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Effects of different doses of trehalose supplementation in egg yolk extender in frozen–thawed Angora buck semen

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ABSTRACT

Few studies have been done on the effects of trehalose supplementation in the cryopreservation of Angora buck semen. The objective of present study was to investigate the effects of the addition of trehalose at different doses in semen extenders, on in vitro semen quality parameters, anti-oxidant enzymes activities and DNA damage after the freeze–thaw process in Angora buck semen. Semen samples from 5 mature Angora bucks (3 and 4 years of age) were used in this study. The bucks, belonging to the Livestock Central Research Institute were maintained under uniform breeding condition. A total number of 40 ejaculates were collected twice a week from the bucks using an artificial vagina, during the breeding season and the semen pooled to minimize individual variation. Each pooled ejaculate was split into 7 equal aliquots and diluted (37 °C) with base extenders supplemented with the trehalose (12.5, 25, 50, 75, 100 and 150 mM), and a base extender with no additives (control). Diluted samples were aspirated into 0.25 ml French straws, and equilibrated at 5 °C for 4 h and then were frozen at a digital freezing machine. The freezing extender supplemented with 50 mM trehalose led to the greatest percentages of CASA motility (53.6 ± 4.69), in comparison to the other groups after the freeze–thawing process ($P < 0.05$). The addition of different doses of trehalose did not provide any significant effect on the percentages of post-thaw sperm motion characteristics (VAP, VSL and LIN), compared to the control ($P > 0.05$). The freezing extender with 150 mM trehalose group led to the highest percentages of acrosome abnormalities ($P < 0.05$) and 50 mM trehalose group had the lowest percentages of total abnormalities ($P < 0.001$), in comparison to the others. There were no significance differences in the DNA integrity among treatment groups ($P > 0.05$). The different doses of trehalose did not show any effectiveness on the maintenance GPx, LPO, GSH, CAT and total antioxidant activities, when compared to the control ($P > 0.05$). Therefore, the additions of 50 mM and 75 mM doses of trehalose will be useful in increasing post thaw motility on Angora buck semen.

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1. Introduction

The cryopreservation of mammalian sperm is a complex process that involves many factors in order to produce satisfactory results (Purdy, 2006). Cooling and freeze–thawing

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produce physical, chemical and oxidative stress on the sperm membrane, which result in reduced sperm viability and fertilizing ability (Evans and Maxwell, 1987). Cold shock in sperm is also generally associated with oxidative stress and the generation of reactive oxygen species (ROS), by dead sperm and atmospheric or molecular oxygen in the environment. Oxidative stress is a cellular condition generally characterized by an imbalance between the production of ROS and the scavenging capacity of the antioxidants. When the production of ROS exceeds the available antioxidant defence system, significant oxidative damage occurs to the sperm organelles through the damage of lipids, proteins and DNA (Gomez et al., 1996; Bucak and Uysal, 2008; Bucak et al., 2009).

