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Effects of anti-oxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze–thawing process

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Abstract

The anti-oxidant system of reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), and superoxide dismutase (SOD) has been described as a defense functioning mechanism against lipid peroxidation (LPO) in semen, and is important in maintaining sperm motility and viability. This anti-oxidant capacity of sperm cells may be insufficient in preventing LPO during the freeze–thawing process. The aim of this study was thus to determine the influence of varying doses of anti-oxidant additives on standard semen parameters, lipid peroxidation and anti-oxidant activities after the freeze–thawing of goat semen. Ejaculate samples (artificial vagina) obtained from 4 mature Angora goats were evaluated and pooled at 37 °C. The semen samples diluted with a Tris-based extender, containing taurine (25, 50, 75 mM), trehalose (25, 50, 75 mM), and cysteine (5, 10, 15 mM), and an extender containing no anti-oxidant additives (control) were again evaluated. Diluted semen was cooled down to 5 °C and frozen in 0.25 ml French straws, prior to being stored in liquid nitrogen. Frozen straws were thawed in a water bath (37 °C) for 30 s for microscopic sperm evaluation. Upon evaluation of parameters for semen quality, the use of a Tris-based extender supplemented with anti-oxidant additives was found to cause no significant improvement in sperm mortality, when compared to the controls. Increasing doses of taurine and trehalose decreased ($P < 0.05$) the sperm motility following the freeze–thawing of the goat semen. In biochemical assays, the application of taurine (75 mM) produced the lowest level of malondialdehyde (MDA) (4.46 ± 0.31 nmol/ml), compared to the controls ($P < 0.001$). Lower GSH levels were higher in the groups in which cysteine was included at 10 and 15 mM (3.27 ± 0.11 and 3.45 ± 0.28 nmol/ml) – compared to the group which received 5 mM cysteine, as well as the controls (2.27 ± 0.08 and 2.50 ± 0.08 nmol/ml respectively, $P < 0.001$). Compared to the controls, taurine at a concentration of 25 and 75 mM, and increasing doses (50 and 75 mM) of trehalose, significantly increased the GSH-PX activity ($P < 0.01$). The maintenance of CAT activity was demonstrated to be higher with the addition of 10 and 15 mM cysteine, compared to the other groups ($P < 0.001$). Vitamin A (VitA) levels were significantly higher, compared to the controls (267.34 ± 9.68 mg/dl and 267.34 ± 9.68 mg/dl, respectively), when 25 mM taurine (329.61 ± 6.35 mg/dl) and 10 mM (318.64 ± 6.34 mg/dl) cysteine was added to the extender ($P < 0.001$). The results of this study provide a new approach to the cryopreservation of Angora goat semen and could contribute to the improvement of this technology in the goat industry.
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Keywords: Angora goat; Semen; Anti-oxidants; Cryopreservation; Oxidative stress parameters

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1. Introduction

Semen cryopreservation allows the widespread dissemination of valuable genetic material, even to small flocks by means of AI, leading to an increased rate of genetic gain. However, cervical AI, when using frozen semen, is limited by low fertility rates in goats. The freeze–thawing of spermatozoa being associated with a reduction in cell motility, viability and fertilizing capacity (Evans and Maxwell, 1987; Holt, 1997).

Mammalian sperm membranes incorporate many unsaturated fatty acids and are susceptible to lipid peroxidation (LPO) in the presence of reactive oxygen species (ROS), leading to decreased sperm quality (Bucak et al., 2007; Lenzi et al., 2002). The anti-oxidant system comprising reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), and superoxide dismutase (SOD) has been described as defense functioning mechanisms against the lipid peroxidation of semen, and important in maintaining sperm motility and viability (Bilodeau et al., 2001; Aitken and Baker, 2004; Gadea et al., 2004). This anti-oxidant capacity in sperm cells may however, be insufficient in preventing LPO during the freeze–thawing process. Thus mammalian spermatozoa lack a significant cytoplasmic component, which contains anti-oxidants that counteract the damaging effects of ROS and lipid peroxidation (Aurich et al., 1997; Storey, 1997).

