

Effect of glutamine and sugars after bull spermatozoa cryopreservation

Pürhan Barbaros Tuncer^a, Serpil Sarıözkan^b, Mustafa Numan Bucak^{a,*},¹,
Pınar Alkım Ulutaş^c, Pınar Peker Akalın^d, Serhat Büyükleblebici^a, Fazile Canturk^e

^a Ministry of Agriculture and Rural Affairs, Lalahan Livestock Central Research Institute, Lalahan, Ankara, Turkey

^b Erciyes University, Safiye Cikrikcioglu Vocational College, 38039, Kayseri, Turkey

^c Adnan Menderes University, Faculty of Veterinary Medicine, Department of Biochemistry, Aydın, Turkey

^d Ministry of Agriculture and Rural Affairs, General Directorate of Protection and Control, Ankara, Turkey

^e Erciyes University, Faculty of Medicine, Department of Biophysics, Kayseri, Turkey

Received 4 August 2010; received in revised form 11 November 2010; accepted 7 December 2010

Abstract

The objective of this study was to evaluate the effects of the addition of different sugars (raffinose, sucrose, and trehalose) on bull spermatozoa cryopreserved in a commercial extender (Optidyl) supplemented with glutamine on semen parameters, fertilizing ability and superoxide dismutase (SOD) activity. Nine ejaculates for each bull were used in the study. Semen was frozen in five different extenders: raffinose 25 mM plus glutamine 3 mM (RGO), sucrose 25 mM plus glutamine 3 mM (SGO), trehalose 25 mM plus glutamine 3 mM (TGO), glutamine 3 mM (GO) and control (O). Insemination doses were processed so that each 0.25 mL straw contained 15×10^6 sperm. Groups of GO and RGO resulted in the higher rates of subjective ($54.0 \pm 1.7\%$ and $64.0 \pm 1.1\%$; $P < 0.01$) and CASA motilities ($53.0 \pm 2.7\%$ and $61.0 \pm 4.4\%$; $P < 0.001$), respectively compared to the other groups. The supplementation of additives did not provide an effect on the level of post-thaw sperm CASA progressive motilities, the sperm motion characteristics and pregnancy rates. GO and RGO provided the better protective effect for sperm acrosome ($4.0 \pm 0.5\%$ and $12.0 \pm 0.6\%$) and total abnormalities ($5.0 \pm 0.3\%$ and $13.0 \pm 0.7\%$; $P < 0.001$), respectively. At the HOST values, the additives did not give to result the protective effect in comparison to Optidyl extender without additives ($P > 0.05$). For pregnancy rates, there were no significant differences among the groups. The supplementation of additives did not provide any significant difference on the level of SOD activity ($P > 0.05$). It can be also thought that these sugars might have worked with glutamine in a synergy. Thereby, sugars such as raffinose and sucrose with glutamine in freezing extender may be recommended to facilitate bull semen freezability.

© 2011 Elsevier Inc. All rights reserved.

Keywords: Bovine; Frozen; Sugars; Glutamine; Fertility; Sperm

1. Introduction

Cryopreservation of bull semen is still challenging due to lower fertility when compared to fresh semen because the all processes of cryopreservation including cooling,

freezing and thawing create oxidative stress on the sperm membrane [1–4]. Oxidative stress generally leads to loss of motility, swelling and the blebbing of the acrosomal membrane and disruption or increased permeability of the plasma membrane of spermatozoa [5,6]. It is well known that mammalian spermatozoa contain high concentrations of polyunsaturated fatty acids, and therefore are highly vulnerable to oxidative stress which is responsible for the generation of reactive oxygen species (ROS) [7,8]. Capacitation/acrosome reaction physiologically requires low

¹ Present address: Selcuk University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Konya, Turkey.

* Corresponding author. Tel.: +90 312 8651196/210; fax: +90 312 8651112.

E-mail address: mustafanbucak@yahoo.com (M.N. Bucak).

concentrations of ROS. Although normally being a balance between the production of ROS and the scavenging capacity of the antioxidants, the excessive of ROS deteriorate sperm function and enzymatic activity [9,10]. The main objective of most experiments on successful semen cryopreservation is to determine the appropriate extenders including cryoprotectant and antioxidants.

