RESEARCHES REGARDING THE ULTRASTRUCTURAL MODIFICATIONS OF SPERMS CELLS BEFORE AND AFTER FREEZING IN DIFFERENT MEDIA

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Abstract

Sperm cryopreservation is a method of ex situ preservation of gametes in all domestic animals, which is extensively used in artificial insemination. There are numerous factors on which the success of freezing sperm and of obtaining living products depends. The composition of seminal plasma, extenders used for dilution, cryoprotective concentrations, reactive oxygen species, and also the cooling rate, influence the quality of sperm cells frozen and thawed and of the number of viable products obtained after the insemination of the cryopreserved sperm. The research of recent years confirms that in sheep and goat, fertility outcome is still very variable, regardless of the conditions and media used for freezing-thawing. Researches show that, regardless of the solvent used, the motility and membrane integrity of sperm deteriorates during cooling and storage at low temperature. These degenerative changes may be the result of lipid peroxidation and excessive production of reactive oxygen species (ROS).Our study presents the results of changes in the integrity of sperm cells membranes of Saanen goats after freezing-thawing, after diluting the buck semen with Tris base extender enriched with 10mm L-cysteine (Sigma), 5 mg/ml BSA (Sigma) and 1mM vitamin E (DL-α-Tocopherol, Merck). After thawing, the highest motility was obtained in the vitamin E and cysteine versions (between 51-55%). Goat semen samples diluted with the 3 antioxidants were processed for examination and analyzed in terms of electron microscopic cellular integrity at all levels (head, midpiece, principal piece and end piece). Electron photography analysis (X10.000) shows that full membranes were observed in a proportion of 49% of cells diluted with Tris-vitamin E at all levels of the cell and at a rate of 37% in sperm cells diluted with Tris-cysteine. Swollen but continuous membranes were considered to be normal. The cell sperm diluted with medium supplemented with BSA displays protein deposits with acicular aspect and membrane with small and frequent gaps. Future researches are targeted at in vivo testing of goat sperm cryopreserved with different antioxidants to determine its fecundity.

Key words: goat, sperm cell ultrastructure, cryopreservation, antioxidant additives

INTRODUCTION

Semen cryopreservation is an “ex situ” conservation method of the gametes in all the domestic animals. It is used extensively in the artificial inseminations as it facilitates the dissemination of the valuable genetic material even in the small flocks, leading thus to the increase of the genetic growth.

The semen freezing-thawing process is still associated to the reduction of the cellular motility, of viability and of fertilization capacity [11]. The results of the most recent research confirm that in sheep and goat, the fecundity result is still very variable, regardless of the freezing-thawing conditions and media used. There are many factors on which depends the success of the semen freezing and implicitly, the genesis of living products. The composition of seminal plasma, the media used for dilution, the concentration of cryoprotectors, the reactive oxygen species and also the cooling rate influence the quality of frozen or thawed sperm cells and the number of viable products obtained after the insemination of the frozen semen. The research indicates that, regardless of the extender used, the motility and membrane integrity of the sperm cell

deteriorates during cooling and storage at low temperatures. These degenerative modifications can be the result of lipid peroxidation and of an excessive production of reactive oxygen species (ROS).

The thermal shock of freezing and thawing is a determining factor in the installation of oxidative stress at the level of the sperm cell [6,10,18]. Other studies showed that the freezing-thawing process decreases the antioxidant defensive capacity of the sperm cell. Thus, cryopreservation leads to the reduction of the activity of superoxide dismutase and of the glutathione level by 78%, compared to the fresh semen [5].

All the cellular components, including lipids, proteins, nucleic acids and sugars are potential targets of oxidative stress. The proportion of alterations induced by oxidative stress depends on the nature and concentration of ROS, on the exposure period to ROS, but also on extra-cellular factors such as temperature, oxygen and environmental makeup (ions, proteins, ROS scavengers).

In mammals, the sperm membranes contain many unsaturated fatty acids and are susceptible to lipid peroxidations (LPO) in the presence of reactive oxygen species (ROS), leading to a decrease in the quality of semen [7]. The antioxidant system, made up of reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT) and superoxide dismutase (SOD), acts as a defense mechanism against lipid peroxidation, with considerable importance for the maintenance of semen motility and viability [1]. However, this antioxidant capacity of the semen can be insufficient for the prevention of LPO over the duration of the freezing-thawing process.

Seminal plasma contains a large number of non-enzymatic antioxidants such as ascorbate, α-tocopherol, pyruvate, glutathione, taurine [15]. These are the most important protection elements used by sperm cells against the attack of ROS. The defense mechanisms include three protection levels: 1) prevention, 2) interception, 3) repair.

