

Role of antioxidant additives in the protection of the cryopreserved semen against free radicals

Received for publication, March 10, 2010

Accepted, May 12, 2010

ANGHEL ANDREEA, ZAMFIRESCU STELA

Universitatea Ovidius Constanta , Romania

Mamaia 124, Constanta, 0721621499

biologiecelulara2008@univ-ovidius.ro

Abstract

The aim of this study was to assess the effects of the addition of antioxidants (vitamin E and cysteine) to freezing 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, during normal reproduction season (October-December 2009) and diluted with a Tris-base extender containing vitamin E (1mM), cysteine (10mM) and without antioxidants (control). Diluted semen was cooled to 4°C and frozen in 0.25 ml straws, prior to being stored in liquid nitrogen. Semen parameters were investigated after thawing. The lipid peroxidation level was examined by measuring the production of malondialdehyde using the thiobarbituric acid method. The results show that both vitamin E and cysteine increased the percentage of sperm motility and viability ($p < 0.05$), but reduced the lipid peroxidation (LPO) level. A significant negative correlation was observed between the percentage of motile spermatozoa and the LPO level ($p < 0.05$).

Keywords: cryopreservation, reactive oxygen species, ram, lipid peroxidation

Introduction

Semen cryopreservation has a very important contribution in developing breeding techniques, such as artificial insemination (AI) and IVF. In sheep, although artificial insemination with fresh semen has been practiced for a long time, insemination with frozen-thawed semen results in a low fertility rate, which limits the practical application of this technique. Artificial insemination will reach its full potential by the efficient use of frozen semen.

Cryopreservation involves several steps, such as dilution, cooling, freezing and thawing (LUVONI, [1]). Each of these steps can damage the plasma membrane structure and functions which affect normal sperm function (HAMMERSTEDT & al., [2]), can reduce mobility and fertilizing ability (MAXWELL, & al., [3]), and can induce premature nuclear decondensation (CORMIER & al., [4]).

There is much evidence attesting to the fact that reactive oxygen species (ROS) and their oxidative negative effects are involved in a large number of pathologies. Oxidative Stress installed at the level of tissues, organs or organelles is derived from the imbalance between the production and elimination of reactive oxygen species (DOWLING & al., [5]). Studies on bull sperm (CHATTERJEE & al., [6]) ram (PERIS & al., [7]) and stallion sperm (BALL & al., [8]) showed that during the freezing-thawing process an overproduction of reactive oxygen species takes place (ROS) and there is a reduced activity of enzymes involved in the antioxidant defense (ALVAREZ & al., [9]), which results in damages to the plasma membrane. The beneficial effects of antioxidants in maintaining cell structure and function of sperm after thawing indirectly proves the hypothesis that oxidative stress is installed during cryopreservation (AGARWAL & al., [10]).

One of the major biological processes associated with the production of ROS is lipid peroxidation (LPO). In mammals, the sperm cell is very likely to suffer lipid peroxidation because cellular and internal organelles membranes have a content rich in polyunsaturated easily-peroxidable acids.

Lipid peroxidation leads to the loss of structural and functional integrity of membranes, causing the growth of cellular permeability, the inactivation of cell membranes enzymes, DNA structural damage and cell death (HSIEH & al., [11]). Given the importance that the process of peroxidation has on normal sperm cell functions, quantifying this process may have diagnosis significance. Currently, the largest part of the studies regarding lipid peroxidation involves analysis of the malondialdehyde (MDA) concentration, a product of degradation resulting from the process of peroxidation, with low molecular weight, which can be measured on the basis of its property to form adducts with thiobarbituric acid (SANOCCA & al., [12]).

Unlike somatic cells, in which antioxidant defense is provided by cytoplasmic enzymes such as catalase, superoxide dismutase and glutathione peroxidase, spermatozoa lose much of the cytoplasm immediately after maturation and therefore lose some of this protection (JONES & al., [13]). Nonenzymatic antioxidants present in seminal plasma (ascorbic acid, α -tocopherol, taurine, glutathione and albumin) are the most important forms of protection of spermatozoa against reactive oxygen species (ALVAREZ & al., [14]; AITKEN, [15]). Their defense mechanism has three levels of protection: prevention, capture and repair.

