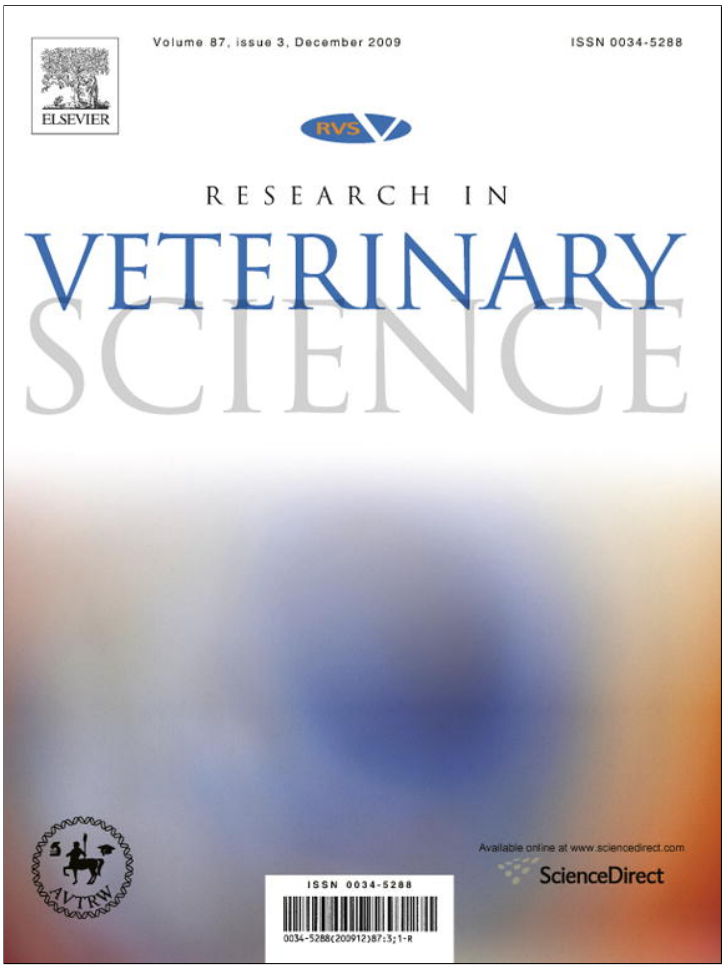

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# Effects of hypotaurine, cysteamine and aminoacids solution on post-thaw microscopic and oxidative stress parameters of Angora goat semen

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ABSTRACT

This study was conducted to determine the effects of cysteamine, hypotaurine and aminoacids solution (BME) on standard semen parameters, lipid peroxidation and antioxidant activities of Angora goat semen after the freeze–thawing process. Ejaculates collected from four Angora goats were evaluated and pooled at 37 °C. Semen samples, which were diluted with a Tris-based extender containing the antioxidants hypotaurine (5 mM) and cysteamine (5 mM), and an aminoacid solution (13%), and an extender containing no antioxidants (control), were cooled to 5 °C and frozen in 0.25-ml French straws in liquid nitrogen. Frozen straws were thawed individually at 37 °C for 20 s in a water bath for evaluation.

Supplementation with cysteamine, hypotaurine and BME caused significant ($P < 0.05$) increases in sperm motility, and significant ($P < 0.05$) decreases in total abnormality rates in comparison to the control group. While all in vitro treatments did not affect the acrosomal abnormality rates, hypotaurine and BME but not cysteamine significantly ($P < 0.05$) increased the HOST results as compared to the control group. Supplementation with antioxidants and BME did not significantly affect MDA levels and CAT activity in comparison to the control group ($P > 0.05$). The antioxidants hypotaurine and cysteamine decreased SOD activity when compared to the BME group and controls ($P < 0.001$).



