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RESEARCH ARTICLE

Effect of Cryopreservation on DNA Damage and Various Sperm Parameters in the Post-Mortem Obtained Buffalo Sperm

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ABSTRACT

This study aimed to investigate the effects of freezing on DNA damage and some sperm parameters in epididymal sperm obtained from the buffalo testicles after slaughtering. Epididymal semen examination of 50 male Anatolian buffaloes (three years old or older) was performed after being obtained from slaughterhouses. The semen samples were divided into two groups: the control group (the fresh semen samples) and the research group (frozen-thawed semen samples). Sperm progressive motility (%), viability (%), abnormal sperm (%), and sperm DNA damage (%) were analyzed. Significant differences ($p<0.001$) between fresh and frozen semen on the progressive motility, viability, and mid-piece, tail, and total abnormality rates were found in the study. It was determined that the difference between the DNA damage values in fresh and thawed semen was significant ($p<0.0001$). Progressive motility rates of fresh and thawed semen were consistent with the studies conducted. It is thought that epididymal buffalo semen obtained post-mortem can be used in biotechnological methods. This research will contribute to further studies to increase the Anatolian buffalo population. More comprehensive studies should be performed to determine the impact of fertility due to the semen DNA damage and minimize the DNA damage in the freezing-thawing semen samples.

Keywords: Anatolian buffalo, DNA damage, epididymal, semen freezing, semen parameters

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Mandalardan Postmortem Elde Edilen Spermalarda Dondurmanın DNA Hasarı ve Bazı Spermatolojik Parametreler Üzerine Etkisi

ÖZ

Bu çalışmada, kesim sonrası manda testislerinden elde edilen epididimal spermada dondurma işleminin DNA hasarı ve bazı spermatolojik parametreler üzerindeki etkisinin araştırılması amaçlanmıştır. 50 adet erkek Anadolu mandasının (3 ve üzeri) testisleri mezbahanelerden kesim sonrası temin edildikten sonra elde edilen epididimal sperma progresif motilite (%), canlılık (%), anormal spermatozoa oranı (%) ve spermatozoon DNA hasarı (%) yönünden analiz edildi. Sperma örnekleri kontrol grubu (taze sperma örnekleri) ve araştırma grubu (dondurulmuş-çözünmüş sperma örnekleri) olmak üzere iki gruba ayrıldı. Taze ve dondurulmuş sperma arasında progresif motilite, canlılık, orta kısım, kuyruk ve toplam anormallik oranlarında önemli farklılıklar bulundu ($p<0,001$). Taze ve çözdürülmüş spermada arasındaki DNA hasarı sonuçlarının anlamlı derecede farklı olduğu belirlendi ($p<0,0001$). Taze ve çözdürülmüş spermadaki progresif motilite oranları yapılan çalışmalarla uyumluydu. Çalışma sonucunda elde edilen veriler ışığında postmortem olarak elde edilen epididimal manda spermasının biyoteknolojik yöntemlerde kullanılabileceği düşünülmektedir. Bu araştırma, Anadolu manda popülasyonunun artırılmasına yönelik daha sonraki çalışmalara katkı sağlayacaktır. Spermatozoonlarda dondurma işlemi sonucunda şekillenen DNA hasarının belirlenmesi ve en aza indirilmesi için daha kapsamlı çalışmalar yapılmalıdır.

Anahtar Kelimeler: Anadolu mandası, DNA hasarı, epididimal, sperma dondurma, sperma parametreleri

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INTRODUCTION

Anatolian (water) buffalo, including the northern part of Central Anatolia, still exists in Turkey. Although more common on the Black Sea coast, it is also found in Eastern Anatolia. Taxonomically, it is classified as a "*Mediterranean*" type; however, all buffaloes belong to the *'River'* species in Europe and the Near East countries, which have similar phenotypes but varying appearances (Borghese and Mazzi 2005).

Artificial insemination is one of the methods that can be used to increase the pregnancy and fertility rates in buffaloes. On this technique, one of the most important factors is the quality of the frozen semen (Saacke 1984). Buffalo semen has a low freezing capacity compared to cattle semen. It has been reported that the main reason for the difference between these two types of semen can be the difference in lipid ratios of plasma membrane (Tatham 2000).

