# Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen

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**Abstract**

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Free radicals are known to be involved in lipid peroxidation as well as DNA and sperm membrane damages that may lead to decreased sperm motility or cell death. The balance between free radical production and their detoxification may be an important factor in sperm survival and function before, during and after cryopreservation. The aim of this study was to determine the effects of the addition of the antioxidants of oxidized glutathione (GSSG), bovine serum albumin (BSA), cysteine and lycopene to freezing media on the post-thawing sperm characteristics, including motility, morphology, acrosome integrity, viability and membrane integrity. A total number of 42 ejaculates were collected using the artificial vagina from 4 Akkaraman rams and 10 replicates of the ejaculates were diluted with a Tris-based extender containing additives and no additives as control.

GSSG (5 mM), BSA (20 mg/ml), cysteine (10 mM) and lycopene (800 μg) showed more positive effects than other concentrations of the supplements and controls in protecting sperm characteristics after the freezing-thawing process ($P < 0.001$).

Many aspects of sperm protection, e.g. sperm motility, viability and membrane stabilisation of the sperm cells during relative cryopreservation, are the key factors in determining the preservation of sperm function. The results of this study provide a new approach to the cryopreservation of sperm from rams and related breeds, and thereby contribute to the improvement of these breeds for the world sheep industry.

*Antioxidants, ram semen, freezing, extender*

Frozen-thawed ram sperm demonstrates serious cryopreservation damage and thus a highly reduced fertilizing capacity (Maxwell and Watson 1996). Cervical artificial insemination, in which frozen semen is used, is limited by low fertility rates in sheep (Salamon and Maxwell 1995). For this reason, it requires application of the intrauterine technique to achieve an acceptable result in sheep fertility. On the other hand, alternative methods for the freezing of ram sperm cells require further improvements regarding membrane damage (King et al. 2004).

Sperm cells have a high content of unsaturated fatty acids in their membranes and they lack a significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) by free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radical, which lead to the structural damage of sperm membranes during the freezing-thawing process (Sinha et al. 1996). The freezing process produces physical and chemical stress on the sperm membrane which in turn reduces sperm viability and fertilizing ability. The cold shock of sperm cells during the freezing-thawing process is associated with oxidative stress induced by free radicals (Salvador et al. 2006; Sanacka and Kurpisz 2004). These free radicals are eliminated by antioxidant systems. Antioxidants play an important role in scavenging free radicals

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which may cause lipid peroxidation of sperm plasma membranes (Baumber et al. 2000). The addition of antioxidants is well known to improve the viability and motility of liquid storage or cryopreserved ram sperm cells (Baumber et al. 2005; Maxwell and Stojanov 1996).

Evidence has shown that the rapid decline in intracellular GSH concentrations, that occurs during the incubation of spermatozoa under aerobic conditions, is not associated with an increase in GSSG concentrations (Bilodeau et al. 2000). This phenomenon can be caused by thiol reacting with other molecules and occurring as hidden GSH (Bernard and Balasubramanian 1995). The fact that proteins bind with glutathione and form mixed disulphides (protein-S-S-glutathione) is well known. These disulphides can protect proteins against oxidative insult (Luberda 2005).

According to Bilodeau et al. (2001), thiols such as glutathione and cysteine, prevented the loss of sperm motility in frozen-thawed bull semen. Cysteine is a low molecular weight amino acid containing thiol; it is a precursor of intracellular glutathione biosynthesis, and increases the GSH level. Funahashi and Sano (2005) and Szczesniak-Fabianczyk et al. (2006) reported that a semen extender with cysteine improved the viability, chromatin structure and membrane integrity of boar sperm cells during liquid preservation.

One of the most important features of bovine serum albumin (BSA) is known as the elimination of free radicals generated by oxidative stress, and the protection of membrane integrity of sperm cells from heat shock during freezing-thawing of canine semen (Uysal et al. 2005). Matsuoka et al. (2006) reported that BSA can be substituted for egg-yolk in the ram semen diluent, and that it enhances the motility and viability of ram sperm cells following the freezing-thawing process.

Carotenoids, such as beta-carotene and lycopene, are important components of antioxidant defense against lipid peroxidation in living cells (Agarwal et al. 2005). Lycopene, an aliphatic hydrocarbon, has received particular attention as a result of studies indicating that it has highly efficient antioxidant and free radical scavenging capacity (Türk et al. 2006).

## Materials and Methods

### Animals and semen collection

Semen samples from 4 mature Akkaraman rams (3 and 4 years of age) were used in this study. The animals were housed at the Education Research and Practice Farm of Ankara University, Faculty of Veterinary Medicine, and maintained using conventional feeding, housing and lighting conditions.

