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ORIGINAL ARTICLE

Laser treatment contributes to maintain membrane integrity in stem cells from human exfoliated deciduous teeth (shed) under nutritional deficit

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Abstract

This study aimed to analyze the effects of laser irradiation on the membrane integrity and viability of stem cells from human exfoliated deciduous teeth (SHED) that were kept in serum starvation. Nutritional deficit was used to mimic the cellular stress conditions of SHED isolation for regenerative dental approaches, where laser therapy could be beneficial. SHED were cultured under serum starvation (MEMα + 1%FBS) for 1 or 24 h pre-irradiation (protocols A and B, respectively). Then, cells received low-level laser therapy (LLLT; 660 nm) at 2.5 J/cm^2 (0.10 W; groups I and V), 5.0 J/cm^2 (0.20 W; groups II and VI), 7.5 J/cm^2 (0.30 W; groups III and VII), or remained non-irradiated (groups IV and VIII). During irradiation, cells were maintained in 1% FBS (groups I–IV) or 10% FBS (normal culture conditions; groups V–VIII). Membrane integrity was evaluated by quantifying lactate dehydrogenase (LDH) release (immediately after irradiation), and cell viability was assessed by the MTT assay (24, 48, and 72 h post-irradiation). Serum starvation did not alter LDH release by non-irradiated SHED, while LDH release decreased significantly in groups irradiated in 1% FBS (I and III), but not in groups irradiated in 10% FBS (V–VII), regardless the pre-irradiation conditions (protocols A/B). Cell viability was significantly higher 24 h after irradiation, in most protocol A groups. In contrast, cell viability remained mostly unaltered in protocol B groups. LLLT contributed to maintain membrane integrity in SHED subjected to nutritional deficit before and during irradiation with 0.10 or 0.30 W. Short serum starvation before irradiation improved SHED viability at 24 h post-irradiation.

Keywords Cell culture techniques · Cell survival · L-lactate dehydrogenase · Low-level light therapy · Stem cells

Introduction

Photobiomodulation therapy (PBMT) is the direct application of light to stimulate cell responses capable of inducing analgesia, reducing inflammation, and promoting tissue healing [1, 2]. Under stress conditions, several cell types seem to be more sensitive to the effects of PBMT, having their regenerative potential further stimulated by this therapy [3]. Therefore, in vitro models have tested the efficacy of PBMT by laser irradiation after nutritional deficit by serum starvation, which induces cellular stress, simulating clinical conditions in which a pH reduction occurs, such as tissue inflammation [3–6]. However, variations in the protocols of nutritional deficit and the parameters of laser irradiation have hindered the comparison of studies on the effect of PBMT in combination with cellular stress [3–5]. Thus, the standardization of in vitro assay

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conditions may clarify the potential of PBMT to treat conditions where cellular stress is involved [7].

The current alternatives to treat irreversibly inflamed or necrotic pulp tissue involve endodontic treatment and prosthetic reconstruction, which may present a limited lifespan, thus requiring replacement over time. Therefore, dentistry has sought therapeutic alternatives to conventional treatments for the healing of inflamed tissues or the replacement of necrotic pulps [8]. Tissue engineering may offer an alternative to conventional rehabilitative treatment of stomatognathic system structures, by regenerating injured tissues through the differentiation of stem cells into specialized cell lines [6–12]. Stem cells from exfoliated deciduous teeth (SHED) have high rates of cell proliferation, differentiation, and growth factor secretion [8]. If properly stimulated, SHED are able to differentiate into odontoblasts and endothelial cells [7–12], representing useful candidates for dental tissue regeneration. Although mesenchymal cells from various sources have been subjected to laser irradiation to stimulate their regenerative potential [8–12], the effect of PBMT on SHED has been poorly studied [6, 10, 12, 13]. In particular, the assessment of SHED viability after laser irradiation under different culture conditions has been limited [6].

In this study, we tested the effect of low-level laser therapy (LLLT) on cultured SHED that had been subjected to serum starvation in vitro, to mimic the stress conditions found when SHED are isolated from inflamed dental pulp for regenerative purposes. We analyzed the effect of different doses of LLLT on the cell membrane integrity and viability (metabolic activity) of SHED, to help define optimal conditions for laser therapy that would improve the potential of SHED for dental tissue regeneration.

