ORIGINAL ARTICLE
KLİNİK ARAŞTIRMA

Effects of antioxidants on motility and DNA integrity in frozen-thawed sperm

Antioksidanların sperm kriyoprezervasyonu sonrası motilite ve DNA bütünlüğü üzerine etkileri

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SUMMARY

Aims: This study aims to investigate whether the addition of the antioxidant complex including taurine, ascorbic acid, and glutathione into the cryopreservation medium affects the damage to sperm during the freezing process.

Material and Methods: Ejaculate samples of patients who applied for semen analysis to the Assisted Reproduction Unit of Private Adatip Hospital were used. Fresh samples were analyzed for standard semen quality parameters according to the World Health Organization guidelines. Samples within the normal range were evaluated for sperm DNA fragmentation using the Halosperm technique. Remaining ejaculates were washed with the gradient method before cryopreservation. Sperm samples of each patient were divided equally for freezing in a cryopreservation medium with or without the antioxidant supplementation. One month later, the samples were thawed. Post-thaw total motility and DNA fragmentation were determined for each sample.

Results: Semen samples of 40 patients were analyzed. We observed decreased total motility (34.8 ±5.32 % vs. 65.5 ±6.42 %, P= 0.002) and increased sperm DNA fragmentation (52.3±5.42 % vs. 26.4±3.12 %, P= 0.002) in post-thaw semen samples following cryopreservation in comparison to fresh samples. The addition of antioxidants to the freezing medium did not have a statistically significant effect on sperm motility (38.3± 6.22 % vs. 34.8 ±5.32 %, P =0.07) and DNA damage (47.5±4.7 % vs. 52.3±5.42, P =0.08) when compared to control samples following the freezing process.

Conclusions: We observed increased sperm DNA fragmentation and decreased total motility following cryopreservation. No significant improvement in sperm motility or DNA integrity was obtained after the addition of 5 μM of the antioxidants taurine, ascorbic acid, and glutathione to the freezing media.

Keywords: halosperm, DNA fragmentation, antioxidants, sperm freezing, motility, cryopreservation

ÖZET

Amaç: Bu çalışmanın amacı sperm dondurma medyumuna taurin, glutatyon ve askorbattan oluşan antioksidan kompleksi eklenmesinin, dondurma işleminde oluşacak sperm hasarına etkisini incelemektir.

Materyal ve Metodlar: Özel Adatıp Hastanesi Üremeye Yardımcı Tedavi Merkezi'ne semen analizi için başvuran hastaların ejakülat örnekleri kulanıldı. Taze numuneler, Dünya Sağlık Örgütü kriterlerine göre analiz edildi. Normal aralıktaki numuneler Halosperm tekniği kullanılarak sperm DNA fragmantasyonu açısından değerlendirildi. Kalan ejakülatlar, kriyoprezervasyon öncesinde gradient yöntemiyle hazırlandı. Her hastanın sperm örnekleri, bir kriyoprezervasyon ortamında antioksidan takviyesi ile veya antioksidan takviyesi olmadan dondurmak için eşit olarak bölündü. Örnekler dondurulduktan 1 ay sonra çözdürme işlemi yapıldı. Çözülme sonrası motilite tayini yapıldı ve DNA fragmantasyon analizi yapıldı.

Bulgular: 40 hastanın semen örnekleri analiz edildi Dondurma çözdürme sonrası, antioksidan eklenen ve eklenmeyen grupların ikisinde de dondurma öncesine oranla total motilitede anlamlı bir düşüş görüldü (34.8 ±5.32 vs 65.5 ±6.42, P= 0.002; 38.3± 6.22 vs 65.5 ±6.42, P= 0.003). Kontrol ve deney gruplarının ikisinde de dondurma öncesine oranla DNA fragmantasyonunda anlamlı bir artış görüldü (52.3±5.42 vs 26.4±3.12, P= 0.002; 47.5±4.7 vs 26.4±3.12, P= 0.003). Ancak, kriyoprezervasyon ortamına eklenen antioksidanların total motilite (38.3± 6.22 % vs. 34.8 ±5.32 %, P =0.07) ya da DNA fragmantasyonuna (47.5±4.7 % vs. 52.3±5.42, P =0.08) anlamlı bir etkisi bulunmadı.

