Ankara Üniv Vet Fak Derg, **65**, 123-128, 2018

Effects of boron added bull semen extender on post-thaw spermatological parameters

Mehmet Borga TIRPAN[1], Hakan GÜRLER[2], Kemal Tuna OLĞAÇ[1], Ali DAŞKIN[1]

[1]Ankara University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Ankara, Turkey;
[2]University of Veterinary Medicine Hannover, Institute of Reproductive Biology, Bünteweg 2, Hannover, Germany.

Summary: In this research, post-thaw effects of bull semen extenders including different amounts of boron (sodium pentaborate) on total motility (TMOT), progressive motility (PMOT), kinetic parameters of spermatozoa (VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF), plasma membrane and acrosome integrity (PMAI) and DNA fragmentation index (DFI) were evaluated. Eight ejaculates were examined from four different bulls. Each ejaculate was split in to five aliquots and extended with standard Tris extender (Control) and 0.4 g boron with 0.5 g glucose (Group 1) 0.5; 0.7; 0.9 g boron without glucose (Group 2, Group 3 and Group 4 respectively) were used as experimental groups. After thawing TMOT, PMOT, kinetic traits of spermatozoa, PMAI and DFI were determined. According to motility and progressive motility (66.04%, 50.79%), Group 1 had higher values than the others, but differences between groups were not statistically significant (P>0.05). The highest values of VSL, LIN, STR and WOB were determined (28.15 µm/s, 42.98%, 71.69%, 59.71%) in Group 1 (P<0.05). While other kinetic traits of spermatozoa were higher numerically in Group 1 than the other groups, differences between groups were not statistically significant (P>0.05). The highest PMAI value was determined as 60.84% in Group 1 (P>0.05). The DFI rates were found similar in all control and experimental groups (P>0.05). In conclusion, it was observed that the boron (sodium pentaborate) has positive effects on post-thaw movement and structural traits. In addition, boron has no detrimental effect on DNA fragmentation index in semen freezing. Thus, boron could be used as an alternative semen extender compound.

Keywords: Bull semen, CASA, flow-cytometry, frozen semen, sodium pentaborate.

Boğa sperması sulandırıcısına eklenen borun çözüm sonu spermatolojik parametreler üzerine etkisi

Özet: Bu çalışmada, farklı dozlarda bor (sodyum pentaborat) içeren boğa sperması sulandırıcılarının çözüm sonu toplam motilite (TMOT), progresif motilite (PMOT), spermatozoa hareket parametreleri (VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF), plazma membranı ve akrozom bütünlüğü (PMAI) ve DNA fragmentasyon indeksi (DFI) parametreleri üzerine etkisi değerlendirildi. Dört baş boğadan toplam 8 ejakülat incelendi. Her ejakülat 5 eşit parçaya bölündü ve kontrol grubu olarak standart Tris sulandırıcısıyla sulandırıldı. Deney grubu sulandırıcıları olarak 0,5 g glikoza ek olarak 0,4 g bor eklenmiş Tris sulandırıcısı (Grup 1) ve glikoz yerine 0.5; 0.7; 0.9 g bor eklenmiş Tris sulandırıcıları (Grup 2, Grup 3, Grup 4) kullanıldı. Spermalar çözdürüldükten sonra TMOT, PMOT, spermatozoa hareket parametreleri, PMAI ve DFI değerleri belirlendi. Toplam motilite ve progresif motilite verilerine göre (%66.04, %50.79) Grup 1 sulandırıcısının daha yüksek değerlerde olmasına rağmen gruplar arası farklılıklar istatistiki açıdan önemsiz bulundu (P>0.05). En yüksek VSL, LIN, STR ve WOB değerlerinin (28.15 µm/s, %42.98, %71.69, %59.71) Grup 1'de olduğu görüldü (P<0.05). Diğer hareket özelliklerinin de Grup 1'de diğer gruplara göre yüksek olduğu belirlenirken, gruplar arası farklılıklar istatistiki açıdan önemsiz bulundu (P>0.05). En yüksek PMAI değerinin Grup 1'de olduğu, DFI değerlerinin ise tüm gruplarda benzer olduğu sonucuna ulaşıldı (P>0.05). Sonuç olarak borun çözdürme işleminden sonra spermatozoanın kinetik ve yapısal özellikleri üzerinde yararlı etkileri olduğu ortaya konmuştur. Ek olarak DFI açısından zararlı bir etkisi olmadığı gözlemlenmiştir. Bu veriler değerlendirildiğinde borun (sodyum pentaborat) alternatif bir sperma sulandırıcısı bileşeni olarak kullanılabileceği sonucuna ulaşılmıştır.