## 1. Introduction

The Angora goat originated on the Central Anatolia plains, and is reported to have existed since 2400 BC. Until the mid 19th century the breed was well preserved. By that time, mohair production and trade had developed around Ankara, capital of Turkey, to serve the export markets. In the second half of the 19th century the British mohair industry was established, as the country wished to produce its own mohair. At this time, the first Angora goats and bucks were taken out of Anatolia to England and then to South Africa, where local goats were upgraded to Angora goats. Other Angora goats were sent to Texas and California. The breed multiplied rapidly and, with the application of modern animal breeding techniques (Gürsoy, 2005).

Semen cryopreservation offers many advantages to the livestock industry, particularly in conjunction with allowing the widespread dissemination of valuable genetic material by means of artificial insemination (AI). However, cryopreservation produces cold shock and oxidative attack on the sperm membrane, which both decrease sperm survival and fertilizing ability, thus leading to sperm death and negative impact on the preservation of semen for AI (Evans, 1988; Maxwell and Watson, 1996). Cold shock of spermatozoa is partly associated with oxidative attack and the generation of reactive oxygen species (ROS). ROS-induced damage of spermatozoa involves an oxidative attack of the bis-allylic methylene group of sperm membrane phospholipids that leads to lipid peroxidation (LPO) (Yanagimachi, 1994; Irvine, 1996; Upreti et al., 1998; Chatterjee et al., 2001).

The progress in semen freezing techniques entails in-depth research of sperm physiological and biochemical processes occurring during the freeze-thawing process (Holt, 1997; King et al., 2004). The plasma membrane of mammalian spermatozoa presents high concentrations of polyunsaturated fatty acids, which make it more susceptible to ROS-induced peroxidative damage, especially following cryopreservation, with a subsequent loss of sperm functions (Aitken et al., 1989a,b; Alvarez and Storey, 1995; Lenzi et al., 2002). However, in vivo, physiological concentrations of ROS are involved in the maintenance of the fertilizing ability and acrosome reaction of spermatozoa (Aitken and Fisher, 1994).

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Mammalian semen contains a variety of antioxidants, such as reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), superoxide dismutase (SOD), taurine and hypotaurine, as a defense functioning mechanism against peroxidative damage (Alvarez and Storey, 1989; Beconi et al., 1991; Zini et al., 1993; Bilodeau et al., 2000; Agarwal and Saleh, 2002). This antioxidant capacity in spermatozoa may be insufficient to prevent LPO and maintain sperm functions during the freeze-thawing process (Aurich et al., 1997; Storey, 1997).

Hypotaurine, a precursor of taurine that also exists in mammalian sperm, is the main end product of cysteine metabolism. It is essential for sperm functions such as capacitation, motility, fertilizing ability and early embryonic development. It is known to neutralize hydroxyl radicals produced during LPO and thus, to protect the thiol groups in the sperm plasma membrane, preventing sperm from damage due to oxidation (Feilman et al., 1987; Barnett and Bavister, 1992).

Cysteamine stimulates GSH synthesis during the in vitro maturation of ovine oocytes, promoting embryonic development and quality (de Matos et al., 2002). Additionally, it enhances post-thaw motility, elevating the antioxidant capacity of ram sperm (Bucak et al., 2007).

Certain organisms are known to accumulate aminoacids to resist cold temperatures. Aminoacids (e.g., glutamine, cysteine) have important roles in preventing the denaturation of fish muscle proteins during frozen storage, and in the cryoprotecting potentiality for goat sperm (Noguchi and Matsumoto, 1971; Kundu et al., 2001). Aminoacids have been demonstrated to act at the extra-cellular level and to improve sperm motility, acrosome integrity and fertilizing potential in stallion, monkey and human studies, after the freeze-thawing process (Renard et al., 1996; Li et al., 2003; Khlifaoui et al., 2005).

The present study was conducted to investigate the effects of hypotaurine, cysteamine and BME on microscopic sperm parameters (motility, acrosome and total abnormalities, HOST), LPO and antioxidant activities (SOD and CAT), following the freeze-thawing process.

