THE EFFECTS OF CYSTEIN, BOVINE SERUM ALBUMIN AND VITAMIN E ON THE CALITATIVE PARAMETERS OF FROZEN-THAWED RAM SEMEN

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Summary

The aim of this study was to determine the effects of the addition of antioxidants (bovine serum albumin, vitamin E, cysteine) to freezing media on the cytological parameters of cryopreserved sperm cells. Ejaculate samples were collected with artificial vagina from Merinos of Palas rams, on normal season of reproduction and were cryopreserved according to the technology developed in our laboratory. Semen samples from the 2 animals were frozen and analyzed separately to account for the freezing adaptability of each animal. Semen parameters including motility, the membrane structural integrity (viability by eosin-nigrosin stain), morphological abnormalities and functional membrane integrity (hipoosmotic test) were investigated after thawing. In general, the variation is repeated for both animals.

The best results on the protection of plasma membranes were obtained from cysteine addition to the two concentrations tested (5 mm and 10mM). A significant increase (p<0.05) of the sperm characteristics (between 7% and 14%) was obtained. Also, bovine serum albumin (BSA) in 5mg/ml and the concentration of vitamin E in both tested concentrations (0.1 mM and 1.0 mM) offer protection against membrane oxidative stress. For BSA in concentration of 10mg/ml these parameters decreased, a fact attributed the excessive growth of freezer osmolarity. The results bring new knowledge in the field of ram semen cryopreservation, helping to improve the quality of freezing-thawed semen.

Keywords: cryopreservation, semen, antioxidants, ram, oxidative stress.

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. However, the use of cervical insemination with frozen semen in sheep do not always lead to acceptable fertility rates (Salamon & Maxwell, 1995; Zamfirescu et al., 2003) The freezing-thawing of spermatozoa is associated with a reduction in cell motility, viability and fertilizing capacity (Evans & Maxwell, 1987; Holt, 1997). Studies have shown that the main steps of cryopreservation, such as cooling and freezing-thawing carries both a physical and chemical stress on membranes (Chatterjee, 2001), but also an oxidative stress (Bilodeau et al., 2001; Salvador et al., 2006). Normally there is a balance between reactive oxygen species (ROS) generating and scavenging systems. However, sperm processing (centrifugation, cryopreservation, refrigeration), high generation of ROS by immature and abnormal spermatozoa, contaminating leukocytes, accompanied by low scavenging and antioxidant levels in seminal plasma and/or sperm-processing media will induce a state of oxidative stress. High levels of ROS (superoxide, hydroxyl, hydrogen peroxide) endanger sperm motility, viability and function by interacting with membrane lipids, proteins and nuclear DNA (Hellstrom et al., 1994). The possible explanation of this damaging process is based of the fact that mammalian spermatozoa membranes are
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rich in polyunsaturated fatty acids. Orientation of unsaturated fatty acids in the plasma membrane creates the fluidity needed by the spermatozoa to perform normal physiological functions (Bell et al., 1993), but at the same time, make them very susceptible to free radical-induced peroxidative damage (Sikka, 2004). In general, the most significant effect of membrane lipid peroxidation (LPO) in all cells is the perturbation of membrane structure and function.

To reduce the intensity of this peroxidation process, several studies have tested the effect of adding antioxidants additives in freezing extenders on the protection of plasma membranes during cryopreservation (Beconi et al.,1993; Szczesniak–Fabianczyk et al.,2006; Zamfirescu et al.,2004).

Most studies have been conducted on human sperm and few studies have been done on the effects of antioxidant supplementation in the cryopreservation of ram semen. The aim of this study was to investigate the influence of varying doses of cysteine, bovine serum albumin and vitamin E on standard quality parameters of the sperm cell after freezing-thawing.

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 42 ejaculates were collected 2 times weekly by artificial vagina, during the breeding season (October – December 2008). The ejaculates were evaluated and accepted for experiments if the following criteria were met: volume greater than 0.75ml; minimum sperm concentration of 3x10^9 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 and evaluation

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 supplemented with anti-oxidant additives, depending on different treatments, to a final concentration of approximately 4x10^8 spz/ml.

a) 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].

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

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

Vitamin E was diluted first into ethylic alcohol and then homogenized by vortex. Processing of semen was made according to the cryo-preservation technology developed in the Reproduction Biotechnologies Laboratory (Zamfirescu & Nadolu, 2001).

Diluted semen samples were drawn into 0.25 ml French straws (Minitub, Germany), sealed with polyvinyl alcohol powder and equilibrated at 5°C for a period of 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°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 (Bearden & Fuquay, 2000).

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). 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) (Schafer & Holzmann, 2000).

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), fitted with a warm stage at 37°C (Buckett et al, 1997; Zamfirescu et al., 2001).

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 (Zamfirescu & Sonea, 2004).

4. Statistical analysis

The study was repeated 6 times for each treatment and for each ram. The results are expressed as the mean±SEM. Differences between the experimental group were analyzed by the t-Student test. The differences with values of p<0.05 were considered statistically significant (Daniel, 1991).