Sonuç: Kriyoprezervasyondan sonra sperm DNA fragmantasyonunda artış ve toplam motilitede azalma gözlendi. Kriyoprezervasyon ortamına 5 uM taurin, glutatyon ve askorbattan oluşan antioksidan kompleksi ilave edildikten sonra sperm motilitesinde veya DNA bütünlüğünde anlamlı bir fark gözlenmedi.

Anahtar kelimeler: halosperm, DNA fragmantasyonu, antioksidan, sperm dondurma, motilite, kriyoprezervasyon

INTRODUCTION

Since the first attempts to preserve human spermatozoa at sub-zero temperatures in 1776 (1), considerable research has been conducted to improve the methods of cryopreservation for optimum cryosurvival and to overcome the associated physical and chemical damage to the sperm (2, 3). Some indications for storage of sperm comprise cytotoxic treatments such as chemotherapy and radiotherapy, sperm donation, obstructive surgeries such as vasectomy, and reproductive problems such as anejaculation, severe oligozoospermia, and obstructive azoospermia (4). The structural and functional integrity of the spermatozoa determines the capability to fertilize an oocyte and therefore is a key for success in assisted reproductive techniques (ARTs) (5).

Frozen-thawed semen can be used for ARTs including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Several clinical reports presented live births with the successful use of human spermatozoa stored for longer than 20 years (6, 7). Although sperm cryopreservation is an effective route to preserve male fertility, some limitations still exist. Despite the advantages spermatozoa membrane presents, cryopreservation leads to several damaging processes such as thermal shock with the formation of intracellular and extracellular ice crystals, cellular dehydration, and osmotic shock (8). A well-controlled cryopreservation process, therefore, is critical to maintaining the original pre-freezing sperm quality parameters. The cryopreservative medium is one of the factors contributing to optimum cryosurvival, egg yolk, and glycerol being the most common constituents (9). Several studies have examined cryodamage in spermatozoa and indicated oxidative stress as one of the main causes of alterations in function due to an imbalance between reactive oxygen species (ROS) and the antioxidant system of spermatozoa (10). Although the effect of cryopreservation on sperm DNA integrity is controversial, many studies propose that cryopreservation induces DNA fragmentation (11-13). Production of ROS has been suggested to be mainly responsible for DNA damage in post-thaw spermatozoa (14, 15).

Seminal fluid as an important source of antioxidants is thought to be crucial in protecting spermatozoa from oxidative injury (16). Vitamin C is one of the principal antioxidants in seminal plasma of fertile men, with a major role in the chain-breaking antioxidant capacity of the semen (17). Decreased seminal glutathione was reported to be implicated in low sperm quality (18). Taurine was identified in mammalian sperm and reproductive tract and was proposed to be essential for fertilization (19). The requirement of antioxidants to overcome the damages mediated by oxidative stress prompted us to investigate the effects of ascorbic acid (vitamin C), glutathione and taurine on post-thaw sperm motility and DNA integrity. This study aims to examine the motility and DNA fragmentation in frozen-thawed sperm when the cryopreservation medium is supplemented with the antioxidants ascorbic acid, glutathione, and taurine.

MATERIALS AND METHODS

Patients

Forty volunteer non-smoking men in early adulthood (between ages 20–40) with normozoospermia according to the World Health Organization (WHO) 2010 criteria (20) were involved in the study. The patients had no history of any systemic disease, febrile disease at childhood, testicular trauma, varicocele, or other diseases that affect reproductive function. Informed consent was obtained from patients. This study was approved by the local clinical ethics board of Maltepe University Faculty of Medicine, Istanbul.