Methods

Cell culture

SHED, kindly provided by Dr. Bruno N. Cavalcanti (DDS, MSc, PhD; Institute of Science and Technology, São Paulo State University, São José dos Campos, SP, Brazil), were isolated by a standard enzymatic digestion protocol and characterized according to Miura et al. [8]. All procedures involving human samples were approved by the Institutional Review Board (protocol no. 46420). Cells were maintained in Eagle's minimum essential medium alpha modification (MEMα; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Invitrogen, Grand Island, NY, USA) and 1% penicillin and streptomycin solution (penicillin–streptomycin, Gibco, Invitrogen) at 37 °C and 5% CO_2 in a high-humidity incubator, and split at a ratio of 1:3 when they reached 80% confluence. The culture medium was changed every 2 days. SHED at passages 10 to 15 were used in all experiments.

Nutritional deficit and low-level laser therapy conditions

SHED were seeded in 96-well plates (1×10^4 cells per well) with MEMα supplemented with 10% FBS and allowed to attach overnight. Then, cells were subjected to different experimental conditions, as described in Table 1. Briefly, the medium in all wells was replaced by fresh culture medium supplemented with 1% FBS (serum starvation) for 1 h (protocol A) or 24 h (protocol B) before irradiation, and then the cells were subjected to LLLT at the doses of 2.5 J/cm^2 (groups I and V), 5 J/cm^2 (groups II and VI) or 7.5 J/cm^2 (groups III and VII), or remained non-irradiated (groups IV and VIII). Laser irradiation was carried out for 10 s in the dark, using an indium-gallium-aluminum phosphide (InGaAlP) red laser (660 nm: MMOptics, São Carlos, São Paulo, Brazil) [14–16]. The irradiation was punctual and in contact with the bottom of the wells, as described previously [10–16]. For groups I to IV, cells were kept under nutritional deficit (1% FBS) during irradiation and for the remaining time of the experimental period (24, 48, or 72 h). For groups V to VIII, cells returned to regular nutritional conditions (10% FBS) immediately before irradiation and maintained in this medium for the remaining time of the experimental period. In each experiment, all conditions were performed in triplicate, and three independent experiments were performed.

Extracellular lactate dehydrogenase assay

The integrity of the cell membrane after irradiation was estimated by quantifying the extracellular lactate dehydrogenase (LDH) levels [17], using the LDH UV Kit (Bioclin, Belo Horizonte, Minas Gerais, Brazil), and the results were expressed in units per liter.

Briefly, 20 μL of culture medium were retrieved from each well (corresponding to a total of 60 μL per group) immediately after nutritional deficit and laser irradiation, and each supernatant sample was added to 1.0 mL of working reagent, transferred to a pre-warmed cuvette at 37 °C, and then incubated for 1 min at 37 °C. Then, the absorbance at 340 nm was read in a Zenyth 200rt microplate reader (Anthos), after 1, 2, and 3 min, and the values were used to calculate the LLD levels, according to the formula: LLD (U/L) = ΔA/min. × 8016, where ΔA/min represents the mean differences in absorbance per minute.

MTT assay

SHED viability in the different experimental groups was evaluated using the methyltetrazolium (MTT) assay, performed

Table 1 Experimental groups divided according to the low-intensity laser irradiation parameters and the fetal bovine serum (FBS) concentration in the culture medium used at the time of SHED irradiation and over the experimental period

Group	FBS concentration (%) during irradiation	Energy density (J/cm^2)	Power (mW)	Time (s)	Total energy (J)
I	1	2.5	10	10	0.10
II		5.0	20	10	0.20
III		7.5	30	10	0.30
IV		Non-irradiated	____	____	____
V	10	2.5	10	10	0.10
VI		5.0	20	10	0.20
VII		7.5	30	10	0.30
VIII		Non-irradiated	____	____	____

24, 48, and 72 h after irradiation. At the end of the respective incubation period, the supernatants were discarded and cells were washed with phosphate-buffered saline (PBS), and 110 μl of MTT solution was added to each well, for a final concentration of 0.5 mg ml^{-1}. After incubation for an additional 4-h period at room temperature, the supernatant was discarded and 200 μl dimethyl sulfoxide (DMSO; Fisher Scientific, Hampton, NH, USA) was added to each well, to solubilize the formazan crystals [14]. Then, the absorbance was read in a Zenyth 200rt microplate reader (Anthos) at 570 nm.