When comparing cattle and buffalo semen during short-term storage at +4°C, it was shown that buffalo semen was more prone to oxidative stress due to the high lipid peroxidation dependent on the decreased activity of antioxidant enzymes (Nair et al. 2006).

Cooling-freezing-thawing processes lead to physical and chemical stresses, which cause a decrease in semen viability and fertilization capacity (Chatterjee et al. 2001). The most prominent stress is lipid peroxidation. It was shown that the effects of lipid peroxidation led to irreversible motility loss, respiratory inhibition, intracellular enzyme leakage, and sperm deoxyribonucleic acid (DNA) damage (White 1993).

Semen diluents are prepared for ejaculated semen samples; therefore, dilution of epididymal semen with commercial diluents and use of seminal plasma may result in motility loss. In the freezing process, semen samples are mixed with seminal plasma before dilution. Therefore, it may not be right to wash the epididymal semen samples with diluents before mixed with seminal plasma. In this case, adding a few drops of diluent is recommended. Seminal plasma can be added after this step and semen samples should be diluted only with semen diluents (Herold et al. 2004a).

The spermatozoon DNA integrity is vital for the embryo, fetus, normal offspring development, and fertilization success, which can be achieved using natural or assisted reproductive techniques (Morris et al. 2002).

It has been reported that storing frozen samples leads to DNA damage and abnormal embryo development, and infertility in humans, mice, fishes, and oysters. Semen with DNA damage negatively affects embryonic development and increases the genetic disease probability (Zini et al. 2001).

Comet test is used in somatic cells to determine the genotoxic damage and mainly single and double-strand breaks (Singh et al. 1988). This test is a gel electrophoresis method used to visualize and measure the DNA damage in cells using a microscope (Olive 2002). It is both a cheap and sensitive method known as reliable in detecting DNA damage (Morris et al. 2002). It has been shown that the Comet test is a more sensitive method compared to others, such as Sperm chromatin structure assay (SCSA) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Donnelly et al. 2000).

The purpose of this research was to investigate the effects of freezing on DNA damage and some sperm parameters in epididymal sperm obtained from buffalo testicles taken from slaughterhouses after slaughtering.

MATERIALS and METHODS

Epididymal semen examination of a total of 50 male *Anatolian buffaloes* (three years old or older) was performed after obtained from slaughterhouses.

Obtaining the Testicles

Testicles of buffaloes were cut immediately after the slaughter as Lambrechts et al. (1999) explained. The scrotum was incised with a knife and removed with epididymis by cutting the spermatic cord.

Obtaining Semen Samples and Processing of Semen

The cauda epididymis was incised with a sterile lancet. The sperm in the ductus epididymis was allowed to pull out. Semen was obtained by pulling out with a sterile syringe (Guerrero 2006). The semen samples were divided into two groups: the control group (the fresh semen samples) and the research group (frozen-thawed semen samples). Sperm examinations (progressive motility, viability, and head-acrosome, mid-piece, tail, total abnormalities) were performed for these groups (Tekin 1994). Furthermore, samples of both groups were examined for DNA damage. The control group-fresh semen samples semen samples were diluted with the commercial Bioxcell® extender (IMV Technologies, L"Aigle, France), and then sperm parameters and DNA damage were examined. Samples were frozen according to the instructions of the company to form the research group. Then, samples were stored at least for 24 hours in liquid nitrogen (-196°C). Samples were thawed in the water bath (at 37°C) for 25-30 seconds (Arriola and Foote 1987) and examined in terms of sperm parameters and DNA damage. Dead/live spermatozoa ratios were determined as percentages by staining method (2% Eosin). This process was performed with a slide, coverslip, and 2% Eosin dye adjusted to body temperature. One drop of semen was mixed with two drops of Eosin and a smear was drawn on a slide. The slides were left to dry for 15 seconds and 400 spermatozoa were counted under the microscope at 40X magnification, and the