Study Design

Semen samples were collected in sterile containers by masturbation after a sexual abstinence period of 2-7 days. The liquefied fresh semen samples were used to analyze standard semen quality parameters according to the WHO guidelines (20) and sperm DNA fragmentation. Remaining aliquots were prepared for cryopreservation with or without the addition of antioxidants for (i) a direct comparison of fresh and frozen semen from the same ejaculate and (ii) evaluation of the effect of antioxidants in cryopreservation media on sperm motility and DNA fragmentation.

Semen Analysis

Semen analysis was performed following a period of incubation at room temperature for 10-60 minutes to allow for liquefaction. After complete liquefaction, the volume and the pH were measured. Ten microliters of each sample were used to fill a Makler counting chamber (Sefi-Medical Instruments, Israel) and the specimens were assessed for sperm count and motility using a phase-contrast microscope (CH30, Olympus Corp., Japan) under magnification of 20x.

The semen smear was prepared, air-dried, and stained with Diff-Quick (Polysciences, USA) to evaluate the

sperm morphology. Briefly, slides were fixed with Diff-Quick fixative agent and stained with cationic and anionic solutions. At least, 200 sperms were counted under 100x magnification according to Kruger's strict criteria (21).

Assessment of DNA Fragmentation

Halosperm Technique

Sperm DNA fragmentation was detected using the Halosperm kit according to the manufacturer's instructions (Halotech DNA SL, Spain). The Halosperm kit facilitates DNA fragmentation analysis based on the sperm chromatin dispersion technique (SCD test)(22). SCD test enables the discrimination of spermatozoa with fragmented DNA following acid denaturation and removal of nuclear proteins. In this way, sperm with intact DNA create halos of dispersed DNA loops while those with degraded DNA will generate either no halos or small halos.

The low melting point agarose gel supplied with the kit was placed in a microwave for 5 minutes to melt and was transferred to a water bath at 370C for 5 minutes. 25μL of the semen sample was then immersed in the liquefied agarose gel and spread onto the slide. The coverslip was gently removed after a subsequent incubation for 5 minutes on a cold plate at 40C for agarose polymerization and sample embedding. Next, the sample was immersed in an acid denaturation solution for 7 minutes, followed by 25 minutes of incubation in lysing solution. Samples were rinsed and dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%). Finally, the slides were air-dried, stained with Diff-Quick and the halos of dispersed DNA loops were observed under 100x magnification. At least 200 spermatozoa were evaluated in this test.

Classification of Sperm

The criteria to determine intact and fragmented DNA in spermatozoa were described by Ferna´ndez et al (22). Five SCD patterns are possible as illustrated in **Figure 1**: (i) large halo with a thickness greater than or equal to the minor diameter of the core (ii) medium halo with a halo size between that of big halo and small halo (iii) small halo with a thickness equal to or smaller than 1/3 of the minor diameter of the core (iv) without halo where no halo is formed (v) degraded where no halo is formed and a weakly or irregularly stained core is demonstrated.

Determination of DNA Fragmentation Ratio

Spermatozoa were classified for possible SCD patterns mentioned above. DNA fragmentation ratio was determined by calculating the total percentage of spermatozoa with DNA fragmentation including degraded spermatozoa and those with small halo and without halo.

Cryopreservation

Density gradient centrifugation

Spermatozoa were selected by density gradient centrifugation method before cryopreservation. First, 50% and 90% of gradient solutions were prepared by diluting the gradient solution (SpermFilter, Gynotec, Netherlands) with a sperm-washing medium (GM501 SpermAir, Gynemed, Germany). The gradient layers were prepared in conical tubes (1 mL of 50 % top layer gradient solution and 1 mL of 90% lower layer gradient solution). The liquefied semen sample was gently layered on top of the gradient. Following centrifugation at 1600 rpm for 10 min, the pellet containing spermatozoa was resuspended and washed in the sperm-washing medium. After another round of centrifugation, spermatozoa were recovered in sperm-washing medium and were ready for subsequent cryopreservation procedures.