Anahtar sözcükler: Akım sitometri, boğa sperması, CASA, dondurulmuş sperma, sodyum pentaborat.

Introduction

The aim of semen cryopreservation is the long-term protection of sperm cells as viable and to be used for assisted reproductive techniques. The harmful product outputs decrease with low metabolic activity by reducing the temperature of the cells. Also, intracellular enzyme activity leads to decrease Adenosine triphosphate level and thus increase Adenosine monophosphate/Adenosine diphosphate level (29). However, varied biochemical and morphological structures of sperm cells (plasma membrane, acrosome, nucleus, axoneme and mitochondria) may be damaged during freezing and thawing processes (3). As a result, decrease in viability and motility values is occurred and this situation affects the fertility. Therefore, fertility of frozen semen is lower than fresh semen. Membranous structures around the spermatozoa are

extremely sensitive to freezing and thawing. The structures, which have fluid mosaic and thermodynamic feature, are formed unsaturated phospholipids. Cooling of the sperm cells may be concluded with irreversible phase changes in the membranous structures, that is, they may be changed from fluid phase to gel phase (16). These changes may be caused to destabilisation by leading the distribution in enzyme kinetics. Also, cooling of the sperm cells causes to increase lipid peroxidation in its membrane which is rich in unsaturated fatty acids (32). Consequently, the main purpose of sperm-freezing is to prevent lethal intracellular changes, to protect membranous structures and reduce the damage during and after cryopreservation.

Boron is an element commonly present as various compounds in earth, stone and water (9), and 72.1% of world total boron reserves are in Turkey (12). Boron and its compounds have various areas of usage in industry and agriculture (9). Although many studies on toxicity of boron have been conducted through the world, any toxic effect on reproductive system of human has not been found yet.

In some researches, it has been recorded that boron has an important role in membranous structure and metabolic activity. However, effects of boron on testicles, which are admitted as the most sensitive organ to boron, in terms of sperm parameters, semen freezability and fertilisation were not examined (19, 20, 26). Frequently, in different studies, effects of boric acid on the reproductive system of rats and mice have been tried to reveal based on dosage and time, high boric acid concentration intake results with antioxidant mechanism improvement (6, 11). In another research, it was determined that low doses of boron application supported antioxidant mechanism, when high concentrations have no genotoxic effect on cellular level (31). In addition decrease in oxidative stress parameters by increasing boron doses was detected. On the contrary, in the studies conducted on humans who live in boron-rich regions, there is neither a negative effect on reproductive system nor semen (5, 21, 30).

In the present study, it was aimed to evaluate the post-thaw effects of bull semen extenders including different amounts of boron (sodium pentaborate), on some sperm parameters (total motility, progressive motility, spermatozoa kinetic parameters, plasma membrane and acrosome integrity and DNA fragmentation index) by using current methods.

Materials and Methods

Animals: Ejaculates were collected from four fertile bulls that were already held and used for routine examinations at the clinic for cattle at the University of Veterinary Medicine Hannover. The bulls were kept under standard conditions of feeding and management. The animals showed no disturbances in general condition and had no sexual dysfunction or disease of the sexual organs during the period of investigations. Sperm was collected two times per week by using an artificial vagina. (Model Hannover, Minitüb, Tiefenbach, Germany).

Extenders and groups: Each ejaculate was split into five aliquots and one was diluted with standard Tris as a Control group and the other four were diluted with Tris extenders including four different amount of boron as experiment groups. Experimental groups which are shown in Table 1 were modelled on the study that researched by Tırpan and Tekin (29). The most beneficial values of sodium pentaborate of that study were selected for the study.

