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Effect of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation

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ABSTRACT

The aim of this study was to determine the effects of the antioxidants glutamine and hyaluronan and the inclusion of different levels on microscopic semen parameters, lipid peroxidation and the antioxidant activities following the freeze–thawing of Angora goat semen. Ejaculates collected from three Angora goat bucks, were evaluated and pooled at 37 °C. The semen samples which were diluted with a Tris-based extender containing additives including glutamine (2.5; 5 mM) and hyaluronan (500; 1000 μl/ml), and an extender containing no antioxidants (control) were cooled to 5 °C and frozen in 0.25 ml French straws and stored in liquid nitrogen. Frozen straws were thawed individually (37 °C) for 20 s in a water bath for microscopic evaluation. Freezing extenders supplemented with 2.5 and 5 mM glutamine led to higher sperm motility and hypo-osmotic swelling test (HOST) values, compared to the control ($P < 0.05$) following the freeze–thawing process. The addition of 500 μl/ml hyaluronan resulted in a higher HOST percentage, compared to the addition of 1000 μl/ml hyaluronan and the control ($P < 0.001$). No significant difference was recorded in the percentage acrosome and total sperm abnormalities, following supplementation with antioxidants. The addition of antioxidants did not prevent malondialdehyde (MDA) formation, compared to the controls. Antioxidant treatment however decreased ($P < 0.01$) the superoxide dismutase (SOD) activity. The maintenance of catalase (CAT) activity was demonstrated to be insignificant following addition of antioxidants. Further studies are required to obtain more repeatable results regarding the characterization of the enzymatic and non-enzymatic antioxidant systems in cryopreserved goat sperm.

1. Introduction

Artificial insemination (AI) with frozen–thawed semen has become an important technique in goat breeding. However cervical AI, with frozen semen, is characterised by low fertility rates in goats. Some of the causes for these low fertility rates encountered with the use of AI in this species include a, decrease in the motility, membrane integrity and fertilizing potential of sperm during cryopreservation and altered membrane responses of sperm to physiological stimuli (Evans, 1998; Maxwell and Watson, 1996). Alternative methods for the cryopreservation of goat sperm require the improvement, as well as the development of a greater understanding of the cellular changes that occur during the freeze–thawing processes in sperm (Holt, 1997; King et al., 2004). Cooling and freeze–thawing produce physical, chemical and oxidative stress on the sperm membrane, which result in reduced sperm viability and

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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 defense system, significant oxidative damage occurs to the sperm organelles through the damage of lipids, proteins and DNA. Thus ultimately leading to sperm death. ROS-induced damage to sperm is mediated by the oxidative attack of sperm phospholipid-bound polyunsaturated fatty acids, resulting in lipid peroxidation (LPO) (de Lamirande and Gagnon, 1992; Gomez et al., 1996; Irvine, 1992).

A characteristic feature of biological membranes is the asymmetrical arrangement of lipids within the bilayer. The lipid composition of the plasma membrane of mammalian sperm is markedly different from those of mammalian somatic cells. Sperm cells contain a high content of polyunsaturated fatty acids. The ratio of unsaturated to saturated fatty acids in small ruminant sperm membranes is also higher than in other species, making the membranes more susceptible to peroxidative damage in the presence of ROS—with a subsequent loss of membrane integrity in the acrosomal region, impaired cell function and decreased motility of the sperm (Aitken et al., 1989; White, 1993; Alvarez and Storey, 1995). However, the controlled and spontaneous release of molecular oxygen, forming low concentrations of ROS, is physiologically involved in the maintenance of the fertilizing ability and capacitation/acrosome reaction of sperm (Alvarez and Storey, 1982, 1984).

The antioxidant system in the cell is comprised of reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), superoxide dismutase (SOD), and metal chelators (transferrin, lactoferrin and ceruloplasmin) and has been described as a defense functioning mechanism against lipid peroxidation (LPO) in semen. It is also an important system in maintaining sperm motility and viability (Bilodeau et al., 2001; Gadea et al., 2004; Agarwal et al., 2007). This antioxidant capacity in the sperm cell may however be insufficient to prevent LPO during the freeze–thawing process. Mammalian sperm thus generally lack a significant cytoplasmic component, which contains enough antioxidants to counteract the damaging effects of ROS and LPO (Aurich et al., 1997; Storey, 1997).

Certain organisms are known to accumulate amino acids (AA's) in order to resist cold temperatures. These AA's (e.g., glutamine, cysteine) have important roles to play in preventing the denaturation of e.g. fish muscle proteins during the frozen stage, and the cryoprotection potential of living cells (Noguchi and Matsumoto, 1971; Kundu et al., 2001). Glutamine acts at an extra-cellular level and improves the post-thaw sperm motility and fertilizing potential (Renard et al., 1996; Khlifaoui et al., 2005). It is the initial substrate of hyaluronic acid synthesis and an effective precursor for GSH synthesis in many cell types. Furthermore, the addition of glutamine to the media for in vitro maturation and culture improves the rate of cumulus expansion, morula and blastocoele formation (Furnus et al., 1998; Kito and Bavister, 1997; Johnson et al., 2003; Masuda and Koyanagi, 2006).