Sugar maintains the osmotic pressure of the diluents by inducing cell dehydration and less ice crystal formation into the spermatozoa (Leibo and Songsasen, 2002; Purdy, 2006). Moreover, sugar has the ability to form a glass (vitrification) by depressing the membrane phase transition temperature of dry lipids. It also interacts with phospholipid membranes at low hydration and thus causes stabilization of the membranes (Aisen et al., 2002). Furthermore, sugar is utilized by spermatozoa as an energy source through glycolysis and mitochondrial oxidative phosphorylation to support sperm motility and movement (Naing et al., 2010). Many researchers have studied the effect of sugar supplementation in semen extender on the quality of cryopreserved spermatozoa. Glucose was suggested to be more suitable sugar than fructose, lactose or raffinose in Tris-based extender in ram sperm (Salamon and Visser, 1972). Trehalose is a nonreducing disaccharide in which the two glucose units are linked in an a,a1,1-glycosidic linkage. Trehalose is able to protect the integrity of cells against a variety of environmental stresses such as dehydration, heat, cold and oxidation (Chen and Haddad, 2004). It had the remarkable stabilizing properties due to the formation of a nonhygroscopic glass state and protected protein and lipids membranes from degradation during the freeze–drying process. Furthermore, trehalose had been extensively used to improve sperm quality parameters in semen cryopreservation and its protective effects significantly improved the freezability of goat spermatozoa due to increase in membrane fluidity resulting from the depression of membrane transition temperature, allowing the sperm membrane to tolerate low-temperature effects (Aboagla and Terada, 2003; Hu et al., 2010). The extender containing trehalose improved antioxidant action and reduced the oxidative stress provoked by cryopreservation (Aisen et al., 2005). Antioxidant treatment with trehalose significantly elevated and improved post-thawed ram sperm motility (Bucak et al., 2007). Comet assay (Single Cell Gel Electrophoresis – SCGE) is one of the most popular cytogenetic methods to detect the DNA damage in a single cell. First published in 1980s as a method using micro-gel electrophoresis of immobilized cells lysed at high salt concentrations, which had been embedded in agarose. When an electrophoretic field applied with pH conditions less than pH 10, tails were observed where the damaged DNA migrated faster than the nuclear DNA (Ostling and Johanson, 1984). High alkaline conditions (pH > 13) for DNA unwinding and electrophoresis were incorporated later (Singh et al., 1988) and allowed the detection of single and double strand breaks as well as alkali-labile sites. Thus the alkaline COMET assay can provide a comprehensive measure of DNA damage (Lewis et al., 2008).

A few studies have been done on the effects of trehalose supplementation in the cryopreservation of Angora buck semen. Thus, the objective of present study was to investigate the effects of the addition of trehalose at different doses, on in vitro semen quality, anti-oxidant enzymes activities [glutathione (GSH), lipid peroxidase (LPO), glutathione peroxidase (GPx), catalase (CAT), total antioxidant] and DNA damage after the freeze–thaw process in Angora buck semen.

2. Material and methods

2.1. Chemicals

All chemicals used in this study were obtained from Sigma–Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

2.2. Animals and semen collection

Semen samples from 5 Angora bucks (3 and 4 years of age), were used in this study. The bucks, belonging to the Livestock Central Research Institute (39°58′ 23.56″ N, 33°06″ 28.51″ E) were maintained under uniform breeding conditions. They were housed in a dirt lot with an in door feeding area. Bucks received a mixed ration balanced to meet minimum nutritional requirements according to NRC (National Research Council 2001) and had free access to water. A total number of 40 ejaculates were collected at the morning time (9:00 am) twice a week intervals from the bucks using an artificial vagina, during the breeding season (autumn to early winter during two months) and the semen pooled to minimize individual variation. Ejaculates which met the following criteria were evaluated: volume of 0.5–2 ml; minimum sperm concentration of 3×10^9 sperm/ml; motility of 80%. Immediately following collection, the ejaculates were placed in a water bath (35 °C), until evaluation in the laboratory. Semen assessment was performed within approximately 10 min following collection. Each group was replicated eight times. The experimental procedures were approved by the Animal Care Committee of Lalahan Livestock Central Research Institute.

2.3. Semen processing

A Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% (v/v), glycerol 5% (v/v), pH 6.8) was used as the base extender. Each pooled ejaculate was split into 7 equal aliquots and diluted with base extenders supplemented with the trehalose (12.5, 25, 50, 75, 100 and 150 mM), and a base extender with no additives (control) for a total of 7 experimental groups to a final concentration of 200×10^6 spermatozoa/ml (single step dilution), in a 15 ml plastic centrifuge tube. Actual sperm concentrations were calculated with the aid of a haemocytometer (Smith and Mayer, 1955). Diluted samples were filled in 0.25 ml French straws, and equilibrated at 5 °C for a period of 4 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). After being stored for at least 24 h, straws were thawed individually (37 °C), for 30 s in a water bath for sperm evaluation. Sperm evaluation was performed on all semen samples immediately after thawing.