## 2. Material and methods

### 2.1. Animals and semen collection

The animals (3–4 year-old goats) were housed at the Lalahan Livestock Central Research Institute, under uniform nutritional conditions. Semen collected by an artificial vagina from four mature Angora goats during the breeding season was used in this study. Immediately after collection, the ejaculates were immersed in a warm water bath at 37 °C until their assessment in the laboratory. Semen assessment was performed within approximately 20 min.

### 2.2. Semen processing

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals. The sperm concentration was determined by means of a haemocytometer (Smith and Mayer, 1955), and sperm motility was estimated using phase-contrast microscopy (400×). Only ejaculates between 1 and 2 ml in volume, spermatozoa with >80% progressive motility and a concentration higher than $2.5 \times 10^9$ spermatozoa/ml were pooled to eliminate individual differences. 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 base extender (freezing extender). Each mixed ejaculate was split into four equal aliquots and diluted at 37 °C with the base extender containing hypotaurine (5 mM), cysteamine (5 mM), BME (13%) and no antioxidant (control), respectively-for a total of four experimental groups, with a final concentration of approximately $4 \times 10^8$ spermatozoa/ml (in a single step), in a 15 ml-plastic centrifuge tube. Diluted semen samples were aspirated into 0.25-ml (medium-sized) French straws 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 plunged into liquid nitrogen for storage. After stored for one month, the frozen straws were thawed individually at 37 °C for 20 s in a water bath for microscopic evaluation.

### 2.3. Post-thaw semen evaluation

#### 2.3.1. Analysis of microscopic sperm 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. Sperm motility estimations were performed in three different microscopic fields for each semen sample, and the mean of the three successive estimations was recorded as the final motility score.

For the assessment of sperm abnormalities, three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 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 put on a slide and covered with a cover slip. The percentage of total sperm abnormality (acrosomal and caput abnormalities, detached heads, abnormal mid-pieces and tail defects) was determined by counting a total of 200 spermatozoa under phase-contrast microscopy (magnification 1000×, oil immersion).

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 hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of 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 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded (Revell and Mrode, 1994; Buckett et al., 1997).

#### 2.3.2. Biochemical assays of post-thaw semen

Biochemical assays were performed in semen samples immediately after thawing and following centrifugation and washing. An aliquot (500 μl) of semen from each sample was centrifuged at 800×*g* for 10 min. The sperm pellet was separated and washed and resuspended in PBS for recentrifugation (three times). After the last centrifugation, 1 ml of deionized water was added to spermatozoa. The LPO and antioxidant enzymatic activity in sperm was calculated for $2 \times 10^8$ cells (Dandekar et al., 2002; Kasimanicham et al., 2007).

*2.3.2.1. Malondialdehiyde (MDA) concentrations.* MDA concentrations, as indices of LPO in sperm samples, were measured using the thiobarbituric acid reaction and according to the method described by Yoshioka et al. (1979). Quantification of thiobarbituric acid reactive substances was performed 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/$2 \times 10^8$ spermatozoa.

*2.3.2.2. SOD and CAT activities.* SOD was analyzed with the technique of Sun and Zigman (1978). A volume of 0.4 ml sample, 0.1 ml of 0.9% NaCl and 0.4 ml of chloroform: ethanol (3:5) 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 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 and this mixture was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by means of adding 1 ml 0.8 mmol/L $CuCl_2$ to the test. The absorbance was recorded at 560 nm by a spectrophotometer. SOD activity was expressed as $U/2 \times 10^8$ spermatozoa.

The method described by Goth (1991) was used for the determination of CAT activity in sperm samples. 0.2-ml sperm 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 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 and hydrogen peroxide was measured at 405 nm using a spectrophotometer. Values of CAT activity were expressed as $kU/2 \times 10^8$ spermatozoa.

### 2.4. Statistical analysis

The study was repeated five times and results were expressed as 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 with values of $P < 0.05$ were considered to be statistically significant (Daniel, 1991).

