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Helium-neon laser irradiation of cryopreserved ram sperm enhances cytochrome c oxidase activity and ATP levels improving semen quality

N. Iaffaldano [a,*,1], G. Paventi [b,1], R. Pizzuto [b], M. Di Iorio [a], J.L. Bailey [c], A. Manchisi [a], S. Passarella [b]

[a] Department of Agricultural, Environmental, and Food Sciences, University of Molise, Campobasso, Italy
[b] Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, Campobasso, Italy
[c] Département des Sciences Animales, Centre de Recherche en Biologie de la Reproduction, Université Laval, Québec, Canada

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ABSTRACT

This study examines whether and how helium-neon laser irradiation (at fluences of 3.96–9 J/cm^2) of cryopreserved ram sperm helps improve semen quality. Pools (n = 7) of cryopreserved ram sperm were divided into four aliquots and subjected to the treatments: no irradiation (control) or irradiation with three different energy doses. After treatment, the thawed sperm samples were compared in terms of viability, mass and progressive sperm motility, osmotic resistance, as well as DNA and acrosome integrity. In response to irradiation at 6.12 J/cm^2, mass sperm motility, progressive motility and viability increased ($P < 0.05$), with no significant changes observed in the other investigated properties. In parallel, an increase ($P < 0.05$) in ATP content was detected in the 6.12 J/cm^2-irradiated semen samples. Because mitochondria are the main cell photoreceptors with a major role played by cytochrome c oxidase (COX), the COX reaction was monitored using cytochrome c as a substrate in both control and irradiated samples. Laser treatment resulted in a general increase in COX affinity for its substrate as well as an increase in COX activity (Vmax values), the highest activity obtained for sperm samples irradiated at 6.12 J/cm^2 ($P < 0.05$). Interestingly, in these irradiated sperm samples, COX activity and ATP contents were positively correlated, and, more importantly, they also showed positive correlation with motility, suggesting that the improved sperm quality observed was related to mitochondria-laser light interactions.

1. Introduction

The widespread application of artificial insemination depends largely on the use of cryopreserved sperm. However, the cryopreservation of sperm usually results in reduced fertility because of repercussions of this procedure on sperm quality [1]. In some mammalian species such as sheep [2,3], cryopreserved semen usually gives rise to unacceptably low conception rates [4]. Freezing and/or thawing destabilize sperm membranes leading to mitochondrial damage, which impairs both sperm motility and their ability to survive in the female reproductive tract [5]. Consistently, the reduction in ram sperm motility produced in response to freezing/thawing has been linked to a sharp decrease in ATP contents [3,6]. Thus, to obtain acceptable fertility levels [7], techniques aimed at improving the quality of cryopreserved ram semen, including sperm motility, are required [8]. To date, research efforts have mainly focused mostly on trying to improve freezing

* Corresponding author. Tel.: +390874404697; fax: +390874404855.
E-mail address: nicolaia@unimol.it (N. Iaffaldano).
[1] These authors contributed equally to this work.

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procedures by finding suitable freezing extenders ([9] and references therein) and/or assessing the use of different additives ([3,10] and references therein) to protect sperm during the freezing-thawing process. However, recent findings indicate that certain cryoprotectants, such as the soy lecithin, could negatively affect sperm mitochondrial function [11], such that other strategies aimed at improving mitochondrial function must be considered. In this regard, photobiostimulation using a low-intensity helium-neon (He-Ne) laser has been shown to improve sperm motility (for a review see [12]). Indeed, photobiostimulation, firstly reported in 1969 [13], has been proven in mouse [14], human [15], sheep [16], dog [17,18], avian [19], and rabbit [20] sperm. This procedure applied to cryopreserved sperm has, nevertheless, only been investigated in cattle [21] and birds [22], thus, studies conducted in other species are needed. In addition, the mechanism whereby sperm photobiostimulation occurs remains to be clearly established. What has been established is that mitochondria are the main light targets in the cell, and photobiostimulation usually results in an increase in ATP synthesis (for references see [23]). It should also be mentioned that sperm motility is dependent on levels of cell ATP, which may be synthesized via both mitochondrial respiration and glycolysis [24–26]. Thus, to expand our preliminary work [27], this study was designed to determine whether mitochondrial stimulation through He-Ne laser irradiation could improve the quality of cryopreserved ram sperm. To this end, we assessed the effects of laser irradiation on certain qualitative parameters in thawed ram sperm, and in a parallel investigation, we also examined the effects on ATP contents and cytochrome c oxidase (COX) activity.