Experimental design: Eight ejaculates were examined from four different bulls. The percentage of progressively motile sperm was determined objectively using a phase contrast microscope. Ejaculates with >70% progressively motile sperm were used in the experiments. Each ejaculate was split in to five aliquots and diluted with different extenders as indicated in Table 1. Final concentration was determined as $60x10^6$ sperm/mL. Samples were evaluated by measuring the total motility (TMOT), kinetic parameters [Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), lateral amplitude (ALH), beat frequency (BCF)] and progressive motility (PMOT) with Computer Assisted Sperm Analyser (CASA) system. In addition plasma membrane and acrosome integrity (PMAI) and DNA fragmentation index (DFI) were evaluated with flow cytometry (Epics XL-MLC flow cytometer, Beckman Coulter, Fullerton, California, USA).

Table 1. Extender components for 100 ml solution.
Tablo 1. 100 ml'lik solüsyon için sulandırıcı bileşenleri.

	Groups				
Components	Control	Group 1	Group 2	Group 3	Group 4
Tris (g)	3.63	3.63	3.63	3.63	3.63
Citric acid (g)	1.82	1.82	1.82	1.82	1.82
Glucose (g)	0.5	0.5	-	-	-
Boron (g)	-	0.4	0.5	0.7	0.9
Egg yolk (%)	10	10	10	10	10
Glycerol(%)	5	5	5	5	5

Cryopreservation and thawing procedure: Semen were diluted at 37°C by one step dilution. After sperm dilution, samples were equilibrated at 4°C for 24 hours (14) and were sealed in 0.25 ml straws (IMV Technologies; L'Aigle, France). Before the straws are placed into the freezing chamber, the freezer was run with the chamber temperature already at 4°C. The straws were organized on racks and placed into the freezing chamber. The computer controls the temperature inside the chamber and in the straw and decreasing it from 4°C to the lowest temperature chosen (-140°C) at the 10°C/min. The computer monitor displays the temperature curves for the freezing chamber and for the straws being frozen. The temperature was controlled with the computer by varying the amount of liquid nitrogen flowing to the freezing chamber from the nitrogen source. It was cooled down to -140°C with 10°C per min using Ice cube 14S Computer Controlled freezing system with integrated PC (Minitüb GmbH, Tiefenbach, Germany) and stored in liquid nitrogen. Cryopreserved samples were thawed by immersing the straws for 30 seconds in a water bath at 37°C.

Examinations: Immediately after thawing, TMOT, PMOT, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF were examined by CASA system Sperm Class Analyser (SCA® v.4.2, Barcelona, Spain) supplied with a phase-contrast microscope (Olympus BX41, Olympus Europe GmbH, Hamburg, Germany). Chambers of 20 µm (Leja; Nieuw Vennep, The Netherlands) were loaded with semen and were kept at 37°C. The percentage of motility, progressive motility and kinetic parameters were determined by observing a minimum of 300 sperm in at least ten different microscopic fields per sample, with a frequency of 60 frames per second.

Plasma membrane and acrosome intact sperm (PMAI) was determined by using FITC-PNA (fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin)/PI (propidium-iodide) assay. Sperm samples were diluted to a concentration of $5x10^6$ sperm/mL with Tyrode's medium. Five microliters of FITC-PNA (100µg/mL) and 3 µl PI (2.99mM) were added to 492 µL of diluted sperm suspension. Sperm samples were incubated at 37ºC for 30 min and remixed just before measurement.

The percentage of sperm with a high DNA fragmentation (DFI) was assessed by using the SCSA™ as described by Evenson and Jost (13). Sperm samples were diluted to a concentration of $2x10^6$ sperm/mL with Tris-NaCl-EDT (TNE) buffer (stock solution; 0.1M Tris–HCl, 0.15M NaCl and 1mM Ethylenediaminetetraacetic Acid (EDTA) with pH: 7.4). TNE buffer was diluted to 10x with distilled water and stored at 4°C until assessed. The sperm suspension (200 µl) was treated with 400 µl acid-detergent solution (pH: 1.2, 0.08N HCl, 0.15M NaCl, 0.1% Triton X-100) for 30 sec, and then stained with 1.2 mL (6 mg/L) purified acridine orange (AO) in a phosphate–citrate buffer (0.2M Na2HPO4, 0.1M citric acid, 0.15M NaCl, 1mM EDTA, pH: 6.0). Samples were examined after 3 min. incubation.

