EFFECTS OF ARGININE AND TREHALOSE ON POST-THAWED BOVINE SPERM QUALITY

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The present study was conducted to examine the protective role of arginine and trehalose on post-thaw bull sperm and oxidative stress parameters. Five ejaculates for each bull were used in the study. Each ejaculate, split into three equal aliquots and diluted at 37 °C with base extenders containing 2 mM arginine, 25 mM trehalose and no antioxidant (control) was cooled to 5 °C and then frozen. Frozen straws were thawed in a water bath for evaluation. Supplementation of the semen extender with arginine decreased the percentages of post-thawed subjective motility ($29 \pm 8.21\%$), CASA motility ($12.2 \pm 5.69\%$) and progressive motility ($3.52 \pm 2.13\%$), compared with the controls ($43 \pm 2.73\%$, $55.4 \pm 6.78\%$ and $33.48 \pm 4.14\%$, respectively, $P < 0.05$). Supplementation of the semen extender with trehalose produced a higher mitochondrial activity and sperm viability ($36.3 \pm 3.99\%$ and $44.1 \pm 2.18\%$) compared with the control (13 ± 8.15 and $31.7 \pm 3.94\%$, respectively, $P < 0.05$). It was established that trehalose (95.1%) and arginine (92.8%) protect DNA integrity compared to the control (90.4%) ($P < 0.05$). Trehalose supplementation in semen extenders provided great benefit in terms of viability, mitochondrial activity, and intact sperm DNA on frozen-thawed bull sperm.

Key words: Arginine, bull sperm, COMET, fluorescent staining, trehalose, oxidative stress

Sperm production is an important factor influencing the reproductive capacity of males of economically important species (Berndtson, 2008). The application of artificial insemination (AI) has been shown to have the ability to disseminate genes from super genetic males for improving productive characteristics (El-Sheshtawy et al., 2015).

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Sperm cryopreservation contributes to the expansion of reproductive techniques, such as AI and *in vitro* fertilisation (Gholami et al., 2010). The factor most affecting conception rate is the quality of frozen semen. The composition of the extender in which semen is diluted before freezing is one of the most important factors affecting cryopreservation (El-Sheshtawy et al., 2015). Mammalian spermatozoa have very specific lipid composition and a high ratio of polyunsaturated fatty acids. This unconventional structure of the sperm membrane is responsible for its flexibility and the functional skill of sperm cells. However, the lipids of spermatozoa are the main cause of peroxidation, which may cause severe functional disorders of sperm. Oxidative stress can cause pathological lipid peroxidation of the sperm membrane (Sanocka and Kurpisz, 2004). Cryopreservation induces damage to spermatozoa that may result in loss of motility, plasma membrane integrity, and fertilising capacity (Aitken et al., 1998).

Oxidative stress can induce DNA fragmentation in the sperm cell (Aitken et al., 2009), and artificially created oxidative stress can induce DNA damage (Aitken et al., 1998). During cryopreservation the level of reactive oxygen species (ROS) generation in the spermatozoa increases (Wang et al., 1997) and this leads to cryo-injury to sperm DNA (Thomson et al., 2009*b*).

Cryopreservation causes cold shock, oxidative stress, osmotic changes and lipid-protein reorganisations within the cell membranes, thus inducing cell damage (Bailey et al., 2000). L-arginine is often referred to as a nonessential amino acid. This amino acid found in the seminal plasma has an important role in spermatozoal metabolism and motility. L-arginine plays an important role in modulating host defence and cellular immunity (Patel et al., 1998). Sugars have a function in protecting the post-thaw viability of sperm cells. Sugars such as raffinose and trehalose have a cryoprotective role by decreasing intracellular ice crystal formation, thus maintaining the osmotic balance of the diluent (Garde et al., 2008).

Trehalose is a non-penetrating disaccharide, which has a protective action on cells both by increasing the tonicity of the extender and by stabilising the plasma membrane, possibly due to specific interactions with head groups of membrane phospholipids (Crowe et al., 1987). Trehalose inserts itself into the membrane phospholipids bilayer, thus modulating membrane fluidity and therefore rendering the membrane more stable during freezing (Aboagla and Terada, 2003). The positive effect of trehalose has reported by many authors in different species, such as ram (Tonieto et al., 2010), goat (Aboagla and Terada, 2003) and bull (El-Sheshtawy et al., 2015).