Results

a) BSA effects

The effect of different concentrations of BSA on semen parameters after freezing-thawing are presented in Table 1. For ram A, at 5mg/ml BSA concentration, there have been insignificant increases of motility and viability (with approx. 5%) and a significant increase (p<0.05) of functional integrity (from 47.41 to 53.57%). For the second male too there were significant increases in motility and functional integrity (to 5-7%) and an insignificant increase of viability. However, for 10mg/ml BSA concentration a significantly decrease of all these parameters is notable in both animals. For this concentration it was remarked too an significant increase of the percentage of morphological abnormalities in one of the animals (B) (from 14.81% to 16.98%).

Table 1. Spermatological characteristics in frozen-thawed semen – BSA effects (mean %± SEM)

<table>
<thead>
<tr>
<th>Ram</th>
<th>Group</th>
<th>Motility(%)</th>
<th>HOST (%)</th>
<th>Viability (%)</th>
<th>Abnormality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±SEM</td>
<td>Mean±SEM</td>
<td>Mean±SEM</td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>A</td>
<td>Control</td>
<td>48.57±2.10</td>
<td>47.41±0.97</td>
<td>45.80±3.28</td>
<td>14.11±0.83</td>
</tr>
<tr>
<td></td>
<td>BSA 5 mg/ml</td>
<td>54.28±2.02</td>
<td>53.57±1.0</td>
<td>51.81±1.79</td>
<td>13.04±0.81</td>
</tr>
<tr>
<td></td>
<td>BSA10mg/ml</td>
<td>35.71±1.70</td>
<td>39.28±2.50</td>
<td>33.58±1.34</td>
<td>16.25±1.0</td>
</tr>
<tr>
<td>B</td>
<td>Control</td>
<td>56.66±1.66</td>
<td>55.83±1.53</td>
<td>60.00±1.82</td>
<td>14.81±0.63</td>
</tr>
<tr>
<td></td>
<td>BSA 5 mg/ml</td>
<td>62.50±1.11</td>
<td>63.33±1.05</td>
<td>61.66±1.66</td>
<td>16.96±0.60</td>
</tr>
<tr>
<td></td>
<td>BSA10mg/ml</td>
<td>45.00±2.58</td>
<td>46.66±1.66</td>
<td>50.83±3.00</td>
<td>16.98±0.60</td>
</tr>
</tbody>
</table>

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

Results on the effect of adding different concentrations of cysteine in freezing extender are presented in Table 2. In this case a significant increase in all cytological parameters in both males can be seen: motility increase by 9-12%, viability by 7-12%, functional integrity (HOST) by 7-14%. Also we found that this increase varies linearly with the concentration of added cysteine. For 10 mM cysteine we obtain higher values of viability, membrane integrity and motility compared with both the control and with the 5 mM cysteine group. The percentage of abnormalities recorded a slight increase from one animal and a decrease for another.

Table 2. Spermatological characteristics in frozen-thawed semen – cysteine effects (mean %± SEM)

| Ram | Group   | Motility(%) | HOST (%) | Viability (%) | Abnormality (%) |
|-----|---------|-------------|----------|--------------|----------------|-----------------|
| A   | Control | 51.42 ±2.60<sup>ab</sup> | 52.55±1.13<sup>ab</sup> | 53.38±1.10<sup>ab</sup> | 13.84±0.72     |
|     | Cys 5mM | 60.00 ±2.18<sup>a</sup> | 60.88±0.90<sup>a</sup> | 60.51±1.39<sup>a</sup> | 12.54±1.23     |
|     | Cys10mM| 62.14 ±1.84<sup>b</sup> | 64.20±1.11<sup>b</sup> | 65.80±1.130b<sup>b</sup> | 12.28±0.77     |
| B   | Control | 59.16 ±1.53<sup>ab</sup> | 53.50±1.70<sup>ab</sup> | 60.83±1.53<sup>ab</sup> | 15.98±0.55     |
|     | Cys 5 mM| 69.16 ±1.53<sup>a</sup> | 61.66±1.05<sup>a</sup> | 69.16±1.53<sup>a</sup> | 16.08±1.14     |
|     | Cys10mM| 72.50 ±1.18<sup>b</sup> | 68.33±1.66<sup>b</sup> | 70.00±1.82<sup>b</sup> | 16.86±0.44     |

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

c) Vitamin E effects

Results are presented in Table 3. A significant increase (p<0.05) for viability and membrane integrity (with 5-8%) were obtained for both concentrations of vitamin E (0.1mM and 1.0mM respectively) and for both animals. Although a linear growth in terms of concentration is no longer found, one of the animals (B) has an increase in motility for the concentration of 1.0 mM compared with the concentration of 0.1 mM.

