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ORIGINAL ARTICLE

Comparison of photobiomodulation using either an intraoral or an extraoral laser on oral mucositis induced by chemotherapy in rats

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Abstract

Purpose The aim of the present study was to compare the effect of intraoral (IO) and extraoral (EO) diode laser irradiation on oral mucositis (OM) induced by 5-fluorouracil (5-FU) in rats.

Methods Animals ($n = 78$) were divided into the following groups: negative control (NC), positive control (PC), IO 6 J/cm^2, EO with 6 J/cm^2 (EO 6 J/cm^2), and 12 J/cm^2 (EO 12 J/cm^2). OM was induced with an intraperitoneal injection of 5-FU and scarification of the buccal mucosa. Over the following 14 days, animals received photobiomodulation (PBM) daily. Clinical and histological evaluation was done by scores at days 8, 10, and 14. The redox state was evaluated by reactive species levels, antioxidant network, and immunohistochemistry analysis.

Results Clinically, on day 8, PBM groups showed lower scores of OM with EO 6 J/cm^2 presenting a significantly lower degree compared to PC ($p < 0.05$). On days 10 and 14, all PBM groups exhibited improvement of OM compared to PC ($p < 0.01$). On day 8, all PBM groups exhibited an accelerated healing process compared to PC ($p < 0.01$) and reduction of reactive species ($p < 0.001$). Also, all PBM groups demonstrated higher levels of antioxidant GPx compared to PC ($p < 0.001$). Analysis of nitrotyrosine revealed that on day 14, this protein damage marker was significantly reduced in the EO 6 J/cm^2 group ($p > 0.05$).

Conclusions An EO diode laser protocol promoted positive effects in the clinical, histopathological, and redox state in OM induced by 5-FU in rats. Among the EO protocols, EO 6 J/cm^2 showed the most encouraging results.

Keywords Diode lasers · Oral mucositis · Low-level laser therapy · Oxidative stress

Introduction

OM is a common acute complication of antineoplastic treatment, consisting of an inflammatory response that can affect patients receiving oncologic treatment [1]. Its incidence ranges from 15% among patients receiving low-risk treatments to 60–100% among patients being treated with high-dose chemotherapy (CT) and head and neck radiotherapy (RT) [2]. Clinically, OM is characterized by erythematous, erosive, and/or ulcerative sores. Severe forms of OM are associated with painful lesions, impairment of function, a need to change the diet or dependence on parenteral nutrition, and risk of infection. Additional costs of care and interruption or discontinuation of treatment could negatively interfere with the patient's prognosis [2–4].

The pathogenesis of OM is a multifactorial and multistage process that involves damage to oral tissues. It has been

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reported to have five stages: initiation, upregulation, and activation leading to the generation of messengers, signal amplification, ulceration, and healing. Oxidative stress (OS), generation of reactive oxygen species (ROS), direct DNA and non-DNA damage, and activation of the innate immune response occur during the initiation phase. Then, the release of endogenous damage-associated molecules, regulated by transcription factors such as nuclear factor kappa B (NF-κB), is observed. After the elimination of damaging stimuli, the healing stage occurs with an increase of epithelial proliferation, migration, and differentiation associated with extracellular matrix reorganization [4–6].

Several treatments have been proposed to minimize the damage that OM can cause to oncological patient. Among them, photobiomodulation (PBM) has been recommended by the Multinational Association of Supportive Care in Cancer (MASCC/ISOO) [7]. PBM is a therapy that uses lasers, light-emitting diodes (LED), and broadband light. It is a non-thermal process involving the absorption of light photons by chromophores, triggering responses by different biological pathways depending on the protocol used, type of cell, and other factors [8]. The physiological effects observed following irradiation and light absorption by cytochrome c oxidase are related to a shift in overall cell redox potential in the direction of greater oxidation and increased ROS production, modulating cell redox activity [9, 10]. These mechanisms promote activation of transcription factors such as NF-κB and synthesis of proteins, growth factors, and cytokines. Consequently, PBM has been associated with modulation of inflammation, a decrease in pain, acceleration of cellular proliferation, and wound healing [11].

Positive effects of different intraoral (IO) laser protocols in the prevention and treatment of OM have been reported [12–14]. However, only a few studies have demonstrated a reduction in the severity of OM using extraoral (EO) appliances [15–19]. An effective EO approach could potentially offer a more comfortable solution for patients since it does not require opening an inflamed mouth with wounds. In addition, it may be used in oropharyngeal mucositis, an area that cannot be reached with an IO laser [19, 20]. Thus, the aim of the present study was to compare the clinical and histopathological effect of PBM using either an IO or EO laser on OM induced by 5-FU in rats. In addition, we explored the effect of PBM in important mechanisms involved in the redox state.

