

INFLUENCE OF VITAMIN E ON MICROSCOPIC AND OXIDATIVE PARAMETERS OF FROZEN-THAWED RAM SEMEN

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Abstract

Cryopreservation is associated with the production of reactive oxygen substances (ROS), which lead to lipid peroxidation of sperm membranes, resulting in a loss of sperm motility, viability and fertility. The aim of this study was to assess the effects of vitamin E adding to the dilution media on standard qualitative parameters (motility, viability, HOST) and oxidative parameters (lipid peroxidation) of frozen-thawed ram semen. Ejaculate samples were collected with artificial vagina from 2 adult Merino Palas rams, on normal season of reproduction (October-December 2009) and diluted with a Tris-base extender containing vitamin E (1 and 2mM) and without antioxidants (control). Diluted semen was cooled to 4°C and frozen in 0.25 ml straw, prior to being stored in liquid nitrogen. Semen parameters including motility, the plasmatic membrane structural integrity (viability by eosin-nigrosin stain), functional membrane integrity (hypoosmotic test) and lipid peroxidation (LPO) were investigated after thawing. Lipid peroxidation level was examined by measuring the production of malondialdehyde using the thiobarbituric acid method. The results show that vitamin E has positive protection effects on the semen characteristics consequently to the freezing-thawing process. Both doses of vitamin E increased the percentage of sperm motility and viability ($p < 0.05$), but reduced the lipid peroxidation (LPO) level. Most effective occur at the 2mM dose, though differences among the doses were non-significant ($p \geq 0.05$).

Key words: ram, semen, cryopreservation, vitamin E, lipid peroxidation

INTRODUCTION

Sperm cryopreservation contributes to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro fertilization. Sperm cryopreservation involves several steps such as cooling, freezing and thawing [17]. Each of these steps can cause sperm damage to the plasma membrane which impairs normal sperm structure and function [14], reduces motility and the fertilizing ability of spermatozoa [18] and induces premature capacitation and nuclear decondensation [12]. Even in the presence of cryoprotectants such as glycerol and egg yolk, significant structural alterations take place.

In recent years, antioxidants have been tested in combination with basic common cryoprotectants to minimise the damage caused by freezing and thawing. The beneficial effects of antioxidants provide

indirect evidence that an oxidative stress occurs during cryopreservation [1, 4, 22]. This has been confirmed by studies which showed that reactive oxygen species (ROS) are produced during freezing and thawing of bull [9, 11], ram [19] and equine spermatozoa [6].

One of the major biological processes associated with ROS is lipid peroxidation (LPO). Mammalian sperm cells are particularly susceptible to lipid peroxidation due to the fairly low activity of the enzymatic anti-oxidative system and because cellular and intracellular sperm membranes are rich in polyunsaturated fatty acids, that are easily peroxidisable. Unlike somatic cells that rely on cytoplasmic enzymes, such as catalase, superoxid dismutase and glutathione peroxidase, for their antioxidant defense, spermatozoa discharge most of their cytoplasm immediately before spermiation

and, as a consequence, lose this protection [16]. Lipid peroxidation triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and structural damage to DNA, and cell death. In view of the importance of lipid peroxidation in defective sperm function, quantification of this process is of some diagnostic significance [15]. At present, the most widely used assay for lipid peroxidation involves the measurement of malondialdehyde (MDA), a small molecular mass degradation product of peroxidative process that can be measured by virtue of its capacity to form adducts with thiobarbituric acid [3].

Vitamin E is believed to be the primary component of the antioxidant system of the spermatozoa and is one of the major membrane protectants against ROS and LPO attack [30]. It appears to be the first line of defence against the peroxidation of polyunsaturated fatty acids contained in the cellular and sub-cellular membrane phospholipids because of its lipid solubility [7, 21].

The objective of this study was to determine the efficacy of vitamin E, a biological antioxidant, in reversing the free radical-mediated oxidative damage induced by freezing-thawing process, on sperm motility, viability and lipid peroxidation.

