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ADVANCED STUDIES ON IMPROVING SHEEP FERTILITY BY USING ARTIFICIAL MEANS OF REPRODUCTION

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Abstract

Artificial insemination (AI) in livestock is used to optimize reproduction efficiency. Compared to other semen preservation methods, cryopreservation is an established industry used worldwide for performing AI. Adequate protocols for semen collection and freezing and then for the use in the AI are set up for all the animal species. In sheep, AI with frozen-thawed semen resulted low fertility rate, which limits the practical application of this technique. Progressive sperm motility, sperm viability, sperm plasma membrane integrity and NAR were significantly ($P < 0.05$) higher for BIOX, MILK, and TEY extenders. Progressive motility increased significantly ($p < 0.01$) using licorice extract 10, 50 and 100 g/ml. Diluter type had a significant effect ($p < 0.01$) on sperm motility. The percentage of progressive motility in all extenders media containing LDL was also higher compared with 20% EY (control) during dilution and equilibration stages. All extenders containing LDL reduced the percentages of abnormalities after dilution as compared to control 20% egg yolk. The percentages of intact Acrosome in all other extenders containing LDL were significantly higher than 20% egg yolk extender. The highest percentage of post-thaw progressive motility was recorded in extender containing 20mm glutamine. After dilution and equilibration, supplementation of glutamine at concentration of 40 and 60mm caused a significant increase in plasma membrane intact compared with control and all other concentrations tested. No significant difference between the control and the irradiated samples for viability However, the semen samples irradiated with 6.12 J/cm^2 showed a slight increase in sperm progressive motility, viability, osmotic resistance, Acrosome and DNA integrity, respect to the semen samples irradiated at low energy doses and control semen samples. Cysteine effected on the ultra-structure of the ram sperm cell within the freezing- thawing dynamics. The positive effect of Cysteine could be a result of its interraction with membranes phospholipids during the freezing, giving it a better Cryopreservation.

Key words**:** *acrosome, cryopreservation, cysteine, freezing-thawing dynamics, ultra-structure*

INTRODUCTION

Reproduction is directly affected by various management related factors. Manipulation of these factors can cause changes in reproductive performance. The control and manipulation of the sheep reproduction has been the objective of scientists around the world for many years. High levels of reproductive performance can only be achieved under optimum management conditions, (including nutrition), [17]. This is one factor that determines the dramatic differences in reproductive efficiency between the developed and the developing countries (where the nutrition and general management of the flocks are not quite good). The factors affecting improving fertility in sheep are genetics (breeds and crossbreds), heat stress and management, food nutrition and energy supply, semen dilution and using some additives and cryopreservation methods [2, 16]

Artificial insemination (AI) in livestock has made it possible to optimize reproduction efficiency. AI is undoubtedly the management

technique that has most contributed to the genetic improvement of livestock in modern animal production. By comparison to the other currently available preservation methods of semen, cryopreservation is an established industry used worldwide for performing AI, as it can preserve cells life un definitely [21] and [20] The main disadvantage of semen cryopreservation is that freezing and thawing induce several forms of cellular lesions, which dramatically reduce the quality and subsequent fertilizing ability of the semen. Sperm motility, viability, membrane integrity and biochemical parameters are all routinely used tests to assess the quality of frozen-thawed semen [14, 19]. However, acceptable motility and viability of spermatozoa do not necessarily lead to acceptable conception rates. By this research project, we aim at increasing the conception rate in ewes following AI, by using frozen-thawed semen. In order to achieve this goal, we have the following objectives: to improve the freezing technologies, to evaluate the cytological and cryobiological indexes of the spermatozoa, to obtain accurate biochemical and electro microscopically profiles of the cryopreserved cells, and to test, in vivo, the efficiency of different preserving and AI technologies, on the cryobiological parameters and reproductive indexes. The low fertility registered in AI when used cryopreserved semen is due to the harmful processes during freezing. Between 10 and 50 % of the spermatozoa die because of the processes. When natural mating occurs, the sperm is exposed to anaerobic conditions, which limits the production of ROS. This does not happen with the cryopreserved sperm, which is exposed to oxygen and to other harmful processes during freezing (e.g. seminal plasma dilution, enzymatic damage), that lead up to an increase in ROS production and lower the antioxidative defense of cells.

MATERIALS AND METHODS

The following tasks were conducted in Kafrelshiekh University farm in Egypt, using the same methods and technique (tasks 1, 2, 3 and 4), while tasks 5 and 6 were conducted at Ovidius University.

