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The effects of antioxidants on the cytological parameters of cryopreserved buck semen

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Abstract

In mammals, the semen membrane contains a large number of unsaturated fatty acids, facilitating thus the lipid peroxidation in the presence of species reactive to oxygen. The consequence of this process is the reduction of the quality of the seminal material or even the death of the sperm cell. The antioxidant capacity of the sperm cell is insufficient to prevent the lipid peroxidation during the freezing-thawing process. The purpose of this study was to determine the influence of antioxidant additives (BSA, Cysteine, vitamin E) added in different concentrations to the freezing media on the semen cytological parameters post-thawing (motility, viability, membrane integrity, anomalies). The experiments were done on 60 ejaculates collected by artificial vagina from 3 bucks (Alpine) during the normal reproduction season. After collecting, the samples were washed of the seminal plasma and diluted in medium based on Tris in which antioxidants were added (6 experimental versions) or in medium without antioxidants (control). The diluted semen was cooled at 4° C, placed in vials and frozen in fine 0.25 ml French vials and then stored in liquid nitrogen. The 6 experimental versions were tested for each buck taking into account how suited each animal is for the freezing of the seminal material. The results show that adding BSA in a concentration of 5 mg/ml, cysteine (10mM) and vitamin E (1mM) have positive protection effects on the semen characteristics consequently to the freezing-thawing process for 2 of the 3 bucks, leading to the improvement of the technology to freeze buck semen. Since the international research regarding the involvement of the oxidative stress associated to freezing thawing in the reduction of the fertility of buck semen post-thawing is at its beginnings, other studies are necessary to confirm the obtained results.

Keyworlds: buck, semen, cryopreservation, antioxidants

Introduction

Semen cryopreservation allows the widespread dissemination of valuable genetic material, even to small flocks by means of artificial insemination, leading to an increased rate of genetic gain. The freeze-thawing of spermatozoa is associated with a reduction in cell motility, viability and fertilizing capacity (HOLT, [1]. Many studies in the area of male infertility have been focused on oxidative stress-related mechanisms of sperm damage (BILODEAU & al., [2]; GADEA & al., [3]; TSELKAS & al., [4]. Normally, there is a balance between free radical generating and scavenging systems. However, high generation of reactive oxygen species (ROS) by sperm processing (e.g. excessive centrifugation, cryopreservation/thawing), accompanied by low scavenging and antioxidant levels in serum, seminal plasma, and/or sperm-processing media will induce a state of oxidative stress (OS). High levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) endanger sperm motility, viability, and function by interacting with membrane lipids, proteins, and nuclear and mitochondrial DNA.

All cellular components including lipids, proteins, nucleic acids and sugar are potential targets of OS. The extent of OS-induced damage depends on the nature and amount of ROS involved and on the duration of ROS exposure and extra-cellular factors such as temperature, oxygen concentration and the composition of the surrounding environment (ions, proteins, ROS scavengers).

Other studies show that the freezing-thawing process decreased the antioxidant defense capacity of sperm cell, the loss of superoxid dismutase activity and the decrease in glutathione levels with 78% given fresh semen (LASSO & al., [5].

Mammalian sperm membranes incorporate many unsaturated fatty acids and are susceptible to lipid peroxidation in the presence of ROS, leading to decreased sperm quality (BUCAK & al., [6]. The anti-oxidant system comprising reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT) and superoxide dismutase (SOD) has been described as defense functioning mechanism against the lipid peroxidation of semen (GADEA & al., [3]; LASSO & al., [5]. In addition, it contains a variety of nonenzymatic antioxidants such as ascorbate, urate, α-tocopherol, pyruvate, glutathione, taurine, and hypotaurine (SALEH & AGARWAL, [7]. The antioxidants present in the seminal plasma are the most important form of protection available to spermatozoa against ROS (SIKKA & al, [8, 9]; SHARMA & AGARWAl, [10]. They provide defense mechanisms through three levels of protection: 1) prevention, 2) interception, and 3) repair. Because ROS generation is a major source of sperm DNA damage, antioxidants may play a role in decreasing apoptosis during spermatogenesis, sperm storage, and transit in the genital tract (SIKKA, [11].

This antioxidant capacity in sperm cells however may be insufficient in preventing lipid peroxidation during the freeze-thawing process.

