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LED-based red light photostimulation improves short-term response of cooled boar semen exposed to thermal stress at 37°C

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Abstract

Pre-treatment of boar semen with a red light photostimulation procedure increases its "in vivo" fertilising ability. However, "in vitro" conducted studies shown contradictory results regarding the ability of photostimulated spermatozoa to react against strong stress and to achieve the capacitation status. The aim here was to determine the effect of photostimulation on the response to short-term moderate thermal stress of boar semen. Boar semen was exposed to red LED light regime emitting a 620–630 nm during 10 min of light, 10 min of rest and 10 min of light after 3 hr since semen was collected. An aliquot without photostimulation was included as a control. After the photostimulation, the sperm cells were incubated for 15 min at 37°C. Afterwards, motility, viability, intracellular Ca^{2+} level and production of reactive oxygen species (ROS) and peroxynitrite were analysed. The results showed that the photostimulated group maintained total motility throughout the time, whereas a significant decrease in total motility was observed in the nonphotostimulated control group. Furthermore, for kinetic parameters of motility, a significant increase was observed in LIN, STR and WOB in photostimulated spermatozoa. Peroxynitrite production was significantly increased in the photostimulated spermatozoa, whereas viability, ROS production and intracellular Ca^{2+} levels were not affected by photostimulation. In conclusion, photostimulation of commercial boar semen has a positive effect on motility of spermatozoa subjected to a short-term moderate thermal stress, which was concomitant with an increase in peroxynitrite production.

KEYWORDS
boar, LED, photostimulation, reproduction, spermatozoa, thermal stress

1 | INTRODUCTION

Artificial insemination (AI) utilising refrigerated, extended semen doses has been routinely applied in pigs for decades (Roca et al., 2015). This technique yields very good results regarding fertility and prolificacy (Yeste, Rodríguez-Gil, & Bonet, 2017) compared with cryopreserved spermatozoa (Johnson, Weitze, Fiser, & Maxwell, 2000). However, AI can become suboptimal depending on several conditions, such as bad semen handling during storage (Lopez Rodriguez, Soom, Arsenakis, & Maes, 2017). Various factors can determine semen quality during storage, and strategies aimed to improve the yield of in vivo fertility parameters are important in boar farming.

Regarding the factors that can affect the stored seminal sample, one of the most important is the sperm alterations

associated with oxidative stress caused by high concentrations of free radicals which are highly unstable and react with other molecules (Bansal & Bilaspuri, 2011). They are produced in harmless quantities and naturally by the cell, and are involved in a series of physiological processes such as capacitation, hyperactivation, acrosome reaction and union to the zona pellucida (de Lamirande, Jiang, Zini, Kodama, & Gagnon, 1997). The increase in the amounts of peroxynitrite increases the oxidation of thiol groups which have been associated with the loss of motility (Vignini *et al*., 2006).

An important sperm metabolite, is the intracellular Ca^{2+}, is involved in modulating the exocytosis of the acrosome and in processes modulated by the mitochondrion like motility hyperactivation (Gunter & Sheu, 2009; Lishko, Botchkina, & Kirichok, 2011; Yeste et al., 2015). A non physiological increase of intracellular Ca^{2+} in boar semen utilised in AI negatively correlates with litter size (Pinart, Yeste, Puigmulé, Barrera, & Bonet, 2013), pointing out thus the importance of this parameter to maintain boar sperm "in vivo" fertilising ability.

