Lasers Med Sci
DOI 10.1007/s10103-017-2254-2

ORIGINAL ARTICLE

Photobiomodulation therapy action in wound repair skin induced in aged rats old: time course of biomarkers inflammatory and repair

Franciane Barbieri Fiorio[1] · Solange Almeida dos Santos[1] · Caroline Sobral de Melo Rambo[1] · Camila Guerra Dalbosco[2] · Andrey Jorge Serra[3] · Brunno Lemes de Melo[4] · Ernesto Cesar Pinto Leal-Junior[1] · Paulo de Tarso Camillo de Carvalho[1,3]

Received: 8 March 2017 / Accepted: 5 June 2017

Abstract Previous studies have discussed an inverse correlation between age and wound healing, because it relates to the association of aging with a gradual decrease in healing capacity. Treatment with photobiomodulation therapy (PBMT) improves wound healing by inducing increases in mitotic activity, numbers of fibroblasts, collagen synthesis, and neovascularization. Therefore, this study aimed to evaluate the effects of PBMT in cutaneous wound healing in aged rats. A punch biopsy of 8 mm in diameter was performed to produce a skin wound. The study included 45 male rats, of which 15 were young (30 days) and 30 were elderly (500 days). The 45 animals were distributed into 3 experimental groups, which were subjected to skin wounds and 1 aged group received PBMT, with a 30-mW laser beam (power density of 1.07 W/cm^2), beam area of 0.028 cm^2, and λ660 nm produced through active phosphide Gallium-Aluminum-Indio (InGaAIP). The PBMT application took the form of a single-point transcutaneous method, with a total energy of 2 joules per wound site, energy density of 72 J/cm^2, and time of 1 min and 7 s. Analysis was performed to verify the effect of PBMT on the quantity of collagen I and III, metalloproteinase 3 and 9 (MMP-3 and MMP-9), tissue inhibitor of metalloproteinase-2 (TIMP-2) and of vascular endothelial growth factor (VEGF) at the wound site by immunohistochemistry, cytokine-induced neutrophil chemoattractant (CINC)-1, by enzyme-linked immunosorbent assay (ELISA) and interleukin (IL)-6 real-time polymerase chain reaction (RT-PCR). That we conclude LLLT is effective in the modulation of inflammatory mediators IL-6, CINC-1, VEGF, MMP-3, MMP-9 and TIMP-2 as well as increased collagen production in aged animals during different phases of the tissue regeneration process. However, the effects of PBMT obtained in the aged animals (aged LLLT group) suggest that new dosimetries should be tested to achieve better results.

Keywords Wound healing · Photobiomodulation therapy · Inflammatory mediators · Age

Introduction

Successful wound healing requires function of many cellular and molecular events that are coordinated to result in tissue repair or regeneration. Disturbances in any of these functions result in impaired wound healing. Wound repair can be thought of as the culmination of three major overlapping phases: inflammation, proliferation, and remodeling [1, 2].

Delayed wound healing in the aged is associated with impaired inflammatory responses with alterations in chemokine production, reduced macrophage phagocytic capacity, enhanced proteolysis and degradation of matrix constituents due to excessive leukocytes and inflammation, delayed re-epithelialization and neovascularization and impaired

✉ Paulo de Tarso Camillo de Carvalho
paulo.tarso@uninove.br

[1] Postgraduate Program in Rehabilitation Sciences, Universidade Nove de Julho (UNINOVE), São Paulo, SP, Brazil

[2] Health Sciences Center, Chapecó University (UNOCHAPECÓ), Chapecó, SC, Brazil

[3] Postgraduate Program in Biophotonics Applied to Health Sciences, Nove de Julho University (UNINOVE), Rua Vergueiro 235, São Paulo, SP, Brazil

[4] Postgraduate Program in Medicine (Cardiology), Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil

Published online: 05 July 2017

fibroblast migration are other characteristics of wound healing in elderly subjects [3, 4].

Overall, there are global differences in wound healing between young and aged individuals [5]. It has long been thought that pro-inflammatory cytokines, including interleukins (IL) 1α and 1β, IL-6, and tumor necrosis factor (TNF)-α, play an important role in wound repair. They likely influence various processes at the wound site, including stimulation of keratinocyte and fibroblast proliferation, synthesis and breakdown of extracellular matrix proteins, fibroblast chemotaxis, and regulation of the immune response [6].

Chemokines among which IL-8 are active participants in the healing process because it stimulates the migration of various cell types into the wound site, particularly inflammatory cells. IL-8 expression is increased in acute wounds and plays a role in re-epithelialization by the increase in migration and proliferation of keratinocytes. It also induces the expression of matrix metalloproteinases (MMPs) by leukocytes, and it is a strong chemoattractant for neutrophils, participating thus the inflammatory response. However, IL-8 at high levels decreases the proliferation of keratinocytes and the contraction of the collagen by fibroblasts structure [7].

MMPs play an important role in all stages of wound healing by degrading all components of the extracellular matrix (ECM) and have the capacity to synthesize collagen type III and IV (MMP-3), collagen type IV, V, VII, X, denatured collagen (MMP-9) and other MEC members who contribute to the remodeling of the wound [8]. MMPs have its release controlled by TIMPs [9]. Many MMPs can also regulate the activity of chemokines and are also involved in neovascularization process due to interaction with vascular endothelial growth factor (VEGF) in the formation of new blood vessels [10].

Angiogenesis is another complex process that could play a role in wound healing. The aging-induced decrease in tissue perfusion and impairment of angiogenesis are known to affect wound healing [4].

Various treatments to reduce the delay of repair and problems in healing that occur with age have been studied both under normal conditions and under conditions involving altered wound healing [7, 11]. PBMT has been shown to be able to change the delay time and normalize wound healing parameters, mainly involving the modulation of inflammatory cytokines [12, 13] and enhanced production of collagens type I and III [14, 15].

We propose, therefore, that PBMT can positively influence the healing process of cutaneous wounds during aging and can also positively modulate mediators of this process, such as vascular endothelial growth factor (VEGF), MMPs, TIMP, collagen, and the pro-inflammatory cytokines IL-6 and CINC-1 (a homolog of human IL-8).

Materials and methods

Animals

The study animals consisted of 45 male Wistar rats (*Rattus norvegicus albinos*), of which 15 were young (30 days) with body weights ranging from 130 to 150 g and 30 were aged (500 days) with body weights ranging from 400 to 450 g. The animals were obtained from the animal housing facility of the and kept under controlled conditions of light and temperature, with free access to water and chow. All experimental procedures were approved by the Institutional Research Ethics Committee (AN 0028/2014), and they were conducted according to the guidelines of the Brazilian College for Animal Experimentation as well as the standards of the International Council for Laboratory Animal Science.

Experimental groups

Animals (15 young and 30 elderly) were randomly divided into a total of 3 groups each of 15 animals, which were further separated to 3 subgroups each containing 5 animals, according to the experimental time points of 3, 7, and 14 days. The groups were split as follows: "control aged," elderly animals that only underwent skin wounding; "LLLT aged," elderly animals that underwent skin wounding and LLLT; and "control young," young animals that only underwent skin wounding.