Hyaluronan (which is an essential component of the extracellular matrix), a non-sulfated glycosaminoglycan and an additive with antioxidant properties, contributes to cell recognition, cellular adhesion and growth regulation. It is involved in important physiological functions such as the post-thaw motility, capacitation ability and antioxidant capacity of the sperm cell (Erlinger, 1995; Ghosh and Datta, 2003; Bucak et al., 2007). In recent years, antioxidants such as cysteamine, trehalose and cysteine have become known to improve post-thaw sperm motility and antioxidant capacity (Aisen et al., 2005; Bucak et al., 2007, 2008).

The present study thus envisaged to investigate the effects of glutamine and hyaluronan, at different levels, on microscopic parameters, lipid peroxidation, and antioxidant activities (SOD, CAT) during the cryopreservation of Angora goat sperm.

2. Materials and methods

2.1. Chemicals

The antioxidants (glutamine and hyaluronan) and other 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 three mature Angora goat bucks (3 and 4 years of age), of superior genetic merit and proven fertility were used in this study. The goats, belonging to the Lalahan Livestock Central Research Institute, were maintained under uniform nutritional conditions. Ejaculates were collected twice a week from the bucks with the aid of an artificial vagina during the breeding season (autumn to early winter). Immediately after collection, the ejaculates were immersed in a water bath (33 °C), until microscopic assessment in the laboratory. Semen assessment was performed within approximately 20 min following collection.

2.3. Semen dilution, freezing and thawing

The volume of semen ejaculates was measured in a conical tube graduated at 0.1 ml intervals, the sperm concentration determinated using a haemocytometer (Smith and Mayer, 1955), and sperm motility estimated using phase-contrast microscopy (400×). Only ejaculates with a volume of 1–2 ml sperm with >80% progressive motility and a concentration higher than 2.5×10^9 sperm/ml were pooled—balancing the sperm contribution of each male and to eliminate individual differences. Six pooled ejaculates were included in the study.

A Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% (v/v), glycerol 6% (v/v), pH 6.8) was used as the base extender (cryodiluent). Each pooled ejaculate was divided into five equal aliquots and diluted (37 °C) with the base extender containing glutamine (2.5; 5 mM) and hyaluronan (500; 1000 μl/ml) and no antioxidant (control), respectively—for a total of five experiment groups, with a final concentration of approximately 4×10^8 sperms/ml (single step dilution), in a 15 ml plastic centrifuge tube. Diluted semen samples were aspirated into 0.25 ml French straws and sealed with polyvinyl alcohol powder and equilibrated at 5 °C for 3 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen for 15 min, and then plunged into the liquid nitrogen for storage. After storage for 1 month, the frozen straws were thawed individually (37 °C) for 20 s in a water bath for microscopic evaluation.

2.4. Sperm evaluation

2.4.1. Analysis of microscopic semen parameters

Progressive sperm motility was assessed using a phase-contrast microscope (100× magnification), on a warm stage at 37 °C. A wet mount

Table 1
Mean (±S.E.) percentage motility, acrosome and total abnormalities and hypo-osmotic swelling test (HOST) in frozen–thawed Angora goat semen.

Groups	Motility	Acrosome abnormality	Total abnormality	HOST
Glutamine 2.5 mM	62.9 ± 3.4[a]	8.4 ± 1.7	17.3 ± 2.3	46.0 ± 2.5[a,b]
Glutamine 5 mM	57.9 ± 1.5[a,b]	6.8 ± 0.9	19.8 ± 2.1	44.4 ± 1.6[a,b]
Hyaluronan 500 μl/ml	55.0 ± 1.5[b,c]	7.8 ± 2.4	18.0 ± 3.4	51.6 ± 3.5[a]
Hyaluronan 1000 μl/ml	57.1 ± 1.8[a,b,c]	5.8 ± 1.0	13.8 ± 1.6	40.4 ± 2.3[b,c]
Control	50.7 ± 3.0[c]	12.0 ± 2.4	25.0 ± 4.7	30.6 ± 2.2[c]
P	*	–	–	***

Different superscripts (a, b and c) within the same column are significantly different.
* $P < 0.05$.
*** $P < 0.001$.

was made using a 5 μl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each pooled semen sample. The mean of the three successive estimations was recorded as the final motility score.