The project researches included the following:

Task 1.1: Obtaining diluting/freezing extenders with the addition of different doses of antioxidants

Task 1.2: *In vitro* assessment of the added antioxidants' effect on certain cytological parameters of cryopreserved spermatozoa, such as motility, structural and functional integrity of plasmatic membrane – vitality, and functional integrity of the membrane

Task 1.3: *In vivo* assessment of the added antioxidant influences on the reproductive parameters (fecundity, fertility).

Task 1.4: Sperm cryopreservation with different extenders, in straws and glass vials.

Task 2.1: The quality of ram semen cryopreserved according to different procedures coming from the FEAS biobasis, will be evaluated by qualitative tests, by using phase contrast and fluorescent microscopy. The scope is to assess some important functional and structural parameters such as motility, viability, osmotic-resistance, Acrosomeial and DNA intactness. Some biochemical parameters like energy charge and COX activity, assumed to be correlated with sperm motility, will be also investigated.

Task 2.2: Samples of cryopreserved ram semen was irradiated post-thawing with Helium-Neon laser at different energy doses. Functional, structural and biochemical parameters was investigated, in order to find the mechanism by which the laser acts on the mitochondria and the cellular membranes, and whether laser irradiation might improve the quality of semen compared to the control (non-irradiated).

Task 3.1: Ram semen was collected and frozen according to the technology developed within the laboratory. Freezing technology of ram semen comprises 9 stages. The technology starts with sperm collection and finishes with liquid nitrogen storage of the straws or the glass vials. After collection, the semen was diluted and cooled at 4 c. In order to protect the spermatozoa against cooling damage, the semen was diluted in a saline extender supplemented with egg yolk. The

lipid fraction from egg yolk, mainly lipoproteins of LDL type, ensures a degree of membrane equilibrium when the cells are set at low temperatures. The cryopreserved (glycerol) will be then included into the semen. Since glycerol is toxic for spermatozoa when used at 37oC, this stage will be performed at 4oC.

Task 3.2: The quality of thawed semen was evaluated, using modern techniques of analysis, in order to assess viability and functional integrity of the mitochondria. Light microscopy (fluorescence and phase contrast microscopy) was used Viability was assessed by using flow cytometry and viability of the thawed spermatic cells was studied with the method of double staining, using 2 fluorochromes to dye nuclear acids: SYBR-14 and propidium iodide (PI). SYBR-14 dyeds spermatozoa, which are alive and have intact membranes, whereas, PI dyeds dead cells with damaged membranes. Quantitative analysis of the fluorescent – marked cell populations was performed by using flow cytometry. Functional integrity of the mitochondrial chain was studied by using the Rhodamine and PI marking method with fluorescence microscopic and flow cytometric analyses. The parameters of spermatozoa established through these methods, was statistically correlated with the reproduction indexes.

Task 4.1: A study on the biochemical profile of the cryopreserved semen. In order to establish the biochemical profile of the cryopreserved semen, there were two evaluated parameters of the oxidative stress, malondialdehyde (MDA) and total antioxidative capacity (TAC), as well as, the concentrations of calcium, sodium and potassium ions. The study of the two parameters focused on the seminal plasma, diluting/freezing extenders and the cryopreserved spermatozoa. The concentrations of MDA and of other substances which react with tiobarbituric acid (TBARS) were determined by using the TBARS method, described by [18]. TAC was evaluated on fresh seminal plasma before freezing and on seminal plasma following freezing-thawing, into different diluting-freezing extenders. The activity was accomplished by using the ABTS method of spectrophotometer [13]

Task 5.1: Characterization of the membrane changes observed at the head, Acrosome and flagel levels (the intermediate, principal and terminal pieces). Ultra structural changes of the cell membranes following thawing was assessed by transmission electron microscopy (TEM), using the classical technique [14] and [23]

Task 6.1: In vivo testing of the tested semen, by using AI. This activity was conducted on ewes with known reproductive cycle (which gave birth 5-6 months before testing). In order to identify the estrus, the ewes were evaluated 2 times a day, in the morning and in the evening. The insemination was performed at around 18 hours from the beginning of estrus. AI was performed intracervically or laparoscopically, using as witness sample diluted fresh semen 1:1. The diluents should be the same used for preparing the cryopreserved semen [12].

RESULTS AND DISCUSSIONS

Progressive sperm motility, sperm viability, sperm plasma membrane integrity and NAR were significantly ($P < 0.05$) higher for BIOX, MILK, and TEY extenders at 1st, 3rd and 5th day of storage compared to EYC extender.

Table 1. The integrity of plasmatic membrane and Acrosome (%, medium±es, n=10), after freeze-thaw process (Merinos of Palas ram, the meat line).