Egg yolk (EY) is a strong cryoprotectant agent that has been widely used in the extenders [11]. Since its strong cryoprotective effect, commercial extenders, such as Optidil, have been produced by the incorporation of EY.

Sugars play a role in the protection of post-thaw sperm viability. Sugars such as raffinose, trehalose, when in a high concentration solution, promote the excretion of water out of the cell so as to decrease intracellular ice crystal formation, maintaining osmotic pressure of the extender and have a cryoprotective role. However, their cryoprotective effects are dependent on storage temperature, their molecular weight and the buffer used in the extender [12–14]. The beneficial antioxidative and cryoprotective effects of sugars on cold shock and freeze-thaw damage have been reported in some studies [15–18].

Aminoacids (AAs) ensure to protect the structure of biological membranes of sperm cells during the freeze-thaw process. Some plants and animals accumulate aminoacids in response to exposure to freezing temperatures. Amongst the aminoacids used in freezing extender, glutamine has been reported in several species such as Angora goat [19,20], ram [21], stallion [22] and bull [23] sperm cells against freeze-thawing damage.

This research is the first evaluation of the influence of different sugars in amino-acid supplemented extender on freezing/thawing process of bull semen. The objective was to determine the effects of sugars such as trehalose, sucrose, and raffinose in commercial Optidil® extender supplemented with glutamine on sperm parameters, fertilizing ability, and SOD activity after the freeze-thawing process.

2. Materials and methods

2.1. Chemicals

The Optidil® extender and additives were obtained from IMV Technologies (Şark Kemikal, Turkey) and Sigma-Aldrich (Interlab, Turkey), respectively.

2.2. Animals and semen collection

Ejaculates from three Brown-Swiss bulls (3 and 4 years of age) were used in the study. The bulls, belonging to the Lalahan Livestock Central Research Institute

(Ankara, Turkey), were maintained under uniform feeding and housing conditions. A total number of 30 ejaculates (10 ejaculates for each bull) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. Immediately after collection, the ejaculates were immersed in a warm water bath at 37 °C until their assessment in the laboratory. Only ejaculates containing spermatozoa with > 80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/mL were used.

2.3. Semen extending, freezing and thawing

The groups established in the study were as follows:

RGO: Experiment group which was frozen in an Optidil extender with raffinose (25 mM) plus glutamine (3 mM).

SGO: Experiment group which was frozen in an Optidil extender with sucrose (25 mM) plus glutamine (3 mM).

TGO: Experiment group which was frozen in an Optidil extender trehalose (25 mM) plus glutamine (3 mM).

GO: Experiment group which was frozen in an Optidil extender with glutamine (3 mM).

O: Experiment group which was frozen in an Optidil extender without additives.

The volume of ejaculates and sperm concentrations were measured using a conical tube graduated at 0.1 mL intervals and Accucell photometer (IMV, L'Aigle, France), respectively. The freezing extender used was a commercial extender (Optidil®). Each ejaculate was divided into five equal parts and diluted to a final concentration of 60×10^6 /mL spermatozoa with the above mentioned extenders, respectively. Diluted semen samples were loaded into 0.25 mL French straws and cooled to 4 °C in 2 h, and then were frozen at a programmed rate of -3 °C/min from +4 to -10 °C; -40 °C/min from -10 to -100 °C; -20 °C/min from -100 to -140 °C using a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. At least after 24 h, frozen straws were thawed in a 37 °C water bath for 20 s.

2.4. Semen evaluation

2.4.1. Analysis of in vitro and in vivo semen parameters

Subjective motility was assessed using a phase-contrast microscope (100x magnification), with a warm stage maintained at 37 °C. A wet mount was made using a drop of semen placed directly on a microscope

slide and covered by a cover slip at same temperature. Sperm motility estimations were performed in three different microscopic fields for each semen sample. Besides estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyse sperm motion characteristics. Thawed semen was diluted in a Tris-based extender (without egg yolk and glycerol). A semen sample (volume) was diluted and put onto a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and sperm motility characteristics were determined with a 10x objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), total sperm motility (%), VAP (average path velocity, $\mu\text{m/s}$), VSL (straight linear velocity, $\mu\text{m/s}$), and VCL (curvilinear velocity, $\mu\text{m/s}$). For each evaluation, 10 microscopic fields were analysed to include at least 300 cells.