Glutathione, due to the relatively high concentrations in seminal plasma has an important role in cellular antioxidant defense. Because of its thiol grouping, it can react directly with hydrogen peroxide, superoxid anion and hydroxyl radicals, but also with hydroperoxides and alkoxyl radicals (STEWART, [16]). Other compounds containing sulfhydryl groups (cysteine, ergothionein) play an important role in maintaining metabolic functions and cell motility of sperm (LENZI & al., [17]).

Vitamin E is considered to be the main component of the antioxidant system of spermatozoa, one of the major protectors of the membranes against ROS and LPO attack (YOUSEF & al., [18]). Because its liposolubility, it occurs in the first line of defense against the peroxidation of the polyunsaturated fatty acid of membranous phospholipids structure (BANSAL & al., [19]; SHARMA & al., [20]).

The objective of this study was to determine the effect of vitamin E and cysteine in fighting the oxidative damage mediated by reactive oxygen species on motility, structural and functional integrity of plasmatic membrane and lipid peroxidation in sperm cells following freezing-thawing.

Materials and methods

Experiments took place at the ICDCOC Palas, Constanța, in the Laboratory of Biotechnology of Reproduction in the normal breeding season.

Animals: in this study we used semen samples from two adult Merinos of Palas rams, with proven fertility. The animals were maintained in uniform conditions of shelter, feeding and lighting. 41 ejaculates were processed.

Semen collection

Semen samples were collected three times a week by artificial vagina. Immediately after collection the ejaculates were placed in a water bath (37°C) until the laboratory evaluation. Semen assessment was performed within 10 minutes.

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 ram were divided into three equal parts and diluted with Tris-based extender without antioxidants (control) and extender supplemented with vitamin E (1mM) and cysteine (10mM), to a final concentration of approximately 4×10^8 spz/ml.

The cryopreservation technology for semen processing was developed by the Laboratory of Biotechnology of Reproduction (ZAMFIRESCU & al., [21]).

Diluted semen samples were drawn into 0.25 ml French straws (MINITUB, Germany), sealed with polyvinyl alcohol powder and balanced at 4°C for a period at 2h. After equilibration, the straws were frozen in liquid nitrogen vapors and then stored in liquid nitrogen (-196°C). After 24 h the straws were thawed individually in a water bath (37°C), for 30 seconds. Sperm evaluation was performed on all semen samples immediately after thawing.

Progressive motility is an indicator of semen quality and was analyzed under microscope (x100) (NOVEX, Holland) equipped with a heating plate maintained at 37°C. Semen motility was estimated by the analysis of three microscopic fields, different for each sample, the final score being the mean of three successive estimations (BARIL & al., [22]).

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 immersion (x1000) (BARIL & al., [22]).

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 20 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) (ZAMFIRESCU & al., [23]).

Measurement of lipid peroxidation: MDA levels were analyzed according to the thiobarbituric acid methods, described by RAO [24]. After thawing 0.5 ml of semen, the spermatozoa were separated from the seminal plasma by centrifugation (1000g for 10 minimum, at room temperature). The supernatant was used for the determination of seminal MDA and the pellet was used for the determination of spermatic MDA. The pellets were washed two times in Tris-HCl (pH 7.1), resuspended in 0.5 ml distilled water and subjected to rapid freeze-thawing three times to lyse 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. As standard, dimethyl acetal malondialdehyde bis (dimethyl acetal) (SIGMA, Germany) was used. The results were expressed as nmol MDA/ml seminal plasma and nmol MDA / 10^8 sperm.

Statistical analysis

The results are expressed as mean± standard error. For the determination of the significant differences among the parameters of the experimental versions, the means were analyzed by the t-Student test. Descriptive statistics used Microsoft Excel.

Correlation: For continuous variables, level correlation was established by calculating the Pearson correlation coefficient using the Excel statistical program. Significance level: $p < 0.05$.

To demonstrate the relationship between different variables, we used simple linear regression. For a correlation coefficient greater than 0.5, a good-very good correlation is appreciated.

Results and discussion

The aim of our study was to determine the influence of antioxidant additives added on the dilution-freezing media on cytological and oxidative parameters after thawing the ram semen.

The mean values of the cytological parameters (motility, viability and functional integrity of HOST membranes) are presented in Table 1 for ram A and in Table 2 for ram B.