The aim of this study was to determine the effects of arginine and trehalose on sperm motility, viability, acrosome integrity, mitochondrial activity, DNA integrity, lipid peroxidation (LPO) and total antioxidant activity (AOP) on bull semen.

Materials and methods

Animals and semen collection

Five healthy Holstein bulls (3–4 years of age) housed at a private dairy farm in Konya and maintained with standard feeding and management practices were used. Semen of these bulls was collected twice a week with the aid of an artificial vagina. In total, 25 ejaculates (five ejaculates from each bull) were collected. Sperm motility was estimated subjectively using phase-contrast microscopy with a warm stage maintained at 37 °C at ×400 magnification. Only ejaculates having $\geq 80\%$ sperm motility and concentrations higher than 800×10^6 spermatozoa/ml were cryopreserved. After collection, the ejaculates were immersed in a warm water bath at 34 °C. The ejaculates were pooled to balance the sperm contribution of each bull.

Semen processing

The pooled semen was divided into three equal fractions and diluted to a final concentration of 80 million spermatozoa/ml with a Tris-based extender [Tris 297.58 mM, citric acid 96.32 mM, fructose 82.66 mM, egg yolk 15% (v/v), glycerol 5% (v/v), pH 6.8] used as the base extender (freezing extender). Each mixed ejaculate was split into three equal aliquots and diluted at 37 °C with base extenders containing 2 mM arginine and 25 mM trehalose, and no antioxidant (control). Diluted semen samples were loaded into 0.25-ml (medium-sized) plastic straws, sealed with polyvinyl alcohol powder, and equilibrated at 5 °C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapour, 4 cm above the liquid nitrogen, for 15 min and plunged into liquid nitrogen for storage. After storage for 1 month, the frozen straws were thawed individually at 37 °C for 20 s in a water bath for microscopic evaluation.

Evaluation of microscopic sperm parameters

To analyse sperm motility and various kinematic parameters the Sperm Class Analyzer (SCA®) CASA system (Microptic S.L., Barcelona, Spain) was used. A 5-μl sample of diluted semen was put onto a pre-warmed slide covered with a coverslip and sperm motility characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), total sperm motility, VAP (average path velocity, l μm s^{-1}), VSL (straight linear velocity, l μm s^{-1}), VCL (curvilinear velocity, l μm s^{-1}), ALH (amplitude of lateral head displacement, l μm) and LIN [linearity index, LIN = (VSL/VCL) × 100]. For each evaluation, seven microscopic fields, each including at least 250 cells, were analysed.

Assessment of sperm plasma membrane integrity (viability)

This assessment was performed by staining with a sperm viability kit (SYBR-14/PI Molecular Probe: L 7011 Invitrogen, Carlsbad, CA, USA). The staining protocol was modified from a study of Garner and Johnson (1995). Thawed straws were diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 30 µl of the diluted semen was mixed with 6 µl of SYBR-14 and 2.5 µl of propidium iodide (PI). The sample was gently mixed, incubated at 37 °C in the dark for 20 min and then 10 µl of Hancock's solution was added for semen fixation. A wet mount was made using a 2.5 µl drop of the sample placed directly onto a microscope slide and covered with a cover slip.

Assessment of sperm acrosome integrity

Sperm acrosome status was assessed using fluorescein isothiocyanate conjugated to *Arachis hypogaea* (peanut) (L7381 FITC-PNA, Sigma-Aldrich Co., St. Louis, MO, USA) and by PI staining as described by Nagy et al. (2003) with modifications. Thawed straws were diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 60 µl of the diluted semen was mixed with 10 µl of FITC-PNA and 2.5 µl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min and 10 µl of Hancock's solution was added for semen fixation. A wet mount was made using a 2.5 µl drop of the sample placed directly onto a microscope slide and covered with a cover slip.

Assessment of sperm mitochondrial activity

Sperm mitochondrial activity was assessed with a staining protocol modified from Garner et al. (1997). Thawed straws were diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 300 µl of the diluted semen was mixed with 2.5 µl JC-1 and 2.5 µl PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min and 10 µl of Hancock's solution was added for semen fixation. A wet mount was made using a 2.5 µl drop of the sample placed directly onto a microscope slide and covered with a cover slip.

