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STUDY ON THE QUALITY OF FROZEN-THAWED SEMEN OF BUCK EXPOSED TO DIFFERENT DOSES OF ENERGY GENERATED BY A He-Ne LASER

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Abstract. The aim of the research was to determine whether and how the two doses of laser irradiation energy (3.96 and 6.12 J / cm2) can improve the quality characteristics of buck sperm after freeze-thaw process.
In this regard semen straws were thawed in water bath at 39oC for 120 seconds. After thawing, semen was divided into three samples: one representing control and the other two were exposed to He-Ne laser irradiation at two different doses of energy (3.96 and 6.12 J / cm2) and mitochondrial activity, cell viability (by flow cytometry), motility and function of plasma membrane integrity test (HOST)were analyzed. He-Ne laser action on thawed buck semen leads to an improvement of motility, viability and mitochondrial function for the dose of 6.12 J/cm2, in contrast to the dose of 3.96 J/cm2 which decreases the quality of semen parameters relative to the control sample.

Key words: Flow cytometry, mitochondrial function, He-Ne laser, motility, viability.

Introduction

In the freezing process any biological activity is stopped until thawing and fertilizing sperm that depends on the thawing technique (Jondet [14]). The straw containing frozen semen has become the universally accepted unit for storage and genetic transfer in goat, procedure that depends on maintaining the functional activity of the spermatozoa (viability and fertilizing capacity).

Cryopreservation modifies the behavioral and functional capacity of spermatozoa, leading to a reduction in motility, in a reduced capacity of

spermatozoa to pass through the cervix and a decrease of viability in the female reproductive tract (Salamon [24]). It istherefore essential to find new procedures to improve the quality of cryopreserved sperm. Sperm irradiation using a Helium-Neon laser (He-Ne) is a new method, photo-stimulating effect of laser irradiation of different biological systems have already been demonstrated (Lubart[15]). For example, in somatic cells, the irradiation stimulates the release of fibroblast growth factor (Yu [26]) and accelerates their mitosis (Lubart [16]), skeletal muscle regeneration and bones repair (Bibikova[3]). He-Ne laser irradiation can also improve the potential of sperm fertilising (Ocana-Quero [20]; Cohen [5]). This could occur due to a variety of factors, including accelerating Ca^{2+} transport throughsperm mitochondria and sperm plasma membrane of sperm cells irradiated (Lubart [17], Breitbart[4], Cohen [5]) and formation of reactive oxygen species (Zan-Bar [30]). In addition, since He-Ne laser irradiation induces an increase in the electrochemical potential and ATP supplementary synthesis in isolated mitochondria (Passarella [21]) and because mitochondria appear to play a key role in energy production and maintaining the sperm motility (Ruiz-Pesini[23], Corral-Baqués [6],[7]), stimulation could depend on energy availability growth. It has been shown that laser irradiation improved the quality of frozen semen for rabbits and turkeys (Iaffaldano [12], [13]).

The purpose of this study is to investigate whether the irradiation with laser energy at different irradiation doses (3.96 and 6.12 J /cm^2) can improve the qualitative characteristics of buck sperm after freezing-thawing process.

Materials and methods

The activity of freezing buck semen was performed according to the freezing technology developed in the Laboratory of Biotechnology of Reproduction, I.C.D.C.O.C. Palas Constanta. Experiments were conducted in the normal breeding season, during October 2012 - December 2012. Thawing and testing semen samples was performed in the Laboratory of Cell Biology, University Ovidius, from February to May 2013.

As dilution medium a diluent of Tris base 20% (v/v) egg yolk was used. The cryoprotectant used for freezing buck semen was glycerol (7% final concentration).

Animals: sperm samples were collected from five adult Saanen buck with known fertility. Collection was made with an artificial vagina, 2 times per week. Sperm samples from each animal were analyzed separately in order to take into account the variability in the individual. Cryopreserved semen samples were thawed in a water bath at 39 ° C and were subjected to two different doses of irradiation energy. A Melles Griot laser with He-Ne was used with a wavelength of 632.8 nm, power 6 mW and the diameter of the aperture (beam diameter) of 0.65 mm. Exposure time required was calculated using the formula:

$$t = \frac{D \cdot A}{P} \cdot (1 + d)$$

where: t - exposure time (s)
D-dose (J / cm^2)
A- beam area (cm2)
P - laser power (W)
d - depth (cm)

Methods for assessing the cryobiological indices after thawing

1. Assessment of sperm motility

Manual evaluation in wet preparation technique (Zamfirescu [29]) was used for assessing sperm motility using a Novex optical microscope with hot plate (x100 magnification).