Statistical analysis

Data were analyzed by two-way ANOVA and Tukey's post hoc test ($P < 0.05$), using the R statistical software.

Results

Serum-starved SHED have reduced cell membrane damage after laser treatment.

Serum starvation has been used efficiently to evaluate the effects of laser irradiation on cellular metabolism [6, 14, 18–20]. The concentration of serum in the culture medium that is considered nutritional deficiency can vary between cell types. As described by Souza et al. [6], different studies employed culture medium supplementation with 0.5% FBS [21], 2.5% FBS [22], 5% FBS [3, 13, 15, 16, 18, 20, 23, 24] and even 10% FBS [19] for the purpose of establishing nutritional deficit, although in different cell types from the one used here. The establishment of nutritional deficit prior to SHED irradiation using culture medium supplemented with 1% FBS has been used routinely by our research group [6–10].

In this study, we evaluated two measures of cell viability—cell membrane permeability and cell metabolism—to produce an overall picture of the effect of laser treatment combined with serum starvation on SHED viability. Cells were subjected to different protocols of serum starvation (1 or 24 h of starvation before irradiation, corresponding to protocols A and B, respectively) and, then, were subjected to LLLT at doses of 0.10 W (2.5 J/cm^2), 0.20 W (5 J/cm^2), or 0.30 W (7.5 J/cm^2). These irradiation doses and energy levels were chosen based on data from previous studies [10, 14, 15].

LDH is a cytoplasmatic enzyme present in essentially all human body tissues, and extracellular LDH is released after cell damage or death [25, 26] caused by ischemia, excess heat or cold, starvation, dehydration, injury, exposure to bacterial toxins, the ingestion of certain drugs, and from chemical poisonings [25]. Since LDH release serves as indicator of disturbances of in-cell membrane integrity induced by pathological conditions [25–27], we evaluate this parameter in the culture medium to verify if the nutritional deficit (starvation) for 1 or 24 h followed by laser irradiation had deleterious effects on SHED membrane integrity. The levels of LDH released in the culture medium after treatment were used to estimate changes in cell membrane permeability. Extracellular LDH levels in SHED culture medium were similar in the groups subjected to starvation for 1 h (protocol A; Fig. 1) or 24 h (protocol B; Fig. 2) before LLLT, at the same energy densities and FBS concentrations during irradiation.

Using protocol A (1 h serum starvation before irradiation), LDH levels were significantly lower ($p < 0.05$) in the supernatant of cells irradiated with 2.5 and 7.5 J/cm^2 LLLT that had been kept in medium containing 1% FBS during irradiation (groups I and III, respectively), compared with a non-irradiated control (group IV) (Fig. 1). There were no significant differences between the groups maintained in 10% FBS during irradiation (groups V to VII) and the non-irradiated group maintained in the same nutritional condition (positive control; group VIII) (Fig. 1). When comparing the groups irradiated with the same energy density, the cells treated with 7.5 J/cm^2 under serum starvation released less LDH than those irradiated in 10% FBS medium (group III) (Fig. 1).

In protocol B (24 h serum starvation before irradiation), significantly lower extracellular LDH levels were found in

