*Pure Appl. Biol., 5(3): 399-405, September, 2016*
*http://dx.doi.org/10.19045/bspab.2016.50052*

**Research Article**

# Effect of in-vitro supplementation of polyunsaturated fatty acids on frozen-thawed bull sperm characteristics using Bioxcell® extender

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**Citation**
Asmatullah Kaka, Wahid Haron, Riaz Ahmed Leghari, Muhammad Ismail Memon, Ubedullah Kaka, Amjad Hussain Mirani, Mohammed Naeem and Qudratullah Kalwar. Effect of in-vitro supplementation of polyunsaturated fatty acids on frozen-thawed bull sperm characteristics using Bioxcell® extender. Pure and Applied Biology. Vol. 5, Issue 3, pp399-405. http://dx.doi.org/10.19045/bspab.2016.50052

Received: 30/01/2016 Revised: 05/04/2016 Accepted: 16/04/2016 Online First: 05/05/2016

**Abstract**

Cryopreservation is considered that it decreases sperm quality about 50 percent therefore research is being conducted to increase the frozen-thawed quality of bull sperm. The object of study was to examine the effect of polyunsaturated fatty acids on the frozen-thawed bull semen sperm characteristics. Semen was collected from three bulls with an average of 8 collection from each bull at interval of 3 days. Semen ejaculates with motility and normal morphology 70% and 80% respectively were processed in extender (BioXcell®) having combined supplementation 0 to 15ng/ml of alpha linolenic acid (ALA) and docosohexaenoic acid (DHA). Extended sample was cooled at 5ºC for 2 hours, packaged and frozen into straws and in liquid nitrogen for 24 hours. Sequentially, straws were examined for sperm cytological characteristics, lipid peroxidation, fatty acid concentration and super oxide dismutase (SOD) after thawing. Sperm cytological characteristics decreased in the supplemented groups in comparison to control group. Docosahexaenoic acid improved in all supplemented groups, whereas ALA improved at 5ng/ml of ALA. Superoxide dismutase and lipid peroxidation were improved linearly by increasing fatty acids. Conclusion, combination of ALA and DHA has detrimental effect on frozen-thawed bull sperm parameters.

**Key words**: Omega-3 fatty acids; DHA; ALA; Semen; Cryopreservation

## Introduction

Artificial insemination (AI) mainly depends on cryopreserved semen which is achieved with semen extender. Main role of extender is to provide energy, prevent contamination from bacterial and provide nourishment to

the spermatozoa during freezing. Artificial insemination (AI) is based upon the good quality frozen semen. It is reported that liveability of frozen sperm is lesser than fresh semen in bulls. Freezing decreases fifty percent survival ability and fertility is decreased by seven fold of sperm [1, 2].

There are series of processes such as freezing and thawing during cryopreservation which may affect sperm physically and chemically [3]. It is unclear that how sperm damage occurs, however, it is confirmed that decrease in liveability and fertility may occur due to the scratch in spermatozoa membrane [4]. Sperm membrane is main structure which regulates cellular processes, which are important for fertilization process. Lipids are main constituent of sperm membrane, mainly polyunsaturated fatty acids (PUFAs) [5].

Formation of ice crystals reorganize lipid and modify bonds of membrane and alters membrane fluidity. Lipids are important for lipid bilayer to control ionic exchanges, help for the sperm during development and acquire viability and regulate membrane physiology. Therefore, changes in sperm membrane decrease sperm fertilizing ability [6]. In addition, reactive oxygen species (ROS) are main production during freezing, which burns PUFAs by increasing the process of oxidation [4].

Role of PUFAs during cooling and storage is still being debated. The increase in concentration of PUFAs is believed to increase membrane fluidity, decreases resistant to cold shock due to the fatty acids presence and make sperm susceptible to the oxidation of lipids [7, 8]. However, higher amount of PUFAs increases the frozen-thawed quality of sperm [9]. Dietary DHA improved motility of the fresh semen collected from bulls [10]. In vitro addition of DHA did not enhance the viability of frozen boars sperm [11]. Similarly, dietary addition of PUFAs produced negative effect on motility and morphology of crude and stored equine semen [12]. Likely to dietary, in vitro addition of PUFAs in semen extender decreased frozen thawed quality of bull sperm in citrate extender [4, 6], however, fish oil supplemented in egg yolk-lactose extender has enhanced frozen-thawed quality of boar sperm [13]. However, less data available on in-vitro supplementation of PUFAs effects on qualityof frozen-thawed bull semen. Therefore, this study was planned, to examine the effect of combination ALA and DHA on frozen thawed sperm parameters, fatty acid concentration, Melondialdehyde (MDA) and SOD on semen of bull extended into BioXcell®