The animals were anesthetized by an intramuscular injection of a 7% ketamine solution (Cetamin, Syntec, Cotia, SP, Brazil) and a 0.3% xylene solution (Xilazin, Syntec, Cotia, SP, Brazil) at a proportion of 2:1 and a total injection volume of 0.2 mL per 100 g body weight. All possible care was taken to avoid any discomfort to the animals. Once anesthetized, the animals were placed in the prone position; the site was sterilized with alcohol-iodine, and the dorsum of the animal was shaved. Skin wounding was performed using an 8 mm diameter "punch" with a circular blade, allowing the removal of a circular area of skin. Four wounds were inflicted on each animal, with the site of wounding located in the middle portion of the median sagittal plane. After wounding, the injured animals were placed in clean cages, 5 animals in each cage, and freely provided with water and chow. The analgesic dipyrone was administered for 2 days after wounding at a dose of 0.1 mL/animal, 4 times daily, with a minimum of 4 h between doses.

Photobiomodulation therapy application

We used the Photon Laser III® (DMC Equipment's LTDA, SP, Brazil) for PBMT, with a λ660 nm laser beam produced through active phosphide Indio-Gallium-Aluminum (InGaAIP) according to the following parameters: frequency

continuous, power density 1.07 W/cm^2, power output 30 mW, spot size 0.028 cm^2, energy density 72 J/cm^2, total energy delivered 2 J, irradiation time per treatment 67 s. The application of PBMT was initiated immediately after skin wounding, and then on alternate days until the day of euthanasia for each group. The control group underwent the same experimental procedures, but did not receive PBMT.

Euthanasia

At the end of each period of 3, 7, and 14 days, the animals were weighed and then euthanized via intracardiac injection with thiopental sodium (Cristália Lab, SP, Brazil) at the dose of 0.05 mL per 100 g body weight. After euthanasia, following the indicated times, the wounded areas were surgically removed with a 1-cm margin of skin around the lesion depth to the fascia. The resulting samples, which were taken from 2 animals per time point, were frozen in liquid nitrogen and stored at −80 °C. Of the 2 samples per time point, 1 sample was allocated for immunohistochemical procedures and the other sample was allocated for the analysis of protein expression by enzyme-linked immunosorbent assay (ELISA) and gene expression by RT-PCR.

Histological procedures and quantification of collagen

Samples frozen in liquid nitrogen were placed in OCT™ (Sakura, Finetechnical Co. Ltd., Japan) and 4 mm sections were prepared using a cryostat (CM 1850, Leica, Germany) and histological sections stained not willing to slides silanized for immunohistochemistry.

Immunohistochemistry

The frozen sections were washed in phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. The sections were washed in PBS (6 × 5 min washes) and mounted with 1% normal goat serum in PBS for 30 min. Subsequently, the slides were incubated in the presence of primary antibody applied overnight at 4 °C. Primary antibodies used were VEGF: mouse anti-rat VEGF antibody (VG-1; Abcam, Tokyo, Japan), TIMP-2: goat anti-TIMP-2 antibody (sc6835, Santa Cruz Biotechnology, Inc), MMP-3: rabbit anti-MMP-3 antibody (ab-53015, Abcam, Tokyo, Japan), MMP-9: goat anti-MMP-9 antibody (sc-6840, Santa Cruz Biotechnology, Inc.), collagen I: mouse antibody (Col-1) (sc-59772, Santa Cruz Biotechnology, Inc.), and collagen III: rabbit antibody (S-17)-R (sc-8780 R, Santa Cruz Biotechnology, Inc.). After washing in PBS (6 times, 5 min), they were incubated with secondary antibody, agreement with primary antibody used, for 30 min. After washing in PBS (6 × 5 min washes), a coloring reaction was carried out with diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) and nuclei were counterstained with hematoxylin. Areas of positive staining for each of the tags were observed under a light microscope (E200, Nikon, Japan), and images were captured by a microcomputer equipped with IC Capture 2.2 software (The Imaging Source, Germany). From each sample, 4 images were recorded of different fields of view, including each part of the stained, using a ×10 objective so that the length was captured. Once captured, the images were analyzed using a software-based image analysis system (Image-Pro Plus® 4.5, Media Cybernetics, MD, USA).

Gene expression quantification

Total RNA was extracted from skin wound samples and RT-PCR assay was performed for mRNA quantification. Thawed tissues were homogenized in 1 mL of TRIzol® reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis and RT-PCR gene expression analysis. Initially, contaminant DNA was removed using DNase I (Invitrogen) at a concentration of 1 unit/μg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM $MgCl_2$ for 15 min at 37 °C, followed by incubation at 95 °C for 5 min for enzyme inactivation. Then, the reverse transcription (RT) reaction was carried out in a 200-μl reaction volume in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen, CA, USA). The reaction conditions were 20 °C for 10 min, then 42 °C for 45 min, and finally 95 °C for 5 min.

The reaction product was amplified by real-time PCR on the 7500 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA, USA) using the SYBR Green® core reaction kit (Applied Biosystems). The thermal cycling conditions were 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles, each at 95 °C for 15 s and then 60 °C for 1 min. Experiments were performed in triplicates for each data point. Target gene mRNA abundance was quantified as a relative value compared with an internal reference, GAPDH, whose abundance was believed not to change between the varying experimental conditions. Primers used for RT-PCR were IL-6; GenBank accession number E02522, forward primer 5′-TCCTACCCCAACTTCCAATGCTC-3′ and reverse primer 5′-TTGGATGGTCTTGGTCCTTAGCC-3′. One microliter of RT reaction mix was used for RT-PCR.

Evaluation of the inflammatory mediators CINC-1

The amount of CINC-1 in skin wounds was quantified by ELISA (R&D Systems, USA) as per the manufacturer's instructions. For this purpose, 96-well plates were coated with 100 mL of monoclonal antibody for each cytokine: anti-CINC-1 diluted in sodium carbonate buffer (0.1 M, pH 9.6). The plates were incubated at 4 °C for 18 h. For blocking, the plates were washed 4 times with PBST (PBS containing 0.05% Tween®-20) and then filled with 300 μL/well of blocking solution (3% gelatin in PBST, Sigma) at 37 °C for 3 h before being subjected to a new cycle of washes. Next, 100 μL/well of diluted samples or recombinant cytokine standards were added to the plate and incubated for 18 h at 4 °C. After washing, 100 μL/well of the respective biotinylated antibody specific for the detection of each cytokine was added and left for 1 h at room temperature (RT). After washing the plates, 100 μL/well of streptavidin–peroxidase was added and incubated for 1 h at room temperature (22 °C) followed by further washes. The reaction was visualized by adding 100 μL/well of 3,3′5,5′-tetramethylbenzidine solution and stopped by adding 50 μL/well of sulfuric acid (2 N). The absorbance of each well was read using a SpectraMax® Plus 384 spectrophotometer (Sunnyvale, CA, USA) at a wavelength of 450 nm with correction at 570 nm. The sample concentrations were calculated from standard curves obtained with recombinant cytokines.

Statistical analysis

The data were tabulated using Excel 2007 software (Microsoft Corporation, WA, USA) and initially assessed for normality using the Shapiro–Wilk test. Since a normal distribution was observed, ANOVA with Tukey's post hoc test was used for comparisons between experimental groups. All of the data were expressed as mean and standard deviation values. Prism® 5 software (GraphPad, CA, USA) was used. Differences from the null hypothesis were considered to be significant when $p < 0.05$.

