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Biological effects of helium–neon (He–Ne) laser irradiation on acrosome reaction in bull sperm cells

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Abstract

The purpose of this study was to determine the effect of He–Ne laser irradiation (632.8 nm, 10 mW) on the induction of acrosome reaction and mortality in bull sperm cells in comparison with two important capacitation agents; calcium and heparin. Frozen-thawed bull sperm cells were washed in percoll gradient and suspended at a concentration of 1×10^6 ml^{-1} in sp-TALP medium, capacitated in the presence of 2 mM $CaCl_2$, 10 μg ml^{-1} heparin, or irradiated at fluences from 2 to 16 J cm^{-2}, and incubated for 0, 30, 60 and 90 minutes. At the end of the incubation period, the percentage of sperm that were acrosome-reacted and dead was determined. The results obtained indicated that laser irradiation at all fluences produced a significant increase ($p < 0.001$) in the percentage of sperm cells that were acrosome reacted, and a significant decrease ($p < 0.001$) in the percentage of dead sperm at 90 minutes of incubation in comparison to other capacitation agents and the control group. The percentage of sperm cells with acrosome reaction was increased with increasing fluences of laser irradiation and time of incubation. It is conclude that the application of He–Ne laser irradiation at fluences from 2 to 16 J cm^{-2} induced the acrosome reaction and decreased the sperm mortality percentage in vitro of bull sperm cells. © 1997 Elsevier Science S.A.

Keywords: He-Ne Laser irradiation; Acrosome reaction; Bull sperm cells

1. Introduction

To date, a rich and extensive literature exists on the application of low-power laser irradiation to various experimental biological models. A great number of authors have reported numerous photostimulating effects, such as: procollagen production in cultured human skin fibroblasts [1]; DNA synthesis in osteoblasts in vitro [2]; cytokine production in cultures of human peripheral blood mononuclear cells [3], calcium transport in bull sperm cells [4], cell cycle phase of human colon adenocarcinoma cells [5]. Conversely, other workers reported an inhibitory action of this type of radiation [6–8].

Since the initial reports of Austin [9] and Chang [10] of a need for spermatozoa to reside in the female reproductive tract for some hours before gaining the ability to penetrate an oocyte, many efforts have been directed towards eliminating the requirement of the female reproductive tract in the process. Capacitation of mammalian spermatozoa in vitro has been reported following a variety of treatments, including exposure to bovine serum albumin (BSA), serum, follicular fluid, cumulus cells, adrenal extracts and Sendai virus [11,12]. However, one of the greatest concerns about the laser effect is whether or not laser irradiation has a direct effect on motility and acrosome reaction of sperm cells in vitro which play a prominent role in assisted fecundation programmes as therapy for resolving infertility problems in domestic animals.

The objective of this work was to study whether He–Ne laser irradiation photostimulates the acrosome reaction and viability of bull sperm cells in vitro, in comparison with calcium and heparin which are two important capacitation agents.

2. Materials and methods

2.1. Laser specifications and irradiation parameters

The physical parameters of He–Ne laser used (Medical Investigation Laser System, Model TM 1083) in this study were the following: wavelength 632.8 nm; average power

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output 10 mW; area of spot size with active head positioned 1 cm above cell suspension 0.125 cm^2. The fluence rate on the preparation 0.08 W cm^{-2} was calculated using the equation:

$$\text{Fluence rate} = \frac{\text{Power output}}{\text{Application surface}}$$

The irradiation times for fluence from 2 J cm^{-2} to 16 J cm^{-2} were calculated between 5 and 40 s [13]. These fluences, employed in our study, were selected at random below and above therapeutic doses used in veterinary medicine (4 to 8 J cm^{-2}).

2.2. *Sperm preparation*

For washing spermatozoa, 30 and 45% Percoll solutions were prepared by the dilution of 90% isotonic Percoll solution (Percoll, Pharmacia LBK Biotecnology AB, Uppsala, Sweden). Then, 2 ml of 30% Percoll solution was placed on 2 ml of 45% Percoll in a 10 ml test tube. For the preparation of spermatozoa, two 0.5 ml straws of frozen semen were thawed in a 37 °C water bath for 30 s, and the semen was deposited on the upper layer of the Percoll gradient solution. The semen was centrifuged for 30 min at 700*g*. The sedimented spermatozoa displaying good motility in the bottom of tube were resuspended in 3 ml of sp-TALP medium (Tyrode's salt solution with albumen, lactate and piruvate).