Statistical analysis: The statistical significance of the difference between the extenders with existing variables was tested with one-way analysis of variance (ANOVA). The descriptive measurements of variables were given in the tables as "Arithmetic Mean (Av.) ± Standard Deviation(SD)". SPSS® for Windows 14.1 (Licence No: 9869264) package programme was used in analysis of the data.

Results

The highest values of VSL, LIN, STR and WOB were found in Group 1 (28.15 µm/s, 42.98%, 71.69% and 59.71%), and the lowest values were found in Control groups (22.06 µm/s, 33.80%, 61.94% and 54.48%) ($P<0.05$). VCL, VAP, ALH, BCF values were highest in Group 1, but differences between groups were not statistically significant ($P>0.05$). In terms of TMOT and PMOT, the highest values (66.04%, 50.80%) were determined in Group 1, and the lowest values (49.79%, 34.38%) were determined in Control ($P>0.05$). PMAI was observed as 60.84% in Group 1 and this value, was higher numerically than the Control and the other groups ($P>0.05$). The DFI values were determined similar in all of the groups. All results are shown in Table 2 and Table 3.

Discussion and Conclusion

In this study, it was aimed to evaluate the effects of boron (sodium pentaborate) on motility (TMOT), kinetic traits (VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF), progressive motility (PMOT), plasma membrane, acrosome integrity (PMAI) and DNA fragmentation index (DFI) of frozen-thawed bull semen. When the highest values were determined in Group 1, Control group had the lowest values in VSL, LIN, STR and WOB parameters, and differences between groups were statistically significant ($P<0.05$). Considering TMOT, PMOT, VCL, VAP, ALH and BCF although Group 1 had higher values, differences between groups were not statistically significant ($P>0.05$). Likewise PMAI rates were higher in Group 1 than the other groups ($P>0.05$). On the contrary, the percentages of DNA fragmentation in all control and the experimental groups were similar (3.18–3.57%) and differences between groups were not statistically significant. As a result of the examinations, it was observed that, the boron added extenders have not any detrimental effect in fragmentation of DNA. Although statistical difference is not present, Group 1 has higher PMAI value numerically than the Control group ($P>0.05$). This result suggests that, boron may have a protective

Table 2. Plasma membrane and acrosome integrity, DNA fragmentation index averages.
Tablo 2. Plazma membran ve akrozom bütünlüğü, DNA fragmentasyon indeksi ortalamaları.

Analysis	Groups	n	Av. ± SD	P
Plasma Membrane and Acrosome Integrity (%)	Control	8	48.27±3.83	0.142*
	Group1	8	60.84±2.90	
	Group 2	8	57.89±3.39	
	Group 3	8	58.67±5.18	
	Group 4	8	59.45±2.58	
DNA Fragmentation Index (%)	Control	8	3.18±0.47	0.956*
	Group1	8	3.20±0.38	
	Group 2	8	3.48±0.43	
	Group 3	8	3.49±0.46	
	Group 4	8	3.57±0.47	

* P>0.05. Differences between values are not statistically significant.
* P>0.05. Gruplar arası farklılıklar istatistiksel olarak önemsizdir.

Table 3. Results of CASA evaluations.
Tablo 3. CASA değerlendirmesi sonuçları.