Trehalose is a cryoprotectant known to protect the sperm membrane structure from oxidative and cold shock damage during the freeze–thawing process. It has a protective action related to both the osmotic effect and specific interactions with membrane phospholipids, rendering a hypertonic media, causing cellular osmotic dehydration before freezing, and then decreasing the degree of cell injury by ice crystal formation (Liu et al., 1998; Storey et al., 1998). So trehalose added to semen extenders is known to improve the viability and motility of liquid or cryopreserved ram sperm (Aisen et al., 2002; Bucak and Tekin, 2007). Of the compounds in the epididymal and oviduct fluids, sulfonic amino acids (e.g. taurine) are important protectors of the cells against the accumulation of ROS – in the case of exposure to aerobic conditions and the freeze/thawing process (Alvarez and Storey, 1989; Holmes et al., 1992; Bucak et al., 2007). Mammalian cells can only utilize thiol compounds such as cysteine and GSH, which have been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both in vitro and in vivo, and protecting the membrane lipids and proteins by direct radical-scavenging properties. Cysteine has been shown to prevent the loss in motility of frozen–thawed bull semen (Bilodeau et al., 2001), ram semen (Uysal and Bucak, 2007) and goat semen (Bilodeau et al., 2001; Bucak and Uysal, 2008), and to improve the viability, the chromatin structure and membrane integrity of boar sperm during liquid preservation (Szczesniak-Fabianczyk et al., 2006).

Few studies have been done on the effects of anti-oxidant supplementation in the cryopreservation of goat semen. Thus the present study envisaged to investigate the effects of trehalose, taurine and cysteine on standard sperm quality parameters (motility, acrosome abnormality and membrane integrity), LPO, and anti-oxidant activities (GSH, GSH-PX, CAT, VitE), following the freeze–thawing process in Angora goats.

2. Materials and methods

2.1. Angora goats and semen collection

Semen samples from 4 mature Angora goats (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 feeding, housing and lighting conditions. A total number of 42 ejaculates were collected twice a week from the bucks using an artificial vagina, during the breeding season (autumn to early winter) and the semen pooled to minimize individual variation. Ejaculates which met the following criteria were evaluated: volume of 0.75–2 ml; minimum sperm concentration of 3×10^9 sperm/ml; motility of 80%. Eight pooled ejaculates were included in the study. Immediately following collection, the ejaculates were placed in a water bath (37 °C), until evaluation in the laboratory. Semen assessment was performed within approximately 10 min.

2.2. Semen processing and evaluation

Salomon's Tris solution (375 mM Tris, 124 mM citric acid, 41.6 mM glucose, 9% (v/v) egg yolk, 5% (v/v) glycerol, pH 6.8) was used as the basic semen diluent (freezing extender). Each pooled ejaculate was split into 10 equal aliquots and diluted (37 °C) with base extenders supplemented with the anti-oxidant taurine (25, 50, 75 mM), trehalose (25, 50, 75 mM), or cysteine (5, 10, 15 mM) and a base extender with no additives (control) for a total of 10 experimental groups (37 °C) – to a final concentration of approximately 4×10^8 sperm/ml. Actual sperm concentrations were calculated with the aid of a hemocytometer (Smith and Mayer, 1955).

Diluted samples were aspirated into 0.25 ml (medium-sized) French straws, sealed with polyvinyl alcohol powder and equilibrated at 5 °C for a period of 2.5 h. After equilibration, the straws were frozen in liquid nitrogen vapour (4 cm above liquid nitrogen) for 15 min and the straws plunged into liquid nitrogen for storage. After being stored for 1 month, 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.

2.3. Semen evaluation

2.3.1. 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.

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).

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).

2.3.2. Biochemical assays

Biochemical assays for the analyses of oxidative parameters were performed on the semen samples, immediately after thawing and without washing of the sample.

2.3.2.1. Malondialdehyde concentrations. Malondialdehyde concentrations, as indices of lipid peroxidation in the sperm samples, were measured using the thiobarbituric-acid reaction and in accordance with the method as described by Placer et al. (1966). All MDA concentrations were expressed as nmol/ml.

2.3.2.2. GSH levels and GSH-PX, and CAT activities. The GSH content of sperm was measured, using the method of Sedlak and Lindsay (1968). The samples were precipitated with 50% trichloracetic acid and then centrifuged at 1000 × *g* for 5 min. The reaction mixture contained 0.5 ml supernatant, 2.0 ml Tris-EDTA buffer (0.2 mol/l; pH 8.9) and 0.1 ml 0.01 mol/l 5,5′-dithio-bis-2-nitrobenzoic acid.

The concentration of the solution was determined with the aid of a spectrophotometer (412 nm). GSH values were expressed as nmol/ml.

