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The Effect of Boron Addition on Spermatological Parameters, Oxidative Stress and DNA Damage After Frozen-Thawed Process in Ramlic Ram Semen *

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The aim of the present study was to investigate the effects of different doses of Boron added to extender on some spermatological parameters, oxidative stress, and DNA damage during post-thawed of ram semen. The ejaculate samples were collected by artificial vagina from five Ramlıç rams. After determining the primary spermatological properties, those having normospermie quality were pooled. The samples were evaluated as a mix ejaculate in the experiment and control groups. The samples were extended with Tris extender containing 5% glycerol and different Boron doses (1, 2 and 4 mM). Control group was formed with no Boron. After dilution and dosing of the samples, withdrawn in 0.25 mL straws were frozen at vapour of liquoid nitrogen. The frozen semen was thawed in a water bath at 37 ºC for 25 seconds. After thawing; motility, morphology, DNA integrity (COMET assay) and oxidative stress parameters (TAS and TOS) of frozen thawed ram sperm were determined. In the thawed semen according to subjective motility (57.00±1.528 %), 1 mM boron dose group indicated the highest value (P<0.05). Head, tail piece and TOS values were found highest in 4 mM group compared to the control group (P<0.05). In terms of DNA damage, 1 mM (34.80±1.289 AU) had the lowest value compared with the control group (48.10±3.093 AU). In terms of mitochondrial activity, the highest value was achieved in 1 mM (34.60±1.035 %) compared with the control group (26.40±1.740 %).

In conclusion, it is thought that using boron might have a positive impact on energy metabolism, DNA damage in freezing, and thawing phase of spermatozoa, and therefore sperm may increase the ability of fertilization.

Key Words: *Boron, DNA damage, ram semen, oxidative stress, cryopreservation*

Ramlıç Irkı Koç Spermasına Katılan Bor'un Dondurma ve Çözdürme Sonrası Spermatolojik Parametreler, Oksidatif Stres ve DNA Hasarı Üzerine Etkileri

Çalışmada koç ejakülatlarının dondurulması ve çözdürülmesi sürecinde sperma sulandırıcısına katılan farklı dozlarda Borun spermatolojik değerler, oksidatif stress ve DNA hasarı üzerine etkilerinin araştırılması amaçlanmıştır. Araştırmada toplam 5 Ramlıç koçtan sun'i vagina ile alınan ejekülatlar toplandı. Başlıca spermatolojik özellikleri belirlenen normospermi kalitesindeki ejekülatlar birleştirilerek kullanıldı. Araştırma ve kontrol gruplarında spermalar mix ejekülat biçiminde değerlendirildi. Spermaların sulandırılması ve dozlanması farklı Boron dozları (1, 2 ve 4 mM) ve % 5 glyserol içeren Tris ana sulandırıcısı ile yapıldı. Ayrıca Boron içermeyen kontrol grubu da oluşturuldu. Sulandırma ve dozlama işlemleri sonrasında 0.25 ml lik payetlere çekilen spermalar sıvı azot buharında dondurulduu. Dondurulan spermalar su banyosunda 37 ^{0} C de 20 saniye tutularak çözdürüldü. Çözdürme sonrası koçlarda spermatozoon motilitesi, morfolojik muayene, membran bütünlüğü (HE testi), DNA hasarı ve oksidatif stres parametreleri (TAS ve TOS) belirlendi. Çözdürülen spermalarda subjektif motilite (% 57.00 ± 1.528) açısından 1 mM bor katılan grupta en yüksek (p<0,05) değerler tespit edildi. Anormal spermatozoon baş ve kuyruk anomal oranı ve TOS değerleri açısından 4mM lık bor grubunun kontrol grubuna göre en yüksek değerler elde edildi. DNA hasarına bakıldığında ise 1mM (34.80 ± 1.289 AU) bor grubunun kontrol grubu (48.10 ± 3.093 AU) ile karşılaştırıldığında en düşük değerler göstererek koruma sağlamıştır. Mitokondrial aktivite yönünden bakıldığında kontrol grubuna göre (26.40±1.740 %) en yüksek değer (34.60±1.035 %) 1mM lık Bor grubunda elde edilmiştir.