## 3. Results

### 3.1. Analysis of microscopic sperm parameters (percentages of motility, acrosome and total abnormalities, HOST)

The effects of various antioxidants on sperm characteristics are set out in Table 1. Supplementation with cysteamine, hypotaurine and BME caused significant ($P < 0.05$) increases in sperm motility, and significant ($P < 0.05$) decreases in total abnormality rates in comparison to the control group. While all in vitro treatments did not affect the acrosomal abnormality rates, hypotaurine and BME but not cysteamine significantly ($P < 0.05$) increased the HOST results as compared to the control group.

### 3.2. Biochemical parameters of post-thaw sperm

The effects of various antioxidants on LPO and antioxidant activities following the freeze–thawing of Angora goat sperm are shown in Table 2. Supplementation with antioxidants and BME did not significantly affect MDA levels and CAT activity in comparison to the control group ($P > 0.05$). The antioxidants hypotaurine and cysteamine decreased SOD activity when compared to the BME group and controls ($P < 0.001$).

## 4. Discussion

Cryopreservation produces chemical oxidative stress on the sperm membrane, causing irreversible damage to sperm structure, and changes in membrane fluidity and enzymatic activity of sperm, associated with a reduction in sperm motility, viability and fertilizing ability (Aitken et al., 1989a,b; de Lamirande and Gagnon, 1992). Additionally, reduction of temperature during the freezing of semen can cause a significant ROS generation by spermatozoa, contributing to damage of sperm function, e.g. sperm motility, membrane integrity and fertility through functional oxidative stress (Aitken et al., 1989a,b; Alvarez and Storey, 1989; Aitken et al., 1993). These findings are in contrast with those reported by Bucak et al. (2007) and Atessahin et al. (2008), who demonstrated a marked improvement in the motility of ram and goat semen in the presence of MDA with increased levels, following the freeze–thawing process. Damage on sperm function can be minimized by addition of antioxidants to semen prior to cryopreservation, resulting in improved post-thaw sperm function (Renard et al., 1996; Bucak et al., 2007). However the question regarding the inconsistency of antioxidative effect on sperm freezing remains in flagrant contradiction. In the present study, antioxidants did not demonstrate any effectiveness in the prevention of MDA formation, similar to studies carried out in ram, goat and canine semen (Bucak et al., 2007; Michael et al., 2007; Atessahin et al., 2008). These indicate that LPO is apparently not a major factor influencing sperm survival after the thawing of sperm.

**Table 1**
Percentages of motility, acrosome and total abnormalities and HOST in frozen-thawed Angora goat sperm (means% ± S.E.M).

| Groups | Motility | Acrosome abnormality | Total abnormality | HOST |
|---|---|---|---|---|
| Cysteamine 5 mM | 61.00±3.67 [a] | 9.75±1.31 | 15.50±2.47 [b] | 34.25±1.11 [bc] |
| Hypotaurine 5 mM | 63.33±4.41 [a] | 7.67±4.06 | 19.67±3.84 [b] | 43.00±4.24 [ab] |
| BME (13%) | 64.17±1.54 [a] | 7.00±1.53 | 14.00±1.15 [b] | 45.00±2.68 [a] |
| Control | 50.00±3.42 [b] | 13.0±3.06 | 29.33±2.24 [a] | 30.25±2.78 [c] |
| P | * | - | * | * |

(a,b,c): Different superscripts within the same column demonstrate significant differences.
* $P < 0.05$.
- No significant difference.

**Table 2**
Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels in frozen-thawed Angora goat sperm.

| Groups | MDA (nmol/2X10$^8$ spermatozoa) | SOD (U/2X10$^8$ spermatozoa) | CAT (kU/2X10$^8$ spermatozoa) |
|---|---|---|---|
| BME (13%) | 1.63±0.36 | 1.44±0.21 [ab] | 6.180±1.00 |
| Hypotaurine 5 mM | 1.47±0.19 | 1.02±0.09 [bc] | 5.365±0.38 |
| Cysteamine 5 mM | 1.90±0.84 | 0.81±0.04 [c] | 4.880±1.44 |
| Control | 1.54±0.18 | 1.64±0.16 [a] | 6.063±0.65 |
| P | - | *** | - |

(a,b,c): Different superscripts within the same column demonstrate significant differences.
*** $P < 0.001$.
- No significant difference.