## Materials and methods

### Collection of Semen and experimental design

An electro-ejaculator was used to collect semen ejaculates from three Brangus-Simmental cross-bred bulls (Electro Jac 5, Ideal Instruments Neogen Corporation, Lansing, Michigan, USA). Before collection each bull was prepared washing and cleaning of perpetual area and rectal massage for about 15minuts before collection. Eight collection from each bull total twenty-four ejaculates were collected at interval of 3 days. After collection semen was transported immediately at 37ºC to the laboratory for evaluation and further processing. Samples having motility of ≥70% and normal morphological of≥80 % and were used in this study.

Each ejaculate was added into BioXcell® consist of equal concentrations (control), 3, 5, 10 and 15ng/ml levels of DHA and ALA were added (Sigma Chemical Co., St. Louis, MO, USA). Ethanol (0.05%) was used to dissolve the fatty acids in water [14]. The samples extended were kept warm at 37ºC for 15 minutes to assimilation of fatty acids into membrane of sperm [15, 16]. Then, diluted samples were cooled at 5ºC (2 hours) [17], and then packed in 0.25ml straws with average of 20 x 106 and successively, frozen into liquid nitrogen for 24 hours. The

frozen straws were thawed at 37oC for 30 seconds in water bath **[18, 15, 16]**, and then investigated for sperm characteristics, fatty acid concentration, SOD and MDA production.

**Evaluation of semen**

Motility was determined by automatic semen analyzer, (Computer assisted semen analyser, CASA, IVOS Hamilton Thorne bioscience, Beverly, Massachusetts USA). Twenty µl of diluted semen was placed on glass slide CASA 2X-cell (20 µm) cover slip was placed to cover (20mm x 20mm) after Loaded on the CASA for motility calculation. An average Superoxide dismutase test was calculated by (Cayman chemicals company USA) assay kit. Two hundred fifty µl of semen were placed in Eppendorf tube and centrifuged for 10 minutes at 1000 x g. The upper (seminal plasma) layer was discarded while lower (sperm rich ) layer was vortex- for 10 seconds for 3 times with 0.5 ml of 0.1 % Triton X-100 (Fisher, Montre´al, PQ, Canada) and centrifuged again for 3 minutes at 1000 x g **[19]**. Other process was followed as the company recommended.

Peroxidation of lipid was calculated by melondialdehyde (MDA) production using thiobarbituric acid reactive substances (TBARS) according to the method explained by **[15, 20]**.

**Statistical analysis**

Data of sperm cytological characteristics, Superoxide Dismutase, fatty acid concentration and melondialdehyde were analysed using the general linear model (GLM) with SAS 9.2 version. Duncan multiple range test was used to differentiate among the means. P.values were considered to be statistically significant at ($P<0.05$).

**Results**

Table 1 presents percentages of cytological characteristics of frozen-thawed spermatozoa extended in Bioxcell® consist of fatty acids. The results of this study shows that all sperm cytological characteristics decreased ($P\geq0.05$) in supplemented groups than control. Though, SOD increased significantly in supplemented groups than control.

Fatty acid concentration of frozen samples is shown in Table 2. Results of DHA concentration was higher ($P<0.05$) at 10 and 15ng/ml. A non-significant difference ($P>0.05$) other supplemented and control groups. Alpha linolenic acid was higher at 5ng/ml. There was no significant difference among n-3 PUFAs ($P>0.05$). Figure 1 presents results of the MDA production. According to the graph MDA production was higher ($P<0.05$) in supplemented groups than control.

