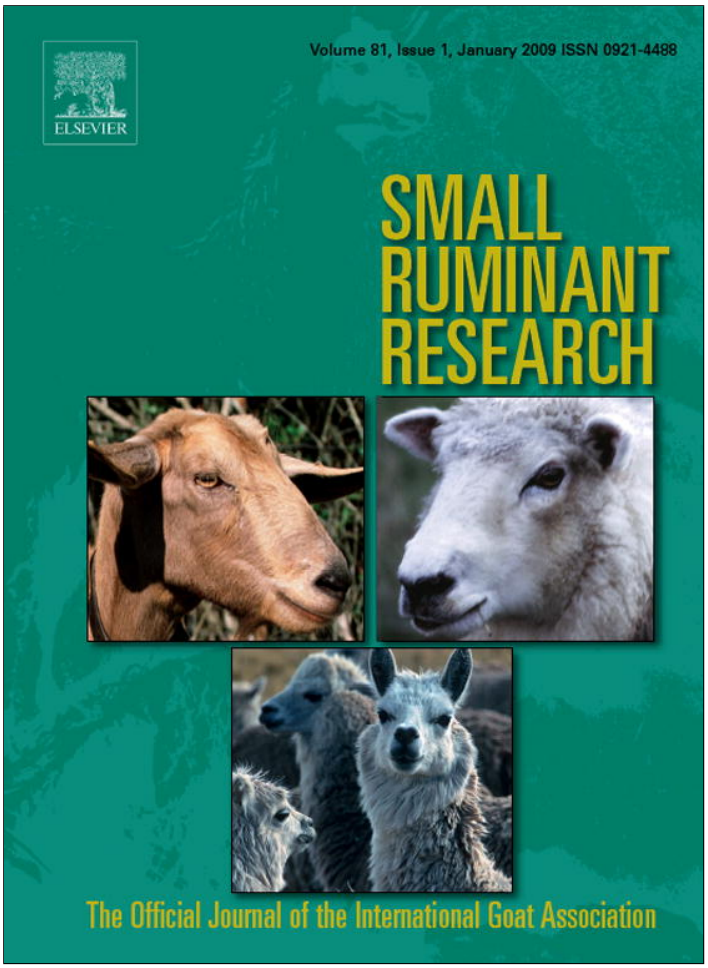

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Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities

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ABSTRACT

Ram semen contains sufficient quantities of superoxide dismutase (SOD) and much lower concentrations of glutathione peroxidase (GSH-PX) and catalase (CAT) to prevent oxidative damage. The anti-oxidant capacity of the sperm cell is limited, due to a small cytoplasmic component, which contains these anti-oxidants to scavenge the oxidants. However, the concentration of these anti-oxidants may decrease considerably by the dilution of the semen. The aim of the present work was to study the effect of two anti-oxidants, namely, glutamine and an amino acid solution (BME) in a Tris-based extender on ram sperm parameters, lipid peroxidation and anti-oxidant capacity after the cryopreservation/thawing process. Ejaculates collected from 4 Akkaraman rams were evaluated and pooled at 37 °C. Semen samples which were diluted with the tris-based extender containing glutamine (2.5 or 5 mM), BME (13 or 26%), and no anti-oxidants (control) were cooled to 5 °C and frozen in 0.25-ml French straws and stored in liquid nitrogen. Frozen straws were thawed individually at 37 °C for 20 s in a water bath for evaluation. The freezing extender supplemented with 5 mM glutamine led to higher motility rate (68.0 ± 4.4%) and hypo-osmotic swelling test (HOST) (64.1 ± 5.5%), when compared to glutamine (2.5 mM) and BME (13 and 26%) ($P < 0.05$). No significant differences were observed regarding sperm motility and HOST, following the supplementation of the freezing extender with glutamine 2.5 mM and BME (13 and 26%) after thawing. CAT activity remained significantly higher following the addition of glutamine 5 mM (6.4 ± 0.9 kU/g protein), compared to the other treatments ($P < 0.01$). The anti-oxidants at different levels were not effective in the elimination of malondialdehyde (MDA) formation and maintenance of SOD activities, when compared to the control ($P < 0.05$). Findings showed that glutamine (5 mM) supplementation in semen extenders, was of greater benefit to frozen–thawed ram sperm. Future efforts are needed to find the appropriate anti-oxidants and their effective concentrations to improve post-thaw sperm parameters (e.g. motility, membrane integrity, fertility) and anti-oxidant activities when frozen–thawed ram sperm is used.

