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ORIGINAL ARTICLE

Lycium barbarum polysaccharide fraction associated with photobiomodulation protects from epithelium thickness and collagen fragmentation in a model of cutaneous photodamage

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Abstract

Ultraviolet radiation (UVR) is the major etiologic agent of cutaneous photoaging, and different strategies are used to prevent and treat this condition. The polysaccharide fraction (LBPF) isolated from *Lycium Barbarum* fruits (goji berry) contains several active ingredients with antioxidant, immune system modulation, and antitumor effects. In addition, the photobiomodulation (PBM) is widely applied in photoaging treatment. This study investigated the effects of LBPF and PBM against the UVR-induced photodamage in the skin of hairless mice. The mice were photoaged for 6 weeks in a chronic and cumulative exposure regimen using a 300-W incandescent lamp that simulates the UVR effects. From the third to the sixth week of photoaging induction, the animals received topical applications of LBPF and PBM, singly or combined, in different orders (first LBPF and then PBM and inversely), three times per week after each session of photoaging. After completion of experiments, the dorsal region skin was collected for the analysis of thickness, collagen content, and metalloproteinases (MMP) levels. A photoprotective potential against the increase of the epithelium thickness and the fragmentation of the collagen fibers was achieved in the skin of mice treated with LBPF or PBM singly, as well as their combination. All treatments maintained the skin collagen composition, except when PBM was applied after the LBPF. However, no treatment protected against the UVR-induced MMP increase. Taken together, we have shown that the LBPF and PBM promote a photoprotective effect in hairless mice skin against epidermal thickening and low collagen density. Both strategies, singly and combined, can be used to reduce the UVR-induced cutaneous photoaging.

Keywords Lycium barbarum · Polysaccharides · Photobiomodulation · Photodamage · Hairless mice

Introduction

The skin is regularly exposed to conditions of direct oxidative damage, including ultraviolet radiation (UVR) from the sun [1]. UVR-induced photodamage causes undesirable changes in skin appearance that over time lead to increased or accelerated physiological reserve loss and protection damage [2]. The UVR initiates a complex cascade of biochemical reactions in

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the skin by activating keratinocyte and fibroblast cell surface receptors. This event triggers a signal transduction cascade, which promotes a variety of molecular changes such as breakdown and inhibition of collagen synthesis in the extracellular matrix (ECM) [3]. UVR is the main physical agent promoting skin carcinogenesis, which induces DNA damage by activating signaling pathways and altering the cell cycle, leading to apoptosis [4].

Several secondary plant metabolites have been studied to prevent, delay, and control the development of age-related pathologies [5]. Some natural compounds exhibit UVR absorption properties and act as antioxidants, reducing the harmful effects of photo exposure and increasing the antioxidant capacity of skin cells [6].

Lycium barbarum fruits, also called as goji berry or wolfberry, are used in traditional Chinese medicine for over 2500 years [7]. Several pharmacological and phytochemical studies using polysaccharide fractions extracted from the fruit of *Lycium barbarum* (LBPF) demonstrated that they have antioxidant [8, 9] and antitumor effects [10], besides participating in the modulation of the immune system [11].

Physical agents are also employed as adjuncts to facilitate tissue repair. They include microcurrent application [12] and photobiomodulation (PBM) [13]. The therapeutic effects of PBM on various biological tissues have been shown in both in vitro and in vivo studies, demonstrating the reduction of inflammatory cells [14], increase of fibroblasts number [15], and increase in collagen synthesis [13].

The PBM was discovered in the late 1960s but has been extensively applied in dermatology [16]. This therapy has proven to be a useful tool for the modulation of cellular metabolism [17], and its association with natural antioxidant extracts appears to be a valuable strategy in protecting the skin against UVR-induced photodamage.

Currently, great clinical interest has been given on the non-invasive therapies that may solve or minimize the UVR effects on cutaneous aging process, which can cause tumor lesions. In this sense, the purpose of this study was to investigate the isolated and associated effects of PBM (red wavelength) and a polysaccharide fraction extracted from the fruit of *Lycium barbarum* (LBPF) in the reduction of UVR-induced photodamage of the skin of hairless mice.