Table 1. The variation of the cytological parameters in the frozen-thawed semen of ram A

Extender	Motility %		Viability %		HOST %	
	mean±SE	limits	mean±SE	limits	mean±SE	limits
control	49.79±1.10 ^a	40-60	53.74±0.98 ^a	46-62	53.02±0.98 ^a	45-63
Vitamin E 1.0mM	58.12±1.03 ^a	50-65	61.11±0.97 ^a	52-69	58.97±0.95 ^{ab}	50-63
Cysteine 10mM	60.2±1.25 ^a	45-70	64.13±1.07 ^a	50-73	62.67±1.09 ^{ab}	49-71

The superscript letters (a, ab) in the column of each version symbolize the significant differences (a: $p < 0.001$; ab: $p < 0.05$)

Table 2. The variation of the cytological parameters in the frozen-thawed semen of ram B

Extender	Motility %		Viability %		HOST %	
	mean±SE	limits	mean±SE	limits	mean±SE	limits
control	55.83±0.77 ^a	50-60	58.30±0.64 ^a	52-64	57.87±0.79 ^a	51-65
Vitamin E 1.0mM	60.20±0.70 ^a	55-65	62.83±0.75 ^{ab}	55-69	60.10±0.77 ^{ab}	51-66
Cystein 10mM	63.33±0.65 ^a	60-70	67.02±0.76 ^{ab}	62-69	64.89±0.64 ^{ab}	60-71

The superscript letters (a, ab) in the column of each version symbolize the significant differences (a: $p < 0.001$; ab: $p < 0.05$)

For both rams, significant increases over control are found in all the morphological parameters tested for extender supplementation with antioxidants. In the first ram, where the control cytological parameters have lower values than those of the second ram, more pronounced increases occur in both experimental versions. Motility and viability values were significantly increased by 8-10% compared to control ($p < 0.001$). Functional integrity expressed by HOST recorded significant increases ($p < 0.001$) for both experimental versions compared to control, but there is a significant difference ($p < 0.05$) between the two versions, the cysteine extender leading to a higher value, compared to the vitamin E medium (62.67 ± 1.09 to 58.97 ± 0.95). In the second ram there are significant increases (by 4-7%) for motility, viability ($p < 0.001$) and HOST ($p < 0.05$). Also, significant differences ($p < 0.05$) between the experimental versions for viability and functional integrity were recorded, the extender with

cysteine leading to higher values. Changes in oxidative parameters are shown in Table 3 for the first ram and in Table 4 for ram B.

Table 3. The variation of oxidative parameters in the frozen-thawed semen of ram A

Extender	Conc. MDA nmol/10 ⁸ spz		Conc. MDA nmol/ml plasma	
	mean±SE	limits	mean±SE	limits
control	5.55±0.55 ^a	1.89-11,3	16.16±0.64 ^a	10.3-20.4
Vitamin E 1.0mM	3.89±0.41 ^a	1.56-7.16	13.44±0.67 ^a	8.8-18.7
Cystein 10mM	4.06±0.35 ^a	1.12-7.01	14.01±0.65 ^a	8.9-19.4

The superscript letters (a, ab) in the column of each version symbolize the significant differences (a: p<0.001; ab: p<0.05)

Table 4. The variation of oxidative parameters in the frozen-thawed semen of ram B

Extender	Conc. MDA nmol/10 ⁸ spz		Conc. MDA nmol/ml plasma	
	mean±SE	limits	mean±SE	limits
control	5.61±0.51 ^a	2.9-11.3	15.69±0.79	3.2-20.4
Vitamin E 1.0mM	4.19±0.43	1.8-7.2	13.76±0.74	9.6-18.9
Cysteine 10mM	3.72±0.31 ^a	1.1-7.01	13.93±0.43	9.8-17.8

The superscript letters (a, ab) in the column of each version symbolize the significant differences (a: p<0.001; ab: p<0.05)

The mean concentration of malondialdehyde control has a value of 5.55 ±0.55 nmol/ml for the first ram and 5.61 ±0.51nmol/ml for the second ram, for a concentration of 10⁸spermatozoa and values of 16.16 ±0 64 and 15.69 ±0.79 nmol/ml, respectively, in seminal plasma. The first ram (Table 3) has registered a significant decline (p<0, 05) in the MDA concentration value for both the seminal plasma and for the suspension of cells, in both experimental versions. The second ram (Table (4) recorded a significant decline in the concentration of MDA only for the suspension of cells and only when cysteine was used as an additive antioxidant in the freezing media.