2.4. Semen evaluation

2.4.1. Analysis of standard semen parameters

Progressive motility as an indicator of semen quality was assessed using a phase-contrast microscope (×100 magnification), fitted with a warm stage maintained at 37 °C (Bearden and Fuquay, 2000). Sperm motility estimations were performed in 3 different microscopic fields for each semen sample and the mean of the 3 successive estimations recorded as the final motility score. Besides recording the subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS,

Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyze sperm motility, progressive sperm motility and sperm motion characteristics. CASA was set up as follows: phase contrast; frame rate – 60 Hz; minimum contrast – 70; low and high static size gates – 0.6–4.32; low and high intensity gates −0.20–1.92; low and high elongation gates 7–91; default cell size – 10 pixels; default cell intensity −80. Thawed semen was diluted (5 μL semen + 95 μL extender) in a Tris-based extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4 μL sample of the diluted semen sample was then put on a pre-warmed 20 μm slide (Leja Products B.V., Nieuw-Vennep, Holland) and the sperm motility characteristics determined using a 10× objective, at 37 °C. The following sperm motility values were recorded: subjective and CASA motility (%), CASA progressive motility (%), VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s) ALH (amplitude of lateral head displacement, μm) and LIN (linearity index (LIN = (VSL/VCL) × 100). For each evaluation, 10 microscopic fields were analyzed to include at least a total of 300 sperm cells.

2.4.2. Assessment of sperm acrosome abnormalities

For the evaluation of acrosome abnormalities in the semen samples, at least 3 drops of the semen were pipetted into Eppendorf tubes, containing 1 ml Hancock's solution (62.5 ml formalin, 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml double-distilled water) (Schafer and Holzmann, 2000). One drop of this mixture was placed on a microscope slide and covered with a cover slip. The percentage of sperm acrosome abnormalities was recorded by counting a total of 200 sperm under a phase-contrast microscope (×1000 magnification; oil immersion).

2.4.3. Assessment of the hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This test was performed by incubating 30 μl semen with a 300 μl 100 mOsm hypo-osmotic solution at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. A total of 400 sperm were evaluated (magnification 1000×) using bright-field microscopy. Sperm with swollen or coiled tails were recorded (Revell and Mrode, 1994; Buckett et al., 1997).

2.5. Assessment of biochemical assays

Semen samples were thawed in 37 °C water for 20 s and they were centrifuged at 4 °C at 1000 × *g* for 15 min in order to separate spermatozoa. Pellet was washed 3 times with a 0.5 ml of PBS. This final solution was homogenized 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 μl of homogenate was mixed with 10 μl 0.5 mM butyl hydroxyl toluene (BHT) and kept in −80 °C until the analysis. The rest of the homogenate was centrifuged at 8000 × *g* for 15 min and the supernatant was separated and kept in −80 °C for the other enzyme analysis.

2.5.1. Enzyme analysis

The levels of lipid peroxidase (LPO) were assessed with the commercial LPO-586TM Oxis research kit, glutathione peroxidase (GPx) levels with GPx-340TM Oxis research kit, superoxide dismutase with Sigma–Aldrich Fluka FL 19160 kit, catalase (CAT) with OxisresearchTM Catalase-520TM kit, GSH (glutathione) with Oxis research-420TM kit and antioxidant capacity with Sigma–Aldrich Antioxidant assay CS 0790 kit with spectrophotometric analysis.