The photostimulation has been applied to different fields. In studies of neuroscience, has been shown the existence of an activating effect of light irradiation on brain tissue, which in turn is able to modify the whole neural activity of the irradiated area (Senova et al., 2017; Valley, Wagner, Gallarda, & Lledo, 2011). Otherwise, the use of low-level laser therapy (600–700 nm) is a very useful tool for improving the proliferation rate of various cell lines, being applied for this purpose in fields such as tissue engineering and regenerative medicine (AlGhamdi, Kumar, & Moussa, 2012). Regarding spermatozoa, Light irradiation it has been reported to have beneficial effects on motility and kinetic parameters in tilapia fish, ram, dog and buffalo spermatozoa (Abdel-Salam et al., 2011; Corral-Baqués, Rigau, et al., 2005; Zan-Bar et al., 2005), and also, an increase in the long-term storage longevity o turkey spermatozoa was reported (Iaffaldano, Meluzzi, Manchisi, & Passarella, 2005). With respect to boar spermatozoa, a study reported no differences in the photostimulation of 90 ml seminal doses (Luther, Thi, Schäfer, Schulze, & Waberski, 2018). However, another report indicated that the photostimulation of boar semen stored in commercial extender in refrigeration conditions increased the main "in vivo" fertility parameters when this semen was utilised for an "in-farming" standard AI procedure (Yeste et al., 2016). This result seemed to be related with an increase in the rate of "in vitro" capacitation rhythm (Yeste et al., 2016).

After AI, the sperm cells will be subjected to thermal stress when enter into the female genital tract, and the hypothesis here is that the photostimulation will improve the response of boar spermatozoa to short-term thermal stress, that could explain the "in vivo" improving effects that photostimulation has shown to induce in "in-farming" conditions. Thus, the aim of this study was to determine the effect of photostimulation on motility, viability, calcium levels and production of reactive oxygen species (ROS) and peroxynitrite in boar spermatozoa subjected to short-term moderate thermal stress.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All the protocols were approved by the Scientific Ethics Committee of the Universidad de La Frontera and were conducted in compliance with Chilean Animal Protection Statute No. 20.380.

2.2 | Study site

The photostimulation experiments were performed in the Reproductive Medicine and Molecular Endocrinology Laboratory of the Center of Excellence in Translational Medicine (CEMT) at the Universidad de La Frontera. The sperm motility assays were conducted at the Center of Excellence in Reproductive Biotechnology (CEBIOR), which is part of the Faculty of Medicine at the Universidad de La Frontera.

2.3 | Semen samples

The samples were provided by the Sociedad Agrícola y Ganadera Pehuén Ltda., Victoria, Chile. The glove-hand technique was used to obtain one ejaculate from each of six boars with proven fertility. The sperm-rich fraction from each ejaculate was diluted in the commercial extender Androstar® Plus (Minitube, Germany) for storage in liquid and divided into semen doses of 60 ml with a concentration of 2×10^9 spermatozoa per dose. The semen doses were stored at 17°C and transported to the laboratory within 1 hr. Only sperm samples with a total motility over 80% and viability >85% were used. The viability was assessed by flow cytometry using propidium iodide (PI; 9.6 μM; Sigma-Aldrich Inc., St Louis, MO, USA) (Yeste, 2017; Yeste, Estrada, Casas, Bonet, & Rodríguez-Gil, 2013; Zhang et al., 2015).

2.4 | Photostimulation

A red LED was used with a chamber designed by MaXipig® (Barcelona, Spain) for the photostimulation. Semen samples were processed before 3 hr post-collection. The procedure was conducted as described by Yeste et al. (2016). Briefly, 1 ml of semen stored in liquid at 17°C from each of the ejaculates was deposited in a 15 ml polypropylene tube and subjected to a specific in vitro photostimulation pattern that consisted of 10 min of light, 10 min of rest and 10 min of light. For the control, 1 ml of semen from the same ejaculate not subjected to the photostimulation process was used. The room temperature for the experimental groups was the same, and the spermatozoa from both groups underwent a 15-min incubation period at 37°C prior to the analyses.

2.5 | Sperm function analysis

2.5.1 | Sperm motility

Sperm motility was analysed using the CASA system (computer-assisted sperm analysis, ISAS V1.0; Proiser SL; Valencia, Spain). Sperm motility and its kinetic parameters were evaluated at 15, 30

and 45 min (T15, T30 and T45). The ISAS system is based on the analysis of 25 consecutive digitised photographic images obtained from a single field at a magnification of 10×. Negative contrast was used for each test using the adjustments for assessing boar spermatozoa. The parameter settings were as follows: 10–80 µm for particle area and average path velocity (VAP) of 10 µm/s to classify a spermatozoon as motile. A 45% of straightness (STR) was considered as a progressive spermatozoon, and 10 images were used to calculate lateral head displacement (ALH). The photographs were taken at a capture speed of 40 mseg. Five different fields were taken, and a minimum of 500 spermatozoa per replica was reached.

