates that the temperature negatively affects sperm production. (1,2,3).

Sperm DNA is packed tightly and densely in the nucleus (4,5). While somatic cell DNA fills only one part of the nucleus, sperm DNA covers almost the entire nucleus. This arrangement in the sperm DNA enables the packaging of the genetic information to be transferred to the oocyte and thus the development of the embryo (6,7). The sperm chromatin packaging in spermatozoa occurs in four steps: DNA binding to the nucleus membrane, DNA cluster formation after binding of the nucleus, replacement of histones with protamine and chromosomal position (8). 90-95% of the histones involved in the packaging of DNA in the sperm cell are replaced by protamines specific to the sperm cell (7). Protamines; they are proteins that are half the size of histones, rich in arginine, located in the sperm nucleus and synthesized in the advanced stages of spermatogenesis. Protamination is epigenetic regulation specific to sperm cells (9). One of the most important mechanisms of sperm DNA damage is the packaging of abnormal or irregular chromatin (10,11).

During the spermatogenesis process, at the spermatid stage, chromatin is enriched with protamine, and sperm performs chromatin condensation. Abnormal chromatin condensation process causes developmental abnormalities in sperm (12). Therefore, in the andrology laboratory, an estimation of sperms to be used in IVF / ICSI application can be provided by examining the sperm DNA fragmentation index and maturation defect rates.

On the other hand, sperm manipulations are performed intensively in IVF applications (13). Density gradient application or swim-up method has taken its place as a routine procedure. However, there is no consensus yet on how these procedures affect sperm quality.

Gradient and swim up methods are routine sperm preparation techniques before IVF-ICSI application routinely used in IVF laboratories. While the gradient method separates abnormal and normal sperm by taking advantage of density differences, the swim up method allows the sperm to swim towards the upper

phase and separate the fast ones (14). Both methods are aimed at obtaining the highest quality sperm with high motility and normal morphology, it allows dead, immotile sperm to separate from the semen together with white cells (15). In IVF applications, sperm selection has been a subject that has been emphasized in recent years and the presence of normal maturation and DNA integrity in selected sperm directly affects the implantation adequacy of the embryo. Therefore, in this study, we aimed to investigate the effects of ambient temperature and preparation procedures on the DNA structure and maturation of sperm.

MATERIALS AND METHODS

Our study was conducted in 80 cases (40 normospermia and 40 oligoasthenoteratospermia) who applied to Biruni University Hospital between January 2018 and June 2018 to perform semen analysis. For this study, the approval of the ethics committee numbered 2018-12-9 and dated 29.01.2018 was obtained from the ethic committee of Biruni University. Male patients between the ages of 27 and 45 were included in the study, and patients with endocrinological and / or metabolic disorders and varicocele and / or prostate diagnosis were excluded as criteria for exclusion.

Standard semen analysis was performed according to World Health Organization Criteria (WHO) in both groups (16). The smear preparation was prepared for the application of acidic aniline blue dye and fixation was performed in 2.5% glutaraldehyde (Merck, Germany) solution for 30 minutes. For 10 minutes it was stained with acidic aniline blue, washed with distilled water, dried and evaluated with a 100x immersion objective. Sperm stained with aniline blue, condensation defect of Henkel and Irez, unstained spermatozoa were evaluated according to the rule that they had a healthy and normal chromatin structure (17,18). Sperm maturation defect rates were calculated by counting 200 sperms. The second smear preparation was prepared for the staining application of acridine orange and fixation was made in 1/3 acetic acid / methanol (Carnoy) fixative for 1 hour. After 5 minutes of treatment with Acridine Orange stain, it was evaluated with a fluorescent microscope after washing with distilled water. Red, yellow fluorescent cells were considered carry fragmented DNA , green stained cells were considered healthy and DNA fragmentation rate was calculated by counting 200 sperms (19).

For the swim-up process, the semen sample was placed in two falcon conical tubes and added medium (1: 1), centrifuged at 1600 rpm for 8 minutes. Supernatant was taken with a pipette. The tubes were placed at a 45 degree angle and 1 ml medium was added on the pellet. The first tube was incubated for 1 hour at 37ºC and the second tube at room temperature. By taking some amount of supernatant, sperm concentration and motility were re-examined and recorded. Each sample was also smeared and placed in fixatives after drying for 10 minutes for analysis of maturation defect and DNA fragmentations.

The gradient solution was diluted with sperm wash medium to 80% using 100% Grad (Lifeglobal, AllGrad 100%). Single phase 80% gradient was used. 1 ml gradient solution was added to 15 ml falcon tube and 1 ml of semen sample was carefully added on it. Centrifuge was carried out for 15 minutes at 2000 rpm. 1 ml medium was added by discarding the supernant and washed by centrifugation at 1600 rpm for 8 minutes. The upper phase was discarded and 2 ml medium was added and incubated for 1 hour at 37 °C and room temperature (22 °C).