MATERIALS AND METHODS

1. Animals and semen collection

Semen samples from 2 mature Merino of Palas rams (2 and 3 years of age) (A, B), with proven fertility, were used in this study. The rams, belonging to the Palas Constanta Research and Development Institute for Sheep and Goat Breeding, were maintained under uniform feeding, housing and lighting conditions. A total number of 36 ejaculates were collected 2 times weekly by artificial vagina, during the breeding season (October – December 2009). The ejaculates were evaluated and accepted for experiments if the following criteria were met: volume greater than 0.75ml; minimum sperm concentration of 3×10^9 sperm/ml; motility higher than 80%. Immediately after collection, the ejaculates were placed in a water bath (37°C), until

evaluation in the laboratory. Semen assessment was performed within 10 minutes.

2. Semen processing

In this study a Tris-based extender (Salomon's) was used (375mM Tris; 124 mM citric acid; 41.6mM glucose, 20% (v/v) egg yolk, 5% (v/v) glycerol, pH=6.8). After evaluation, the ejaculates of each ram were divided into 3 equal aliquots and diluted with Tris-based extender (control) and extender supplemented with 1.0mM and 2.0mM vitamin E, to a final concentration of approximately 4×10^8 spz/ml.

Processing of semen was made according to the cryopreservation technology developed in the Reproduction Biotechnologies Laboratory [29]. Diluted semen samples were drawn into 0.25 ml French straws (Minitub, Germany), sealed with polyvinyl alcohol powder and equilibrated at 4°C for a period of 2. After equilibration, the straws were frozen in liquid nitrogen vapours and then stored in liquid nitrogen (-196°C). The straws were thawed individually in a water bath (37°C), for 30s. Sperm evaluation was performed on all semen samples immediately after thawing.

3. Semen evaluation

Progressive motility as an indicator of semen quality was assessed using a phase-contrast microscope (Novex, Holland) (x100 magnification), fitted with a warm stage at 37°C. Sperm motility estimation was performed in 3 different microscopic fields for each semen sample and the mean of the 3 successive estimations recorded as the final motility score [8].

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. The test was performed by incubating 30μl semen with 300 μl hypo-osmotic solution (100mOsm) at 37°C for 60 min. After incubation 0.2 ml of the mixture was spread with a cover slip on a warm slide. At least 400 spermatozoa were evaluated using bright-field microscopy on a Novex microscope (x1000 magnification) [10, 27].

The viability was evaluated by eosin-nigrosin staining. At least 200 sperm cells

were assessed at a magnification of 1000x under oil immersion. The white sperm (unstained) was classified as alive and that that showed any pink coloration was classified as dead [28].

Measurement of lipid peroxidation: MDA levels were analyzed according to the thiobarbituric acid methods, described by Rao [20]. After thawing of 1 ml semen, spermatozoa were separated from seminal plasma by centrifugation (1000g for 10 min at room temperature). The supernatant was used for determination of seminal MDA and the pellet was used for determination of spermatic MDA. The pellets were washed 2 times in Tris-HCl (pH 7.1), resuspended in 1 ml distillate aqua and subjected to rapid freeze-thawing three times to lyses the cells. To each tube (spermatozoa and seminal plasma), 0.5 ml of thiobarbituric acid reagent (TBA)(0.67 g of 2-thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 g NaOH and 100 ml glacial acetic acid added) was added and then heated for 1 h in a boiling water bath. After cooling, each tube was centrifuged for 10 min at 4,000 x g and the supernatant absorbance was read on a spectrophotometer at 532 nm. The molar extinction coefficient for MDA is $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. The results were expressed as nmoles MDA/ml seminal plasma and nmoles MDA / 10^8 sperm.

4. Statistical analysis

The study was repeated 9 times for each treatment and for each ram. The results are expressed as the mean±standard error (mean±SE). Significant differences between the

experimental group were analyzed by the unpaired t-Student test. The differences with values of $p < 0.05$ were considered statistically significant [13].

RESULTS AND DISCUSSION

In sperm, as with other live cells, damage results from the cryopreservation/thawing process. The success of any technology involving cryopreserved sperm depends upon the ability to recover large numbers of progressively motile sperm that have intact, functional membranes. The focus of our present study is to test the potential benefits of adding an additive antioxidant, vitamin E, to control oxidative stress induced by cryopreservation.

The effect of vitamin E on post-thawing sperm motility, membrane integrity by HOST, viability by eosin-nigrosin staining and malondialdehyd concentrations in spermatozoa and seminal plasma for ram A are shown in table 1 and for ram B in table2.

