Original Research Article

Photobiomodulation Mitigates Oxidative Stress Induced by *Bothrops jararacussu* Venom in C2C12 Myoblast Cells

ABSTRACT

Myonecrosis is a significant complication of Bothropic envenoming, and standard antivenom treatment is ineffective in mitigating local tissue damage. Photobiomodulation has demonstrated potential in reducing the local effects induced by *Bothrops* snakebite envenoming, though the underlying mechanisms remain poorly understood. This study investigated the effects of photobiomodulation on oxidative stress in C2C12 myoblast cells exposed to *Bothrops jararacussu* venom. Cells were incubated with venom (12.5 µg/mL) and immediately irradiated with lasers at wavelength of red 660 nm (red) and 780nm (infrared), with energy densities of 4 and 5 J/cm^2, respectively. Key indicators of venom-induced oxidative stress were analyzed, including nitric oxide (NO) levels, hydrogen peroxide (H_2O_2) production, antioxidant enzyme activities (superoxide dismutase [SOD] and glutathione peroxidase [GPx]), and lipid peroxidation (TBARS). Results showed that PBM significantly reduced reactive oxygen species (ROS) generation and lipid peroxidation while enhancing SOD activity. These findings suggest that PBM provides cellular protection, at least in part, by mitigating oxidative stress.

Keywords: Bothops venom; *Bothrops jararacuss*; *low level laser*; *oxidative stress*; *photobiomodulation*

1. INTRODUCTION

Snakebite is a neglected global health issue that affects thousands of individuals every year mainly in tropical and subtropical areas of the world (Gutiérrez et al., 2010). In Brazil, 90% of venomous snakebites are attributed to the Bothrops genus (Silva et al., 2018). Bothrops venom induces severe local tissue damage, marked by intense pain, swelling, leukocyte infiltration, bleeding, and myonecrosis, which can result in permanent tissue loss and functional impairment. (Gutiérrez et al. 2009). The myotoxic effects of snake venoms trigger ion influx and protein release into the extracellular space, leading to muscle fiber necrosis and initiating an acute inflammatory response (Gutiérrez and Lomonte 1995, Díaz et al., 1991, Paiva-Oliveira et al., 2012).

The standard treatment for snake envenomation is antivenom therapy, which can prevent death but fails to mitigate local tissue damage and resulting functional disabilities, highlighting the need to explore alternative therapeutic approaches (Gutierrez et al., 2021). Photobiomodulation therapy (PBMT) is an emerging, noninvasive alternative treatment that has been used to promote healing, reduce pain and inflammation, and prevent tissue death (Chen et al., 2011). The irradiation using low-level laser (LLL) or light emitting diode (LED) to biological tissues contributes to tissue regeneration, healing, and pain relief by stimulating cellular processes, enhancing the activation of biochemical mechanisms, and reducing inflammation (Glass, 2021). Furthermore, the literature suggests that PBMT could be an alternative therapy for the treatment of local effects caused by snakes of the *Bothrops* genus due to its ability to attenuate the local damage related to snakebite (Barbosa et al., 2008; 2009; Doin-Silva et al., 2009; Dourado et al., 2003; Nadur-Andrade et al., 2012; 2016). However, the mechanisms involved in this muscle protection caused by PBMT are not yet fully understood.

Envenomation by various snake genera, such as *Bothrops*, has been shown to trigger oxidative/nitrosative stress (ONS), marked by an imbalance between pro-oxidant and antioxidant systems (Strapazzon et al., 2014). ONS can have highly detrimental effects on living organisms and is linked to numerous pathophysiological conditions, including hematological disorders and inflammatory responses involving leukocyte activation. During envenomation, the rise in reactive species is attributed to both ischemia-reperfusion tissue injury and the inflammatory response caused by venom injection (Strapazzon et al., 2014).

Considering that hemostatic disorders caused by Bothrops envenomation result in an imbalance in the redox state, which may further exacerbate these conditions, we hypothesize that ONS generation could be attenuated by PBM. Therefore, in this study, we focused on analyzing the effects of PBM therapy on oxidative stress in C2C12 myoblast cells following exposure to *Bothrops jararacussu* venom.

2. MATERIAL AND METHODS

2.1 *B. jararacussu* venom

The venom of *B. jararacussu* (BjssuV) was obtained from the Center for the Study of Nature at UNIVAP. It was lyophilized, stored at 4°C, and diluted in culture medium immediately prior to use.