The analysis of the correlations between various sperm parameters showed the existence of significant negative correlations (p <0.05) between sperm motility and MDA sperm concentration in both rams (Fig. 1 a and b), for the vitamin E version.

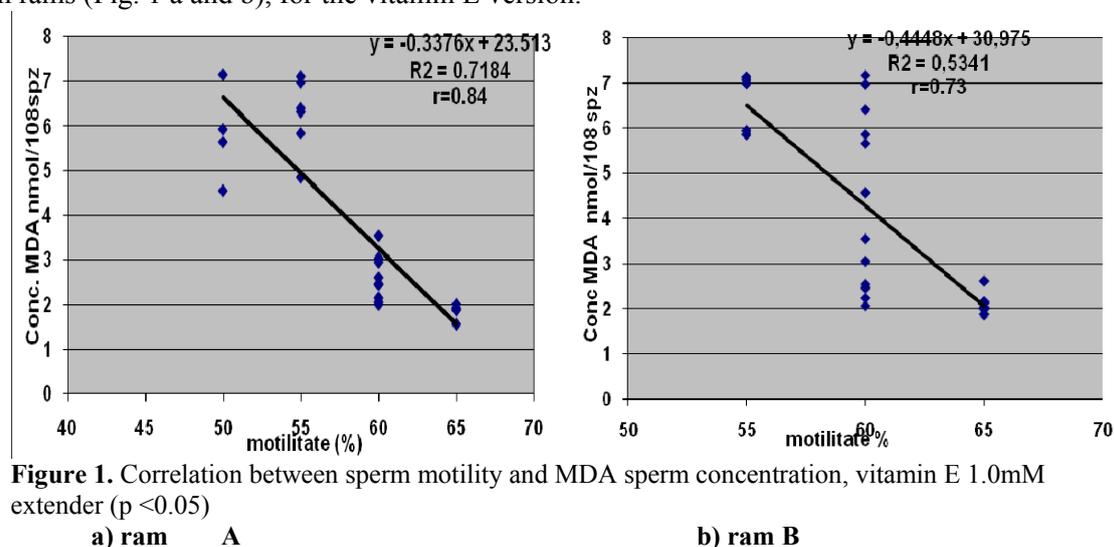


Figure 1. Correlation between sperm motility and MDA sperm concentration, vitamin E 1.0mM extender (p <0.05)

a) ram A

b) ram B

In case of cysteine, we obtained significant negative correlations ($p < 0.05$) between sperm motility and MDA spermatozoa concentration for both rams (Fig. 2a and b), although for the control samples there are no significant correlations. MDA seminal concentration was negatively correlated with sperm motility only for ram A, in both experimental versions (Fig. 3a and b).