number of spermatozoa receiving dye (dead cells) was determined as a percentage. The fluid fixation method was used to determine the abnormal spermatozoa ratio. The rate of head-acrosome, middle part, tail anomalies and total spermatozoa anomalies were determined as percent. The sperm was fixed in Hancock solution and a drop of this prepared solution was placed on the slide, the coverslip was covered, and a drop of immersion oil was dropped on the covered coverslip, and their morphology was determined by counting 400 spermatozoons at 100X magnification (Tekin 1994). Epididymal semen, diluted with Bioxcell® diluent, a commercial diluent, to a density of 120-150x10^6 sp/ml (Sansone et al., 2000), was manually drawn into 0.25 ml straws. It was equilibrated at +5°C for 4 hours in accordance with the recommended procedure. After equilibration, the straws were frozen by keeping them in liquid nitrogen vapor at an average temperature of −80°C to −120°C for approximately 20 minutes, and finally they were immersed in liquid nitrogen at -196°C and stored until thawing.

Sperm DNA Damage Detection
Spermatozoon DNA damage was detected by using a gel electrophoresis method named "Comet test" (Single Cell Gel Electrophoresis) (Olive 2002).

Washing Semen Samples
Semen samples were centrifuged two times with Mg^{+2} and Ca^{+2} free phosphate buffer solution (PBS) at 4°C (800 g for 10 minutes) and samples were diluted with PBS (20×10^6 sp/ml) after washing steps (Fraser and Strzezek 2004).

Slide Preparation and Gel Placement
Fully frosted slides were covered with 100µl of 0.75% normal melting point agarose, a coverslip added and the agarose allowed to solidify at room temperature for 5 minutes. The coverslip was removed and approximately 1×10^5 sperm cells in 5µl of PBS were mixed well with 75µl of 0.5% low melting point agarose was used to form the second layer. A coverslip was added again and stored at +4°C for solidification. Then, slides were ready after removing the coverslip (Singh et al. 2003).

Cell Lysis
The cold lysis solution (+4°C) (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, 1% Triton X-100, pH: 10) was prepared and slides were put in this solution for 1 hour at +4°C. Then, 40mM of DTT (Dithiothreitol) was added and samples were left for an additional 1 hour at +4°C. In the end, the lysis solution was mixed with Proteinase K (100 µg/ml) and slides were incubated in the solution at 37°C for 15 hours (Singh et al. 2003).

Electrophoresis
Following the lysis step, slides were incubated in freshly prepared and cooled (0-4°C) electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH: 12.5) for 20 minutes to ensure the separation of the DNA strands from each other. After the incubation, samples were run for 20 minutes in this buffer solution (300mA and 20V) (Singh et al. 2003).

Neutralization
After the electrophoresis step, slides were washed three times with Tris buffer (40 mM Tris HCl, pH: 7.4) to remove the detergents and to change the alkalinity (Singh et al. 2003).

Staining the Samples
Following the neutralization process, slides were stained with fluorescent ethidium bromide (5µg/ml) and DNA structures of samples were evaluated in four hours upon the staining (Hu et al. 2008).

Comet Image Analysis
Camera attachment fluorescence microscope (Olympus BX51) with an excitation wavelength of 580 nm and image analysis system (TriTek CometscoreTM version 1.5 software) were used to evaluate the Comet parameters from 100 DNA images for each sample (Xu et al. 2013).

Thawing of Frozen Semen
Straws were thawed in the water bath (37°C) for 25-30 seconds after 24 hours storage period in liquid nitrogen (-196°C) (Arriola and Foote 1987).

Statistical Analysis
The mean, standard deviation, and mean standard error values were calculated by using the SPSS statistical program (Version 21, IBM Crop., USA). Results were represented as mean±SEM. Fresh and thawed semen samples were examined in terms of sperm parameters and results were analyzed by using a paired t-test. Comet DNA analysis results were compared using Student's t-test.

RESULTS

In this study results showed that progressive motility, viability, and mid-piece, tail, total abnormalities (Tekin 1994) were significantly different between fresh and thawed semen samples ($p<0.001$). Only head-acrosome abnormality was not significantly different between fresh and thawed semen samples (Table 1).

In this study, the percentages of DNA damage in the Comet head and the Comet tail were significantly different in thawed semen samples than in fresh samples ($p<0.0001$) (Table 2).

Singh et al. (2003) described, the tail moment parameter quantified the amount of DNA damage, as it explained the distance the DNA had migrated and

the amount of DNA that had migrated from the head region. The results of this measurement can be seen in Figure 1.