Analysis	n	Control	Group 1	Group 2	Group 3	Group 4
TMOT	8	49.79 ± 6.71	66.04 ± 5.95	60.47 ± 5.45	62.11 ± 4.90	61.99 ± 3.26
PMOT	8	34.38 ± 4.56	50.80 ± 4.71	44.77 ± 4.10	46.92 ± 3.90	47.02 ± 2.79
VCL	8	65.46 ± 3.33	65.81 ± 2.27	64.55 ± 3.40	64.05 ± 2.15	62.08 ±1.59
VSL	8	22.06^{b} ± 1.17	28.15^{a} ± 1.08	23.90^{ab} ± 1.26	24.26^{ab} ± 0.96	24.50^{ab} ± 1.05
VAP	8	35.68 ± 1.91	39.31 ± 1.49	35.70 ± 1.88	35.49 ± 1.20	34.85 ± 1.00
LIN	8	33.80^{c} ± 1.16	42.98^{a} ± 1.31	37.14^{bc} ± 1.31	37.96^{abc} ±1.15	39.44^{ab} ±1.20
STR	8	61.94^{b} ± 1.05	71.69^{a} ± 1.16	66.99^{ab} ± 1.48	68.33^{a} ± 1.25	70.13^{a} ± 1.31
WOB	8	54.48^{b} ± 1.09	59.71^{a} ± 1.06	55.35^{b} ± 0.87	55.45^{b} ± 0.73	56.15^{ab} ± 0.71
ALH	8	3.04 ± 0.11	2.86 ±0.07	3.00 ± 0.12	3.00 ± 0.09	2.93 ±0.08
BCF	8	8.71 ± 0.27	9.73 ± 0.17	9.60 ± 0.28	9.55 ± 0.24	9.60 ± 0.30

a,b,c: Averages in groups in the same row with different superscripts are statistically important (P<0.05).
a,b,c: Aynı satırdaki farklı harfleri taşıyan grup ortalamaları arasındaki farklar istatistiksel açıdan önemlidir (P<0.05).

effect on cell plasma membrane against the harmful effects of cold. In addition, according to motility and kinetic data, it can be considered that the boron added extenders may provide the energy necessary for the intracellular metabolism of spermatozoa during the freezing and the thawing processes.

In a study (29), Angora buck semen was frozen with boron added extenders. According to results 0.7 and 0.9 g sodium pentaborate used instead of glucose was beneficial for motility and progressive motility, respectively (P<0.001). When these results are compared with the current study, it can be said that boron addition to standard extender, may be beneficial for cryopreservation of semen.

Spermatozoon motility is known as the most important parameter in evaluation of fertility (10). This strong relationship between motility and fertility can be related to variable calcium ion (Ca^{+}) permeability of plasma and acrosome membranes. In some researches (1, 4, 24), progressive motility of bull semen frozen with standard Tris extender was found at the range of 40-60%, total motility of the cryopreserved bull semen was found around 50-75%. When the results of the study were taken into consideration, it was seen that results were consistent with the other studies. Although statistical difference is not present, all groups that contain boron have numerically higher total and progressive motility results than the Control group (P>0.05). These results revealed that addition of boron to Tris extender with or without glucose has no detrimental effect on motility and progressive motility of bull semen. Furthermore, these data suggests that, boron may have the potential to increase the motility.

Several studies indicate that there are correlations between fertility and different sperm kinetic traits evaluated by CASA. While velocity parameters like VCL, VSL and VAP can be useful for prediction of in vivo

fertility (15, 23), STR, LIN and WOB have positive correlation with in vitro fertilization rates (27, 28). ALH and BCF have poorly negative correlation with spermatozoa fertility. In lights of all these information, it can be said that sodium pentaborat has some benefits on sperm motion traits. We can see in results demonstrated, sodium pentaborate increased VSL, LIN, STR and WOB significantly ($P<0.05$). In terms of VCL, VAP and ALH, it can be seen boron also has beneficial effects, though there are no important differences ($P>0.05$). Although the only negative effect of sodium pentaborat occurred in BCF averages, differences between control and the experimental groups are not statistically significant ($P>0.05$).

Degeneration of membranes resulting from cold shock can cause decrease in fertility indirectly. Plasma membrane and acrosome integrity are in charge of healthy semen production, and they are mostly overlooked. On the other side, crystallization is archenemy for this parameter. Therefore, it is necessary to evaluate the semen in terms of plasma and acrosomal membrane integrity in addition to motility (22). In researches (1, 2, 4, 25), PMAI of cryopreserved bull semen was found at around 60%. When the results of the study were taken into consideration, the results were seen that were consistent with the other studies. Although statistical difference is not present, all groups that contain boron have numerically higher PMAI results than the Control group ($P>0.05$). These results revealed that addition of boron to Tris extender has no detrimental effect on plasma and acrosomal membranes of bull semen. Furthermore, these data suggests that, boron may have the potential to protection of these membranous structures.