Results

Quantification of collagen I

Significant differences in the percentage of collagen I deposition at the wound site were observed among groups at 3 days post-wounding. There was a significant difference in the means of the percentage of collagen deposition at the wound site between the control young and aged (control and LLLT) groups. At 7 days, groups had similar behavior at 3 days, with significant differences between groups. At 14 days, the control young group showed significantly higher expression that the control aged and LLLT aged groups. There was also a significant difference between the control aged and LLLT aged groups, showing the biomodulatory effect of laser in deposition of collagen type I (Fig. 1).

Quantification of collagen III

At 3 days post-wounding was found significant lower percentage of collagen III deposition at the wound site in the aged (control and LLLT) groups compared with control young group, however the LLLT aged group had higher percentage than the control aged group. At 7 days aged (control and LLLT) groups also had percentage of collagen III lower than the control young group and the LLLT aged group obtained higher percentage than the control aged group. However, at 14 days the control young group had lower amount of collagen than the control aged and LLLT aged groups, and the LLLT aged group had a lower percentage than the control aged group (Fig. 2).

Effect of PBMT on metalloproteinase 3 (MMP-3)

At 3 and 7 days post-wounding the control young group showed a significant lower percentage of MMP-3 compared the control aged and LLLT aged groups, However, the LLLT aged group showed a significant decrease compared the control aged group. At 14 days, the aged (control and LLLT) groups showed a significantly higher percentage than the control young group, however LLLT aged group showed significantly lower percentage than the control aged group (Fig. 3)

Effect of PBMT on metalloproteinase 9 (MMP-9)

The percentage of MMP-9 in control young group showed to be significantly lower that the control aged and LLLT aged groups at 3 and 7 days post-wounding, however LLLT reduced significantly the percentage of the LLLT aged group compared with control aged group. At 14 days, the control young group showed a significant decrease percentage compared aged (control and LLLT) groups and LLLT aged group showed significant decrease compared control aged group, proving the LLLT biomodulatory effects (Fig. 4).

Effect of PBMT on TIMP-2

At 3 days post-wounding, the control young group showed significantly higher expression of TIMP-2 than aged (control and LLLT) groups and LLLT aged group showed higher expression than the control aged group. At 7 and 14 days, control young group had significantly higher expression than the control aged and the LLLT aged groups.

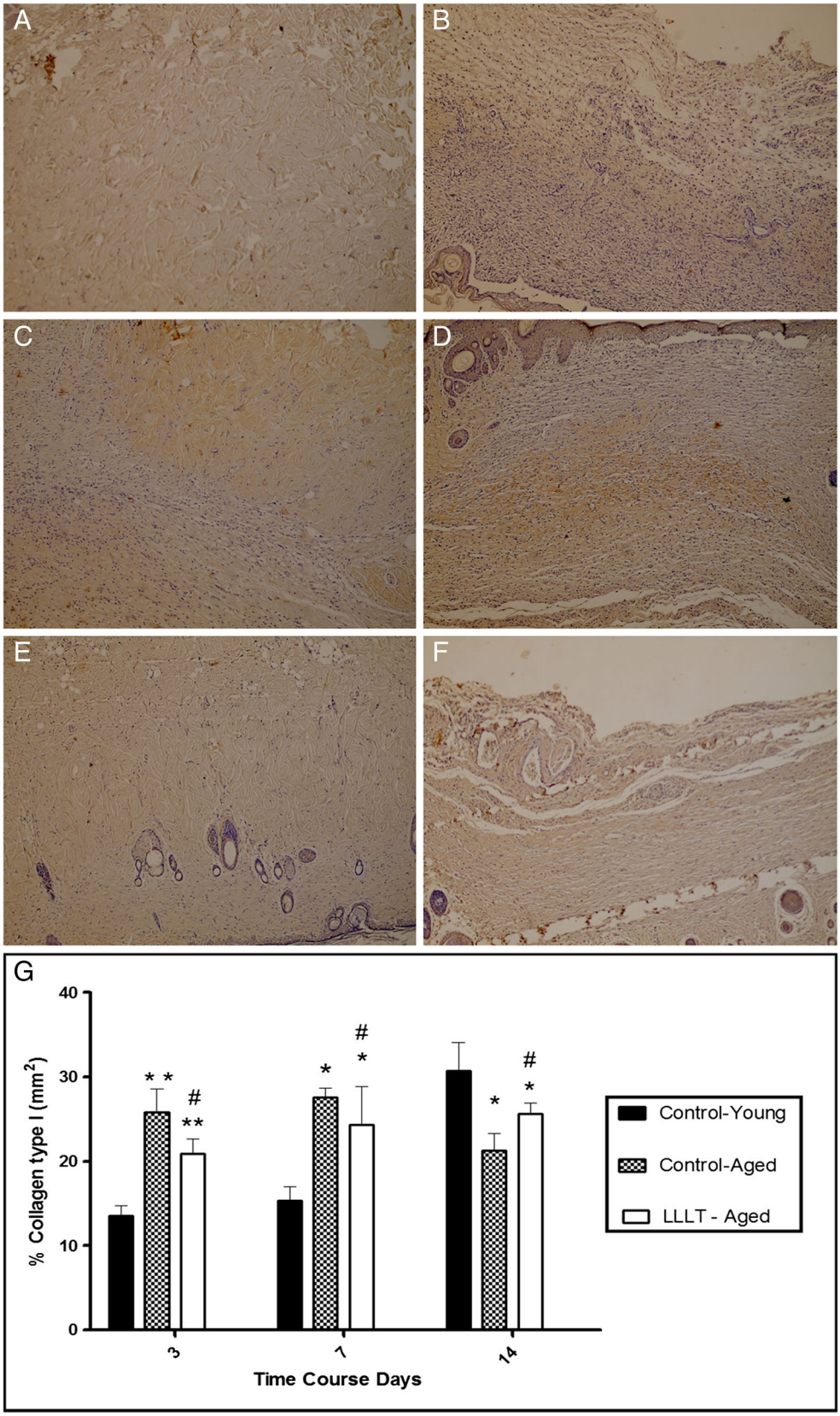

Fig. 1 **a** (control-young) and **b** (LLLT-aged) represent concentrations of collagen type I in wounds 3 days after injury. **c** (control-young) and **d** (LLLT aged) represent concentrations in wounds 7 days after injury. **e** (control young) and **f** (LLLT-aged) represent concentrations in wounds 14 days after injury. **g** The comparisons of the mean and standard deviation concentrations of collagen type I over 3, 7, and 14 days after preparation of the wound healing. $^{*}p < 0.05$, $^{**}p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group. The sections were viewed with a ×10 objective. *Scale bar*, 0.01 mm

However, despite the aged groups have lower expression than the control young group, the LLLT aged group showed significantly higher expression of TIMP-2 than the control aged group (Fig. 5).