For the assessment of sperm abnormalities, a minimum of three drops of each sample were added to Eppendorf tubes containing 1 ml Hancock solution (62.5 ml formalin (37%), 150 ml saline solution, 150 ml buffer solution and 500 ml double-distilled water) (Schafer and Holzmann, 2000). One drop of this mixture was put on a slide and covered with a cover slip. The percentage of total sperm abnormalities (acrosomal abnormalities, detached heads, abnormal mid-pieces and tail defects) was recorded by counting a total of 400 sperm under phase-contrast microscopy (1000× magnification, oil immersion).

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) 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 cells were evaluated (magnification 1000×) using bright-field microscopy. Sperm cells with swollen or coiled tails were recorded (Revell and Mrode, 1994; Buckett et al., 1997).

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 semen sample was centrifuged at 800 × *g* for 10 min. The sperm pellet was separated and washed by resuspension in PBS and recentrifuged three times. After the last centrifugation, 1 ml deionized water was added to the sperm sample (Dandekar et al., 2002; Kasimanicham et al., 2007).

2.4.2.1. MDA concentrations. Malondialdehyde (MDA) concentrations, as indices of LPO activity in the semen samples, were measured using the thiobarbituric acid reaction, in accordance with the method described by Yoshioka et al. (1979). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption with the standard curve of malondialdehyde equivalents generated by the acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The MDA concentrations were expressed as nmol/l.

2.4.2.2. SOD and CAT activities. SOD was analysed as described by Sun and Zigman (1978). A volume containing 0.4 ml of the sample, 0.1 ml 0.9% NaCl and 0.4 ml chloroform was centrifuged for 10 min at 2000 × *g*. The reaction mixture contained 20 ml 3 mmol/l xanthine, 10 ml 0.6 mmol/l EDTA, 10 ml of 0.15 mmol/l NBT, 6 ml 400 mmol/l sodium carbonate, and 3 ml 1 g/l BSA for 20 tests. An aliquot (50 μl) of xanthine oxidase (20 μmol/2 ml) was added to 2.45 ml of the reaction mixture, followed by the incubation at 25 °C for 20 min. Subsequently, the reaction was terminated by adding 1 ml of 0.8 mmol/l $CuCl_2$ to the test. The absorbance was recorded at 560 nm in a spectrophotometer. SOD activity was expressed as U/g protein.

The method described by Goth (1991) was used for the determination of CAT activity in the semen samples. Sperm samples (0.2 ml) were incubated in 1 ml substrate (65 mmol/ml) hydrogenperoxide in 60 mmol/l sodium–potassium phosphate buffer (pH 7.4) at 37 °C for 60 s. One unit of CAT decomposes 1 μmol of hydrogenperoxide per min, under the indicated conditions. The enzymatic reaction was terminated with 1 ml 32.4 mmol/l ammonium molybdate and hydrogenperoxide was measured at 405 nm using a spectrophotometer. Values of CAT activity were expressed as kU/g protein.

2.5. Statistical analysis

The study was repeated six times and the results were expressed as the mean ± S.E.M. Means were analyzed using a one-way analysis of variance, followed by Tukey's post hoc test to determine significant differences in all the parameters between all groups using the SPSS/PC computer program (Version 12.0; SPSS, Chicago, IL). Differences with confidence values of $P < 0.05$ were considered to be statistically significant (Daniel, 1991).

3. Results

3.1. Microscopic sperm parameters (percentage motility, acrosome, total abnormalities, and HOST)

The influences of glutamine and hyaluronan on the standard semen parameters following the freeze–thawing process were evaluated in six independent experiments. As shown in Table 1, the freezing diluents supplemented with 2.5 and 5 mM glutamine led to higher motility and

Table 2
Mean (±S.E.) malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels in frozen–thawed Angora goat semen.

Groups	MDA (nmol/l)	SOD (U/g protein)	CAT (kU/g protein)
Glutamine 2.5 mM	2.1 ± 0.3[a]	1.1 ± 0.1[b,c]	4.1 ± 0.2
Glutamine 5 mM	0.6 ± 0.1[b]	1.2 ± 0.1[b]	3.7 ± 0.6
Hyaluronan 500 μl/ml	1.7 ± 0.3[a,b]	0.7 ± 0.0[c]	5.0 ± 1.3
Hyaluronan 1000 μl/ml	1.7 ± 0.4[a,b]	0.8 ± 0.1[c]	4.5 ± 1.3
Control	0.6 ± 0.2[b]	1.7 ± 0.2[a]	6.8 ± 0.9
P	**	***	–

Different superscripts (a, b, and c) within the same column are significantly different.
** $P < 0.01$.
*** $P < 0.001$.

HOST rates, compared to the controls ($P < 0.05$), following freeze–thawing. The addition of 500 μl/ml hyaluronan resulted in a higher HOST value, compared to the addition of 1000 μl/ml hyaluronan and the controls ($P < 0.001$). No significant difference was observed in the percentage of acrosome and total sperm abnormalities, following supplementation with the antioxidants at different levels.