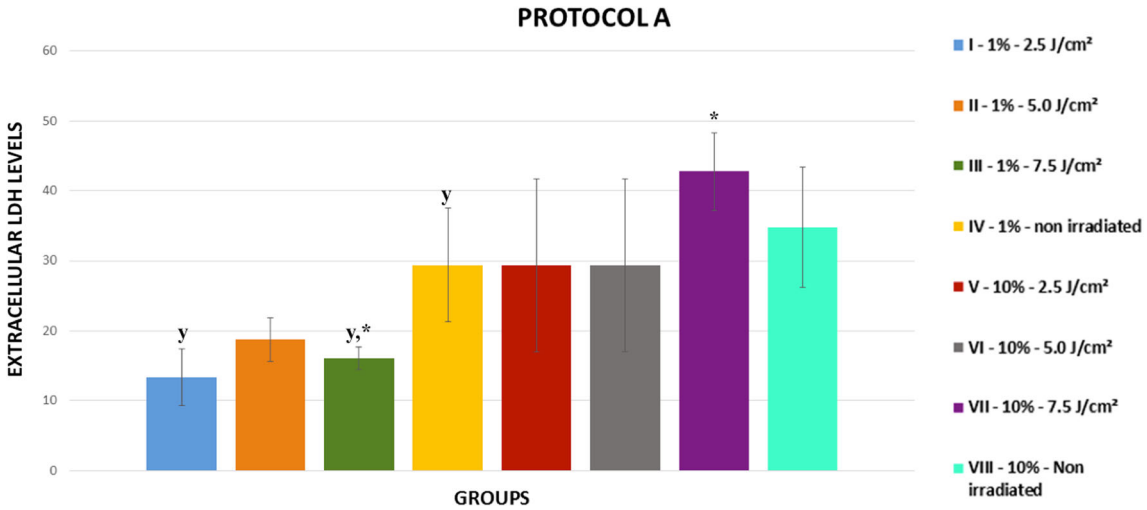

Fig. 1 Lactic dehydrogenase (LDH) in the culture medium of SHED kept under nutritional deficit (1% FBS) for 1 h prior to laser irradiation (protocol A). Groups I–III were irradiated in medium containing 1% FBS, while groups V–VII were irradiated in medium containing 10% FBS. Groups IV and VIII were non-irradiated controls (kept in medium with 1 and 10% FBS, respectively). The lower case letter (y) indicates statistically significant differences relative to the control (non-irradiated) group in the same nutritional condition, and the asterisk symbol indicates statistically significant differences relative to the group irradiated with the same dosimetry, but maintained in a different nutritional condition (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$)

all cultures irradiated in 1% FBS (groups I to III) in comparison with the corresponding non-irradiated group (group IV) (Fig. 2). LDH release was significantly higher in the group treated with the highest LLLT dose tested while in 10% FBS medium (group VII), when compared with all other groups irradiated in 10% FBS (groups V to VIII) (Fig. 2). When comparing groups irradiated with the same energy density, cells maintained in culture medium supplemented with 1% FBS released less LDH than those maintained in 10% FBS during irradiation (Fig. 2).

Brief serum starvation before laser treatment generates a temporary improvement in cell viability after laser treatment.

We evaluated the effect of laser treatment on the viability of serum-starved SHED using the MTT assay (which assesses the metabolic activity of cells) up to 3 days post-irradiation (24, 48, and 72 h). Most statistically significant changes in cell viability were observed at 24 h post-irradiation, between groups kept in serum starvation for 1 h prior to irradiation

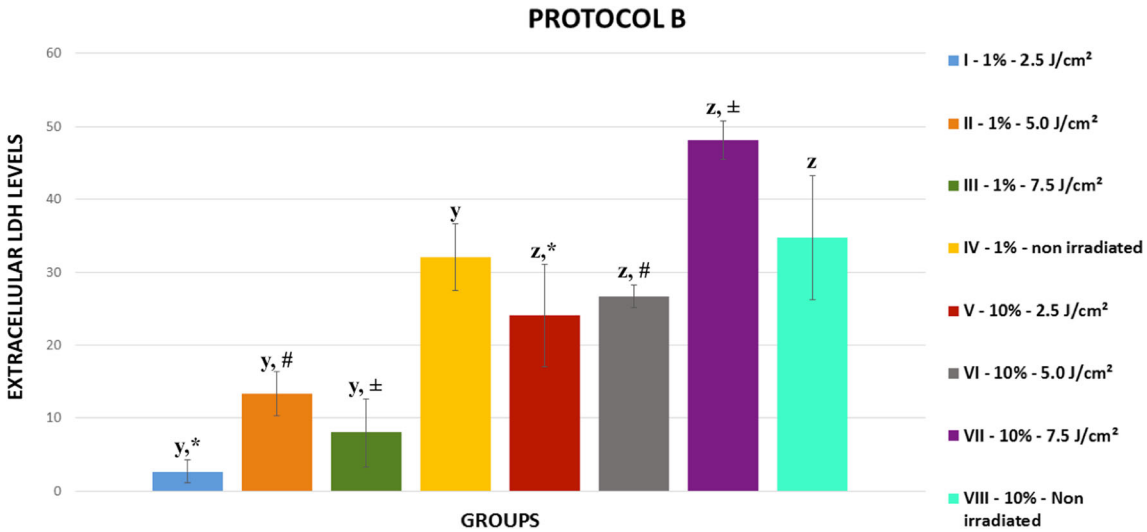

Fig. 2 Lactic dehydrogenase (LDH) levels in the culture medium of SHED kept under nutritional deficit (1% FBS; groups I–IV) for 24 h prior to laser irradiation (protocol B). Groups I–III were irradiated in medium containing 1% FBS, while groups V–VII were irradiated in medium containing 10% FBS. Groups IV and VIII were non-irradiated controls (kept in medium with 1 and 10% FBS, respectively). Lower case letters (y, z) indicate statistically significant differences relative to the control (non-irradiated) group in the same nutritional condition, and the asterisk, number sign, and plus-minus sign symbols indicate statistically significant differences relative to the group irradiated with the same dosimetry, but maintained in a different nutritional condition (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$)