Sperm cryopreservation

To determine the role of antioxidants in sperm freezing, prepared sperm suspension was divided into two equal portions. Both groups of the sperm suspensions were mixed with an equal volume (1:1) of Freezing Medium TEST-Yolk Buffer (Irvine Scientific, USA) and were exposed to nitrogen liquid-vapor (5 cm above liquid nitrogen) for 10-20 minutes followed by plunging into liquid nitrogen (–196 °C). The same batch of the freezing medium was used for all samples used in this study. Additionally, the freezing medium of the second group was supplemented with the antioxidant compounds taurine (Merck, Germany), ascorbate (Merck), and glutathione (GSH) (Merck) with a final concentration of 5 μM. The first group did not include any antioxidant supplementation in the freezing medium and therefore represents the control group.

Sperm Thawing

Thawing was performed one month after the sperm samples were frozen. The samples were thawed at 37°C for 1 minute. After gentle addition of 1mL of

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preheated sperm washing medium, the freezing medium was removed by centrifugation at 1800 rpm for 5 minutes. The pellet containing spermatozoa was resuspended in the sperm washing medium.

Statistical Analysis

All statistical analyses were performed using SPSS software (version 13.0, SPSS Inc). The demographic characteristics of the patients were expressed as mean and min-max. Data on DNA fragmentation and motility analyses were presented as mean ± standard deviation. The Mann-Whitney U test was used to compare the differences between the groups. The paired sample t-test was used to compare the differences before-and-after the procedures. A p-value of less than 0.05 is statistically significant.

RESULTS

Characteristics of the study population

Semen samples from 40 male patients were analyzed. The mean age and body mass index (BMI) of the patients were calculated as 34.25 (between 20-40) years, and 28 (between 25 and 34) kg/m2, respectively. Semen parameters (mean, range) were evaluated for sperm volume (mL), sperm count (106/mL), and sperm total motility (%) as presented in **Table 1.**

Effect of antioxidants on post-thaw total motility

Each sample was prepared through density gradient centrifugation and the final volume was divided equally for cryopreservation in the absence or presence of antioxidants. Total motility (%) was determined before and after cryopreservation (**Table 2**). A significant decrease in total motility was found in post-thaw control samples when compared to fresh samples (34.8 ±5.32 % vs. 65.5 ±6.42 %, P= 0.002). Similarly, total motility was significantly reduced in post-thaw samples frozen with antioxidant supplementation in comparison to fresh samples (38.3± 6.22 % vs. 65.5 ±6.42 %, P =0.003).

To determine any cryoprotective effect of the antioxidants used in this study, the total motility of post-thaw samples frozen with antioxidant supplementation was compared to that of control samples. Spermatozoa cryopreserved with or without the addition of antioxidants demonstrated total motility of 38.3± 6.22 % and 34.8 ±5.32 %, respectively, without a statistically significant difference (P =0.07; **Table 2**).

Effect of antioxidants on post-thaw DNA integrity

The images of halos generated with the Halosperm technique enabled the classification of spermatozoa for DNA fragmentation. Cryopreservation led to an increased sperm DNA fragmentation ratio (**Table 3**). Post-thaw samples frozen in the absence and presence of antioxidants showed a DNA fragmentation ratio of 52.3±5.42 % and 47.5±4.7 %, respectively, statistically higher than that of fresh samples (26.4±3.12 %, P =0.002 and P =0.003, respectively). Antioxidants did not cause a significant difference in DNA fragmentation ratio when compared to control samples (P =0.08; **Table 3**) (**Figure 2**).

Table 1: Demographic characteristics of the patients.

Age (yr)	34.25 (20-40)
Semen volume (mL)	2.1 (1,6-4,1)
Sperm count (10^6/mL)	62 (25-120)
Sperm total motility (%)	65.5 (52-84)
BMI (kg/m^2)	28 (25-34)

[a] Data are presented as mean (range).

Table 2: Comparison of total motility (%) in fresh and post-thaw spermatozoa. Cryopreservation was performed with or without the addition of antioxidants. [a]*P* =0.002 and [b]*P* =0.003 in comparison to fresh samples, [c]*P* =0.07 in comparison to control samples.