Materials and methods

Ultraviolet irradiation on the hairless mice skin

The procedure of ultraviolet irradiation on the skin of the animals was performed with a chronic and cumulative exposure regimen using an incandescent lamp *Ultra Vitalux* 300 W (OSRAM, Augsburg, Germany). The lamp was subjected to irradiance testing with the aid of Ocean optics spectrasuite® software. In this study, an irradiance of 0.1 mW/cm^2 of UVB (280 to 315 nm), 0.8 mW/cm^2 of UVA (315 to 480 nm) and 3.89 mW/cm^2 of lamp total irradiance (280 to 886 nm), with a distance of 70 cm between the lamp and the dorsal region of the animals, was applied during the sessions. All experimental groups, except for the CTRL group, were exposed to 100 mJ/cm^2 (1 erythematous minimum dose = 100 mJ/cm^2), seven times per week during the first week for 16 min/session, and then at 200 mJ/cm^2 three times per week for 5 weeks for 33 min/session, totaling 6 weeks of photo exposure, according to a previously described protocol adaptation [18, 19].

Animals and treatments

HRS/J female mice, aged approximately 8 weeks, from the Federal University of Mato Grosso do Sul—Brazil, were kept in individual polycarbonate cages at constant temperature (23 ± 2 °C) and humidity (55%), under light/dark cycle of 12:12 h, with free access to standard commercial feed and potable water. The animals were randomly divided into 6 subgroups (n = 8 per group): CTRL—animals that were not photoaged; PC—animals that were photoaged; LBPF—animals that were photoaged and treated with the LBPF; PBM—animals that were photoaged and treated with photobiomodulation (red wavelength); LBPF + PBM—animals that were photoaged, treated with LBP fraction and then with PBM; and PBM + LBPF—animals that were photoaged, treated with PBM and then LBPF. During the first and second week of ultraviolet irradiation, the animals did not receive any type of treatment; they occurred from the third week of irradiation and finished in the sixth week. Treatments of LBPF, PBM, LBPF + PBM, and PBM + LBPF groups were performed three times per week after each session of photoaging induction for 4 weeks (from 3rd to 6th week).

The gel formulation of the LBPF at 5% was performed by the addition and homogenization of 5 g of polysaccharides isolated from *Lycium barbarum* fruit and 2.5 g of hydroxyethylcellulose in 95 mL of ultrapure water. A dose/time (50 mg) of the LBPF 5% was applied to an area of 3 cm × 2 cm on the dorsal region skin of the animals with the aid of a spatula. Then, it was spread gently, without rubbing, with the tip of the index finger, until its total absorption. Each PBM treatment session was performed in the same area following parameters: Photon Lase III (DMC, São Carlos, SP, Brazil), properly calibrated, with a wavelength of 660 nm (red wavelength); beam area of 0.028 cm^2; 40 mW/cm^2 of power; and fluence of 40 J/cm^2, with application time of 28 s and 1.12 J/point (6 points). Two days after completion of the treatments, all animals were euthanized, and the dorsal region skin of each animal was collected and fixed in 10% buffered formalin for

24 h, then washed in running water for 1 h and stored in alcohol 70%.

Histology

The sections were dewaxed and stained with hematoxylin-eosin for the analysis of epidermal thickness and structural organization and picrosirius red for the quantification of birefringent collagen fibers. The images were captured and scanned through of a photomicroscope (Olympus SC30), and the measurements of epidermal thickness were performed with the Cellsens Standard® software, and the quantitative analysis of the area percentage of birefringent collagen fibers was performed using ImageJ® software.