2.5.2 | Intracellular ROS and cell viability

The reagent dihydroethidium (DHE, Molecular Probes, Life Technologies) was used to detect O_2−, since the mean fluorescence intensity (MFI) of the oxidised DHE is proportional to the amount of intracellular O_2− (Guthrie & Welch, 2010). For the simultaneous analysis of sperm viability, SYTOX Green (Molecular Probes; Life Technologies) was used. The procedure was performed according to Guthrie and Welch (2010) with some modifications. Briefly, 48 µl aliquots of sperm suspension (2×10^6 ml^{-1}) diluted in the Androstar® Plus extender were incubated with 4 µM of DHE and 0.04 µM of SYTOX Green at 25°C for 20 min. Then, they were re-suspended in 500 µl of extender for their later analysis by flow cytometry.

2.5.3 | Intracellular Ca^{2+}

In order to determine the level of intracellular Ca^{2+}, Fluo-4 a.m. (Invitrogen™, Oregon, USA) was used, an indicator that accumulates intracellularly and increases its green fluorescence when it bonds to Ca^{2+} (Fernández-Gago, Domínguez, & Martínez-Pastor, 2013). The procedure was conducted as described by Fernández-Gago et al. (2013) with modifications. 40 µl aliquots of sperm suspension (2×10^6 ml^{-1}) were incubated with 5 µM of Fluo-4 a.m. at 37°C for 1 hr. Once incubation was complete, the spermatozoa were re-suspended in 500 µl of extender and PI was added to exclude dead spermatozoa in the analysis. The MFI of Fluo-4 in the cells was determined by flow cytometry.

2.5.4 | Production of peroxynitrite

The compound dihydrorhodamine 123 (DHR; Enzo Life Science Inc., Farmingdale, NY, USA) was used to detect peroxynitrite. The procedure was conducted as described by Uribe, Boguen, Treulen, Sánchez, and Villegas (2015) with some modifications. 48 µl aliquots of sperm suspension (2×10^6 ml^{-1}) were diluted in Androstar® Plus extender and incubated with 1 µM of DHR at 37°C for 15 min. Finally, the cells were re-suspended in 500 µl of extender and 9.6 µM of PI was added to exclude dead spermatozoa in the analysis. The MFI of DHR was determined by flow cytometry.

2.5.5 | Flow cytometry analysis

The fluorescence analyses were performed in a FACS Canto II flow cytometer (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA). The sperm population was gated based on its size versus internal complexity by using the forward side-scatter pulse area (FSC-A and SSC-A) and also the forward scatter pulse width (FSC-W), which allow us to discriminate and discard debris and doublets. The cells were excited at a 488 nm wavelength using an argon laser, and each analysis included 10,000 spermatozoa, which were quantified simultaneously for each fluorescence. The data were provided on a logarithmic scale and digitised using the FACS DiVa 6.0 software (Becton, Dickinson and Company).

2.6 | Statistical analysis

The software GraphPad Prism® v. 5.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. D'Agostino's K2 test was applied to evaluate the Gaussian distribution, and numeric results were transformed to a logarithmic scale because they did not pass the normality test. A two-way ANOVA with Bonferroni's post-test was used to analyse sperm motility. For analysis of sperm viability, intracellular ROS, peroxynitrite production and intracellular Ca^{2+} levels, a *t* test was applied. The results are presented as the average ± standard deviation (*SD*), and *p*-values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Effect of photostimulation on motility and kinetic parameters of motility on boar sperm

The effect of photostimulation and incubation time on sperm motility was assessed. A time-dependent decrease ($p < 0.01$) in total sperm motility was observed. The decrease in total motility was statistically significant only in the nonphotostimulated control group at 45 min compared to 15 min ($p < 0.001$; Figure 1a), while the photostimulated group maintained the total sperm motility during the time analysed (Figure 1a). The progressive motility does not present variation in either group (Figure 1b). The different kinetic parameters in the photostimulated spermatozoa and the control group at 15, 30 and 45 min were evaluated. In the photostimulated spermatozoa, a significative increase in linearity (LIN), straightness (STR) and wobble (WOB) was determined, associated with a reduction in curvilinear velocity (VCL) and amplitude of lateral head (ALH), whereas in the control group decrease the beat-cross frequency (BCF) (Table 1).