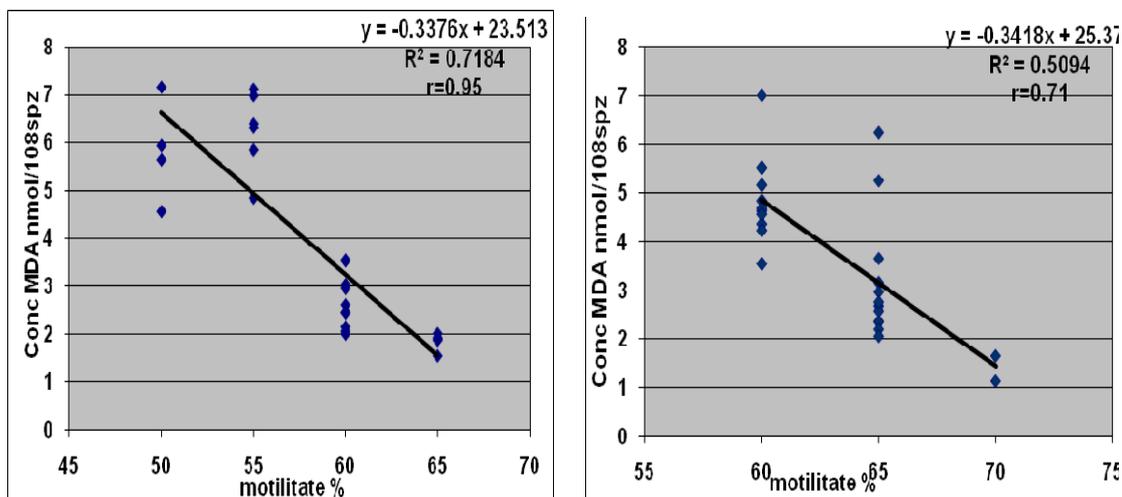


Figure 2. Correlation between sperm motility and sperm MDA concentration, cysteine 10.0mM extender ($p < 0.05$)

a) ram A

b) ram B

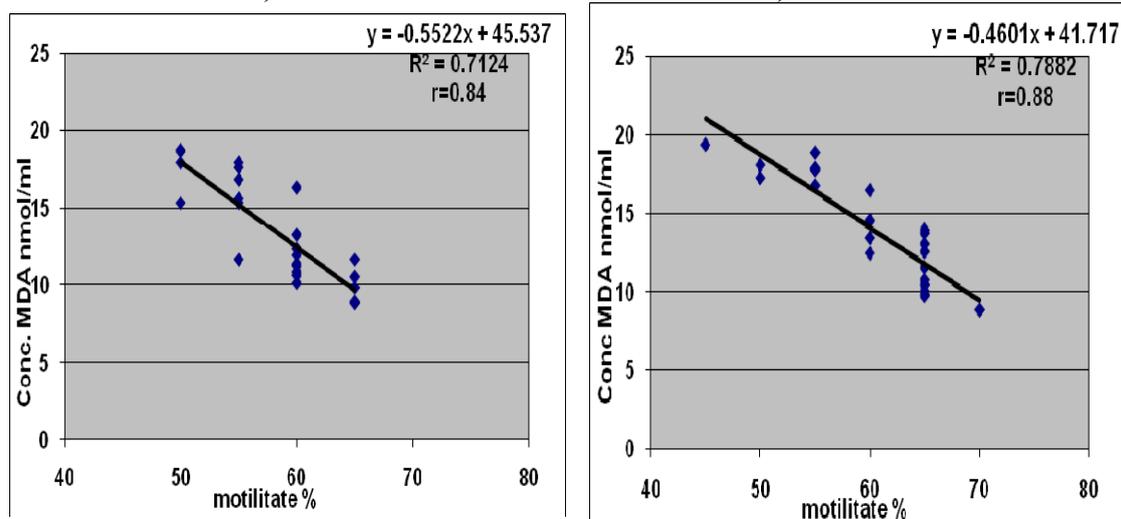


Figure 3. Correlation between sperm motility and seminal MDA concentration, ram A ($p < 0.05$)

a) vitamina E extender

b) cisteyne extender

The cryopreservation process affects the spermatozoa cell, an explanation being the increase in ROS production and / or decreased antioxidants levels during freezing and after freezing-thawing (ALVAREZ, [9]). Our results show a decrease in all cytological parameters after freezing-thawing; similar observations were reported by the literature (PERIS & al., [7]).

After thawing, antioxidants added on dilution media reduce the risk of initiating peroxidation of lipid by membrane sperm cell (BALL & al, [8]). Our results showed a high protective effect on post-thaw cytological parameters given by the cysteine-enriched medium dilution, similar to results obtained by BILODEAU [25] in bull sperm. Cysteine is part of the thiols and an amino acid precursor of glutathione. In mammals, reduced glutathione (GSH)

(L-glutamyl-L-cysteinyl-Glycine) is the most important endogenous antioxidant involved in maintaining the antioxidant-prooxidant balance in the tissue.

Other endogenous thiols are cysteine (Cys), homocysteine (Hcy) and cysteinylglycine (CGS). Thiols are involved in eliminating ROS, thus they have an important role in maintaining the function of sperm cells (ESKIOCAK & al., [26]. DNA present in the sperm head is highly compacted as a result of disulfide bridges between the cysteine residues of protamine molecules. The oxidation of thiols has a role in maintaining motility, in the stabilization of the flagellum structure and protection of DNA against physical and chemical deterioration.

