Received: 8 July 2020 | Revised: 25 September 2020 | Accepted: 29 September 2020

DOI: 10.1002/jbio.202000274

FULL ARTICLE

JOURNAL OF BIOPHOTONICS

Photobiomodulation therapy drives massive epigenetic histone modifications, stem cells mobilization and accelerated epithelial healing

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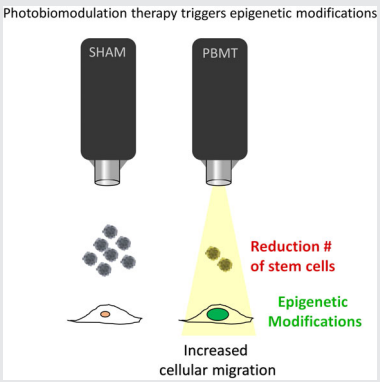
Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Grant/Award Number: 001; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 2013/07276-1; Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul, Grant/Award Number: 17/0045-5; National Institute of General Medical Sciences, Grant/Award Number: R01GM120056

Abstract



Emerging evidence indicates the clinical benefits of photobiomodulation therapy (PBMT) in the management of skin and mucosal wounds. Here, we decided to explore the effects of different regiments of PBMT on epithelial cells and stem cells, and the potential implications over the epigenetic circuitry during healing. Scratch-wound migration, immunofluorescence (anti-acetyl-Histone H3, anti-acetyl-CBP/p300 and anti-BMI1), nuclear morphometry and western blotting (anti-Phospho-S6, anti-methyl-CpG binding domain protein 2 [MBD2]) were performed. Epithelial stem cells were identified by the aldehyde dehydrogenase enzymatic levels and sphere-forming assay. We observed that PBMT-induced accelerated epithelial migration and chromatin relaxation along with increased levels of histones acetylation, the transcription cofactors CBP/p300 and mammalian target of rapamycin. We further observed a reduction of the transcription repression-associated protein MBD2 and a reduced number of epithelial stem cells and spheres. In this study, we showed that PBMT could induce epigenetic modifications of epithelial cells and control stem cell fate, leading to an accelerated healing phenotype.

KEYWORDS
epigenetics, histone acetylation, low-level laser therapy, photobiomodulation, wound healing

1 | INTRODUCTION

Wound healing is a dynamic process that coordinates cellular events essential for tissue repair and the reestablishment of the epithelial barrier function [1]. The healing process consists of three main overlapping phases: the inflammatory phase, followed by cellular proliferation, and the tissue remodeling phases [2]. During the proliferative phase, keratinocytes undergo proliferation followed by migration and ultimately undergo differentiation and stratification [3]. When considering the importance of the wound healing process in the maintenance of tissue homeostasis, the speed by which epithelial cells close the wound is key in the reduction of water loss and in the mitigation of infections. There are multiple therapeutic approaches currently in use in the clinic that facilitate tissue healing. Among them, the use of antibiotics, anti-inflammatories drugs, herbal medicines, surgical debridement of necrotic tissues in case of burn wounds, and, more recently, the application of photobiomodulation therapy (PBMT) to improve healing [4, 5].

The PBMT is defined as the application of non-ionizing forms of light such as lasers, light-emitting diodes and broadband light to promote physiological changes and therapeutic benefits [6]. PBMT has been considered an important tool in accelerating wound healing in both experimental [7, 8] and clinical settings [9, 10]. Most studies have used different sources of laser light, and the benefits in tissue healing are related to the effects of laser irradiation directly to the cells. PBMT triggers cellular biostimulation via the excitation of intracellular chromophores such as mitochondrial and membrane cytochromes, endogenous porphyrins and flavoproteins [11]. Nonetheless, the effects of PBMT on tissues are dependent on the irradiation along with the treatment parameters that include wavelength, output power, exposure time and irradiated area [4]. Despite the positive results demonstrated by PBMT in epithelial cells [12–14], a number of variables, including the application parameters and the optimal dose, remain unknown, and the molecular mechanisms involved in the stimuli of epithelial tissues and its stem cells. There are already some studies identifying different mechanisms of action of PBMT in irradiated tissues, such as the activation of TGF-β1 and mammalian target of rapamycin (mTOR) signaling, and the modification of histone acetylation and NFκB expression [10, 15, 16]. However, the effects of PBMT therapy on epigenetic modifications of epithelial cells and its potential implication over stem cell maintenance remain poorly understood.

Epigenetics refers to the study of changes in gene expression without the alteration of the nucleotide sequence of the genetic code [17]. Epigenetic modifications include DNA methylation, histone modifications and non-coding RNA, also known as micro-RNAs [18]. Covalent modifications in the histone tails directly influence the chromatin organization and the DNA packaging. More specifically, histone acetylation on the residue of lysine 9 in the tail of histone H3 (H3K9ac) is considered an epigenetic marker for active chromatin. Increased acetylation levels of H3K9ac result in relaxed chromatin, increased binding of transcription factors, and significantly increased gene expression. These epigenetic mechanisms promote dynamic alterations in the cell transcriptional potential and regulate many biological processes, including cell proliferation, migration and differentiation, signaling pathway activation or inhibition and cell senescence. During wound healing, the involvement of epigenetic processes like the loss of the polycomb group proteins and the methylation of histones has been described in specific developing stages of injured tissues [1]. The activation of selective genes at very particular moments determines the phases of cell proliferation, differentiation and migration.