Table 1. Spermatological examination of fresh and frozen semen samples

Semen parameters		Fresh Semen $\overline{X} \pm S_{\overline{X}}$	Frozen Semen $\overline{X} \pm S_{\overline{X}}$	P
Progressive motility (%)		50.10 ± 1.78[a]	27.50 ± 1.41[b]	p<0.001
Viability (%)		16.58 ± 0.48[a]	36.76 ± 0.95[b]	p<0.001
Abnormal spermatozoa (%)	Head-acrosome	3.38 ± 0.26	3.50 ± 0.23	p>0.05
	Mid-piece	15.00 ± 0.84[a]	22.10 ± 1.11[b]	p<0.001
	Tail	13.68 ± 1.10[a]	17.30 ± 1.07[b]	p<0.001
	Total	32.06 ± 1.62[a]	42.90 ± 1.64[b]	p<0.001

a, b: For each trait, means with the different letters in the same row were significantly different.

Table 2. Comet parameters of fresh and frozen semen

Comet Parameters	Fresh Semen $\overline{X} \pm S_{\overline{X}}$	Frozen Semen $\overline{X} \pm S_{\overline{X}}$	P
Comet Head (%)	89.44 ± 1.0[a]	32.47 ± 1.3[b]	p<0.001
Comet Tail (%)	10.58 ± 1.0[a]	67.29 ± 1.3[b]	p<0.001

a, b: For each trait, means with the different letters in the same row were significantly different.

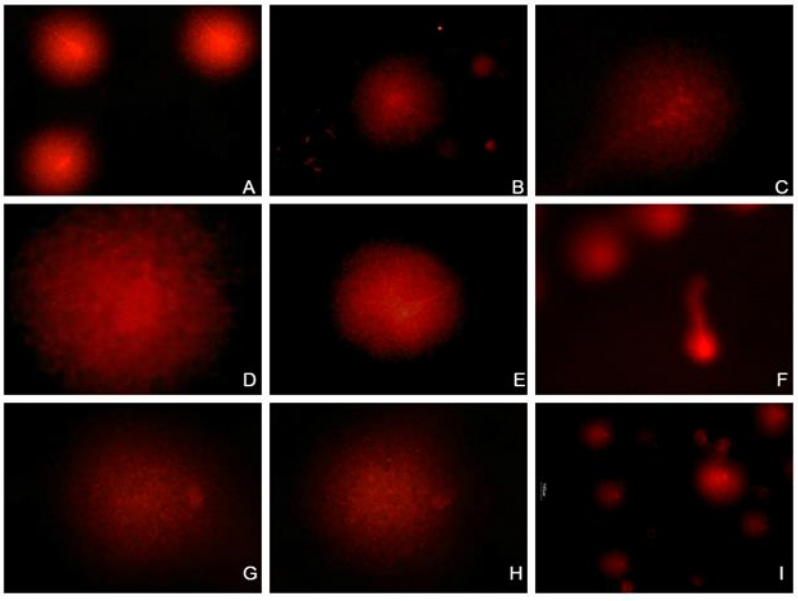

Figure 1: Comet images. (A, B, C: Comet images of the control group the spermatozoon. D, E, F: Spermatozoon with less DNA damage. G, H, I: Spermatozoon with DNA damage)

DISCUSSION

Many researchers have studied fresh Buffalo epididymal sperm parameters. In this research, the progressive motility rate of the fresh semen samples was higher than by Herold et al. (2004b; 2006) and lower than by Hiron et al. (2006). Morphology expresses the physical structure of spermatozoa, and during analysis, the different types of abnormalities in the sample are detected. Abnormalities are categorized according to head, midpiece and tail

abnormalities resulting from either environmental conditions or genetic factors. The ratio of abnormalities in a sample may be affected by the season, gender, frequency of ejaculation, and stress encountered before ejaculation. The sample's capability to be used for AI or IVF or to be used for cryopreservation can drastically impact a high percentage of abnormalities (Vale 1994). Various studies examined the viability and abnormal spermatozoa parameters in fresh epididymal buffalo semen samples. According to this research's results, viability was compatible with the results of studies performed by Kumar et al. (2008) and Singh et al. (2007). On the other hand, the results related to viability were lower compared to the rates detected in the research of Lambrechts et al. (1999). The rates of abnormal spermatozoa were lower than the values in the literature mentioned above.