2. Determination of viability of sperm cells by flow cytometry

To determine the percentage of viable sperm cells a Live-Dead Sperm viability kit (Invitrogen) was used that allows flow cytometric analysis of viability, but can also be used to determine viability by fluorescence microscopy technique.

The method used is the double staining, in which, in order to determine the viability of sperm cells 2 fluorochromes are used that stain the nucleic acids. SYBR-14, which staines the spermatozoa with intact membranes and the propidium iodide, which stains cells with damaged membranes were used. The method was used to determine the viability in most species of mammals (Garner [9]).

3. Assessment of mitochondrial function by flow cytometry with Rhodamine (R123)

A Beckton-Dickinson FACS Calibur flow cytometer was used for quantitative analysis of fluorescent labeled spermatozoa, the inputs were registered and processed using a Apple computer and the specialized software CellQuest Pro.

The lipophilic fluorochrome Rhodamine 123 has a positive charge at physiological pH which favors its concentration in the mitochondria under the influence of potential difference generated by the respiratory function. This fluorochrome is typically used in the assessment of mitochondrial activity, but may also be used to determine dead cells in the population, since these accumulate in small quantity Rhodamine 123 (Ronot [22]).

The red fluorescence emitted by the dead cells stained using propidium iodide is captured by the FL2 detector and the green fluorescence emitted by cells with functional mitochondria stained with Rhodamine 123 was captured by the FL1 detector. Interpretation of results was done through dot-plot graphs statistics FL1/FL2, where each cell read is represented as a point and each population is represented as a cloud of points.

Statistical analysis of experimental data

IBM SPSS, version 17.01 was used for descriptive statistics. The results are expressed as mean ± standard error. To determine the normal distribution of the results and therefore the choice of using parametric or nonparametric tests for significant differences of means we used the Kolmogorov-Smirnov test and for added security, because the number of samples was small, the Shapiro-Wilk test. To determine significant differences, the means were analyzed using paired Student T-test.

Results and discussions

The aim of the research was to determine whether and how the two doses of laser irradiation energy (3.96 and 6.12 J/cm^2) can improve the quality characteristics of buck sperm after the freeze-thaw process

In this regard semen straws were thawed in a water bath at 39^{o}C for 120 seconds. After thawing, semen was divided into three samples: one representing control and the other two were exposed to He-Ne laser irradiation at two different doses of energy (3.96 and 6.12 J /cm^2) and mitochondrial activity, cell viability (by flow cytometry) and were analyzed.

Table 1 Variation of quality parameters of thawed semen irradiated with He-Ne laser

Semen characteristics	Samples		
	Control	Sample 1 irradiated (3.96 J/cm^2)	Sample 2 irradiated (6.12 J/cm^2)
Motility (%)	46.39 ± 3.23^a	41.3 ± 2.44^a	47.9 ± 3.67^a
Viability (%)	53.8 ± 3.12^a	47.28 ± 2.53^b	54.93 ± 3.24^a
Mitochondrial activity (%)	43.51 ± 2.13^a	43.07 ± 2.57^a	47.38 ± 3.23^b

Within rows different small letters significant at ($p<0.05$)

Motility: The best values were obtained by He-Ne laser irradiation at a dose of 6.12 J/cm^2. The exposure at lower doses of energy lead to a reduced motility compared to the other irradiated samples and control. There are no statistically significant differences ($p <0.05$) between motility values.

Viability: The percentage of viable spermatozoa is statistically significantly higher ($p <0.05$) for the control sample and the sample irradiated with a dose of energy of 6.12 J/cm^2 compared to the sample irradiated with the dose of 3.96 J/cm^2.

Similar results were obtained for the assessment of mitochondrial activity. The best results were obtained for sample 3 (6.12 J/cm^2), the values being significantly higher ($p <0.05$) compared to the other alternatives.

The results demonstrated that following irradiation with He-Ne laser (in particular the energy dose of 6.12 J /cm^2) of the cryopreserved buck sperm cells the motility, viability and mitochondrial activity of the sperm membrane is improved. Similar results were obtained on turkey semen in which was found that the laser irradiation (at doses ranging between 3.24 - 5.40 J /cm^2) resulted in a increase of the quality parameters after thawing (Iaffaldano [13]). Ocana- Quero [20] showed an increase in acrosome reaction of bull semen and a decrease mortality of spermatozoa after irradiation with doses ranging from 2 to 16 J/cm^2. Wenbin [25] found that the laser irradiation lead to an increased sperm fructose fermentation, respiration, ^{32}P absorption capacity and the absorption of Ca^{2+}, thereby increasing the motility and time survival of the buck spermatozoa.