For the assessment of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 mL of Hancock solution [24]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of sperm acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (magnification 1000x, oil immersion).

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails. A sample (volume) of each treatment was incubated with 300 μL of a 100 mOsm hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37 °C for 60 min. After this time a sample (volume) was spread with a cover slip on a warm slide at 37 °C. A total of 200 sperm were evaluated with bright-field microscopy (100x magnification) and sperm with swollen or coiled tails were recorded [25,26].

The fertilizing ability of spermatozoa was calculated based on the pregnancy rates at 60 d by rectal palpation. A total number of 270 Brown-Swiss cows (between 2 and 5 y of age) were artificially inseminated with sperm treated with additives or not, each comprising the semen of one bull.

2.4.2. Biochemical assays

Biochemical assays were performed on sperm samples immediately after thawing following centrifugation and washing. An aliquot (500 μL) of semen from each sample was centrifuged at 800x g for 10 min, and the supernatant was removed and the pellet was washed with PBS. This procedure was repeated for

three times. After the last centrifugation, Triton X-100 (0.1%) was added to the sperm pellets. The superoxide dismutase (SOD) activities, which were calculated for 2×10^8 sperm cells, were determined from supernatants [27,28].

2.4.3. Determination of SOD activity

The SOD activity was analysed with the technique of Sun and Zigman [29]. A volume of 0.4 mL sperm sample, 0.1 mL of 0.9% NaCl and 0.4 mL of chloroform was centrifuged for 10 min at 2000g. 0.5 mL of supernatant was used for the test. The reaction mixture contained 20 mL of 3 mmol/L xanthine, 10 mL of 0.6 mmol/L EDTA, 10 mL of 0.15 mmol/L nitroblue tetrazolium, 6 ml of 400 mmol/L sodium carbonate, and 3 ml of 1 g/L bovine serum albumin for 20 tests. 2.45 mL of this reaction mixture was added into 0.5 mL of supernatants in test tubes. An aliquot (50 μL) of xanthine oxidase (20 $\mu\text{mol}/2 \text{ mL}$) was added into this mixture and was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by adding 1 mL of 0.8 mmol/L CuCl_2 to the test tubes. Then the absorbance was recorded at 560 nm using a UV/VIS spectrophotometer. SOD activity was expressed as U/g protein.

2.5. Statistical analysis

The results were expressed as mean \pm SEM. Means were analyzed using a repeated measurement model of ANOVA, followed by Duncan's post hoc test to determine significant differences among the groups using the SPSS/PC computer program (version 12.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered to be statistically significant. Pregnancy rates were evaluated using the chi-square test.

3. Results

As shown in Table 1, Group GO and RGO resulted in the higher rates of subjective ($54.0 \pm 1.7\%$ and $64.0 \pm 1.1\%$; $P < 0.01$) and CASA motilities ($53.0 \pm 2.7\%$ and $61.0 \pm 4.4\%$; $P < 0.001$), respectively compared to the other groups. The supplementation of additives did not provide an effect on the level of post-thaw sperm CASA progressive motilities, the sperm motion characteristics and pregnancy rates. GO and RGO provided the better protective effect for sperm acrosome ($4.0 \pm 0.5\%$ and $12.0 \pm 0.6\%$) and total abnormalities ($5.0 \pm 0.3\%$ and $13.0 \pm 0.7\%$; $P < 0.001$), respectively. At the HOST values, the additives did not give to result the protective effect in comparison to Optydil extender

Table 1
Mean \pm SEM sperm parameters in frozen-thawed bull semen.

