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Effect of Nonenzymatic Antioxidants on Sperm Motility and Survival Relative to Free Radicals and Antioxidant Enzymes of Chilled-Stored Ram Semen

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Abstract

The effect of inclusion of three antioxidants (Vitamin E, cysteine and glutathione) in ram semen extender on the release of antioxidant enzymes and free radicals was studied. A 3 × 3 factorial experiment was conducted to test effects of supplementing ram semen extender with Vitamin E (1, 5 and 10 IU), cysteine (1, 5 and 10 mM) or glutathione (0.5, 1 and 2 mM) on the sperm survival and release of superoxide dismutase (SOD), glutathione peroxidase (GPX) and thiobarbituric acid reactive substances (TBARS). Eighty ejaculates of eight fertile Najdi rams were collected, assessed for the gross examination and the good ejaculates (≥90% motility) were pooled and sperm count was assessed. Therefore ten extenders; control (C) and 9 treated (C plus antioxidant) were tested. Extended semen was stored at 5˚C for 96 h, examined for motility and survival and sperm cells were separated from plasma, sonicated, homogenized and exposed to the determinations of SOD, GPX and TBARS. The highest sperm survival was found in diluents containing 5 IU Vitamin E/ml, 1 and 2 mM glutathione (55.5% survival), while the lowest survival was found in 10 mM cysteine (11.1%). TBARS concentration was highest ($P < 0.05$) in control than other treatments, however, Vitamin E and glutathione exhibited low values. Contrariwise, activities of SOD and GPX increased ($P < 0.05$) within sperm cells and seminal plasma in diluents containing 5 IU E, 1 mM and 2 mM glutathione. The enzymatic activities were generally higher in seminal plasma than in sperm cells. It was concluded that supplementing ram semen extender during chilled storage with 5 IU Vitamin E per ml or 1 - 2 mM glutathione enhanced sperm survival and reduced free radicals.

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Keywords

Ram, Semen Storage, Free Radicals, Antioxidant Enzymes

1. Introduction

Semen contains various unsaturated fatty acids which are oxidized during preservation resulting in reactive oxygen species (ROS). These oxygen species are very active on the cellular level resulting in various degrees of damage to the sperm cells. Sperm cells are very susceptible to lipid peroxidation by free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radical which could later lead to the structural damage of sperm membranes during the aerobic storage of sperm [1]. Cold shock during semen preservation poses stress on the sperm cell membrane via the free radicals leading to sperm damage [2] [3]. The mechanism by which the free radicals do their damage was ascribed to their ability to catch the electrons from the nucleic acids, lipids and proteins causing the cell damage [4]. Much work has been done to protect the integrity of the sperm cells of different species from physical and chemical damage during processing and preservation. Enzymatic and non enzymatic antioxidants have been tested on this aspect. The ROS can be neutralized by an antioxidant system, comprised of reduced glutathione (GSH), GPX, catalase (CAT) and SOD. Added to that the various non-enzymatic molecules such as glutathione, thioredoxin and other thiol-containing molecules, as well as Vitamins D, E and C which serve as a defense mechanism against the lipid peroxidation of semen and maintaining sperm motility and viability. Vitamin E (α-tocopherol) was found to owe the ability to maintain a steady-state rate of peroxyl radical reduction in the plasma membrane depending on the recycling of α-tocopherol by external reducing agents such as ascorbate or thiols [5] [6].

Glutathione is a tripeptide naturally occurring in semen and providing intracellular defense to the sperm against the oxidative stress caused by an over-production of ROS during the freezing and thawing process. Glutathione supplementation in semen extender up to a level of 2 mM was found to be protective against the damaging effects of free radicals, beyond that no protective effects were found in bull spermatozoa [7]. The addition of glutathione to the semen extender prevented the loss of sperm motility by inhibition of lipid peroxidation caused by ROS in frozen thawed bull semen [7] and goats [1]. Moreover, glutathione increased the viability, plasma membrane and acrosomal integrity after thawing buffalo spermatozoa [8].