Although there is a body of data stating the importance of PBMT in the process of wound healing, the effects of laser irradiation on epigenetic mechanisms of reepithelization and in the modulation of stem cells are not yet totally elucidated. In this sense, our study aimed to evaluate the impact of different PBMT protocols on the behavior and epigenetic modifications of epithelial cells and stem cells.

2 | MATERIALS AND METHODS

2.1 | Cell lineages and materials

Keratinocyte cell line (HaCaT) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B. The cells were maintained in a 5% CO_2-humidified incubator at 37°C.

Cells were seeded on tissue culture plates and cultured until achieving 70% of cellular confluency. Cells were cultured until 100% of confluency exclusively during wound assay. Cell stress was induced by culturing

cells with a low concentration of FBS (DMEM with 2% FBS, nutritional deficit) [19, 20]. The laminar flow (biosafety class II) was used to perform all the cell culture experiments, and cells were monitored daily using a phase-contrast microscope (Nikon Eclipse Ts2, Nikon, Melville, New York).

2.2 | Photobiomodulation therapy

All laser irradiations were executed using continuous wave indium-gallium-aluminum-phosphide (InGaAlP, 660 nm) diode laser (Twin Laser, MM Optics, Sao Paulo, Brazil) in a punctual mode with a spot size of 0.04 cm^2). Five groups with different PBMT parameters were established using different output power (40 and 100 mW) and energy densities (4 and 20 J/cm^2) (Table 1). All laser application was carried out at a 90° angle in relation to the tissue-culture plates. The sham control group was manipulated identically to the treatment conditions omitting the laser irradiation (laser off). Each culture condition was seeded in a different culture dish to avoid exposure of cells due to cross-irradiation. The laser therapy was administered in close contact with the base of the optically clear plates to maintain optimal irradiation. The process of cellular irradiation was conducted under partially dark conditions to avoid the potential interference of other light sources. The output power of the laser was continuously monitored using a power meter (Laser Check; MM Optics LTDA, Sao Paulo, Brazil).

2.3 | Scratch-wound migration assay

The ability of epithelial cells to migrate upon different parameters of PBMT irradiations was accessed using an in vitro scratch-induced wound model. Keratinocytes were seeded on 60 mm culture plates and kept at 37°C with FBS supplement until complete cellular confluence. The culture medium was replaced by a nutritional-deficient media (2% FBS) in all groups 2 hours before the scratch, as previously described [12, 19]. Two perpendicular crossing scratches (lines) were performed in the center of the confluent monolayer of epithelial cells utilizing a 200 μL pipette tip (Figure 1A). After scratch, time zero was established and used as the baseline for all future measurements. We applied four irradiations sessions with 6 hours of intervals between each procedure. Each 60 mm culture plates received five distinct irradiation points (Figure 1A) [19]. The first irradiation time point followed the scratching (T0). In order to standardize and guide the irradiation time points, circles were drawn at the bottom of each culture dish using a permanent marker, as illustrated in Figure 1A. Wound closure was assessed and photographed at 0-, 12-, 24-, 48-, 60-, 72-, 84- and 96-hour time points using an inverted microscope. Wound closure was mensurated by using software to analyze the images, and the percentage of the closed area was calculated (AxioVision 4.8.1, Carl Zeiss, New York). All scratch assays were conducted in replicate with an analysis of eight areas in each group.

2.4 | Immunofluorescence and nuclear size analyzes

Keratinocytes cells were seeded on glass coverslips in individual 60 mm culture plates and maintained for 12 hours at 37°C with normal growth. The culture media was replaced by nutritional-deficit media in all groups (0% FBS). After 48 hours of starving condition, each coverslip was exposed to three cycles of PBMT irradiation respecting intervals of 6 hours (0, 6 and 12 hours) with different parameters, as described in Table 1. After the last PBMT session, cells were fixed with absolute methanol at −20°C for 5 minutes, followed by immunofluorescence staining. Briefly, Triton X-100, 0.5% (vol/vol) in PBS, was used to block cells in combination with 3% (wt/vol) bovine serum albumin following incubation with anti-acetyl-histone H3 (acH3 Lys9, 1:100) and anti-acetyl-CBP (Lys1535)/p300 (Lys 1499, 1:100) both antibodies from Cell Signaling Technology, Danvers, Massachusetts. After a wash in PBS (×3), cells were incubated with Alexa 488 (green) and 546 (red)-conjugated

TABLE 1 PBMT parameters used

	Wavelength (nm)	Power (mW/cm^2)	Energy density (J/cm^2)	Irradiation time (s)	Energy (J)
SHAM	0	0	0	0	0
Group 1	660	40	4	4	0.16
Group 2	660	40	20	20	0.8
Group 3	660	100	4	1.6	0.16
Group 4	660	100	20	8	0.8

Abbreviation: PBMT, photobiomodulation therapy.