2.2 Cell Culture

The murine C2C12 cell line (ATCC) was used as the venom target. For maintenance of C2C12 myoblast, cells were cultured in growth medium consisting of Dulbeccos modified Eagles medium (DMEM, Cultilab, Campinas, SP, Brazil) supplemented with heat-inactivated 10% fetal bovine serum and 1% antibiotic-antimycotic solution in a humidified atmosphere of 5 % CO2 at 37 °C. Growth medium was changed every two days.

2.3 Experimental groups

Myoblast C2C12 cell cultures were used in all experiments and all measurements were obtained from triplicate cultures. The following groups were studied: (1) Control (cells non-irradiated); (2) Venom (cells incubated with *B. jararacussu* venom); (3) venom + 660 nm (cells incubated with venom and immediately irradiate with laser at 660 nm); (4) venom + 780 nm (cells incubated with venom and immediately irradiate with laser at 780 nm). The venom dose used was 12.5 µg/mL and was chosen on the basis of previous study from our group which showed that a dose of 12.5 µg/mL decrease 50-60 % cell viability in the period of 15 to 60 min (Silva et al., 2012).

2.4 Laser irradiation

The experiments utilized semiconductor lasers, specifically gallium aluminum arsenide (Ga-AlAs) at 780 nm in the near-infrared range, and aluminum gallium indium phosphide (InGaAlP) at 660 nm, in red (MM Optics Ltd., São Carlos, SP, Brazil). The parameter settings are detailed in Table 1. Cells were irradiated immediately upon addition of the venom in the culture. The beam was positioned perpendicularly of the lower surface plate and irradiation was performed by one point at the center of each culture well, without moving the laser tip. The experiments were conducted in an environment with partial obscurity to not suffer interference from external light. The optical power output of the laser was measured using a Newport multifunction optical meter (model 1835C, Newport Corp., Irvine, CA, USA).

Table 1. Laser parameter setting

Parameter	***Red laser***	***Near-infrared laser***
Wavelength (nm)	660	780
Average radiant Power (mW)	16	20
Effective radiant Power (mW)	14.8	17.6
Operating mode	Continuous	Continuous
Beam spot size	0.04	0.04
Area irradiated (cm^2)	0.04	0.04
Irradiance (mW/cm^2)	400	500
Radiant exposure (J/cm^2)	4	5
Effective radiant exposure (J/cm^2)	3.52	4.4
Exposure duration (s)	10	10
Total energy (J)	0.16	0.2
Number of points irradiated	1	1
Application technique	Contact	Contact

2.5 NO Levels

The release of NO from C2C12 cells was determined by the accumulation of nitrite (NO) in the cell culture supernatants which was measured by the Griess reaction (Green et al., 1982). Aliquots of cell supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% n-(1-naphthyl)ethynedianime dihydrochloride in 2.5% phosphoric acid), incubated during 10 min at room temperature and the resulting absorbance was measured at 550nm. Nitrite concentration values were extrapolated from a calibration curve constructed using sodium nitrite as standard and were expressed as $\mu mMNO^{-}_{2}$.

2.6 H_2O production

The production of H_2O_2 by C2C12 cells was determined in cell suspensions collected 1 h after BjssuV or medium (control) incubation. Total cell number was adjusted to 2 x 10^6 cells/mL, centrifuged for 10 min and the cell pellet subsequently resuspended in 1 ml of phenol red solution (140 mM NaCl, 10 mM potassium-phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.56mM phenol red) containing 8.5U/ml of horseradish peroxidase, as previously described by Pick and Keisary (1980). Using 96-well flat-bottom tissue culture plates, 100 ml of the cell suspension were combined with 10 ml of phorbol myristate acetate solution (PMA, 10 ng/well) and incubated at 370ºC in a 5% CO atmosphere for 1 h. At the end of the incubation period, 10 ml of 1 N NaOH were added to each well to stop the reaction. Hydrogen peroxide-dependent phenol red oxidation was spectrophotometrically determined by measurement of absorbance at 620 nm (Titertek Multiscan reader, Labsystems, USA). H_2O_2 concentration values were extrapolated from a standard H_2O_2 calibration curve and expressed as nmoles H_2O_2/2 x 10^6.