2. Materials and methods

2.1. Chemicals

The LIVE/DEAD Sperm Viability Kit was purchased from Molecular Probes Inc. (Eugene, OR, USA). Other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Semen treatments

Cryopreserved semen from four mature Merinizzata Italiana rams was obtained from the Breeding Association APA (Potenza, Italy). The semen was frozen in straws (about 230×10^6 sperm/straw) according to FAO, 2012 guidelines. Seven semen pools were prepared each by thawing (in a water bath at 37 °C for 30 seconds) and mixing 16 straws (four straws per ram). Thus, in seven different experiments, we used a total of 112 straws of cryopreserved semen.

2.3. Sperm irradiation

Each semen pool was divided into four aliquots subjected to the treatments: no irradiation (control) or irradiation, as reported by Iaffaldano et al. [19], at room temperature, using a He-Ne laser (wavelength 632.8 nm; 6 mW, 1 cm^2 light spot size) for 11 (t1), 17 (t2), or 25 (t3) minutes. The three irradiation treatments corresponded to energy doses of 3.96, 6.12, and 9 J/cm^2, respectively.

2.4. Sperm quality assays

In both control (nonirradiated) and irradiated ram sperm samples, the sperm variables motility, viability, osmotic resistance, acrosome integrity, and DNA integrity were assessed in duplicate as described in Rosato et al. [28], with minor adaptations as described below.

Sperm motility was microscopically assessed 30 seconds after irradiation treatment. A drop of 10 μL of semen was deposited on a clean glass slide and covered with a coverslip. The preparation was then examined on a warmplate at × 400 magnification using a phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany). Sperm mass motility and forward progressive motility were determined in five micrographs. Sperm mass motility was defined as the percentage of spermatozoa showing any form of sperm head movement. Forward progressive motility was recorded as the percentage of spermatozoa showing linear movement.

The combination of stains, SYBR-14 and propidium iodide, in the LIVE/DEAD Sperm Viability Kit was used to assess sperm viability, as described in Rosato et al. [28]. In brief, sperm aliquots (5 μL) were added to 39-μL PBS solution containing 2 μL of SYBR-14 (diluted 1:100 in dimethyl sulfoxide) and incubated at 37 °C for 10 minutes. Next, 5 μL of propidium iodide (diluted 1:100 in PBS) were added, and the mixture incubated at 37 °C for a further 5 minutes. Then, 10 μL of the suspension were placed on microscopic slides, covered with coverslips and viable and/or nonviable spermatozoa detected by epifluorescence microscopy (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany): blue excitation $\lambda = 488$ nm (× 1000 magnification using a 100 × oil immersion objective, two slides/sample; 200 sperm/slide).

A hypo-osmotic water test was used to assess sperm osmotic resistance. In this test, 10 μL of semen were mixed with 40 μL of distilled water in an 1.5-mL eppendorf tube and incubated for 5 minutes at 37 °C. Ten microliters of the mixture were placed on a clean glass slide, covered with a thin cover slip and examined under a phase-contrast microscope. The typical “coiled tail” sperm osmotic reaction was easily detected. Hypo-osmotic water test–positive cells were identified in 200 cells counted in at least five fields at × 800 total magnification [29].

Acrosome integrity was determined in duplicate air-dried smears of a drop of semen from each treatment group. After fixation in methanol for 30 minutes, slides were washed with water and air-dried. Slides were incubated with fluorescein isothiocyanate–*Pisum sativum* agglutinin (FITC–PSA) for 30 minutes at room temperature, mounted with 50% glycerol (v:v) and coverslipped [30]. Acrosome intact and damaged cells were identified by counting 200 sperm in each sample at × 1000 magnification using an oil immersion objective under epifluorescence illumination. Using this stain, acrosome-intact sperm show a uniform applegreen fluorescence, whereas acrosome-damaged sperm show little or no green fluorescence in the anterior head. The percentage of