Immunofluorescence

Paraffin sections with a thickness of 5 μm were deparaffinized, hydrated, and mounted in silanized slides. Antigenic recovery was performed by incubating the slides in citrate buffer (pH 6.0) with the aid of a steam pan for 40 min. Thereafter, the slides were cooled, washed three times (PBS buffer—5 min/time), dried, and incubated with 0.5% BSA and 10% donkey serum in PBS for 2 h to block non-specific binding sites. After three washes with PBS buffer for 5 min each, the slides were incubated in primary antibodies: anti-MMP1 (Abcam Ab137332, 1:100), anti-MMP2 (Abcam Ab37150,1:100), and anti-MMP9 (Abcam Ab38898, 1:100), diluted in 0.5% BSA (PBS) in a humid chamber at 4 °C (18 h). Afterwards, the slides were washed and incubated with the secondary antibodies: Alexa Fluor 488 (Life Technologies A11034, 1:500) and Alexa Fluor 568 (Life Technologies A11011, 1:500), diluted in 0.5% BSA (PBS) in a light-protected wet chamber, for 1 hour at room temperature. The slides were then washed three times with PBS and mounted with a solution containing DAPI (Ab104139, Abcam) to stain nuclei. Images for the quantification of the markers by immunofluorescence were obtained with a fluorescence microscope (BX61, Olympus) and Cellsens Dimension® software. The light exposure in the images was standardized, and the measurements, based on pixel integrated density (IntDen), were performed in three different fields per section on each slide using the ImageJ® software.

Statistical analysis

To compare the groups, the results were analyzed by ANOVA followed by Tukey post-test ($p < 0.05$) for parametric data, or Kruskal-Wallis with Dunn post-test for non-parametric data, using the Graph Pad Prism® 7.0 software.

Results

After the treatments, skin samples from animals were collected and submitted to histological processing. Representative sections of the skin from dorsal region of hairless mice were photographed and analyzed by light microscopy. The group of animals that were photoaged (PC) showed histological changes, such as discrete fiber fragmentation in the dermis region and thickening of the epidermis (Fig. 1a, b), when compared with the treated groups and the CTRL group. Results indicate significant differences ($p < 0.0001$) between photoaged control (PC) and treated groups (LBPF, PBM, LBPF + PBM, PBM + LBPF) (Fig. 1b). The treatments applied, both isolated (LBPF and PBM) and associated with PBM (LBPF + PBM and PBM + LBPF), presented a protective effect against increased epithelium thickness, a type of damage caused by chronic photo exposure to UVR. The results obtained after the treatments were similar to the CTRL group. The group treated with LBPF had a thinning of the epidermis and more organized collagen fibers on reticular dermis, with parallel bundles to the epidermis and longer, less fragmented fibers. In the group treated with the PBM (red wavelength), a discrete but not significant thickening and an increase of the density of collagen fibers in the dermis was observed, with a greater amount of mature fibers (Fig. 1a). In the groups that received association of treatments (LBPF + PBM and PBM + LBPF), the dermis assumed characteristics of each isolated treatment (LBPF and PBM), representing an intermediary phase between them regarding collagen formation and density, especially in the LBPF + PBM group in which the order of treatments may have induced a biomodulation of birefringent collagen fiber synthesis and degradation, as this was observed in picrosirius red staining (Fig. 2a). In general, all treatments seem to protect and preserve the dermis and the epidermis characteristics from photodamage induced by UVR, except for the LBPF + PBM group that promoted biomodulation in the reticular dermis region.

Histological analysis of the total dermal region sections stained with picrosirius red showed a difference in the quantification of birefringent collagen fibers by percentage of area, with a significantly less amount of collagen fibers in the PC group compared with the CTRL group ($p < 0.05$) (Fig. 2a, b). This result confirms one of the deleterious effects of UVR on skin, which is the increase in degradation and decrease in the amount of collagen fibers. On the other hand, except for the group of animals treated with the LBPF before PBM (LBPF + PBM), all the treatments applied during the photoaging process have effects on the maintenance of the amount of birefringent collagen fibers in the same levels as the control non-photoaged group (Fig. 2a, b). The group LBPF + PBM presented a smaller amount of birefringent collagen fibers when compared with the control group, which can indicate an inhibitory effect of collagen synthesis or increased collagen