Materials and methods

Experimental procedure

This study was approved by the Ethics Committee on Animal Use (CEUA, 2018-0096).

There were 78 male rats (*Rattus norvegicus albinus*, Rodentia Mammalia Wistar line) used, between 8 and 12 weeks of age and weighing 275 ± 25 g following animal maintenance according to Curra et al. [21].

The animals were randomly divided into five groups:

- Negative control (NC, $n = 6$): without intervention
- Positive control (PC, $n = 18$): OM induction, no treatment, only daily handling
- IO 6 J/cm^2 ($n = 18$): OM induction and IO laser with 6 J/cm^2
- EO 6 J/cm^2 ($n = 18$): OM induction and EO laser with 6.11 J/cm^2
- EO 12 J/cm^2 ($n = 18$): OM induction and EO laser with 12.22 J/cm^2

OM was induced in the rats across all study groups (except for NC) using an intraperitoneal injection of 5-FU at days 0 (60 mg/kg) and 2 (40 mg/kg) followed by bilateral buccal mucosa scarification on days 3 and 4 [21]. On day 5, all animals were evaluated for clinical identification of OM. PBM treatment was started on day 0 of OM induction, up to 14 days, and the output power of the equipment was confirmed using a power meter.

Parameters of IO PBM

IO PBM was delivered with a continuous indium–gallium–aluminum–phosphide (InGaAlP) diode laser (MMOptics Ltda, São Carlos, Brazil), and all of the parameters are described in Table 1 [21, 22]. Irradiation was performed once daily, perpendicularly to the mucosa, at one central point on the oral mucosa (Fig. 1a).

Parameters of EO laser

EO irradiations were performed with a pulse diode laser (Gemini® manufactured by Azena Medical, LLC, distributed by Ultradent Products, Inc.) with dual wavelength 810 + 980 nm and two distinct protocols (Table 1). A daily EO application was performed perpendicularly and in contact with the skin of the right and left cheeks at a central point (Fig. 1b). Power output was checked using a power meter (Coherent Inc., Santa Clara, CA). The laser irradiations were done following biosafety rules.

Euthanasia

Six animals from each group were euthanized using overdose of isoflurane at D0 (only animals from NC) and at days 8, 10, and 14, animals from other groups. Buccal mucosa was photographed and removed. One buccal mucosa was fixed in 10% buffered formalin solution for histopathological and

Table 1 Extraoral diode laser parameters

Protocol	IO 6 J/cm^2	EO 6 J/cm^2	EO 12 J/cm^2
Center wavelength (nm)	660 nm ± 10 nm	810 nm + 980 nm (50%/50%)	810 nm + 980 nm (50%/50%)
Operating mode	Continuous	Pulsed	Pulsed
Frequency (Hz)	~ 50/60 Hz	50 Hz	50 Hz
Pulse duration (ms)	Continuous	2 ms	2 ms
Duty cycle (%)	–	10%	10%
Peak power (W)	0.01 W	20 W	20 W
Average power (mW)	100 mW	2000 mW	2000 mW
Polarization	Yes	No	No
Spot size (cm^2)	0.04	4.91	4.91
Beam shape	Round	Round	Round
Beam profile	–	Gaussian	Gaussian
Irradiance at target (mW/cm^2)	2500 mW/cm^2	407 mW/cm^2	407 mW/cm^2
Exposure duration (s)	2.4	15	30
Radiant exposure (J/cm^2)	6	6.11	12.22
Total radiant energy (J)	0.24	30	60
Number of points irradiated	1	1	1
Area irradiated (cm^2)	0.04	4.91	4.91
Application technique	Contact	Contact	Contact
Number and frequency of treatment sessions	1× day/14 days	1× day/14 days	1× day/14 days

immunohistochemical study. The other was conditioned in liquid nitrogen and later in the freezer − 80 C for evaluation of the redox state.

Clinical and histopathological evaluation

For OM clinical analysis, photos from all groups were analyzed by calibrated and blinded oral medicine professional based on the method described by Lima et al. [23] (Table 2). Samples embedded in paraffin were cut into 5-μm slices and stained with hematoxylin–eosin. The evaluation of the material was performed according to the inflammatory parameters shown in Table 2 [23].

Immunohistochemical evaluation

Immunohistochemistry with nitrotyrosine was performed using the streptavidin–biotin–peroxidase method following a routine protocol. Sections were incubated overnight (4 °C) with a nitrotyrosine primary antibody diluted 1:100 in PBS plus bovine serum albumin (PBS-BSA), incubated with avidin–biotin–peroxidase conjugate and counterstained with Harry's hematoxylin. Only cytoplasmic expression in epithelial cells was considered positive. Each case was classified according to percentage of positive cells (PP). The PP was scored as follows: 1 (0–4%), 2 (5–19%), 3 (20–39%), 4 (40–59%), 5 (60–79%), and 6 (80–100%) [24].