Assessment of sperm DNA integrity

Sperm DNA integrity was investigated using the single cell gel electrophoresis (COMET) assay, which is generally performed under neutral conditions. Our method was similar to several protocols applied in previous studies and involved few modifications (Duty et al., 2002; Li et al., 2008). The straws were thawed by gentle shaking in a 37 °C water bath for 10 s, and centrifuged once at 600 g for 10 min at 4 °C. The remaining sperm cells were washed with PBS (Ca^{2+}- and Mg^{2+}-free). Each pre-cleaned slide was pre-coated with a layer of 1% normal melting point agarose in PBS (Ca^{++}-and Mg^{++}-free) and then dried at

room temperature. Approximately 100,000 sperm cells (18 μl) were mixed with 0.75% low melting point agarose (50 μl) at 37 °C, and this suspension was poured onto the first agarose layer. The slides were allowed to solidify for 20 min at 4 °C. The coverslips were removed, and the slides were immersed in freshly prepared cold lysis buffer. The slides were then incubated at 37 °C in lysis buffer with 20 μg ml^{-1} of proteinase K for 2 h. The slides were removed from the lysis buffer, drained and placed into a horizontal electrophoresis unit filled with fresh neutral electrophoresis buffer at 4 °C for a 20-min incubation to allow the DNA to unwind. Electrophoresis was performed at room temperature, at 25 V for 20 min. Following electrophoresis, the slides were air-dried, stained with 50 μl of 8 μl ml^{-1} ethidium bromide, and covered with a coverslip. The images of 200 randomly chosen nuclei per sample were analysed visually. Observations were made at a magnification of ×400 using a fluorescence microscope (Olympus, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were considered to be undamaged.

Oxidative stress parameters

Briefly, thawed semen samples were centrifuged at 800 *g* for 20 min at 4 °C to separate the cells from the diluted seminal plasma, and spermatozoa were washed twice with PBS at 800 *g* for 20 min. After centrifugation, the supernatant was discarded and the pellet was completed to 500 μl with PBS. Subsequently, the sperm suspension was transferred into a 2 ml beaker filled with ice water and sonicated with a probe (Bandelin Sonopuls, Bandelin Electronic, Gerate-Typ: UW 2070, Pro-Nr. 51900037369.004, Berlin) for 10 s on ice, such that the process was repeated six times at intervals of 30 s to separate the sperm head and tail. For LPO analysis, 10 μl of 0.5 mM butyl-hydroxytoluene (BHT) was added to 120 μl of the homogenate samples and stored at −86 °C until analysis. The remaining homogenate was centrifuged at 8000 *g* for 15 min at +4 °C and the supernatant was collected and stored at −86 °C for total antioxidant activity (AOP) analysis.

Determination of lipid peroxidation (LPO) level

LPO level was determined using commercial kits of LPO 586TM Oxis Research (Oxis Research^{TM}, Bioxytech, CA, USA) by spectrophotometry (UV 2100 UV-VIS Recording Spectrophotometer, Shimadzu, Japan). The assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole with MDA and 4-hydroxyalkenals (LPO) at 45 °C. One molecule of either MDA or 4-hydroxyalkenal reacts with two molecules of N-methyl-2-phenylindole in acetonitrile, to yield a stable chromophore with maximal absorbance at 586 nm. The results are expressed as μmol for 10^9 cells ml^{-1}.

Determination of total antioxidant activity (AOP)

AOP activity was determined with an AOP-490TM Oxis Research kit (Oxis Research[TM], Bioxytech) by spectrophotometry. The assay was based on the reduction of Cu^{++} to Cu^{+} by the combined action of all the antioxidants present in the sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2:1 complex with Cu^{+}, which has a maximum absorbance at 490 nm. A standard of known uric acid (a water-soluble antioxidant) concentration is used to create a calibration curve. The results are expressed as mmol for 10^{9} cells ml^{-1}.

Statistical analysis

Results were expressed as mean ± SEM. Sperm motility, motion characteristics and abnormality were analysed by analysis of variance, followed by Tukey's *post hoc* test to determine significant differences between the groups. Spermatozoa with damaged DNA were evaluated by the chi-square test. Differences with values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed by using the SPSS 21 package programme.