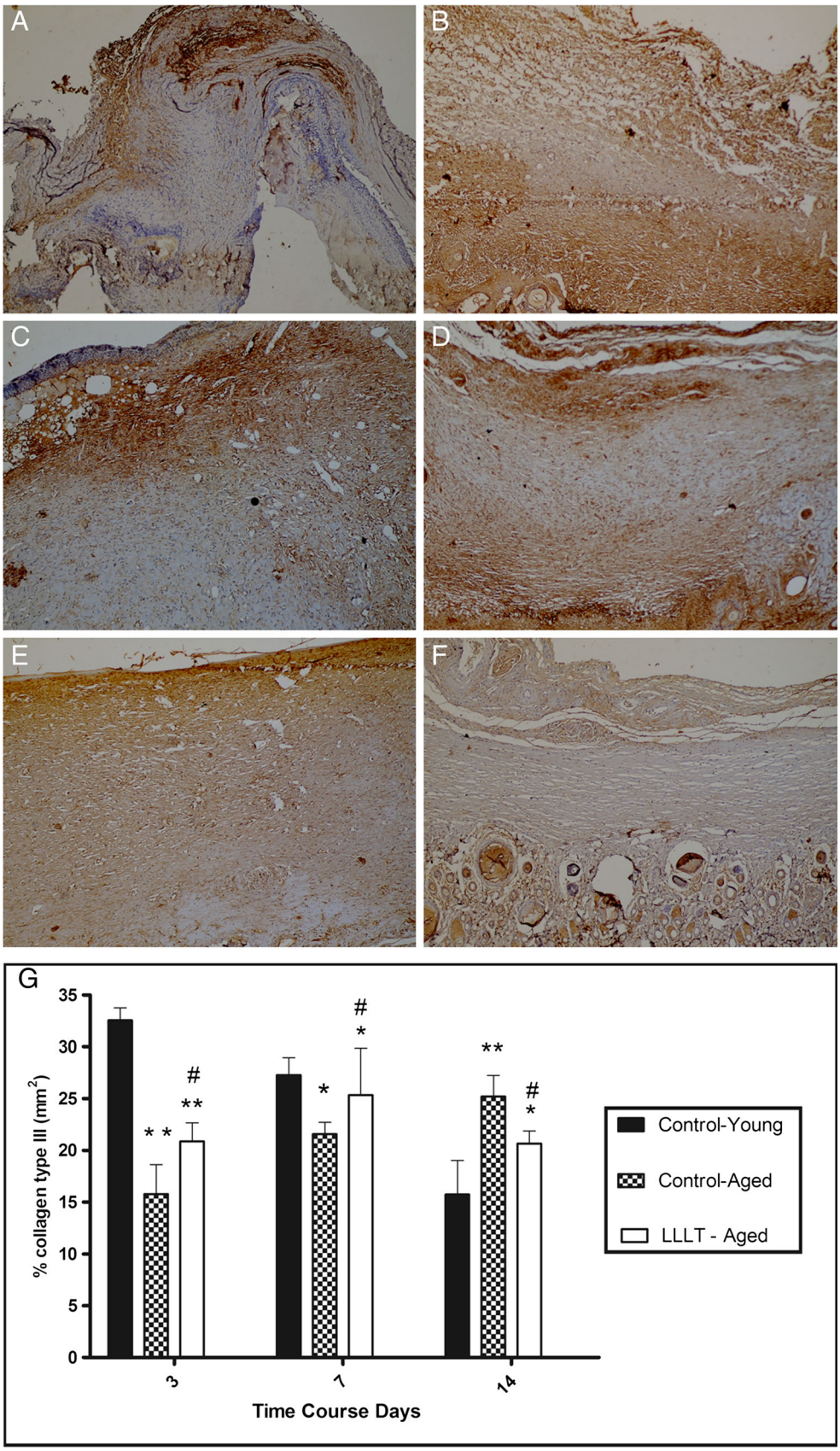

Fig. 2 **a** (control-young) and **b** (LLLT-aged) represent concentrations of collagen type III in wounds 3 days after injury. **c** (control-young) and **d** (LLLT aged) represent concentrations in wounds 7 days after injury. **e** (control young) and **f** (LLLT-aged) represent concentrations in wounds 14 days after injury. **g** The comparisons of the mean and standard deviation concentrations of collagen type III over 3, 7, and 14 days after preparation of the wound healing. $^{*}p < 0.05$, $^{**}p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group. The sections were viewed with a ×10 objective. *Scale bar*, 0.01 mm