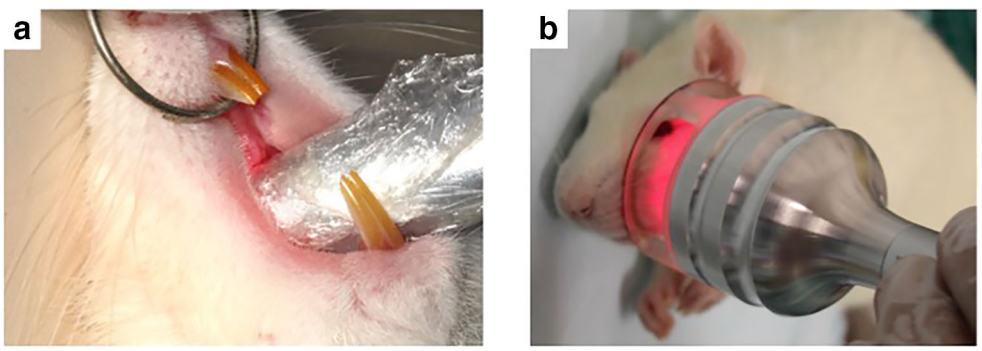

Fig. 1 Illustrative photographs of intraoral (**a**) and extraoral (**b**) diode laser application

Table 2 Methods of clinical and histopathological evaluation

Score	Clinical evaluation	Histopathological evaluation
0	• Normal oral mucosa	• Normal epithelium and conjunctive tissue
	• Absence of or slight erythema and hyperemia	• No vasodilatation
	• No areas of bleeding, ulceration, or abscesses	• Absence or discreet inflammatory infiltrate
		• Absence of bleeding, ulceration, and abscesses
1	• Moderate erythema and hyperemia	• Mild vascular hyperemia
	• No areas of bleeding, ulceration, or abscesses	• Areas of reepithelialization
		• Discreet inflammatory infiltrate
		• Prevalence of mononuclear infiltrates
		• Absence of bleeding, ulceration, and abscesses
2	• Severe erythema and hyperemia	• Moderate vascular hyperemia
	• Presence of areas of bleeding	• Areas of hydropic epithelial degeneration
	• Small ulcers or eschars	• Inflammatory infiltrate
	• No abscesses	• Prevalence of neutrophils
		• Areas of bleeding, edema
		• Occasional ulceration
		• Absence of abscesses
3	• Severe erythema and hyperemia	• Severe vascular hyperemia and vasodilatation
	• Presence of areas of bleeding	• Inflammatory infiltrate
	• Extensive ulcers	• Prevalence of neutrophils
	• Abscesses	• Areas of bleeding, edema
		• Extensive ulcers and abscesses

Redox state analysis

Sample preparation

The specimens were submitted to redox state evaluation protocols. For biochemical analysis, each buccal mucosa was individually homogenized in 10 volumes (1:10 *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, 1 mM EGTA, and 1 mM PMSF. Homogenates were centrifuged at 3000 rpm for 10 min at 4 °C to discard nuclei and cell debris. The pellet was discarded, and the supernatant was taken for biochemical assays.

Oxidant level measurement

Levels of ROS were measured fluorimetrically, following the dichlorofluorescin (DCFH) oxidation method [25]. Briefly, 50 μL of the diluted sample were incubated at 37 °C/30 min, in the dark, with the addition of 200 μL of H2DCF-DA. H2DCF-DA is cleaved by cellular esterases, and the DCFH formed is eventually oxidized by the reactive oxygen and nitrogen species present in the samples, producing a fluorescent compound, dichlorofluorescein (DCF). A standard curve of DCF (0.25–10 mM) was performed in parallel with the samples.

Antioxidant parameters

Reduced glutathione (GSH) concentration was determined fluorimetrically [26]. Supernatant was precipitated with meta-phosphoric acid (1:1, *v*/*v*) and centrifuged at 5000*g* for 10 min at 25 °C. GSH present in the supernatant reacts with the fluorophore o-phthaldialdehyde 7.5 mM prepared in 100 mM sodium phosphate buffer, pH 8.0, with 5 mM EDTA. The fluorescence was read in 350 nm and 420 nm, respectively, using the SpectraMax Gemini XS Fluorescence. Standard GSH curve ranging from 0.001 to 1 mM was prepared, and a blank sample was performed in parallel.

Glutathione peroxidase activity (GPx, EC 1.11.1.9) was determined according to Wendel [27], with modifications. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was subtracted from the overall reaction ratio.