The sperm antioxidant system predominantly includes SOD, CAT, glutathione and GSH-PX to offset LPO (Alvarez and Storey, 1989; Aitken and Baker, 2004; Sikka, 2004). SOD is an important component of the sperm enzymatic system in mammalian spermatozoa (Holland et al., 1982; Beconi et al., 1991). Increased SOD activity in spermatozoa was detected due to altered membrane integrity following the cryopreservation process (Laso et al., 1994). The presence of CAT in sperm has been demonstrated for mammals, and its potential role is in the aging processes and control of oxidative stress in cells, mainly resulting from $H_2O_2$ (Upreti et al., 1998; Bilodeau et al., 2001).

In some reports, cysteamine conferred better cryoprotection on frozen ram sperm (Bucak et al., 2007), and higher embryo development rates when added to the maturation medium of goat oocytes (Rodriguez-Gonzalez et al., 2003). In this study, while cysteamine resulted in higher rates of motility, lower rates of abnormal spermatozoa and decreased SOD activity, it did not cause a significant effect on CAT activity. These findings are in agreement with the results of our previous study that was performed in ram semen (Bucak et al., 2007).

Hypotaurine, present in mammalian sperm, is essential for sperm functions, such as capacitation, motility, fertilizing ability and early embryonic development (Meizel et al., 1980; Boatman et al., 1990). Hypotaurine, when applied at a dose of 5 mM, improved sperm motility, morphology and functional membrane integrity (HOST) during cryopreservation, and did not display antioxidative properties without elevating the CAT and SOD levels. The result for motility agrees with studies carried out in rabbit (Alvarez and Storey, 1983), hamster (Boatman et al., 1990) and human (Donnelly et al., 2000) semen, where improvement in sperm motility in the presence of hypotaurine, during sperm liquid storage or in the unfrozen state, was recorded. While hypotaurine did not cause a significant effect on MDA levels, it significantly decreased SOD activity. This finding, with respect to MDA, is in agreement with the results of a study conducted on human sperm (Donnelly et al., 2000), in which a decrease was not observed in the level of ROS, when hypotaurine was added. As regards BME, it significantly improved post-thaw sperm motility, morphology and membrane integrity. However, it was ineffective in the elimination of MDA and elevation of antioxidant activities. Changes in the preservation protocol and experimental methodology, extender formula, antioxidant concentrations and animal species-breeds may explain different behaviours of antioxidant capacities and why additives do not prevent MDA. It can be hypothesized that the addition of antioxidants does not elevate antioxidant activity, when the extending and freeze–thawing of semen leads to low antioxidant activity in the presence or absence of antioxidants (Bilodeau et al., 2000). Additionally, this may partly explain the lower fertility of frozen-thawed semen, in comparison to fresh semen in goat breeding. The axosoma and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility (Garner and Hafez, 1993). Cryopreservation can induce axonemal and mitochondrial damage, resulting in the deterioration of sperm motility and morphological-functional integrity. Based on the current results, it can be hypothesized that antioxidants showed cryoprotective influence on the integrity of the axosoma and mitochondria, improving post-thawed sperm motility, morphological integrity over the head, middle and tail pieces.

In conclusion, it seems that, compared to the controls, antioxidants can improve sperm motility, morphology and functional membrane integrity evaluated with HOST, without influencing ROS formation and elevating antioxidant capacity, after freeze–thawing. Due to contradictory results, further studies are required to obtain more information on the determination of LPO and antioxidant capacities, and to determine the most effective additives (e.g. antioxidants) in the freezing of goat semen. In the future, a success of goat semen freezing will contribute to the widespread use of AI, and increased performance of studies on improvement in goat breeding.

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