Sonuç olarak çalışmada kullanılan Bor'un spermanın dondurulması ve çözdürülmesi aşamasındaki enerji metabolizmasına ve DNA hasarına olumlu etki yaptığı ve buna bağlı olarak spermanın fertilizasyon yeteneğini arttırabileceği düşünülmektedir.

Anahtar Kelimeler: *Bor, DNA hasarı, koç sperması, oksidatif stres, kryopreservasyon*

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Introduction

In animal breeding, artificial insemination (AI) is the first important biotechnical step taken to improve reproduction and genetics of farm animals, and the second step is freezing of sperm successfully. Artificial insemination with frozen sperm in ram is not as successful as in bull. According to O'Hara et al. (1), this is because the spermatological

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parameters, the diluents used, the semen freezing and insemination techniques have not been sufficiently developed. Research should be carried out to ensure that the sperm washing, dilution, and freezing techniques used for ram spermatozoa have an optimum performance. Ramlıç is a type of sheep which combines the high viability of Dağlıç with the meat and fleece yield characteristics of the Rambouillet breed. The Ramlıç sheep is a significant breed for which research and applications must be carried out in order to develop the freezability of spermatozoa, protect the genes, and increase the production of Ramlıç (2).

Boron is an element commonly found as various compounds in Earth, water, and stone; and 72.1% of the world's total boron reserves are in Turkey (3). Boron and its compounds have various areas of usage in the industry and farming (3, 4). Toxic effects on the human reproductive system have not been found in the studies conducted on boron around the world yet. In some researches, it was recorded that boron has an important role in membranous structure and metabolic activity. In contrast to the adverse effects of boron particularly on the reproductive system, Duydu et al. (3) and Başaran et al. (4) performed a study with the employees in a boron mine, and Saylı et al. (5), Korkmaz et al. (6), and Korkmaz et al. (7) carried out a study about people living in regions which were rich in boron minerals and reported that no negative effect on the reproductive system or semen was encountered. Studies have shown that the boron element plays an important role as a macromineral in the human cell membrane and in the cellular mechanism. It has been also noted that it has an anticarcinogenic effect on some types of cancer. Mostly, the effects of the boron element on the reproductive system of rats and mice have been endeavoured to be manifested with various studies based on dosage and duration. The testes considered as the most sensitive organ for the boron element have not been studied in terms of spermatological parameters, sperm's freezing ability, and effects on fertilization. It is important to study the effects of using reconstituted boron in semen freezing in the reproduction parameters of farmed livestock which have an economic value such as rams because it plays an important role in the cell membrane and cellular mechanism (8, 9).

The objective of the current study was to manifest the usability of the boron element in freezing of spermatozoa by studying the spermatozoa motility, live spermatozoa ratio, abnormal spermatozoa ratio and acrosomal abnormal spermatozoa ratio, mitochondrial activity, DNA damage and total antioxidant and oxidant capacity after the addition of boron element to Tris diluent used for freezing the semen of Ramlıç rams and to investigate the fertilization ability of the frozen sperm after thawing.

Materials and Methods

Five heads of 2-3 years old Ramlıç (Dağlıç x Rambouillet) rams raised at the Afyon Kocatepe University Livestock Application and Research Center were used in the present study. The study's experimental design was approved by the Animal Care Committee Afyon Kocatepe University Veterinary Medicine Faculty in terms of ethics with the authorisation number B.30.2.AKÜ.0.9Z.00.00/189. Sperm was collected from the rams with an artificial vagina. During the mating season, ejaculate was regularly collected from the rams twice per week to a total of 10 ejaculates from each ram. Tris was used as a diluent in the study, and then four different groups were formed by adding boron at different concentrations (1 mM, 2 mM, and 4 mM) and no antioxidants (control). Sperm specimens collected in separate tubes were pooled in one tube and subsequently divided into four equal volumes after spermatological examinations before freezing. Sperm samples were reconstituted by dosing with pre-prepared diluents containing 5% glycerol and different antioxidants as $150x10^6$ mL. Following the dilution, the specimens were drawn in 0.25 mL different colour aliquots and subjected to 3 hours of equilibration at 5 ºC followed by freezing in 15 minutes in nitrogen vapour (~ –110 ºC), and the specimens were stored frozen in liquid nitrogen (–196 ºC) until in vitro evaluation. After being stored for six months, the aliquots were individually thawed in a water bath at 37 ºC for 25 seconds for microscopic evaluation.