2.6. Determination of sperm DNA damage using comet assay

In our study, the most commonly used alkaline comet assay parameters have been used, which are; Tail Intensity (percentage of DNA in the tail compared to the percentage in the 'head' or unfragmented DNA); Tail length (the length of the tail measured from the leading edge of the head) and Tail moment (percentage of DNA in the tail – tail DNA – times the distance between the means of the tail and head fluorescence measures). Each of these parameters describes endogenous DNA damage corresponding to DNA strand breakage and/or alkali-labile sites. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. Semen samples were thawed in 37 °C water for 20 s and then were centrifuged at 600 × *g* for 10 min at room temperature. Seminal plasma was removed and remaining sperm cells were washed with PBS (Ca^{2+} and Mg^{2+} free) two times to yield a concentration of 1×10^5 spermatozoa/ml. Each microscope slide was pre-coated with a layer of 0.65% high melting point agarose (HMA) in distilled water and thoroughly dried at room temperature. 75 μl of 0.65% low melting point agarose (LMA) at 50 °C was mixed with 25 μl of the cell suspension then dropped on top of the first layer, and covered with coverslips. Slides were allowed to solidify for 10 min at 4 °C in a moist box. The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na^2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothreitol (pH 10) for 1 h at 4 °C. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis (25 V and was adjusted to 300 mA) was performed for 20 min at room temperature. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents. After neutralization, the slides were stained with 65 μl of 20 μg/ml ethidium bromide and covered with a coverslip. All these steps were conducted under dimmed light to prevent the occurrence of additional DNA damage. The images of 100 randomly chosen nuclei were analyzed using a fluorescent microscope at a magnification of 400× (Zeiss, Germany). Nucleotide DNA extends under electrophoresis to form "comet tails," and the relative intensity of DNA in the tail images DNA break frequency. The percentage of the total DNA in the comet tail was taken as a measure of DNA break frequency. Tail DNA (%) was assessed in 100 cells by using Comet Assay III image analysis system (Perceptive Instruments, UK). Analysis was performed blindly by one slide reader.

2.7. Statistical analysis

Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared. The test revealed that the variances were homogeneous. After that, comparisons between the groups were made using analysis of variance with Duncan post hoc test. $P > 0.05$, not significant; $P < 0.05$; $P < 0.01$ and $P < 0.001$.

3. Results

As shown in Table 1, the freezing extender supplemented with 150 mM trehalose led to the lowest percentages of subjective motility (28.1 ± 5.59; $P < 0.001$), and the highest percentages of acrosome (8.3 ± 3.61; $P < 0.05$) in comparison to the other groups. 50 mM trehalose provided significance effect on the percentages of CASA motility (53.6 ± 4.69; $P < 0.05$). On the other hand, 50 mM and 75 mM trehalose led to significance effect on the percentages of post-thaw CASA progressive motilities compared to control (16.1 ± 2.74; 15.5 ± 1.70; $P < 0.001$, respectively). The addition of different doses of trehalose did not provide any significant effect on the some sperm motion characteristics (VAP, VSL and LIN), compared to the other groups ($P > 0.05$). The lowest VCL value was recorded (146.6 ± 5.35; $P < 0.01$) on 150 mM trehalose group. There were significance differences in the percentages of plasma membrane functional integrity among treatment groups ($P < 0.01$), the lowest and highest values were recorded (42.5 ± 2.44 and 56.1 ± 2.57) 150 and 25 mM trehalose groups. As shown in Tables 2 and 3, DNA damage was not affected by supplementation of different doses of trehalose as well as antioxidant activity ($P > 0.05$).

4. Discussion

When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress that arise through ice crystallization and LPO due to membrane changes (Bilodeau et al., 2000). Trehalose can improve the antioxidant action in semen extenders resulted in better