GSH-PX activity was determined according to Lawrence and Burk (1976). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 EU/ml oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM H_2O_2. An enzyme source (0.1ml) was added to 0.8 ml of the respective mixture, and this mixture was incubated at 25 °C for 5 min, prior to the initiation of the reaction induced by the addition of 0.1 ml peroxide solution. The absorbance, using a spectrophotometer (412 nm) was recorded for 5 min. GSH-PX activity was expressed as international unit (IU)/g protein for the semen samples.

The method described by Goth (1991) was used for the determination of the CAT activity in the sperm. 0.2 ml-sperm samples were incubated in 1 ml of substrate (65 μmol/ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) at 37 °C for 60 s. The yellow-coloured molybdate and hydrogen peroxide complex was measured (405 nm) using a spectrophotometer. Values of CAT activity were expressed as *k*U/l, where *k* is the first-order rate constant.

2.3.2.3. Vitamin E (VitE) levels. Beta-carotene levels in the semen samples were determined in accordance with the method described by Suzuki and Katoh (1990). Hexane (2 ml) was mixed with 0.5-ml of sperm and the absorbance measured at 453 nm, using a spectrophotometer. Vitamin A (VitA) values were expressed as mg/dl.

2.4. Statistical analysis

The study was repeated 8 times and the results expressed as the mean ± S.E.M. Means were analyzed using a one-way analysis of variance, followed by a Tukey's post hoc test to determine the significant differences in all the parameters between the different groups, using the SPSS/PC computer program (Version 12.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered to be statistically significant (Daniel, 1991).

3. Results

3.1. Sperm characteristics (percentage motility, acrosome abnormalities and HOST)

The effect of anti-oxidant additives on standard semen quality parameters in frozen Angora goat semen were evaluated in 8 independent experiments. As set out in Tables 1–3 (compared to the controls), the use of a Tris-based extender supplemented with anti-oxidant additives did not lead to any significant improvement in semen quality. Increasing the doses of taurine and trehalose decreased ($P < 0.05$) the sperm motility in goat semen. Cysteine (15 mM) resulted with the highest motility rate (as shown in Table 2), although no significant improvement occurred following the freeze–thawing process.

Table 1
Mean (±S.E.) percentages of motility, acrosome abnormalities, HOST and MDA, GSH, GSH-PX, CAT and VitA levels in Angora goat semen frozen with different taurine doses

	Control	Taurine 25 mM	Taurine 50 mM	Taurine 75 mM	*P*
Motility (%)	51.88 ± 2.76^{a}	44.29 ± 2.95^{ab}	47.14 ± 2.95^{ab}	37.86 ± 2.95^{b}	*
Acrosome abnormalities (%)	8.75 ± 0.88	10.57 ± 1.46	9.16 ± 1.62	7.33 ± 0.98	NS
HOST (%)	34.43 ± 3.24	32.20 ± 4.03	34.00 ± 4.44	31.71 ± 3.42	NS
MDA (nmol/ml)	6.27 ± 0.18^{b}	5.54 ± 0.31^{bc}	9.80 ± 0.51^{a}	4.46 ± 0.31^{c}	***
GSH (nmol/ml)	2.50 ± 0.08^{c}	1.88 ± 0.07^{b}	2.27 ± 0.07^{a}	2.35 ± 0.03^{a}	***
GSH-PX (IU/g prot)	6.56 ± 0.17^{b}	14.77 ± 1.40^{a}	8.05 ± 0.23^{b}	16.37 ± 1.26^{a}	***
Catalase (*k*U/l)	638.2 ± 10.2	744.9 ± 78.6	866.7 ± 129.5	856.4 ± 101.4	NS
VitA (mg/dl)	267.3 ± 9.6^{b}	329.6 ± 6.3^{a}	223.4 ± 9.6^{c}	120.8 ± 6.3^{d}	***

a, b, c, d: Different superscripts within the same column demonstrate significant differences (*$P<0.05$; ***$P<0.001$), NS: no significant.

Table 2
Mean (±S.E.) percentages of motility, acrosome abnormalities, HOST and MDA, GSH, GSH-PX, CAT and VitA levels in Angora goat semen frozen with different cysteine doses