Version	n	Plasmatic membrane Head	Flagellum	Acrosome
Witness	10	37,2±1,65	39,25±1,59	42,40±3,18
Cysteine 10,0mM	10	49,0±3,02	49,90±2,36	52,70±3,82

Moreover, progressive sperm motility, sperm viability and sperm plasma membrane integrity were not affected up to third day of storage in BIOX extender and at 5th day of storage the values for these parameters remained significantly ($P<0.05$) higher in BIOX compared to other extenders. Sperm abnormalities (head, mid piece and tail) did not differ among the different extenders at 1st, 3rd and 5th day of storage1, 5, Progressive

motility increased significantly ($p < 0.01$) in levels of using licoric extract 10, 50 and 100 .g / ml in both diluters as antioxidant, during all storage periods.

The means of progressive motility were 72.5 ± 1.02 %, 72.08 ± 1.05, 70.90 ± 2.05 % and 66.25 ± 3.15 % respectively, compared to the control (0) 61.45 ± 16.2 %. . Levels 1, 5 and 10 .g /ml were superior ($p < 0.01$) to levels 50 and 100 .g /ml (fig1). Diluter type had a significant effect ($p < 0.01$) on sperm motility. Overall the percentage of motile sperm in EYT diluter (66.48 ± 1.21 %) was higher than that in yolk-glucose citrate diluter (64.37± 1.44 %). Sperm motility tended to decline significantly ($p < 0.01$) as the length of storage period increased. The means of progressive motility were 80.00 ± 2.04 % after dilution (0h), 68.75 ±3.15 % 61.25 ± 4.27% and 50.62 ±4.61 %, at 24, 48 and 72 h after cooling [10] respectively. Three experiments also were conducting as follows**:**

Experiment 1: Assessment of the cryoprotective effect of LDL on the freezing of Buffalo semen [11].

Experiment 2: Assessment of the cryoprotective effect of Glutamine on the Buffalo semen.

Experiment 3: Assessment of the cryoprotective effect of the best low concentration of Glutamine when combined with 12 % LDL on the freezing of Buffalo semen [15].

The percentage of progressive motility in all extenders media containing LDL was higher compared with 20% EY (control) during dilution and equilibration stages [12]. The percentage of post-thaw progressive motility was twofold higher ($P<0.05$) in 12% LDL extender than control extender containing 20% EY (63.3 vs. 35%, respectively). The post-thaw motility after 30 days storage were more twofold higher in 10, 12 and 15% LDL (51.67, 61.67 and 52.5%, respectively) than control extender (20% EY, 26.67%). In cooling semen at 5°C, the percentage of progressive motility in 8, 10 and 12% LDL stayed approximately constant between 0 h and 24 h. The percentage of progressive motility in Ram spermatozoa preserved at room temperature (20 - 25oC) was significantly ($P<0.05$) different among different storage periods and the rate reduction of progressive motility was:

1.Significantly smaller ($P<0.05$) in LDL extenders as compared to control 20% EY. [13]

2.The percentage of live spermatozoa did not differ between dilution and after equilibration stages in extenders containing 8, 10 and 12% LDL, and significantly ($P<0.05$) lower in control and other extenders containing LDL (4, 6 and 15%) after equilibration [14]. The percentage of post-thaw live sperm in 12% LDL extender was twofold higher ($P<0.05$) than extender with 20% EY (76.0 vs. 38.67%, respectively). In cooling semen at 5° C, after 96 h of storage, the percentage of live spermatozoa significantly decreased. The highest value was recorded in 12% LDL when compared to control extender containing 20% EY. The percentage of live spermatozoa in all LDL extenders were significantly ($P<0.05$) higher compared to control at any storage period at room temperature and the highest values of livability were recorded in 12% LDL [15].

3-All extenders containing LDL reduced the percentages of abnormalities after dilution as compared to control 20% egg yolk in a buffalo semen extender and continued in low levels after equilibration, freezing and after one month storage in liquid nitrogen. In cooling semen at 5° C for 4 days, the greatest improvement rate due to LDL replacement was recorded in 10 and 12 % LDL followed by 8 and 15% LDL. The percentage of sperm abnormalities in extenders containing LDL especially 8, 10 and 12% LDL, was significantly ($P<0.05$) lower than control 20% EY in any storage period at room temperature [15].