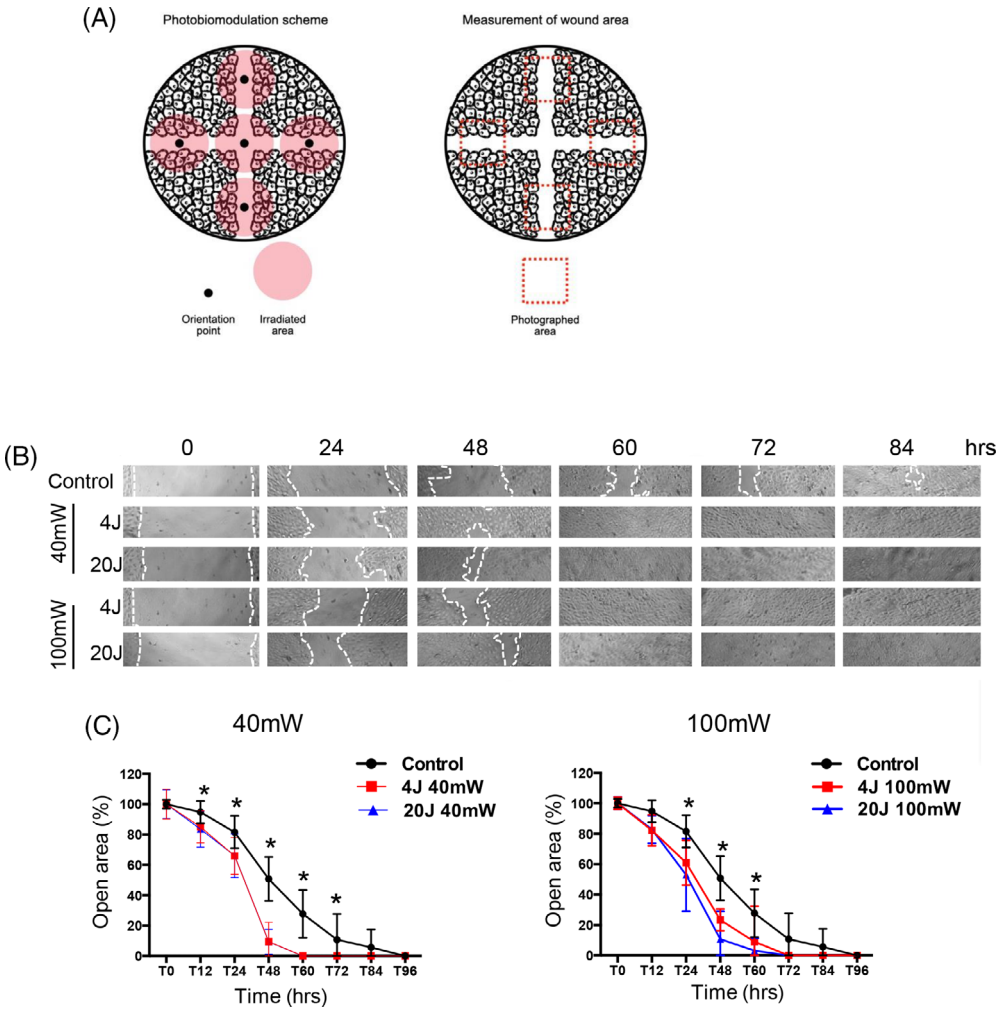


FIGURE 1 Scratch-wound migration assay. A, Illustration of the irradiated area following the orientation points marked on the bottom of each culture dish well. Representation of the photographed areas within each scratch-wound assay. B,C, Photobiomodulation therapy (PBMT) accelerated keratinocyte migration. In the first 12 hours, only the PBMT groups with 40 mW output power showed a significant acceleration of migration compared to control. At 24- and 48-hour time points, all the PBMT groups receiving 40 and 100 mW of output power demonstrated accelerated wound healing (*$P < .05$)

secondary antibodies and stained with Hoechst 33342 (DAPI) for visualization of DNA content.

Images of 10 fields of each group were captured at ×400 magnification using a QImaging ExiAqua monochrome digital camera attached to a Nikon Eclipse 80i microscope (Nikon) and visualized with QCapturePro7 software, as previously described [21]. Grayscale images were captured separately after fluorescence excitation using FITC_HYQ and TRITC_HYQ filters. Cells receiving 300 nM of the histone deacetylase inhibitor Trichostatin A (TSA) diluted in media for 24 hours were used as positive controls for histone acetylation. CBP/p300 morphometric analyses were performed using the ImageJ (Version 1.38s; NIH, Bethesda, Maryland). For nuclear evaluation, 50 cells of each group stained with DAPI were measured using ImageJ software.