Groups	O	GO	RGO	SGO	TGO	P
Subjective motility (%)	39.0 \pm 4.5 ^a	54.0 \pm 1.7 ^b	53.0 \pm 2.7 ^b	38.0 \pm 1.5 ^a	39.0 \pm 4.3 ^a	**
Progressive motility (%)	18.0 \pm 3.1	22.0 \pm 1.3	23.0 \pm 2.4	20.0 \pm 2.0	16.0 \pm 2.6	—
CASA motility (%)	51.0 \pm 3.2 ^a	64.0 \pm 1.1 ^b	61.0 \pm 4.4 ^b	47.0 \pm 2.5 ^a	49.0 \pm 2.9 ^a	***
VAP (μ m/s)	116.1 \pm 6.4	115.8 \pm 5.5	112.6 \pm 7.0	113.9 \pm 5.6	101.3 \pm 13.3	—
VSL (μ m/s)	85.9 \pm 2.0	85.9 \pm 3.2	87.8 \pm 3.9	87.6 \pm 3.2	86.8 \pm 2.6	—
VCL (μ m/s)	219.6 \pm 15.4	217.2 \pm 13.5	201.3 \pm 14.7	210.9 \pm 13.8	213.0 \pm 15.1	—
Acrosome abnormality (%)	10.0 \pm 0.6 ^{bc}	4.0 \pm 0.5 ^a	5.0 \pm 0.3 ^a	11.0 \pm 0.6 ^c	9.0 \pm 0.6 ^b	***
Total abnormalities (%)	21.0 \pm 1.1 ^b	12.0 \pm 0.6 ^a	13.0 \pm 0.7 ^a	21.0 \pm 1.4 ^b	19.0 \pm 1.5 ^b	***
HOST (%)	63.0 \pm 1.9 ^b	62.0 \pm 2.3 ^b	63.0 \pm 2.0 ^b	64.0 \pm 1.3 ^b	55.0 \pm 1.1 ^a	*
Pregnancy rate (%)	87.8 (65/74)	75.6 (34/45)	78.7 (37/47)	91.1 (41/45)	74.6 (44/59)	—
SOD (U/mg protein)	0.49 \pm 0.03	0.5 \pm 0.034	0.6 \pm 0.04	0.5 \pm 0.07	0.6 \pm 0.03	—

^{a-c} Different superscripts within the same row demonstrate significant differences among groups (* P < 0.05, ** P < 0.01, *** P < 0.001).

— No significant difference (P > 0.05).

without additives (P > 0.05). The supplementation of additives did not provide difference on the level of SOD activity (P > 0.05).

4. Discussion

Mammalian spermatozoa are susceptible to LPO which destroys the structure of the lipid matrix of spermatozoa membrane, due to the attacks of ROS formed from reduction of oxygen, during cryopreservation. The damage of lipid matrix finally causes loss of membrane integrity, membrane deterioration-due to membrane phase transitions, decreased sperm motility, loss in fertility and damage of the sperm DNA, through the oxidative stress and the production of cytotoxic aldehydes [5–10]. SOD is an enzyme, which scavenges ROS, such as superoxide anion and hydroxyl radicals, and thus protects mammalian sperm from oxidative damage [30]. In this study, additives did not increase SOD activity. Various factors complicate the cryopreservation of semen, like mechanical and biochemical stresses, oxygen-derived free radicals, high sensitivity to osmotic pressure changing, cooling, freezing and thawing processes.

Other important factors in sperm freezing protocols are cryoprotectants (e.g., glycerol) [12–14]. It was reported that glycerol alone is less effective than sugar/glycerol mixtures [27,28]. Adding sugars as cryoprotectant was suggested to ameliorate the complications. Non-permanent sugars can contribute to cellular dehydration before freezing where di- and tri- saccharides may be more influential than monosaccharides by increasing osmotic dehydration [31,29]. Raffinose plays a cryoprotective role, causing cellular osmotic dehydration during cryopreservation by interacting with membrane lipids and proteins and decreasing the risk of

intracellular ice crystal formation [32,33]. The cryoprotective properties of various sugars against freezing-thawing induced damages have been well documented in various cell types [32,34,35]. The effect of raffinose related to oxidative stress has been considered as indirect effects of sugar signalling and triggering the production of specific ROS scavengers [36]. Raffinose also reported to be used in most procedures for blocking the extent of extracellular ice formation, enhancing the formation of a metastable glass or microcrystalline state and providing hypertonicity [31,33]. In this study, there were significant differences in sperm parameters of post-thawed bull semen in response to raffinose and glutamine supplemented to the extender (RGO). The results comprising subjective and CASA motilities and acrosome integrity represented clearly that using raffinose plus glutamine or alone glutamine in the extender might give better post-thawed sperm quality. Disaccharides were reported to provide superior protection for motility, plasma membrane integrity and fertilizing capability of post-thawed sperm against cryodamage in different species [13,16,28,29,33].

