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Original article

Evaluation of the effects of photostimulation on freeze-thawed bull sperm cells in terms of reproductive potential

A.D. Ömür[1,2,3]

[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Atatürk University, 25240, Yakutiye/ Erzurum, Turkey
[2] Department of Biochemistry, Faculty of Veterinary Medicine, Atatürk University, 25240, Yakutiye/Erzurum, Turkey
[3] Department of Molecular Biology and Genetics, Faculty of Science, Atatürk University, 25240, Yakutiye/Erzurum, Turkey

Abstract

The aim of this study was to determine the time-dependent effectiveness of photo-stimulation against bovine sperm cells using a multi-wavelength LED (Light Emitting Diode). Spermatological parameters were evaluated for viability, acrosome structure and motility. In addition, the effect of photo-stimulation on frozen-thawed sperm cells subjected to in vitro capacitation was evaluated in terms of changes in mitochondrial membrane potential. The study consisted of two separate experiments and a total of 32 sperm samples obtained from separate bulls were used. All sperm samples were obtained from Holstein bulls using an artificial vagina. Semen was diluted to a final concentration of 92 x 10^6 spermatozoa per ml in 0.25 ml straws. The sperm cells were frozen using the conventional method. Straws were kept in a 37°C water bath for 20 seconds and diluted 1:4 in phosphate buffered saline (PBS) to eliminate the potentially deleterious effect of glycerol, the main permeable cryoprotectant in the freezing medium for bull sperm. This dilution also helped in the evaluation of sperm quality parameters. In the first experiment, whereas the 15-10-15 showed no differences with the control, other treatments such as 10-10-10, 5-5-5, and 3-1-3 exhibited significantly higher percentages of viable spermatozoa at 24h. The results obtained for acrosome integrity were pretty much similar to those observed in the sperm viability assessment. In effect, while the treatment consisting of 15-10-15 had no positive effects, shorter treatments exerted a much more positive effect. The percentages of acrosome-intact spermatozoa in 2-1-2 and 1-1-1 were significantly higher than those obtained in the control. The significant differences in mitochondrial membrane potential were observed at 0, 2, 4 and 24h post-photo-stimulation in all treatments, except 15-10-15. The highest increase in the percentage of spermatozoa exhibiting high mitochondrial membrane potential was found in 10-10-10, 5-5-5 and 3-1-3 treatments. With regard to total and progressive motility, whereas 10-10-10 was the best regime, 5-5-5 and 3-1-3 treatments also had a positive effect. However, 15-10-15 appeared to have a stimulating effect upon progressive motility at 2h and 4h but later declined and showed no significant differences with regard to the control at 24h. In the second experiment, not immediately after thawing but after having been kept at room temperature for up to 24h, it was observed that there was no statistical difference in terms of viability, acrosome integrity and total/progressive motility between photostimulation and the control group. This indicates that photo-stimulation is less able to exert a beneficial effect when post-thawed sperm are not immediately stimulated. As a result it was determined that photo-stimulation at a pattern of 10-10-10, 5-5-5, 3-1-3 and, to a lesser extent 2-1-2, increases the resilience of frozen-thawed bull sperm when applied upon thawing.

Key words: in vitro analysis, laser, LED (Light Emitting Diode), photostimulation, sperm cell

Correspondence to: A.D. Ömür, e-mail: alidogan@atauni.edu.tr

Introduction

Nowadays, it is an important issue to keep genetic resources and transfer them to the future generations. The process of cryopreservation of semen, which has a wide range of uses such as reproductive biotechnology, preservation of species and clinical applications, is of great importance in this aspect. In addition, the sustainability of animal protein, which is an indispensable source of human nutrition, depends on the successful preservation of gene resources and transfer to future generations. Storage of semen by freezing is of particular importance in this respect. Freezing the semen in a way to provide sufficient fertility will make artificial insemination practices more practical and economical, as well as keeping more accurate and reliable pedigree records. In addition, it will be possible to use animals with high genetic capacity in large populations. Structural deformations in membrane structures, cell organelles and fragments in DNA occur due to membrane lipid phase change, osmotic-mechanical stress and free oxygen radicals developing in the environment during the freezing-thawing of semen, and these negative effects can be reduced by the addition of antioxidants, various cryoprotectants and chemicals to sperm extenders (Aitken and West 1990, Bucak et al. 2010, Maia et al. 2010, Wright et al. 2014, Rashid and Sil 2015, Omur and Coyan 2016). Developments made in the light of recent research have been in the direction of increasing fertility potential by irradiating sperm cells in the cryopreservation process in different spectrum areas (Fernandes et al. 2015, Iaffaldano et al. 2016, Yeste et al. 2016). Additionally, light application (photobiomodulation) has areas of use in terms of phototherapy (Desan et al. 2007, Saltmarche 2008, Avci et al. 2013, Soares et al. 2021, de Rezende et al. 2022).

Laser irradiations in general take place in many areas of our lives due to their characteristics such as linearity, single wavelength, stable radiation and high power. Thanks to their narrow focusing advantages, laser sources have gained increasing acceptance in various industries (Kim et al. 2012, Cui et al. 2013, You et al. 2014). Light-emitting diodes (LEDs) have low-intensity illumination and preferred stabilizations in terms of light intensity and wavelength than other sources (Dall Agnol et al. 2009).

In this study, we demonstrated the effects of photostimulation on freeze-thawed bull sperm cells.

Materials and Methods

Sperm samples

The study consisted of two separate experiments and a total of 32 sperm samples from separate bulls were used. All sperm samples came from Holstein bulls and were from different sources, including the United States (confidential), Catalonia, Galicia and Asturias. In all cases, sperm were cryopreserved either using the conventional method or following the protocol described by Muiño et al. (2008). The conditions were set to obtain a final concentration of 92×10^6 spermatozoa per mL in straws of 0.25 mL each. Straws were shaken vigorously for 20 sec at 37ºC and diluted 1:4 in phosphate buffered saline (PBS) to eliminate the potentially deleterious effect of glycerol, the main permeable cryoprotectant in the freezing medium for bull sperm. This dilution also benefitted in the evaluation of sperm quality parameters.