**Table. 1. Effects combination of Docosahexanoic acid (DHA) and Alpha-linplenic acid (ALA) in Bioxcell® extender on frozen thawed sperm parameters in bulls (Mean%±SEM)**

| Sperm | DHA+ALA ng/ml | | | | |
|---|---|---|---|---|---|
| Parameters% | 0 | 3 | 5 | 10 | 15 |
| Motility | 40.5±0.2$^{a}$ | 38.50±2.0$^{ab}$ | 34.75±0.9$^{b}$ | 36.25±1.7$^{ab}$ | 35.00±1.7$^{b}$ |
| Morphology | 61.25±3.4$^{a}$ | 57.00±0.4$^{ab}$ | 57.25±0.6$^{ab}$ | 51.00±0.9$^{b}$ | 52.25±1.3$^{bc}$ |
| Acrosome integrity | 64.25±2.7$^{a}$ | 59.00±3.0$^{ab}$ | 57.75±3.2$^{ab}$ | 57.00±3.0$^{ab}$ | 51.25±1.4$^{b}$ |
| Membrane Integrity | 55.25±2.7$^{a}$ | 50.50±0.8$^{abc}$ | 52.00±1.9$^{ab}$ | 48.50±0.8$^{bc}$ | 45.75±1.4$^{c}$ |
| Viability | 65.75±2.7$^{a}$ | 57.75±2.8$^{ab}$ | 56.25±1.4$^{ab}$ | 51.75±0.7$^{b}$ | 49.75±6.3$^{b}$ |
| SOD U/ml | 22.29±0.5$^{d}$ | 39.33±10.4$^{cd}$ | 51.76±1.6$^{bc}$ | 66.63±3.3$^{b}$ | 85.05±0.9$^{a}$ |

$^{a,b,c}$ Values with different superscripts within rows show significant difference $p < 0.05$,
DHA: Docosahexanoic acid, ALA: Alpha-linplenic acid, ng/ml: nanograms per milliliter
SOD: Superoxide dismutase, U/ml: Units per milliliter

**Table. 2. Comparison of fatty acid composition in different concentrations of Docosahexanoic acid (DHA) and Alpha-linolenic acid (ALA) combination in BioXcel® extender frozen-thawed sperm (Mean % ± SEM)**

| Fatty Acids | DHA & ALA Concentration ng/ml | | | | |
|---|---|---|---|---|---|
| | 0 | 3 | 5 | 10 | 15 |
| C14:0 | 0.58+0.12 | 0.98+0.65 | 0.53+0.11 | 0.63+0.10 | 0.66+0.03 |
| C16:0 | 24.26+0.11 | 23.86+1.86 | 22.25+1.28 | 23.36+2.14 | 25.44+2.39 |
| C16:1 | 0.78+0.26 | 1.01+0.47 | 1.62+0.34 | 2.11+0.65 | 2.19+0.56 |
| C17:0 | 0.57+0.03$^{b}$ | 0.86+0.23$^{ab}$ | 0.73+0.03$^{ab}$ | 0.76+0.24 $^{ab}$ | 1.60+0.55$^{a}$ |
| C18:0 | 5.88+0.43 | 7.61+1.19 | 8.87+0.31 | 7.41+1.88 | 6.99+0.77 |
| C18:1n-9 | 14.82+0.51 | 15.44+1.96 | 14.93+1.61 | 15.23+0.78 | 14.95+0.36 |
| C18:2n-6 | 48.30+1.02 | 45.37+1.86 | 45.79+0.42 | 45.71+3.75 | 42.16+2.64 |
| c18:3n-6 | 0.57+0.17 | 0.42+0.21 | 0.43+0.01 | 0.49+0.16 | 1.13+0.64 |
| C18:3n-3 ALA | 0.99+0.04$^{ab}$ | 0.89+0.07$^{b}$ | 1.07+0.03$^{a}$ | 0.94+0.05$^{ab}$ | 0.97+0.04$^{ab}$ |
| C20:4n-6 | 0.66+0.19 | 0.81+0.04 | 1.08+0.02 | 0.71+0.15 | 0.73+0.13 |
| C20:5n-3 | 0.77+0.02$^{a}$ | 0.67+0.06$^{ab}$ | 0.49+0.05$^{c}$ | 0.55+0.04$^{bc}$ | 0.78+0.06 $^{a}$ |
| C22:5n-3 | 0.38+0.10 | 0.59+0.14 | 0.70+0.11 | 0.45+0.06 | 0.68+0.16 |
| C22:6n-3 DHA | 1.44+0.04$^{b}$ | 1.49+0.05$^{b}$ | 1.50+0.03$^{b}$ | 1.67+0.08$^{a}$ | 1.73+0.05$^{a}$ |
| SFA | 31.28+0.46 | 33.31+3.46 | 32.38+1.21 | 32.16+3.77 | 34.68+2.88 |
| MUFA | 15.60+0.70 | 16.45+2.42 | 16.56+1.28 | 17.33+0.58 | 17.14+0.49 |
| n-3PUFA | 3.58+0.06 | 3.65+0.32 | 3.77+0.09 | 3.60+0.09 | 4.16+0.20 |
| n-6PUFA | 49.53+1.17 | 46.60+1.93 | 47.29+0.39 | 46.90+3.93 | 44.02+3.05 |
| PUFA | 53.11+1.13 | 50.24+2.08 | 51.06+0.34 | 50.50+3.88 | 48.18+2.87 |