Catalase (CAT) (EC 1.11.1.6) activity was evaluated by measuring the decrease of hydrogen peroxide (H_2O_2) at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer, pH 7.0.

Statistical analysis

The results were expressed as the mean and standard deviation of the mean. The data of clinical, histopathological, and redox

state were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The software used was GraphPad Prism 5 (GraphPad Software Inc., San Diego, California), and the level of significance was 5% ($p < 0.05$).

Results

PBM promotes the clinical reduction of OM

All animals developed OM on day 5. Figure 2 illustrates the clinical aspects of OM in all groups during the experimental period (8, 10, and 14 days). On day 8, the PC group presented higher scores of OM. PBM groups showed lower scores of OM; however, only EO 6 J/cm^2 presented a significantly lower degree compared to PC ($p < 0.05$). At day 10, all PBM groups showed improvement in OM compared to PC ($p < 0.01$). At day 14, PC maintained OM while PBM groups showed no more lesions ($p < 0.01$). No differences were observed among irradiated groups on days 10 and 14 ($p > 0.05$).

PBM accelerates reepithelization and resolution of inflammation in OM

The epithelial aspect and severity of inflammation among the experimental groups at different periods of time were evaluated with histopathological criteria (Fig. 3). On day 8, the PC group presented with the highest scores (2.8 ± 0.4) that differed significantly from all the irradiated groups ($p < 0.01$) (Fig. 3a and 3b). The PC group presented with a predominance of ulceration with moderate to severe vascular hyperemia and an inflammatory infiltrate with neutrophils in the areas of abscess, (scores 2 and 3) (Fig. 3c). All irradiated groups revealed accelerated OM healing showing similar results to each other ($p > 0.05$). They presented with a preponderance of reepithelialization, slight hyperemia, moderate to slight chronic inflammatory infiltrate, and an absence of ulceration and abscesses (score 1).

On day 10, some animals in the PC group still exhibited small ulcerations with chronic inflammatory infiltrate while other animals presented with reepithelization and slight inflammation (mean score 1.3 ± 0.5). All irradiated groups had a total reepithelization with an absence of inflammatory infiltrate, a predominance of new fibroblasts and appearance of immature skeletal muscle cells. The statistical analysis of mean scores showed no differences among the groups ($p > 0.05$). At day 14, all groups presented with complete OM lesions healing ($p > 0.05$) (Fig. 3a).

EO PBM reduces protein damage

The immunohistochemical staining with nitrotyrosine appeared strongly pronounced in the irradiated groups that received CT, on days 8 and 10 when compared with the NC group (Fig. 4a), indicating protein damage in all groups that received CT. On day 14, a significant reduction of nitrotyrosine was detected in the EO 6 J group achieving levels similar to the NC group ($p > 0.05$).

PBM modulates the redox state in OM

We analyzed the impact of different protocols of PBM in DCF and the antioxidant network (GPx, CAT, and GSH) during 5-FU induced OM (Fig. 4). DCF analyzes the intracellular generation of reactive species. On day 8, the PC group showed an increase of this biomarker compared to the NC group (without OM) ($p < 0.001$). PBM groups, in general, exhibited a reduced level of this biomarker compared to PC. However, EO 6 J/cm^2 and EO 12 J/cm^2 groups presented significantly less oxidative damage than the PC group as indicated by the lower level of DCF ($p < 0.001$, $p < 0.01$, respectively). In addition, these groups showed a similar level of this biomarker when compared to the NC group ($p > 0.05$). On days 10 and 14, there were no differences in DCF levels as detected among all groups ($p > 0.05$) (Fig. 4b).

GPx is part of the enzymatic defense system that attempts to control the occurrence of oxidative damage, in order to balance the production of ROS, by converting H_2O_2 to H_2O as a form of cellular adaptation and protection [28]. Our results demonstrated that on day 8 some differences existed among the groups. The PC group presented a lower level of this antioxidant enzyme activity compared to NC ($p < 0.001$). All PBM groups demonstrated higher levels of GPx compared to PC. Furthermore, IO 6 J/cm^2 and EO 6 J/cm^2 showed similar GPx level to NC ($p > 0.05$). On days 10 and 14, no differences were detected ($p > 0.05$) (Fig. 4c).

CAT and GSH results are presented in Fig. 4d, e, respectively. No difference among groups on days 8, 10, and 14 were observed for both antioxidants.