20 microliters were taken from the incubated samples, sperm count and motility were re-examined and recorded. Finally, with the help of a pipette, a total of 1 ml of sperm suspension was drawn from the surface of the tube, and 2 samples (0,5 ml) were transferred to the tube to be kept at 37 °C and 22 °C. Prepared sperm incubated at specified temperatures for 1 hour was used for maturation defect and DNA fragmentation analysis.

Statistical Methods

Mann-Whitney Test, Friedman Test, Wilcoxon Test were used by using SPSS 22 statistics package.

RESULTS

Table 1 shows the semen parameters of normospermic and oligoasthenoteratospermic (OAT) cases. Higher DNA fragmentation and chromatin condensation defects are seen in the OAT group. DNA fragmentation occurred when the sperm reacted with a red or yellow color reaction with the Acridine orange test (figure 2)

Table 2 shows the average of histone positive (immature) sperm percentages in normospermic cases with acidic aniline

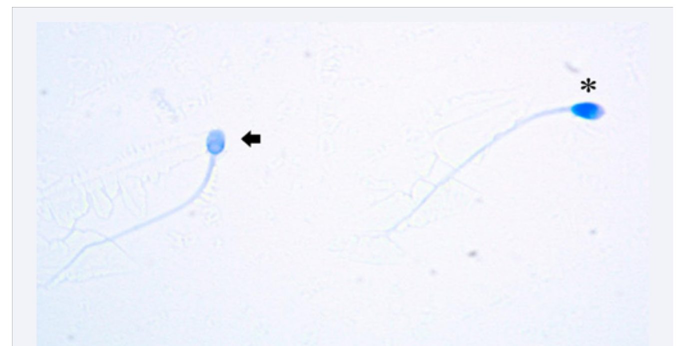

Figure 1 Sperms stained with or without staining of acidic aniline blue stain are evaluated as negative (-), and dark blue stained sperms are evaluated as positive (+) and show maturation defect. (100x immersion lens with light microscope image) Arrow AB negative, * AB positive.

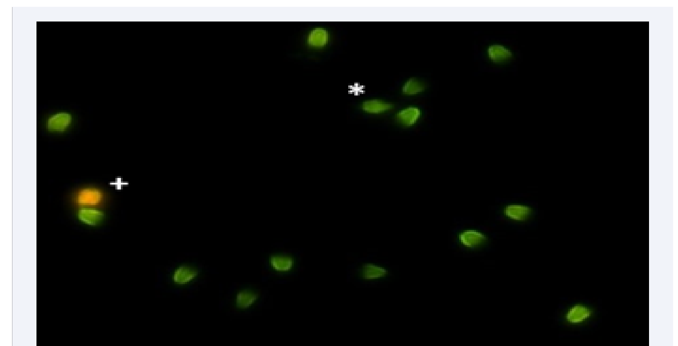

Figure 2 Acridin orange staining A) shows that there is no DNA fragmentation in the sperms displayed as green (normal), B) shows DNA fragmentation in the sperms displayed as yellow or orange (damaged). (100 x immersion lens fluorescent microscopy image).

Table 1: Demographic data of normospermic and OAT cases.			
	Normospermia (x±SD)	**OAT (x±SD)**	**p**
Age	32,45±6,23	33,49±4.96	0,276
Normal	6,25±1,88	2,55±1,91	<0,001
Head anomalies	53,55±7,57	53,55±7,99	1,000
Middle piece	21,95±5,52	19,16±8,84	0,239
Tail	18,35±4,77	22,40±5,56	0,018
Progressive motility (%)	44,60±7,58	22,75±7,21	<0,001
Concentration (mil/ml)	69,60±41,68	10,27±3,36	<0,001
Sperm Cond. defect%	21,05±7,19	50,55±10,73	<0,001
DFI %	21,50±6,60	30,85±10,64	0,002

Table 2: Aniline Blue Test positive sperm results and comparison in the normospermia group.			
	Mean±Std. Dev. (n=20)		**p**
ABtest [2,3]	21,05±7,19	(1)	0,001*
ABGradRT [1,3]	16,30±7,03	(2)	
ABGrad37 [1,2]	15,35±6,86	(3)	
ABSwimRT	16,75±6,97		0,053
ABSwim37	15,25±7,02		

blue method. It has been observed that both swim up and gradient methods decrease the percentage of AB positive sperm. However, the change in temperature did not affect these results. The acidic aniline blue test is shown in (figure 1). Sperm stained in dark blue show histone positivity. This indicates chromatin condensation error.

In (Table 3), sperm maturation defect was decreased by sperm preparation methods in oligospermic cases. The reduction was higher in the Gradient application. It was understood that the change of temperature did not differ in terms of maturation.