**$^{a,b,c}$ Values with different superscripts within rows show significant difference p < 0.05, HA: Docosa hexanoicc acid, ALA: Alpha linolenic acid, SFA: Saturated fatty acids: sum of (C14:0+ C16:0+C17:0+ C18:0), MUFA: Monounsaturated fatty acids: sum of (C16:1+C18:1n-9), PUFA: Polyunsaturated fatty acids: sum of (C18:2n-6+C18:3n-6+C18:3n-3+C20:4n-6+C20:5n-3+C22:5n-3+C22:6n-3), n-6 PUFA: sum of (C18:2n-6+C18:3n-6+C20:4n-6), n-3 PUFA: sum of (C18:3n-3+C20:5n-3+ C22:5n-3+C22:6n-3)**

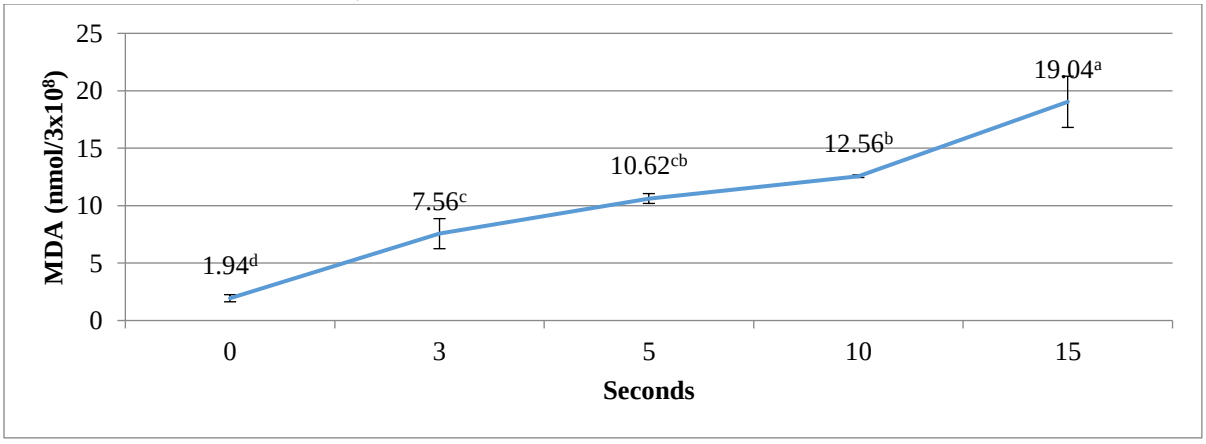


**Figure 1. Melondialdehyde (MDA) production in frozen thawed bovine semen treated with combination of Alpha linolenic acid (ALA) and docosahexanoic acid (DHA) in BioXcell® extender**

## Discussion

Present study, addition of fatty acids reduced characteristics frozen thawed sperm such as motility, membrane integrity, morphology, acrosome integrity and viability. Earlier studies are in line with this study, which reported that PUFAs reduced quality of frozen thawed bull semen in citrate extender in **[6]**. Similarly, other experiment also reported that PUFAs supplementation lowered sperm viability; motility and morphology **[4]**. Moreover, the PUFAs with an antioxidant in ovine semen improved the frozen-thawed quality parameters **[5]**. It is proven before that that cryopreservation reduces the sperm quality **[11]**. In the current study, PUFAs reduced the quality of frozen-thawed bull semen. Polyunsaturated fatty acids affects positively and negatively on fertility usually depend on only the type of fatty acids **[21]**.