(protocol A). Using this protocol, the viability (MTT absorbance) of cells irradiated with 7.5 J/cm^2 in normal nutritional conditions (group VII) was similar to that of group VIII (positive control), and both were significantly higher than that of group III (7.5 J/cm^2 under serum starvation), 24 h after irradiation (Fig. 3). In contrast, for protocol B, there were no statistically significant differences in cell viability between the groups, at 24 h post-irradiation (Fig. 4). Interestingly, cell viability was higher in groups IV through VIII from protocol A than in the corresponding groups from protocol B, at 24 h post-irradiation.

At later time-points post-irradiation (48 and 72 h), statistically significant differences in cell viability were only observed for protocol A at 72 h, when the cell viability was significantly higher in group VIII (positive control) than in group II (Fig. 3). No statistically significant differences in cell viability between groups were detected at 48 h post-irradiation, irrespective of the protocol. In addition, at 48 h post-irradiation, there were no significant differences in cell viability between corresponding groups from protocols A and B.

When comparing cell viability in protocol A groups over time, cells were significantly more viable at 24 h than at 48 and 72 h in all groups, except for group II, where a significant decrease in viability was only observed at 72 h post-irradiation. In protocol B groups, cells were significantly more viable at 24 h than at 72 h in groups I and III, while cell viability in group II did not vary between 24 and 48 h post-irradiation, but decreased significantly between 48 and 72 h. In the remaining groups (IV to VIII), cell viability did not vary significantly over time.

Discussion

Several studies have evaluated the effects of PBMT on different cell types and processes of cellular repair, analgesia, epithelization, and protein synthesis, through in vitro [18, 19, 22, 28] and in vivo assays [29, 30], and in clinical reports [31]. However, the effect of PBMT on SHED has been poorly studied. Here, we evaluated the effects of laser treatment on SHED under different nutritional conditions and produced an overall picture of the combined effects of laser treatment and nutritional deficit on SHED viability, an essential property to be maintained if cells are to be used in dental tissue regeneration.

The analysis of LDH release to the medium—an indicator of cell membrane permeability—suggested that, in the absence of laser irradiation (groups IV and VIII—negative and positive controls, respectively), SHED released similar amounts of LDH when cultured in medium supplemented with either 1% FBS or 10% FBS, as well as when subjected to 1 or 24 h of nutritional deficit prior to irradiation (protocols A or B, respectively). These results suggest that nutritional deficit did not alter the cell membrane permeability of SHED, in the experimental period tested here. Importantly, our results indicate that serum starvation protects the cell membrane against damage, as SHED cultured in medium supplemented with 1% FBS during irradiation released less LDH after laser treatment, compared with the non-irradiated control, and this effect was not apparent in cells irradiated in medium containing 10% FBS, irrespective of the period of cellular stress prior to irradiation (protocols A or B). Also, when comparing the groups irradiated with the same energy