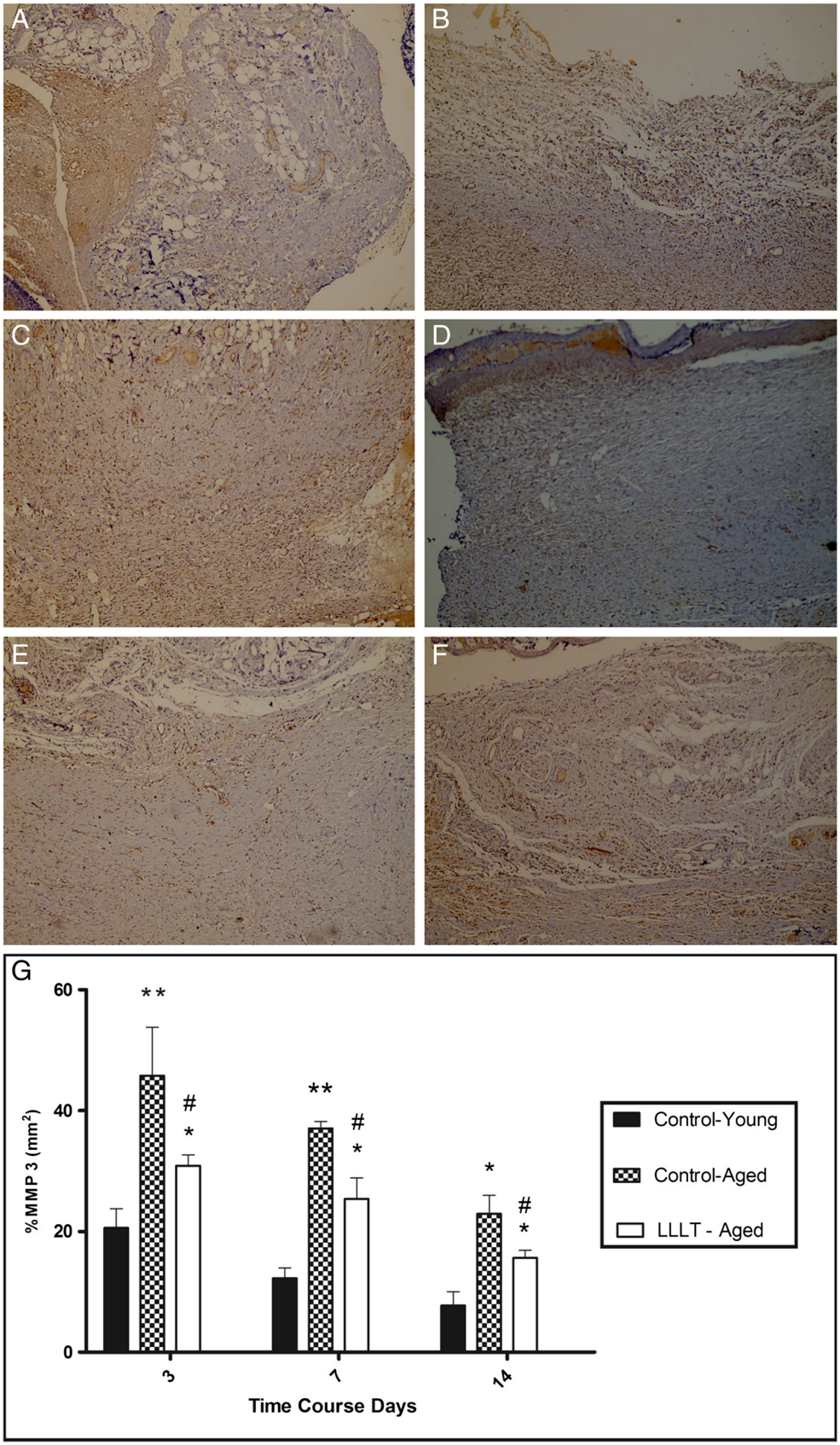

Fig. 3 **a** (control-young) and **b** (LLLT-aged) represent concentrations of MMP-3 in wounds 3 days after injury. **c** (control-young) and **d** (LLLT-aged) represent concentrations in wounds 7 days after injury. **e** (control-young) and **f** (LLLT-aged) represent concentrations in wounds 14 days after injury. **g** The comparisons of the mean and standard deviation concentrations of MMP-3 over 3, 7, and 14 days after preparation of the wound healing. $*p < 0.05$, $**p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group. The sections were viewed with a ×10 objective. *Scale bar*, 0.01 mm

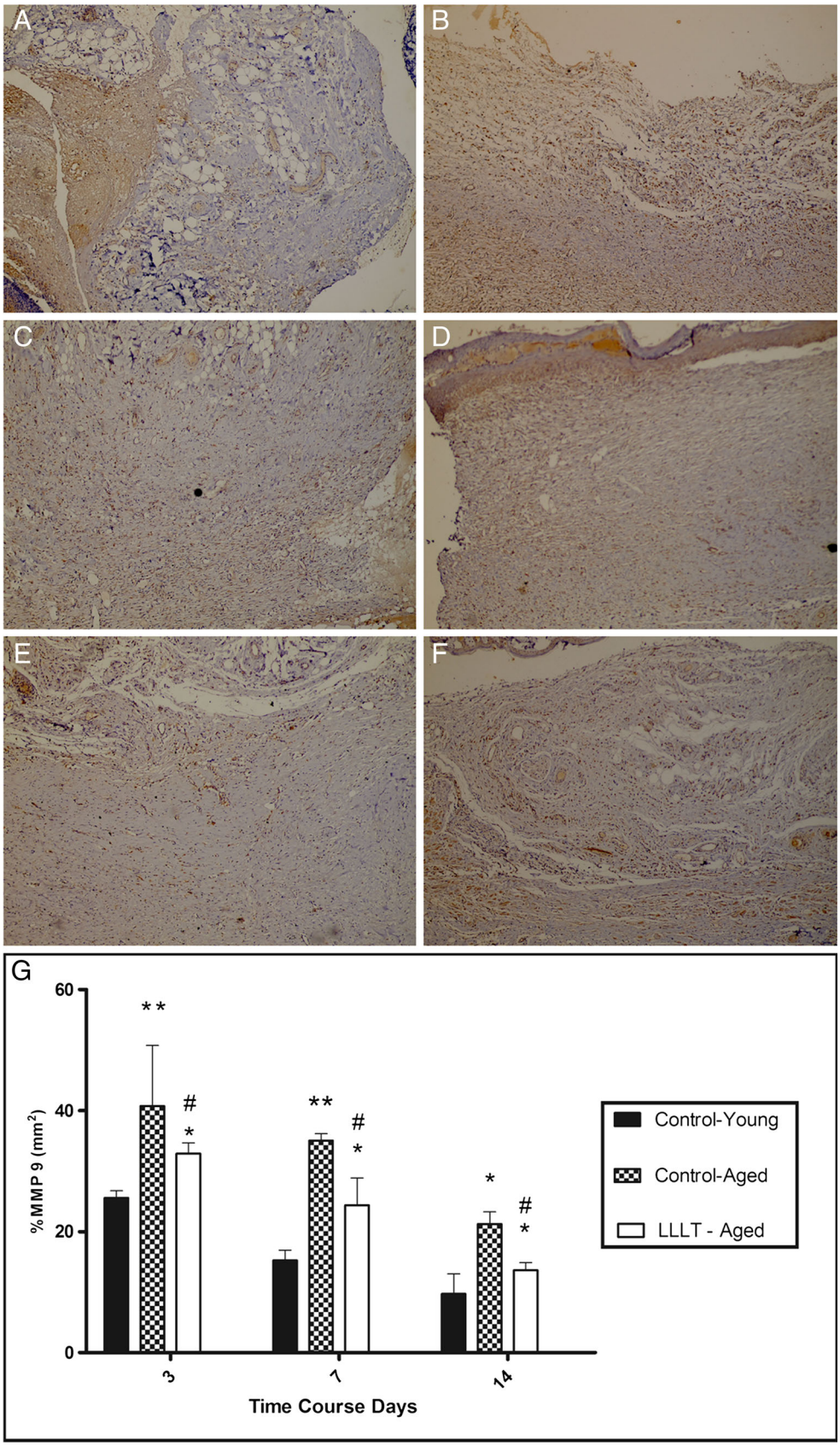

Fig. 4 **a** (control-young) and **b** (LLLT-aged) represent concentrations of MMP-9 in wounds 3 days after injury. **c** (control-young) and **d** (LLLT-aged) represent concentrations in wounds 7 days after injury. **e** (control-young) and **f** (LLLT-aged) represent concentrations in wounds 14 days after injury. **g** The comparisons of the mean and standard deviation concentrations of MMP-9 over 3, 7, and 14 days after preparation of the wound healing. $*p < 0.05$, $**p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$ using Tukey's test with comparisons against the control age group. The sections were viewed with a ×10 objective. *Scale bar*, 0.01 mm