2.7 Antioxidant enzymes

Superoxide dismutase (SOD) activity was measured spectrophotometrically in C2C12 cell homogenate by rate inhibition of pyrogallol autooxidation at 420 nm [23]. Enzyme activity was reported as U/mg protein. The results are expressed as nmol of reduced H2O2/min/mg protein. Glutathione peroxidase (GPx) activity was assessed in LV homogenates by adding to the assay a mixture of 1 U/mL glutathione reductase and 2 mmol/L glutathione in 1 mL phosphate buffer. Mixtures were preincubated at 37°C for 30 minutes. Subsequently, NADPH and tert-butylhydroperoxide were added, and the change in absorbance at 340 nm was recorded to calculate GPx activity, as previously described (Flohe and Gunzler, 1984, Del Maestro, 1985).

2.8 Thiobarbituric acid reaction (TBARS)

For the TBARS assay, trichloroacetic acid (10%, w/v) was added to the C2C12 cell homogenates to precipitate proteins and to acidify the samples (Buege nad Aust, 1978). This mixture was then centrifuged (10006 g, 3 minutes), the protein-free sample was extracted, and thiobarbituric acid (0.67%, w/v) was added to the reaction medium. The tubes were placed in a water bath (100°C) for 15 minutes. The absorbances were measured at 535 nm using a spectrophotometer. Commercially available malonyldialdehyde (MDA) was used as a standard, and the results are expressed as nmoles/mg protein.

2.9 Statistical analysis

Data are reported as means ± SEM. After confirming that all continuous variables were normally distributed using the Kolmogorov-Smirnov test. One-way ANOVA followed by the Tukey test was used to compare groups. Differences were considered significant at $P \leq .05$ for all tests.

3. RESULTS

3.1 Effect of PBM on the production of nitric oxide (NO) and hydrogen peroxide (H_2O_2) induced by *B. jararacussu* venom.

To evaluate the effect of *B. jararacussu* venom (VBjsu) on C2C12 cells, nitric oxide (NO) production (Fig. 1A) and hydrogen peroxide (H_2O_2) release (Fig. 1B) were analyzed after a 1-hour incubation with the venom. As shown in Fig. 1A, cells treated with the venom released significantly higher levels of NO compared to the control group. Photobiomodulation (PBM) treatment markedly reduced NO production induced by the venom. Similarly, Fig. 1B indicates that venom exposure led to a statistically significant increase in H_2O_2 release compared to the control. PBM effectively reduced H_2O_2 levels in the venom-treated group.