Discussion

PBM has been recommended for the prevention and treatment of OM [3, 18]. Studies have been performed with IO diode lasers devices applied directly to the oral mucosa [29, 30]. However, some other forms of phototherapy, such as an LED EO diode laser [19, 20] or an IO defocused high-power diode laser, have shown satisfactory results [29, 31, 32]. In the

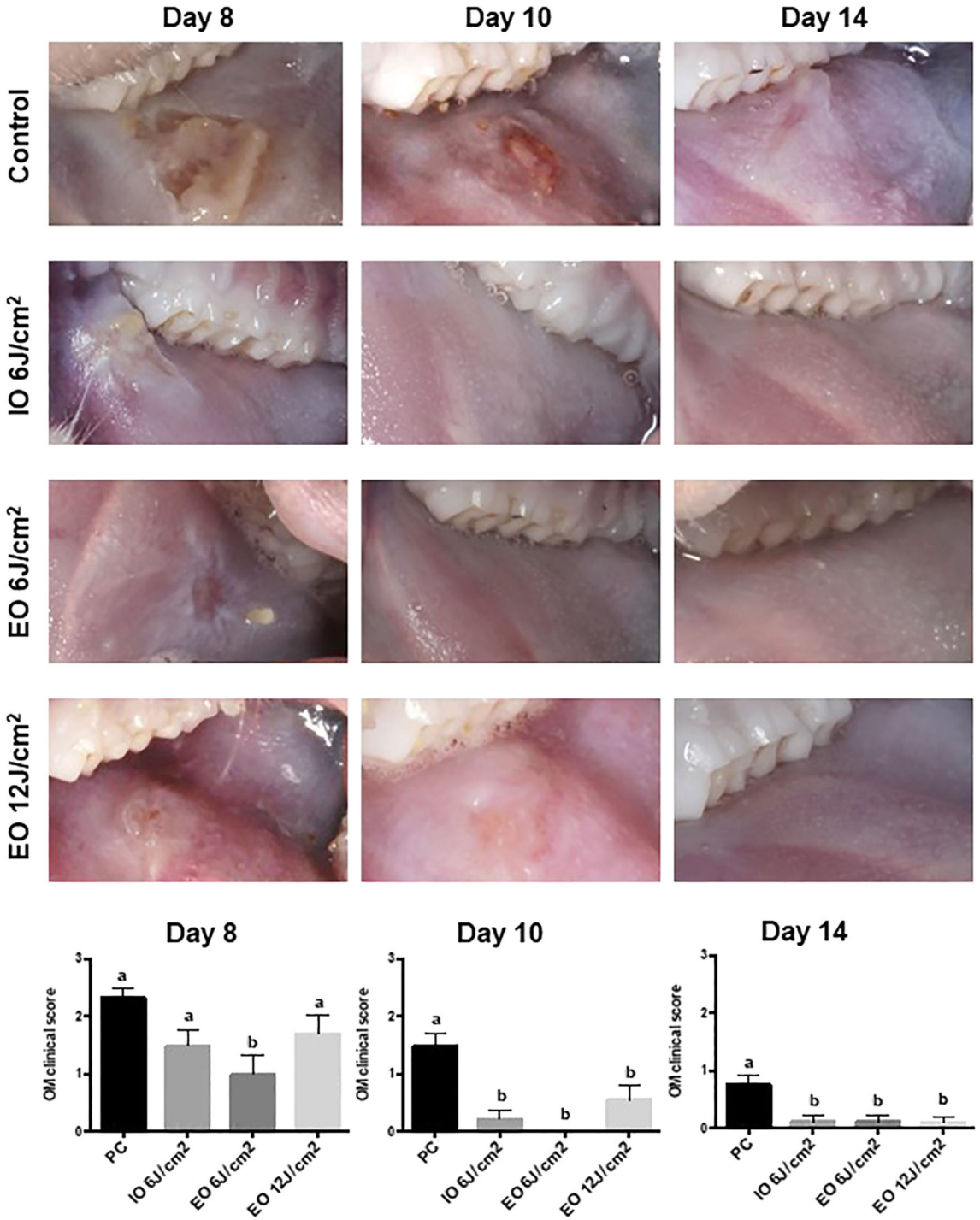

Fig. 2 Clinical aspects of OM in all groups during the experimental period. Clinical analysis expressed by mean and standard deviation. On day 8, EO 6 J/cm^2 showed lower clinical scores compared to other groups ($p < 0.05$). On days 10 and 14, all irradiated groups presented better clinical response compared to PC. Different lowercase letters ("a" and "b") in columns (intergroup analysis) denote significant difference ($p < 0.05$)

present study, we evaluated the effect of IO and EO diode lasers on 5-FU-induced OM in rats. Our results have demonstrated that all laser protocols promoted OM reduction. However, the clinical positive effects of EO 6 J/cm^2 occurred earlier compared to other laser parameters tested. The clinical improvement of OM lesions with different protocols of irradiation was associated with a decrease of the inflammatory process and faster reepithelization. In parallel, PBM altered the redox state regulated by reactive species and GPx antioxidant enzyme activity.