Microscopic Evaluation of Sperm: Phase-contrast microscopy was performed on a heating plate (200 X) set at 37 ºC for the spermatozoa motility assay. Five different areas were examined for each semen sample. The motility averages in the field were recorded as %. The abnormal spermatozoa ratio in semen samples was determined by the Giemsa staining method. The prepared slides were counted as 200 spermatozoa for each sample in the immersion lens (1000 X), and the percentage of abnormal spermatozoa ratio was determined as % (10).

Spermatozoa Viability: SYBR-14 / PI (SYBR-14 / PI Molecular Probe: L 7011 Invitrogen, Carlsbad, CA.) fluorescence staining was applied by modifying the Garner and Johnson (11) method for the detection of spermatozoa viability.

Spermatozoa Acrosomal Integrity: Fluoresinisocyanate (FITC-PNA) / Propidium Iodide (PI) fluorescence staining was applied with the method of Nagy et al. (12) for acrosomal assessment.

Spermatozoa Mitochondrial Activity: JC-1 / PI fluorescence staining was carried out to determine mitochondrial activity previously described by Bucak et al. (13) which was modified from a study of Garner et al. (14).

Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) Analysis: Sperm TAS measurements was carried out according to the method developed by Erel (15), and TOS measurements were also performed considering the method developed by Erel (16).

Determination of Spermatozoa DNA Damage: The comet assay method as reported by Hughes et al. (17) was used to detect DNA damage in spermatozoa. Evaluation was done using the visual scoring method. As the length of migration varied depending on the amount of fragments, DNA chain breaks, and the level of alkaline-labile regions, DNA images were graded in five subcategories according to the severity of the damage. DNA with no damage was rated as 0 while DNA with damage was given a score from 1 to 4 depending on the degree of damage. The results for each ram were collected and evaluated as an arbitrary unit (AU) (18).

Statistical Analysis: One-way analysis of variance (ANOVA) was performed in the statistical evaluation of the obtained data. Multiple post hoc Duncan test was used to determine the difference between groups. Analyses were transferred in a computer environment using SPSS (13.0) package program. The results were given as mean and standard error (± S.E.M). The level of significance was evaluated as P<0.05.

Results

Subjective motility and abnormal spermatozoa ratios obtained after freezing-thawing are presented in Table 1. In terms of higher motility, values of the group with 1 mM of boron compared to the control group were statistically significant (P<0.05). However, it was noted that motility decreased significantly in the group containing 4 mM of boron (P<0.05). When abnormal spermatozoa was examined, it was determined that head and tail anomalies increased significantly (P<0.05) in the group containing 4 mM of boron. No difference was observed between the groups in terms of mid-piece anomalies. Live spermatozoa ratio, spermatozoa acrosomal integrity, and spermatozoa mitochondrial activity are given in Table 2. Although there is a relative increase in terms of spermatozoa viability and acrosomal integrity when group with 1 mM of boron is compared with the control group and the other groups, there are not significant differences about statistically.

The results of the present study on spermatozoa mitochondrial activity revealed that the group with 1 mM of boron had a higher activity level than the control group and the groups with 2 and 3 mM of boron, and there was statistically significant (P<0.05). TAS, TOS analysis, and DNA damage parameters manifested after freezing-thawing are given in Table 3, and the TAS value shows that although there was a numerical increase in the group with 1 mM of boron, no statistical significance was identified. When TOS value was taken into consideration, it was determined that stress significantly increased (P<0.05) in the group with 4 mM of boron compared to the control group and the group with 1mM of boron. When the level of spermatozoa DNA damage was examined, it was noted that the group with 1mM of boron was protected at a significant level against DNA damage in the spermatozoa (P<0.05) compared to the control group and the other groups.