The percentages of intact Acrosome in all other extenders containing LDL were significantly higher than 20% egg yolk extender. Also, intact Acrosome was superior in medium containing 12 % LDL ($P<0.05$) than either control 20% EY or other extenders containing different concentrations of LDL. Percentage of Ram abnormal spermatozoa

1.Stored in extender containing 20% EY was significantly (P<0.05) higher than spermatozoa stored in all extenders containing different LDL concentrations at any storage period at 5oC. [15]

2.The highest membrane integrity value was recorded in 12% LDL followed by 10%, 15%, 8% and 6% LDL after dilution. The sperm membrane integrity in 12% LDL was 87.67, 84.67, 71.33 and 68.50% after dilution, equilibration, freezing and 30 days of storage in liquid nitrogen, respectively. In cooled semen at 5o C, the percentage of plasma membrane integrity of Buffalo spermatozoa decreased over time on to 96 h and all values were significantly (P<0.05) better in different LDL concentrations than control 20% EY and significantly (P<0.05) higher in all extenders containing LDL compared to control egg yolk at any storage period in buffalo semen stored at room temperature.

The effects of He-Ne laser at various energy doses, on the quality of cryopreserved ram semen

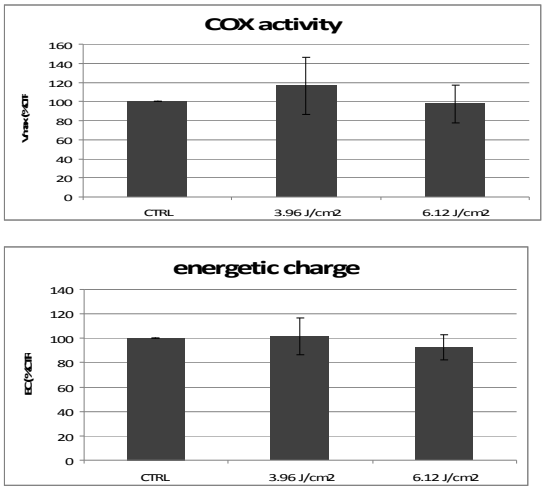

Fig.1. COX activity and Energetic charge of cryopreserved and irradiated ram semen.

The lower dose of laser energy resulted to be ineffective (P<0.05) than other irradiated samples and control.

No significant difference between the control and the irradiated samples for viability (47.96 ± 2.18 vs 45.77 ± 1.81 and 49.06 ± 1.66), osmotic resistance (37.94 ±3.08vs 36.45 ± 2.85 and 39.43 ± 1.87), Acrosome integrity (37.89 ± 2.83 vs 36.68 ± 2.68 and 40.68 ± 1.07) and DNA integrity (98.50 ± 0.29 vs 97.79 ± 0.35 and 98.74 ± 0.20) was found. However, the semen samples irradiated with 6.12 J/cm2 showed a slight increase in sperm progressive motility, viability, osmotic resistance, Acrosome and DNA integrity, respect to the semen samples irradiated at low energy doses and control semen samples. In parallel, the effect of irradiation on biochemical parameters of samples was evaluated by measuring the activity of cytochrome oxidase (COX) and the energetic charge (Fig 1) [9].

As for parameters reported, no significant difference in mean values for both COX activity and Energetic charge between control and laser treated sperm samples was found. This could be mostly due to the extreme variability of semen samples which resulted in an unpredictable effect of laser treatment [10].

"Effect of glutamine supplementation to extender on frozen-thawed buffalo semen"

1-When compared with the control, and the 20, 40 and 60mm Glutamine significantly improved occurred in progressive motility (P<0.05) after different stages of cryopreservation of semen. The highest percentage of progressive motility was recorded at concentrations of between 20 and 40 mm Glutamine at any stage of cryopreservation of ram semen. Post-thaw motility decreased when the Glutamine concentration increased from 60 to 80 and 100mm. At any stage of storage after cooling at 5^{o} C, the use of 20mm Glutamine caused an increase of progressive motility than control extender. Glutamine at concentrations of 20 and 80mm caused a slightly improvement in sperm motility at any storage period at room temperature (18 – 20^{o} C) compared with the control.

2-Addition of 20, 40 and 60mm Glutamine resulted in significant (P<0.05) increase in live spermatozoa after different stages of cryopreservation compared with control. At concentration between 80 and 100mm glutamine significantly (P<0.05) reduced the percentage of live spermatozoa compared with concentrations of 20 and 40mm at any

stages of cryopreservation. Moreover, at 100mm concentration, the live spermatozoa decreased significantly (P<0.05) compared with the control at any stage of cryopreservation. The highest percentage of live Buffalo spermatozoa was obtained at 40mm for glutamine at any storage period at 5° C, while, at room temperature was achieved in the presence of 40 and 60mm glutamine but, at concentration of 100mm significantly (P<0.05) reduced compared to the control.