3.2 | Effect of photostimulation on viability, ROS and peroxynitrite production and intracellular Ca^{2+} levels in boar spermatozoa

The percentage of viability was not significantly affected by exposing the spermatozoa to LED lights, with sperm viability being maintained around 90% in both groups (Figure 2a).

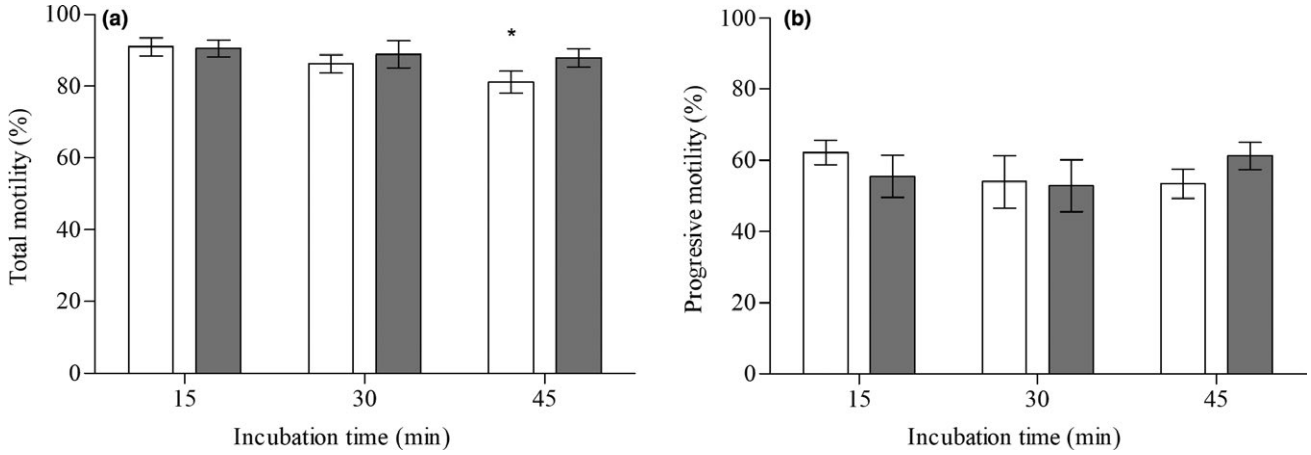

FIGURE 1 Percentages of total motility (a) and progressive motility (b) in boar spermatozoa subjected to photostimulation. The spermatozoa were subjected to a photostimulation protocol, and the motility was evaluated at 15, 30 and 45 min of incubation at 37°C. The control corresponds to spermatozoa not subjected to photostimulation. The results are shown as the mean ± standard deviation of 6 different experiments. The white bars correspond to the control group and the grey bars to the photostimulated group. The asterisk indicates significant differences with respect to the 15 min

Likewise, when ROS production in spermatozoa was evaluated after photostimulation, no statistically significant differences were demonstrated compared to the control group (Figure 2b). However, a significant ($p < 0.05$) increase in peroxynitrite production was observed in photostimulated samples when compared to the control ones (Figure 2c). Finally, no significant differences were detected in intracellular Ca^{2+} levels when the photostimulated group was compared to the control group (Figure 2d).

4 | DISCUSSION

Our results indicate that the precise photostimulation procedure performed by a commercial device improves the response of boar spermatozoa against a short and moderate thermal stress similar to that find by spermatozoa in the first stages of the commercial AI procedure. This is important considering that AI with liquid semen preserved at 17°C is the most widespread and prominent procedure applied at this moment in pig farming worldwide. This, in turn, explains the importance to evaluate photostimulation as a relevant technique to improve pig farming and, perhaps, animal production in a whole.