In recent years, antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation. Cysteine has been shown to prevent the loss in motility of frozen-thawed bull semen (BILODEAU & al., [12], ram semen (UYSAL & BUCAK, [13], and buck semen (BUCAK & UYSAL, [14] and to improve the viability, chromatin structure and membrane integrity of boar sperm during liquid preservation (SZCZESNIAK-FABIANCZYK, [15]. Semen extender supplemented with natural antioxidants (α–tocopherol and ascorbic acid) has protective effect on metabolic activity and viability of bull spermatozoa cryopreserved (BECONI & al., [16].

In addition, human serum albumin in freezing media acts as a powerful antioxidant that prevents oxidative stress-induced damage (ARMSTRONG & al., [17]; AITKEN & al., [18].
Few studies have been done on the effects of antioxidant supplementation in the cryopreservation of goat semen. Thus, the present study envisaged to investigate the effects of bovine serum albumin, cysteine and vitamin E on standard sperm quality parameters (motility, total abnormality, viability and membrane integrity).

Materials and methods

Animals and semen collection: Semen samples from 3 mature Alpine bucks (2 and 3 years of age) (bucks A, B, C), with proven fertility, were used in this study. A total number of 62 ejaculates were collected 3 times weekly by artificial vagina, during the breeding season. The ejaculates were evaluated and accepted for experiments if the following criteria were met: volume of 0.75-2ml; minimum sperm concentration of $3x10^9$ sperm/ml; motility higher than 80%. Immediately following collection, the ejaculates were placed in a water bath (37^oC), until evaluation in the laboratory. Semen assessment was performed within 10 minute. The spermatozoa were separated from seminal plasma through twice centrifugation (10min, 3500rpm).

Semen processing and evaluation

In this study a Tris-based extender 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 goat were divided into 3 equal aliquots and diluted with Tris-based extender supplemented with anti-oxidant additives, depending on different treatments, to a final concentration of approximately $4x10^8$ spz/ml.

BSA effect: 3 groups (1.control-Tris extender without additives, 2. Tris- extender supplemented with 5 mg/ml BSA (bovine serum albumin, fr V, Sigma) and 3. Tris- extender supplemented with 10 mg/ml BSA

Cysteine effect: 3 groups (1. control, 2. Tris +5mM L-cysteine (Sigma),3. Tris + 10 mM L-cysteine

Vitamin E effect: 3 groups (1. control, 2. Tris +0.1mM vitamin E (DL-α-tocopherol, Merck), 3. Tris + 1.0mM vitamin E

Vitamin E was diluted first into ethylic alcohol and than homogenized by vortex**.**

Diluted semen samples were drawn into 0.25 ml French straws (Minitub, Germany), sealed with polyvinyl alcohol powder and equilibrated at 5^oC for a period at 2.5 h. After equilibration, the straws were frozen in liquid nitrogen vapors (12cm and 4cm above liquid nitrogen) and then stored in liquid nitrogen (-196^oC). The straws were thawed individually in a water bath (37°C), for 30s. Sperm evaluation was performed on all semen samples immediately after thawing.

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 (ZAMFIRESCU & SONEA, [19]. 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.

For the evaluation of morphological and acrosomal abnormalities in the semen sample, 3 drops of the semen were dripped into Eppendorf tubes containing 1 ml Hancock′ solution (62.5 ml formaldehyde; 150 ml physiological serum; 150 ml buffer and 500 ml double-distilled water) (SCHAFER & HOLZMANN, [20]. One drop of this mixture was placed on a microscope slide and covered with a cover slip. The percentage of sperm abnormalities was recorded by counting 200 spermatozoa under a phase-contrast microscope (x 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. The test was performed by incubating 30µl semen with 300 µl hypo-osmotic solution (100mOsm) at 37°C for 60 min. 400 spermatozoa were evaluated using bright-field microscopy (ZAMFIRESCU & al, [21].

The viability was evaluated by eosin-nigrosin staining. At least 200 sperm cells were assessed at a magnification of 1000x under oil immersion. The sperm that was white (unstained) was classified as alive and that which showed any pink coloration was classified as dead (ZAMFIRESCU, [22].