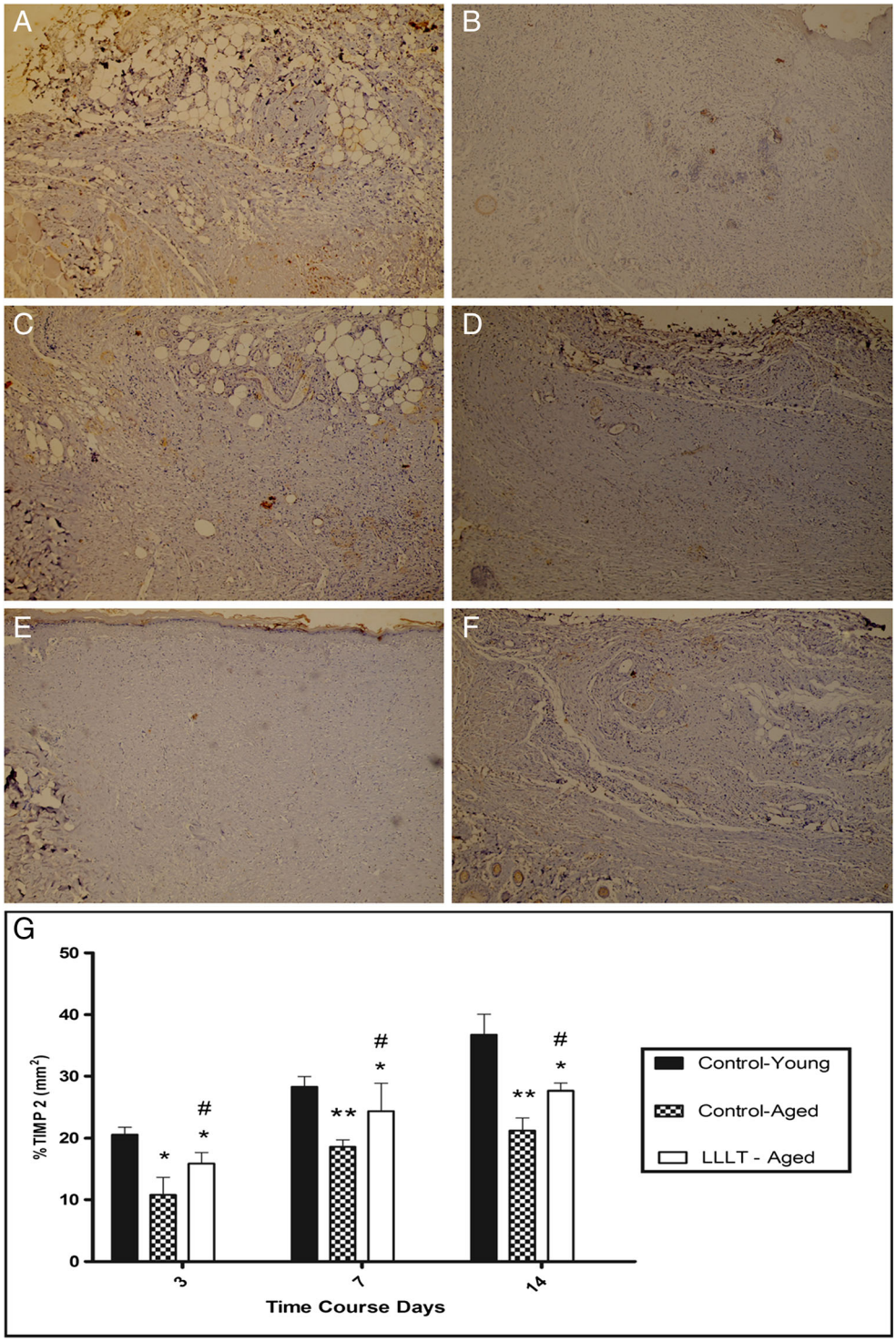

Fig. 5 **a** (control-young) and **b** (LLLT-aged) represent concentrations of TIMP-2 in wounds 3 days after injury. **c** (control-young) and **d** (LLLT-aged) represent concentrations in wounds 7 days after injury. **e** (control-young) and **f** (LLLT-aged) represent concentrations in wounds 14 days after injury. **g** The comparisons of the mean and standard deviation concentrations of TIMP-2 over 3, 7, and 14 days after preparation of the wound healing. $*p < 0.05$, $**p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group. The sections were viewed with a ×10 objective. *Scale bar*, 0.01 mm

Effect of PBMT on vascular endothelial growth factor (VEGF)

The control young group showed a significantly higher percentage of VEGF compared with control aged group in 3 and 7 days, and at 14 days showed significantly lower percentage compared with the same group. When compared with LLLT aged group showed significant increase in 3 and 7 days, and at 14 days showed no statistical difference. The LLLT aged group showed a significant increase in the percentage of VEGF at 3 and 7 days compared with control aged group, and at 14 days showed a significant decrease compared with same group, getting approximate values to the control young group (Fig. 6).

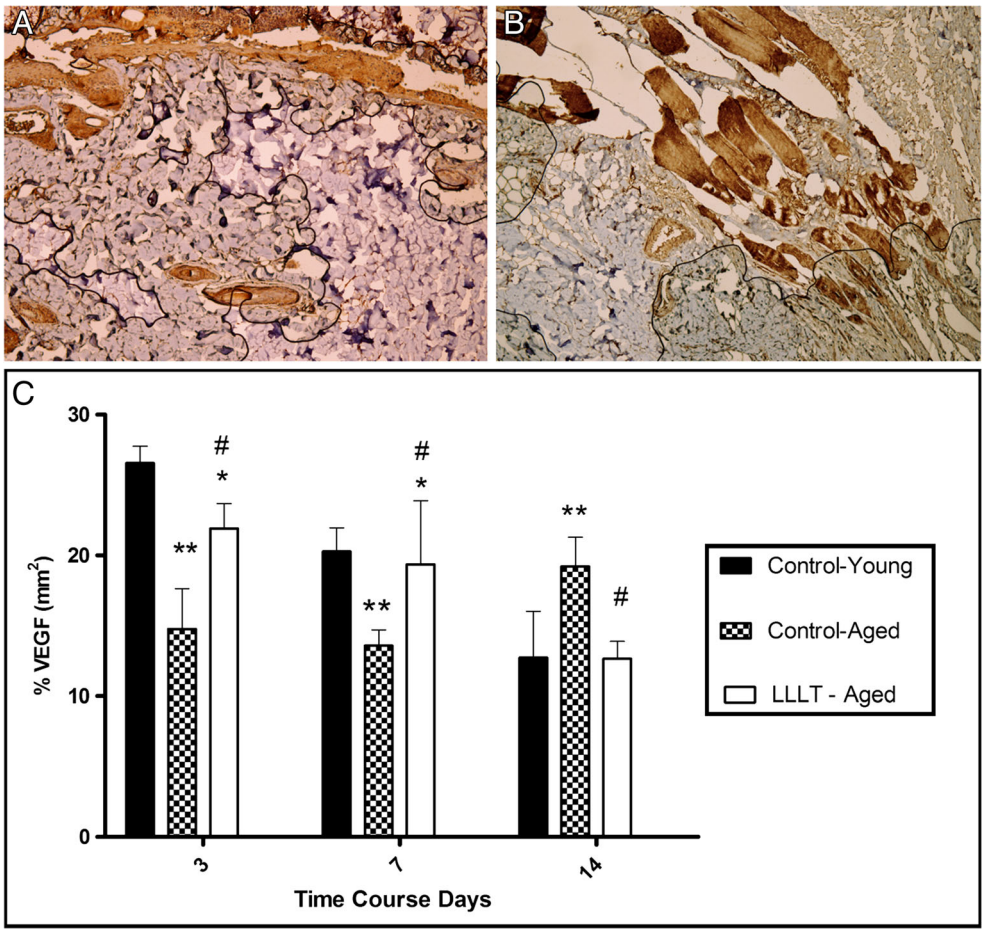

Fig. 6 **a** (control-young) and **b** (LLLT-aged) represent concentrations of VEGF in wounds 7 days after injury. **c** The comparisons of the mean and standard deviation concentrations of VEGF over 3, 7, and 14 days after preparation of the wound healing. *$p < 0.05$, **$p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group. The sections were viewed with a ×10 objective. *Scale bar*, 0.01 mm

Effect of PBMT on CINC-1 protein expression

The control young group showed a significant decrease in the concentration of IL-8 (CINC-1) compared with the control aged and LLLT aged groups at 3 and 7 days post-wounding. At 14 days showed a significant decrease compared to aged (control and LLLT) groups, however, LLLT administration led to a significant decrease in expression of IL-8 protein when comparing the aged (control and LLLT) groups, in 3, 7, and 14 days after injury (Fig. 7).