Specifically, our results showed that photostimulation enabled a better preservation of total motility. This improvement was associated with an increase in peroxynitrite production in the photostimulated spermatozoa without affecting sperm viability. Similar results with respect to motility have been reported in human, buffalo and turkey spermatozoa exposed to different laser irradiation systems (Abdel-Salam et al., 2011; Iaffaldano et al., 2005; Salman Yazdi et al., 2014).

In our study, the incubation time had a negative effect on total motility, which was significant only in the control group, which agrees in part with what was described by Yeste et al. (2016), who reported an exponential decrease in the total motility of photostimulated and nonphotostimulated boar spermatozoa using the same red LED light exposure regime. In dog spermatozoa irradiated with laser light (655 nm), total motility is maintained up to 45 min after exposure (Corral-Baqués et al., 2005). On the other hand, using the same photostimulation system as in our study, a gradual decrease in the time for total motility was reported, which was significant after 90 min of incubation at 38°C, using semen doses of 95 ml stored in transparent plastic tubes, concluding that the photostimulation does not improve the in vitro quality of diluted boar semen (Luther et al., 2018). These differences can be explained by the sample volumes used during the procedure and also by the handle of the doses that were submitted to a stressed by hypothermic storage and/or thermic stress.

There is no clarity with regard to the mechanism by which LED light modulates sperm activity; however, in other cell models, it is suggested that there is an interaction of light and elements of the electron transport chain in the mitochondrion, such as the cytochrome C oxidase complex, reducing the inflammation in age-related macular degeneration in mice (Begum, Powner, Hudson, Hogg, & Jeffery, 2013) and increasing the energy metabolism in neural cells exposed to LED lights at 670 nm (Wong-Riley, Bai, Buchmann, & Whelan, 2001; Wong-Riley et al., 2005). This increase in mitochondrial activity was corroborated by Yeste et al. (2016), with an immediate increase in the percentage of photostimulated boar spermatozoa with high mitochondrial membrane potential, which remained up to the 90 min of incubation; this could explain the significant increase observed in this study in terms of the kinetic parameters (LIN, STR and WOB), which increased over time. This pattern was also reported by Corral-Baqués et al. (2005) and Yeste et al. (2016).

In relation to ROS production, no significant differences were detected in photostimulated boar spermatozoa compared with the control group. A reduction in ROS production and an increase in

TABLE 1 Kinetic parameters of motility in photostimulated boar spermatozoa and in the control group without photostimulation

	Time (min)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
Control	15	62.9 ± 21.5	24.0 ± 2.7	38.6 ± 7.05	41.6 ± 13.0	63.7 ± 12.1	64.1 ± 11.0	2.7 ± 1.0	6.9 ± 1.58[a]
	30	54.7 ± 17.5	21.6 ± 5.42	34.2 ± 1.9	43.8 ± 18.7	63.7 ± 17.1	65.7 ± 13.5	2.5 ± 0.8	6.0 ± 1.83[b]
	45	54.5 ± 19.0	22.2 ± 4.95	33.6 ± 5.85	44.2 ± 15.3	66.6 ± 12.5	64.5 ± 12.3	2.6 ± 0.94	5.76 ± 1.52[b]
Photostimulated	15	71.5 ± 20.8[a]	21.2 ± 2.58	39.1 ± 5.89	31.8 ± 9.81[a]	55.0 ± 8.5[a]	56.7 ± 9.4[a]	3.2 ± 1.07[a]	6.50 ± 1.67
	30	67.4 ± 20.9[a]	23.5 ± 2.28	40.2 ± 4.21	37.9 ± 12.3[a]	59.3 ± 10.6[a]	62.4 ± 11.3[ab]	3.06 ± 1.06[a]	6.60 ± 1.10
	45	54.1 ± 8.36[b]	24.5 ± 5.75	36.3 ± 4.63	46.4 ± 13.0[b]	67.0 ± 9.41[b]	68.1 ± 10.3[b]	2.5 ± 0.53[b]	6.0 ± 1.52

Note. The results are shown as average ± *SD*. Different letters indicate statistically significant differences between the evaluation times for each of the groups.

motility and fertilisation after exposure to visible red light at 660 nm have been previously reported in sheep spermatozoa (Zan-Bar et al., 2005). Boar spermatozoa produce less ROS than other species (Guthrie & Welch, 2012). The semen samples used in this study were stored with the seminal plasma, and it could be inferred that the antioxidant mechanisms contributed by the seminal plasma (Li et al., 2018) protected the boar spermatozoa subjected or not to the photostimulation regime.