It was demonstrated that high levels of glutathione in seminal plasma are associated with high motility, while low levels are associated with a high percentage of spermatozoa with morphological abnormalities and lack of motility. Also, the concentration of cysteine is negatively correlated with the percentage of spermatozoa with abnormalities (ESKIOCAK & al., [26]. Cryopreservation significantly decreases reduced glutathione (GSH), with approximately 78% (ALVAREZ & al., [9]. Therefore, we can assume that the supplementation of freezing extender with cysteine thiols helps to maintain the cytological parameters after thawing.

Our results showed that vitamin E has a protective effect on sperm cells against the harmful effects of oxidative stress induced by freezing. Results were confirmed by other studies that showed increases of the antioxidant capacity of human semen during preservation after vitamin E addition (ASKARI & al., [27]. It also protects bull sperm against oxidative stress (BANSAL & al., [19]. Vitamin E captures and eliminates oxygen radicals within the membranes and also the peroxyl and alkoxyl radicals that are generated during the conversion of lipid hydroperoxides, which are fuel for the peroxidation chain reaction (AITKEN & al., [28].

The lipid peroxidation in the spermatozoa cell is accompanied by irreversible damage to the plasma membrane, which leads to the loss of some components in the cytosol and then premature death (STOREY, [29]. Lipid peroxidation levels in seminal plasma can be considered a good indicator of several types of sperm problems (MORTE & al., [30].

In humans, it has been demonstrated that there was a significant correlation between the level of lipid peroxidation expressed by plasma MDA concentration and a rapid motility decrease (SHARMA & al., [20]; AITKEN, [15]. Observations on the damage of the plasma membrane is confirmed by our results which show the existence of significant negative correlation between MDA seminal plasma concentration and sperm motility, which strengthens the hypothesis that lipid peroxidation is responsible for the significant reduction of motility.

Membrane damage may explain the decrease in sperm viability recorded after freezing-thawing because they allow the vital dye eosin-nigrosin within sperm cells, indicating death. Methods of determining the concentration of MDA are not yet standardized and ram sperm studies are few, but our results are confirmed by those made on human sperm (LAUDAT & al., [31].

Conclusions

The addition of antioxidants (vitamin E, 1.0 mM and 10.0 mM cysteine) on dilution-freezing media leads to increased motility and structural and functional integrity of membrane sperm cells, but also reduces the level of lipid peroxidation, confirming that antioxidants are effective in preventing the fast decrease of motility that normally occurs on cooling spermatozoa and maintains sperm motility under conditions of oxidative stress. Our study

confirms the recent experiments which have demonstrated that membrane lipid peroxidation is involved in the decreased motility of the sperm cell after freezing-thawing.

So far there is no universally accepted international method for estimating oxidative stress at sperm level. Because of the many variables that can influence its quantification, studies are needed to standardize protocol analysis and to define clinical changes between which oxidative stress can be defined accurately.

Acknowledgements

This research was realized within the National Research Program PN2 no. 62082, subsidized by CNMP within the Ministry for Education and Research, Bucharest. The authors thank assistant professor Olivia Chirobocea for the translation of the article into English.