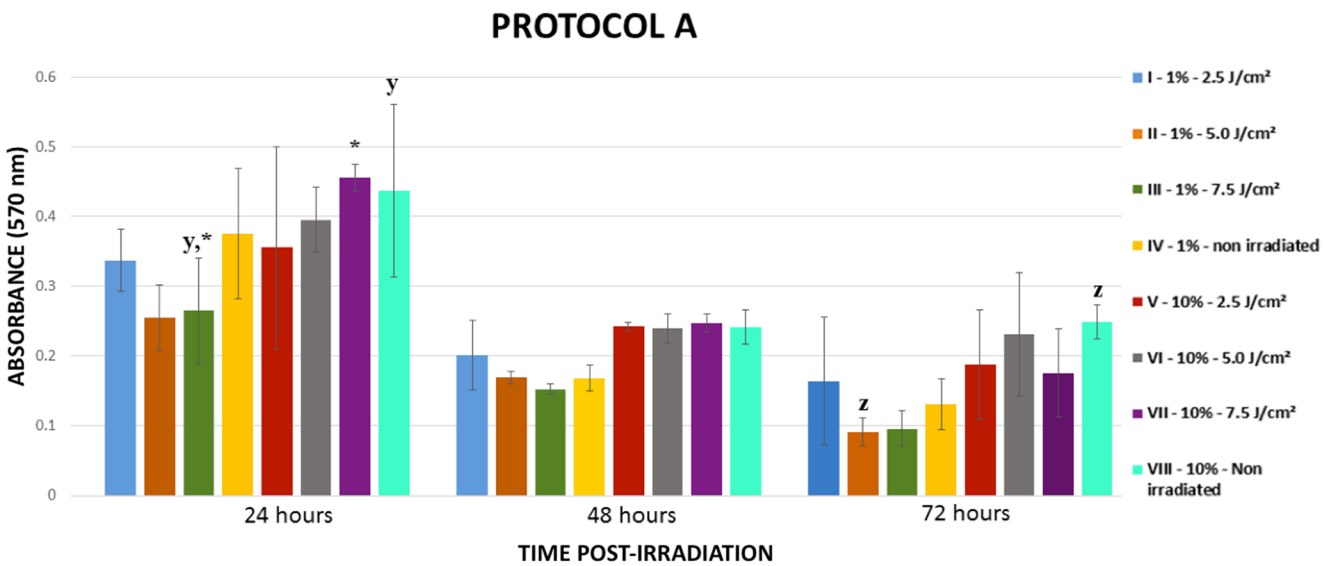

Fig. 3 Viability of SHED cultured under nutritional deficit (1% FBS) for 1 h prior to laser irradiation (protocol A). Groups I–III were irradiated in medium containing 1% FBS, while groups V–VII were irradiated in medium containing 10% FBS. Groups IV and VIII were non-irradiated controls (kept in medium with 1 and 10% FBS, respectively). Cell viability was estimated using the MTT assay, at 24, 48, and 72 h post-irradiation. For each time-point, lower case letters (y, z) indicate statistically significant differences relative to the control (non-irradiated) group in the same nutritional condition, and the asterisk symbol indicates statistically significant differences relative to the group irradiated with the same dosimetry, but maintained in a different nutritional condition (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$)