a

	PC	IO 6J/cm²	EO 6J/cm²	EO 12J/cm²
Day 8	2.8 ± 0.4^{A}	1.2 ± 0.5^{B}	1.0 ± 0.6^{B}	1.5 ± 0.5^{B}
Day 10	1.3 ± 0.5^{A}	0.0 ± 0.0^{A}	0.3 ± 0.5^{A}	0.6 ± 0.5^{A}
Day 14	0.6 ± 0.5^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}

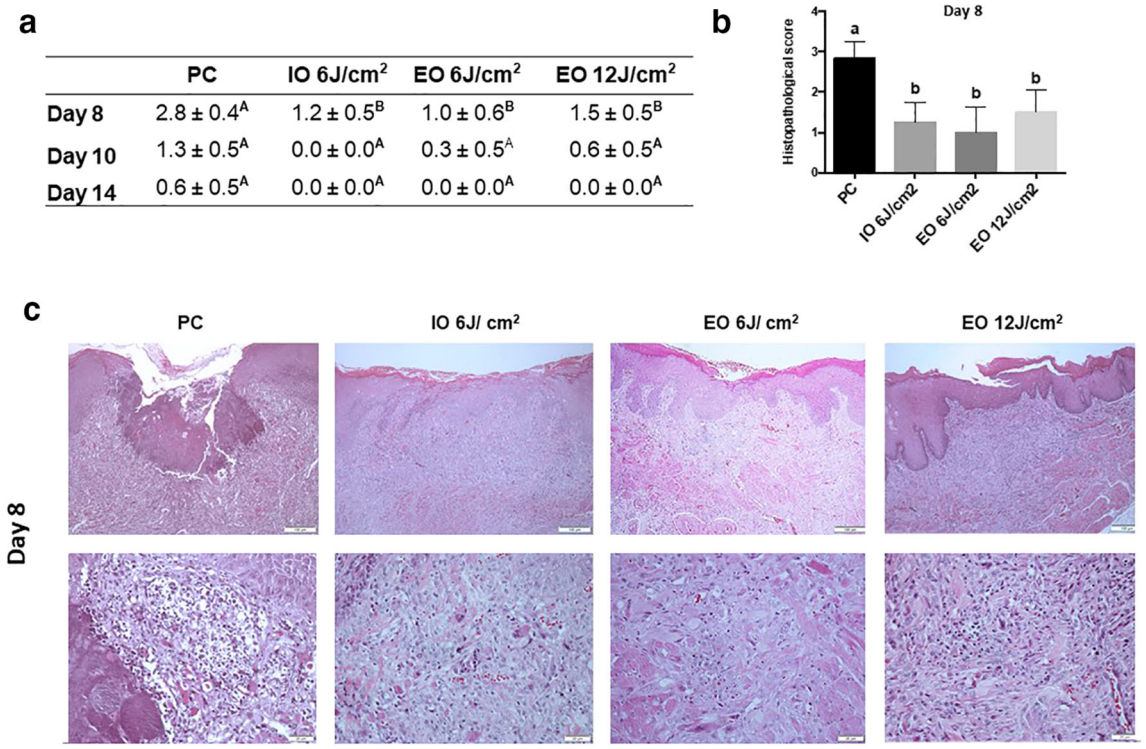

Fig. 3 Histopathological evaluation of OM healing. **a** Mean and standard deviation observed in all experimental groups on days 8, 10, and 14. (**a**) Different uppercase letters ("A" and "B") on lines (intergroup analysis) denote significant difference ($p < 0.05$). (**b**) On day 8, all irradiated groups revealed accelerated OM healing compared to PC. (**c**) Photomicrographs of experimental groups on day 8. PC exhibited ulceration and neutrophils inflammatory infiltrate. All irradiated groups showed reepithelization and slight/moderate chronic inflammatory infiltrate (HE, original magnification, × 100 and × 400)