1. Introduction

Semen cryopreservation is an important procedure which allows specific advantages to the livestock industry, particularly in conjunction with the dissemination of

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genetic material—limiting the spread of sexually transmitted diseases, collaborating in breed improvement programs, and the banking of genome resources to preserve valuable transgenic lines. Despite comprehensive studies on the cryopreservation of ram sperm, the fertilization rate is markedly decreased following transcervical insemination using frozen sperm, compared to fresh semen (Evans and Maxwell, 1987; Salamon and Maxwell, 1995; Bailey et al., 2000). An in-depth research of the ram sperm physiology and biochemistry is required to overcome these low fertility rates achieved and gain insight of the cell changes that occur during the freeze–thawing of sperm (Yoshida, 2000).

Mammalian sperm characteristically contain a high concentration of polyunsaturated fatty acids linked to phospholipids, asymmetrically distributed over the lipid bilayer of the plasma membrane (Aitken et al., 1993; Aitken, 1995; Gadella et al., 1999). It is well known that ram sperm (Fiser and Fairfull, 1989) is more sensitive to cold shock than those of other species such as the bull, rabbit, or even the human (Watson, 1981). The different responses to temperature stress are mainly due to differences in membrane lipid composition. Thus, ram sperm have a higher polyunsaturated/saturated fatty acids ratio and a lower cholesterol/phospholipid molar ratio than other species (Evans and Maxwell, 1987). This can account for membrane disarrangement, making the sperm more susceptible to peroxidative damage in the presence of ROS (e.g. superoxide anion radical, hydrogen peroxide) with subsequent loss of membrane integrity of the acrosomal region, impaired cell function of the human sperm (Alvarez and Storey, 1989; Aitken and Fisher, 1994) and decreased motility of turkey sperm (Wishart, 1984).

Cryopreservation disrupts the transbilayer-phospholipid asymmetry in the plasma membranes of the ram sperm. Ultrastructural damage of the plasma membranes then increases the susceptibility to lipid peroxidation (LPO), when high production of the reactive oxygen species (ROS) occurs during the freeze–thawing process—predisposing the ram sperm to gross morphologic defects, decreased motility and fertilizing capability (Hinkovska-Galcheva et al., 1989; Maxwell and Watson, 1996; Muller et al., 1999). However, in vivo physiological concentrations of ROS are involved in providing membrane fluidity, and maintaining the fertilizing ability and acrosome reaction of the sperm (Bell et al., 1993; Aitken and Fisher, 1994).

Living organisms are known to accumulate amino acids to resist cold shock. Amino acids (e.g. glutamine) have important roles in preventing the denaturation of muscle proteins in fish (Noguchi and Matsumoto, 1971) and show cryoprotective potential in goat sperm during the frozen stage (Kundu et al., 2001). So for instance, glutamine acts at the extracellular level, improving post-thaw human sperm motility and fertilizing potential (Renard et al., 1996). The addition of glutamine to the culture medium for mouse pre-implantation embryos has also led to improvement in the rate of blastocoele formation and hatching (Masuda and Koyanagi, 2006). Additionally, it has altered the oocyte nuclear maturation and cumulus expansion in hamsters (Kito and Bavister, 1997).

Ram semen possesses sufficient amounts of superoxide dismutase (SOD) and much lower concentrations of glutathione peroxidase (GSH-PX) and catalase (CAT) to prevent oxidative damage. This anti-oxidant capacity in sperm cells, due to the small cytoplasmic component containing anti-oxidants for scavenging oxidants, is limited. Thus, the concentration of anti-oxidants may decrease considerably by the dilution of the semen. Mammalian sperm may however be incapable in preventing LPO during the freeze–thawing process (Abu-Erreish et al., 1978; Mann and Lutwak-Mann, 1981; Aurich et al., 1997; Storey, 1997). Additives showing anti-oxidative properties could reduce the impact of ROS-induced damage due to LPO (Alvarez and Storey, 1983; Donnelly et al., 2000)—thus improving ram (Aisen et al., 2002) and canine (Michael et al., 2007) semen parameters (e.g. motility, membrane integrity) after thawing. The aim was therefore to study the effect of anti-oxidant supplementation in a tris extender on ram semen parameters, lipid peroxidation and anti-oxidant capacity, after thawing.