A total number of 42 ejaculates were collected from the rams using an artificial vagina twice a week during the breeding season (autumn to early winter). Semen samples were pooled to eliminate individual differences. Ten pooled ejaculates were included in the study. For the purpose of collecting ejaculates, rams were penned with ewes in oestrus, in the presence of a handler with an artificial vagina (Ollero et al. 1996; Paulenz et al. 2002). The ejaculates were evaluated and accepted for evaluation if the following criteria were met: volume varying between 0.75 - 2 ml; sperm concentration of $3 \times 10^9$ sperm/ml; the motile sperms percentage higher than 70% and less than 10% abnormal sperm in total.

### Semen processing and evaluation

All the reagents used were analytical grade (Sigma, St. Louis). Since the additives (Sigma Chemical Co.), GSSG, BSA, cysteine, and lycopene were available in different purities, the following samples of stated catalogue designations were used throughout the study: GSSG, G-2140; BSA, Sigma Fraction V, A-9647; cysteine, C-7352; lycopene, L-9879. In this trial, a Tris-based extender (CTR) was used (Tris 3.63 g/100 ml, fructose 0.50 g/100 ml, citric acid 1.99 g/100 ml, egg yolk 10 ml/100 ml, penicillin 100.000 iu/100 ml, streptomycin 100 mg/100 ml - pH 6.8, 300 mOsm) for all the ejaculates. Each pooled ejaculate was split into 13 equal aliquots and diluted with the CTR extender with GSSG (5, 10 or 20 mM), BSA (5, 10 or 20 mg/ml), cysteine (5, 10 or 20 mM), lycopene (800, 1600 or 3200 µg/ml) or no additives (controls) for a total of 13 experimental semen groups (37 °C) at a final concentration of 4 × 108 spermatozoa per ml. Sperm concentrations were determined with the aid of a hemocytometer (Smith and Mayer 1955). Diluted semen samples were drawn into 0.25 ml French straws and frozen in liquid nitrogen vapour (-100 to -120 °C) and then stored in liquid nitrogen (-196 °C). Post-thawing sperm motility, morphologic sperm abnormalities, acrosome damages, viability and the functional membrane integrity of sperm cells were determined in samples.

Progressive motility was assessed using a phase-contrast microscope (× 100 magnification), with a warm stage maintained at 37 °C. A wet mount was made using a 5 µl drop of semen ($2 \times 10^6$ sperm) placed directly

on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in 3 different microscopic fields for each semen sample. The mean of the 3 successive estimations was recorded as the final motility score.

For the sperm morphology assessment, at least 3 drops of semen were added to Eppendorf tubes, containing 1 ml of Hancock's (62.5 ml formalin, 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml double-distilled water) solution (Schafer and Holzmann 2000). One drop of this mixture was put on a microscope slide and covered with a cover slip. The percentage of abnormal sperm (detached heads, acrosomal aberrations, abnormal mid-pieces and tail defects) was recorded by counting a total of 200 spermatozoa under phase contrast microscopy (× 1000 magnification; oil immersion).

The sperm viability of the samples was assessed by means of the nigrosin-eosin staining (Evans and Maxwell 1987). The stain was prepared as eosin-Y 1.67 g, nigrosin 10 g, sodium citrate 2.9 g, dissolved in 100 ml distilled water. The sperm suspension smears were prepared by mixing a drop of the semen sample with 2 drops of the stain on a warm slide and spreading the stain with a second slide immediately. The viability was assessed by counting 200 cells under the phase-contrast microscope (× 1000 magnification). Sperm showing partial or complete purple colouring were considered non-viable and only sperm showing strict exclusion of the stain were considered to be alive.

The Hypoosmotic Swelling Test (HOST) was used as a complementary test to the viability assessment protocol to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to the loss of permeability barriers under stress conditions of stretching in a hypoosmotic medium (Buckett et al. 1997). Sperm cells with resistant membranes exhibited a swelling around the tail, such that the flagella became curled and the membrane maintained a swollen 'bubble' around the curled flagellum. The assay was performed by mixing 30 µl of semen with a 300 µl 100 mOsm/kg hypoosmotic (9 g fructose plus 4.9 g sodium citrate per litre of distilled water) solution (Revell and Mrode 1994). This mixture was incubated (37 °C) for one hour; 0.2 ml of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated (× 400 magnification) under the phase-contrast microscope. A total of 200 spermatozoa were counted in at least 5 different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

### Statistical analyses

The sperm evaluations were repeated 10 times and the results were expressed as the mean ± S.E.M. Data were evaluated by analysis of variance (ANOVA), followed by the Duncan test to determine significant differences between the 12 experimental groups and the control group - with additives or no additive after the freezing-thawing process for sperm characteristics using the SPSS/PC version 12.0 software (SPSS, Chicago). Differences with values of $P < 0.05$ were considered to be statistically significant (Daniel 1991).