Table 4 shows the normospermia group sperm DNA fragmentation rates. Although sperm preparation methods have positive results in terms of DNA fragmentation, negative effects of temperature increase have been observed.

In (Table 5), in oligospermic cases, sperm preparation methods were successful in isolating sperms with fragmented DNA but negative effects of 37°C degrees temperature were observed.

When the groups were compared in terms of sperm maturation defect in (Table 6), it was found that the sperm maturation defect in the seminal fluid was always high, but the different temperature did not show a significant difference in the isolated sperm groups. In terms of DNA fragmentation, the values in semen were always high, and it was understood that when the samples were incubated at 37°C degrees or room temperature, 37°C degrees statistically increased DNA damage.

Percentage changes of the AB and AO test [(RT value-AB before) / AB before] compared to the previous value were calculated and averaged in both groups. The averages of change percentages from the previous value were compared with the Mann-Whitney Test.

DISCUSSION

In this study, in the normospermic patient group, compared with the oligoasthenoteratospermic (OAT) group, lower fragmentation and lower maturation defects were observed in terms of sperm maturation and DNA fragmentation. It has been observed that the density gradient or swim up method has a similar effect in reducing sperm DNA fragmentation and maturation defects but the ambient temperature has a negative effect in terms of DNA fragmentation.

In many mouse species, it has been understood that when fertility-inhibiting mutants appear, the shaping of the sperm head and nuclear condensation is related to the modeling of the chromatin structure (20,21).

The basis of sperm DNA damage is the regulation of spermatogenesis by decreasing DNA repair mechanisms in the late period (22). When the mechanisms responsible for DNA fragmentation in human sperm, including those that occur during spermatogenesis and transport along the reproductive tract, are reviewed, defects in chromatin remodeling during the process of spermiogenesis, DNA damage caused by oxygen radicals, sperm caspases during the transition from the seminiferous tubules

Table 3: Aniline Blue Test positive sperm results in oligospermia group and comparison.			
	Mean±Std. Dev. (n=40)		**p**
ABtest [2,3]	50,55±10,74	(1)	0,001*
ABGradRT [1,3]	42,60±9,83	(2)	
ABGrad37 [1,2]	40,70±9,59	(3)	
ABSwimRT	46,50±10,22		0,059
ABSwim37	45,50±9,67		

Table 4: DNA fragmentation results and comparison in the normospermia group.			
	Mean±Std. Dev. (n=40)		**p**
AObefore [2,3]	21,50±6,61	(1)	0,001*
AOGradRT [1,3]	18,00±6,22	(2)	
AOGrad37 [1,2]	19,55±6,86	(3)	
AOSwimRT	16,60±6,40		0,01**
AOSwim37	17,60±6,07		

Table 5: DNA fragmentation results and comparison in oligospermia group.			
	Mean±Std. Dev. (n=20)		**p**
AOunprocessed [2,3]	30,85±10,64	(1)	0,001
AOGradRT [1,3]	28,35±10,95	(2)	
AOGrad37 [1,2]	32,55±10,89	(3)	
AOSwimRT	25,50±10,46		0,0001
AOSwim37	30,20±10,34		

Table 6 : Percentages of change and comparison between groups according to the previous measurement.				
		Normospermia	**Oligospermia**	**p***
AB	ABunpro_RT_difference	-0,19±0,38	-0,16±0,09	**0,024**
	ABunpro_37_difference	-0,24±0,35	-0,19±0,09	**0,028**
	AB_RT_37_difference	-0,05±0,11	-0,04±0,02	0,489
AO	AOunpro_RT_difference	-0,17±0,07	-0,09±0,06	**0,001**
	AOunpro_37_difference	-0,10±0,06	0,06±0,05	**0,001**
	AO_RT_37_difference	0,09±0,09	0,18±0,12	**0,002**

to the epididymis and activation of endonucleases, the effect of environmental toxicants gain importance (23,24). Sperm DNA can explain the difference between single and double DNA strand breakage, fertile and infertile men. Sperm DNA fragmentation may be caused by structural factors such as abortive apoptosis, deficiencies in recombination, protamine deficiencies or oxidative stress (23,25). Damage may also occur due to external factors such as storage temperatures, elongation agents, conditions of use, duration after ejaculation, infections and reactions to drugs or oxidative stress after testis (23).

Two features distinguish sperm from somatic cells: Protamination and no DNA repair. DNA repair in sperm ends after transcription and spermiogenesis, so these cells do not have a mechanism to repair the damage that occurs during the transition to the epididymis and during post-ejaculation (26). Oocytes and early embryos have been shown to repair sperm DNA damage, so it has been suggested that the effect of sperm DNA fragmentation is related to the combined effects of sperm chromatin damage and capacity (27).