Disaccharides, which mammalian cells are impermeable to, prevent the irreversible alteration in membranes and stabilise membrane bilayers during cooling-freezing states [37,38]. The concentration of sucrose is directly proportional to the amount of ice crystal and the temperature reduction occurred during freezing [31]. In this study, SGO did not play an important role in maintaining sperm motility and acrosome integrity, in contrast with a study carried out in Iberian red deer epididymal spermatozoa [39]. But SGO gave a favorable effect on pregnancy rates, compared to that of other groups. However this difference was not statistically significant. Based on our results, we can suggest

that raffinose displayed cryoprotection on the sperm motility and morphology.

Trehalose showed better cryoprotection and post-thaw fertilizing ability in mouse, ram, and bull sperm in some reports, leading to diminished death and damage of sperm [33,40,41]. In this study, TGO did not provide a positive effect on maintaining semen quality parameters. This finding was in contrast with the results of the study of Aboagla and Terada performed in goat [42] who found significant improvement in goat sperm freezability with trehalose containing extender. Difference of the extender content and animal species may explain contradictory results.

Aminoacids may play a role in the maintenance of the sperm membrane lipids which are especially vulnerable to LPO damage [43]. The supplementation of various aminoacids to freezing media has been found to be beneficial for spermatological properties of many species [19–22,44]. Glutamine, a tripeptide, plays a regulatory role in several cell-specific processes, including the metabolism (e.g., oxidative fuel, lipogenic precursor), cell integrity (apoptosis, cell proliferation), protein synthesis and degradation, redox potential, respiratory burst, gene expression and extracellular matrix synthesis [43]. In regard to sperm functions, glutamine acts at the extra-cellular level, improving the motility of human and stallion sperm [45,46] and the fertilizing ability of human sperm [45] following the freeze-thawing process. In this study, the supplementation of the extender with glutamine led to higher motility rates, functional and acrosomal integrity of sperm membrane. These results were similar to those previously reported for frozen-thawed sperm of the Angora goat, stallion and bull where glutamine addition also resulted in a significant increase in sperm motility and membrane integrity after thawing [20,22,23]. We may suggest that glutamine provide cryoprotection in mammalian sperm by decreasing cryodamage of sperm.

The HOST test assessed the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling driven by the hypo-osmotic treatment. This, thus provided a form of a membrane stress-test, which is particularly useful for testing the membrane-stabilising action of antioxidants [34]. In regard to HOST values, the additives did not provide protective effect in comparison to Optydil extender without additives ($P < 0.05$) in this study.

The main aim of artificial insemination with frozen bull semen is to achieve successful pregnancy rate. In

this study, fertility rate of SGO (91.1%) were slightly higher compared to other groups even though these differences were not statistically significant. Regarding our fertility results, it may be remarkable that sufficient pregnancy rates were obtained from all groups following cryopreservation/thawing of semen.

In conclusion, the percentages of post-thawed sperm motility, acrosome and functional plasma membrane integrity were found to be significantly higher in the groups of GO and RGO, compared to other groups. It can be suggested that these sugars might have worked with glutamine in a synergy. Sugars such as raffinose and sucrose with glutamine in freezing extender may be recommended to facilitate bull semen freezability. Further studies are required to evaluate the influence of sugar and aminoacid type on the sperm parameters and fertilizing ability in cryopreserved bull sperm.

Acknowledgments

This study was supported by the Republic of Turkey, Ministry of Agriculture and Rural Affairs, Lalahan Livestock Central Research Institute, Ankara, Turkey.