## Results

The effects of different concentrations of GSSG on sperm characteristics following the freezing-thawing process are presented in Table 1. The anti-oxidant GSSH at 5 mM had a significant ($P < 0.001$) effect in maintaining post-thaw sperm motility, sperm morphology, acrosome integrity, viability and membrane integrity when compared to the control group.

Table 1. Percentages of post-thawing motility, total abnormality, acrosome damage, viability and HOST in frozen-thawed ram spermatozoa (means % ± S.E.M)

| Groups | Motility | Total abnormality | Acrosome damage | Viability | HOST |
|---|---|---|---|---|---|
| Control | 39.5 ± 2.73a | 30.1 ± 5.30b | 13.4 ± 2.32b | 50.7 ± 6.05a | 36.9 ± 4.40a |
| GSSG 5 mM | 60.6 ± 1.63b | 10.9 ± 2.45a | 3.0 ± 0.73a | 78.6 ± 4.48c | 65.2 ± 3.81c |
| GSSG 10 mM | 48.0 ± 2.81a | 12.3 ± 2.50a | 10.7 ± 1.69ab | 74.4 ± 3.99bc | 44.8 ± 6.04ab |
| GSSG 20 mM | 44.5 ± 2.12a | 19.8 ± 3.21a | 8.4 ± 3.41ab | 65.7 ± 4.25b | 49.3 ± 3.72b |
| *P* | *** | ** | ** | * | * |

(a, b, c): Different letters within the same column showed significant differences among the groups (* $P < 0.05$, ** $P < 0.01$, ** * $P < 0.001$)

The effect of the various concentrations of BSA on post-thawing sperm characteristics is set out in Table 2. BSA (20 mg/ml) showed a more significant positive effect than other concentrations of BSA and the control group in protecting sperm characteristics during the freezing-thawing process of ram semen ($P < 0.001$).

Table 2. Percentages of post-thawing motility, total abnormality, acrosome damage, viability and HOST in frozen-thawed ram spermatozoa (means % ± S.E.M.)

| Groups | Motility | Total abnormality | Acrosome damage | Viability | HOST |
|---|---|---|---|---|---|
| Control | 39.5 ± 2.73a | 30.1 ± 5.30b | 13.4 ± 2.32b | 50.7± 6.05b | 36.9 ± 4.40a |
| BSA 5 mg/ml | 45.0 ± 1.29bc | 15.2 ± 2.70a | 9.4 ± 2.95ab | 73.2 ± 7.42a | 41.5 ± 5.87ab |
| BSA 10 mg/ml | 48.5 ± 3.88bc | 17.1 ± 3.93a | 8.6 ± 2.30ab | 69.3 ± 6.18a | 48.5 ± 6.40bc |
| BSA 20 mg/ml | 51.2 ± 3.62c | 11.8 ± 3.21a | 3.6 ± 3.12a | 78.2 ± 6.30a | 55.2 ± 4.21c |
| *P* | ** | *** | * | *** | * |

(a, b, c): Different letters within the same column showed significant differences among the groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Table 3. Percentages of post-thawing motility, total abnormality, acrosome damage, viability and HOST in frozen-thawed ram spermatozoa (means % ± S.E.M.)

| Groups | Motility | Total abnormality | Acrosome damage | Viability | HOST |
|---|---|---|---|---|---|
| Control | 39.5 ± 2.73a | 30.1 ± 5.30c | 13.4 ± 2.32b | 50.7 ± 6.05a | 36.9 ± 4.40a |
| Cysteine 5 mM | 45.0 ± 3.42a | 19.2 ± 4.76b | 11.7 ± 3.17b | 70.5 ± 5.29b | 42.7 ± 5.74a |
| Cysteine 10 mM | 59.0 ± 4.35b | 9.8 ± 4.05a | 2.9 ± 2.52a | 73.5 ± 6.72b | 40.7 ± 4.74 a |
| Cysteine 20 mM | 39.5 ± 3.31a | 14.2 ± 3.52ab | 8.1 ± 4.21ab | 58.6 ± 5.38a | 32.4±5.37 a |
| *P* | *** | *** | ** | *** | - |

(a, b, c): Different letters within the same column showed significant differences among the groups ( ** $P < 0.01$, *** $P < 0.001$)
- : No significant difference ($P > 0.05$)

The effect of the different concentrations of cysteine on the protection of sperm characteristics is set out in Table 3. Cysteine at 10 mM had a significant ($P < 0.001$) effect on maintaining post-thawing sperm characteristics, when compared to the other treatments.