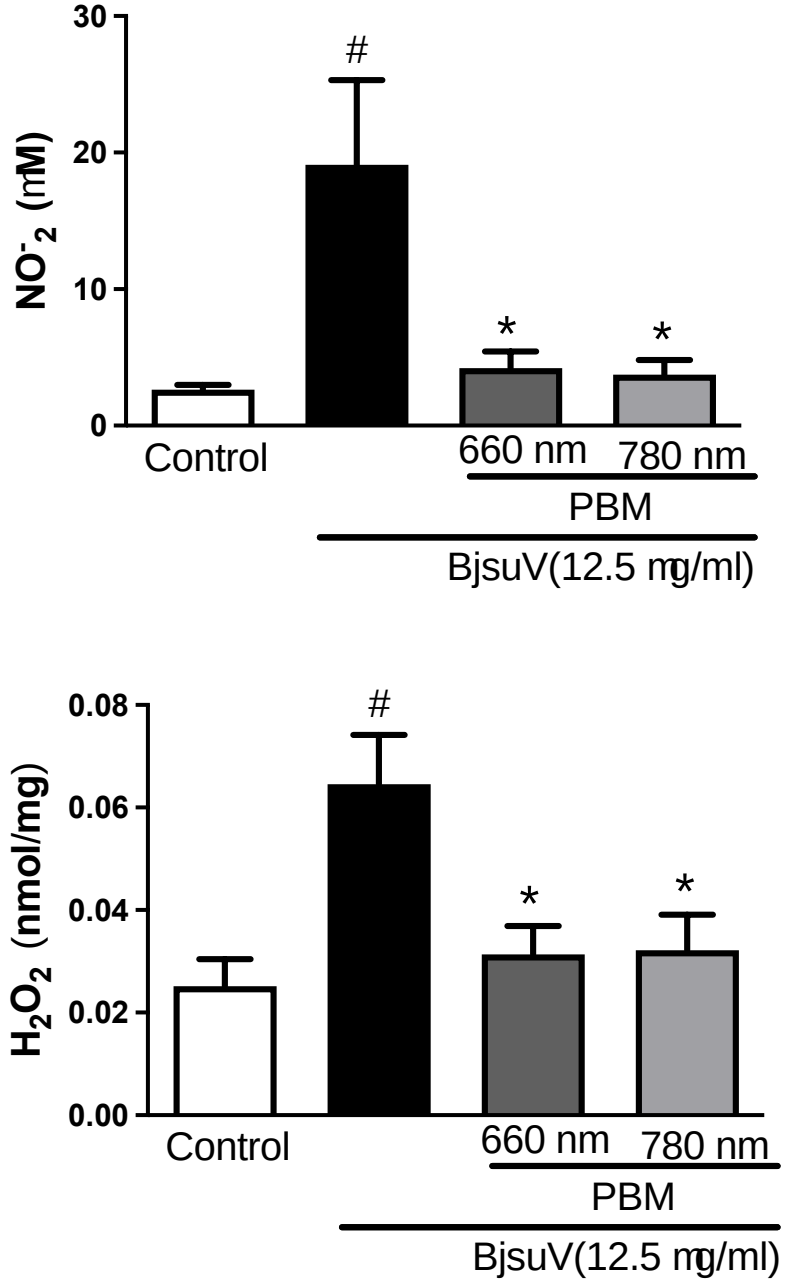

Figure 1. Photobiomodulation reduces NO and H2O2 production induced by *B. jararacussu* venom in C2C12 muscle cells.

C2C12 cells were seeded in 96-well plates and allowed to adhere for 24 hours. Cells were then treated with *B. jararacussu* venom (12.5 µg/mL) and immediately irradiated with photobiomodulation (PBM), followed by a 1-hour incubation. (A) Nitric oxide (NO) levels in the supernatant were measured using the Griess method, and (B) hydrogen peroxide (H2O2) levels were quantified using the phenol red method. Data represent the mean ± SEM of three independent experiments. #p < 0.05 compared to the control group; *P < 0.05 compared to the venom-only group.

3.2 Effect of PBM on the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) by C2C12 cells incubated with *B. jararacussu* (VBjsu) venom.

Analysis of the antioxidative enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) revealed that venom incubation reduced SOD activity, whereas PBM irradiation at a wavelength of 660 nm significantly increased SOD activity (Fig. 2A). In contrast, GPx activity showed no statistically significant differences among the experimental groups (Fig. 2B).