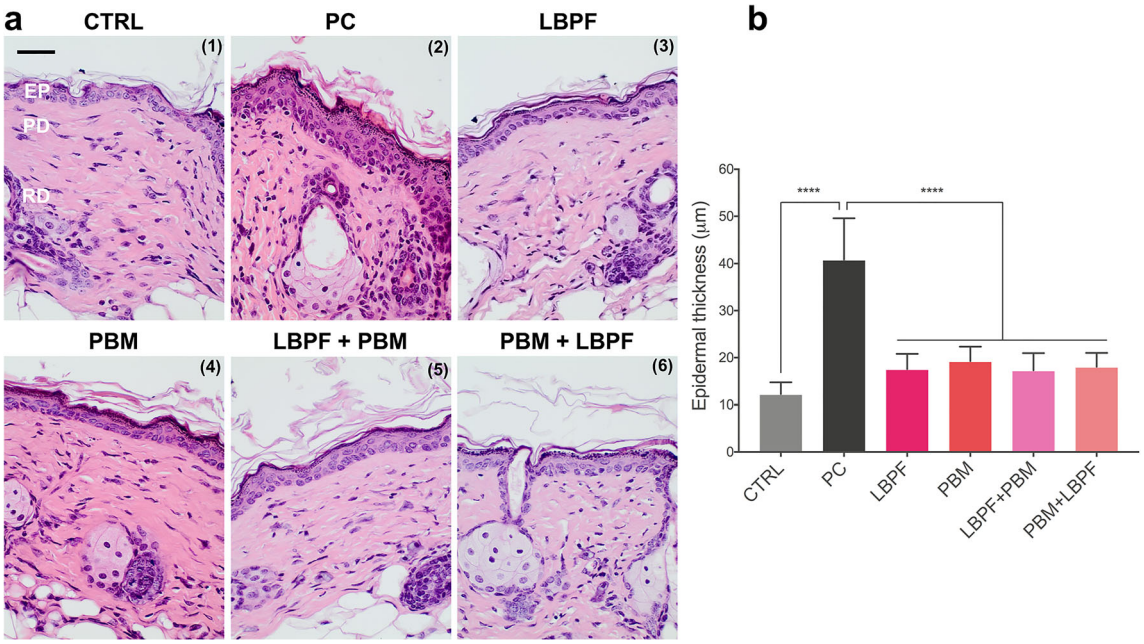

Fig. 1. Photoprotective potential of the LBPF and PBM (red wavelength). **a** CTRL (1)—animals that were not photoaged, PC (2)—animals that were photoaged, LBPF (3)—animals that were photoaged and treated with the LBPF, PBM (4)—animals that were photoaged and treated with photobiomodulation (red wavelength), LBPF + PBM (5)—animals that were photoaged and treated with LBP fraction and then with PBM, and PBM + LBPF (6)—animals that were photoaged and treated with PBM and then LBPF. Treatments of LBPF, PBM, LBPF + PBM, and PBM + LBPF occurred from the third week of irradiation and finished in the sixth week. HE, EP = epidermis, PD = papillary dermis, RD = reticular dermis, Bar = 20 µm. **b** Epidermis thickness after photoaging induction and treatments. The sections images were captured and scanned through of a photomicroscope (Olympus SC30). Values shown are mean ± SD, **** indicates significant differences ($p < 0.0001$) between photoaged control group (PC) and treated groups (LBPF, PBM, LBPF + PBM, PBM + LBPF). The results were analyzed by ANOVA and Tukey post-test for comparison between groups, using the GraphPad Prism® 7.0 software

fiber degradation due to the application of the LBP fraction before the PBM (Fig. 2a, b).

Despite MMP-1 expression has increased 19.8% in the PC group compared with the group of animals that were not photoaged (CTRL), this difference was not significant. Among the treated groups, there was no significant differences in the level of this enzyme, except for the PBM + LBPF group, where a significant increase in MMP-1 expression ($p < 0.05$) was observed compared with the LBPF and LBPF + PBM treatments (Fig. 3a, d). This difference indicates that the association of the treatments performed probably induced an increase in the expression of the MMP-1 enzyme due to the order of the treatments, since the same treatments applied inversely maintained the expression levels close to the control group (CTRL).

Similarly, the expression levels of the MMP-2 enzyme increased 20.5% in the PC group compared with the control group (Fig. 3b, e), but also without statistical significance. The treated groups maintained the levels close to those of the PC group, except for the group of animals treated with PBM and then with LBPF (PBM + LBPF) that presented a significant increase ($p < 0.0001$) on MMP-2 expression compared with the other treatments and PC group (Fig. 3e).