This antioxidant alters the available free radicals to molecules that have less negative impacts on the cell. Its addition to semen diluents is expected to decrease or prevent the emergence of free radicals that can ruin the plasma membranes [9]. Other compounds containing sulfhydryl groups as cysteine and ergothionein play an important role in maintaining metabolic functions and cell motility of sperm [10]. It has also been shown that addition of L-cysteine up to 2.0 mM in Tris-citric acid extender improved the post-thaw quality of Sahiwal bull semen [11]. Therefore, it was the objective of the current study to evaluate effects of supplementing Najdi ram semen extender with Vitamin E, cysteine or glutathione at various concentrations on sperm motility and survival and on the releases of free radicals and antioxidant enzymes within sperm cells and seminal plasma.

2. Materials and Methods

2.1. Animals and Semen Collection

Eight healthy mature Najdi rams (2 - 3 years of age) were used in this study. The rams were fed on a traditional diet of barley (1 kg/head/day) and alfalfa hay, housed in semi-shaded yards and offered clean drinking water and integrated salt licks as free choice. The study was carried out at Qassim University Experimental Station from September 2013 to January 2014.

Semen was collected twice a week for five consecutive weeks. A total number of 80 ejaculates were collected from the rams using an artificial vagina (10 ejaculates/ram). At semen collection, rams were exposed to estrous ewes and semen was collected after teasing the ram for two times false mounts. Ejaculates were evaluated and included in the study if the following criteria were met: volume of 0.5 - 2 ml and the progressive motile sperms percentage was equal to or higher than 90%. Semen ejaculates of good quality were pooled to avoid the ram effect and equally distributed into 10 diluents.

2.2. Semen Processing and Dilution

2.2.1. Basic Semen Extender

The basic extender (control) comprised of 3.63 g Tris (hydroxy methyl aminometahne), 0.50 g fructose, 1.99 g citric acid (monohydrate), 5 ml egg yolk, 1 ml antibiotics solution containing 100,000 IU penicillin and 100,000 µg streptomycin and glass-distilled water up to 100 ml and pH was adjusted to 6.8 - 7.

2.2.2. Antioxidants-Containing Extenders

The three antioxidants (Vitamin E, cysteine and glutathione) were formulated to be tested at three different levels as follow:

1) Vitamin E at 1, 5 and 10 IU/ml;
2) Cysteine at 1, 5 and 10 mM;
3) Glutathione at 0.5, 1 and 2 mM.

Therefore, control and 9 antioxidant-containing extenders were prepared for semen dilution. Treatment groups (G) were; G1 (Control, basic diluents), G2 (basic diluents containing 1 IU vitamin E/ml), G3 (basic diluents containing 5 IU vitamin E/ml), G4 (basic diluents containing 10 IU vitamin E/ml), G5 (basic diluents containing 1 mM Cysteine), G6 (basic diluents containing 5 mM Cysteine), G7 (basic diluents containing 10 mM Cysteine), G8 (basic diluents containing 0.5 mM Glutathione), G9 (basic diluents containing 1 mM Glutathione) and G10 (basic diluents containing 2 mM Glutathione).

2.3. Semen Dilution, Preservation and Evaluation

Good quality semen ejaculates were pooled and progressive motility and sperm count were assessed. The pooled sample was divided into 10 aliquots to avoid the ram effect. One volume of raw semen was added to 5 equal volumes of the designed extender. Extended semen samples (37˚C) surrounded by warm water jacket (37˚C) were gradually cooled to 5˚C in the refrigerator and stored for 96 hours. A semen sample was taken out after 96 h of storage, then warmed to 37˚C and checked for progressive motility, viability and abnormality.

2.4. Semen Evaluation

Sperm motility was examined by a phase contrast microscope. To evaluate this parameter, one drop of semen was placed on a warm (37˚C) stage and spermatozoa with progressive motility counted used X20 objective lens. Sperm viability was assessed by Eosin Y. (0.5%)—Nigrosin (0.1%) staining mixture. Dead cells were stained by the Eosin, whereas the live cells retained their cellular membrane integrity preventing the stain to enter cells. Nigrosin served as a background stain to provide contrast for the unstained (white) live cells. A total of 200 sperm were assessed under oil immersion with a high-resolution (X100) objective equipped with correct adjustment of the bright field optics. Unstained spermatozoa which appeared white were classified as “live” and those that show any pink or red color were classified as “dead” [12]. Sperm survival after 96 h storage was estimated by dividing the percentage of progressive motility at 96 h by the percentage of initial progressive motility.