Treatments

All photo-stimulation treatments were performed using the PhAST-Red equipment (IUL; S.A.; Barcelona, Spain). In the case of Experiment 1, the following light regimes were assessed: 15-10-15, 10-10-10, 5-5-5, 3-1-3, 2-1-2 and 1-1-1. As the most positive effects in Experiment 1 were observed for 10-10-10 treatment, only that protocol was tried in Experiment 2. In all cases, samples were kept at room temperature following photo-stimulation and up to the time of evaluation.

Evaluation of sperm quality

Evaluation of sperm viability

Sperm viability was evaluated by flow cytometry following the procedure described by Garner and Johnson (1995), modified and adapted to bull sperm (Prieto-Martínez et al., 2017). Briefly, sperm were incubated with 100 nM SYBR-14 for 10 min at 37°C in the dark, and then with 12 M Propidium Iodide (PI) for 5 min at 37ºC. Three replicates of each sample were evaluated, and 10,000 events were examined in each assessment.

Evaluation of acrosome integrity

Acrosome integrity was evaluated following the protocol described by Nagy et al. (2003) and adapted to bull sperm (Prieto-Martínez et al., 2017). In brief, samples were incubated with lectin PNA conjugated with fluorescein isothiocianate (PNA-FITC) and PI. Again, three replicates of each sample were evaluated, and 10,000 events were examined in each assessment.

Evaluation of mitochondrial membrane potential

The potential of mitochondrial membrane was evaluated through staining with JC-1. Briefly, samples were stained with JC-1 at a final concentration of 1 µM.

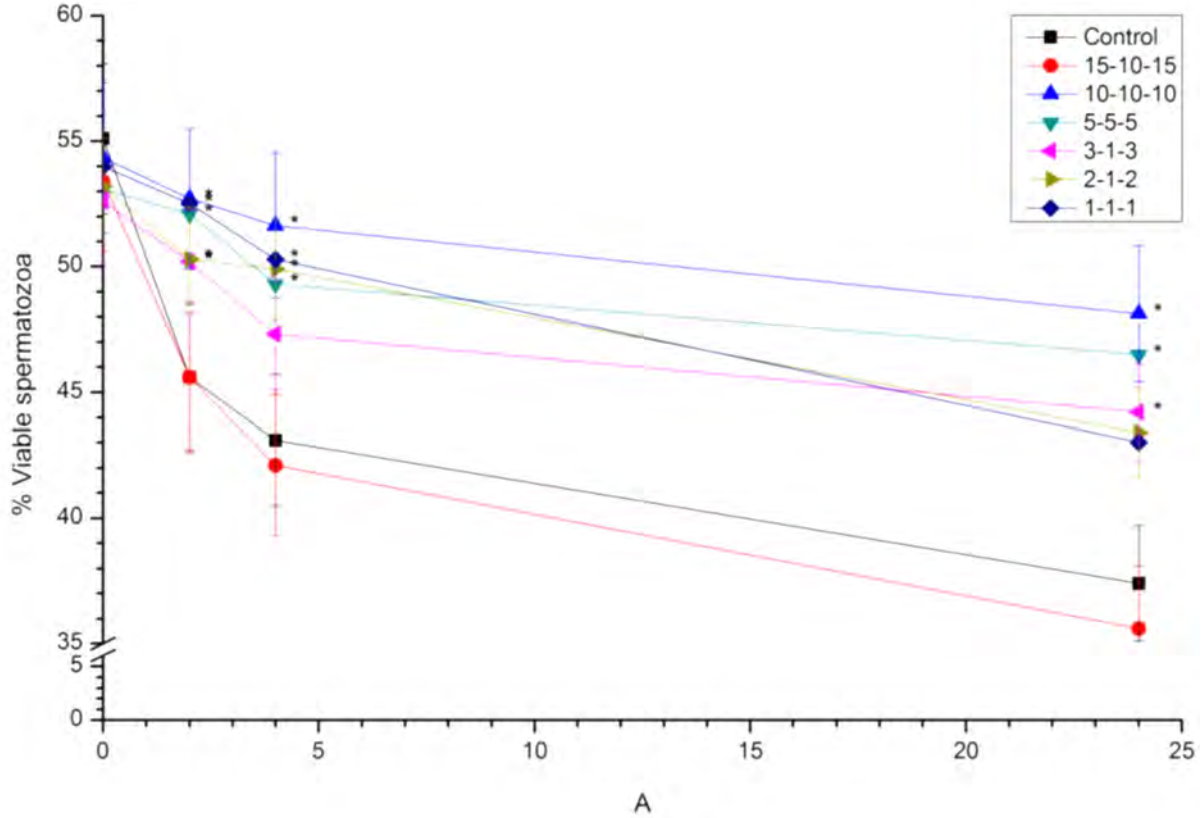

Fig. 1. Bull sperm viability (experiment 1). Data are shown as mean ± SEM. (*) means significant differences of a given treatment with respect to the control at a given time point.

After incubation at 38.5°C for 30 min in the dark, three replicates of each semen sample were evaluated, and 10,000 events were examined in each replicate.

Evaluation of sperm motility

Computer assisted sperm analysis (CASA) was used in terms of sperm motility evaluation. All samples were incubated at 37°C for 15 min prior to evaluation. Determinations were made using a Makler chamber (Sefi-Medical Instruments, Haifa, Israel) under a phase-contrast microscope at 10× magnification (Olympus 10x 0.30 PLAN). Three replicates of each sample (at least 1,000 sperm each) were evaluated and several kinetic parameters were determined, including straight-line velocity, average-path velocity, percentage of linearity, and percentages of total and progressively motile spermatozoa. Cut-off values were specific for bull sperm and were those set by Prieto-Martínez et al. (2017).