References

1. GC LUVONI, Gamete cryopreservation in the domestic cat, *Theriogenology*, 66(1):101-111, (2006)
2. RH HAMMERSTEDT, JK GRAHAM, JP NOLAN, Cryopreservation of mammalian sperm: what we ask them to survive, *J Androl*, 11: 73-88, (1990)
3. WMC MAXWELL, PF WATSON, Recent progress in the preservation of ram semen, *Anim Reprod Sci*, 42: 55-65, (1996)
4. N CORMIER, MA SIRARD, JL BEILEY, Premature capacitation of bovine spermatozoa is initiated by cryopreservation, *J Androl*, 18:461-468, (1997)
5. DK DOWLING, LW SIMMONS, Reactive oxygen species as universal constraints in life-history evolution, *Proc. R. Soc. B*, 276: 1737-1745, (2009)
6. S CHATTERJEE, E DE LAMIRANDE, C GAGNON, Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione, *Mol Reprod Dev*, 60: 498-506, (2001)
7. SI PERIS, JF BILODEAU, M DUFOUR, JL BAILEY, Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation and functional parameters in ram sperm, *Mol Reprod Dev*, 74(7): 878-892, (2007)
8. BA BALL, AT VO, J BAUMBER, Generation of reactive oxygen species by equine spermatozoa, *Am J Vet Res*, 62:508-515, (2001)
9. JG ALVAREZ, BT STOREY, Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode for sublethal cryodamage to human sperm during cryopreservation, *J Androl*, 13: 232-241, (1992)
10. A AGARWAL, RA SALEH, MA BEDAIWY, Role of reactive oxygen species in the pathophysiology of human reproduction, *Fertil Steril*, 79(4): 829-843, (2003)
11. YY HSIEH, CC CHANG, CS LIN, Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility, *Int J Biol Sci*, 2:23-29, (2006)
12. D SANOCKA, D KURPISZ, Reactive oxygen species and sperm cells, *Reprod Biol Endocrinol*, 23:2-12, (2004)
13. R JONES, T MANN, R SHERINS, Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma, *Fertil Steril*, 31:531-537, (1979)
14. JG ALVAREZ, BT STOREY, Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility, *Biol Reprod*, 29:548-555, (1983)
15. RJ AITKEN, The Amoroso Lecture. The human spermatozoon-a cell in crisis? *J Reprod Fertil*, 115:1-7, (1999)
16. I STEWART, Glutathione as a treatment for male infertility, *Reviews Reprod*, 1: 6-12, (1996)
17. A LENZI, M PICARDO, L GANDINI, F DONDERO, Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy, *Human Reprod Upd*, 2(3): 246-256, (1996)
18. MI YOUSEF, GA ABDALLAH, KI KAMEL, Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits, *Animal Reprod Science*, 76: 99-111, (2003)

19. AK BANSAL, G BILASPURI, Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress, *Anim Sci Pap and Rep*, 27(1):5-14, (2009)
20. RK SHARMA, A AGARWAL, Role of reactive oxygen species in male infertility, *J Urology*, 48: 835-850, (1996)
21. S ZAMFIRESCU, A ŞONEA, *Biotehnologii de reproducție la rumegatoarele mici*, Ed Ex Ponto, Constanța, (2004)
22. G BARIL, P CHEMINEAU, JC VALLET, *Manuel de formation pour l'insemination artificielle chez les ovins et les caprines*, FAO, Roma, (1993)
23. S ZAMFIRESCU, V CIUPINA, D NADOLU, Utilizarea testului hipoosmotic pentru evaluarea integrității funcționale a membranei spermatozoizilor de berbec după congelare- decongelare, *Bul. SNBC*, 29: 248, (2001)
24. B RAO, JC SOUFIR, M MARTIN, G DAVID, Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility, *Gamete Res*, 24 : 127-34, (1989)
25. JF BILODEAU, S BLANCHETTE, IC GAGNON, MA SIRAD, Thiols prevent H₂O₂-mediated loss sperm motility in cryopreserved bull semen, *Theriogenology*, 56: 275-286, (2001)
26. S ESKIOCAK, AS GOZEN, SB YAPAR, F TAVAS, AS KILIC, M ESKIOCAK, Glutathione and free sulphhydryl content of seminal plasma in healthy medical students during and after exam stress, *Human Reprod*, 20(9):2595-2600, (2005)
27. HA ASKARI, JH CHECK, N PEYMER, A BOLLENDORF, Effect of natural antioxidants tocopherols and ascorbic acids in maintenance of sperm activity during freeze-thaw process, *Arch Androl*, 33: 11-15, (1994)
28. RJ AITKEN, JS CLARKSON, Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques, *J Androl*, 9:367-376, (1988)
29. BT STOREY, Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa, *Molecular Hum Reprod*, 3 (3): 203-213, (1997)
30. MI MORTE, AS RODRIGUES, D SOARES, AM RODRIGUES, S GAMBOA, J RAMALHO-SANTOS, The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: Seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility, *Anim Reprod Sci*, 106:36-47, (2008)
31. A LAUDAT, K LECOURBE, A PALLUEL, Lipid peroxidation, morphological stress pattern and nuclear maturity of spermatozoo, *Annales de Biol Clin*, 57(1):51-56, (1999)