The studies accomplished so far have demonstrated that cysteine prevents the reduction of the motility of frozen-thawed bull [6], ram [19] or buck [8] semen and improves the viability, chromatin structure and membrane integrity in the bull semen during refrigeration [17]. The use of natural antioxidants in the dilution and freezing media, such as α-tocopherol and ascorbate, has protecting effects on the metabolic activity and on the cell viability of cryopreserved bovine sperm cells [3]. Human serum albumin works as a strong antioxidant, preventing the occurrence of oxidative stress in the human sperm cells [1,2].

The purpose of this study was to compare the protecting effects of antioxidants added in the freezing media on the integrity of plasmatic membrane after thawing.

MATERIALS AND METHODS

1. Animals and semen collection

The research regarding the freezing of semen was accomplished in the Laboratory of Reproduction Biotechnologies of ICDCOC Palas Constanta, while the electrono-microscopic processing of the buck semen was realized in the Laboratory for Electronic Microscopy of "Ovidius" University Constanta. This study used semen samples from 3 adult Saanen bucks (2 years old), marked A, B and C, with known fertility. The bucks belong to the National Association of Goat Breeders (CAPRIROM) and were maintained in uniform conditions of feeding, shelter and illumination. A total of 90 ejaculates were processed. The collections were accomplished by artificial vagina, three times a week, during the normal mating season (October-December 2009). Only the ejaculates that met the following criteria were included in the experiment: volume over 0.75 ml; minimum sperm concentration $3x10^9$ spc/ ml; motility over 80%.

2. Semen processing and evaluation

As extender for freezing, the medium used was based on Tris (375m M Tris; 124 mM citric acid; 41,6m M glucose, 20% (v/v) egg yolk, 5% (v/v) glycerol, pH=6,8). As experimental versions, the following were used: Tris with added 10mM L-cysteine (Sigma), 5 mg/ml BSA (Sigma) and 1mM vitamin E (DL-α-tocopherol, Merck). After

evaluation, the sperm cells were separated from the seminal plasma by washing and centrifuging for 10 minutes at 3500 rpm, in stock medium based on Tris without egg yolk and glycerol. The washing process was repeated twice.

At each collection, the seminal material of each buck was divided into two equal parts: control (diluted with Tris simple) and experimental (diluted with medium with added antioxidants, according to the experimental version, till the final concentration of $4x10^8$ spz/ml. The experiments were repeated 10 times for each experimental version.

The diluted semen was aspirated in 0.25 ml vials (Minitub, Germany), which were sealed with polyvinyl alcohol plugs and equilibrated at 5°C for 2.5 hours. After equilibration, the vials were pre-frozen in liquid nitrogen vapors and then plunged into liquid nitrogen for storage. After 24 hours, the vials were thawed individually (at 37°C) for 30 seconds in water bath. The evaluation of the morphocytological parameters was accomplished immediately after thawing in all the semen samples.

3. Semen evaluation

Progressive motility is an indicator of semen quality and was analyzed under microscope (Novex) equipped with a heating plate maintained at 37°C [3]. Semen motility was estimated by the analysis of three microscopic fields (x100), different for each sample, the final score being the mean of three successive estimations.

Viability, as indicator of the membrane structural integrity, was evaluated by coloration with eosin-nigrosin. As the coloring matter penetrates only the deteriorated membrane of the sperm cell, it is appreciated that pink sperm cells are dead, while white ones are alive. At least 200 sperm cells were counted under the optical microscope in light field (x1000) [22].

The ultrastructural modifications occurred at membrane level after thawing were evaluated by transmission electron microscopy (TEM). For the determination of the sperm cells ultrastructure, the frozen semen was prefixed in Sorensen phosphate buffer solution with glutaraldehyde 2.5%, fixed with osmium tetroxide 1%, dehydrated in seriate baths with ethylic alcohol and encased in Epon 812 resin.

The sectioning operations were realized by Reichert ultramicrotome, type Ultracut R, which allowed the cutting of fine 400-600Å sections. The sections were colored by uranyl acetate and lead acetate. The grids were examined under a Philips-301 electronic microscope and photographed directly on celluloid plates. The electronomicroscopic profile of sperm cells was appreciated by the characterization of modifications occurred at all the levels of cell membrane integrity (head, midpiece, principal piece and end piece). The bloated but continuous membranes were considered normal [22]. At least 200 cells were analyzed for each experimental version.