Significant differences between the control and PC groups were only found in the expression levels of the MMP-9 enzyme ($p < 0.0001$) (Fig. 3c, f). The findings indicate that the artificial induction of photoaging in the hairless mice promoted an increase in the expression of this enzyme. Similar to our findings for the expression of the MMP-2 the PBM + LBPF group presented an increase in the expression levels of the MMP-9, compared with the PC group ($p < 0.0001$). In summary, regarding MMP expression, the LBP fraction associated with PBM does not appear to have a beneficial effect in decreasing the levels of these metalloproteinases in the skin of hairless mice at the doses used in these experiments.

Discussion

Several studies on pharmacology and phytochemistry have demonstrated that one of the main active ingredients of *L. barbarum* is the LBPF [8], estimated to correspond to 5–8% of the dried fruits. LBPF has a molecular mass ranging from 24 to 242 kDa and consists of a complex mixture highly branched and partially characterized of polysaccharides and proteoglycans [20].

It was demonstrated that UVR activates cell surface receptors of keratinocytes and fibroblasts on the skin, inducing a cascade of signal transduction that in turn leads to a variety of

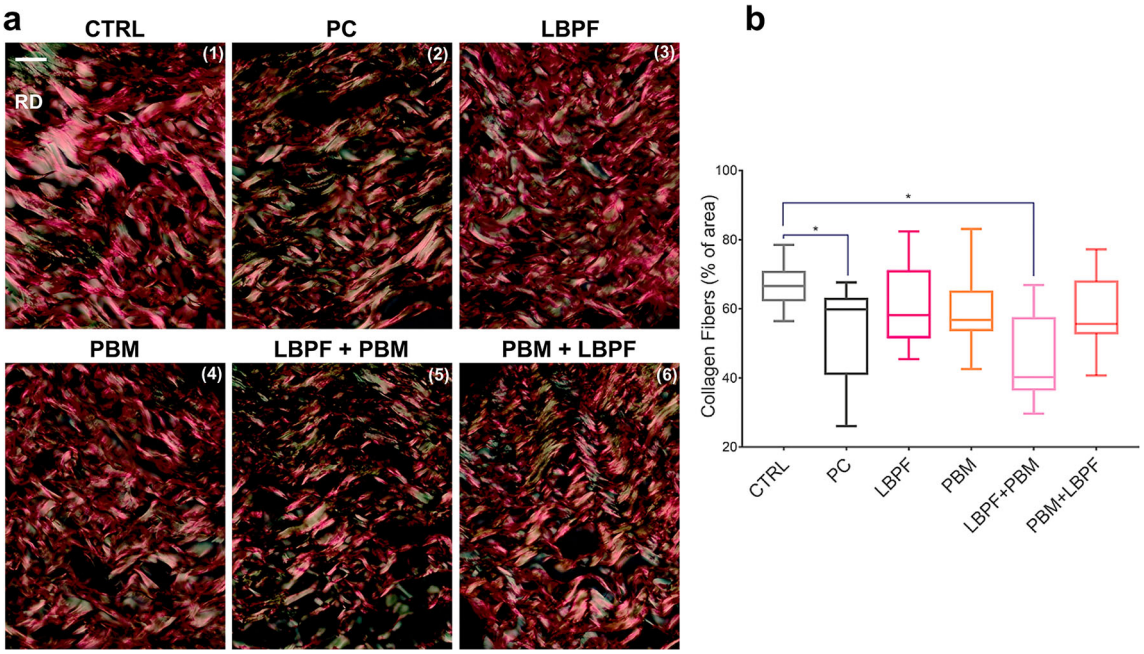

Fig. 2 Photomicrographs of picrosirius red stained sections. **a** CTRL (1)—animals that were not photoaged, PC (2)—animals that were photoaged, LBPF (3)—animals that were photoaged and treated with the LBPF, PBM (4)—animals that were photoaged and treated with photobiomodulation (red wavelength), LBPF + PBM (5)—animals that were photoaged and treated with LBP fraction and then with PBM, and PBM + LBPF (6)—animals that were photoaged and treated with PBM and then LBPF. All experimental groups, except the CTRL group, were photo exposed for 6 weeks and received the treatments from of third week until the sixth week (during photo exposure). Two days after completion of the treatments all animals were euthanized, and the dorsal region skin of each animal was collected. Fixed skins were submitted to histological processing and staining with picrosirius red. RD = reticular dermis, Bar = 10 μm. **b** Percentage of birefringent collagen fibers (%) in dorsal skin of hairless mice. The sections images were captured and scanned through of a photomicroscope (Olympus SC30) and the quantitative analysis of the area percentage of birefringent collagen fibers was performed using Image J® software. Values shown are means ± SDs. * indicates significant differences between groups of animals that were not photoaged (CTRL) and animals that were photoaged (PC) ($p < 0.05$). Values were compared by Kruskal-Wallis with Dunn post-test ($p < 0.05$), using the GraphPad Prism® 7.0 software