2.3. *Irradiation of the sperm cells and in vitro induction of acrosome reaction*

Seven 300 μl samples (one sample per treatment) of the sperm suspension prepared as stated above were placed in seven different 5 ml test tubes. The first and second samples were resuspended in 1 ml of sp-TALP medium respectively, containing 10 μg ml^{-1} heparin and 2 mM $CaCl_2$. The third to the sixth samples were irradiated with He–Ne laser irradiation at 2, 4, 8, and 16 J cm^{-2} of fluence, using a laser pointer of fiber optic of 1.40 m in length, which was positioned exactly 1 cm above the surface of sperm cells layer for delivering the laser irradiation. Then, to each test tube was added 1 ml of sp-TALP medium. The control sperm sample was only resuspended in 1 ml of sp-TALP. Finally, all treatments were incubated for capacitation for 90 min in a 5% CO_2 incubator.

2.4. *Sperm staining and slide preparation*

Aliquots from the different treatments were removed and stained with a simple dual stain procedure [14] at 0, 30, 60, and 90 min. Briefly, a 200 μl aliquot was removed from the sperm suspension and placed into a plastic tube; an equal volume of 0.2% trypan blue (Sigma) in albumin-free sp-TALP medium was added. The tube was then incubated for 10 min in a 37 °C water bath. After incubation the spermatozoa were diluted with 2 ml of sp-TALP medium and then centrifuged for 6 min at 700*g*. The supernatant was discarded and the sperm in the pellet was resuspended in 2–3 ml of sp-TALP medium and centrifuged as before. This step was repeated until the suspension was clear or pale blue (one or two washings were usually enough). A 10–20 μl aliquot of the sperm suspension was placed on a microscope slide and smeared with a second glass slide. The smears were dried quickly on a warning stage (40 °C) under a stream of air. Spermatozoa on the slides were subsequently stained for 30–35 min with a 5% Giemsa solution in Sorensen buffer. Following staining the slides were rinsed under a stream of distilled water and air-dried. Microscope fields were selected at random and all spermatozoa in each field were evaluated with a brightfield microscope (400 × magnification) until 1800 spermatozoa per treatment were examined (3 trials × 2 slides × 300 spermatozoa/slide).

2.5. *Evaluation of stained spermatozoa and analysis of data*

Spermatozoa stained with trypan blue/Giemsa were considered viable if they excluded trypan blue, resulting in a white appearance of the postacrosomal region. Dead spermatozoa stained blue in the postacrosomal region. The acrosome of intact spermatozoa stained purple with Giemsa, whereas lacking spermatozoa and acrosome did not take up the stain.

Differences between the means of the treatments were tested for significance using Turkey's studentized range test [15].

3. Results

The effects of He–Ne laser irradiation and incubation on the percentage of acrosome reaction are presented in Fig. 1. The percentage of spermatozoa that had undergone acrosome reaction varied with the different treatments and, within each treatment, with the period of incubation ($p < 0.001$). The increase of percentages of acrosome-reacted spermatozoa were statistically significant when heparin or calcium were used in comparison with the control group, after 30, 60 and 90 min of incubation. The maximum acrosome reaction percentage was obtained when spermatozoas were irradiated with He–Ne laser irradiation compared with heparin, calcium and control treatments. This acrosome reaction was increased with increasing fluence of He–Ne laser irradiation in all incubation periods.

The effects of He–Ne laser irradiation and incubation on sperm mortality are presented in Fig. 2. The percentage of spermatozoa that died varied with both the different treatments and the duration of incubation ($p < 0.001$). The percentage of sperm mortality obtained in the control group were