Our results show an increase of all cytological parameters in the both rams for both concentrations of vitamin E. For 2.0 mM concentration, an increase of motility, membrane integrity and viability appears significant for both rams($p < 0.05$). For the first ram, that the control cytological parameters are lower than the second ram, a stronger increase of this parameters is observed for vitamin E uses, for both concentrations (approximately 11% versus 5%).

Table 1 Spermatological characteristics in frozen-thawed semen of A ram– (mean %± SE)

Extender	Motility %	HOST %	Viability %	MDA nmol/ml	MDA nmol/ 10^8 spz
Control	40.02±1.75 ^a	39.38±1.96 ^a	37.39±0.86 ^{ab}	17,21±0,41 ^a	2,52±0,07 ^a
Vit E 1.0 mM	45.87±1.84 ^b	44.05±1.61 ^b	46.01±0.76 ^a	14,11±0,69 ^a	1,93±0,06 ^a
Vit E 2.0 mM	51.62±0.38 ^{ab}	50.25±1.30 ^{ab}	51.47±0.30 ^b	11,91±0,50 ^a	1,14±0,08 ^a

Different superscripts (a, b, ab) within the same column of each group demonstrate significant difference ($p < 0.05$)

Table 2 Spermatological characteristics in frozen-thawed semen of B ram– (mean %± SE)

Extender	Motility %	HOST %	Viability %	MDA nmol/ml	MDA nmol/10 ⁸ spz
Control	50.00±1.33 ^a	48.75±1.6 ^a	50.21±0.68 ^a	17,47±1,09 ^a	2,77±0,46 ^a
Vit E 1.0mM	53.12±1.44	51.72 ± 1.08	52.25 ±1.13 ^b	15,65±0,81 ^{ab}	1,9±0.26 ^{ab}
Vit E 2.0 mM	56.5 ±0.96 ^a	54.00 ±0.75 ^a	55.26 ±0.65 ^{ab}	12,76±0,86 ^{ab}	1,13±0,13 ^{ab}

Different superscripts (a, b, ab) within the same column of each group demonstrate significant difference (p<0.05)

These results may be explained based on the fact that vitamin E protects the spermatozoa by preventing from endogenous oxidative DNA and membrane damages, thereby helping the sperm to overcome the oxidative attack. Vitamin E is the major chain-breaking antioxidant in membranes. It scavenges all the three types of free radicals, namely superoxide, peroxy and hydroxyl radicals. These radicals will lead to the peroxidation of phospholipids in the mitochondria of the sperm cell and thus to their ultimate immotility [23]. Thus, by maintaining the membrane integrity and optimum functioning of ram sperm, vitamin E improves the per cent sperm motility. Similar observations have been made on humans [26], buffalo [24], boar [25] and rabbits [30].

Regarding the degree of lipid peroxidation of membranes, is found, in both animals, that the MDA concentration of sperm cell suspension is about 10% of that of the seminal plasma. For control samples the seminal MDA concentrations varies between 10.2 and 20.4 nmol/ml, with an average of 17 nmol/ml, and the sperm MDA concentration between 0.6 and 3.2 nmol/ 10⁸spz with an average of 2.5-2.7 nmol/10⁸spz.

The data indicate that vitamin E addition inhibited the peroxidative damage induced by cryopreservation in sperm as it does in other tissues. Our results are in accord with Singh [24] which reported a drop in MDA production upon addition of 2,5 μM vitamin E to buffalo sperm subjected to oxidative stress induced with FeSO₄/ascorbic acid.

Hence, the present study confirms a dose-dependent reduction in MDA production when various doses of vitamin E are added to

freezing extender and shows the effectiveness of vitamin E in protecting sperm motility and viability by suppressing LPO. Similar observations have been made on bull [7] and humans [2, 5].

CONCLUSIONS

The present study on ram spermatozoa indicates that both tested doses (1,0 respective 2,0mM) of vitamin E increased the percentage of sperm motility and viability, but reduced the lipid peroxidation (LPO) level. Thus, it is suggested that vitamin E may be effective in preventing the rapid loss of motility that normally occurs during cooling of spermatozoa and maintains the motility under oxidative stress conditions.

In summary, this study supports recent observations that membrane LPO is involved in freeze-thaw damage to sperm motility.

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