Table 1. Mean (±SEM) spermatological parameters after frozen–thawed ram semen (n: 10)

Groups	Motility %	Abnormal sperm rate (%)		
		Head	Mid-peace	Tail
Control	52.00±1.33^{b}	5.81±0.778^{b}	0.69±0.148	11.25±1.114^{bc}
1 mM	57.00±1.52^{a}	6.00±0.551^{b}	0.76±0.149	11.90±1.378^{ab}
2 mM	50.00±1.491^{b}	6.16±1.227^{b}	1.43±0.747	12.25±1.344^{ab}
4 mM	45.00±1.66^{c}	10.12±1.436^{a}	0.62±0.144	16.20±1.943^{a}
P	0.000	0.017	0.451	0.049

a-c Different superscripts within the same column demonstrate significant differences (P<0.05)

Table 2. Mean (±SEM) flourescent staining in frozen–thawed ram semen (n: 10)

Groups	Sperm viability %	Acrosome integrity %	High mitochondrial activity %
Control	64.70±9.717	37.60±2.363	26.400±1.740^{bc}
1 mM	69.30±10.334	40.60±7.086	34.60±1.035^{a}
2 mM	60.20±10.776	39.60±3.341	29.30±2.050^{b}
4 mM	57.60 ± 9.963	35.10±6.413	22.90±2.237^{c}
P	0.858	0.883	0.047

a-c Different superscripts within the same column demonstrate significant differences (P<0.05)

Table 3. Mean (±SEM) oxidative stress and DNA damage parameters after frozen–thawed ram semen (n: 10)

Groups	TAS mmol Trolox Equivalent/g-protein	TOS µmol H2O2 Equivalent/g-protein	DNA Damage (AU)
Control	0.353±0.067	30.130±2.077^{b}	48.10±3.093^{b}
1 mM	0.385±0.051	29.950±2.001^{b}	34.80±1.289^{c}
2 mM	0.343±0.065	33.313±1.518^{ab}	57.60±4.969^{a}
4 mM	0.330±0.060	36.274±2.309^{a}	66.10±2.433^{a}
P	0.930	0.019	0.000

a-c Different superscripts within the same column demonstrate significant differences (P<0.05)

Discussion

Freezing ram spermatozoa properly and using it in artificial insemination have a direct impact on sheep breeding and rehabilitation. Ram spermatozoa are very susceptible to freezing due to lipid peroxidation generated by reactive oxygen derivatives in excess unsaturated fatty acids found within the plasma membranes. Sperm freezing and the events occurring during this time cause damage in the functions of the spermatozoa. For this reason, adding antioxidants and cryoprotective substances to semen diluents can minimize the negative effects of the freezing process and accompanying effects.

In the study of Tırpan and Tekin (19), boric acid was added to spermatozoa of the Angora goat and motility values were found similar to our study. These data also showed that the values obtained in 1mM dose

group were in aggrement with our values. They reported that statistically significant reductions in abnormal spermatozoa ratio were observed in group which is supplemented with boric acid instead of glucose and added boric acid diluents compared to the control group. In the current study, spermatozoa with 4 mM doses higher head and tail anomalies were observed in the groups compared to the control group and the difference was significant. No statistically significant difference was determined in live spermatozoa ratios in the control, 1 mM, 2 mM, and 4 mM groups in this study. Regarding the abnormal spermatozoa rates of acrosomal abnormalities, it was reported that the ratio of abnormal spermatozoa due to acrosome was significantly lower in the group supplemented with boron and boron added diluent than in the control group. In our study, no significant differences were detected between the control group and the other groups in terms of spermatozoa acrosomal integrity results. However, it was noted that the values of the 1 mM group were relatively higher than the other groups. However, Tirpan and Tekin (19) reported that the addition of boron into the diluent did not have an adverse effect on post-dissolution motility values and increased progressive motility and spermatozoa mobility values, and this could have positive effects on fertility compared to the control group. This result was in parallel with the increase in mitochondrial activity in the present study, especially in the 1 mM group. It was reported that fertility rates were higher in sperm having higher relative motility values in the association between total, progressive motility, and speed values.