The effects different concentrations of lycopene on sperm characteristics following freeze-thawing process are set out in Table 4. Lycopene at 800 µg exhibited significantly ($P < 0.001$) the best post-thawing spermatological indicators when compared to the other groups.

Table 4. Percentages of post-thawing motility, total abnormality, acrosome damage, viability and HOST in frozen-thawed ram spermatozoa (means % ± S.E.M.)

| Groups | Motility | Total abnormality | Acrosome damage | Viability | HOST |
|---|---|---|---|---|---|
| Control | 39.5 ± 2.73a | 30.1 ± 5.30b | 13.4 ± 2.32a | 50.7 ± 6.05b | 36.9 ± 4.40a |
| Lycopene 800 µg | 57.2 ± 2.91b | 16.3 ± 2.53a | 7.3 ± 2.61a | 70.5 ± 6.91c | 49.1 ± 3.18b |
| Lycopene 1600 µg | 46.5 ± 3.00ab | 25.3 ± 5.04 ab | 12.6 ± 4.62a | 61.5 ± 6.29ac | 55.1 ± 6.12b |
| Lycopene 3200 µg | 33.0 ±3 .09a | 26.2 ± 5.87ab | 11.5 ± 2.68a | 39.4 ± 6.16a | 29.16 ± 3.72a |
| *P* | *** | ** | - | ** | *** |

(a, b, c): Different letters within the same column showed significant differences among the groups (** $P < 0.001$, *** $P < 0.01$)
- : No significant difference ($P > 0.05$)

## Discussion

The sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore susceptible to peroxidative damage with consequent loss of membrane integrity, decreased sperm motility, and eventually loss in fertility, resulting from reactive oxygen species

during aerobic incubation (Alvarez et al. 1987). Therefore, free radicals must be eliminated by supplementation with antioxidants such as GSH, cysteamine and taurine during the freezing-thawing or liquid storage of semen (Bucak and Tekin 2007 in press; Bucak et al. 2007).

Cryopreservation alters the membrane sulfhydryl status of spermatozoa. GSSG reduces the mobility of sulfhydryl-containing proteins in the sperm membrane. Sulfhydryl groups are under redox control and a change in the redox status of the membrane can be linked to the ROS production that occurs during cooling and freezing-thawing of spermatozoa (Mazur et al. 2000). The GSH/GSSG pair plays important roles both as a redox sensor and protector against ROS induced damages in many cell types (Halliwell and Gutteridge 1998).

Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione, as the electron donor, removes peroxyl (ROO.) radicals from various peroxides including H2O2. Glutathione reductase then regenerates reduced GSH from GSSG as shown in the following equation:

2 GSH + $H_2O_2$ peroxidase-(Se) GSSG + 2 $H_2O$
GSSG + NADPH + H+ reductase 2 GSH + NADP+ (Calvin et al. 1981)

Cryopreservation of spermatozoa reduced the GSH level 5-fold, without increasing the GSSG level, which could suggest that the GSH content of spermatozoa may be transported out of the sperm cells (Bilodeau et al. 2000). Therefore, upon addition to the extender, GSSG partially prevents the loss of sperm motility after a freezing/thawing cycle, whereas GSH does not. GSSG, but not GSH, prevents increase in the mobility of sulfhydryl containing proteins due to the freezing/thawing of spermatozoa (Chatterjee et al. 2001). GSSG also interacts with two vicinal free cysteine residues of the active site in protein tyrosine phosphatases and inactivates these enzymes (Gabitta et al. 2000). Such a mechanism could explain the positive effects exerted by GSSG (Hammerstedt et al. 1990).

In the present study, it was observed that post-thawing *in vitro* results from whole concentrations of GSSG were much better than the control group. The concentration of 5 mM GSSG was determined to exert the best protective effect on post-thawing sperm motility, sperm morphology, acrosome integrity, viability and membrane integrity in ram semen. Higher concentrations of GSSG (10 or 20 mM) may have exhibited negative or toxic effect on post-thawing spermatological indicators when compared to the 5 mM concentration of GSSG.