Statistical analysis

The study was repeated 7 times for each treatment and for each buck. The results are expressed as the mean±SEM. Differences between the experimental group (with additive) and the control group (no additive) for sperm characteristics after the freezing-thawing process were analyzed by the t-Student test using OriginPro 7.5 software. The differences with values of $p<0.05$ were considered statistically significant (DANIEL, [23].

Results and discussion

The assessment of motility alone is inadequate for the evaluation of sperm survival after thawing (UYSAL & al., [24]. The integrity and functional activity of sperm membrane are of major importance in the fertilization process and assessment of membrane function may be useful indicators of the fertilizing ability of the spermatozoa (UYSAL & KORKMAZ, [25]. Highly motile cells can be damaged in structure or functions of membranes and conversely, highly immotile sperm cells can have intact plasmalemma and thus viability. Therefore, combined tests (intravital staining, HOST test, motility) are necessary for sperm cells post thawing evaluation.

The effect of various antioxidants on post-thawing sperm motility, total abnormality, membrane integrity by HOST and viability by eosin-nigrosin staining for buck A are shown in table 1, for buck B in table 2 and for buck C in table 3.

Table 1. Spermatological characteristics in frozen-thawed semen in buck A (mean %± SEM)

Groups	Treatment	Motility %	HOST %	Viability %	Abnormality %
1. BSA	Control	50.00 ± .63^{ab}	49.93±1.68^{ab}	50.69 ±2.53^{a}	11.11 ± 1.47^{a}
	BSA 5 mg/ml	55.25 ± .07^{a}	52.51 ± .70^{a}	54.51 ±2.51^{b}	13.28 ± 1.57
	BSA10mg/ml	39.50 ±1.15^{b}	37.9 ± 1.63^{b}	37.15 ± .36^{ab}	14.9 ± 1.12^{a}
1.Cysteine	Control Cys	50.62 ±1.75^{a}	50.35 ± .70^{a}	52.37 ± 2.10	11.47 ± 1.02^{a}
	Cys 5 mM	53.5 ± 1.23^{b}	52.07 ± .09^{b}	54.26 ± 1.24	8.16 ±0.67^{ab}
	Cys10 mM	59.37 ± .13^{ab}	55.5 ±0.82^{ab}	57 .00 ±0.75	13.1 ± 1.20^{b}
1. vit E	Control Vit E	51.00 ±2.33^{a}	49.75 ± 2.6^{a}	49.21 ±1.68^{a}	10.85 ± 0.72^{a}
	Vit E 0.1mM	54.12 ±2.44	52.72 ± 2.08	51.25 ±2.13^{b}	12.38 ± 1.36
	Vit E 1.0 mM	57.5 ±1.96^{a}	55.00 ±1.65^{a}	54.26±1.65^{ab}	15.01 ± 0.94^{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 in buck B (mean %± SEM)

Groups	Treatment	Motility %	HOST %	Viability %	Abnormality %
1. BSA	Control BSA	38.25 ± 1.82^{a}	38.61 ± 2.29	37.58 ± 1.85	15.3 ± 0.85^{ab}
	BSA 5mg/ml	45.25 ± 1.40^{a}	42.07±2.068^{a}	41.3 ± 2.01^{a}	18.26 ± 0.98^{a}
	BSA 10mg/ml	33.75 ± 2.17	34.32 ± 2.29^{a}	34.01 ± 1.96^{a}	19.07 ± 1.91^{b}
1.Cysteine	Control Cys	42.5 ± 1.89^{ab}	39.40 ±2.18^{ab}	38.95 ±1.90^{ab}	14.8 ± 0.85^{ab}
	Cys 5mM	49.5 ± 1.44^{a}	48.21 ± 1.38^{a}	49.2 ± 1.48^{a}	9.96 ± 1.51^{a}
	Cys 10mM	54.37 ± 1.47^{b}	51.67 ± 1.26^{b}	53.12 ± .38^{b}	10.56 ± 1.70^{b}
1. vit E	Control Vit E	39.02 ± 0.75^{a}	38.48 ± 1.96^{a}	38.39 ± .86^{ab}	14.5 ± 0.75
	Vit E 0.1 mM	44.87 ± 1.84^{b}	43.15 ± 1.61^{b}	47.01 ± .76^{a}	16.95 ± 1.31
	Vit E 1.0 mM	50.62 ±0.18^{ab}	49.35 ±1.30^{ab}	52.47 ± .30^{b}	16.3 ± 0.93

