ANTIOXIDANT ADDITIVES EFFECT ON CYTOLOGICAL PARAMETERS OF REFRIGERATED RAM SEMEN

Andreea Hortanse Anghel[1], Stela Zamfirescu[2], D. Coprean[2], Dorina Nadolu[1]

[1]The Research and Development Institute for Sheep and Goat Breeding - Palas Constanta
[2]Ovidius University, Constanta
e-mail: palaslrb@canals.ro

Abstract

The aim of this study was to assess the influence of antioxidant additives on standard ram semen parameters during storage at 5ºC for 0, 24, 48, 72, 96 and 120 h after collection and dilution. Experiments were carried out in extra-season of reproduction (March-June 2009) at Laboratory of Biotechnology for Reproduction from Institute of Research and Development for Sheep and Goats, Palas-Constanta. A total of 36 ejaculates from 2 Merino rams was collected by artificial vagina and were extended with egg yolk-tris buffer, which was containing vitamin E (1mM), cysteine (10mM) and control group without any antioxidant. After diluting sperm is kept in the fridge (5 ^{o}C) and for 5 days, at intervals 24 h is checked for qualitative parameters (motility, viability). According to the obtained results for 5 consecutive days, the viability of sperms for control decreased with approximately 70%, while for experimental lots viability was with 59% lower for vitamin E and 60% less when applied cysteine. In the first 72 h the changes in viability of control and the two experimental variants are not important. After 96h, for p<0, 05, viability of control is 39.59±2.78% related to 43.11±2.53%, respectively 43.82±3.56% for extender containing antioxidants vitamin E and cysteine, respectively. Our results show that both vitamin E and cysteine had protective effect over membrane integrity (viability) and motility for the both rams. These finding have practical importance because the seminal material was sampled in extraseason of reproduction when the quality of sperm is lower.

Key words: ram, refrigerated semen, antioxidants

INTRODUCTION

Since the development of large scale artificial insemination (AI) programs in the twentieth century, the need to inseminate large numbers of females with semen from genetically superior males required transport of semen from the collection point or centre to the site of the female and sperm preservation. The aim of storing spermatozoa is to prolong their fertilizing capacity. The preservation of semen for short-term (liquid) storage was been achieved by reducing the metabolism of spermatozoa through reductions in storage temperatures, and for long-term (frozen) storage by arresting the metabolism by storage at sub-zero temperatures. Although the fertilizing capacity of spermatozoa may be prolonged by storage in a liquid or frozen state, the storage processes inevitably reduce the proportion of motile spermatozoa and cause degenerative changes to sperm membrane integrity, which ultimately reduces fertilizing capacity after AI [14, 26].

The survival of ejaculated sperm in seminal plasma alone is limited to a few hours. To maintain sperm for longer periods and to cool or cryopreserve semen, dilution with a protective solution is necessary. The extenders must preserve fertilization capacity of spermatozoa during "in vitro" storage at low temperatures. Regardless of extenders nature, motility and membrane integrity of spermatozoa are deteriorating during the cooling process and storage at low temperatures. The degenerative changes are possible results of lipids peroxidation or excessive production of reactive species of oxygen (ROS) [5].

A series of refrigerated spermatozoids protection methods against ROS were proposed, like storage in low oxygen atmosphere or addition of antioxidants in the storage diluents. There are few experiments of antioxidants effect and in some cases the results are the opposite.

Studies on stallion sperm established the protective effects of bovine serum albumin (BSA) against peroxidation and maintaining

of sperm [11,12]. Addition of superoxid dismutase and catalase in refrigerated Tris media used for sperm ram preservation, resulted in increased motility, acrozome integrity and fertility [15,19].However, for refrigerated seminal material of ram using BSA, results were not positive [13]. Experiments carried out by Upreti [21] showed no preservative effect of Vitamin E on ram sperm, by the contrary it induced motility reduction, while Cerolini [7] proved that vitamin E increased the motility of pig refrigerated spermatozoa. Similar, Breininger [6] demonstrated that vitamin E prevents oxidative stress of pig semen kept at 19ºC.

The aim of this study is to increase the ram sperm refrigeration period at 5ºC and maintaining the qualitative parameters of spermatozoa collected in extra-season mating by adding antioxidant additives in dilution media (cysteine and vitamin E).

MATERIALS AND METHODS

Experiments were carried out in extra-season of reproduction (March-June 2009) at Laboratory of Biotechnology for Reproduction from The Research and Development Institute for Sheep and Goat Breeding - Palas Constanta.

Animals: Semen samples from two mature Merino rams (4 and 5 years of age) (rams A, B), with proven fertility, were used in this study. The semen samples were collected two times weekly, by artificial vagina. Sperm samples from each ram were analysed separately to take in consideration the variability of animals. From each male two ejaculates were collected at period of 10-15 min, and then the samples were pooled and designed to experiments. A total of 36 ejaculates were processed.

Sperm processing

After collecting, sperm samples were divided in 3 parts and diluted according to each experimental method applied.