4. Statistical analysis

The experiments were repeated 10 times for each experimental version and for each animal, and the results are expressed in percentages mean ± error, standard mean (mean ± SEM). For the determination of the significant differences among the parameters of the experimental versions, the means were analyzed by the t-Student test. The differences with p values < 0.05 were considered statistically significant [9].

RESULTS AND DISCUSSIONS

During the cryopreservation process, the sperm cells undergo ultrastructural modifications (of the plasmatic, mitochondrial and acrosomal membranes), biochemical and functional modifications [13, 23]. The alterations can occur at any stage of the process, less during storage at -180°C, but in larger proportion during the cooling periods at 0°C and at thawing. The sperm cell structures and organelles respond differently in various stages of the process. This is why the optimum cryopreservation procedures must represent a compromise used to protect the integrity of different structures with various cryobiological requirements [23]. The integrity and functional activity of sperm membranes have a major role in the fertilization process. Thus,

the appreciation of membrane ultrastructure and function is an important indicator for the prediction of semen fertility [20].

Our tests were accomplished on three Saanen bucks, the experimental versions being realized on each male in order to take into account how fit for freezing is the seminal material of each buck [22].

The effects of the three antioxidants on the motility and viability of the frozen-thawed semen are presented in Table 1.

Table 1 Morphocytological parameters of frozen-thawed semen, depending on the experimental version (mean± SE)

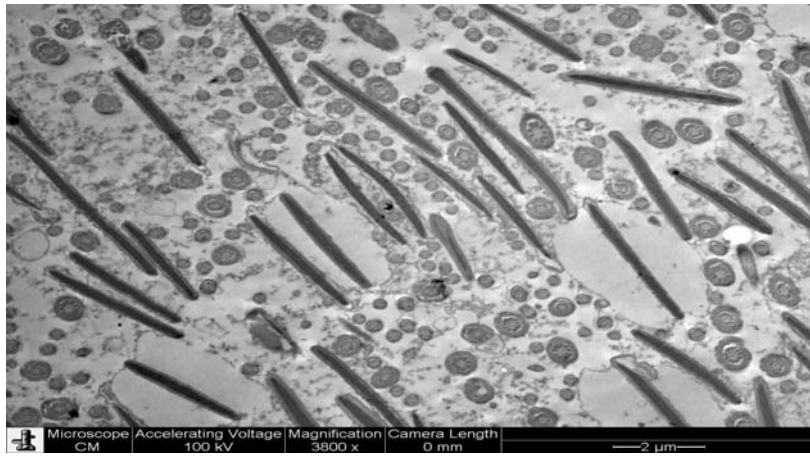
Treatment	Buck A		Buck B		Buck C	
	Motility %	Viability %	Motility %	Viability %	Motility %	Viability %
Control BSA	40.69±2.53	39.93±1.68	39.25±2.82	38.58± 1.85	45.12±1.21	47.25±1.33
BSA5mg/ml	44.51±2.51	42.51± 1.70	45.25±3.40	41.30± 2.01	46.06±1.16	46.51±0.41
Control Cys	41.33±1.35	42.37± 2.10	42.5 ± 1.89[a]	38.95±1.90[a]	46.81±0.21[a]	46.91±1.25
Cys 10 mM	43.50±1.23	44.26±1.24	49.5 ± 1.44[a]	49.20±1.48[a]	51.31±1.08[a]	51.50±0.89
Control VitE	39.03±0.33[a]	40.21±0.68[a]	40.02±0.55[a]	37.39±0.86[a]	45.08±0.78[a]	48.52±2.48
Vit E 1.0mM	46.50±1.02[a]	45.26±0.65[a]	51.62±0.38[a]	51.47±0.30[a]	51.50±1.36[a]	50.44±1.35

The superscript letter (a) in the column of each version symbolizes the significant differences ($p<0.05$)

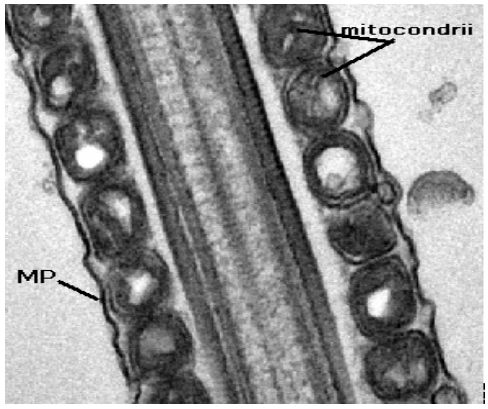
Vitamin E provided the best results regarding the protective effect on motility after thawing. In all three animals, significant rises were recorded, compared to the witness ($p<0.05$). Also, for two of the animals, there was a significant increase of viability. The analysis of the electronomicrophotographs, at direct enhancements between 3000-100000x, shows that the integrity of the cell membrane is preserved in a percentage of 49% in the cells diluted with Tris-vitamin E (fig. 1A). These observations correspond to the results of motility and viability, which registered the highest values post-thawing when vitamin E was used. The integrity of plasmatic membrane is maintained both at the level of the main piece (fig. 1B, C) and at the level of the nucleus (fig. 1D) and acrosome (fig. 1E).