3-Glutamine improved sperm abnormalities at all concentrations tested (20 to 100mm) and at any stage of cryopreservation (after equilibration and post-thawing process.

4-At concentrations of between 20 and 80mm, Glutamine significantly (P<0.05) decreased the sperm abnormalities compared with the control from 24 to 96 h of preserved at 5ºC and at room temperature (18-20° C) from 24 to 48 h .

5-The percentage of Buffalo spermatozoa with an intact Acrosome after stage of equilibration, was significantly (P<0.05) lower in extenders [3]

6.Containing glutamine at concentration of between 20 and 80mm compared to the control extender, and also after freeze-thawing process the highest value were at 20 and 40mm glutamine. In preserved semen at 5° C the Acrosome integrity of semen supplemented with glutamine at concentration of 20, 40 and 60mm were significantly (P<0.05) higher than in the control-without glutamine and 100mm glutamine at any storage period. At room temperature, addition of glutamine to control extender from 20 to 60mM significantly (P<0.05) increased Acrosome integrity after 0 h and 24 h.

6-After dilution and equilibration, supplementation of glutamine at concentration of 40 and 60mm caused a significant increase in plasma membrane intact compared with control and all other concentrations tested. 7-Also, after freezing, the post-thaw plasma membrane integrity percentage was significantly (P<0.05) higher in 20, 40 and 60mm glutamine compared with control and other extenders. In cooling semen at 5° C, the rate damage of membrane integrity was lowest in 60, 40 and 20mm respectively; while, glutamine at 100mm was ineffective and plasma membrane integrity was slightly lower than that in control. At room temperature, only 40 and 60mm glutamine significantly (P<0.05) improved intact plasma membrane when compared with the control.

Effect of LDL and glutamine supplementation to the extender on frozen-thawed buffalo semen

1-The low concentrations of Glutamine used showed that 10mm, 20mm and 30mm glutamine +12% LDL were significantly (P < 0.05) better than 50mm or 60mm Glutamine and non significant with 40mm after 30 days of storage in LN. The highest percentage of post-thaw progressive motility was recorded in extender containing 20mm glutamine (68.33%). In preserved semen at 5° C at 24, 48, 72 and 96 h, the mean forward motility of Ram spermatozoa was highest (P < 0.05) due to addition of Glutamine at 10 and 20mm as compared to control and other Glutamine concentrations. The forward motility of buffalo spermatozoa diluted in 10mm and 20mm Glutamine. Was significantly (P < 0.05) higher than in the control at any storage period at room temperature, while, 60mm led to decrease sperm motility in comparison with the control [4].

2-The post-thaw live spermatozoa and After 30 days of storage in liquid nitrogen, for 10 and 20mm glutamine were significantly (P < 0.05) higher compared with the other concentrations tested respectively). After 30 days of storage in liquid nitrogen, the post-thaw live spermatozoa for 10 and 20mm glutamine were significantly (P < 0.05) higher compared to the other concentrations. The highest live spermatozoa of ram-cooled semen at 5ºC was recorded for 10 and 20mm glutamine with 12% LDL at each of storage periods in comparison with control except 6mm Glutamine at 96 h, which it significantly (P < 0.05) decreased compared to the control. The addition of glutamine at concentrations 10, 20, 30, 40 and 50mm led to improve live spermatozoa compared to the control and 60mm Glutamine this difference was significant (P < 0.05) at 24, 48 and 72 h of storage.

3-The percentage of sperm abnormalities of ram spermatozoa slightly improved due to supplementation of glutamine at different concentration after dilution compared to the control, but differences were not significant. When compared to the control, only 10, 20 and 30mm glutamine + 12 % LDL significantly ($P < 0.05$) decreased sperm abnormalities of spermatozoa after equilibration and the lower value was observed in 10mm Glutamnie + 12% LDL. The highest sperm abnormalities of spermatozoa ($P<0.05$) were observed for 60mm Glutamine + 12% LDL compared with all medium tested after freeze-thawing process or following 30 days of storage in LN . It was observed that the addition of Glutamine at different concentrations led to reduce sperm abnormalities percentage in comparison to the control-without glutamine at each storage period at 5 ° C. At room temperature glutamine at 10 and 20mm significantly ($P < 0.05$) improved the percentage of sperm abnormalities at any storage time when compared to the control.