Results

Supplementation of the semen extender with arginine decreased the percentages of post-thawed subjective motility (29 ± 8.21), computer-assisted sperm analysis (CASA) motility (12.2 ± 5.69) and progressive motility (3.52 ± 2.13), compared with the controls (43 ± 2.73, 55.4 ± 6.78 and 33.48 ± 4.14%, respectively, $P < 0.05$). Trehalose increased the percentages of post-thawed subjective motility (50 ± 3.53), CASA motility (64.7 ± 6.79%) and progressive motility (34.36 ± 4.77), compared with the controls (43 ± 2.73, 55.4 ± 6.78 and 33.48 ± 4.14%, respectively, $P > 0.05$) (Table 1). Significant differences were observed between the arginine and the control groups for sperm motion characteristics ($P < 0.05$) except for the ALH value. Arginine provided a lower VAP (43.8 ± 3.37 µm/s), VSL (29.9 ± 3.35 µm/s), VCL (81.4 ± 4.93 µm/s) and LIN (36.82 ± 3.46%) in comparison to the control group (74 ± 6.74 µm/s, 59.5 ± 6.06 µm/s, 105.7 ± 9.44 µm/s, 56.3 ± 1.71%, $P < 0.05$) (Table 2). Trehalose increased sperm viability, mitochondrial activity and DNA integrity (44.1 ± 2.18, 36.3 ± 3.99 and 95.1%, respectively), compared with those of the control group (31.7 ± 3.94, 13 ± 8.15, 90.4%, $P < 0.05$) (Table 3). Arginine decreased sperm viability (22.1 ± 3.28) despite protecting DNA integrity (92.8%) compared to the control group (31.7 ± 3.94%, $P < 0.05$) (Table 3). No significant differences were observed between the groups for sperm acrosome integrity ($P > 0.05$) (Table 3). In the comet assay, it was determined that both antioxidants protected DNA integrity and re-

sulted in a lower percentage of sperm with damaged DNA than that of the controls ($P < 0.05$) (Table 3). In terms of biochemical assays, no significant difference was observed in LPO and AOP between groups ($P > 0.05$) (Table 4).

Table 1

Sperm motility (mean ± SEM) in frozen-thawed bull semen

Groups	Subjective motility (%)	CASA motility (%)	Progressive motility %
Control	43 ± 2.73[b]	55.4 ± 6.78[b]	33.48 ± 4.14[b]
Arginine 2 mM	29 ± 8.21[a]	12.2 ± 5.69[a]	3.52 ± 2.13[a]
Trehalose 25 mM	50 ± 3.53[b]	64.7 ± 6.79[b]	34.36 ± 4.77[b]
P	*	*	*

[a,b,c]Different superscripts within the same column demonstrate significant differences ([*]$P < 0.05$)

Table 2

Sperm motion characteristics (mean ± SEM) in frozen-thawed bull semen

Groups	VAP	VSL	VCL	ALH	LIN
Control	74 ± 6.74[b]	59.5 ± 6.06[a]	105.7 ± 9.44[a]	3.86 ± 0.42[a]	56.3 ± 1.71[b]
Arginine 2 mM	43.8 ± 3.37[a]	29.9 ± 3.35[b]	81.4 ± 4.93[b]	4.38 ± 0.61[a]	36.82 ± 3.46[a]
Trehalose 25 mM	64.8 ± 10.27[b]	50.8 ± 9.67[a]	97.9 ± 13.29[ab]	3.8 ± 0.31[a]	51.76 ± 4.44[b]
P	*	*	*	–	*

−: No significant difference; [a,b,c]Different superscripts within the same column demonstrate significant differences ([*]$P < 0.05$)

Table 3

Flourescent staining (mean ± SEM) in frozen-thawed bull semen

Groups	Acrosome integrity (%)	High mitochondrial activity (%)	Sperm viability (%)	Damaged DNA (%)
Control	43.5 ± 4.46[ab]	13 ± 8.15[a]	31.7 ± 3.94[a]	9.6[a]
Arginin 2 mM	37.4 ± 4.43[a]	7.6 ± 4.89[a]	22.1 ± 3.28[b]	7.2[b]
Trehalose 25 mM	48.2 ± 8.70[b]	36.3 ± 3.99[b]	44.1 ± 2.18[c]	4.9[c]
P	*	*	*	**

[a,b,c]Different superscripts within the same column demonstrate significant differences ([*]$P < 0.05$; [**]$P < 0.01$)

Discussion

Cryodamage to spermatozoa caused by the cryopreservation procedure results in impaired fertility and decreased survival of spermatozoa in the female reproductive system (Singh et al., 2012).