In this research, the spermatozoa motility rate of frozen semen samples was higher and partially compatible with the rate detected by Herold et al. (2004b). However, it was higher than Herold et al. (2006) and lower than Hiron et al. (2006). The viability and abnormal spermatozoa rates of frozen buffalo semen samples were examined in this research. According to the results, viability was compatible with the results of studies performed by Kumar et al. (2008) and Hiron et al. (2006). On the other hand, the viability results were lower than Herold et al., (2006). The rates of abnormal spermatozoa were lower than the values in the literature mentioned above.

Factors such as breed, age, methods of animal care, nutrition conditions, geographical position of the region in which the research is conducted, and the climate are the reasons for this research's different results related to the sperm parameters. It was detected that the differences between the fresh and the thawed semen samples in terms of the progressive motility, viability, and mid-piece, tail, total abnormality rates ($p<0.001$). Only head-acrosome abnormality rates were not significantly different between fresh and thawed semen samples.

Abnormalities in the male genome are the potential reason for post-fertilization failures and problems (Sakkas et al. 2002; Sergerie et al. 2005). Saleh et al. (2002) showed that DNA damage in the spermatozoon negatively affected fertility. It is believed that the oxidative damage in the spermatozoon DNA leads to problems in the genetic material transfer, infertility, congenital disabilities, genetic diseases, and cancer development in infants by increasing the mutations and abnormalities (Ames et al. 1994; Cummins et al. 1994). Various studies determined that freezing and thawing of the buffalo semen samples prominently increased the DNA damage (Fraser and Strzezek 2004, El-Kon and Darwish 2011). DNA damage results of fresh semen are compatible with the results of Kumar et al. (2011) whereas the results of frozen semen were higher compared to the results of El-Kon and Darwish (2011) and Kumar et al. (2011). The reason for the higher DNA damage rates detected in this research can be that the alkaline Comet assay was used, which detected various damages such as base damages, single-strand breaks, and crosslinks. Furthermore, DNA damage rates of thawed semen samples were also higher than other studies and the reason can be the use of the epididymal semen samples. The diluents used in this research are not prepared for the epididymal semen samples and the lack of antioxidants can be the reason for these higher rates of DNA damage.

Cooling-freezing-thawing processes lead to physical and chemical stresses on the spermatozoon membrane, thus decreasing fertilization ability (Chatterjee et al. 2001). Furthermore, it is commonly accepted that these steps cause a more than 50% decrease in spermatozoon vitality (Watson 1979). Buffalo semen is richer in polyunsaturated fatty acids than bull semen (Nair et al. 2006). The damage is comparatively higher in buffalo sperm during freezing and thawing cycles, and buffalo semen has lower motility and pregnancy rates. Therefore, buffalo semen samples are more prone to oxidative damage. These risks can be minimized by optimizing the cooling and freezing rates and using appropriate diluents for the frozen spermatozoa (Kumar et al., 1992).

Assisted reproductive techniques can be used to minimize the errors during the manipulations and freezing-thawing steps, determine new strategies, take precautions on *in vivo* and *in vitro* conditions, and stabilize the possible DNA damage in the spermatozoon DNA (Türk et al. 2006).

CONCLUSION

Consequently, progressive motility rates of fresh and thawed semen samples are in line with other studies and it is possible to use the postmortem epididymal buffalo semen samples by using biotechnological techniques. Furthermore, in case a male buffalo with a high fertility rate dies because of any reason, epididymal semen samples can be obtained, and its genetic material can be used with the assisted reproductive techniques. This research concluded that the semen freezing-thawing process leads to prominent damage and problems in buffalo epididymal semen samples as it is commonly observed in other animal races. This research reports the spermatozoon DNA damage levels during the freezing-thawing process, which can negatively affect the fertility rates in buffaloes, and it is believed that our results will be efficiently used to increase the buffalo population in Turkey. More comprehensive studies should be performed to determine the impact of fertility due to the semen DNA damage and minimize the DNA damage in the freezing-thawing semen samples.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: The authors declare that they contributed equally to the article.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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