2. Material and methods

2.1. Reagents

The anti-oxidants used in this study such as L-Glutamine (G8540) and Amino acids Solution (BME, B6766), and other chemicals were obtained from Sigma–Aldrich (Interlab Ltd., Ankara, Turkey).

2.2. Semen collecting and processing

The 4 animals (2–4-year-old rams) were housed at the Lalahan Livestock Central Research Institute under uniform feeding, housing and lighting conditions. A total number of 32 ejaculates were collected from the 4 mature Akkaraman rams, of proven fertility after natural mating, using an artificial vagina twice a week during the breeding season (autumn). Immediately after collection, the ejaculates were transferred to the laboratory, and immersed in a water bath (33 °C), until semen evaluation. The sperm concentration of each ejaculate was determined by means of a haemocytometer (Smith and Mayer, 1955). The percentage progressive sperm motility was estimated using phase-contrast microscopy (×100 magnification). Only ejaculates between 1 and 2 ml in volume, sperm with >80% progressive motility and a concentration higher than 2.5×10^9 sperm/ml were pooled, in order to eliminate individual ram differences. Five pooled ejaculates were frozen in the study. A Tris-based extender (Tris 3.07 g/100 ml, citric acid 1.64 g/100 ml, fructose 1.26 g/100 ml, egg yolk 15% (v/v), glycerol 5% (v/v), pH 6.8) was used as the basis extender (Kumar et al., 2003). Each mixed ejaculate was split into 5 equal aliquots and diluted at 37 °C with the base extender containing glutamine (2.5 or 5 mM), BME (13 or 26%), and no anti-oxidants (control), respectively—with a final concentration of approximately 4×10^8 sperm/ml (in a single step), in a 15 ml-plastic centrifuge tube. Diluted semen samples were aspirated into 0.25 ml French straws, sealed with polyvinyl alcohol powder and cooled horizontally from 37 to 5 °C, at a rate of 0.2–0.3 °C/min in a cold cabinet, and maintained at 5 °C for 2 h. The straws were frozen in liquid nitrogen vapor (4 cm above liquid nitrogen) for a 15 min period and plunged into liquid nitrogen for storage. After being stored for one month, 15 frozen straws from each group were thawed individually at 37 °C for 20 s in a water bath for microscopic evaluation.

2.3. Semen evaluation

2.3.1. Analysis of semen parameters

Progressive sperm 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. Sperm motility estimations were performed in three different microscopic fields for each semen sample and the mean of the three successive counts recorded as the final motility score (Evans and Maxwell, 1987).

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, and was performed by incubating 20 μl of semen with 200 μl of a 100 mOsM hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate/1 distilled water) at 37 °C for 60 min. After incubation, 100 μl of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm were counted in at least 5 different microscopic fields. The percentage of sperm with swollen and curled tails was then recorded (Revell and Mrode, 1994; Buckett et al., 1997).

2.3.2. Biochemical assays of thawed semen

Biochemical assays were performed on the semen samples immediately after thawing and following centrifugation and washing. An aliquot (500 μl) of semen from each sample was centrifuged at 800G for 10 min. Sperm pellets were separated, and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation, 1 ml of deionized water was added to the spermatozoa. The samples were thawed before the LPO and anti-oxidant enzyme activities analyses. The LPO and anti-oxidant enzymatic activities in sperm were calculated for 2×10^8 sperm cells (Dandekar et al., 2002; Kasimanicham et al., 2007).

2.3.2.1. Malondialdehyde (MDA) concentrations. The concentrations of malondialdehyde (MDA), as indices of LPO activity in the sperm samples, were measured using the thiobarbituric acid reaction and according to 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. MDA concentrations were expressed as nmol/l.