	Control	Cysteine 5 mM	Cysteine 10 mM	Cysteine 15 mM	*P*
Motility (%)	51.88 ± 2.76^{ab}	50.00 ± 2.76^{b}	55.00 ± 2.76^{ab}	61.43 ± 2.95^{a}	*
Acrosome abnormalities (%)	8.75 ± 0.88	9.00 ± 1.31	9.50 ± 1.56	6.67 ± 0.80	NS
HOST (%)	34.43 ± 3.24	35.71 ± 4.26	41.28 ± 4.06	42.00 ± 4.64	NS
MDA (nmol/ml)	6.27 ± 0.18^{b}	7.54 ± 0.48^{ab}	9.02 ± 0.51^{a}	8.66 ± 0.69^{a}	**
GSH (nmol/ml)	2.50 ± 0.08^{b}	2.27 ± 0.08^{b}	3.27 ± 0.11^{a}	3.45 ± 0.28^{a}	***
GSH-PX (IU/g prot)	6.56 ± 0.17^{b}	8.27 ± 1.23^{ab}	6.85 ± 0.58^{b}	11.5 ± 1.90^{a}	*
Catalase (*k*U/l)	638.2 ± 10.2^{b}	373.2 ± 12.1^{c}	945.9 ± 77.0^{a}	872.8 ± 89.1^{a}	***
VitA (mg/dl)	267.3 ± 9.6^{b}	234.4 ± 13.2^{b}	318.6 ± 6.3^{a}	263.6 ± 12.6^{a}	***

a, b, c: Different superscripts within the same column demonstrate significant differences (* $P<0.05$; ** $P<0.01$; *** $P<0.001$), NS: no significant difference.

3.2. Analysis of oxidative stress parameters

The effects of the anti-oxidant additives added to the freezing extender on lipid peroxidation and anti-oxidant activities in thawed buck semen is set out in Tables 1–3. Taurine (75 mM) caused the lowest level of malondialdehyde (4.46 ± 0.31 nmol/ml) production, compared to the control group ($P<0.001$) (Table 1). Glutathione levels were higher in the group treated with cysteine at 10 and 15 mM, when compared to the group which was treated with cysteine (5 mM) and the control group ($P<0.001$) (Table 2). Taurine at 25 and 75 mM, and increasing doses (50 and 75 mM) of trehalose, significantly increased the GSH-PX activity, compared to the controls ($P<0.01$) (Tables 1 and 3). The maintenance of catalase activity was demonstrated to be higher upon the addition of 10 and 15 mM cysteine, compared to the other groups ($P<0.001$) (Table 2). In this study, Vitamin A levels were significantly higher than the controls, when 25 mM taurine and 10 mM cys-

Table 3
Mean (±S.E.) percentages of motility, acrosome abnormalities, HOST and MDA, GSH, GSH-PX, CAT and VitA levels in Angora goat semen frozen with different trehalose doses

	Control	Trehalose 25 mM	Trehalose 50 mM	Trehalose 75 mM	*P*
Motility (%)	51.88 ± 2.76^{a}	55.00 ± 2.95^{a}	52.50 ± 2.95^{a}	28.33 ± 4.51^{b}	***
Acrosome abnormalities (%)	8.75 ± 0.88	8.00 ± 1.13	9.40 ± 1.54	6.33 ± 1.33	NS
HOST (%)	34.43 ± 3.24	34.33 ± 2.03	34.28 ± 3.64	36.33 ± 5.24	NS
MDA (nmol/ml)	6.27 ± 0.18^{b}	10.85 ± 0.88^{a}	7.35 ± 0.18^{b}	6.99 ± 0.48^{b}	***
GSH (nmol/ml)	2.50 ± 0.08^{a}	2.14 ± 0.09^{b}	2.13 ± 0.01^{b}	2.27 ± 0.08^{ab}	**
GSH-PX (IU/g prot)	6.56 ± 0.17^{c}	9.87 ± 1.30^{bc}	19.11 ± 3.21^{a}	17.14 ± 2.90^{ab}	**
Catalase (*k*U/l)	638.2 ± 10.22^{b}	617.2 ± 136.9^{b}	988.6 ± 41.4^{a}	714.4 ± 16.1^{ab}	**
VitA (mg/dl)	267.3 ± 9.6^{a}	238.0 ± 3.6^{bc}	216.0 ± 3.6^{c}	245.3 ± 3.6^{ab}	***

a, b, c: Different superscripts within the same column demonstrate significant differences (** $P<0.01$; *** $P<0.001$), NS: no significant.

teine were added to the freezing extender ($P < 0.001$) (Tables 1 and 2).