3.2. Analysis of lipid peroxidation and antioxidant activities

The influence of antioxidants on lipid peroxidation and antioxidant activities in thawed Angora goat sperm are set out in Table 2. The addition of antioxidants did not prevent MDA formation when compared to the controls. Antioxidant treatment decreased ($P < 0.01$) SOD activity, compared to the controls. The maintenance of CAT activity was demonstrated to be insignificant following the addition of antioxidants.

4. Discussion

The mammalian sperm plasma membrane is particularly rich in polyunsaturated fatty acids (PUFA). This predominance of PUFA renders sperm highly susceptible to LPO, which occurs as a result of the oxidation of the membrane lipids, by partially reduced oxygen molecules such as superoxide, hydrogenperoxide and hydroxyl radicals. Spontaneous LPO of the membranes of mammalian sperm destroy the structure of the lipid matrix, as a result of the invasion by reactive oxygen species (ROS). These attacks then ultimately lead to the impairment of sperm function (sperm motility, functional membrane integrity and fertility), and damage to the sperm DNA, through oxidative stress and the production of cytotoxic aldehydes (Aitken, 1984; Alvarez and Storey, 1989; Aitken et al., 1993). In the current study, glutamine and hyaluronan did not achieve any effectiveness in the prevention of MDA formation, when compared to the controls. The current findings relating to LPO are also in agreement with the results performed on ram and goat sperm in which a decrease was not recorded in the level of MDA in the presence of hyaluronan and thiols, following the freeze–thawing process (Bucak et al., 2007, 2008).

Glutamine is known to play a regulatory role in several cell specific processes, including the metabolism (e.g., oxidative fuel, lipogenic precursor), cell integrity, protein synthesis and degradation, redox potential, gene expression, and extracellular matrix synthesis (Curi et al., 2005). With respect to the microscopic sperm parameters evaluated, the supplementation of the extender with 2.5 and 5 mM glutamine led to higher motility rates and functional membrane integrity, compared to the other groups. This result was similar to that previously reported for frozen–thawed sperm of the Poitou jackass, cynomolgus monkey and stallion, where glutamine addition also resulted in a significant increase in sperm motility and membrane integrity after thawing (Trimeche et al., 1996; Li et al., 2003; Khlifaoui et al., 2005). Glutamine may therefore provide cryoprotection in mammalian sperm via decreased damage to 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 when testing the membrane-stabilising action of antioxidants (Bucak and Tekin, 2007). In this study, the highest HOST rates were obtained when sperm samples were cryopreserved with glutamine and 500 μl/ml hyaluronan. It can be hypothesized that antioxidants may facilitate the maintaining of post-thawed sperm membrane stability against oxidative stress, without eliminating the production of MDA (malondialdehyde formation).

The sperm antioxidant system predominantly includes SOD, CAT and GSH-PX activities to restrict lipid peroxidation (Alvarez and Storey, 1989; Aitken and Baker, 2004; Sikka, 2004). SOD (superoxide dismutase) is thus an important component of the enzymatic antioxidant system in mammalian sperm (Holland et al., 1982; Beconi et al., 1991) and increased SOD activity in the sperm cell was recorded as a result of altered membrane integrity, following the cryopreservation process (Laso et al., 1994). The presence of CAT (catalase activity) in sperm has been demonstrated in mammals, and its potential role is involved in the aging processes and the control of oxidative stress in cells—mainly resulting in the formation of H_2O_2 (Upreti et al., 1998; Bilodeau et al., 2001). In this study, the antioxidants supplemented did not elevate the antioxidant capacity, and therefore did not improve the motility and functional membrane integrity of sperm following thawing. This is in contrast with the findings of Bucak et al. (2007, 2008) who demonstrated a marked improvement in ram sperm motility associated with the increased CAT activity of frozen sperm supplemented with the antioxidants taurine and cysteine. This study is also contradictory to studies evaluating antioxidant enzymatic activity where a positive effect of SOD activity on the sperm microscopic parameters and a negative effect on lipid peroxidation were reported (Marti et al., 2003; Cassani et al., 2005). These reports and the current findings possibly indicate that LPO and antioxidant capacity are apparently not major factors influencing sperm survival following cryopreservation thawing.

5. Conclusions

It may be stated that the addition of antioxidants (glutamine and hyaluronan) improve sperm motility, and functional membrane integrity as evaluated by HOST—demonstrating a cryoprotective effect, after cryopreservation and thawing. These antioxidants did not eliminate the MDA production, or elevate antioxidant capacity. In order to confirm the contradictory results, further studies are warranted to obtain more concrete results regarding the characterization of enzymatic and non-enzymatic antioxidant systems in cryopreserved goat sperm.

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