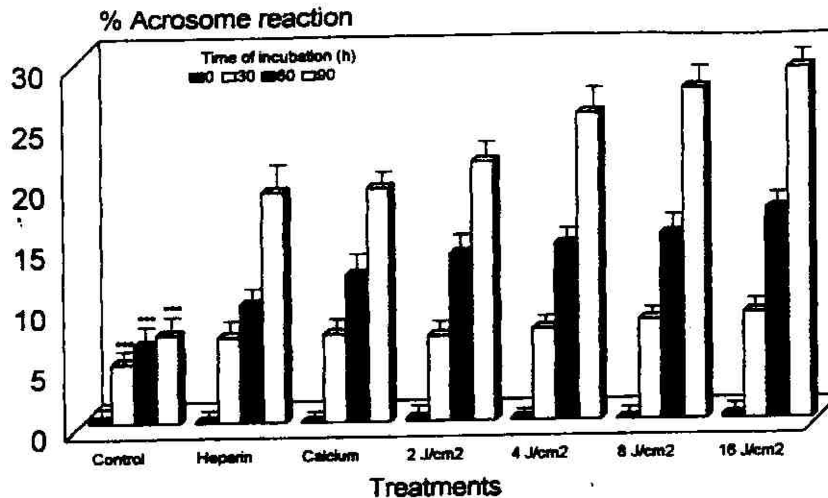

Fig. 1. The effect of calcium, heparin, and different fluences of He–Ne laser irradiation on the induction of the acrosome reaction in bull sperm cells. Frozen-thawed sperm were washed in Percoll gradient and resuspended in sp-TALP. Sperm were exposed to different agents for capacitating, such as: 2 mM $CaCl_2$, 10 $\mu g\ ml^{-1}$ heparin and He–Ne laser irradiation at 2, 4, 8, and 16 J cm^{-2}, and incubated for 0, 30, 60 and 90 minutes. At the end of the incubation period, the percentage of sperm that were acrosome-reacted was determined. Distribution of the different capacitation agents at various time periods are the means ± SEM from 1800 cells (3 replicates × 2 slides × 300 cells/slide). The dead cells were not counted, only the acrosome reaction of the live cells. Values with asterisks (significative differences at 30, 60 and 90 minutes of incubation times) differ from different treatments $p < 0.001$.

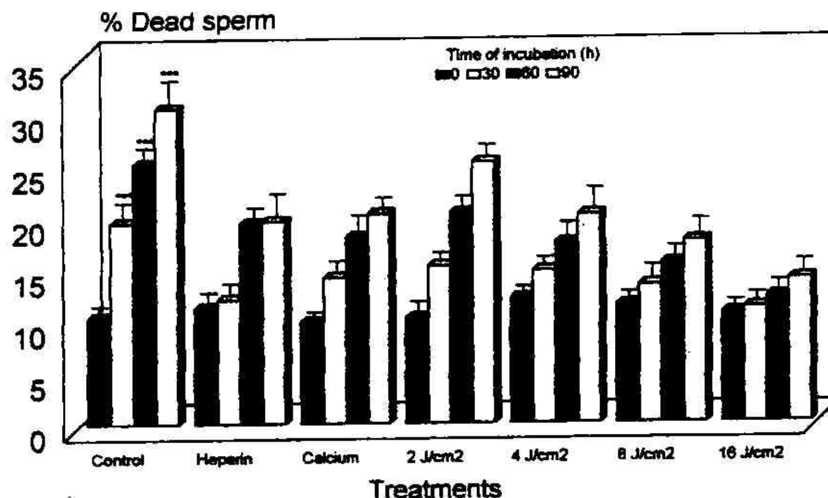

Fig. 2. The effect of calcium, heparin, and different fluences of He–Ne laser irradiation on sperm mortality in bull sperm cells. Frozen-thawed sperm were washed in Percoll gradient and resuspended in sp-TALP. Sperm were exposed to different agents for capacitating, such as: 2 mM $CaCl_2$, 10 $\mu g\ ml^{-1}$ heparin and He–Ne laser irradiation at 2, 4, 8, and 16 J cm^{-2}, and incubated for 0, 30, 60 and 90 minutes. At the end of the incubation period, the percentage of dead sperm was determined. Distribution of different capacitation agents at various time periods are the means ± SEM from 1800 cells (3 replicates × 2 slides × 300 cells/slide). Values with asterisks (significative differences at 30, 60 and 90 minutes of incubation times) differ from different treatments $p < 0.001$.

higher ($p < 0.001$) than those obtained in the rest of treatments, after 30, 60 and 90 min of incubation. When the spermatozoa were irradiated with He–Ne laser irradiation, the percentage of sperm mortality decreased with increasing fluence. The fluence of 8 and 16 J cm^{-2} of He–Ne laser irradiation produced significantly lower sperm mortality than all other treatments, after the different incubation times. The fluence of 2 J cm^{-2} produced a significantly higher sperm mortality than those produced by calcium or heparin treatments. However, the percentage of sperm mortality observed in calcium or heparin treatments was similar to that observed at 4 J cm^{-2} of laser radiation.