First dilution, 1:1 is made with diluents at 37^{o}C immediately after ejaculate collection. Before analysis, samples were maintained on water bath at 37^{o}C. Sperm was analyzed in about 10 min time after sampling. Following first dilution, routine sperm control concerning ejaculate volume, motility and concentration of spermatozoids is made. In experiments were included only ejaculates that fulfilled the following requirements: volume over 0.5 ml, minimum sperm concentration of $3x10^9$ sperm/ml; motility higher than 70%.The second dilution is performed at room temperature (20^{o}C), according to the ejaculate volume and concentration, until a concentration of $1\text{-}1.6x10^9$ spz/ml [23].

The basic semen extender was Tris media, having the following composition: 375 mM Tris (hydroxymethyl-aminomethane); 124 mM citric acid anhydrous; 41.6 mM glucose, 20% egg yolk(v/v), pH=6.8-6.9).

Experimental treatment

1- Tris media enriched with 10 mM L-cysteine (Sigma)
2- Tris media enriched with 1mM vitamin E (DL-α-tocopherol, Merck)

Refrigerated semen quality evaluation

After diluting sperm is kept in the fridge (5^{o}C) and for 5 days, at

24h intervals, is checked for qualitative parameters (motility, viability).

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 10 different microscopic fields for each semen sample [24].

Viability evaluation was estimated by supravital staining technique using eosin-nigrosin stain mixture (5% w/v nigrosin; 0.6% w/v eosin; 3% w/v sodium citrate dehydrate). Considering that the dye penetrates only damaged spermatozoid membranes it is appreciated that pink sperms are dead and the whites (colourless) are viable. At least 200 spermatozoids are counted by microscope in lighted field (x1000) [4].

Statistical analysis

Experiments were repeated 6 times for each variant and each animal and results are expressed as mean ± standard deviation. For semificative differences evaluation of experimental parameters, media were analysed by test of pair differences (Origin Prolab 8.0). Differences with $p< 0,05$ were considered semnificative statisticaly [9].

RESULTS

The results concerning the effect of vitamin E and cysteine on quality of ram A refrigerated semen are found in table 1 (viability) and in table 2 (motility).

According to the obtained results on sperm for 5 consecutive days, the viability of sperms for control decreased with approximately 70%, from 92.93%±2.94% to 31.25%±1.57%, while for experimental lots viability was with 59% lower for vitamin E and 60% less when applied cysteine. In the first 72 h the changes in viability of control and the two experimental variants are not significant. After 96h, for p<0, 05, viability of control is significant decreased, from 39.59±2.78% related to 43.11±2.53% and 43.82±3.56% for extender containing antioxidants vitamin E and cysteine, respectively. There are no significant differences in experimental variants. Results are maintained for 120 h refrigeration times, where it was remarked too a significant decrease from control (31.25±1.57%) to vitamin E (34.20±1.97%. 10 mM Cysteine has no significant effect on viability comparing to control lot.

Table 1
Antioxidants effect on viability (%) for ram A

Refri-geration time (h)	Control	Vitamin E (1mM)	Cysteine (10mM)
0	92.93±2.94	93.13±2.13	92.74±2.11
24	82.92±4.56	84.39±2.74	83.40±0.94
48	60.79±3.41	62.67±5.02	62.43±1.94
72	50.51±5,56	52.70±3.57	52.65±2.01
96	39.59±2.78[a]	43.11±2.53[a]	43.82±3.56[a]
120	31.25±1.57[a]	34.20±1.97[a]	32.22±3.58

Results are expressed as mean± standard deviation. The superscript shows the significant differences on the same row (p<0, 05).

Over 5 days of monitoring, motility decrease with 66% for control and 57-60% for experimental lots (Table 2). For the first 72h of refrigeration at 5°C, like for viability, the differences in motility are not significant (p>0, 05) for control and antioxidants experiments. However, after 96h, the mean motility for control (33.0±4.83%) is significant lower (p<0, 05) related to values of experimental lots, 44.5±2.83% and 43.0±2.58%. After 120h refrigeration the difference recorded for control and the three experiments is significant (25.5±3.68% control and 34.0±2.10%, 32.5±3/53%, respectively).

Table 2
Antioxidants effect on motility (%) for ram A

Refri-geration time (h)	Control	Vitamin E (1mM)	Cysteine (10mM)
0	91.3±4.37	93.5±1.44	92.3±3.36
24	81.0±5.67	83.0±2.58	81.5±4.11
48	61.0±3.94	70.5±3.68	69.0±5.16
72	45.5±4.97	57.5±5.40	53.5±6.68
96	33.0±4.83[a]	44.5±2.83[a]	43.0±2.58[a]
120	25.5±3.68[a]	34.0±2.10[a]	32.5±3.53[a]

Results are expressed as mean± standard deviation. The superscript shows the significant differences on the same row (p<0, 05).

For the second ram (B) viability varies as for the first ram (A) (Table3). In the first refrigeration 72h period the results of control and experimental variants are almost the same. After 96h the results are significantly improved for all variants comparing to the control. Also, after 120h viability is higher in the presence of antioxidants.