protection of sperm plasma membrane in semen cryopreservation by decreasing LPO (Aisen et al., 2005). The cryoprotective capacity of trehalose varied depending on the concentration of supplementation in the extenders (Naing et al., 2010). In this study supplementation of 50 mM of trehalose increased CASA motility and besides this supplementation of 25 mM trehalose had positive effect of subjective motility, but a supplementation of 150 mM trehalose had negative effects on subjective motility. Subjective sperm motility in this study was greater than the study which was reported in different species by Hu et al. (2010) and Tuncer et al. (2010). Although subjective and CASA motility were lower than the study on Japanese miniature Shiba goat which was reported by Aboagla and Terada (2003). Trehalose has a protective action related to the osmotic effect and the specific interactions with membrane phospholipids, which renders the media hypertonic, thereby it minimizes the degree of sperm cell injury during the freeze–thaw process (Hu et al., 2010). Atessahin et al. (2008) have found that supplementation of 75 mM trehalose had negative effect on motility but our study was in contradiction to this. Our study is also inconsistent with another study on the freezing ability of boar spermatozoa that supplementation of 100 mM trehalose significantly improved sperm motility, membrane integrity and acrosome integrity (Hu et al., 2009). It is considered that the differences between our study and the reported other studies above, may depend on the different content of sperm extenders or using different freezing protocol. Aisen et al. (2002) and Hu et al. (2010) observed a favourable effect on sperm motility when trehalose concentration was reducing on semen extender. On the other hand, when the greater (200–400 mOsm) trehalose concentration was used a deleterious effect was observed on sperm motility.

Cryopreservation disrupts the transbilayer-phospholipid asymmetry in the plasma membrane of mammalian sperm. Structural damage of the plasma membrane increases the susceptibility to LPO when high production of ROS occurs during the freeze–thawing process. This was stated for human (Alvarez and Storey, 1989), boar (Gadea et al., 2004; Funahashi and Sano, 2005), ram (Upreti et al., 1998; Bucak et al., 2007), bull (Sarıozkan et al., 2009) and buck (Tuncer et al., 2010) sperm. In spite of the beneficial effect of trehalose, not all reports have found the same results (Malo et al., 2010). In our study, trehalose group which was added up to 100 mM did not give different results when compared to control group, but when trehalose was added more than 100 mM, the membrane integrity was affected in a negative way. Parallel to current findings, Hu et al. (2010) reported that the functional integrity of sperm acrosomal membrane and plasma membrane associated with sperm motility can be expected to have been destroyed by large doses of trehalose. Besides this, it has been revealed with the studies on different species that using trehalose doesn't have effects on protecting membrane integrity (Chen et al., 1993; Squires et al., 2004; Fernández-Santos et al., 2007a, 2007b). Different from our findings, in a ram sperm study, it has been reported that trehalose added to sperm extender had positive effect on protecting membrane integrity (Aisen et al., 2000). At the studies on different

Table 1
Mean (±SEM) sperm motility, sperm motion parameters, hypo-osmotic swelling test (HOST), acrosome and total abnormalities in frozen–thawed Angora buck semen.