Protection of biochemical, structural and metabolic quality of spermatozoa are essential for fertility, but they cannot reveal the other important defect that occurred by fractionated DNA. Although, there is low correlation between DNA fragmentation and the current parameters, it is known that the sperm chromatin status can influence fertilization and embryonic survival rate (7). So, DNA fragmentation analyse can be useful to differentiate the infertility with normal spermatological characteristics (8). Various studies have shown that the DFI has significant correlation with fertility (17, 18). They found that DFI has low average in bulls while individual variations are disregarded. And it is realised that if DFI values become above 7% to 10%, fertility index will decrease. In the study, DFI was between 3.18% and 3.57%. These values are below the subfertility limit and similar to bull DFI average. According to the results in the study, we only can conclude that boron has no positive (or negative) effect on protection of DNA integrity.

In conclusion, it can be said that boron (sodium pentaborate) has beneficial effects on kinetic traits of frozen thawed bull semen. According to the CASA data it can be considered that the boron added to extenders may protect the intracellular metabolism of spermatozoa during the freezing and the thawing processes. In addition, it is seen that the boron has no negative effect on plasma and acrosomal membranes and DNA fragmentation in semen freezing and thawing process. It can be concluded that sodium pentaborate could be an alternative compound for semen extenders. However, further studies, supported with the fertility data, should be conducted in order to understand the exact effects of sodium pentaborat in bull semen cryopreservation.

References

1. **Ahmad M, Ahmad N, Riaz A, et al.** (2015): *Sperm survival kinetics in different types of bull semen: Progressive motility, plasma membrane integrity, acrosomal status and reactive oxygen species generation.* Reprod Fert Develop, **27**, 784-793.
2. **Almadaly E, Farrag F, Shukry M, et al.** (2014): *Plasma membrane integrity and morphology of frozen-thawed bull spermatozoa supplemented with desalted and lyophilized seminal plasma*. Glob Vet, **13**, 753-766.
3. **Amirat L, Tainturier D, Jeanneau L, et al.** (2004): *Bull semen in vitro fertility after cryopreservation using egg yolk LDL: A comparison with Optidyl®, a commercial egg yolk extender.* Theriogenology, **61**, 895-907.
4. **Anzar M, Kroetsch T, Boswall L** (2011): *Cryopreservation of bull semen shipped overnight and its effect on post-thaw sperm motility, plasma membrane integrity, mitochondrial membrane potential and normal acrosomes.* Anim Reprod Sci, **126**, 23-31.
5. **Bakırdere S, Örenay S, Korkmaz M** (2010): *Effect of boron on human health.* TOMPJ, **3**, 54-59.
6. **Başaran N, Duydu Y, Bolt MH** (2012): *Reproductive toxicity in boron exposed workers in Bandırma, Turkey.* J Trace Elem Med BioI, **26**, 165-167.
7. **Bochenek M, Smorag Z, Pilch J** (2001): *Sperm chromatin structure assay of bulls qualified for artificial insemination.* Theriogenology, **56**, 557-567.
8. **D'Occhio MJ, Hengstberger KJ, Johnston SD** (2007): *Biology of sperm chromatin structure and relationship to male fertility and embryonic survival.* Anim Reprod Sci, **101**, 1-17.
9. **Demirtaş A** (2006): *Bor bileşikleri ve tarımda kullanımı.* Atatürk Univ J Agricultural Faculty, **37**, 111-115.
10. **Donnelly ET, Lewis SE, McNally JA, et al.** (1998): *In vitro fertilization and pregnancy rates: The influence of sperm motility and morphology on IVF outcome.* Fertil Steril, **70**, 305-314.
11. **Duydu Y, Başaran N, Üstündağ A, et al.** (2011): *Reproductive toxicity parameters and biological monitoring in occupationally and environmentally boron-exposed persons in Bandırma, Turkey.* Arch Toxicol, **85**, 589-600.
12. **Eti Mine Works General Directorate** (2016): World Boron Reserves. Available at http://etimaden.gov.tr/tr/page/uretim-dunya-bor-rezervleri. (Accessed Feb 09, 2016).
13. **Evenson D, Jost L** (2001): *Sperm chromatin structure assay for fertility assessment.* 1-7. In: JP Robinson (Ed),