Table 3
Antioxidants effect on viability (%) for ram B

Refri-geration time (h)	Control	Vitamin E 1mM	Cysteine 10mM
0	90.09±2.30	91.10±1.31	90.99±1.80
24	76.03±1.71	77.80±1.38	76.43±0.88
48	61.81±1.96	64.75±1.06	63.35±0.77
72	42.04±2.22	45.17±0.78	44.68±0.52
96	30.04±0.79[a]	34.45±0.96[a]	33.88±0.56[a]
120	20.54±1.17[a]	24.06±0.92[a]	22.82±0.82[a]

Results are expressed as mean± standard deviation. The superscript shows the significant differences on the same row (p<0, 05).

For the second ram motility decreased more drastically comparing to the first ram, with 74% for control and 66-68% for experimental variants (Table 4). The motility has the same tendency as for the first ram: no change by 72h refrigeration time, but at 96h and 120h, respectively the motility was significantly greater in the presence of antioxidants than control.

Tabel 4.
Antioxidants effect on motility (%) for ram B

Refri-geration time (h)	Control	Vitamin E (1mM)	Cysteine (10mM)
0	88.0±3.49	89.3±3.49	88.5±3.28
24	73.5±4.74	74.0±4.59	72.5±2.63
48	59.0±3.94	62.0±4.21	60.5±2.83
72	39.5±2.83	43.0±3.49	41.0±3.94
96	25.0±4.08[a]	31.5±2.41[a]	30.0±4.08[a]
120	14.5±4.97[a]	21.0±2.10[a]	19.5±3.68[a]

Results are expressed as mean± standard deviation. The superscript shows the significant differences on the same row (p<0, 05).

DISCUSSIONS

The necessity to use ram semen all over the year stimulated the research on storage of spermatozoa under artificial conditions. There are many reports showing that fertility of sheep inseminated intra cervical with refrigerated sperm decrease dramatically related to the refrigeration time: decrease in fertility was at a rate of 10-35% per day of storage. Thus, while 75-85% of ewes lambed from insemination with fresh semen, the lambing rates for semen stored for 24, 48 and 72h were 45-50%, 25-30% and 15-20% respectively [17, 25].

Oxidative stress is one of the most important factors associated with fertility decrease over sperm preservation. The plasmatic membrane of spermatozoa contains a great number of unsaturated fatty acids which are susceptible to lipid peroxidation, and the consequences are numerous, ranging from membrane damage, cell functions disorder and low motility [1,3].

Addition of antioxidants prevents or diminishes the peroxidation process. In this study the effects of vitamin E and cysten over motility and membrane integrity of ram sperm diluted and refrigerated at 5ºC were analyzed.

Vitamin E is the major chain-breaking antioxidant in membranes. Addition of vitamin E in preservation media had however opposite results. According to Ball [3], motility is not significant influenced by vitamin E adding in stallion semen after refrigeration for 72 or 96 h. In contrast, Aguero [2], reported that motility and viability results were positive by adding vitamin E during 24h refrigeration of stallion semen. Studies on ram semen kept at 15ºC, showed that adding vitamin E in refrigeration media reduced the sperm motility [22].

Our results show that 1 mM vitamin E had protective effect over membrane integrity (viability) and motility. The values of these parameters are higher then those of the control after 96 and 120h refrigeration at 5ºC. These results are in accord with findings of Beconi [5], and Sarlos [18], which reported the protective effect against lipid peroxidation of plasmatic membranes of 1mg/ml vitamin E for bovine and ram spermatozoa.

As concerning cysteine effect, our study proved that by adding 10mM cysteine in dilution/refrigeration media the motility and viability of spermatozoa refrigerated for 96 and 120 h increased significantly (p<0.05).

The positive effect of cysteine over spermatic parameters is due of a series of factors. Cysteine is included in Thiols (-SH) group which is a large class of antioxidants. Cysteine is an α-amino acid and is a precursor in the production of intracellular glutathione (GSH) which inactivates the reactive species of oxygen and catalyzes hydrogen or other superoxide detoxification [23], as well as it functions as a cofactor of glutathione peroxidase to destroy hydrogen peroxide (H_2O_2) [16]. In addition, it has been shown that glutathione (GSH) can denote hydrogen atoms to repair damaged DNA why glutathione (GSH) and the other Thiols (-SH) compounds such as cysteine or N-acetyl-L-cysteine may be important substances to protect cells from DNA damage.

Our results are in accord also with other studies that demonstrated that cysteine increased pig spermatozoa viability during preservation by refrigeration [8, 10, 20].

CONCLUSIONS

The susceptibility of spermatozoa to suffer lipid peroxidation of plasmatic membrane differs among species even tough among the individuals of the same species.

Our results demonstrated that by supplementing the Tris dilution/refrigeration media with cu 1mM vitamin E or 10mM L-cysteine the quality of refrigerated ram semen

is increased. These finding have practical importance because the seminal material was sampled in extra-season of reproduction when the semen quality is lower.

Taking in consideration the importance of fresh or preserved semen for artificial insemination are required as well in vivo studies for testing the fertility rate after artificial insemination with semen processed with antioxidants.

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