Sperm parameters	Control	Trehalose 12.5 mM	Trehalose 25 mM	Trehalose 50 mM	Trehalose 75 mM	Trehalose 100 mM	Trehalose 150 mM	*P*
Sub. motility (%)	46.3 ± 6.32^{b}	54.4 ± 5.21^{bc}	61.9 ± 3.40^{c}	61.9 ± 4.11^{c}	55.0 ± 4.01^{bc}	48.1 ± 2.30^{bc}	28.1 ± 5.59^{a}	<0.001
CASA motility (%)	35.5 ± 4.73^{a}	46.5 ± 4.08^{ab}	46.0 ± 3.68^{ab}	53.6 ± 4.69^{c}	50.9 ± 5.31^{b}	38.6 ± 3.40^{ab}	34.3 ± 3.99^{a}	<0.05
Progressive motility (%)	10.3 ± 2.08^{ab}	13.0 ± 1.91^{bc}	13.8 ± 2.42^{bc}	16.1 ± 2.74^{d}	15.5 ± 1.70^{d}	10.6 ± 1.65^{bc}	7.4 ± 1.78^{a}	<0.001
VAP (μm/s)	86.3 ± 4.74	93.1 ± 4.78	91.2 ± 4.57	89.4 ± 4.23	87.8 ± 5.29	82.5 ± 3.52	75.2 ± 3.30	–
VSL (μm/s)	67.7 ± 4.24	74.0 ± 5.08	70.5 ± 4.40	71.3 ± 3.78	68.4 ± 4.73	65.5 ± 3.42	56.8 ± 2.80	–
VCL(μm/s)	177.0 ± 8.22^{ab}	187.5 ± 5.67^{a}	185.0 ± 7.92^{a}	176.4 ± 7.71^{ab}	170.9 ± 9.17^{ab}	159.5 ± 4.87^{ab}	146.6 ± 5.35^{c}	<0.01
ALH (μm)	8.4 ± 0.24^{c}	8.7 ± 0.16^{c}	8.5 ± 0.25^{c}	8.1 ± 0.27^{bc}	7.7 ± 0.34^{abc}	7.3 ± 0.13^{ab}	7.0 ± 0.20^{a}	<0.001
LIN (%)	37.3 ± 1.08	38.5 ± 1.81	37.0 ± 1.09	39.9 ± 0.74	39.6 ± 1.05	41.4 ± 1.07	38.8 ± 0.88	–
HOST (%)	51.4 ± 3.35^{bc}	50.9 ± 2.74^{bc}	56.1 ± 2.57^{c}	53.9 ± 1.89^{c}	51.3 ± 3.53^{bc}	44.0 ± 2.08^{a}	42.5 ± 2.44^{a}	<0.01
Acrosome (%)	2.5 ± 0.33^{a}	2.6 ± 0.38^{a}	1.3 ± 0.16^{a}	1.8 ± 0.31^{a}	2.0 ± 0.38^{a}	3.3 ± 0.37^{a}	8.3 ± 3.61^{b}	<0.05
Total morphology (%)	14.1 ± 1.81^{bc}	12.6 ± 0.84^{bc}	11.1 ± 1.82^{bc}	8.8 ± 0.82^{a}	13.0 ± 1.04^{bc}	18.0 ± 1.64^{d}	19.9 ± 2.18^{d}	<0.001

a–d: Different superscripts within the same row demonstrate significant differences among groups.

Table 2
Mean (±SEM) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioxidant levels in frozen–thawed Angora buck semen.

Antioxidant	Control	Trehalose 12.5 mM	Trehalose 25 mM	Trehalose 50 mM	Trehalose 75 mM	Trehalose 100 mM	Trehalose 150 mM	*P*
GPx (mU/ml–10^9 cell/ml)	11.4 ± 0.20	11.4 ± 0.26	11.4 ± 0.13	11.8 ± 0.07	11.6 ± 0.29	11.6 ± 0.12	11.5 ± 0.20	>0.05
LPO (mU/ml–10^9 cell/ml)	18.1 ± 3.12	15.7 ± 2.03	13.9 ± 2.35	13.4 ± 2.66	8.1 ± 1.59	21.3 ± 3.87	14.6 ± 4.16	>0.05
GSH (mU/ml–10^9 cell/ml)	83.9 ± 10.63	74.9 ± 15.05	94.4 ± 5.75	108.1 ± 11.11	85.9 ± 14.14	96.1 ± 12.92	90.5 ± 14.66	>0.05
Catalase (mU/ml–10^9 cell/ml)	18.1 ± 0.71	22.4 ± 2.29	20.8 ± 1.52	30.0 ± 8.15	16.9 ± 0.86	18.4 ± 1.03	36.2 ± 11.36	>0.05
Total antioxidant (mmol/trilox/ml–10^9 cell/ml)	1.3 ± 0.30	0.6 ± 0.25	1.4 ± 0.40	1.0 ± 0.27	0.9 ± 0.30	0.8 ± 0.34	1.0 ± 0.29	>0.05

Table 3
Mean (±SEM) endogenous DNA damage corresponding to DNA strand breakage and/or alkali-labile sites detected by comet assay in frozen–thawed Angora buck semen.