Statistical analyses

Results were analyzed using a statistical package (IBM SPSS for Windows, ver. 21.0; Chicago, IL, USA) and data are shown as mean ± standard error of the mean (SEM). Experiments 1 and 2 were evaluated separately. In both cases, data were first checked for normality and homoscedasticity through Shapiro-Wilk and Levene tests. When required, data were transformed through arcsin x prior to run a linear mixed model (with repeated measures) in which the inter-subject factor was the treatment (i.e. control vs. photo-stimulated) and the intra-subject factor was the time during which samples were kept at room temperature. Friedman and Wilcoxon tests were used when, even having been corrected, data did not match with parametric assumptions. Level of significance was set at 5%.

Results

Experiment 1

Sperm viability

Figure 1 shows the percentages of viable spermatozoa when sperm were photo-stimulated upon thawing and then kept at room temperature for a 24 h-period. Whereas the 15-10-15 showed no differences with the control, other treatments such as 10-10-10, 5-5-5, and 3-1-3 exhibited significantly higher percentages of viable spermatozoa at 24 h.

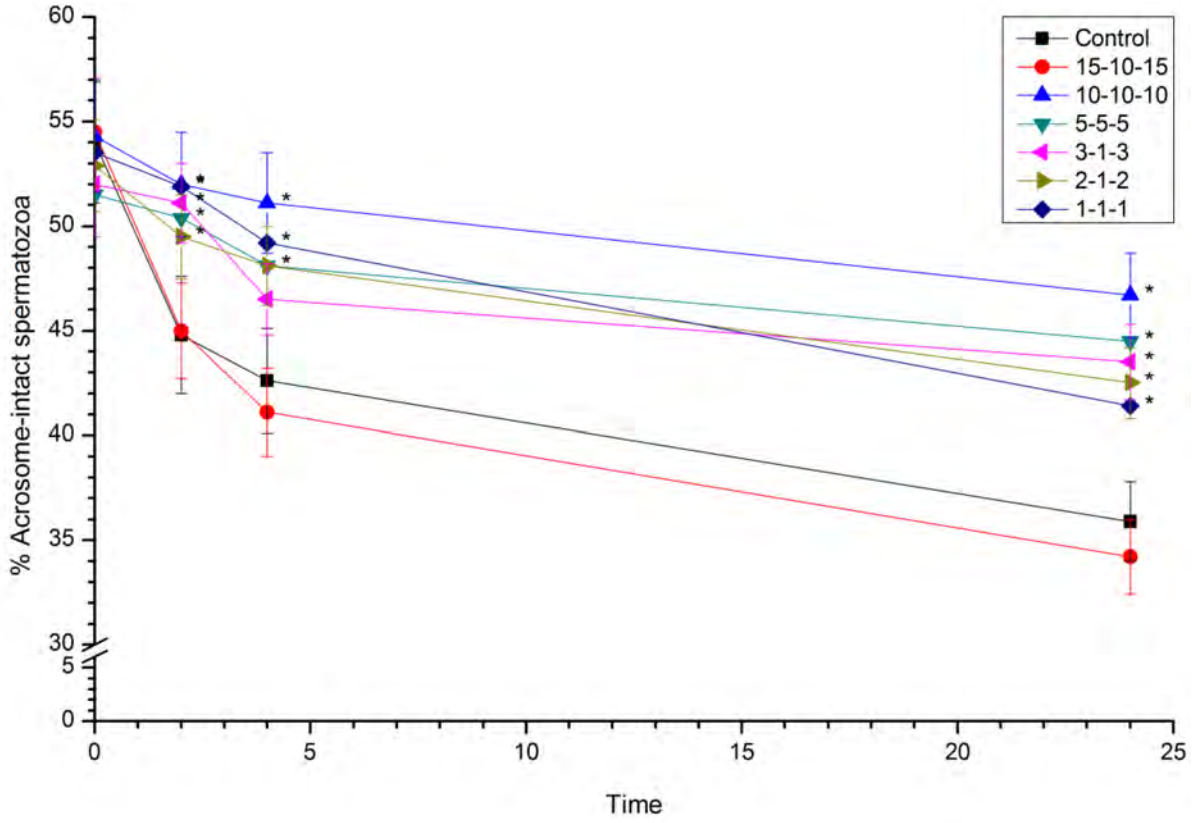

Fig. 2. Acrosome integrity (experiment 1). Data are shown as mean ± SEM. (*) means significant differences of a given treatment with respect to the control at a given time point.

Acrosome integrity

The results obtained for acrosome integrity were very similar to those observed in the sperm viability assessment (Fig. 2). In effect, while the treatment consisting of 15-10-15 had no positive effects, shorter treatments exerted a much more positive effect. One should note that, whilst the shortest treatments (2-1-2 and 1-1-1) had no clear effect when sperm viability was evaluated at 24h, the percentages of acrosome-intact spermatozoa in 2-1-2 and 1-1-1 were significantly higher than those obtained in the control.

Mitochondrial membrane potential

As shown in Fig. 3, significant differences in mitochondrial membrane potential were observed at 0, 2, 4 and 24 h post-photo-stimulation in all treatments, except 15-10-15. Interestingly, the highest increase in the percentage of spermatozoa exhibiting high mitochondrial membrane potential was found in 10-10-10, 5-5-5 and 3-1-3 treatments.

Sperm motility

Figure 4a shows that whereas 10-10-10 was the best regime, 5-5-5 and 3-1-3 treatments also had a positive effect on total motility. Nonetheless 15-10-15 appeared to have a stimulating effect upon progressive motility at 2 h and 4 h but later declined and showed no significant differences with regard to the control at 24 h (Fig. 4b).

Experiment 2

Only 10-10-10 treatment was tried in the second experiment.