Variable	Fresh	Post-thaw	
		Control	*Antioxidants*
Total motility (%)	65.5 ±6.42	34.8 ±5.32 [a]	38.3± 6.22 [b, c]

Table 3: Comparison of DNA fragmentation ratio (%) in fresh and post-thaw spermatozoa. Cryopreservation was performed with or without the addition of antioxidants. [a]*P* =0.002 and [b]*P* =0.003 in comparison to fresh samples, [c]*P* =0.08 in comparison to control samples.

Variable	Fresh	Post-thaw	
		Control	*Antioxidants*
Sperm DNA Fragmentation (%)	26.4±3.12	52.3±5.42 [a]	47.5±4.7 [b, c]

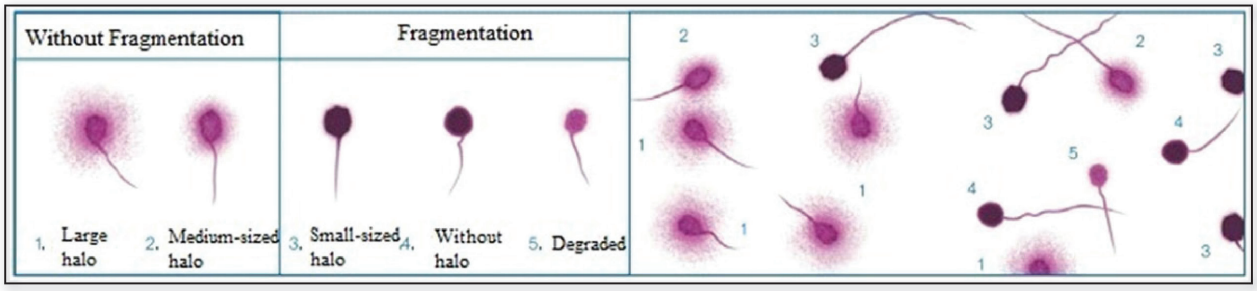

Figure 1: Classification of human sperm DNA fragmentation using Halosperm test (39).

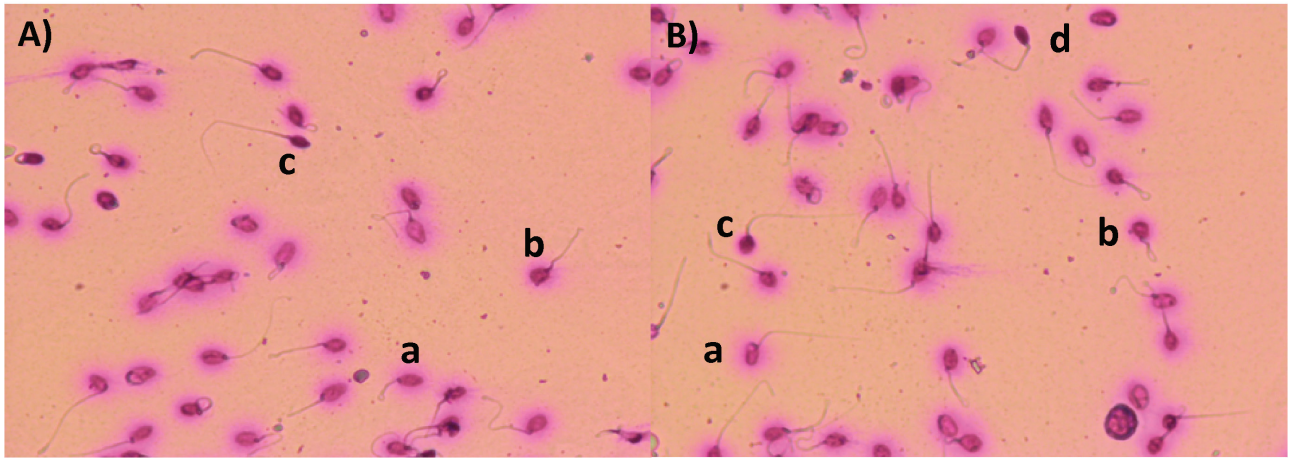

Figure 2: Illustrates the patterns of DNA loop halos after cryopreservation with or without the antioxidant supplementation. Semen sample of patient 20 after cryopreservation A) without any antioxidant supplementation B) with antioxidant supplementation: a. large halo b. medium halo c. small halo d. without halo.