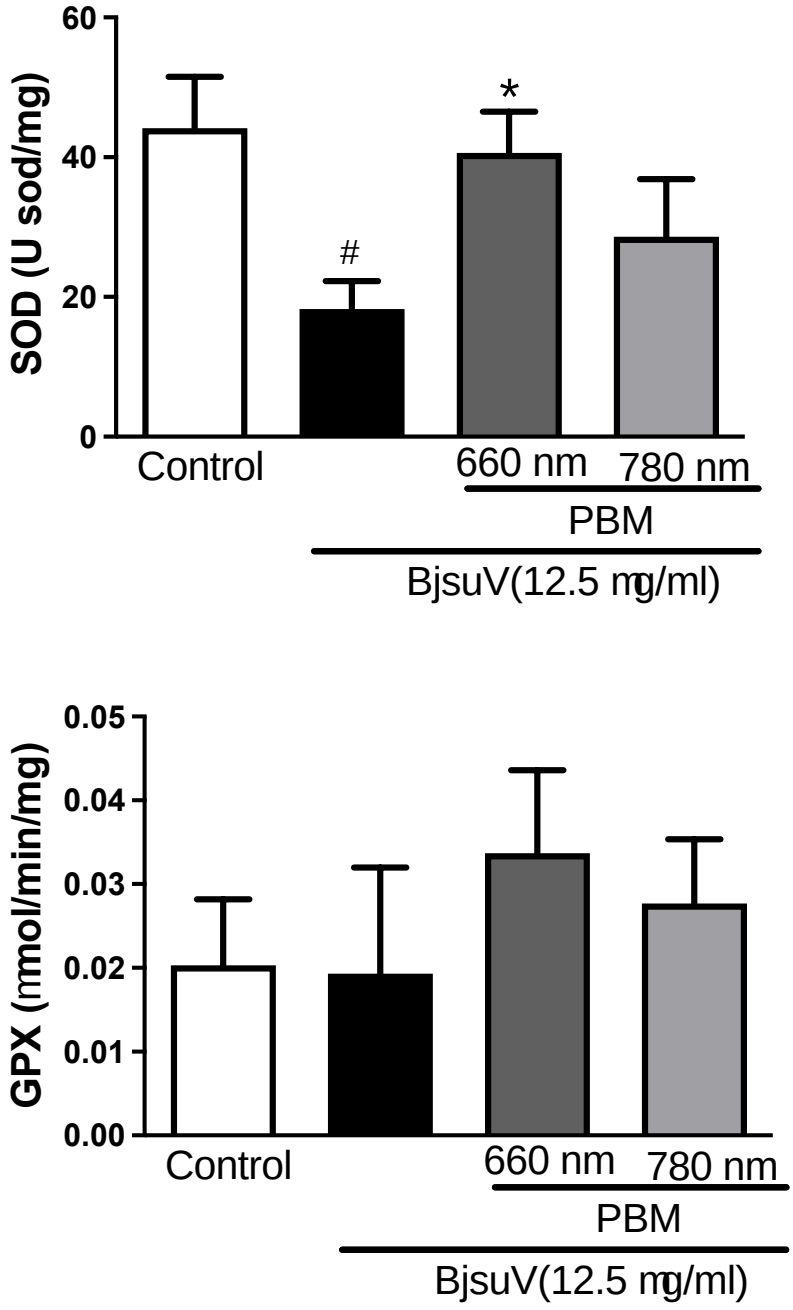

Figure 2. Photobiomodulation effect on the production of antioxidant enzymes SOD and GPx in C2C12 cells treated with *B. jararacussu* venom.

C2C12 muscle cells were seeded in 96-well plates and incubated for 24 hours to allow cell adhesion. Following this, cells were treated with *B. jararacussu* venom (12.5 µg/mL), immediately irradiated with photobiomodulation, and incubated for 1 hour. (A) Superoxide dismutase (SOD) and (B) glutathione peroxidase (GPx) levels in the supernatant were quantified using a spectrophotometric method. Data represent the mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared to the control group; $^{*}P < 0.05$ compared to the venom-only group.

3.3 The effect of PBM on the expression of lipid peroxidation (TBars) by C2C12 cells incubated with *B. jararacussu* venom (VBjsu).

Our results indicate that the 780 nm wavelength significantly reduced lipid peroxidation compared to both the control and VBjsu venom-treated groups. However, no significant differences in lipid peroxidation were observed in myoblasts under the 660 nm treatment (Fig. 3).

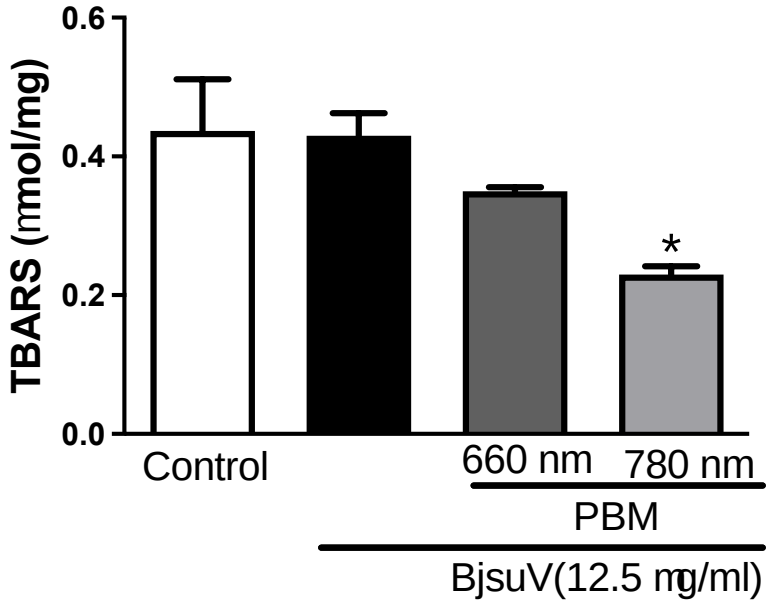

Figure 3. Photobiomodulation effect on lipid peroxidation induced by B. jararacussu (VBjsu) venom in C2C12 cells.