molecular changes, promoting collagen fragmentation in the extracellular matrix and a disruption of new collagen synthesis [21]. Importantly, the oral consumption of goji berry juice was able to inhibit the cutaneous UV-induced photodamage in mice [22]. We demonstrated here that LBPF applied to the skin, both isolated and associated with PBM (red wavelength), presented effects against UVR-induced damage showing a protection against epidermal thickening, fragmentation of collagen fibers in the dermis, and a biomodulation of the synthesis and degradation of birefringent collagen fibers of the reticular dermis in the LBPF + PBM group. Hwang and colleagues [18] observed a similar effect for LBPF on the protection against increased epithelium thickness and changes in collagen density. However, in our study, the application of LBPF followed by PBM promoted a decrease in birefringent collagen fibers. Therefore, an inhibitory effect may have occurred due to PBM-associated fraction composition (LBPF) in this order of application. Also, the application followed by the two treatments in this order, without time interval, may have been the cause of these effects. This set of variables may have promoted inhibition or increased collagen fiber degradation. Silva et al. [17] have shown that combining PBM with natural extracts may be a useful strategy, but choosing a natural extract is very challenging due to work concentration and other properties, such as photosensitivity, which may bring unwanted results.

In our study, quantitative analysis of birefringent collagen fibers revealed promising results in the groups treated with LBPF, PBM, and PBM + LBPF. These treatments reduced the degradation of collagen, as their levels were similar to the levels of non-photoaged animals (CTRL). Thus, there is a need for further studies in the same experimental model to investigate the effects of different LBPF concentrations and PBM parameters to avoid the deleterious effects of UVR on the skin.

Chronic exposure of the skin to UVR causes photo-oxidation, resulting in the upregulation of MMPs and loss of dermal collagen [1, 23]. In our findings, the analysis of MMP-1, MMP-2, and MMP-9 expression levels demonstrated that the treatments appear to produce different responses depending on the order of the application and the isolated or associated form. We found that all the treatments carried out conserved the expression levels of the MMP enzymes close to the PC group. On the other hand, the PBM + LBPF group, in which the PBM application was made prior to the application of LBPF, had even higher MMP levels and possibly the order