2.5. Sperm Cells Sonication

At the 96 h storage, extended semen was centrifuged (~3000 rpm/15min/5˚C). Diluted seminal plasma was aspirated and transferred to a labeled tube and pellet of the sperm cells was resuspended in normal saline solution at a dilution rate resulting in a concentration of 1×10^9 sperm/ml. Sperm cells were exposed to 2 cycles of sonication (Q-Sonic7, USA) at 1350 rpm for 49 sec each [13]. Seminal plasma and sperm cellular contents were used later in the determination of free radicals and antioxidant enzymes.

2.6. Superoxide Dismutase (SOD) Activity Determination

The method of Marklund *et al.* was adopted to determine SOD activity by the commercial kit (SOD Assay kit, Cayman Chemical Co., Ann Arbor, MI, USA) [14]. This method utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals. In this assay, total SOD was measured. Briefly, the assay procedure was done by pipetting 200 µl of the diluted radical detector and 10 µl standard or

samples into each well. The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all wells except in background wells, 20 µl sample buffer replaced xanthine oxidase. The plate was covered and gently shaken for a few seconds to thoroughly mix the samples and incubated on a shaker for 20 minutes at room temperature. Absorbance was read at 450 nm using a plate reader (Mindray, China). Activity of SOD in samples was derived from the standard curve using the equation obtained from the linear regression of the standard curve substituting the linear rate (LR) for each sample. Enzyme activity (U/ml) was attributed to each milliliter of seminal plasma and to each 1×10^9 sperm in the sperm suspension.

2.7. Glutathione Peroxidase (GPX) Activity Measurement

According to Paglia and Valentine [15], the GPX activity was assessed by using a commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA). The procedure depends on measurement of GPX activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) produced upon reduction of hydroperoxide by GPX, and is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in the absorbance at 340 nm. Under a condition in which the GPX activity is rate limiting, the rate of decrease in the absorbance is directly proportional to the GPX activity in the sample. The GPX activity in seminal plasma was expressed as nmol/min/ml, and in sperm cells as nmol/min/10^9 spermatozoa.

2.8. Lipid Peroxidation (LPO) Measurement

The LPO levels in seminal plasma and sperm suspension were determined according to Armstrong and Browne [16] using a commercial kit (TBARS Assay kit, Cayman Chemical Co., Ann Arbor, MI, USA). The method was performed on the basis of the reaction of the thiobarbituric acid (TBA) with malondialdehyde (MDA) under high temperature (90˚C - 100˚C), then the resultant color was measured at 540 nm. The sample concentration of TBARS was calculated from the standard curve and expressed as µM MDA/ ml seminal plasma or µM MDA/10^9 spermatozoa.

2.9. Statistical Analysis

Data of sperm motility and survival were analyzed by the least square analysis of variances (ANOVA, SAS 2000) [17]. Data of TBARS and enzymatic activities were subjected to one-way analysis of variances. Differences between treatments were compared by the Duncan Multiple Range Test (DMRT) [18]. Significant level was considered at $P < 0.05$.

3. Results

3.1. Semen Characteristics

The highest post-thaw motility and sperm survival (50% and 55.5%) 96 h after storage at 5˚C (**Table 1**) were attained in the basal diluents containing 5 IU Vitamin E, 1 mM and 2 mM glutathione. Likewise, the correspondent percentages of live spermatozoa were 65%, 65% and 68%, respectively. Contrariwise, percentages of sperm abnormalities for the three previous treatments were 20%, 15% and 15%, respectively. The lowest ($P < 0.05$) survival (11.1%, **Table 1**) was found in the basal diluents containing 10 mM cysteine followed by 5 mM cysteine. The only improvement on sperm survival that cysteine has done over the control (22.2%) was found at 1 mM concentration (33.3%). Inclusion of Vitamin E up to 5 IU/ml in the diluents improved the viability and survival of ram sperm. Beyond 5 IU Vitamin E, there appears to exert adverse effects on sperm viability and survival (**Table 1**).