Polyunsaturated fatty acids are found in sperm membrane man, ram and bull. Fatty acids improve the membrane fluidity, and provide resistance to the sperm against cold shock **[22]**. However, PUFAs such as DHA are important to maintenance of the membrane fluidity and its fertilizing ability. **[23]**. Nevertheless, omega-3 fatty acids also make sperm susceptible by ROS production and lipid peroxidation which compromise the functional integrity of the sperm.

The present study with addition of ALA and DHA improved fatty acids the in frozen sperm of bull. Alpha linolenic acid, EPA and DHA was significant difference among treatment and control. However, total n-3 fatty acids were non-significant. This may be due to the ALA is considered precursor of DHA and other n-3 fatty acids. These results are in range of results obtained after addition of n-3 fatty acids, improved DHA concentration and total PUFAs in ram semen **[5]**, in bulls **[5, 14]**, in goats **[24]**. However, about boars' results reported after of in -*vivo* and *vitro* addition of PUFAs enhanced n-3 PUFAs of frozen semen **[25]**. Melondialdehyde is the main concern after addition of ALA and DHA for decreases the quality of bull semen, in the present experiment the MDA increased after addition of ALA and DHA which resulted in decreased frozen thawed quality of bull semen. The supplementation of PUFAs decreased resistance against lipid peroxidation **[6]**. Moreover, it is reported that supplementation of PUFAs decreases resistance against cold shock and increases lipid peroxidation in the semen **[4]**. SOD level was improved that were similar to results reported in chicken **[26]**, and similarly PUFAS from fish oil increased SOD **[27]**. In current study enzymatic (SOD) activity was enhanced but it was not enough to prevent the frozen thawed sperm damage. Being the most important defense enzyme in the sperm but due to less concentration in the cytoplasm it is unable neutralize the peroxidation in sperm during freezing **[28]**.

## Conclusion

Conclusively, current study revealed that combined supplementation of DHA and ALA into BioXcel® reduced quality of frozen thawed bull sperm. All sperm parameters were decreased in relation to control.

## Authors' Contributions

Conceived and designed the experiments: A Kaka & W Haron, Performed the experiments: A Kaka, Analyzed the data: MI Memon & U Kaka, Contributed reagents/ materials/ analysis tools: AH Mirani, M Naeem, Q Kalwar & W Haron, Wrote the paper: A Kaka & RA Leghari.

## References

1. Lessard C, Parent S, Leclerc P, baileys JL and sullivan R (2000). Cryopreservation alters the levels of the bull sperm surface protein P25b. *J. Androl.* 21 (5):700-707.
2. Watson P (2000). The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.* 60:481-492.