4. Discussion

The effects of lasers at the cellular level seem to be quite diverse, affecting many different types and producing several responses. Numerous studies have demonstrated the biostimulating in vitro [16]. By contrast, other studies have

reported the detrimental and destructive effects of laser irradiation in biological systems [6,8].

There is currently much interest in the clinical use of low-level lasers with reported applications in dermatology [17,18], surgery [19], and rheumatology [20] fields. One area that has received much attention is the use of low-level He–Ne laser irradiation and visible light on the biostimulation of gametic and embryonic cells in vitro [21,22,4,8].

In our study, we have observed that the application of He–Ne laser irradiation promoted the acrosome reaction of bull sperm cells compared with two important fusogenic agents, such as: heparin and calcium, after 90 min of incubation. Furthermore, the irradiated sperm cells showed a mortality percentage lower than those in the control group in all incubation times. This sperm mortality decreased with increasing fluences. The results showed that by increasing laser stimulation, both reacted acrosome increase and death rate of sperm decreased at 90 min of incubation period. However, We think that this period is too short to evaluate the true effect of the laser on the cell viability. It is possible that over a longer period of time the longevity of the irradiated sperm would be lower.

In literature, there are also other studies of effect of laser irradiation on sperm cells. In this sense, Lubart et al. [4], found that there is an accelerated Ca^{2+} transport in irradiated cells using He–Ne laser irradiation at various fluences. Marin and Velez [23], and Sato [24] also reported that He–Ne laser irradiation increased the motility of irradiated sperm cells at fluences between 4–12 J cm^{-2}.

To explain the biostimulation effect of low-level irradiation at 630 nm, Karu [25] proposed a model in which light is absorbed by components of the respiratory chain (i.e. flavines, cytochromes) which causes an activation of the respiratory chain and the oxidation of the NAD pool, which leads to changes in the redox status of both the mitochondria and the cytoplasm. This may have an effect on membrane permeability transport, with changes in the Na^+/H^+ ratio and increases in Na^+, K^-, ATPase activity which in turn has an effect on the Ca^{2+} flux. The Ca^{2+} flux affects the levels of cyclic nucleotides, which modulates DNA and RNA synthesis, which modulates cell proliferation (i.e. biostimulation).

In our study, we used a staining procedure to detect viability and the true acrosome reaction in spermatozoa of bovine. During the fertilization process, mammalian spermatozoa must undergo a morphological change termed the acrosome reaction that involves a progressive vesiculation between the outer acrosomal membrane and the overlying plasma membrane [26]. This event, occurring only in live spermatozoa, is referred to as the true acrosome reaction. In contrast, the false acrosome reaction occurs in nonviable spermatozoa as a postmortem change; it is characterized by random breakdown and loss of the acrosomal and plasma membranes [27]. When engaged in routine fertilization studies, a simple assay that is able to identify acrosomal status in conjunction with spermatozoan viability would be beneficial for determining directly the percentage of spermatozoa undergoing a true acrosome reaction. Differential interference microscopy has been used by Saacke and Marshall [28] to detect the acrosomal changes of bull spermatozoa; however, assessment of acrosomal morphology is difficult when motility is moderate to high. Many acrosome stains have been used in order to determine the percent of acrosome reacted spermatozoa. However, with these techniques one is unable to determine if the acrosome reacted spermatozoa were live or dead at the time of staining. In our study, we used a dual stain procedure that allowed detection of the true acrosome reaction of bull spermatozoa. This dual stain technique is a simple and fast procedure that could be useful in routine evaluation of spermatozoa viability and acrosomal integrity.

In conclusion, the application of He–Ne laser irradiation at fluences ranging from 2 to 16 J cm^{-2} produced an increase of acrosome reacted spermatozoa and a decrease of the sperm mortality percentage after 90 min of incubation compared with the control group. However, other studies are necessary to analyze whether the irradiated sperm cells with He–Ne laser irradiation undergo important ultrastructural changes in the cytoplasm and nucleus.

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