Effect of PBMT on IL-6 mRNA expression in wound healing

Figure 8 shows a statistically significant increase of IL-6 mRNA expression between aged control group and control young group in 3, 7, and 14 days after injury. When compared the LLLT aged and control young groups, also occurred a significant increase on day 3 and on days 7 and 14. Although the aged groups showed higher expression compared the control young group, LLLT promoted a significant decrease expression in LLLT aged group on days 3, 7, and 14 days.

Discussion

The present study was designed to seek possible explanations as to whether the behavior of the healing process in elderly animals may be similar to that of the young animals already studied in previous work by our research group [16]. We observed that the response to PBMT was different in aged rats compared with young rats. The present study was conducted to analyze the gene expressions of pro-inflammatory cytokine (IL-6) protein expression and a cytokine-induced neutrophil chemoattractant (CINC-1), a chemokine that belongs to the interleukin 8 family, and immunohistochemical expression of VEGF, MMP-3, MMP-9, TIMP-2, collagen type I and III.

The main results of this study included the following: The behavior of the repair process occurred differently between young and aged rats, with an obvious delay in the aged groups, as was verified by analysis of collagen and the expression of inflammatory mediators. The aged group treated with PBMT showed an increased rate of wound repair process, compared with their respective control group; however, the LLLT aged group still showed a lag in repair compared with the control young group, mainly regarding the inflammatory phase and proliferation, angiogenesis, and fibrogenesis.

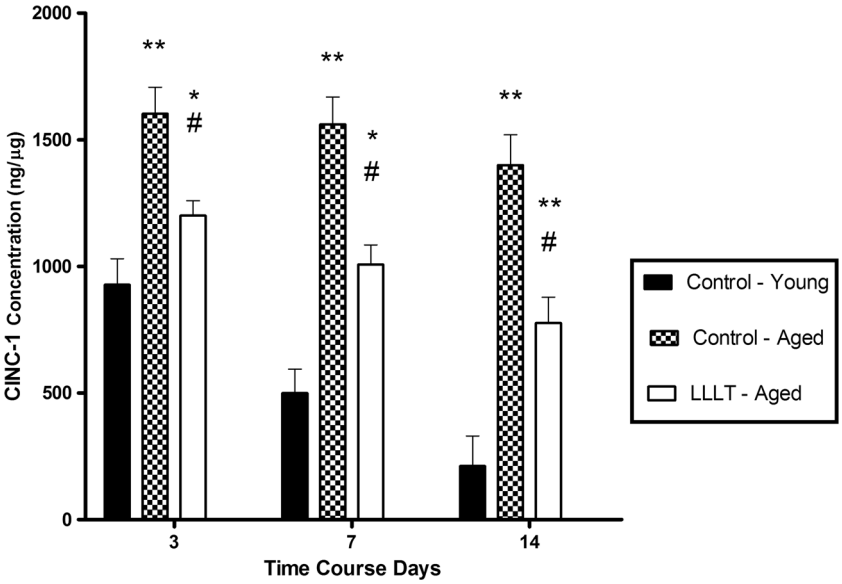

Fig. 7 Comparisons of the mean and standard deviation levels of IL-6 in skin wound tissue over 3, 7, and 14 days after preparation of the wound healing. *$p < 0.05$, **$p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group

The early response to wounding also results in the release of chemokines by keratinocytes, which act as chemoattractants for the migration of immune cells to the site of injury. Neutrophils arrive at the wound within minutes of wounding and become the predominant cells in the wound site for the first 2 days after the injury occurs. Neutrophils and platelets entrapped and aggregated in the blood clot release a wide variety of factors that amplify the aggregation response, initiate the coagulation cascade, and/or act as chemoattractants for cells involved in the inflammatory phase. Among other proinflammatory cytokines, IL-6 is produced by neutrophils and has been shown to be important in initiating the healing response; IL-6 has mitogenic and proliferative effects on keratinocytes and, at the same time, acts as a chemoattractant for neutrophils [17]. CINC-1 (a homolog of human IL8) expression increased the migration and proliferation of keratinocytes, induces the expression of MMPs and it is a strong chemoattractant for neutrophils [7].

According to Ershler and Keller [18, 19], IL-6 is normally expressed at low levels except during infection, trauma or other stress factors. Among the many factors that regulate the expression of the IL-6 gene are estrogen and testosterone. After menopause or andropause, levels of IL-6 are elevated in the absence of infection, trauma or stress.

After tissue injury, IL-6 expression since increases in the 15 to 20 min of injury, with peak levels in 24–48 h, gradually reducing in 7 days [17]. CINC-1 shows increased expression in the first hour after injury, down to 24 h of follow-up, when it starts an increase up to 48 h and from 7 days ago decreased expression [20]. The three groups of our study showed higher expression of IL-6 and CINC-1 in the initial phase of the repair process, to decrease over time, however, the aged groups showed high values IL-6 and CINC-1 and that PBMT was effective in decreasing the expression of these inflammatory mediators, throughout the experimental period. However, it is observed that although PBMT have attenuated the levels of IL-6 and CINC-1 in the irradiate group, the expression was higher than the control young group. This finding suggests a new proposal for LLLT dosimetry, which may be used in aged animals

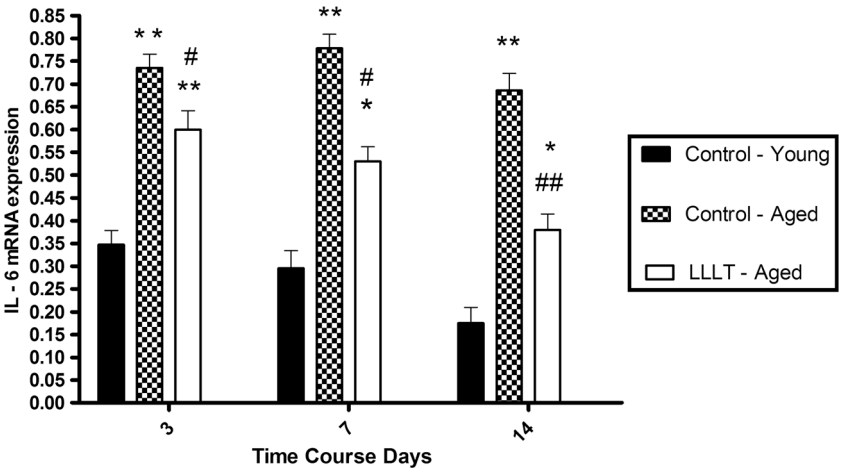

Fig. 8 Comparisons of the mean and standard deviation levels of CINC-1 (a homolog of human IL-8) in skin wound tissue over 3, 7, and 14 days after preparation of the wound healing. *$p < 0.05$, **$p < 0.001$—using Tukey's test with comparisons against the control young group; $^{\#}p < 0.05$—using Tukey's test with comparisons against the control age group

to achieve better results. This finding reinforces the results achieved by Rambo et al. [16] similar to ours in that compared the levels of pro-inflammatory cytokines (IL-1 and TNF) in young and old animals, and these suggest a differentiated dosimetry of PBMT for treatment of young and aged.