2.5 | Western blotting

Keratinocyte cells were seeded into 60 mm culture plates and maintained for 12 hours at 37°C in a humidified incubator with 5% CO_2 in culture media supplemented with 10% FBS. The culture media was substituted by nutritional-deficit media in all groups (2% FBS). After 48 hours of starving condition, each PBMT group received three sessions of PBMT irradiations with 6 hours intervals (0, 6 and 12 hours) with different parameters, as described in Table 1. At the end of the last PBMT session, the cells were washed with cold PBS and lysed with RIPA buffer. After centrifugation at 4°C for 10 minutes, protein lysates were loaded and run in 10% SDS-PAGE gels and transferred to polyvinyl difluoride membrane (Millipore, Billerica, Massachusetts). After protein transfer, the membranes were blocked with 5% milk solution for 1 hour following washed with TBS-Tween and incubated overnight with anti-MBD2 (Bethyl Laboratories, Montgomery, Texas,1:2000), anti-phospho S6 (Cell Signaling, 1:2000) and GAPDH as a loading control (Calbiochem, Gibbstown, New Jersey). The membranes were washed (×3) with TBS-Tween followed by the incubation of the secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. The reactions were visualized using the ECL Super Signal West Pico

Substrate (Pierce Biotechnology, Rockford, Illinois). Optical density (OD) of representative WB bands were quantified using ImageJ. OD of MBD2 and pS6 proteins were normalized to the corresponding OD for the same well for GAPDH to control for small changes in protein loading.

2.6 | Sphere assay

To evaluate the impact of PBMT over epithelial stem cells, 2.5×10^3 keratinocytes were cultured in ultralow attachment plates to generate spheres (Corning, New York, New York). After 5 days of growth, culture media was substituted by nutritional-deficit media in all groups (2% FBS). Then, two groups were established: the Sham group (without irradiation) and the PBMT group. Epithelial spheres received the same PBMT protocol described in Table 1. The PBMT group received three sessions of irradiations with 6 hours intervals during four consecutive days. The sham group received identical treatment conditions omitting the laser irradiation. Cytospin was applied at 1500 rpm, 4°C for 10 minutes, and the slides were stained with hematoxylin-eosin or submitted to immunofluorescence for detection of acetyl-histone H3 (ac.H3 Lys9, 1:100, Cell Signaling), B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1, cloneF6, 1:50, Millipore) and acetyl-CBP (Lys1535)/p300, 1:100, Cell Signaling). The total number of spheres was counted in ×100 magnification using a Nikon Eclipse 80i microscope (Nikon).

2.7 | Fluorescence-activated cell sorting of epithelial stem cells

Aldehyde dehydrogenase (ALDH) activity (Aldefluor kit, StemCell Technologies, Durham, North Carolina) assay was used to identify epithelial stem cell-like cells using flow cytometry. Briefly, 5×10^4 keratinocytes were plated in 60 mm culture plates and maintained for 12 hours at 37°C in a humidified chamber maintained at 5% CO_2 concentration in DMEM supplemented with 10% FBS. The culture media was substituted by nutritional-deficit media in all groups (2% FBS). Two groups were established: Sham (without irradiation) and PBMT. The PBMT group received three sessions of irradiations with 6 hours intervals during four consecutive days using the same protocol described in Table 1. The cells were suspended with activated Aldefluor substrate (BODIPY-amino acetate) or negative control (diethylaminobenzaldehyde, a specific ALDH inhibitor) for 45 minutes at 37°C. Ten thousand events were counted for each sample using the FACSDiVA Cell Sorter (BD Biosciences, Mountain View, California).

2.8 | Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California). Two-way analysis of variance (ANOVA) statistical analysis was applied in the scratch assay and western blot assay, followed by Tukey's multiple comparison tests. Nuclear size measure, acH3 and CBP/p300 stains statistical analyses were performed using a one-way ANOVA and Tukey's multiple comparison tests. Asterisks denote statistical significance ($^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ and ns $P > .05$).

3 | RESULTS

3.1 | PBMT accelerated epithelial wound healing independent of the irradiation parameters

Here, we decided to explore if distinctive PBMT parameters would produce a different effect on epithelial migration. Using a scratch wound healing approach and keratinocyte cells, we observed that all PBMT parameters resulted in accelerated epithelial migration.

Overall, we observed that the application of 40 mW output produced a superior result over the epithelial cells when compared to 100 mW (Figure 1B,C). Cells exposed to 40 mW started to migrate faster than the sham group by 12 hours, while cells receiving 100 mW shown accelerated migration by 24 hours after irradiation (Figure 1C). Consistent with the early start in cellular migration, keratinocytes completely closed the scratch wound after 60 hours when receiving 40 mW of output. Cells receiving 100 mW, however, only achieved complete closure by 72 hours after wound (Figure 1C). The sham group exhibited closure within 84 and 96 hours after wound.

3.2 | PBMT-induced chromatin remodeling associated with epigenetic modification and activation of mTOR pathway in epithelial cells

Following, we decided to evaluate if PBMT was able to induce morphological changes to epithelial cells. Interestingly, we observed that irradiated cells respond to PBMT by increasing the nuclear size independent of the output power and the energy density used (Figure 2A).