In our study, higher DNA fragmentation with higher condensation defect was observed in the advanced OAT group. Shamsi et al. Found high DNA fragmentation in oligozoospermic, asthenospermic and OAT cases in their study in 2009 (28). In these cases, they also detected a lower rate of antioxidant enzymes (28). Several techniques have been developed related to sperm protamination. CMA3, Toluidine blue and aniline blue techniques stain sperm histones and show protamination defects (29). The relation of sperm protamination errors with infertility has been demonstrated by Irez and Aoki et al (29,30). According to the studies conducted by Irez et al. In 2015 and 2018, it has been shown that sperm chromatin condensation values (aniline blue negative sperm rate), that is, protamination, can predict clinical pregnancy in mild male factor and unexplained infertile cases (25,29). Researchers have shown that sperm nuclear proteins, that is, the protamination of histones, play a very important role in the development of embryos after fertilization and that can predict positive pregnancy in intrauterine insemination (25,29). Protamines are the main nuclear proteins in sperm. The human sperm nucleus contains two types of protamine: a family of protamine 1 (P1) and protamine 2 (P2) proteins (P2, P3 and P4) encoded by a single copy gene and a precursor protein. Protamines have been discovered more than a century ago and their functions are not yet fully understood. In reality, different hypotheses have been proposed for protamines, as Oliva R. explains in 2006: condensation of the sperm nucleus into a compact hydrodynamic shape, preservation of the genetic message transmitted by spermatozoa, involvement in processes that maintain the integrity and repair of DNA during and after nucleohiston-nucleoprotamine passage, and taking part in epigenetics functions of spermatozoa.(31,32) . Changes in the expression of P1 and P2 protamine have been found to be associated with infertility in humans (31). Mutations in protamine genes have also been found in some infertile patients (31). Transgenic mice that are defective in the expression of protamines also show various structural defects in the sperm nucleus and have varying degrees of infertility (33). There is evidence that varying levels of protamine can cause increased susceptibility to damage to spermatozoan DNA, and it has also been shown to cause infertility or poor ART results in assisted reproduction (34).

In a study conducted to investigate the effect of ambient temperature on sperm DNA fragmentation, sperm aneuploidy, seminal alpha glucosidase level (NAG), sperm maturation and acrosine activity, the scrotum temperature was warmed for 30-40 minutes per week, up to 43 degrees in volunteers and the results of 3 months were evaluated. (35). After the heated treatment, a decrease in sperm acrosine activity and NAG level and DNA fragmentation was observed. It was also shown to have increased sperm aneuploidy (35). In a study conducted by Wach-Gygax L. et al in 2017, DNA fragmentation and membrane integrity were examined in the annual follow-up of horse sperm (36). In the study, the researchers showed that sperm concentration and motility were highest in spring and decreased in summer. They suggested that the quality of sperm changed seasonally. The 37°C degree incubation we used in our study was found to increase sperm DNA fragmentation. This situation confirmed previous experimental studies (35,36).

Sperm preparation methods are one of the important steps in assisted reproductive techniques. The common purpose of all sperm preparation methods is to get rid of seminal plasma and non-sperm cells and at the same time to obtain the highest capacity sperm (17). Today, density gradient and swim-up techniques are used routinely for sperm preparation in IVF laboratories. Generally, the gradient method is preferred for samples with low concentration and motility, whereas the swim-up method is sufficient for samples with high motility and concentration. In this study, both methods were compared in the normospermia and OAT groups. According to this study, when swim up and gradient methods were used as a sperm preparation method, no significant difference was found in terms of motile sperm, sperm concentration and morphology in the normospermic and OAT groups. Considering the motility and concentration parameters, both methods can be preferred depending on the condition of the

case. According to the studies of Spano et al. In 1999, sperms that have completed their chromatin condensation can be selected by the swim up method (37). There are also studies showing that the density gradient method significantly reduces sperm DNA fragmentation (38). According to other studies, the reason why the density gradient method shows higher DNA damage than the swim-up method is thought to be more centrifuge application (39). Studies have shown that centrifuge applications increase DNA damage (40). In our study, we also found higher DNA fragmentation in both normospermic and OAT cases compared to the swim up method in the gradient application. It has been shown that sperm DNA fragmentation is high in asthenospermic cases (41). Various animal studies have shown that temperature stress causes oxidative stress mechanisms in the testicles and DNA fragmentation by causing ROS production (42). The increase in DNA fragmentation associated with increasing temperature in our study supports this finding.

In conclusion, in this study, sperm maturation defect and DNA fragmentation have been shown to decrease with both density gradient and swim up method. Especially in OAT samples, compared to normospermia, the negative effects of incubation at 37 °C in terms of DNA fragmentation appeared. It has been observed that the increase in temperature increases sperm DNA damage but does not affect sperm histones.

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