Corral - Baqués [6] reported that the irradiation of dog sperm with a laser having a wavelength of 655 nm at doses of 4, 6 and 10 J/cm^2 improves the speed and progressive motility of sperm. Zan-Bar [30], working with tilapia and buck sperm, showed the increase of the motility and viability of tilapia sperm after exposure to red light (630-670 nm) and white light (400-800 nm), while in the case of buck sperm there was a slight increase in motility and viability only in the case of irradiation with red light.

The analysis of cell viability and mitochondrial activity was determined by the technique of flow cytometry. The technique is used for counting, examining and sorting of cells and has the advantage that measurements are made simultaneously for multiple features of a certain cell at a rate between 500 and 4000 cells per second.

After double staining with the two fluorochromes ,the cytometric evaluation of spermatozoa were comparativelyanalyzed from the experimental samples. The biparametric cytograms (figure 1) shows the presence of four subpopulations:

- Subpopulation 1, which represents propidium iodide-labeled spermatozoa, dead spermatozoa;
- Subpopulation 2 stained with SYBR-14 and that are the viable spermatozoa;
- Sub-population 4 which shows a double staining with both fluorochromes;
- Subpopulation 3, which includes other particles.

Subpopulation 3 was not considered because it doesn't represent a sperm population.

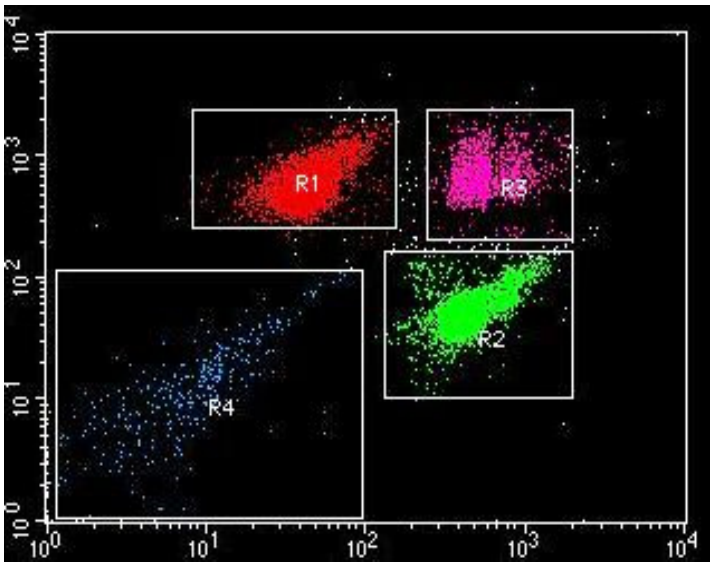

Figure 1 The comparative analysis through the quadrants technique of identifying simultaneously viable cells (green dots), dead (red dots) and dying (double positive - purple dots) and non-sperm population(blue dots) from frozen-thawed sperm. Abscissa: intensity of cells stained with PI red fluorescence (FL2) in logarithmic scale. Ordinate: green fluorescence intensity of cells stained with SYBR-14 (FL1), in logarithmic scale.

The results of this study are similar to those in the literature, which shows that laser irradiation with red light leads to an improvement in thawed semen quality parameters. According to the studies of Zan-Bar [30], light effects are mediated through reactive oxygen species. Indeed, although high levels of reactive oxygen species can lead to cell death (by ATP depletion and lipid peroxidation) at a low level of reactive oxygen species may play an important role in the activation of many cellular processes. In the case of spermatozoa, the reactive oxygen species, including superoxide anion and H_2O_2, and reactive nitrogen species such as nitric oxide (NO) may cause the hyper-capacitation of spermand acrosome reaction (Aitken [1], Aitken [2],Martínez - Pastor [19]). On the other hand, an increase of intracellular Ca^{2+}level and transport was demonstrated in the irradiated bull spermatozoa (Lubart[17],Breitbart[4]), and mouse (Cohen [5]). More recently, it was shown that intracellular movementof Ca^{2+} controls motility, acrosome reaction and sperm capacitation (Darszon[8]).

The results of this study show that irradiation with a He-Ne laser of thawed buck sperm leads to an increase of the motility, viability and mitochondrial activity of the sperm membrane for the dose of 6.12 J/cm^2. For lower dose of energy the result proved to be inefficient compared to the other samples irradiated and the control.

Conclusions

Using flow cytometry techniques leads to more accurate results due to the large number of cells analyzed.

He-Ne laser action on thawed buck semen leads to an improvement of the motility(47.9 ± 3.67 vs 46.39 ± 3.23), viability (54.93 ± 3.24 vs 53.8 ± 3.12) and mitochondrial function (47.38 ± 3.23 vs 43.51 ± 2.13) for the dose of 6.12 J/cm^2, in contrast to the dose of 3.96 J/cm^2 which decreases the quality of semen parameters relative to the control sample.

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