4. Discussion

Mammalian spermatozoa are highly sensitive to lipid peroxidation, which occurs as a result of the oxidation of the membrane lipids by the partially reduced oxygen molecules such as superoxide, hydrogen peroxide and hydroxyl radicals. LPO due to the attacks by ROS, formed through the univalent reduction of oxygen, led to the impairment of sperm function, e.g. sperm motility, functional membrane integrity and fertility through oxidative stress and the production of cytotoxic aldehydes (Aitken, 1984; Alvarez and Storey, 1989; Aitken et al., 1993). In this study, 75 mM taurine had a significant effect on the prevention of malondialdehyde formation, compared to the control group. Results with respect to LPO, is in agreement with the results of a study conducted on rabbit sperm in which a decrease was observed in the level of malondialdehyde in the presence of taurine (Alvarez and Storey, 1983). The addition of the anti-oxidant additives to the freezing extender did not cause any further improvement in the semen quality parameters. Current findings are in contrast with those reported by Bucak et al. (2007) and Bucak and Uysal (2008), who demonstrated a marked improvement in the motility of ram and goat semen following the freeze–thawing process. Increasing doses of taurine and trehalose (75 mM) decreased the post-thaw sperm motility. High doses of anti-oxidant additives can be expected to have destroyed the functional integrity of the axosome and mitochondria of the sperm cells, which are associated with motility. In the current study, the highest protective effect of trehalose was identified at a concentration of 25 mM, and at a much reduced rate at 75 mM. The latter concentration resulted in a high osmolarity of the extender, which in itself is deleterious to the sperm cells. As demonstrated in previous studies, anti-oxidant additives exhibit cryoprotective activity on certain sperm parameters, when Tris-citric formulations are included in moderate doses – but not when increasing doses of anti-oxidant additives with a hypertonic property were applied (Uysal et al., 2005; Bucak et al., 2007).

The anti-oxidant system comprising GSH, GSH-PX, CAT, taurine, and superoxide dismutase, have been described as functioning as a defense mechanism against LPO in semen (Bilodeau et al., 2001; Aitken and Baker, 2004; Gadea et al., 2004). GSH for example is a tripeptide thiol compound which has many important functions in the cellular physiology, including the protection of the cell from oxidative stress, synthesis of protein and DNA, and the fertilization of gametes (Perreault et al., 1984; Nasr-Esfahani and Johnson, 1992; Irvine, 1996). GSH-PX is a selenocysteine – containing an anti-oxidant enzyme that plays a role in the elimination of hydrogen peroxide and is also known to be involved in the detoxification of reactive lipids (Fridovich, 1978; Meister and Anderson, 1983). In the present study, the highest levels of GSH were observed when the freezing extender was supplemented with increasing doses (10, 15 mM) of cysteine – displaying a dose-dependent effect. In addition to contributing to the GSH capacity of sperm, cysteine significantly increased the VitA levels, compared to the control.

It is suggested that taurine promotes the bioavailability of lipid soluble VitA, by probably forming different types of water soluble, easily hydrolyzable complexes. The ability of taurine to convert lipids and lipid soluble substances into a water soluble state is a possible key to understanding the unusually wide diversity of biological phenomena associated with taurine (Petrosian and Haroutounian, 2000). In this study, 25 mM taurine led to the highest levels of GSH-PX activity and VitA levels, when compared to the controls. The fact that the low doses of taurine (except for the application of high doses 50 and 75 mM), provided an increase of VitA level, remains an enigma and is worthy of further investigation.

The elevation of GSH-PX activity by trehalose in the present study was indicative of the anti-oxidative capacity, similar to studies on the freezing of ram semen (Aisen et al., 2005; Bucak et al., 2007). Increasing the doses of trehalose led to the lowest level of MDA and the highest activity of catalase – this phenomenon was not considered to be statistically important. It may be stated that extenders with a hypertonic property and high doses of trehalose confer better protection against oxidative stress to sperm following cryopreservation.

5. Conclusions

It may be stated that, compared to the controls, anti-oxidant additives such as those used, generally maintain acceptable levels of the anti-oxidant systems following the thawing of semen ($P < 0.001$). Anti-oxidant additives did not lead to any significant improvements, compared to the control group. However, 15 mM cysteine resulted in the highest rate of motility (Table 2), and no significant improvement occurred following the freeze–thawing process. The question regarding the consistency between anti-oxidative capacities following the thawing of semen remains unanswered. Changes in extender composition with a hypertonic environment, anti-oxidant additive concentrations and animal species may explain the dif-

ferent reactions regarding anti-oxidant capacity and why anti-oxidant additives do not prevent the MDA and improve the semen quality parameters.

Further studies are required for a better understanding of the biochemical changes and obtain more information on the determination of lipid peroxidation and anti-oxidant capacities in cryopreserved Angora semen. The Angora goat breed, being of great importance to the economy of countries in several parts of the world – with regard to the improvement and dissemination of the breed with desirable characteristics for hair production.

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