3.2. Free Radicals

Free radicals concentrations (**Table 2**) considerably decreased ($P < 0.05$) in all treatments compared with control. The least TBARS concentrations were found in diluents contained 1 and 5 IU Vitamin E and in all glutathione containing-diluents. Cysteine-containing diluents resulted in intermediate levels of TBARS. There found significant ($P < 0.05$) low levels of TBARS in ram sperm cells than in seminal plasma (**Table 2**) in all treatments. The magnitude of the TBARS concentration in control seminal plasma was 2.9 folds that found in sperm cells.

Table 1. Effect of type and concentration of antioxidant in ram semen extender on percentage of sperm progressive motility, abnormality, livability and survival rate after 96 h storage at 5˚C (LSM ± SEM)[**].

Antioxidant-Extender	% Initial Motility	% Progressive Motility after 96 h (5˚C)	% Live Sperm after 96 h (5˚C)	% Abnormality after 96 h (5˚C)	% Sperm Survival
Control (C)[*]	90	$20^{a} \pm 5.0$[**]	$30^{a} \pm 4.0$	$40^{a} \pm 3.0$	22.2
C + 1 IU E/ml	90	$40^{b} \pm 4.0$	$50^{b} \pm 5.0$	$30^{b} \pm 3.0$	44.4
C + 5 IU E/ml	90	$50^{b} \pm 5.0$	$65^{c} \pm 5.0$	$20^{c} \pm 5.0$	55.5
C + 10 IU E/ml	90	$30^{c} \pm 2.0$	$40^{a} \pm 5.0$	$25^{b} \pm 3.0$	33.3
C + 1 mM Cyst.	90	$30^{c} \pm 3.0$	$35^{a} \pm 4.0$	$30^{b} \pm 4.0$	33.3
C + 5 mM Cyst.	90	$20^{a} \pm 3.0$	$30^{a} \pm 3.0$	$30^{b} \pm 5.0$	22.2
C + 10 mM Cyst.	90	$10^{d} \pm 2.0$	$15^{d} \pm 3.0$	$40^{a} \pm 5.0$	11.1
C + 0.5 mM Glut.	90	$40^{b} \pm 5.0$	$50^{b} \pm 3.0$	$20^{c} \pm 5.0$	44.4
C + 1 mM Glut.	90	$50^{b} \pm 5.0$	$65^{c} \pm 5.0$	$15^{c} \pm 2.0$	55.5
C + 2 mM Glut.	90	$50^{b} \pm 4.0$	$68^{c} \pm 5.0$	$15^{c} \pm 2.0$	55.5

[*]Control extender: Tris-Citric-Fructose-Egg Yolk; E = Vitamin E; Cyst. = Cysteine; Glut. = Glutathione. [**]Means in the same column with different superscripts significantly differ ($P < 0.05$).

Table 2. Effect of type and concentration of antioxidant in ram semen extender on the free radicals concentration and antioxidant enzyme activity after 96 h storage at 5˚C (LSM ± SEM)[**].