Table 3. Spermatological characteristics in frozen-thawed semen–Vitamin E effects (mean %± SEM)

| Ram | Group   | Motility(%) | HOST (%) | Viability (%) | Abnormality (%) |
|-----|---------|-------------|----------|--------------|----------------|-----------------|
| A   | Control | 51.42±2.10  | 52.90± 0.93<sup>a</sup> | 53.52 ± 1.01<sup>a</sup> | 13.55±0.45     |
|     | Vit E 0.1mM | 52.14±1.01  | 57.97± 0.69<sup>a</sup> | 59.54 ± 1.27<sup>a</sup> | 16.04±1.14     |
|     | Vit E 1.0 mM | 53.57±1.42  | 55.41± 0.89  | 59.87 ± 0.49<sup>b</sup> | 14.51±0.82     |
| B   | Control | 60.00±1.82<sup>ab</sup> | 60.50± 1.88<sup>ab</sup> | 63.16 ± 1.01<sup>ab</sup> | 15.50±1.09     |
|     | Vit E 0.1mM | 68.33±1.66<sup>a</sup> | 68.30± 0.82<sup>a</sup> | 69.61 ± 0.47<sup>a</sup> | 17.05±0.56     |
|     | Vit E 1.0 mM | 71.66±1.66<sup>b</sup> | 73.51±1.30<sup>b</sup> | 68.48 ± 1.26<sup>b</sup> | 16.48±0.50     |

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

Discussion

Assessment of a single parameter, motility, is not enough to assess post-thawing sperm fertility. The integrity and functional activity of sperm membrane are of major importance in the fertilization process, so appreciation of membrane function is an important indicator for sperm fertility prediction (Zamfirescu et al., 2003). Highly motile cells can be damaged in structure or functions of membranes and conversely, highly nonmotil 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. Our results demonstrate that supplementation with antioxidants may prevent or protect sperm membrane from lipid peroxidation, but did not affect the cell in the same way. Experiments have shown that the highest values of motility, viability and membrane integrity after thawing (increases by 7-12% compared to control) were obtained from cystein, in both concentrations tested. Adding vitamin E also leads to increases of all sperm post-thawing parameters, but it significantly increased viability and integrity of membranes. BSA protects especially the functional integrity but only for the concentration of 5mg/ml, higher concentrations leading to a decrease in all parameters. Albumin is an important extra cellular antioxidant because of its property to link transitional metal ions (Fe$^{2+}$ and Cu$^+$), thus minimizing the formation of OH$^-$ radical, the promoter of sperm lipid peroxidation (Alvarey & Storey, 1983). Human serum albumin acts as a powerful antioxidant that prevents oxidative stress-induced damage in human sperm (Aitken et al., 1994; Armstrong et al, 1998). Our results show an improvement in the sperm parameters post thawing, but only for the concentration of 5 mg/ml BSA. Current findings are according with those obtained in our experiments on goat sperm and those reported by Uysal on ram (2007) and bull semen (2007a) and by Bucak & Uysal (2008) on goat sperm. However, for high concentrations of BSA (10mg/ml), the values of all parameters of sperm quality decrease significantly (to 6-8%) for both males. These results, although they do not correspond to those obtained by Uysal & Bucak (2007), that reported a cryoprotective activity on sperm parameters even at high concentrations of BSA (10 or 20mg/ml), we can put into high osmolarity of the extender becomes hypertonic and affect the sperm cell. Toxic effects of a hypertonic media has been emphasized by our other studies (unpublished). Also the percentage of morphological abnormalities increases for this concentration may be due to hypertonic media.

Cysteine is a thiolic compound that can penetrate the plasma membrane and is a precursor of intracellular glutathione biosynthesis. It has also been known for a long time that cysteine protects the sperm cell from toxic oxygen metabolites causing lipid peroxidation of sperm plasma membranes under "in vitro" conditions (Meister & Tate, 1976). Funahashi and Sano (2005) reported that a semen extender with 5 mM cystein improved the viability and membrane integrity of boar sperm cells during liquid storage. Our studies show that a higher concentration (10mM) leads to increased quality of ram semen after freezing-thawing.

Tocopherol (vitamin E) is the primary lipid soluble small molecule antioxidant in biologic and the major, if not the only, chain-breaking antioxidant in membrane systems. However, its membrane concentration is very low, usually equal to or less than 0.05–0.1 nmol/mg of protein, in seminal plasma as 0.3 to 0.5 μmol/l. On the other hand, the rate of lipid radical generation in membranes can be very high, about 1–5 nmol/mg protein per minute under certain circumstances (Buettner, 1993; Sikka, 2004). Nevertheless, under normal conditions, vitamin E deficiency is seldom found, during freezing-thawing, due to oxidative stress induced by cold shock, centrifuge, thawing, involves the need to supplement environmental dilution of this vitamin. Our studies have shown that a further dilution of the extender, both with 0.1 mM and 1.0 mM of vitamin E leads to improved post-thawing cytological parameters for the ram sperm.

**Conclusion**

In conclusion, this study demonstrated that supplementation with antioxidants of semen extenders, depending on various concentrations, may exert beneficial
effects on the quality of the freezing-thawing ram semen. The results show that antioxidants such as cysteine (5 mm and 10 mM), BSA (5 mg/ml) and vitamin E (1.0 mM) have beneficial effects on cytological sperm parameters (motility, functional integrity of plasma membrane, the membrane structural integrity). Results contribute to increase the quality of ram semen frozen-thawed, but further studies are necessary to confirm present findings.

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