References

- [1] Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennigs Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998;59:1037–46.
- [2] White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod Fertil Dev* 1993; 5:639–58.
- [3] Zhao Y, Buhr MM. Cryopreservation extenders affect calcium flux in bovine spermatozoa during a temperature challenge. *J Androl* 1995;16:278–85.
- [4] Chatterjee S, De Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. *Mol Reprod Dev* 2001;60:498–06.
- [5] Watson PF. The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5 °C and deep-freezing. *J Termal Biol* 1976;1:137–41.
- [6] White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. *Reprod Fertil Dev* 1993;5:639–58.
- [7] Aitken RJ, Fischer H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* 1994;16:259–67.
- [8] Cassani P, Beconi MT, O'Flaherty C. Relationship between total superoxide dismutase activity with lipid peroxidation, dynamics and morphological parameters in canine semen. *Anim Reprod Sci* 2005;86:163–73.
- [9] Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MCG. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial potential and membrane lipid peroxidation. *J Androl* 2000;21:895–902.

- [10] Hammerstedt RH. Maintenance of bioenergetic balance in sperm and prevention of lipid peroxidation: a review of the effect on design of storage preservation systems. *Reprod Fertil* 1993;5:675–90.
- [11] Hu JH, Li QW, Zan LS, Jiang ZL, An JH, Wang LQ, Jia YH. The cryoprotective effect of low-density lipoproteins in extenders on bull spermatozoa following freezing–thawing. *Anim Reprod Sci* 2010;117:11–7.
- [12] Garcia MA, Graham EF. Development of a buffer system for dialysis of bovine spermatozoa before freezing. II. Effect of sugars and sugar alcohols on postthaw motility. *Theriogenology* 1989;31:1029–37.
- [13] Garde JJ, Olmo A del, Soler AJ, Espeso G, Gomendio M, Roldan ER. Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in endangered Cuvier's gazelle (*Gazella cuvieri*). *Anim Reprod Sci* 2008;108:384–401.
- [14] Abdelhakeam AA, Graham EF, Vazquez IA, Chaloner KM. Studies on the absence of glycerol in unfrozen and frozen ram semen: Development of an extender for freezing: Effects of osmotic pressure, egg yolk levels, type of sugars, and the method of dilution. *Cryobiology* 1991;28:43–9.
- [15] Malo C, Gil L, Gonzalez N, Cano R, Blas I. de, Espinosa E. Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender. *Cryobiology* 2010;61:17–21.
- [16] Tuncer PB, Bucak MN, Sariözkan S, Sakin F, Yeni D, Çiğerci IH, Ateşşahin A, Avdatek F, Gündoğan M, Büyükleblebici O. The effect of raffinose and methionine on frozen/thawed Angora buck (*Capra hircus ancyrensis*) semen quality, lipid peroxidation and antioxidant enzyme activities. *Cryobiology* 2010;61:89–93.
- [17] Bucak MN, Ateşşahin A, Varışlı O, Yüce A, Tekin N, Akçay A. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stress parameters after freeze–thawing process. *Theriogenology* 2007;67:1060–67.
- [18] Woelders H, Matthijs A, Engel B. Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* 1997;35:93–105.
- [19] Bucak MN, Tuncer PB, Sarozkan S, Ulutaş PA, Coyan K, Başpınar N, Ozkalp B. Effects of hypotaurine, cysteamine and aminoacids solution on post-thaw microscopic and oxidative stress parameters of Angora goat semen. *Res Vet Sci* 2009;87:468–72.
- [20] Bucak MN, Sariözkan S, Tuncer PB, Ulutaş PA, Akçadağ HI. Effect of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation. *Small Rumin Res* 2009;81:90–5.
- [21] Bucak MN, Tuncer PB, Sariözkan S, Ulutaş PB. Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. *Small Rumin Res* 2009;81:13–7.
- [22] Khlifaoui M, Battut I, Bruya JF, Chatagnon G, Trimeche A, Tainturier D. Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. *Theriogenology* 2005;63:138–49.