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

Table 3. Spermatological characteristics in frozen-thawed semen in buck C (mean %± SEM)

Groups	Treatment	Motility %	HOST %	Viability %	Abnormality %
1. BSA	Control	45.12 ± 1.21	44.10 ± 1.02	47.25 ± 1.33	12.01 ± 1.32
	BSA 5mg/ml	46.06 ± 1.16	45.93 ± 2.30	46.51 ± 0.41	13.88 ± 1.66
	BSA 10mg/ml	46.23 ± 2.24	45.21 ± 1.11	46.15 ± 1.95	13.96 ± 1.22
1. Cysteine	Control Cys	47.81 ± 0.21[a]	46.18 ± 1.30[a]	49.91 ± 1.25	11.18 ± 2.03
	Cys 5mM	48.5 ± 1.56	47.57 ± 1.18	50.32 ± 1.82	10.16± 0.55
	Cys 10mM	51.31 ± 1.08[a]	50.25 ±1.22[a]	51.50 ±0.89	12.15 ± 1.87
1. vit E	Control Vit E	46.08 ± 0.78[a]	44.58 ± 2.01[a]	48.52 ± 2.48	11.35 ± 1.52
	Vit E 0.1 mM	47.82 ± 1.84	46.87 ± 1.78	49.33 ± 2.23	10.35 ± 1.11
	Vit E 1.0 mM	51.5 ± 1.36[a]	49.96 ± 0.23[a]	50.44 ± 1.35	11.01 ± 1.05

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

BSA effects

BSA is known to eliminate free radicals generated by oxidative reactions and therefore to protect the membrane integrity of sperm cell from lipid peroxidation (LEWIS & al., [26]. Our results show that BSA 5 mg/ml significantly increased (P<0.05) the sperm motility for 2 goats (with 5.25% and 7.0% respectively) (tables 1,2) and membrane integrity for one goat (table 1). But 10mg/ml BSA lead to a significantly decreased motility (with 10.5%), viability (with 13%) and membrane integrity for one buck (table 1). But the highest morphological abnormality was determined by this concentration, the differences between control and experimental groups being significant for 2 male (tables 1, 2). These decreases may be due to extender osmolarity increase, which is deleterious for sperm cell.

Cystein effects

Cysteine is a thiol compound that has been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both in vitro and in vivo and it protects the membrane lipids and proteins by direct radical-scavenging properties (UYSAL & BUCAK, [27]. Our studies show positive effects of cysteine for both concentrations (5 and 10mM) for all the males. In addition, an increase of these parameters was noted, compared to the control group, at the same time with the concentration increase.

Cysteine at 5mM had a significant increasing effect (P<0.05) for all the parameters in one buck (table 2), and 10mM cysteine leads to significant increase (with 4-15%) of motility and membrane integrity for all the males. At the same time, for 5mM cysteine it was noted that abnormalities percent decrease against control for all the males.

Vitamin E effects

Vitamin E is the major, if not the only, chain-breaking antioxidant in membranes. It scavenges all the three types of free radicals, namely superoxide, peroxyl 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. Vitamin E supplementation has been shown to restore glutathione levels of red blood cells and inhibit membrane peroxidation (SULEIMAN & al., [28].

Our results show an insignificant increase of all evaluated parameters in all three bucks for 0.1 mM vitamin E. For 1.0 mM concentration, an increase (P<0.05) of motility and membrane integrity for all the three goats by 4-11% appears significant; the viability increase is significant in 2 bucks (table 1, 2). 1.0mM vitamin E caused an insignificant increase of abnormality.

Conclusions

The best effects regarding the viability, motility and membrane integrity maintenance are given by 10 mM cysteine, which does not produce abnormalities increases compared to the other high levels of antioxidants (BSA, Vitamin E).

Except the 10mg/ml BSA, which causes a decrease in the sperm parameters for all males, the other antioxidants confer to the sperm cell protection against the oxidative stress induced by the freezing-thawing process.

The results are according to those obtained by UYSAL & al. [13] which noted the motility and viability increase in case of bull sperm cryopreservation with extenders supplemented by 5mg/ml BSA. At variance with the experiments done by the same authors on ram semen (UYSAL & BUCAK, [27] where it is noted the positives effects of 20mg/ml BSA on sperm parameters after freezing- thawing.

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