The presence of elevated levels of IL-6 and IL-8 in the older age groups was also found by Sansoni et al. [21] showing a chronic inflammatory state.

The potential of PBMT in modulating inflammatory cytokines among them the IL-6 and CINC-1 is clear in the study. Alves et al. [19] found that PBMT reduces IL-6 expression in joint inflammation in rats. On human cell cultures, the red laser increased the migration and proliferation of keratinocytes and the granulation tissue, by modulating IL-8 expression promoting more rapid re-epithelialization [22]. Lima et al. [23] reported that PBMT (GaAlAs, continuous, 9 mW, 670 nm, 0.031 W/cm^2) significantly decreased IL-6 levels, strongly suggesting an inflammatory biomodulation of LLLT.

Ambrosch et al. [24] found that the reduction of inflammation reduces IL-6 concentration, followed by decreased levels of MMPs and TIMP increased. MMPs expression is regulated by interleukins, TIMPs and other factors and play essential roles in all phases of the healing process. They degrade all components of the extracellular matrix, stimulate migration of epithelial cells, fibroblasts, vascular endothelial cells and synthesize collagen and other members of the ECM [8, 10].

MMP-3 has anti-inflammatory effect, increase cell migration into the damaged tissue as well as degrading proteoglycan, fibronectin, collagen type III and IV [10] and can also enhance the proteolytic effect of MMP-9, which acts on the type IV collagen, elastin and collagen denatured [25].

In our study, the MMP-3 and MMP-9 expression was higher in aged groups (control and LLLT) in all phases of repair compared to the control young group, however the aged group treated with LLLT showed expression below its old control showing PBMT capacity of reducing MMP-3 and MMP 9 expression. MMPs long overexpression degrades essential proteins for the healing process, leading to delay the healing process or abnormal scar formation [26]. Thus, MMPs modulation is essential in the process of repair and LLLT is effective in this modulation.

High levels of MMPs can also degrade the growth factors such as PDGF, EGF, VEGF, and consequently there is a decrease or absence of proliferation essential cells for tissue replacement, such as fibroblasts which has function of synthesize collagen [27]. TIMPs function modulates the activity of MMPs and especially TIMP-2 has strong attraction to MMP-3 [10, 28].

In our study, we observed that in the control young group, TIMP-2 expression was increased in relation to age groups (control and LLLT), and against MMP-3 and MMP-9 was decreased in all phases of repair. This proves that the TIMP modulates MMP expression. However, it is observed that although the expression was lower in the control young group, the PBMT induced a higher TIMP-2 expression in the LLLT aged group compared to the control aged group showing the effect of PBMT in biomodulatory TIMP and MMPs expression.

Studies show that IL-6 [29], IL-8 [30] and MMP-9 [31] are involved in angiogenesis due to their interaction with the endothelial growth factor (VEGF) in the formation of new blood vessels.

The angiogenesis is a critical and complex event in the wound-healing process. It depends on both angiogenic growth factors present and ECM components participating in granulation tissue and in microvascular vessels. The cooperative regulation of the activities of growth factors and the production of ECM components is essential for wound repair. Vascular endothelial growth factor (VEGF or VEGFA), which exerts its biological activity predominantly on endothelial cells, is a key mediator of angiogenesis [32].

In our study, the results of an analysis of VEGF immunohistochemistry demonstrated that, although the control young group had a higher VEGF expression than older groups at all stages of repair, PBMT increased expression in the elderly group treated at 3 and 7 days. Another factor observed at 14 days following injury the elderly control group reached levels of VEGF higher than other groups, leading us to believe in the process of these aged animals delay.

Our results for the effects of PBMT on VEGF are similar to those obtained by Colombo et al. [33], who investigated angiogenesis in the dorsal cutaneous wounds of rats treated with laser (λ660 nm, 16 mW, 10 J/cm^2). They concluded that laser treatment (λ660 nm) contributed to increased angiogenesis.

Szymanska et al. [34] evaluated the laser effects on the vascular endothelial proliferation in vitro and VEGF secretion, and they concluded that PBMT with wavelength 635 nm increases endothelial cell proliferation. Significant increase in endothelial cell proliferation and corresponding decrease in VEGF concentration may suggest the role for VEGF in the healing process. In addition, Galiano et al. [35] assert that therapeutic restoration of VEGF has been shown to improve significantly repair outcomes, and PBMT proves efficient in modulating VEGF.

Angiogenesis restores the level of both oxygen and nutrients for the newly forming tissue, supplying the high metabolic demand, favoring protein synthesis as well as cell proliferation and migration, such as fibroblasts, which is one of the functions synthesize collagen [33].

We observed that in the control young group, the collagen I and III showed a normal course in the repair process, with greater deposition of collagen III and consequently less deposition of collagen I at the early phase, and increased deposition of collagen I and lower collagen III in the late phase of the process. In aged groups, we found that there was a delay in the deposition of collagen type I and III when compared to the

control young group, however PBMT was able to reduce this delay in the LLLT aged group, especially in collagen III in the early stages of repair and collagen I at 14 days after injury.

The less collagen deposition in aged groups may be related to the small number of fibroblasts found in aged skin [36]. The improvement in collagen deposition in the LLLT aged group can be explained by the fact that the laser helps the proliferation of fibroblasts [16], so there will be more collagen deposition.

Increased collagen deposition at the beginning of the healing process with PBMT was also found by Biondo-Simões and Busnardo [37], with increased deposition of collagen III in the third postoperative day and by Silva et al. [15] in wounds of diabetic rats with an increase in the total percentage of type III collagen.

Other studies have found increased collagen deposition with laser use. Fiorio et al. [38] found increase collagen deposition in third-degree burns in rats. Carvalho et al. [39] demonstrated that the application of low intensity He-Ne laser (λ632.8 nm) promoted increase in the percentage of collagen in skin of diabetic rats wounds by increasing the amount of collagen fibers similar to process observed in animals non-diabetic efficacy, indicating of PBMT effects in the healing process.

The healing process involves the coordinated efforts of several cell types such as cytokines, chemokines, metalloproteinases and their inhibitors, growth factors and fibroblast that will deposit collagen in the wound. According to the results of this study, we conclude that even in aged tissue there is delayed healing, this tissue satisfactorily responded to PBMT with modulation of inflammatory mediators IL-6, CINC-1, MMP-3, MMP-9, TIMP-1 and VEGF as well as increased collagen production in aged animals during different phases of the tissue regeneration process. In view of the obtained results, we can predict that these results are especially important to the establishment of therapeutic strategies in elderly patients with the imminent risk of presenting problems during the healing of cutaneous lesions. Our results also lead us to believe that PBMT can be used as an important tool in the early stages of tissue repair favoring the proliferation and remodeling phases of these lesions.

Compliance with ethical standards

Role of the funding source This work was supported by grants from the São Paulo Research Foundation (FAPESP, grant number (2015/13677-4) and the National Council for Scientific and Technological Development - CNPq (grant number 309065/2015-1).

Conflict of interest Professor Ernesto Cesar Pinto Leal-Junior receives research support from Multi Radiance Medical (Solon, OH - USA), a laser device manufacturer. Multi Radiance Medical had no role in the planning of this study, and the laser device used was not theirs. They had no influence on study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remaining authors declare that they have no conflict of interests.

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