2.3.2.2. Superoxide Dismutase (SOD) and Catalase (CAT) activities. SOD was analysed using the technique as described by Sun and Zigman (1978). A volume of 0.4 ml semen, 0.1 ml of 0.9% NaCl and 0.4 ml of chloroform: ethanol (3:5) was centrifuged for 10 min at 2000G. The reaction mixture contained 20 ml 3 mmol/l xanthine, 10 ml 0.6 mmol/l EDTA, 10 ml 0.15 mmol/l NBT, 6 ml 400 mmol/l sodium carbonate, 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 and this mixture was incubated at 25 °C for 20 min. Thereafter, the reaction was stopped by adding 1 ml of 0.8 mmol/l $CuCl_2$. The absorbance was read at 560 nm using a spectrophotometer. SOD activity was expressed as U/g protein.

The method as described by Goth (1991) was used in the determination of CAT activity in the semen samples. 0.2-ml semen samples 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 generally decomposes 1 μmol of hydrogenperoxide per min, under these conditions. The enzymatic reaction was terminated with 1 ml of 32.4 mmol/l ammonium molybdate. Hydrogen peroxide was measured at 405 nm using a spectrophotometer. Values of CAT activity were expressed as kU/g protein.

2.4. Statistical analysis

The study was repeated 5 times and the results were expressed as the mean ± SEM. 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 of values with a confidence level of $P < 0.05$ were considered to be significant (Daniel, 1991).

3. Results

3.1. Sperm (motility and HOST) and biochemical (MDA, SOD and CAT) parameters

The effects of anti-oxidants on the sperm characteristics of frozen ram semen were evaluated in 5 independent experiments. As shown in Table 1, a freezing extender supplemented with 5 mM glutamine led to a higher motility rate (68.0 ± 4.4%) and HOST (64.1 ± 5.5%), when compared to the other groups ($P < 0.05$). No significant differences were observed in motility and HOST, following the supplementation of the freezing extender with glutamine 2.5 mM and BME (13 or 26%) after the freeze–thawing process. The effects of anti-oxidants on lipid peroxidation and anti-oxidant activities in thawed ram semen are set out in Table 2. CAT activity remained significantly higher following the addition of 5 mM glutamine (6.4 ± 0.9 kU/g protein), compared to the other treatments ($P < 0.01$). The different doses of the anti-oxidants did not show the ability in the elimination of MDA formation and the maintenance of SOD activities, when compared to the control ($P < 0.05$).

Table 1
Mean (±SE) percentages of motility and HOST in frozen–thawed ram sperm.

Groups	Motility	HOST
Glutamine 2.5 mM	57.5 ± 9.5^{ab}	47.5 ± 6.4^{ab}
Glutamine 5 mM	68.0 ± 4.4^{a}	64.1 ± 5.5^{a}
BME (13%)	44.3 ± 2.0^{b}	47.0 ± 4.1^{b}
BME (26%)	36.7 ± 3.3^{b}	56.1 ± 3.7^{ab}
Control	50.7 ± 2.5^{b}	46.9 ± 2.3^{b}
P	**	*

Different superscripts (a and b) within the same column demonstrate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$).

4. Discussion

The semen cryopreservation process, which includes the decrease in temperature, causes oxidative stress on the sperm membrane. This, in turn, results in irreversible damage to the sperm organelles and changes in enzymatic activity, associated with a reduction in sperm motility, functional membrane integrity and fertilizing ability (Aitken, 1984; de Lamirande and Gagnon, 1992; Maxwell and Watson, 1996). Damage due to oxidative stresses may be bypassed by the inclusion of anti-oxidants in ram semen extender prior to the freezing process (Bucak et al., 2007; Bucak and Uysal, 2008). Alternatively, long-term (freezing) and short-term (liquid) storage of sperm may lead to membrane deterioration—due to membrane phase transitions occurring in the regions of the highly specialized regionalized sperm plasma membrane (Maxwell and Watson, 1996). This study was undertaken to ascertain which anti-oxidant would provide the most effective protection against cold shock and oxidative damages during the cryopreservation process.

In this study, glutamine and BME at different doses were not effective in the prevention of MDA formation,

Table 2
Mean (±SE) malondialdehyde (MDA), superoxide dismutase (SOD) acatalase (CAT) levels in frozen–thawed ram sperm.