Antioxidant-Extender	TBARS (μMMDA)		SOD (U)		GPX (nmol/min)	
	Sperm ($/10^9$)	Plasma (/ml)	Sperm ($/10^9$)	Plasma (/ml)	Sperm ($/10^9$)	Plasma (/ml)
Control (C)[*]	$15.13^{a} \pm 0.45$	$43.68^{a} \pm 3.5$	$15.9^{a} \pm 3.7$	$32.8^{a} \pm 5.6$	$12.5^{a} \pm 2.6$	$22.1^{a} \pm 3.2$
C + 1 IU E/ml	$0.12^{b} \pm 0.06$	$22.86^{b} \pm 3.9$	$25.8^{a} \pm 6.7$	$43.5^{a} \pm 7.8$	$15.6^{a} \pm 2.2$	$35.4^{b} \pm 5.3$
C + 5 IU E/ml	$0.12^{b} \pm 0.03$	$18.78^{b} \pm 4.5$	$47.6^{b} \pm 4.5$	$89.5^{b} \pm 4.6$	$96.8^{b} \pm 6.5$	$137.9^{c} \pm 7.4$
C + 10 IU E/ml	$2.13^{c} \pm 0.19$	$28.01^{c} \pm 6.1$	$20.7^{a} \pm 5.1$	$29.8^{a} \pm 6.7$	$20.8^{a} \pm 8.5$	$30.4^{b} \pm 5.5$
C + 1 mM Cyst.	$1.14^{c} \pm 0.13$	$12.78^{b} \pm 2.6$	$22.6^{a} \pm 6.1$	$36.1^{a} \pm 3.9$	$17.9^{a} \pm 7.6$	$26.8^{b} \pm 5.1$
C + 5 mM Cyst.	$0.94^{bc} \pm 0.22$	$17.28^{b} \pm 3.1$	$16.7^{a} \pm 3.2$	$34.4^{a} \pm 5.3$	$14.5^{a} \pm 6.6$	$22.6^{a} \pm 2.3$
C + 10 mM Cyst.	$2.59^{c} \pm 0.31$	$31.58^{c} \pm 3.8$	$15.7^{a} \pm 4.9$	$38.6^{a} \pm 4.5$	$13.8^{a} \pm 8.1$	$27.9^{b} \pm 6.8$
C + 0.5 mM Glut.	$0.13^{b} \pm 0.04$	$10.17^{b} \pm 4.9$	$31.1^{c} \pm 3.9$	$45.6^{a} \pm 3.6$	$20.7^{a} \pm 7.7$	$40.2^{b} \pm 2.9$
C + 1 mM Glut.	$0.37^{b} \pm 0.09$	$9.12^{b} \pm 2.1$	$57.4^{b} \pm 6.5$	$101.8^{c} \pm 9.1$	$89.9^{b} \pm 4.5$	$143.6^{c} \pm 5.1$
C + 2 mM Glut.	$0.18^{b} \pm 0.12$	$0.26^{d} \pm 0.1$	$66.7^{b} \pm 3.5$	$120.5^{c} \pm 9.2$	$130.4^{c} \pm 3.5$	$156.9^{c} \pm 6.5$

[*]Control extender: Tris-Citric-Fructose-Egg Yolk; E = Vitamin E; Cyst. = Cysteine; Glut. = Glutathione. [**]Means in the same column with different superscripts significantly differ ($P < 0.05$).

However, this magnitude approached about 19 folds in treated samples.

3.3. Antioxidant Enzymes

Superoxide dismutase (SOD) activity was only significantly higher ($P < 0.05$) in antioxidant-supplemented diluents with 5 IU Vitamin E/ml and 1 and 2 mM glutathione compared with control (**Table 2**). The highest SOD activity either in seminal plasma (120.5 ± 9.2 U/ml) or in sperm cells (66.7 ± 3.5 U/10^9 sperm) was obtained in diluents containing 2 mM glutathione with no difference than in 1 mM glutathione. The second high level of SOD (89.5 ± 4.6 U/ml plasma and 47.6 ± 4.5 U/10^9 sperm) was observed in the 5 IU Vitamin E—diluent. Cysteine at all tested levels and Vitamin E at 10 IU didn't enhance the SOD activity than the control diluents.

Similar trend to what was found in SOD activity was obtained with GPX activity (**Table 2**). Also, the best GPX activity was found in the diluents contained 5 IU Vitamin E, 1 and 2 mM glutathione. Cysteine at all molarities didn't enhance this enzyme activity over the control.

4. Discussion

Due to the high speed of the sperm, depending on the generation of energy by the mid-piece mitochondrial oxidative phosphorylation, a high concentration of free radicals are produced inside and outside the sperm cells [19] [20]. The increase of the production of ROS might damage the sperm cell membrane resulting in lower sperm motility and survival after storage at low temperatures [21] which leads to diminishing the sperm penetration of the cervical mucus *in vitro* [22]. Addition of either enzymatic or non-enzymatic specific antioxidants would impact a beneficial reduction to the free radicals. In the current study, three non-enzymatic antioxidants were chosen to be tested at 3 levels each. Vitamin E (α-tocopherol) and Vitamin C (ascorbic acid) were traditionally used for a long time as antioxidants. Vitamins E and C were considered as electron trapping molecules.