Sperm viability

Figure 5 shows the sperm viability of samples photo-stimulated not immediately after thawing but after having been kept at room temperature for up to 24 h. There were no clear differences between both treatments, which indicates that photo-stimulation is less able to exert a beneficial effect when post-thawed sperm are not immediately stimulated.

Acrosome integrity

Similar results were obtained for acrosome integrity in Experiment 2, as treatments did not show any clear difference between them (Fig. 6). Therefore, photo-stimulating sperm after keeping them at room temperature for some time did not show any improvement in the percentage of acrosome-intact spermatozoa.

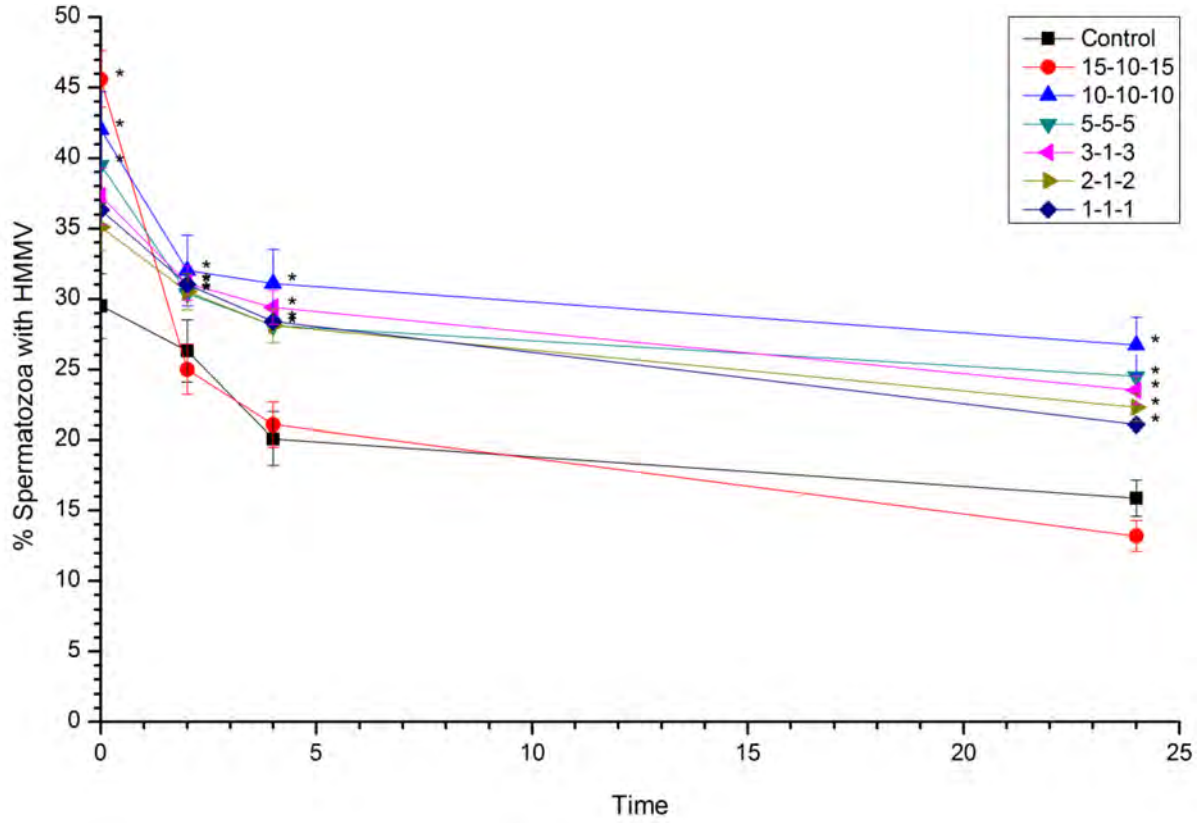

Fig. 3. Mitochondrial membrane potential (experiment 1). Data are shown as mean ± SEM. (*) means significant differences of a given treatment with respect to the control at a given time point.