DISCUSSION

The present study is based on the association of reactive oxygen species (ROS) production with deleterious effects on post-thaw spermatozoa and aims to improve on DNA integrity and functional capabilities including motility by the use of the antioxidants in cryopreservation medium.

Sperm motility and DNA integrity provide useful information for diagnosing male infertility (23). Infertile men are more vulnerable to oxidative damage with reduced enzymatic antioxidant defenses and increased ROS generation in their semen (24). High ROS concentrations were indicated to lead to suboptimal sperm quality by affecting the structural and functional integrity of sperm including the motility and DNA fragmentation (25). Oral antioxidant intake including vitamins and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine, and glutathione have been investigated to counteract the seminal oxidative damage, in the recent years (26). Oxidative stress also represents a concern during cryopreservation (10, 27). Freezing and thawing exert detrimental effects on spermatozoa that lead to poor motility (3, 16, 28). Whilst there is no agreement in the literature on whether cryopreservation induces DNA damage, a number of studies reported DNA fragmentation in post-thaw spermatozoa (11-13). Post-cryopreservation analysis revealed the link between the production of ROS following the changes of mitochondrial membrane properties and loss of motility and increased DNA fragmentation in frozen-thawed sperm (14, 15). Spermatozoa are highly sensitive to ROS-induced damage due to the elevated content of polyunsaturated fatty acids in their membranes (29). Seminal plasma, rich in antioxidants, may have a protective capacity against oxidative damage. Sperm frozen in seminal plasma was reported to be more resistant against cryodamage (16).

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To overcome the effects of oxidative stress in post-thaw spermatozoa, antioxidant molecules or antioxidant enzymes have been introduced in the cryopreservation media during freezing (10, 17). Notably, significant improvements in several sperm parameters including viability, motility, and DNA integrity were demonstrated after supplementation of freezing media with antioxidants such as vitamin E analogous, glutathione and L-carnitine (30-32). On the other hand, some studies failed to demonstrate the same positive effects (10, 17). Vitamin E supplementation of cryopreservation medium significantly improved post-thaw motility, without changing sperm viability or extent of DNA fragmentation. Interestingly, the positive effect of vitamin E supplementation was associated with men's age, in this study (33).

The antioxidant molecules used in the present study are ascorbic acid (vitamin C), glutathione and taurine, important for the proper function of antioxidant enzymes (10). Their combination was evaluated for a possible protective effect during cryopreservation. No significant improvement in sperm motility or DNA integrity was obtained after the addition of 5 μM of the antioxidants to the freezing media. So far, these antioxidants have been used in several studies, where positive effects, as well as no effect on sperm parameters, were observed. The addition of vitamin C to prepared spermatozoa was reported to result in a better recovery rate of sperm parameters and DNA integrity following vitrification (34). However, another study showed that vitamin C along with TEST yolk buffer did not result in any improvement in sperm parameters including motility (35). Moreover, it was claimed that vitamin C has dose-dependent effects during cryopreservation and reduced motility was observed at high doses (36). A recent study evaluated glutathione supplementation and reported higher sperm viability and decreased DNA fragmentation (32). Nevertheless, the addition of glutathione had a partial benefit in another study, as despite reduced levels of ROS in spermatozoa, no effect on lipid membrane disorder or chromatin condensation was detected (37). The studies on the role of taurine in the preservation of human spermatozoa are limited. Researchers demonstrated improved sperm motility, viability, and reduced DNA damage in the presence of taurine in chicken (38).

The protective effects of the antioxidants appear to be dependent on the selection of the molecules (alone or in combination) and the conditions including the dosage and the procedure. The number and the demographic characteristics of the patients might also explain the differences in the results. Thus, future studies are required to determine the ideal antioxidants and optimum conditions for the use of antioxidants in the media for assisted reproductive technologies.

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