4-The highest number of Acrosome integrity of Buffalo spermatozoa, even if not significant, was obtained at 10 and 20mm Glutamine + 12% LDL after equilibration stage comparison with the control. The 20mm Glutamine + 12% LDL medium gave the highest ($P < 0.05$) number of Acrosome integrity of post-thawed Buffalo spermatozoa following freezing or 30 days of storage in LN in comparison with other media. The medium containing 10 and 20mm Glutamine +12% LDL provides the best protection of Acrosome integrity in comparison with other extenders tested at any stage of storage at cooling semen at 5° C and at room temperature (15 – 18° C).

5-After equilibration, glutamine at concentrations of 10 and 20mm gave the best rate of membrane integrity in comparison with the other concentrations tested. The 20mm glutamine +12% LDL gave the best percentage of post thawed plasma membrane integrity of frozen. Spermatozoa following thawing and 30 days of storage in LN in comparison with all other concentrations tested. At 5° C, the percentage of intact plasma membrane in extenders containing 10mm and 20mm Glutamine+ 12% LDL was significantly ($P < 0.05$) higher compared with the control and other concentrations tested at any storage time. In addition, the percentage of membrane integrity in 60mm Glutamine +12% LDL was significantly lower ($P < 0.05$) than control at any storage time. At room temperature, the highest percent of intact plasma membrane for buffalo spermatozoa was at 20mm Glutamine +12 %LDL compared with other extenders tested and the effect of storage period on the membrane integrity was significant in all extenders used in the current study.

Effects of Licorice Extract (Anti Oxidant) on Sperm Motility of Chilled Progressive motility

Increased significantly ($p < 0.01$) in levels of licorice extract 1, 5, 10, 50 and 100 .g / ml in both diluters, during all storage periods. The means of progressive motility were 72.5 ± 1.02 %, 72.08 ± 1.05, 70.90 ± 2.05 % and 66.25 ± 3.15 % respectively, compared to the control (0) 61.45 ± 16.2 %. (fig1). Levels 1, 5 and 10 .g /ml were superior ($p < 0.01$) to levels 50 and 100 .g /ml (fig1). Diluter type had a significant effect ($p < 0.01$) on sperm motility. Overall the percentage of motile sperm in EYT diluter (66.48 ± 1.21 %) was higher than that in yolk-glucose citrate diluter (64.37± 1.44 %). Sperm motility tended to decline significantly ($p < 0.01$) as the length of storage period increased. The means of progressive motility were 80.00 ± 2.04 % after dilution (0h), 68.75 ±3.15 % 61.25 ± 4.27% and 50.62 ±4.61 %, at 24, 48 and 72 h after cooling, respectively [11] and [22].

Cysteine effect on the ultra-structure of the ram sperm cell within the freezing-thawing dynamics

The ultra-structure of freeze-thaw spermatozoa in Tris medium

Generally, all the head sections have membranes with corrugations; sometimes they are excessively bloated and only a percentage of 37.2% of the cells have at their head unlysate plasmatic membranes (fig. 1 and 2). In some areas small vacuoles appear under the internal double membrane (fig. 1). The Acrosome has an intact structure at 42%

of the cells, and the Acrosome external membrane is generally, unaffected. At the flagellum, the plasmatic membrane has a jigsaw aspect and it is partial detached or is completely broken (Photo 2 and 3). In the intermediate piece, the mitochondria has a normal appearance.

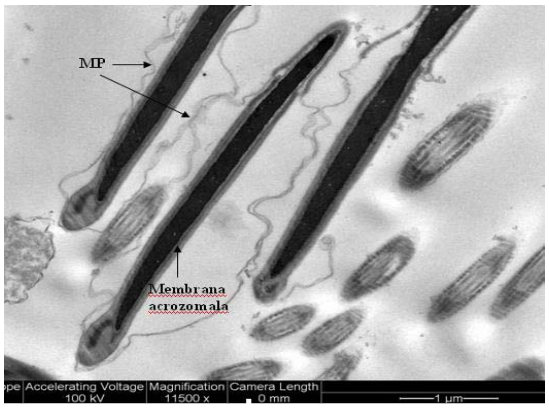

Photo 1. Head sagittal section (x11500)
Ram form the meat line, Tris media

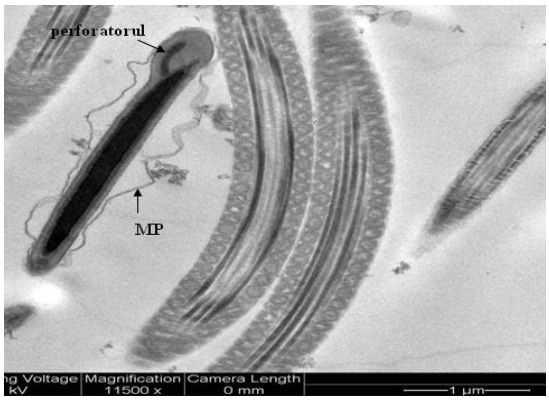

Photo 2. Ram sperm cells frozen into the Tris medium (x11500)

Head Sagittal section; the Acrosome contain an Acrosome matrix with electronodens layout Where one may see the "puncher" (apical ridge) Cross section of the intermediate piece, the Mitochondria are unaffected.