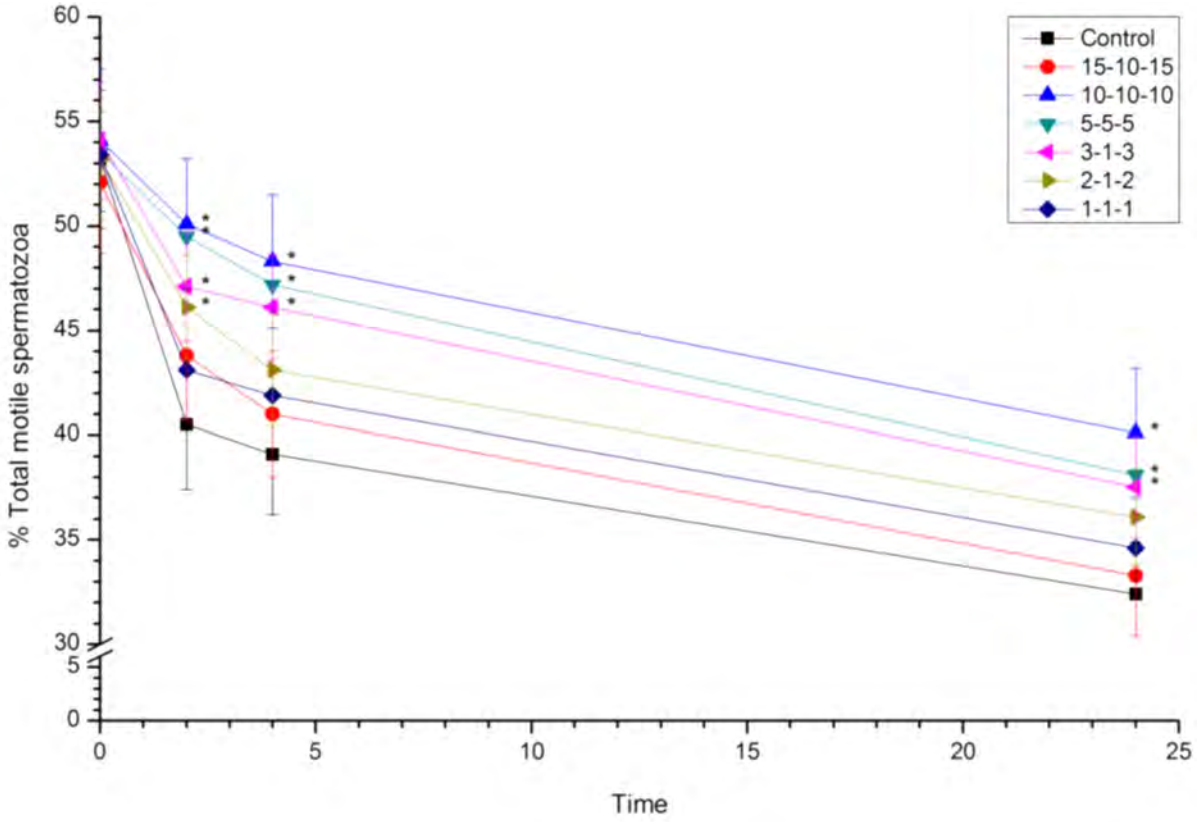

Fig. 4a. Percentages of total motile spermatozoa (experiment 1). Data are shown as mean ± SEM. (*) means significant differences of a given treatment with respect to the control at a given time point.

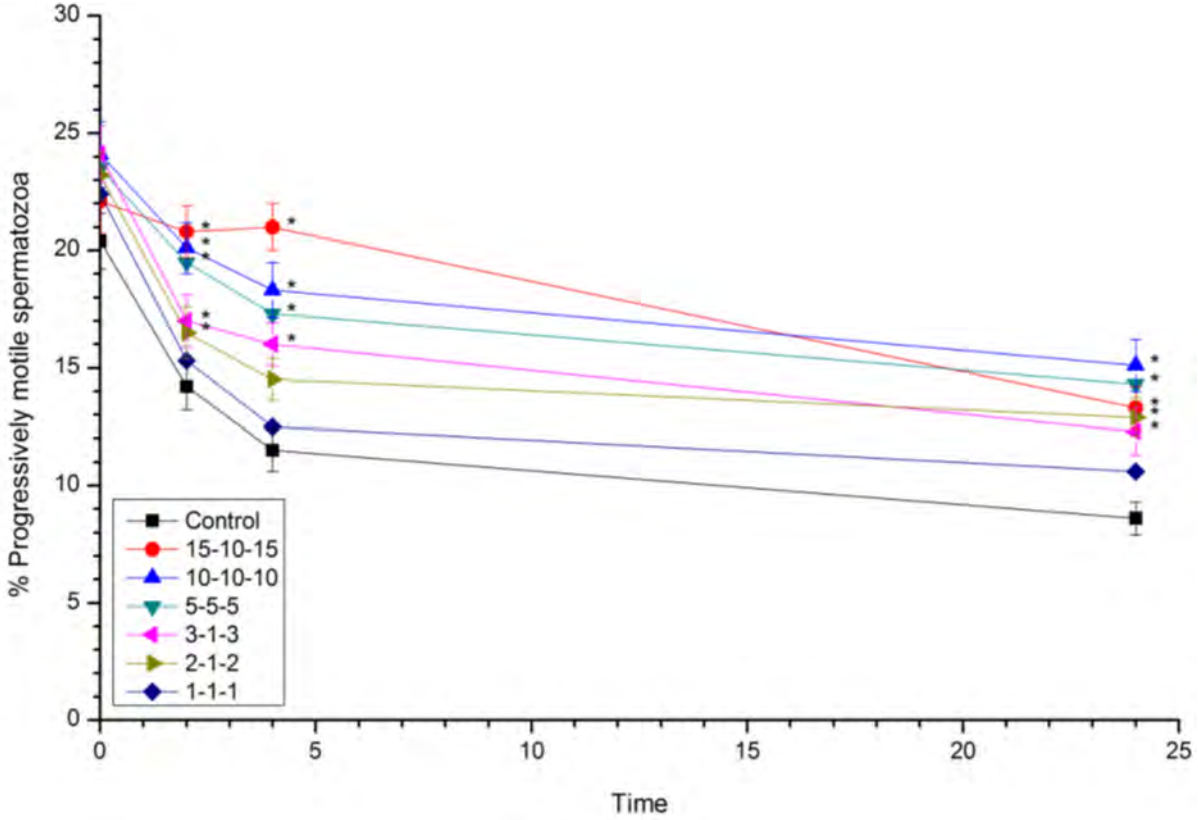

Fig. 4b. Percentages of progressively motile spermatozoa (experiment 1). Data are shown as mean ± SEM. (*) means significant differences of a given treatment with respect to the control at a given time point.