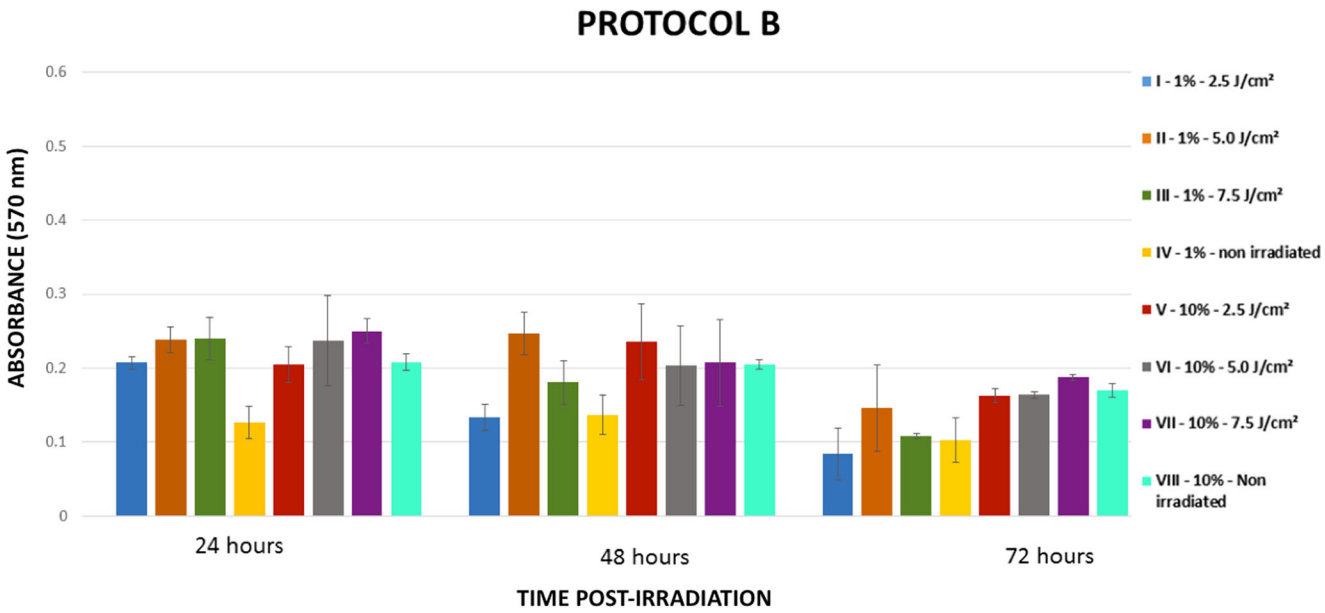

Fig. 4 Viability of SHED cultured under nutritional deficit (1% FBS) for 24 h prior to laser irradiation (protocol B). Groups I–III were irradiated in medium containing 1% FBS, while groups V–VII were irradiated in medium containing 10% FBS. Groups IV and VIII were non-irradiated controls (kept in medium with 1 and 10% FBS, respectively). Cell viability was estimated using the MTT assay, at 24, 48, and 72 h post-irradiation. No statistically significant differences were observed between groups (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$)

density, LDH release was significantly higher when SHED were irradiated with 7.5 J/cm^2 in medium containing 10% FBS than in medium containing 1% FBS, for protocol A, reinforcing the protective effect of nutritional deficit on SHED membrane integrity. This effect was more pronounced when cells were subjected to nutritional deficit for a longer period (24 h) prior to irradiation (protocol B), as irradiation in medium with 1% FBS decreased LDH release compared with 10% FBS, for all three energy densities tested. Similarly, Silva et al. [32] observed that both red (685 nm) and infrared (830 nm) lasers inhibited of LDH released by myoblasts (C2C12) exposed to snake venom, suggesting that laser treatment protects C2C12 cells against the cytotoxic effects of snake venom.

With regard to the viability by MTT, short serum starvation temporarily increased viability (24 h), further decreasing at 48 and 72 h. For cells serum-starved 1 h prior to irradiation (protocol A), SHED viability was higher at 24 h, irrespectively of the culture conditions and laser energy density employed. This effect was temporary, as the viability decreased at 48 and 72 h, particularly for the cells kept in serum starvation. For protocol B, SHED viability was higher at 24 h than at 72 h only for cells cultured in medium supplemented with 1% FBS after irradiation, while no alterations in cell viability over time were found in cells cultured in medium supplemented with 10% FBS after irradiation.

When comparing groups irradiated with the same energy density, we found that SHED were significantly more viable when irradiated with 7.5 J/cm^2 and cultured in medium containing 10% FBS (group VII) than 1% FBS (group III), at 24 h, and for protocol A. For the same groups, LDH release was significantly higher in group VII than in group III, indicating a disruption in membrane integrity when the cells were irradiated with 7.5 J/cm^2 and cultured in medium containing 10% FBS. Our hypothesis is that, although laser irradiation might have a protective effect on SHED membrane integrity when cells are cultured in medium supplemented with 1% FBS during irradiation, cells were less responsive to the effects of PBMT when the nutritional deficit was established only 1 h prior to irradiation. Therefore, although membrane integrity was lower in group VII than in group III, cells from group VII were metabolically more active 24 h after irradiation (in group A), while those from group III were comparatively more quiescent.

Differently from what was reported by Fernandes et al. [10], we did not observe significant differences in the viability of SHED subjected to 1-h starvation and irradiated with 5.0 J/cm^2 in culture medium supplemented with 10% FBS, in comparison with those irradiated with 2.5 J/cm^2 or with non-irradiated cells. Comparisons with data reported in other studies were impaired due to divergences in the parameters of laser irradiation and the protocols of nutritional deficit employed, reinforcing the need to establish standardized parameters and protocols, to clarify the effects of PBMT on SHED.

Conclusion

Red laser irradiation was able to maintain cell integrity in SHED subjected to nutritional deficit before and during

irradiation with 0.10 or 0.30 J. Serum starvation for 1 h prior to irradiation helped to maintain SHED cell viability for a period of 24 h after irradiation.

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Compliance with ethical standards

This study was conducted after approval by the Ethics Committee of Institute of Science and Technology, São Paulo State University (under protocol 46420).

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

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