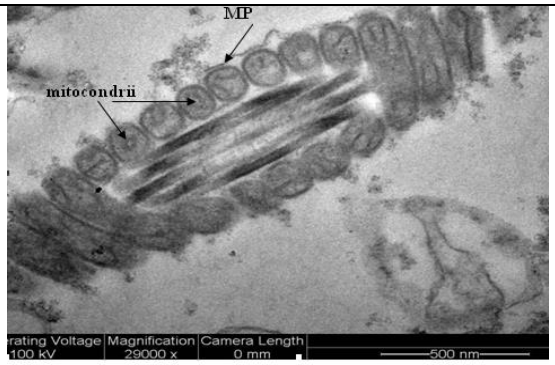

Photo 3. Cross section through sperm cells

One may observe only small parts of the plasmatic membrane through sperm cells of the intermediate and final piece, frozen into the Tris medium.

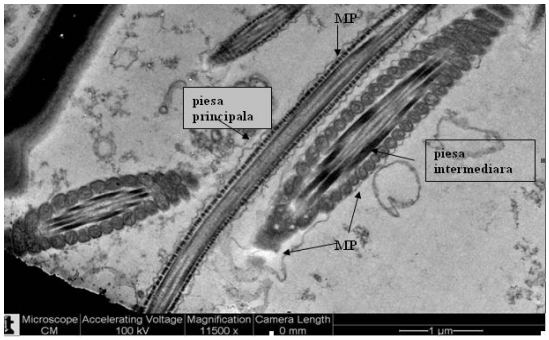

Photo 4. Cross section of the intermediate piece (x29000)

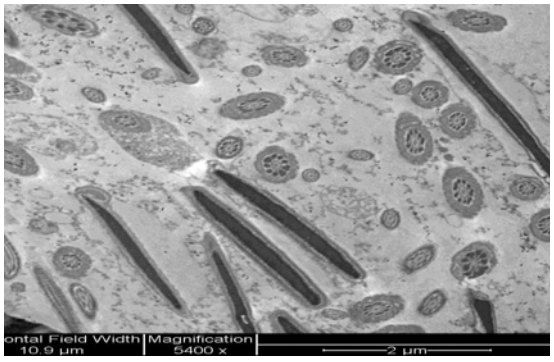

Photo 5. Ram sperm cells freeze into the Tris medium-Cysteine (x5400)

57% of the cells have an intact Acrosome.

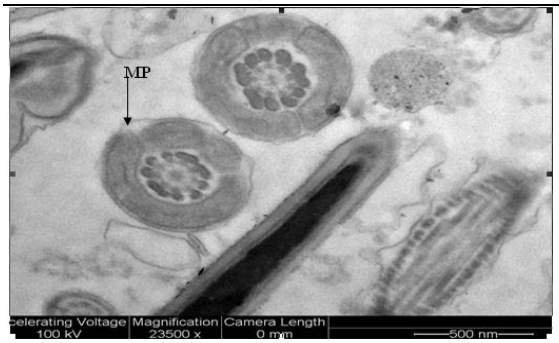

Photo 6. Ram sperm cells freeze into Tris medium-cysteine (X 23500)

Cross section at the middle piece and through Acrosome at ram. The plasmatic membrane has small vacuolations without interruptions.

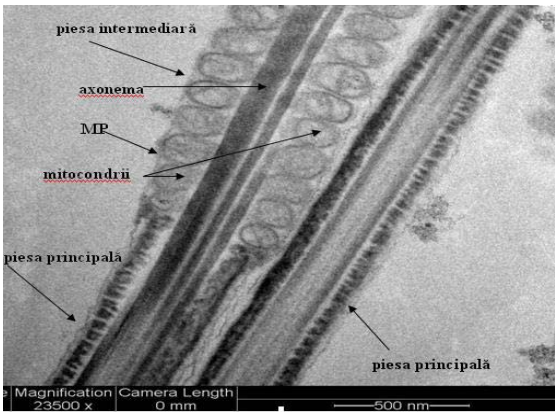

Photo 7. Ram sperm cells freeze into the Tris medium-cysteine, (x23500)

Longitudinal section of the intermediate and main pieces

The plasmatic membrane is attached to internal structures. Mitochondria and axoneme are not changed.