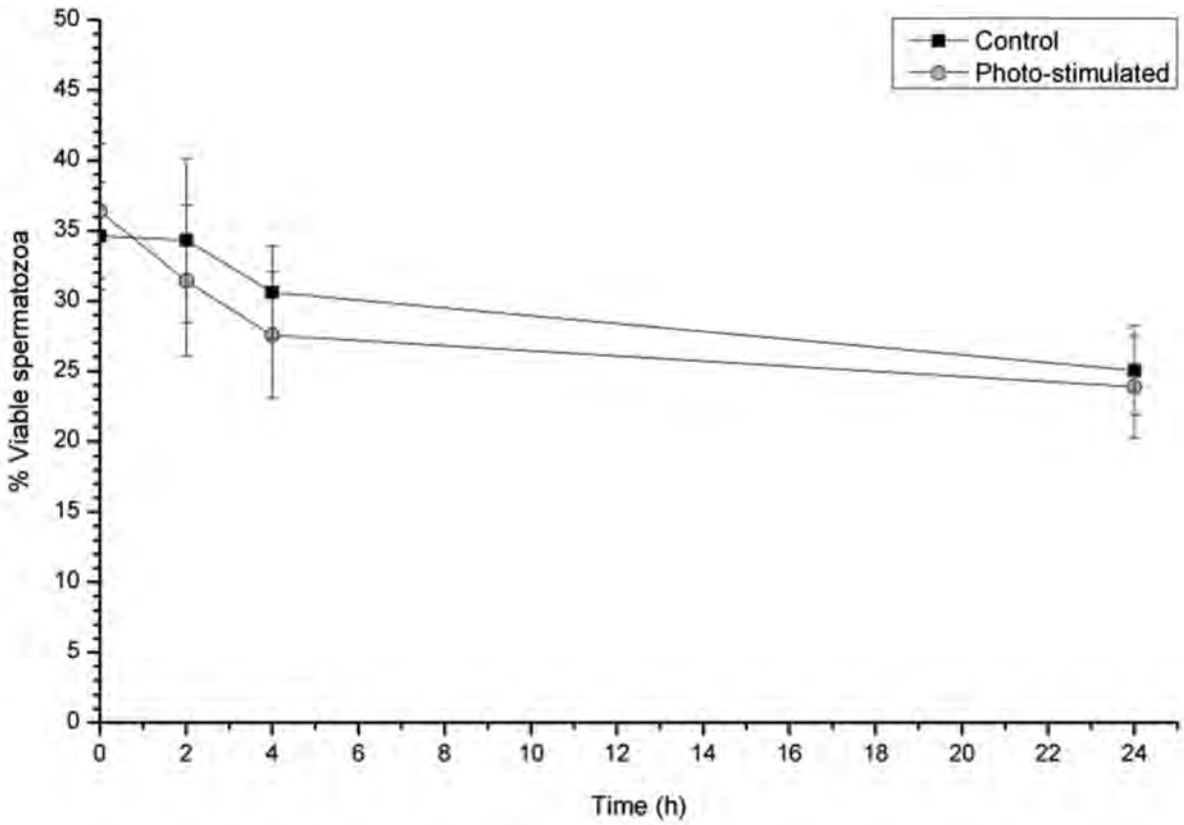

Fig. 5. Percentages of viable spermatozoa (as mean ± SEM) in control and photo-stimulated bull sperm (Experiment 2). Sperm were thawed either photo-stimulated and evaluated (0 h) or kept at room temperature for 2, 4 or 24 h, and then photo-stimulated and evaluated.

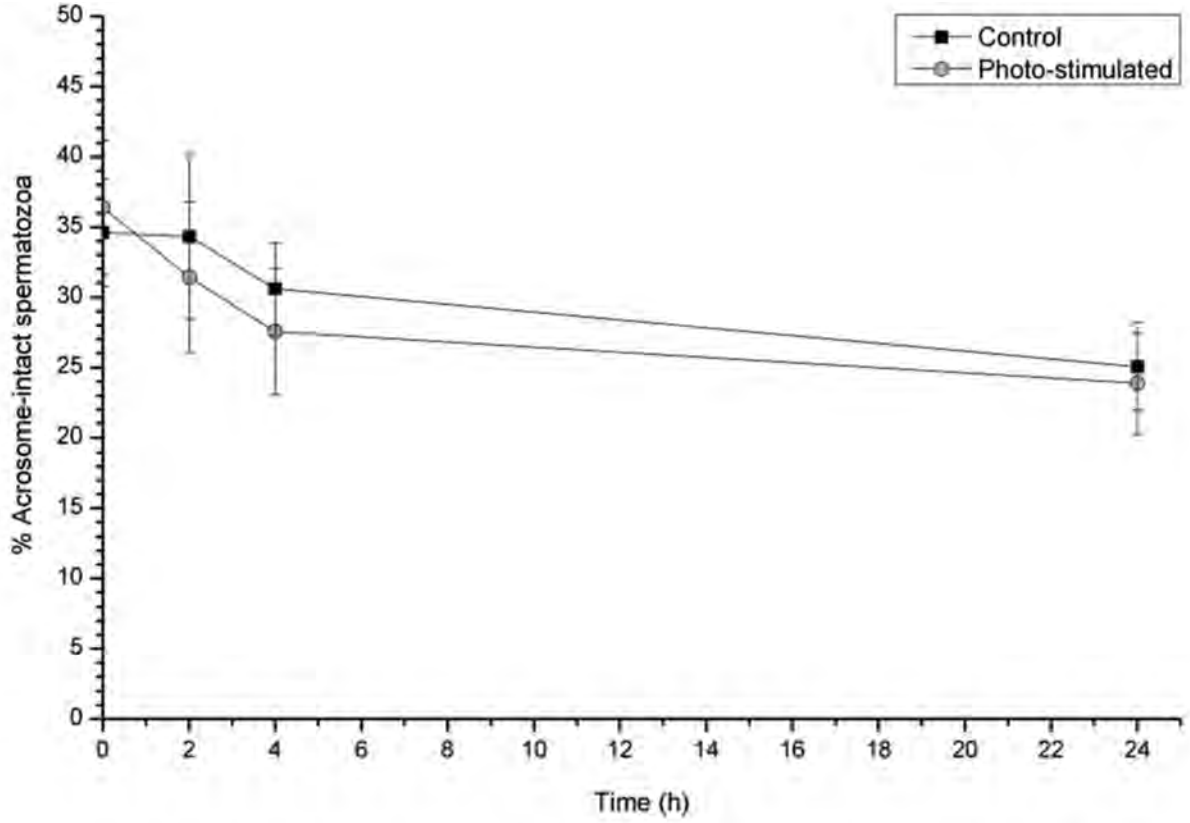

Fig. 6. Percentages of acrosome-intact spermatozoa (as mean ± SEM) in control and photo-stimulated bull sperm (Experiment 2). Sperm were thawed either photo-stimulated and evaluated (0 h), or kept at room temperature for 2, 4 or 24 h, and then photo-stimulated and evaluated.