In terms of peroxynitrite production, this was increased in the photostimulated spermatozoa compared to the control, suggesting that the beneficial effect of photostimulation on motility could be associated with peroxynitrite production. Peroxynitrite is a free radical formed by the reaction of NO with O_2− at a rate controlled by diffusion (Pacher, Beckman, & Liaudet, 2007). Elevated peroxynitrite concentrations are associated with an alteration in sperm function (Uribe et al., 2015), negatively affecting the motility parameters in human spermatozoa (Vignini *et al*., 2006); however, at low concentrations, this molecule modulates sperm functions, emphasising the concept that capacitation is part of an oxidative process (Herrero, Lamirande, & Gagnon, 2001). Yeste et al. (2016) reported that photostimulation accelerates the achievement of "in vitro" capacitation through mechanisms concomitant with a significant increase in the overall mitochondrial activity. Boar spermatozoon seems to have a specific motility pattern when they are incubated in capacitating conditions, characterised by an increase in LIN and STR (García *et al*., 2005), both parameters increased significantly in our study. Consistent with our results, the production of NO, a precursory molecule of peroxynitrite, was increased in bovine spermatozoa exposed to visible light (400–800 nm) (Ankri et al., 2010). Accordingly, we hypothesise that photostimulation caused a physiological increase in peroxynitrite that contributes to maintaining the motility noted in our study. This increase in reactive species after photostimulation could be due to the interaction of light and endogenous photosensitive molecules of the mitochondrion and plasma membrane, like the flavoproteins present in nicotinamide adenine dinucleotide phosphate oxidase ($NADPH^+$) (Yeste, Castillo-Martín, Bonet, & Rodríguez-Gil, 2018).

Other important molecule associate at sperm function is Ca^{2+}, at new is limited information about the effect of photostimulation on the flow of Ca^{2+} in mammalian spermatozoa; however, in mouse spermatozoa radiated with a 630-nm helium–neon laser light, an increase was reported in the intracellular Ca^{2+} and the fertilising potential of these cells, suggesting that the effect of light would depend on the presence of Ca^{2+} while inhibiting the voltage-dependent Ca^{2+} channels present in the membrane (Cohen, Lubart, Rubinstein, & Breitbart, 1998). In bovine spermatozoa exposed to different irradiation systems with different wavelengths (632–780 nm), an increase in Ca^{2+} transport was demonstrated (Lubart, Friedmann, Levinshal, Lavie, & Breitbart, 1992), whereas in the same species if the photostimulation power is increased, an inhibitory effect on the transport of Ca^{2+} is observed (Ankri et al., 2010).

In conclusion, the photostimulation of boar spermatozoa with LED lights at a 620–630 nm wavelength has a positive effect