Table 4

LPO ($\mu M \times 10^9$) and AOP ($mM \times 10^9$) levels (mean ± SEM) in frozen-thawed bull semen

Groups	LPO ($\mu M \times 10^9$)	AOP ($mM \times 10^9$)
Control	19.47 ± 7.36	19.11 ± 6.57
Arginin 2 mM	19.41 ± 8.16	24.09 ± 7.64
Trehalose 25 mM	19.82 ± 8.96	21.27 ± 6.39
P	–	–

–: No significant difference

This study investigated the protective effects of the antioxidants arginine and trehalose against cryoinjury by the evaluation of sperm and oxidative stress parameters following freezing and thawing of bull semen.

Membrane lipid peroxidation has been considered an important problem for sperm survival and fertility, and sperm motility is also inhibited by reactive oxygen species formation through decreased ATP production (Guthrie and Welch, 2012). Oxidative stress causes cellular damage when the production of ROS goes beyond the antioxidant capacity of the organism. ROS damage the sperm membrane and the DNA, which results in decreased sperm motility and reduced ability to fuse with the oocyte (Tremellen, 2008). Antioxidants scavenge free radicals and improve sperm parameters (Gharagozloo and Aitken, 2011).

Sperm freezing increases the percentage of DNA fragmentation. This effect occurs after cryopreservation and has been reported by several authors (Aitken et al., 1998; Thomson et al., 2009a). DNA fragmentation and oxidative DNA damage were found to be related to each other and they correlate with cryopreservation (Thomson et al., 2009b). Potts et al. (2000) has reported DNA damage in human spermatozoa connected with ROS and lipid peroxidation. Like in our study, Ogretmen et al. (2015) also reported that extenders with antioxidant additives had protective effects against sperm DNA damage resulting from cryopreservation.

Trehalose acts like a non-permeating cryoprotectant which causes dehydration of spermatozoa due to the osmotically driven flow of water. Due to this mild dehydration, spermatozoa have less intracellular water which results in reduced intracellular ice crystal formation. This is beneficial for sperm because intracellular ice crystal formation results in cell death and, consequently, reduced fertility of the cryopreserved semen (Chhillar et al., 2012). Supplementation of trehalose to the semen extender significantly improved sperm characteristics such as post-thaw motility, viability, and membrane integrity. The same results verify the findings of earlier studies using the same additive to the semen extender for improving sperm quality after cryopreservation (Reddy et al., 2010; Singh et al., 2012). Ram semen extender including trehalose (100 mM) improved post-thaw

membrane integrity but did not have any positive effect on sperm motility and morphology (Cirit et al., 2013). The addition of 50 mM trehalose to the semen diluent (Tris–citric acid–egg yolk–fructose) had a beneficial effect in chilling bull semen: trehalose ameliorated motility, sperm membrane integrity (HOST), viability, abnormal morphology, and DNA fragmentation compared to control group (El-Sheshtawy et al., 2015). Trehalose is able to protect the DNA integrity of sperm cells and has a protective effect on chromatin status. Trehalose showed protective effects on sperm characteristics and acrosome integrity compared to the controls (Bhattacharyya et al., 2006). In the present study, 25 mM trehalose caused a significant (P < 0.05) increase in post-thaw sperm motility, viability, mitochondrial activity, and DNA integrity compared to the control group.

Patel et al. (1999) showed that L-arginine has an important role in male fertility and can be used in the treatment of male infertility. Arginine resulted in better post-thaw motility, viability, and membrane integrity in buffalo semen compared with those of the control group; in addition, arginine had a protective effect on LPO level compared to the control (Siddique and Atreja, 2013). L-arginine protects sperm from lipid peroxidation by inhibiting the nitric oxide synthase enzyme (Srivastava et al., 2006). Total motility and hyperactivated cells were reduced at an L-arginine concentration of 10 mM, and such doses of L-arginine were found to be harmful to sperm motility patterns in cryopreserved equine sperm (Carvalho et al., 2012). L-arginine is essential for sperm cells and it is useful for artificial insemination with subnormal human semen (Patel et al., 1998). O'Flaherty et al. (2004) suggested that L-arginine has a positive effect on bovine sperm function. This amino acid induces sperm capacitation and acrosome reaction without causing any change in the progressive motility and viability of bovine spermatozoa.

In the present study, it was concluded that trehalose provides a protective effect by improving the spermatological parameters and protecting DNA integrity. However, arginine worsened these parameters with the exception of DNA integrity. The use of extenders including trehalose sugars may be recommended to improve cryopreservation of bovine semen.

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