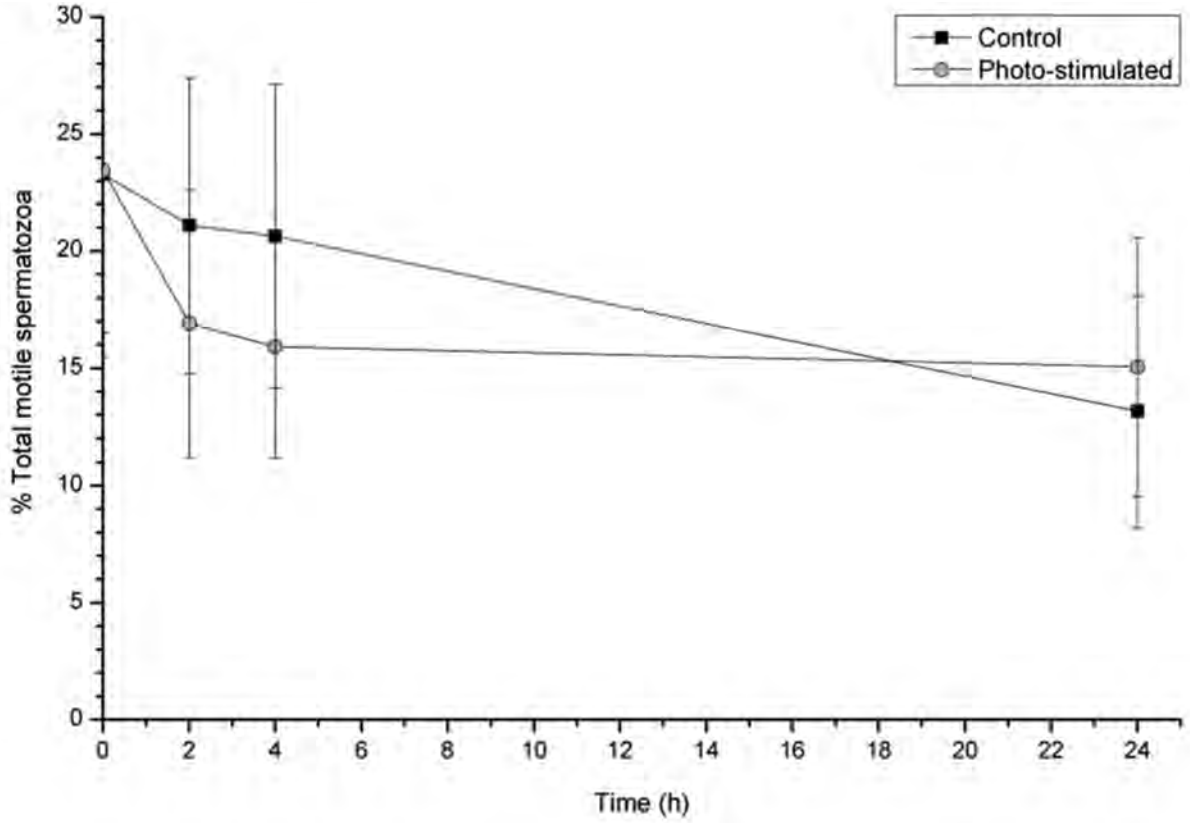

Fig. 7a. Percentages of total motile spermatozoa (as mean ± SEM) in control and photo-stimulated bull sperm (Experiment 2). Sperm were thawed either photo- stimulated and evaluated (0 h) or kept at room temperature for 2, 4 or 24 h, and then photo-stimulated and evaluated.