Elkomy et al. (20) reported that rabbits supplemented with different rates of boron in their diets indicated a positive impact in many spermatological values and behaviours, especially in motility values and sperm quality. It was reported that on the molecular level Boron affected the activities of at least 26 enzymes (21) and that a large number of these enzymes were necessary for the energy substrate metabolism. It was reported that boron had several regulating roles on the macromineral metabolism (22, 23), the energy metabolism (24) and the immune system (25) and that after delivery of boron at different levels, the fructose concentration data in seminal plasma decreased significantly compared to the control group while the seminal plasma fructose concentration in the treatment group decreased, spermatozoa concentration per ejaculate increased which could be linked to the consumption of excess fructose to meet the necessary energy for the metabolism.

Duydu et al. (3) carried out a study on the impact of boron on workers, farmers, and citizens in the vicinity of boron resources in Bandırma and evaluated the levels of FSH and LH in blood, and total testosterone as well as sperm density, motility, and morphological abnormality, which are accepted as indicators of reproductive toxicity caused by air, water, and food-borne exposure. They determined the mean daily boron exposure as 14.45 mg/day. However, no negative impact was encountered. Urine, blood, and semen samples were examined in the study to determine the amount of exposure which turned out to be 4.68 mg of boron/day for the control group, 7.39 mg of boron/day for the low exposure group, 11.2 mg of boron/day for the intermediate exposure group, and 14.45 mg dg boron/day for the high exposure group. Scialli et al. (26) reported that no evidence was found that the reproductive systems of the male workers in the boron mine who were the subject of the study and exposed to excessive boron had been subjected to any adverse effect. However, in the same study, they reported that there was a decrease in the ratio of Y bearing chromosomes in the spermatozoa.

TAS is a biochemical parameter suitable for evaluating the overall antioxidant status of body fluids resulting from antioxidant intake and/or production and their consumption by increased levels of ROS production (27). The capacity of known and unknown antioxidants and the non-synergistic interaction are therefore assessed, giving an insight into the delicate balance between oxidants and antioxidants in vivo (27, 28). When the oxidant/antioxidant balance is tilted towards oxidants and oxidative stress arises, there is a significant negative correlation between the TAS and TOS values (16).

The highest and lowest values obtained in the current study as a TAS value was 0.385±0.051 and a TOS value of 29.950±2.001 with a 1mM dose. It was noted that the 1 mM dose at TOS level was exposed to oxidative stress at a lower level than the 4 mM dose and was statistically significant. In terms of DNA damage, boron added at a dose of 1 mM incurred less damage compared to the other groups which was statistically significant ($P<0.05$). In the study conducted by İnce et al. (29) which supports our study, the rats in their control group were fed with standard rodent feed containing 6.4 mg boron/kg while the other groups were fed with feed supplemented with 100 mg boron/kg of boric acid and borax supplemented feed. As a result, it was observed that a diet with a high concentration of boron reduced lipid peroxidation and improved the antioxidant action mechanism and the vitamin level. In another similar study, peripheral blood samples from humans were cultured. Various tests were conducted to determine the DNA damage and oxidative stress parameters by introducing boron at various doses to the cultures. As a result of the conducted tests, it was revealed that even the lowest dose of boron supported antioxidant enzyme activity in human blood cultures and that even the highest concentrations had no genotoxic effect on the cellular basis, and that increasing doses decreased oxidative stress. The data obtained in the current study supports the results of the above mentioned studies, and the antioxidant effect of boron was manifested.

In conclusion, it can be said that various substances can be added to sperm diluents in different amounts considering that they may have positive effects on the spermatological parameters of frozen and thawed ram sperm. Although the effects of these additives are generally positive, their effects can vary depending on the animal feed, diluent components, and freezing protocols. The conducted study manifested that the

result of adding boron in different amounts to the diluents has a positive effect on spermatological parameters. In particular, as a result of the higher spermatozoa motility manifested in the group supplemented with 1 mM of boron than in the control group as well as mitochondrial activity in particular, DNA damage and differences on the TOS level, it can be said that supplementing diluents with boron on a 1 mM level has a positive effect. In order to achieve better results from the present study, it is crucial to support the work done with a fertility parameter and that new studies should be carried out for this purpose.

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