Vitamin E is one of the most important antioxidant molecules and it is primarily found in the cell membranes, explaining thus its protective role against the lipid peroxidation of the membranal lipids. The role of vitamin E is to interrupt the chain reactions of peroxidation and to eliminate the free radicals generated by the univalent reduction of molecular oxygen. These radicals are responsible for the peroxidation of the phospholipids in the mitochondria of sperm cells, a process which leads to the loss of motility [16], which explains the protective role of vitamin E in the maintenance of motility after thawing.

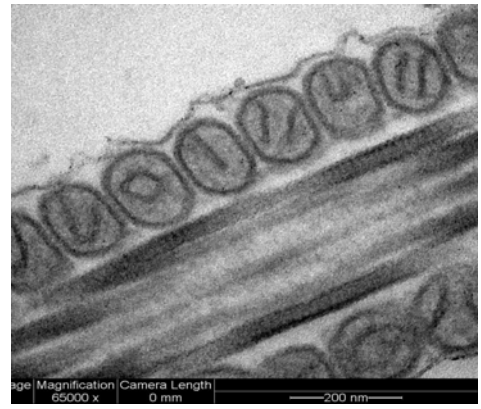
Cysteine is a thiolic compound which can penetrate the plasmatic membrane and is a precursor in the biosynthesis of intracellular glutathione. Also, it has been known for a long time that cysteine protects the sperm cell against the toxic oxygen metabolites which induce the lipid peroxidation of the sperm plasmatic membranes “in vitro” [14]. Our results show the positive effects of cysteine (concentration of 10mM) regarding the maintenance of all parameters post-thawing in all the three bucks. Also, there is a significant increase ($p<0.05$) of motility compared to the witness in two animals and of viability in one animal. The electronomicroscopic analysis shows that the membrane integrity is maintained in 37% of the sperm cells diluted with Tris-cysteine (fig. 2). Even though the plasmatic membranes are more affected than when vitamin E is used, the level of membrane degradation if not very marked. The membrane displays vesicles and detachments especially at the flagellum level.