During the cryopreservation process the spermatozoa have ultra-structural changes (of plasmatic, mitochondrial, Acrosome), biochemical and functional [24]. The damages can occur in any stage of the process, but to a bigger extent during cooling periods at 0oC and in the thaw case and less, during storage -180°C. The structures and sperm organelles respond differently to osmotic or environment temperature changes in process' diverse stages. Ultra-structural deteriorations are accompanied by biochemical changes or even by the loss of cell's vital content. The plasmatic membrane integrity and the mitochondrial function are the main characteristics of a spermatozoon to fertilize an ovule. The deterioration of plasmatic membrane and the mitochondrial function may lead to membrane destabilization and impairment of mitochondrial and energetic metabolism, as well as the one of cell's viability [7]. After freeze-thaw process one may observe filament mitochondrial alterations and in a lower percentage in the axoneme, filaments and flogging fibrils. However the main target of damages caused by cryopreservation is the spermatozoa plasmatic membrane. Because of the temperature's variation and the osmolarity, both the 7 freezing and thawing induce alternations in the cells volume of water, generating a huge mechanical stress onto the cell membranes [8]. Since only spermatozoa with intact membranes may suffer empowerment and Acrosome reaction, it is very important to know the type and the place of changes to optimize the freezing technologies.

The plasmatic membrane surrounds the whole sperm cell and it has the role to protect the cell organelles, and by semi-permeability properties it maintains ions chemical gradient and other soluble components. Specific membrane proteins facilitate the transport of glucose and fructose from the extracellular medium into the cell. These transporters are essential in providing energetic substrates because into the mature spermatozoid 90% of the ATP is provided by glycolysis (anaerobe). If the plasmatic membrane is non functional, the sperm is considered to be damaged because *in vivo* it is not capable to fertilize.

For mammals, the sperm cell membrane has particular lipids content, different from the one of other cells. It has high levels of phospholipids, sterols, saturated and unsaturated fatty acids, plasmalogens and sphingomyelins. This specific structure is responsible for the fluidity, flexibility and functionality ability of sperm cell. An important role in ensuring fluidity and regulating spermatogenesis is the one of the polyunsaturated fatty acids have [1]. Spermatozoa's plasmatic membrane has a heterogeneous structure, in 5 specific fields:

Acrosome, equatorial segment, basal segment, intermediate piece and the final one. The differences between these regions are related to different physiological functions. Before and after semen the plasmatic membrane suffers some changes regarding the lipids integrity, the modification in the fatty acids degree of saturation and the loss of cholesterol from its composition, which leads to a marked decrease of cholesterol/ phospholipids report. Different regions of membranes are different concerning this report.

Cholesterol content of membranes is not different only between species, but also between ejaculates of the same individual, [5]. These differences are the basis of some different capacity processes regarding the fertility and suitability for freezing a male ejaculates.

Microscopic examination of ram sperm labeled with a membrane integrity mark demonstrated that exposure to low temperatures followed by heating, affect in different ways the plasmatic membrane, especially the middle piece and the head's one [7]. Ultra-structural analysis results show that the head's plasma membranes are more affected the flagellum ones. While one may identify several cytoskeleton proteins, their role in maintaining 8 the integrity of plasmatic membrane remains unclear. In our ultra-structural evaluation studies of the ram sperm cells membranes we obtained similar results, on the head membrane being recorded more cells with damaged membranes. Also, the research shows that the Acrosome is less affected, although the plasmatic membrane surrounding the spermatozoid's head has major detachments, vacuolation and even interruptions. Similar results were found [5] that, the plasmatic membrane surrounding the sperm head is considerably more labile than the flagellum's one, and the external Acrosome membrane is more vulnerable than the domestic one.

CONCLUSIONS

The effect of irradiation on biochemical parameters of samples was evaluated by measuring the activity of cytochrome oxidase (COX) and the energetic charge. Freezing-thawing leads to plasma membrane alterations including rupture, especially at head level, and membrane detachment or vacuolation at head or flagellum part [25], [26] and [27].

Results regarding the damage degree of plasmatic membrane after freezing-thawing process in different environments with addition of antioxidants are similar when cells were frozen in Tris medium and with addition of cysteine as antioxidant. The analysis of micro photography shows that the Acrosomes were partially affected by the freezing-thawing process. Although many cells have a swollen Acrosome, the internal Acrosome membrane is intact. The plasmatic membrane has several degenerative changes at head in comparison with the flagellum [18].

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