3. Chatterjee S and Gagnon C (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol. Reprod. Dev.* 59 (4):451-458.
4. Abavisani A, Arshami J, Naserian AA, Kandelousi MAS and Azizzadeh M (2013). Quality of bovine chilled or frozen-thawed semen after addition of omega-3 fatty acids supplementation to extender. *Int. J. Fertili. Steril.* 7 (3):161.
5. Towhidi A and Parks J (2012). Effect of n-3 fatty acids and α-tocopherol on post-thaw parameters and fatty acid composition of bovine sperm. *J. Assist. Reprod. Gen.* 29 (10):1051-1056.
6. Kandelousi MS, Arshami J, Naserian A and Abavisani A (2013). The effects of addition of omega-3, 6, 9 fatty acids on the quality of bovine chilled and frozen-thawed sperm. *Open Vet.* 3 (1):47-52.
7. Giraud M, Motta C, Boucher D and Grizard G (2000). Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. *Hum. Reprod.* 15 (10):2160-2164.
8. White I (1993). Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod. Fert. Develop.* 5 (6):639-658.
9. Nikolopoulou M, Soucek DA and Vary JC (1985). Changes in the lipid content of boar sperm plasma membranes during epididymal maturation. *BBA-Biomembranes.* 815 (3):486-498.
10. Gholami H, Chamani M, Towhidi A and Fazeli M (2010). Effect of feeding a docosahexaenoic acid-enriched nutriceutical on the quality of fresh and frozen-thawed semen in Holstein bulls. *Theriogenology.* 74 (9):1548-1558.
11. Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P and Noble R (2005). Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Theriogenology.* 63 (2):411-421.
12. Grady S, Cavinder C, Brinsko S, Forrest D, Sawyer J and Scott B (2009). Dietary supplementation of two varying sources of n-3 fatty acids and subsequent effects on fresh, cooled, and frozen seminal characteristics of stallions. *PAS.* 25 (6):768-773.
13. Chanapiwat P, Kaeoket K and Tummaruk P (2014). Improvement of the frozen boar semen quality by docosahexaenoic acid (DHA) and L-cysteine supplementation. *AJB.* 11 (15):3697-3703.
14. Nasiri A, Towhidi A and Zeinoaldini S (2012). Combined effect of DHA and α-tocopherol supplementation during bull semen cryopreservation on sperm characteristics and fatty acid composition. *Andrologia.* 44 (s1):550-555.
15. Kaka A, Wahid H, Rosnina Y, Yimer N, Khumran A, Sarsaifi K, Behan AA, Kaka U and Ebrahimi M (2014). Improve the quality of post-cooling and frozen-thawed bovine sperm. *Ani. Rep*-5115; P. 7
16. Kaka A, Wahid H, Rosnina Y, Yimer N, Khumran A, Sarsaifi K, Behan AA, Kaka U and Ebrahimi M (2015). α-Linolenic acid supplementation in BioXcell® extender can improve the quality of post-cooling and frozen-thawed bovine sperm. *Anim. Reprod. Sci.* 153:1-7.
17. Memon AA, Wahid H, Rosnina Y, Goh YM, Ebrahimi M and Nadia F (2012). Effect of antioxidants on post thaw microscopic, oxidative stress parameter and fertility of Boer goat spermatozoa in Tris egg yolk glycerol extender. *Anim. Reprod. Sci.* 136 (1):55-60.
18. Yoshida M (2000). Conservation of sperms: current status and new trends. *Anim. Reprod. Sci.* 60:349-355.
19. Calamera J, Buffone M, Ollero M, Alvarez J and Doncel G (2003).

Superoxide dismutase content and fatty acid composition in subsets of human spermatozoa from normozoospermic, asthenozoospermic, and polyzoospermic semen samples. *Mol. Reprod. Dev.* 66 (4):422-430.

20. Mercier Y, Gatellier P, Viau M, Remignon H and Renerre M (1998). Effect of dietary fat and vitamin E on colour stability and on lipid and protein oxidation in turkey meat during storage. *Meat Sci.* 48 (3):301-318.
21. Castellano C-A, Audet I, Bailey J, Laforest J and Matte J (2010). Dietary omega-3 fatty acids (fish oils) have limited effects on boar semen stored at 17 C or cryopreserved. *Theriogenology*. 74 (8):1482-1490.
22. Wathes DC, Abayasekara DRE, Aitken RJ (2007). Polyunsaturated fatty acids in male and female reproduction. *Biol. Reprod.* 77 (2):190-201.
23. Safarinejad MR, Hosseini SY, Dadkhah F, Asgari MA (2010). Relationship of omega-3 and omega-6 fatty acids with semen characteristics, and anti-oxidant status of seminal plasma: a comparison between fertile and infertile men. *Clin. Nutr.* 29 (1):100-105.
24. Rooke JA, Shao CC, Speake BK (2001). Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen. *Reproduction*. Feb 1; 121 (2):315-22.
25. Ansari M, Towhidi A, Moradi Shahrbabak M, Bahreini M (2012). Docosahexaenoic acid and alpha-tocopherol improve sperm cryosurvival in goat. *Slovak. J. Anim. Sci*.; 45 (1):7-13.
26. Partyka A, Łukaszewicz E and Niżański W (2012). Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. *Theriogenology*. 77 (8):1497-1504.
27. Strzezek J (2002). Secretory activity of boar seminal vesicle glands. *Reprod. Biol.* 2(3):243-266.
28. Bucak MN, Ateşşahin A, Varışlı Ö, Yüce A, Tekin N and Akçay A (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: microscopic and oxidative stress parameters after freeze–thawing process. *Theriogenology*. 67 (5):1060-1067.