- [23] Amirat-Briand L, Bencharif D, Vera-Munoz O, Bel Hadj Ali H, Destrumelle S, Desherces S, Schmidt E, Anton M, Tainturier D. Effect of glutamine on post-thaw motility of bull spermatozoa after association with LDL (low density lipoproteins) extender: preliminary results. *Theriogenology* 2009;71:1209–14.
- [24] Schafer S, Holzmann A. The use of transmigration and spermac stain to evaluate epididymal cat spermatozoa. *Anim Reprod Sci* 2000;59:201–11.
- [25] Revell SG, Mrode RA. An osmotic resistance test for bovine semen. *Anim Reprod Sci* 1994;36:77–86.
- [26] Buckett WM, Luckas MJ, Aird IA, Farquharson RG, Kingsland CR, Lewis-Jones DI. The hypo-osmotic swelling test in recurrent miscarriage. *Fertil Steril* 1997;68:506–9.
- [27] Dandekar SP, Nadkarni GD, Kulkarni VS, Puneekar S. Lipid peroxidation and antioxidant enzymes in male infertility. *F Postgrad Med* 2002;48:186–9.
- [28] Kasimanicham R, Kasimanicham V, Thatcher CD, Nebel RL, Cassell BG. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology* 2007;67:1004–12.
- [29] Sun M, Zigman S. Determination of superoxide dismutase in erythrocytes using the method of adrenaline autooxidation. *Anal Biochem* 1978;90:81–9.
- [30] Fridovich I. Superoxide dismutase, regularities and irregularities. *Harvey Lec* 1985;79:51–75.
- [31] Yoshioka T, Kawada K, Shimada S. Lipid peroxidation in maternal and cord blood and protective mechanism against activated oxygene toxicity in the blood. *Am J Obstet Gynecol* 1979;135:372–6.
- [32] Agca Y, Gilmore J, Byers M, Woods EJ, Liu J, Critser JK. Osmotic characteristics of mouse spermatozoa in the presence of extender and sugars. *Biol Reprod* 2002;67:1493–501.
- [33] Storey BT, Noiles EE, Thompson KA. Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. *Cryobiology* 1998;37:46–58.
- [34] Bucak MN, Tekin N. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Rumin Res* 2007;73:103–8.
- [35] Yıldız C, Kaya A, Aksoy M, Tekeli T. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology* 2000;54:579–85.
- [36] Van den Ende W, Valluro R. Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging?. *J Exp Bot* 2009;60:9–18.
- [37] Aboagla EM, Terada T. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. *Biol Reprod* 2003;69:1245–50.
- [38] De Leeuw FE, De Leeuw AM, Den Daas JH, Colenbrander B, Verkleij AJ. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology* 1993;30:32–44.
- [39] Fernandez-Santos MR, Martinez -Pastor F, Garcia-Macias V, Estes MC, Soler AJ, Pazd P de, Anel L, Gadre JJ. Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa. *Theriogenology* 2007;67:738–53.
- [40] Aisen EG, Medina VH, Venturino A. Cryopreservation and postthawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology* 2002;57:1801–8.
- [41] Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. *Cryobiology* 1993;30:423–31.
- [42] Aboagla EME, Terada T. Effects of the supplementation of trehalose extender containing egg yolk with sodium dodecyl sulfate on the freezability of goat spermatozoa. *Theriogenology* 2004;62:809–18.

- [43] Curi R, Lagranha CJ, Doi SQ, Sellitti DF, Procopio J, Pithon-Curi TC, Corless M, Newsholm P. Molecular mechanisms of glutamine action. *J Cell Physiol* 2005;204:392–401.
- [44] Sariözkan S, Bucak MN, Tuncer PB, Ulutaş PA, Bilgen A. The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. *Cryobiology* 2009;58:134–8.
- [45] Renard P, Grizard , Griveau JF, Sion B, Boucher D, Lannou DL. Improvement of motility and fertilization potential of postthaw human sperm using glutamine. *Cryobiology* 1996;33:311-9.
- [46] Khlifiaoui M, Battut I, Bruyas JF, Chatagnon G, Trimeche A, Tainturier D. Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. *Theriogenology* 2005;63:138–49.