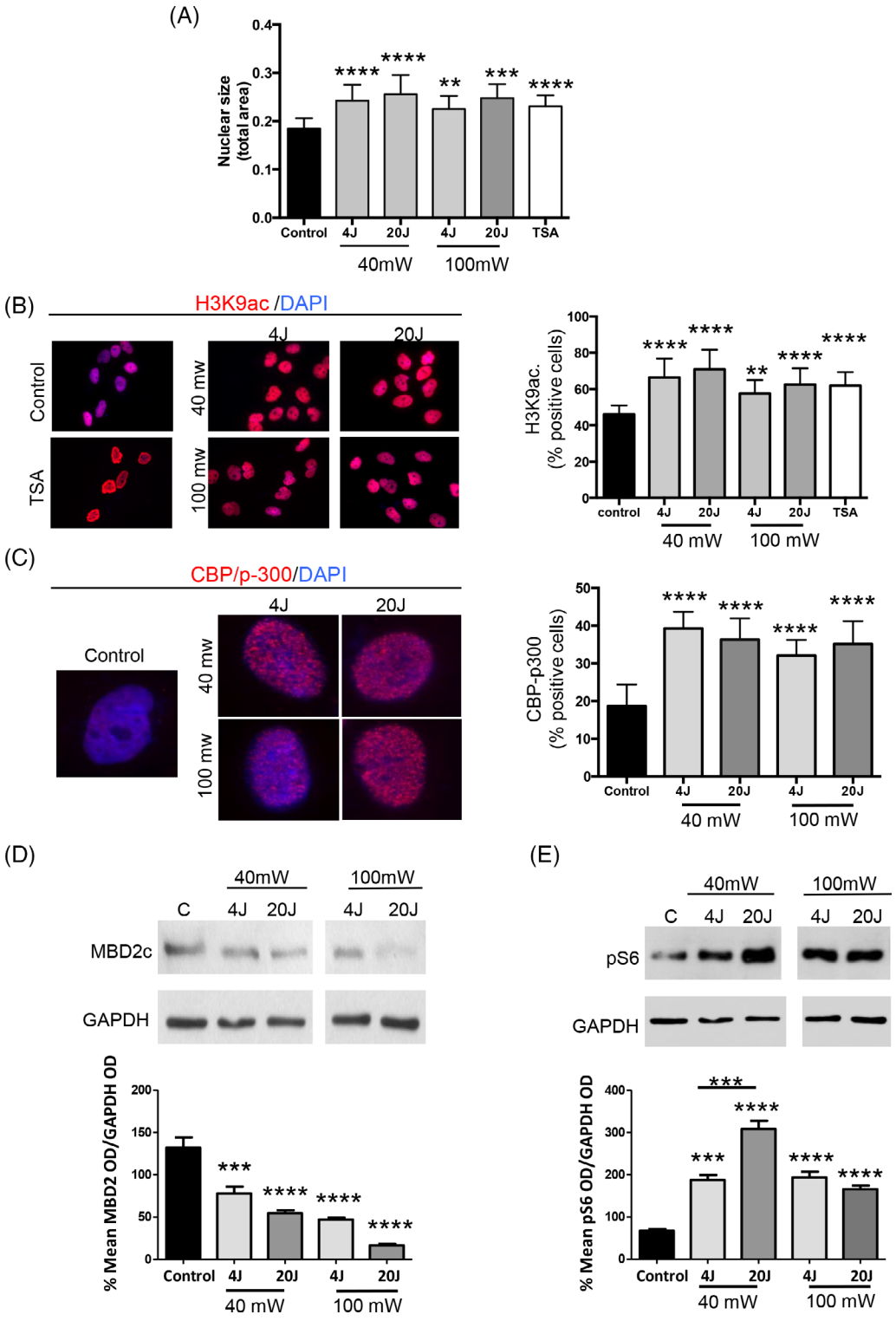


FIGURE 2 Photobiomodulation therapy (PBMT)-induced chromatin remodeling and epigenetic modification in epithelial cells. A, Significant increase in the nuclear size of keratinocyte in all PBMT groups compared with baseline control and Trichostatin A (TSA)-treated cells (positive control). B, PBMT induces chromatin remodeling evident by increased levels of H3K9ac, and, C, accumulation of CBP/p300 in keratinocytes. D,E, Representative Western blots of MBD2c and pS6 after PBMT treatment of keratinocytes. Optical density (OD) of representative WB bands was quantified using ImageJ. OD of MBD2 and pS6 proteins were normalized to the corresponding OD for the same well for GAPDH to control for small changes in protein loading (****$P < .0001$; ***$P < .001$; **$P < .01$)

Chromatin decompaction is a sign of epigenetic modifications. With that in mind, we decided to explore the effects of low power irradiation on histone acetylation. We observed that both energy densities (4 and 20 J/cm^2) resulted in increased acetylation levels of histone H3 at lysine 9 (H3K9ac) independent of the output power used (40 or 100 mW) (Figure 2B). We further decided to explore if PBMT would also have an effect on transcriptional co-activating proteins, therefore, suggesting a mechanism for histone modifications. Indeed, we observed that PBMT is capable of triggering the expression of the transcription cofactors CBP and p300 (Figure 2C). We further decided to explore if MBD2, a protein involved in the recognition of methylated CpG islands and therefore associated with gene transcription repression, would be affected by PBMT. Western blot of irradiated keratinocyte lysates demonstrated that administration of low energy densities (4 and 20 J/cm^2) diminished the protein levels of MBD2, in special at 20 J/cm^2 and output power of 100 mW (Figure 2D). We have previously shown that PBMT influences the expression levels of the mTOR signaling pathway. We observed that PBMT induces upregulation of the mTOR pathway by the activation of pS6 in keratinocytes independent from the output power used (40 or 100 mW) (Figure 2E).