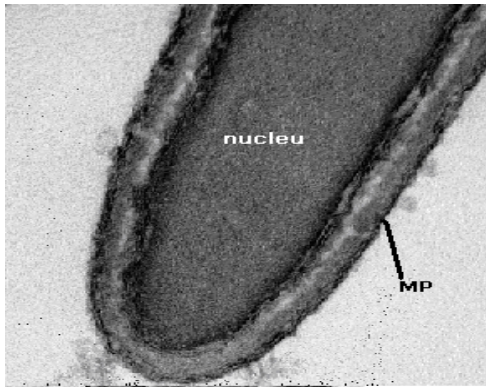
A



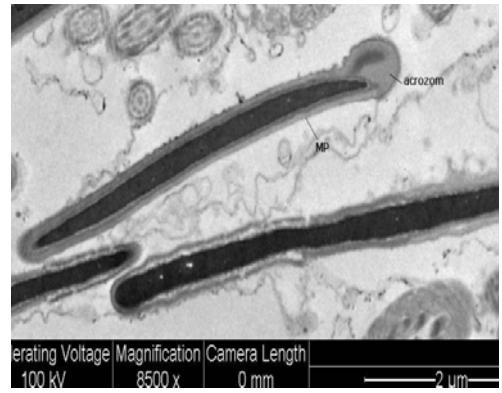
B



C

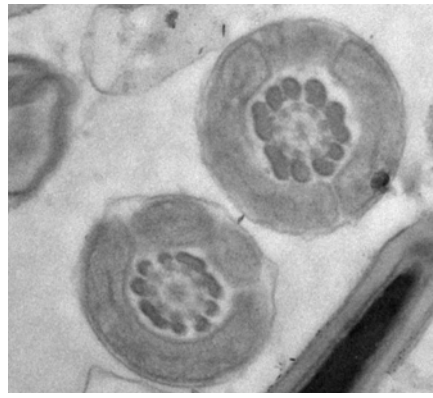


D

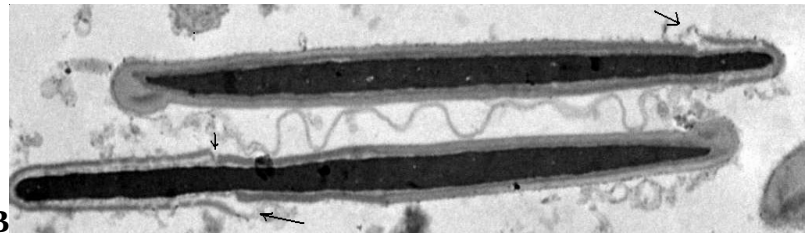


E

Fig 1 Sperm cell frozen in Tris +vitamin E medium.
49% of the cells (A, x3800) display intact plasmatic membranes at all levels: at the level of the main piece B (x50000), C (x65000) and at the level of the nucleus D (x87000) and acrosome E (x8500)



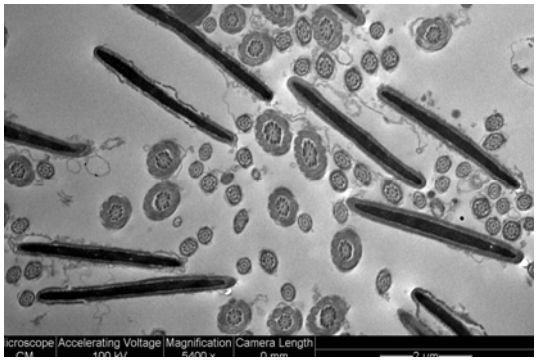
A



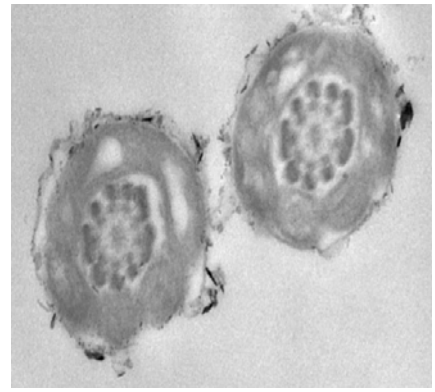
B

Fig 2 Sperm cells frozen in Tris + cysteine medium
37% of the cells display integral membranes
A (23500x) at the level of the main piece, the plasmatic membrane has vacuolisations
B(8500x) at the level of the head, the plasmatic membrane has small gaps (→) and vacuolisations

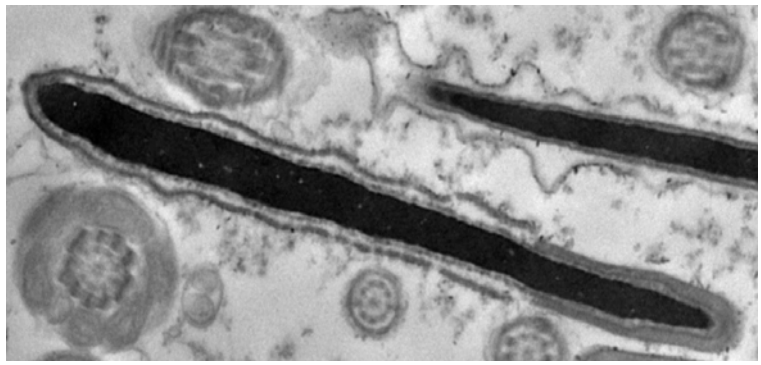
In the case of the semen diluted with medium supplemented with BSA (fig. 3), detachments of the plasmatic membrane were noticed, especially at the level of the head (fig. 3C) and of the midpiece (fig. 3B) and also detachments of the acrosome from nucleus (655 deteriorated cells). Also, there are protein deposits with acicular aspect at the level of the plasmatic membrane of the main piece (fig 3B). The motility and viability do not decrease, but do not register significant differences compared to the control (table 1).



A(x5400)



B(x23500)



C (x40000)

Fig 3 Sperm cells frozen in Tris+ BSA medium
35% of the cells (A, x5400) have intact plasmatic membranes
- at the level of the main piece B (x23500) there are gaps in the membrane and protein deposits
- at the level of the nucleus C (x40000) there are vacuolisations and gaps in the membrane

CONCLUSIONS

The viability and motility of sperm cells vary according to individual and the freezing medium used. Antioxidants have a very important role in the protection of the structure and function of the plasmatic membrane. The best effects regarding viability, motility and the membranal ultrastructure were given by the use of vitamin E in concentration of 1.0mM. Future researches will attempt to test *in vivo* buck semen frozen in media with different additions of antioxidant used to increase its fecundity.

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