Current protocols in cytometry. John Wiley & Sons, Inc., New York.
14. **Fetterolf PM, Rogers BJ** (1990): *Prediction of human sperm penetrating ability using computerized motion parameters.* Mol Reprod Dev, **27**, 326-331.
15. **Fleischa A, Malamaa E, Witschib U, et al.** (2017): *Effects of an extension of the equilibration period up to 96 hours on the characteristics of cryopreserved bull semen.* Theriogenology, **89**, 255-262.
16. **Holt WV** (2000): *Basic aspects of frozen storage of semen.* Anim Reprod Sci, **62**, 3-22.
17. **Karabinus DS, Evenson DP, Jost LK, et al.** (1990): *Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry.* J Dairy Sci, **73**, 2364-2371.
18. **Karoui S, Díaz C, González-Marín C, et al.** (2012): *Is sperm DNA fragmentation a good marker for field AI bull fertility?* J Anim Sci, **90**, 2437-2449.
19. **Korkmaz M, Saylı U, Saylı SB, et al.** (2007): *Estimation of human daily boron exposure in a boron-rich area.* Brit J Nutr, **98**, 571-575.
20. **Korkmaz M, Yenigün M, Bakırdere S, et al.** (2011): *Effects of chronic boron exposure on semen profile.* Biol Trace Elem Res, **143**, 738-750.
21. **Ku WW, Chapin RE, Wine RN, et al.** (1993): *Testicular toxicity of boric acid: relationship of dose to lesion development and lack of recovery in the F344 rat.* Reprod Toxicol, **7**, 305-319.
22. **Lemma A** (2010): *Effect of cryopreservation on sperm quality and fertility.* 191-216. In: M Manafi (Ed), Artificial insemination in farm animals. INTECH Open Access Publisher.
23. **Liu DY, Clarke GN, Baker HG** (1991): *Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro.* J Androl, **12**, 231-239.
24. **Moallem U, Neta N, Zeron Y, et al.** (2015): *Dietary a-linolenic acid from flaxseed oil or eicosapentaenoic and docosahexaenoic acids from fish oil differentially alter fatty acid composition and characteristics of fresh and frozen-thawed bull semen.* Theriogenology, **30**, 1-11.
25. **Nagy S, Jansen J, Topper EK, et al.** (2003): *A triple-stain flow cytometric method to assess plasma-and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles.* Biol Reprod, **68**, 1828-1835.
26. **Saylı BS, Tüccar E, Elhan AH** (1998): *An assessment of fertility in boron-exposed Turkish subpopulations.* Reprod Toxicol, **12**, 297-304.
27. **Sukcharoen N, Keith J, Irvine DS, et al.** (1996): *Prediction of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF.* Hum Reprod, **11**, 1030-1034.
28. **Sukcharoen N, Sithipravej T, Promviengchai S, et al.** (1998): *Sperm morphology evaluated by computer (IVOS) cannot predict the fertilization rate in vitro after intracytoplasmic sperm injection.* Fertil Steril, **69**, 564-568.
29. **Tırpan MB, Tekin N** (2015): *Effects of boron (sodium pentaborate pentaborate), added instead of Tris components, on freezing and post-thaw quality of Angora buck semen.* Vet J Ankara Univ, **62**, 295-302.
30. **Treinen KA, Chapin RE** (1991): *Development of testicular lesions in f344 rats after treatment with boric acid.* Toxicol Appl Pharm, **107**, 325-335.
31. **Türkez H, Geyikoğlu F, Tatar A, et al.** (2007): *Effects of some boron compounds on peripheral human blood.* Z Naturforsch, **62**, 889-896.
32. **Watson PF** (2000): *The causes of reduced fertility with cryopreserved semen.* Anim Reprod Sci, **60**, 481-492.

Geliş tarihi: 11.10.2016 / Kabul Tarihi: 19.01.2017

Address for correspondence:
Mehmet Borga TIRPAN
Ankara University, Faculty of Veterinary Medicine,
Department of Reproduction and Artificial Insemination,
06110, Dışkapı/Ankara, Turkey.
+903123170315/4408
borgat@gmail.com