Study on the effects of exposure to different doses of energy generated by a He-Ne laser on the quality of frozen-thawed semen of ram

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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 ram sperm after freeze-thaw process. In this regard semen straws were thawed in water bath at 39°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 / 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 ram semen leads to an improvement of motility, viability, mitochondrial function and functional integrity of the membrane 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.

Keywords: He-Ne laser, flow cytometry, mitochondrial function, motility, viability

1. Introduction

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

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 to decrease of viability in the female reproductive tract (Salamon [24]). This usually leads to unacceptably low rates of pregnancy in ewes inseminated with frozen semen (Gillan [10]). It is therefore 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 improves the potential of sperm.
fertilization (Ocana-Quero [20]; Cohen [5]). This could occur due to a variety of factors, including accelerating Ca^{2+} transport through mitochondria and 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 ram sperm after freezing-thawing process.

2. Materials and methods

The activity of freezing ram 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 August 2013 - October 2013. Thawing and testing semen samples was performed in the Laboratory of Cell Biology, University Ovidius, from December 2013 to February 2014.

As dilution medium a diluent of Tris base 20% (v/v) egg yolk was used. The cryoprotectant used for freezing ram semen was glycerol (5% final concentration).

**Animals:** sperm samples were collected from five adult Merinos de Palas rams with known fertility. Collection was made with an artificial vagina, 2 times per week. For each male 1-3 ejaculates were collected (every 15-30 minutes), which were subsequently mixed and subjected to experiments. A total of 81 ejaculate were processed. Semen was cryopreserved in 0.25 ml fine straws.

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 wave length 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 (cm^2)  
- 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 (x 100 magnification).
2. Determination of viability of sperm cells by flow cytometry

A Live-Dead Sperm viability kit (Invitrogen) was used in order to determine the percentage of viable sperm cells. This assay allows flow cytometry analysis of viability, but can also be used to determine cell viability by fluorescence microscopy technique.

In order to determine the viability of sperm cells the double staining method is used, in which, the nucleic acids are stained with 2 fluorochromes. SYBR-14, which stains 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 an 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. 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.

4. Functional integrity of the plasma membrane

The hypo-osmotic (HOST) test is used to assess the functional integrity of the sperm membrane. Is based on the semi-permeability of intact cell membranes, that, in the hypo-osmotic conditions, leads to swelling the sperm cell flagella. These is an indication that the transport of water through the membrane is carried out normally.

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/cm²) can improve the quality characteristics of ram sperm after the freeze-thaw process.

After thawing the semen straws in a water bath at 39°C for 120 seconds, semen was divided into three samples. Mitochondrial activity, cell viability (by flow cytometry), motility and function of plasma membrane integrity test (HOST) were analyzed for all three samples:
one representing control and the other two exposed to He-Ne laser irradiation at two different doses of energy (3.96 and 6.12 J/cm²). The results are presented in Table 1.

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>Mitochondrial activity (%)</th>
<th>HOST (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.2 ± 2.18a</td>
<td>50.1 ± 2.74a</td>
<td>40.52 ± 1.47a</td>
<td>42.97 ± 1.62a</td>
</tr>
<tr>
<td>Sample 2 (3.96 J/cm²)</td>
<td>39.5 ± 1.64a</td>
<td>45.22 ± 1.73b</td>
<td>41.2 ± 2.15a</td>
<td>40.23 ± 1.97a</td>
</tr>
<tr>
<td>Sample 3 (6.12 J/cm²)</td>
<td>45.6 ± 2.45a</td>
<td>52.3 ± 2.86a</td>
<td>45.12 ± 2b</td>
<td>46.52 ± 2.07a</td>
</tr>
</tbody>
</table>

Within column different superscript letters represents statistical significant differences at (p<0.05)

**Motility:** It is observed that the best values were obtained by He-Ne laser irradiation at a dose of 6.12 J/cm². The exposure at lower doses of energy lead to a reduced motility compared to the other irradiated samples and control. Statistically significant differences are between motility values (p <0.05).

**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/cm² compared to the sample irradiated with the dose of 3.96 J/cm².

Similar results were obtained for the functional integrity of the membranes (HOST test) and for the assessment of mitochondrial activity. The best results were obtained for sample 3 (6.12 J/cm²), the values being significantly higher (p <0.05) compared to the other alternatives in the case of mitochondrial activity.

The results demonstrated that following irradiation with He-Ne laser (in particular the energy dose of 6.12 J/cm²) of the cryopreserved ram sperm, the cells motility cells viability and functional integrity of the 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/cm²) resulted in an increase of the quality parameters after thawing (Iaffaldano [13]). Ocana-Quero [20] demonstrated an increase in acrosome reaction of bull semen and a decrease mortality of spermatozoa after irradiation with doses ranging from 2 to 16 J/cm². Wenbin [25] found that the laser irradiation leads to an increased sperm fructose fermentation, respiration, ³²P absorption capacity and the absorption of Ca²⁺, 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 wave length of 655 nm at doses of 4, 6 and 10 J/cm² improves the speed and progressive motility of sperm. Zan-Bar [30], working with tilapia and ram 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 ram 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 of cells per second.
After double staining with the two fluorochromes, the cytometric evaluation of spermatozoa were comparatively analyzed 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](image)

**Figure 1** The comparative analysis through the quadrants technique for 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 at different temperatures. 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$_2$O$_2$, and reactive nitrogen species such as nitric oxide (NO) may cause the hyper-capacitation of sperm and acrosome reaction (Aitken [1], Aitken [2], Martinez - 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 movement of 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 ram sperm leads to an increase of the motility, viability and functional integrity of the sperm membrane.
for the dose of 6.12 J/cm². For lower dose of energy, the result proved to be inefficient compared to the other samples irradiated and to the control.

3. Conclusions

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

He-Ne laser action on thawed ram semen leads to an improvement of the sperm motility (45.6 ± 2.45 vs 43.2 ± 2.18), sperm cells viability (52.3 ± 2.86 vs 50.1 ± 2.74), mitochondrial function (45.12 vs 40.52 ± 1.47 ± 2) and functional integrity of the sperm membrane (46.52 ± 2.07 vs 42.97 ± 1.62) 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.

References