3.3 | PBMT promoted modification in keratinocytes stem cells

We have previously shown that changes in the chromatin organization have a profound impact on the population of cancer cells and cancer stem cells [22, 23]; however, little is known on the ability of PBMT in modulating

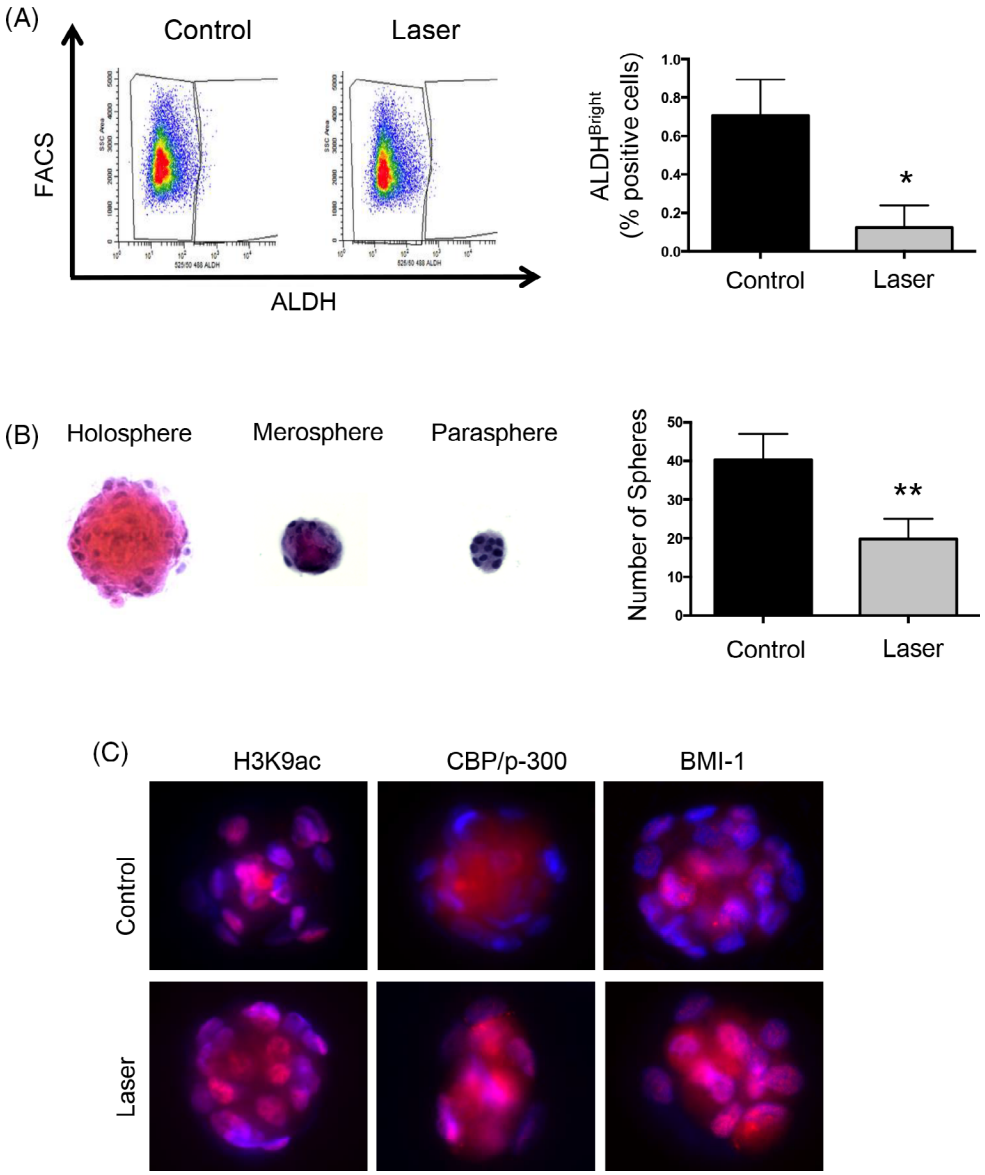


FIGURE 3 Stem cell analysis. A, Photobiomodulation therapy (PBMT) caused a reduction in aldehyde dehydrogenase (ALDH+) cells and, B, in the total number of epithelial spheres. C, Keratinocyte spheres demonstrated an increase in H3K9ac, CBP/p-300 and BMI-1 after PBMT (*$P < .05$; **$P > .01$)

normal epithelial stem cells. Here, we found that PBMT has a significant impact on the number of ALDH positive cells when compared to sham (*$P < .05$) (Figure 3A). Increased enzymatic activity of ALDH is a strong indication of cells presenting enriched stem and progenitor function. Along with the detection of ALDH enzymatic levels, we also explored the impact of PBMT on epithelial spheres. Epithelial cells cultured in low adhesion conditions form spheres presenting enrichment for stem cells. We found a reduction in the total number of spheres in PBMT groups (**$P < .01$) (Figure 3B). Further characterization of irradiated epithelial spheres indicates a relative accumulation of H3K9ac, CBP/p-300 and BMI-1 compared with sham controls (Figure 3C).