C2C12 muscle cells were seeded in 96-well plates and incubated for 24 h to allow cell adhesion. Following this, cells were treated with *B. jararacussu* venom (12.5 µg/mL), immediately irradiated with photobiomodulation, and incubated for 1 hour. The lipid peroxidation concentration was evaluated in the supernatant by ELISA method. Each value represents the mean ± SEM of three independent experiments. *$P < 0.05$ compared to the venom-only group.

4. DISCUSSION

Myonecrosis is a major effect of *B. jararacussu* snake venom, resulting in muscle mass loss and impaired tissue regeneration, which may lead to permanent damage and dysfunction (Milani Junior et al., 1997, Chippaux and Goyffon, 1998). Therefore, alternative therapeutic strategies are needed to treat venom-induced muscle injury and promote tissue repair. Photobiomodulation treatment has been reported to have beneficial effects in attenuating local responses induced by

Bothrops snake venoms, both in preclinical studies and in a clinical trial (Silva et al., 2018, Carvalho et al., 2024). However, the mechanisms involved in these beneficial effects are still poorly understood. In the present study we evaluated the effect of laser photobiomodulation on redox oxidative capacity produced by C2C12 myoblast cells stimulated with *Bothrops jararacussu* venom.

During envenomation, the increase in reactive oxygen and nitrogen species has been attributed to both ischemia-reperfusion tissue injury and the inflammatory reaction that occurs after venom injection (Sachetto et al., 2018). In this sense, it has been demonstrated that *B. jararaca* and *B. asper* venom induces an increase in NO and H_2O_2 and also the formation of peroxynitrite (Zamuner et al., 2001). These authors suggested that the formation of these oxygen and nitrogen radicals would play an important role in the toxicity caused by *Bothrops* venoms. Our findings showed that PBM (λ660nm and 780nm) reduced the generation of NO caused by BjsuV in muscle cells. Thus, it is possible to suggest that the protection caused by PBM at these two wavelengths used (λ660nm and 780nm), at least in part, results from a decrease in these oxygen radicals. The literature demonstrates that envenomation of snakes of the genus *Bothrops* can cause oxidative/nitrosative stress characterized by an imbalance between the pro and antioxidant systems (Sachetto et al., 2018). The imbalance in oxidative stress can lead to extremely deleterious effects on living organisms and has been associated with several pathophysiological conditions, including hematological disorders and inflammatory reactions that result in the activation of leukocytes (Sunitha et al., 2015). Our results showed that the venom did not alter the amount of lipid peroxidation or antioxidant enzymes such as SOD or GPX in our experimental model. However, after irradiation with LLLT (λ660nm and 780nm), the cells incubated with the venom showed a significant increase in SOD. We did not observe an increase in the GPX enzyme; we evaluated this enzyme during the 1-h incubation period with the venom; it is possible that a greater quantity of this enzyme could be produced at later times. Urishi et al., (2009) propose that antioxidant levels are determinant for the regenerative capacity of muscle stem cells. TBARS are markers of oxidative stress, more specifically of lipid oxidation. In this study, we did not observe the increase in TBARS levels when the venom alone was incubated with myoblast cells as compared to cells without venom. Dantas et al. (2018) showed a significantly increase in TBARS in a kidney of mice injected with *Bothrops insularis* after 24 h. It is possible that the time

in which we evaluated is not sufficient to verify the increase in this lipidic oxidation. However, the laser irradiation at a wavelength of 780 nm caused a significantly reduction on TBARS in C2C12 cells, after the venom incubation. Thus, the increase in SOD and the decrease in TBARS following the treatment of cells with LBI may contribute to the muscular improvement observed in *in vivo* studies (Barbosa et al., 2009, Campos et al., 2018).

4. CONCLUSION

This study demonstrated that photobiomodulation (PBM) applied to myoblast cells exposed to *Bothrops jararacussu* venom exhibits significant *in vitro* antioxidant activity. Specifically, PBM effectively reduced ROS generation and lipid peroxidation, accompanied by an increase in antioxidant SOD activity. These findings suggest that PBM can protect cells by mitigating oxidative stress. Therefore, PBM holds potential as a therapeutic candidate for combating oxidative stress-related muscle cytotoxicity induced by *Bothrops* envenomation.

ETHICAL APPROVAL

It is not applicable.

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