BSA is known to eliminate free radicals generated by oxidative reactions, and therefore to protect the membrane integrity of sperm cells from lipid peroxidation during the semen freezing process (Lewis et al. 1997). In our investigation, various concentrations of BSA were observed to improve post-thawing sperm motility and viability, and to protect acrosome and membrane integrity, but the best results were obtained from 20 mg/ml of BSA. It can be concluded that concentrations lower than 20 mg/ml of BSA are not sufficient to protect sperm cells from lipid peroxidation. Post-thawing sperm motility (51.2%), morphologic sperm abnormalities (11.8%), acrosome damage (3.6%), and viable sperm (78.2%) were determined for 20 mg/ml BSA in our study, which are close to the findings of Matsuoka et al. (2006).

Thiol compounds, such as cysteine, are precursors of intracellular glutathione biosynthesis, and cysteine protects sperm cells from toxic oxygen metabolites causing lipid peroxidation of sperm plasma membranes under *in vitro* conditions (Meister and Tate 1976). Funahashi and Sano (2005) reported that a semen extender with 5 mM cysteine improved the viability and membrane integrity of boar sperm cells during liquid storage. On the contrary, in the current study, the best post-thawing spermatological indicators were obtained from goat semen frozen with 5 mM cysteine.

Carotenoids such as beta-carotene and lycopene are also important components of antioxidant defense (Gupta and Kumar 2002). Beta-carotenes protect the plasma membrane against lipid peroxidation (Di Mascio et al. 1989). In this study, the effect of lycopene was similar to results obtained by other researchers, who reported that epididiymal sperm characteristics were protected in rat testes treated by lycopene (Ateşşahin et al. 2006) and structural and functional damage in the testicular tissue and sperm quality of rats caused by oxidative stress were prevented by lycopene supplementation (Türk et al. 2006). However, according to our findings, a lycopene concentration of 3200 μg was determined to lead to deleterious effects on spermatological indicators including a decline in sperm motility, and an increase in sperm abnormality, acrosome damage and dead sperm.

In conclusion, this study demonstrated that supplementation with antioxidants of semen diluents, depending on various concentrations, during semen cryopreservation attempts, may exert beneficial effects on the quality of the freezing-thawing of ram semen. This study has shown that many aspects of sperm protection, e.g. sperm motility, viability and membrane stabilisation of sperm cells during relative cryopreservation, are of prime importance, the antioxidants GSSG (5 mM), BSA (20 mg/ml), cysteine (10 mM) and lycopene (800 μg) provided a near-optimal concentration for improved sperm survival during the freezing-thawing process. The results of this study therefore provide a new approach to the cryopreservation of sperm from rams of different breeds, and so contribute to the improvement of these breeds for the world sheep industry. Further studies are necessary to obtain results to confirm present findings.

## Účinek oxidovaného glutathionu, bovinního sérového albuminu, cysteinu a lycopenu na kvalitu rozmrazeného semene beranů

Volné radikály se účastní peroxidace lipidů stejně tak jako poškozování DNA a membrány spermií, což může vést ke snížení motility spermií nebo smrti buňky. Rovnováha mezi produkcí radikálů a jejich zneškodňováním může být důležitým faktorem pro životaschopnost spermií a jejich funkci před, během a po kryokonzervaci. Cílem této studie bylo zjistit vliv přídavku antioxidantů (oxidovaný glutathion (GSSG), bovinního sérového albuminu (BSA), cysteinu a lycopenu) do konzervačního média na vlastnosti spermatu po rozmražení, jako jsou například motilita, morfologie, integrita akrozomu, životaschopnost a integrita membrány. Celkový počet 42 ejakulátů byl odebrán na umělou vagínu od čtyř beranů plemene Akkaraman a 10 párových vzorků ejakulátů bylo zředěno Tris pufrem s přísadami. Vzorky zředěné Tris pufrem bez přísad sloužily jako kontrola. Po rozmrazení se ukázalo, že nejlepší účinek ze všech koncentrací ve vzorcích a kontrolách na vlastnosti semene měly GSSG (5 mM), BSA (20 mg·ml$^{-1}$), cystein (10 mM) a lycopene (800 μg) ($P < 0{,}001$). Četné aspekty ochrany spermií, jako např. jejich motilita, životaschopnost a stabilizace membrány spermií během kryokonzervace, jsou klíčovými faktory pro uchování funkce spermií. Výsledky této studie předkládají nový přístup ke kryokonzervaci spermatu beranů a příbuzných plemen a tak přispívají ke zlepšení těchto plemen pro světovou produkci ovcí.

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