Comet assay	Control	Trehalose 12.5 mM	Trehalose 25 mM	Trehalose 50 mM	Trehalose 75 mM	Trehalose 100 mM	Trehalose 150 mM	*P*
Tail length (μm)	73.2 ± 7.60	67.3 ± 7.57	57.6 ± 9.45	50.9 ± 8.26	57.9 ± 7.42	59.8 ± 5.58	71.7 ± 10.09	>0.05
Tail intensity (%)	15.1 ± 2.42	14.5 ± 4.03	12.5 ± 2.00	8.6 ± 1.66	10.5 ± 1.14	10.9 ± 1.82	14.3 ± 2.80	>0.05
Tail moment (μm %)	7.1 ± 2.00	5.9 ± 2.20	4.6 ± 1.42	2.8 ± 0.73	3.4 ± 1.05	3.9 ± 1.22	6.7 ± 2.03	>0.05

species, it has been suggested that trehalose did not have positive effect on protecting membrane integrity (Caiza de la Cueva et al., 1997a, 1997b). Adding trehalose at different doses do not have any positive effect on protecting membrane and acrosome integrity, even it may have negative effects when they are added 100 mM and more concentrations. It is considered that this may be related with many factors such as species or individual variation (Holt, 2000; Aisen et al., 2002; Garde et al., 2008), storage temperature, type of buffer and rate of freezing (Naing et al., 2010), or composition of extender including the presence of cryoprotectants (Gutiérrez-Pérez et al., 2009; Kozdrowski, 2009).

DNA damage that occurs during freezing of sperm effects embryo development negatively (Haaf and Ward, 1995). It is known that sperm DNA damage higher than 8% can not be completely repaired in the oocyte and might lead to impaired embryo development and early pregnancy loss (Ahmadi and Ng, 1999). It is therefore very important to have a standardized assay in reproductive toxicology at hand, which can effectively target the male germ cells (Baumgartner et al., 2004). Cryopreservation of spermatozoa enhances oxidative stress, which not only disrupts the motility and fertilizing ability of spermatozoa, but also increases DNA damage (Twigg et al., 1998; Rajesh et al., 2002). DNA damage was not affected by adding different doses of trehalose in this study in which contradict those obtained from Reddy et al. (2010) who reported that addition of trehalose to the freezing extender led to the reduction of cryodamage to the buffalo sperm. Tuncer et al. (2010) reported that raffinose is a different sugar, and it had cryoprotective effect (especially at doses of 5–10 mM), and preserved acrosome integrity and DNA integrity of sperm against cryodamage.

Antioxidant mechanisms exist to maintain defense against oxidative stress-induced damages in semen (Chen et al., 2003; Wai-Sum et al., 2006; Fernández-Santos et al., 2007a, 2007b). However, the antioxidant capacity of sperm cells is insufficient in preventing oxidative stress during the freeze–thawing process (Storey, 1997). In the current study the extender supplemented with trehalose did not achieve significant improvement in GPx, LPO, GSH, CAT and total antioxidant levels in comparison with the control. Aisen et al. (2005) reported that the extender containing trehalose enhanced the level of GSH and decreased the oxidative stress provoked by the freeze–thaw process in ram sperm. Atessahin et al. (2008) found that an extender supplemented with trehalose increased the GSH-Px and CAT activity of frozen–thawed goat semen. Increasing the doses of trehalose resulted in greater activity of CAT and a marked improvement in bovine sperm motility (Hu et al., 2010). Those reports were in contrast with our findings. In harmony with the obtained results, Bucak et al. (2007) demonstrated that the addition of trehalose did not cause significant differences in levels of GSH and GSH-Px of frozen–thawed ram semen.

5. Conclusions

The obtained results showed that, using 50 mM trehalose better than control group that it has positive effects on subjective and CASA motility, and total morphology. However using 100 mM and more trehalose doses have negative effects on plasma membrane integrity and total morphology. The additions of 50 mM and 75 mM doses of trehalose will be useful in increasing post thaw motility on Angora buck semen.

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