4 | DISCUSSION

The effects of PBMT on tissue repair are well documented in a broad range of studies [14, 24, 25]. While some mechanisms of action associated with PBMT were described in the literature, little is known about the relation of PBMT with epigenetic and stem cells [16, 26]. The activation and differentiation of stem cells have an important role in the restorative capacity of the wound healing process [27, 28]. Our study demonstrates for the first time that while PBMT accelerates epithelial migration, it promotes chromatin remodeling and mobilizes the population of stem cells. Here, we investigate the ability of PBMT to stimulate keratinocyte migration and accelerate epithelial wound healing using different parameters. We observed that the lower power output of 40 mW provided faster effects than using 100 mW (Figure 1). Indeed, we previously demonstrated augmented epithelial migration and accelerated wound healing of keratinocytes with 40 mW (4 and 20 J/cm^2) [12]. Nonetheless, the use of higher output power of 100 mW was also shown to be effective in accelerating healing. In this sense, it was previously demonstrated that the power density seems to influence cell growth in an inversely proportional manner [29]. PBMT has shown biostimulatory effects, such as faster epithelial cell migration [12] and proliferation [8, 19], which are important events in the wound healing process. Therefore, PBMT appears very useful in the clinic setting as it accelerates the healing process of different oral mucosal disorders, such as oral mucositis, oral lichen planus, recurrent aphthous stomatitis and herpes infection [30–34].

The ability of PBMT in induced epigenetic chromatin modifications shown here is exciting and demonstrates that low power laser is capable of modulating gene expression in epithelial cells. Indeed, some of the effects of laser irradiation on chromatin properties were previously described in the study of Smol'yaninova et al [35]. In this study, irradiated (He-Ne laser, 632.8 nm, 28-11 J/m2) human peripheral blood lymphocytes presented chromatin activation potentially related to a more responsive behavior of these cells to natural stimulators present in tissues. In this context, the authors concluded that this effect might help to explain the mechanism of wound healing following irradiation. Interestingly, in the present study, PBMT promoted nuclear modification similar or superior to TSA, an histone deacetylases (HDAC) inhibitor capable of inducing broad chromatin acetylation. TSA selectively inhibits HDAC classes I and II, inducing histone modifications while preventing the deacetylation of histone lysine [36]. The acetylation of lysine residues of histones and the recruiting of protein histone acetyltransferases (HATs) are often associated with transcriptionally active genes [36, 37]. Indeed, the use of PBMT in epithelial cells resulted in the acetylation of H3K9ac along with the nuclear accumulation of CBP/p300. CBP/p300 has an important function associated with chromatin remodeling, and it is a transcriptional coactivator factor that plays distinct roles, such as bridging of DNA-binding and general transcription factors and relaxation of chromatin through its intrinsic HATs activity [38]. Interestingly, HATs can also interact and acetylate other substrates besides histones. Although CBP/p300 does not directly acetylate H3K9, it interacts with several transcription factors to regulate gene expression, such as ATF2, E2F, NFkB and HMG protein [39, 40] and CBP/p300 also acetylates and activates non-histone proteins, like the tumor suppressor p53 [41]. These characteristics indicate the importance of CBP/p300 in the regulation of many physiological processes, including cell growth, transformation, development and apoptosis [42].

Our findings, therefore, provide evidence that PBMT promotes epithelium wound healing associated with epithelial migration and epigenetic modifications, such as increases in H3K9 acetylation and CBP/p300. Several epigenetic mechanisms have been shown to be engaged in regulating epithelial homeostasis and wound repair [43, 44]. Antagonism of HDAC using HDAC inhibitor valproic acid promoting acetylation of histones has been shown to improve tissue regeneration, including the spinal cord of mice [45]. In addition, induction of Histone H3 and H4 acetylation promoted by the administration of valproic acid also activates AKT/mTOR pathway, increasing the levels of Rictor, Raptor, pS6 and Akt, suggesting a link between mTOR and the epigenetic machinery in prostate cancer cells [46]. In an animal that undergone digital amputation, the administration of intraperitoneal injections of HDAC inhibitor presented increased proliferation and collagen deposition at the amputation site, leading to improved digital regeneration compared with

controls [47]. In this study, PBMT presented similar behavior to HDACi, promoting chromatin remodeling and histone acetylation. In an animal model of oral tongue ulcer healing, our group demonstrated that PBMT induces epithelial migration during the initial stages of healing along with enhanced keratinocyte differentiation at the late stage of healing. Such a process involved the acetylation of histones and the expression of NFkB [16].

Epigenetic regulation comprises the interaction between histone modifications and DNA methylation through the association of proteins at specific sites of the DNA [48]. MBD proteins coordinate the signaling between cytosine methylation and chromatin structure, acting as transcriptional regulators. Interestingly, MBD2, a member of MBD proteins, recruits the Nucleosome Remodeling and Deacetylase corepressor complex and repress transcription through the methylation of CpG islands of the associated genes [48]. In this study, it was observed that laser irradiation with the energy densities used resulted in diminished levels of MBD2, which was more evident for 20 J/cm^2 using an output power of 100 mW. The reduced levels of MBD2 associated with the accumulation of H3K9ac and CBP/p300 suggest that PBMT induces an epigenetic reprogramming responsible for activating gene transcription while downregulating MBD2 and its ability to repress gene transcription. Among all of these processes, our group has been focused on identifying signaling pathways associated with the effects of PBMT-induced epithelial cell migration. Among several pathways, we have identified the PI3K/mTOR signaling as a major player in the process of epithelial reepithelialization [12]. Here, we decided to verify if the epigenetic modification induced by PBMT was associated with enhanced epithelial migration.