Groups	MDA (nmol/L)	SOD (U/g protein)	CAT (kU/g protein)
Glutamine 2.5 mM	8.2 ± 0.7^{a}	1.3 ± 0.0^{cd}	$4.0 \pm 0.4^{b,c}$
Glutamine 5 mM	6.2 ± 0.7^{ab}	0.9 ± 0.0^{d}	6.4 ± 0.9^{a}
BME (13%)	7.2 ± 0.9^{a}	2.3 ± 0.3^{b}	$5.1 \pm 0.7^{a,b}$
BME (26%)	8.0 ± 1.5^{a}	1.4 ± 0.0^{c}	2.8 ± 0.2^{c}
Control	4.1 ± 0.3^{b}	3.0 ± 0.1^{a}	4.6 ± 0.4^{b}
P	*	***	**

Different superscripts (a, b and c) within the same column demonstrate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

when compared to the control. This finding related to LPO, is in agreement with the results of studies performed on ram (Bucak et al., 2007) and buck (Atessahin et al., 2008) sperm in which no decrease was observed in the level of MDA in the presence of various anti-oxidants, following the freeze–thawing process. This indicates that LPO is apparently not a major factor influencing sperm quality, after thawing. The current results were also contradictory to other studies on rabbit (Alvarez and Storey, 1983) and canine (Michael et al., 2007) sperm, where inhibition of lipid re-oxidation was observed in case of incubation with anti-oxidants during liquid storage or cryopreservation. Glutamine is known to play 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, gene expression, respiratory burst, and extracellular matrix synthesis (Curi et al., 2005). In sperm functions, it acts at the extra-cellular level, improving the motility of human and stallion sperm (Renard et al., 1996; Khlifaoui et al., 2005) and the fertilizing ability of human sperm (Renard et al., 1996) following the freeze–thawing process. In the current study, glutamine, when applied at a dose of 5 mM, improved sperm motility and functional membrane integrity (HOST) during cryopreservation. Current results, with respect to sperm parameters, are in agreement with studies conducted on the Poitou jackass (Trimeche et al., 1996), cynomolgus monkey (Li et al., 2003) and stallion (Khlifaoui et al., 2005) in which glutamine addition resulted in significant increases of sperm motility after freeze–thawing. The presence of the CAT enzyme in sperm has been demonstrated for the ram (Upreti et al., 1998) and bull (Bilodeau et al., 2000; Bilodeau et al., 2001). Its potential role is in the aging processes and control of oxidative stress in cells, mainly by removing superoxide anion radical production, generated by NADPH oxidase in living cells. In this study, the CAT activity in the group of thawed semen with an extender containing glutamine 5 mM, was higher than in the other groups. This finding was supported by studies in sheep (Bucak et al., 2008) and goats (Atessahin et al., 2008), where CAT activities were elevated in the presence of different anti-oxidants following the freeze–thawing process. The axosome and associated dense fibers of the middle pieces of the sperm cell are covered by mitochondria that generate energy from intracellular ATP. These are responsible for sperm motility (Garner and Hafez, 1993). Additionally, large amounts of reactive oxygen species can impair sperm motility, producing axonemal damage as a result of ATP depletion (de Lamirande and Gagnon, 1992). Based on the current results, it can be hypothesized that glutamine displayed an anti-oxidative effect, increasing CAT anti-oxidant activity. So, for example, glutamine may have a cryoprotective effect on the functional integrity of the sperm axosome and mitochondria, improving post-thawed sperm motility due to decreased damage of the sperm cell.

5. Conclusion

Glutamine (5 mM) provided a cryoprotective effect, improving post-thawing motility and membrane integrity of the ram semen, compared to the control, as well as significantly elevating the CAT activity. Lipid peroxidation (LPO) was not reduced post-thawing in anti-oxidant-treated sperm. Looking at previous studies in the rabbit (Alvarez and Storey, 1983), ram (Bucak et al., 2007) and canine sperm (Michael et al., 2007) sperm, the question regarding the non-existence of consistency between anti-oxidant activities following the thawing of semen remains to be answered. Studies on the determination of oxidative stress and anti-oxidant activities of ram sperm are required to better understand the biochemical changes that are important in semen freezing, in vivo/vitro fertilization and in embryo production systems. Current findings showed that glutamine (5 mM) supplementation in semen extenders, was of greater benefit to frozen–thawed ram sperm. It could be concluded that future efforts are needed to find the appropriate anti-oxidants and their effective concentrations, which will improve post-thaw sperm parameters (e.g. motility, membrane integrity, fertility) and anti-oxidant activities even further when frozen–thawed ram sperm is used.

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