Vitamin E is considered to be the main component of the antioxidant system of spermatozoa, one of the major protectors of the membranes against ROS and lipid peroxidation attack [23]. Because of its solubility in lipids, vitamin E might serve as the first line of defense against the peroxidation of the polyunsaturated fatty acids on the membranous phospholipids structure [24] [25]. The present study concluded that vitamin E concentration in ram semen extender must not exceed 5 IU/ml to achieve the best post-thaw motility and survival with the highest antioxidant enzyme activity. At a similar level, Anghel *et al.* found better post-thaw sperm parameters of frozen ram semen [26]. Also, Michael *et al.* found protective effects of low level of Vitamin E (0.3 mM) on frozen dog sperm [27]. Also, the concentration of Vitamin E up to 1.0 mM offer protection against membrane oxidative stress of frozen ram sperm [28].

Natural compounds containing thiol group are considered precursors of intracellular reduced glutathione biosynthesis. Addition of cysteine to the semen extender prevents loss of sperm motility by inhibition of lipid peroxidation caused by ROS in frozen-thawed bull semen [29]. In the current study, cysteine at low level decreased the release of malondialdhyde (TBARS) and increased the activity of SOD and GPX. This effect was attributed to the protective effect of cysteine on the sperm cellular level preventing the toxic oxygen species from causing lipid peroxidation [30]. Supplementing the freezing media of goat and ram semen by cysteine enhanced post-thaw motility [31]-[33] and improved membrane integrity of boar sperm [34]. Using high levels of cysteine did not protect goat sperm membrane and sperm viability during freezing [35]. Uysal and Bucak demonstrated that increasing doses of cysteine (15 mM) decreased post-thaw sperm abnormality and increased acrosomal damage in rams [31]. The best sperm survival in the current tested cysteine levels was found at the lowest concentration (1 mM). Above this level, there found a significant ($P < 0.01$) decline in sperm survival accompanied with increased free radicals and decreased antioxidant enzyme activity. Recently, Kledmanee *et al.* showed that the protective effect of cysteine on carp chilled sperm was attained by using levels up to 1 mM cysteine, however above this level there found deleterious effects on sperm characteristics [36]. This observation was also confirmed on post-thaw quality of Sahiwal bull semen [11].

Kubovicova *et al.* found that addition of glutathione (but not caffeine) has a positive effect on fertilizing ability of ram spermatozoa [37]. Moreover, the cycle of freezing and thawing has been reported to be responsible for a decrease in the level of antioxidants such as glutathione (GSH) or superoxide dismutase (SOD) in human and bovine spermatozoa [29] [38]. This decrease in antioxidants would enable ROS to cause sperm damage during the freezing process. The determinant role of superoxide dismutase (SOD) in the antioxidant defense systems has been known since 1968. It is well known that superoxide ion (O^{2-}) is the starting point in the chain production of free radicals. At this early stage, superoxide dismutase inactivates the superoxide ion by transforming it into hydrogen peroxide (H_2O_2). The latter is then quickly catabolised by catalase and peroxidases into dioxygen (O_2) and water (H_2O). Different studies have confirmed that the production of H_2O_2 under the action of SOD is the triggering factor in the natural antioxidant defense mechanisms. SOD therefore seems to be the key enzyme in the natural defense against free radicals [39]. In the current study, the significant increases of SOD and GPX activities coinciding with the high post-thaw sperm survival in the glutathione-supplemented ram semen were confirmed in the early finding by Michiels *et al.* [40]. The mechanism by which glutathione plays its role is attributed to that the enzyme glutathione peroxidase reduces lipidic or non-lipidic hydroperoxides and H_2O_2 during the oxidation of two molecules of glutathione. Since GSH serves as a hydrogen donor, the enzyme

GPx functions linearly. As GSH concentration increases the reaction accelerates and the sperm cell protection improves [41].

5. Conclusion

For the best protection against the increased free radicals production during chilling preservation of ram semen, extenders must contain 1 - 2 mM glutathione or 5 IU Vitamin E per ml. The intensive production of free radicals in seminal plasma was counteracted by the inclusion of vitamin E or glutathione at the above mentioned concentrations. However, the supplementation of ram semen extender with cysteine hasn't showed the desirable protective effects. Further studies are required to test the impacts of inclusion of such antioxidants in the freezing media on subsequent sperm fertilization *in vivo*.

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