The oral epithelium is an actively renewable tissue presenting a reservoir of stem cells localized at the basal layer. Epithelial stem cells represent a minor population of cells with a strong capability to self-renew, to maintain tissue homeostasis and to repair damaged tissues [49]. Different stimuli, including PBMT, can induce stem cells to proliferate and activate terminal differentiation. To analyze the effects of PBMT in keratinocyte stem cells, we explored the effects of low power laser over ALDH levels, a known detoxifying enzyme involved in the oxidation of intracellular aldehydes [50]. ALDH may have a function in the early differentiation of stem cells through its role in oxidizing retinol to retinoic acid being considered a functional marker for the stem cell population. High ALDH levels have been reported in stem cells from different tissues and to be present at high levels in cancer stem [51, 52]. Following the administration of PBMT, we observed a significant reduction of $ALDH^+$ cells when compared to the sham. Parallel to the use of ALDH levels to identify stem cells, we decided to implement a functional assay to enrich stem cells by inducing the formation of spheres. Upon administration of PBMT, we observed a reduction in the total number of spheres when compared to the sham-treated spheres. Combined, ALDH and sphere-forming assays demonstrate that PBMT promotes a reduction in the total number of epithelial stem cells.

In addition, to verify the effect of PBMT in the expression of epigenetic markers in the stem cell population, it was performed an immunofluorescence labeling in the spheres. The results showed an increase in H3K9ac and CBP/p-300 expression after PBMT, indicating that diode laser promoted chromatin remodeling in keratinocyte stem cells. There is growing evidence that epigenetic alteration may cause specific chromatin changes to distinguish stem and differentiated cells in a wide range of tissues [53]. Histone acetylation has been described as a regulator of differentiation and self-renewal of multiple types of stem [54]. However, the role of histone acetylation in differentiation depends on the specific cell type and phase of development.

The differentiation process indicates that pluripotent stem cells give rise to specialized cells. While differentiating, cells undergo several stages of cellular differentiation and progressively specialized at each step. However, the mechanism associated with the stem cell's decision to self-renew or differentiate still needs to be completely elucidated. A handful of molecular markers involved in this stem cell conundrum have been identified, including the BMI1. While BMI1 is observed in stem cells, its overexpression reduces apoptosis and increases cellular proliferation in mesenchymal stem cells [55]. Such a process involves the repression of p16INK4 and prevents stem cells from undergoing cellular senescence. Indeed, we observed that low power laser induces the accumulation of BMI1 in the epithelial spheres. We also observed that laser therapy also reduced the number of spheres being formed. The remainder spheres are characterized by higher levels of BMI1, suggesting the potential role in the stem cell decision to self-renew or differentiate.

Collectively, our results indicated that PBMT stimulates a pronounced effect on the organization of chromatin in epithelial cells causing relaxation in its configuration around the bound DNA sequences by acetylation of histones, activation of CBP/p300 and reduction of MBD2, leading to an increase in nuclear size. Histone acetylation and CBP/p300 activation have been described as important signaling endpoints involved in cellular plasticity in both normal and tumoral cells, regulating the expression of genes involved in various biological mechanisms, such as cell proliferation, differentiation and survival. The present study is the first one to

demonstrate that PBMT can modulate the epigenetic signature of keratinocytes and its stem cell population, which may justify some of the important effects previously attributed to PBMT, such as cellular proliferation, cellular migration and reestablishment of the cellular metabolism.

ACKNOWLEDGMENTS

This work was funded by the NIH – NIGMS (PIs: Drs C. H. S. and R. M. C.—R01GM120056). This work was entirely performed at the laboratory of Epithelial Biology at the University of Michigan. The funder had no role in study design, data collection, and analysis, decision to publish or preparation of the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. Drs M. D. M. and V. S. B. were funded by CAPES - Finance Code 001, FAPERGS n. 17/0045-5 and FAPESP n. 2013/07276-1 (for scholarship and fellowship).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Manoela D. Martins, **Marco A. T. Martins**, **Cristiane H. Squarize** and **Rogerio M. Castilho**: Contributed equally in the design of the study, acquisition, analysis and interpretation of data, writing the manuscript, revising it critically, approving the final version to be published and agreeing to be accountable for all aspects of the work. **Felipe Martins Silveira**, **Luciana O. Almeida** and **Vanderlei S. Bagnato**: Contributed equally in the analysis and interpretation of data, writing the manuscript, revising it critically, approving the final version to be published and agreeing to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Martins MD, Silveira FM, Martins MAT, et al. Photobiomodulation therapy drives massive epigenetic histone modifications, stem cells mobilization and accelerated epithelial healing. *J. Biophotonics*. 2021;14:e202000274. https://doi.org/10.1002/jbio.202000274