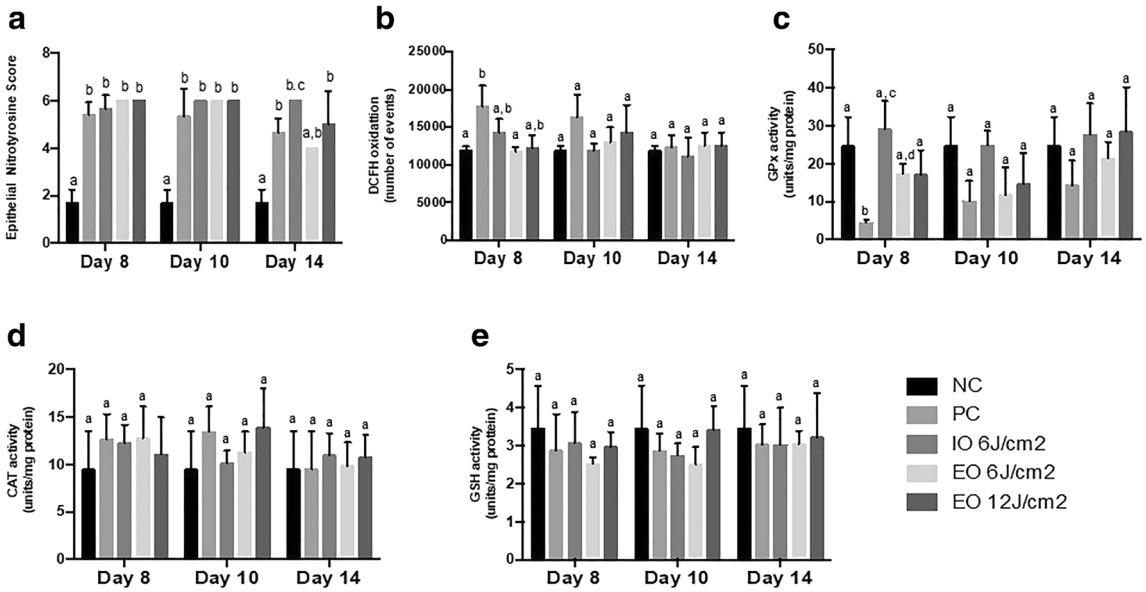

Fig. 4 Oxidative damage biomarker (DCFH) and antioxidant activities in all experimental groups. (**a**) Mean of nitrotyrosine epithelial score observed in all experimental groups on days 8, 10, and 14. Different lowercase letters ("a," "b," and "c") in bars (intergroup analysis) denote significant difference ($p < 0.05$). (**b**) DCFH analysis (nmol DCF/mg protein). On day 8, intracellular ROS generation (DCFH level) was increased in PC and reduced in irradiated groups. (**c**) GPx antioxidant enzyme activity (units/mg protein). A reduction of GPx was detected in PC and irradiated groups presented an increase in this protective enzyme. (**d**) CAT antioxidant enzyme level (units/mg protein) showed no difference among groups. (**e**) GSH levels (nmol GSH/mg protein) showed no variation among groups in each period of time evaluated. Different lowercase letters ("a," "b," "c," and "d") in columns (intergroup analysis) denote significant difference ($p < 0.05$)

We found positive effects from all tested laser protocols compared to the control group. In general, laser groups exhibited accelerated OM healing compared to the PC group in clinical (days 8, 10, and 14) and histopathological analysis (day 8). These results seem to have occurred via stimulation of epithelial migration and proliferation that quickly covered the wounds. Regarding the inflammatory process, laser irradiation promoted a decrease in the inflammatory response generating a reduction in the amplification and ulcerative phases of OM. Also, the animals in the laser group enhanced the healing phase characterized by the increase of fibroblast proliferation and collagen deposition. Similar effects have been reported in the literature following different diode laser protocols [12, 21, 29, 30, 33, 34]. Unfortunately, wide ranges of laser protocols for OM presenting variation in wavelength, irradiance, power output, energy density, energy, and mode of application have been described in the literature. Most evidence from animals and clinical studies recommends a wavelength between 633 and 685 nm or 780–830 nm, power output between 10 and 150 mW, and energy density of 2–3 J/cm^2 and no more than 6 J/cm^2 but the literature does not exclude the efficacy of other settings. A few studies were performed using higher energies and IO, but they had controversial results and some methodological limitations [29, 31]. Ottaviani et al. [31] reported that a 970 nm diode laser with a 2.5 mW in continuous wave and 50% duty cycle at 5 W, 30 s, spot size diameter of 0.5 cm, and an energy density of 375 J/cm^2 presented positive biostimulating and anti-inflammatory effects in a mouse model of OM induced by CT. Campos et al. [29] compared a low-level laser, LED, and high-power laser in a defocused mode with a wavelength of 808 nm, delivered through a 400 μm optical fiber with 1.0-W output power, applied in continuous-wave mode (irradiance of 1 W/cm^2) for 10 s in scanning movements. According to the protocols, the low-level laser and LED therapies generated better results than a high-power laser.