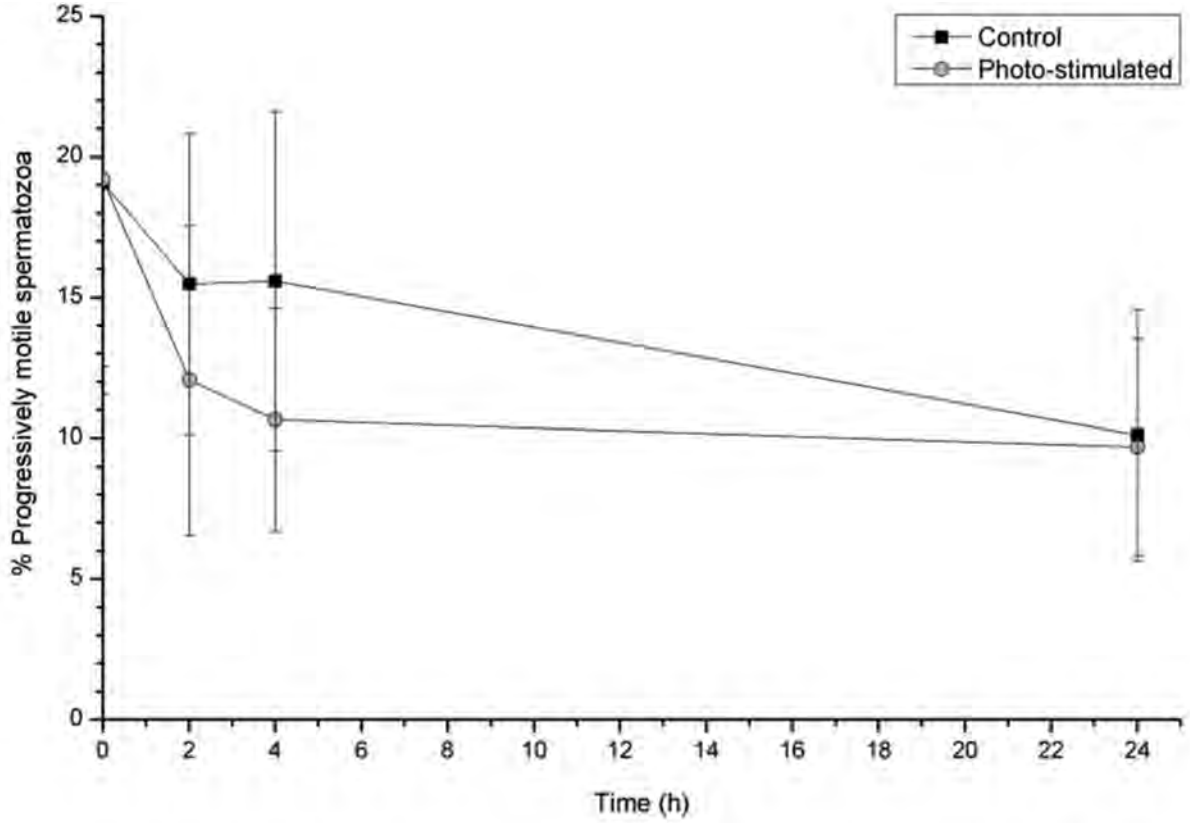

Fig. 7b. Percentages of progressively motile spermatozoa (as mean ± SEM) in control and photostimulated bull sperm (Experiment 2). Sperm were thawed either photo- stimulated and evaluated (0 h) or kept at room temperature for 2, 4 or 24 h, and then photo-stimulated and evaluated.

Sperm motility

Figures 7a and 7b show the percentages of total and progressive motile spermatozoa after keeping samples at room temperature for up to a 24 h-period. No clear differences were observed after photo-stimulation, and results did much differ from those obtained in Experiment 1.

Discussion

As shown in our study, photostimulation application has positive effects in terms of sperm parameters. Therefore, this situation is directly related to fertility rates. Each of the parameters analyzed in our study, such as sperm viability, acrosome integrity, mitochondrial membrane potential and sperm motility, has a distinct importance and function in the fertilization process.

If the basic mechanism is mentioned in order to interpret the effects of photostimulation application in terms of sperm parameters; the light signal reaches the mitochondria, which will control key aspects of sperm function such as motility and lifespan. Light also acts directly on the cytochrome complexes of the mitochondrial electronic chain, which modulate mitochondria-controlled sperm functions (Rodríguez-Gil 2019). Oxidative phosphorylation in sperm mitochondria leads to the formation of ATP, which is primarily required for sperm motility. In relation to this situation, it has been shown that the energy load of the cell increases following laser irradiation (Iaffaldano et al. 2005). Laser irradiation of sperm leads to increased respiration, fructose fermentation, ^{32}P uptake and Ca^{2+} absorption, which increases motility and prolongs sperm survival (Wenbin et al. 1996). On the other hand, studies have shown that high intracellular Ca^{2+} levels are detected in irradiated mice (Lubart et al. 1992) and bull spermatozoa (Breitbart 1996, Cohen 1998). Presence and activity of intracellular Ca^{2+} and increasing of cAMP concentration are related to regulating sperm motility, capacitation, and acrosome reaction (Aitken 1997, Darszon et al. 2007).

There are various studies showing the effects of photostimulation application on bull sperm parameters, as follows:

In a study with preliminary data similar to the present study, Omur and Balcı (2020) determined that the application of red laser diode -635 nm- in 5 mw at the regime of 3-1-3, increases the motility rates of frozen-thawed bull sperm when applied upon thawing. Ocaña-Quero et al. (1997), studied effects of He-Ne

laser irradiation on acrosome reaction with fluences of 2 to 16 J/cm^2 at 632 nm in bull spermatozoa. They revealed that laser irradiation significantly increased the percentage of acrosome-reacted spermatozoa and decreased the percentage of dead sperm in vitro at 90 min incubation, compared to other capacitation agents and the control group. In another study, Fernandes et al. (2015), used an aluminum gallium indium phosphide laser with a wavelength of 660 nm and power of 30 mW, to determine the effect of low-level laser irradiation on sperm motility, plasma membrane integrity and acrosomal integrity in cryopreserved bull semen. When the results obtained in terms of the effects of low-level laser irradiation on sperm cells are interpreted, it is seen that a positive picture emerges in the percentage of membrane intact (live) and acrosome intact spermatozoa and the preservation of sperm motility.

Studies conducted to increase fertility abilities by irradiating sperm cells in the cryopreservation process in different spectrum areas also attract attention. (Fernandes et al. 2015, Iaffaldano et al. 2016, Yeste et al. 2016).

In addition to the studies carried out, the efficiency of photostimulation applications was determined in human (Fekrazad et al. 2014, Frangez et al. 2015), buffalo (Abdel-Salam et al. 2011), turkey (Iaffaldano et al. 2005), horse (Brand˜ao et al. 2008), dog (Corral-Baqués et al. 2009), ram (Iaffaldano et al. 2016), ovine (de Almeida et al. 2019) and rabbit sperm cells (Iaffaldano et al. 2010). When we consider variables such as the duration of the irradiation, wavelength range, applied power, and the characteristics of the sperm cell; the results we have obtained regarding the photostimulation effect and the findings of other researchers show the useful and improvable aspects of this technique. Additionally, in studies evaluating the photostimulation effect, it is observed that the levels of free oxygen radicals decrease or do not change (Huang et al. 2013, Pezo et al. 2018, Antognazza et al. 2019). This result creates a positive situation against the detrimental effects of free oxygen radicals formed during the cryopreservation process.

Therefore, further studies are needed to confirm the positive effects of photostimulation in terms of different sperm parameters.

Conclusions

1) Photo-stimulation at a pattern of 10-10-10, 5-5-5, 3-1-3 and, to a lesser extent, 2-1-2, increases the resilience of frozen-thawed bull sperm when applied upon thawing.

2) The positive effect of these treatments are not only restricted to sperm membrane integrity (i.e. acrosome and plasma membranes) and motility, but also concern the mitochondrial membrane potential.

3) The effects of photo-stimulation are less clear when post-thawed sperm are stimulated later than thawing and, on the basis of the current available data, this approach is discouraged.

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