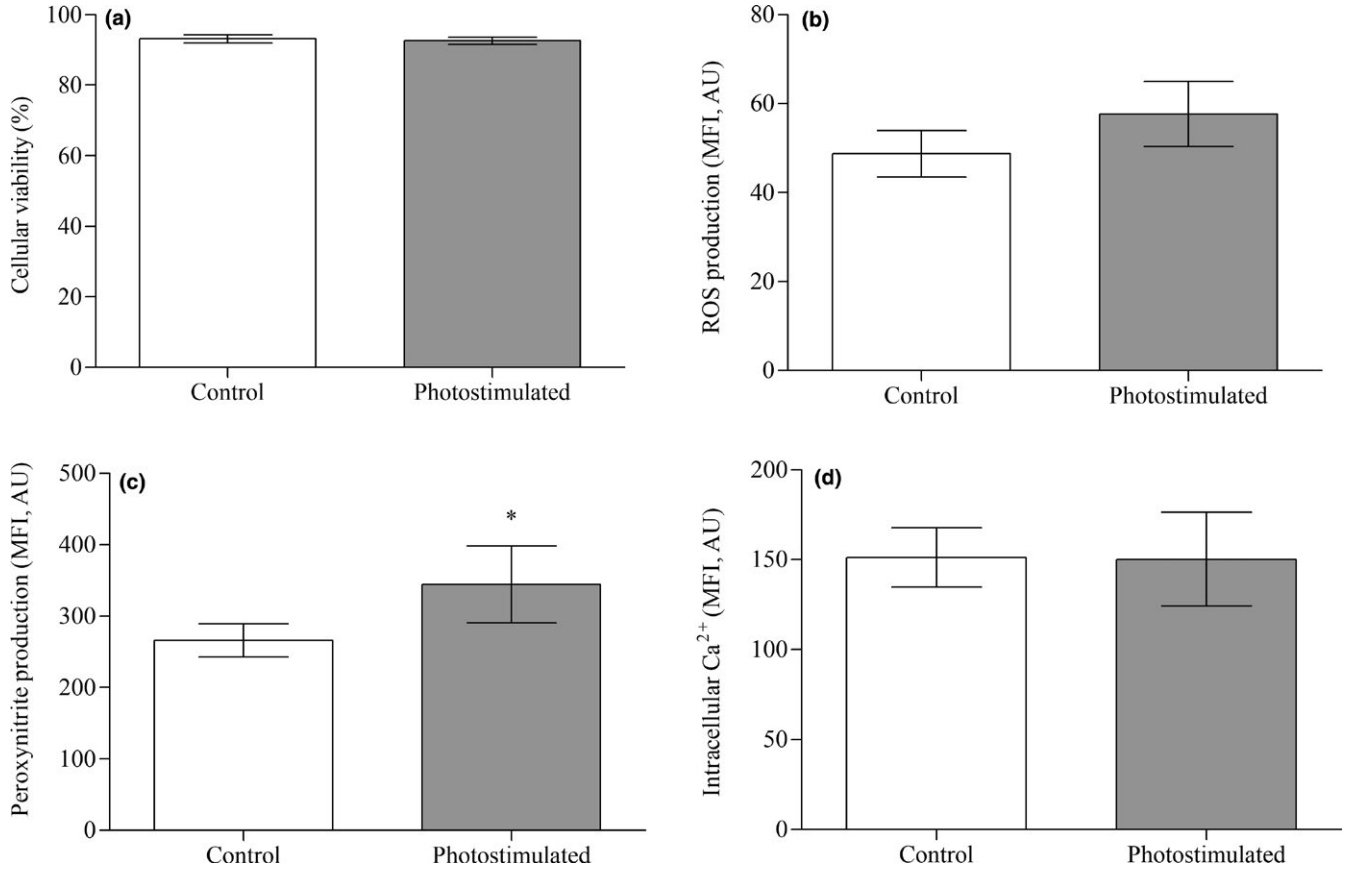

FIGURE 2 Effect of photostimulation on viability (a), ROS production (b), peroxynitrite production (c) and intracellular Ca^{2+} levels (d) in boar spermatozoa. After photostimulation, the spermatozoa were incubated at 37°C for 15 min and the parameters were analysed. The results are presented as average ± standard deviation of 6 different experiments. The asterisk indicates significant differences between groups (*$p < 0.05$). AU: arbitrary units; MFI: mean fluorescence intensity; ROS: reactive oxygen species.

on boar sperm motility subjected to a short-term, 37°C thermal stress. This beneficial effect is associated with an increase in peroxynitrite production, without affecting sperm viability. However, given the limited research on the effect of photostimulation in mammalian spermatozoa, it is important to evaluate the behaviour of the metabolites generated during this process over time, which might make it possible to explain the molecular mechanisms triggered by photostimulation in mammalian spermatozoa.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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