In the present study, we decided to use the IO 6 J/cm^2 protocol, 660-nm diode laser, continuous, 100 mW, 2500 mW/cm^2, 0.04 cm^2 spot size, during 2.4 s resulting in 0.24 J per point as a "gold standard" PBM protocol for OM [12, 21, 22]. Also, we tested two protocols of EO irradiation using 6 and 12 J/cm^2. In comparison, the EO diode protocol utilized a higher pulsed wavelength (810 nm + 980 nm), lower irradiance (407 mW/cm^2), higher output power (2000 mW), spot size (4.91), and energy (20 J and 60 J). EO 6 J/cm^2 was the only group, on day 8, that showed better clinical results compared to the PC group. On day 10, all laser protocols were similar and superior to PC indicating that even protocols with higher energy and lower irradiance applied extraorally can promote faster healing of OM lesions in an animal model. The effect of laser irradiation with different parameters has been explained based on the biphasic dose model (Arndt–Schultz curve) where a low dose of irradiation stimulates, while higher doses inhibit healing. Considering this information, it is not yet known exactly what is considered a high dose and low dose for OM and we believe that our protocols meet the range that promotes positive results. According to Huang et al. [10], most articles considered energy or fluency as an important descriptor of PBM dose, but neglect other important aspects such as wavelength, irradiance, pulse structure, coherence, polarization, and irradiation time. Thus, there is a consensus that remains a need to identify optimal PBM parameters for OM [35].

The investigations of the mechanisms involved in the pathobiology of OM have shown the important role of redox state, focused on OS, as a triggering factor. RT and CT promoted an increase of ROS that will cause an imbalance between the oxidative challenge and the antioxidant defense capacity of an organism, resulting in OS [4, 5, 36]. In the OM model, the initiation phase occurs when animals are exposed to chemotherapeutic drugs, such 5-FU, resulting in the production of a large number of reactive species such as superoxide, H_2O_2, and nitric oxide by epithelial and mucosal cells followed by installation of OS [37]. In the present study, we detected on day 8 in PC an increase of DCFH oxidation, which is a marker of intracellular concentration of reactive species, mainly ROS, when compared to the NC group. It demonstrated that CT infusion (days 0 and 2) and scarification (days 3 and 4) promoted an increase of ROS, which remained until the eighth day after the initiation of OM induction protocol. Concomitantly, at clinical evaluation, all PC animals presented with ulceration. In contrast, at the same time, all irradiated groups showed lower levels of DCFH with EO 6 J/cm^2 and EO 12 J/cm^2 presenting similar levels to the NC, demonstrating less oxidative damage. EO 6 J/cm^2 clinically exhibited biostimulatory results on day 8 with less severe OM compared to the PC group. In parallel, we examined some of the antioxidant network, evaluating the activity of GPx and CAT, as well as the levels of the most important non-enzymatic cellular antioxidant, GSH. On day 8, a decreased activity of GPx was observed in the PC group, suggesting that there was a reduced antioxidant response to H_2O_2. However, in all PBM groups, this cellular adaptation occurred as evidenced by higher levels of GPx. GSH and CAT were not altered in either treatment group. These antioxidants can be produced and consumed at an early stage of oxidative and after 8 days, they may no longer be active. Collectively our results indicated that laser irradiation protocols modulate redox state in OM promoting a better clinical response.

Recent studies have suggested that ROS is a key molecular circuitry that is activated during laser irradiation and promotes tissue stimulation by increasing the "good" ROS. It is able to activate redox-sensitive signal transduction pathways such as Nrf-2, NF-κB, and ERK which act as key redox checkpoints and signaling pathways [9, 38–40]. In addition, Rupel et al. [41] demonstrated that ROS production is regulated by the wavelength used. They showed that 660-nm laser light increased ROS production when applied either before or after

an oxidative stimulus. Instead, near-infrared 970-nm lasers presented a moderate antioxidant activity. The 800 nm or the combination of the three wavelengths exhibited the most marked reduction in the levels of ROS, suggesting that a multiwavelength PBM protocol could represent a promising treatment. In the present study, we used dual wavelength (810 nm + 980 nm) in the EO protocols and our results suggest that the lower energy group (EO 6 J/cm^2) showed acceleration of clinical healing of the OM lesions associated with decreased DCF oxidation, indicating a reduction in reactive species levels, and higher GPx levels. In addition, the potential benefits of oral EO PBM with double and higher wavelengths include rapid, simple, non-IO administration that may be more feasible in young children, patients with a limited oral opening, patients in severe pain, as well as accomplishing treatment in structures that have a lower depth of penetration, such as in oropharyngeal mucositis [17, 19].

In conclusion, an EO diode laser protocol, as well as traditional IO diode laser irradiation, exhibited positive effects on the clinical, histopathological, and redox state in OM induced by 5-FU in rats. In this preclinical study, among the EO protocols, EO 6 J/cm^2 presented the most encouraging results. Since dosimetry is highly complex and an EO with diode laser was rarely studied until now, new studies involving different parameters and their effects in other cellular mechanisms should be performed.

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Compliance with ethical standards

This study was approved by the Ethics Committee on Animal Use (CEUA, 2018-0096).

Conflict of interest The authors declare that they have no conflict of interest.

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