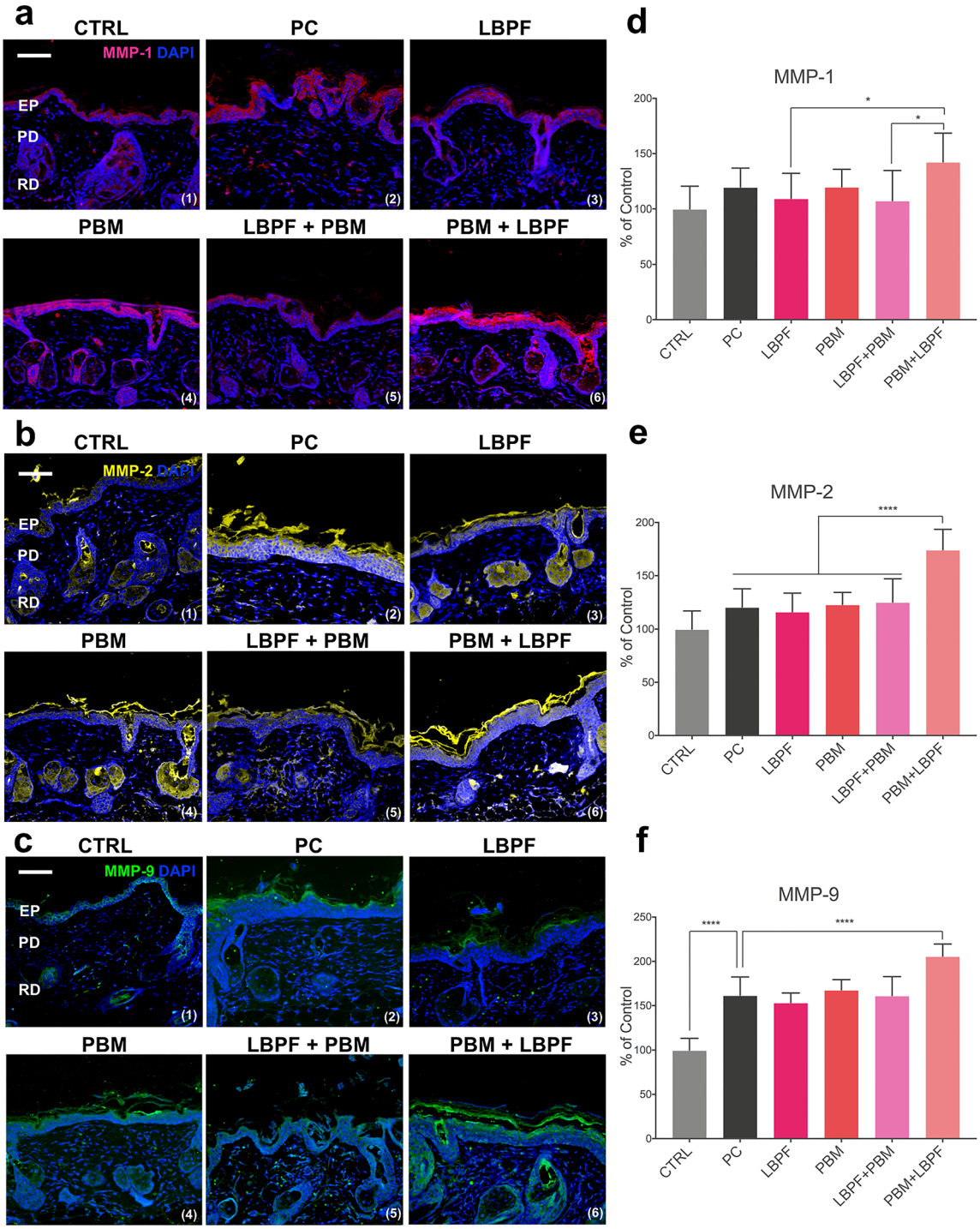

Fig. 3 Photomicrographs of the immunofluorescence technique for the labeling of the enzyme MMP-1 (**a**), MMP-2 (**b**), and MMP-9 (**c**). CTRL (a.1,b.1,c.1)—animals that were not photoaged, PC (a.2, b.2, c.2)—animals that were photoaged, LBPF (a.3, b.3, c.3)—animals that were photoaged and treated with the LBPF, PBM (a.4, b.4, c.4)—animals that were photoaged and treated with photobiomodulation (red wavelength), LBPF + PBM (a.5, b.5, c.5)—animals that were photoaged and treated with LBP fraction and then with PBM, and PBM + LBPF (a.6, b.6, c.6)—animals that were photoaged and treated with PBM and then LBPF. EP = epidermis, PD = papillary dermis, RD = reticular dermis, Bar = 50 μm. Quantitative expression analysis of the enzyme MMP-1 (**d**). * indicate significant differences ($p < 0.05$) between LBPF and LBPF + PBM treatments with PBM + LBPF group. Quantitative expression analysis of the enzyme MMP-2 (**e**). **** indicate significant differences ($p < 0.0001$) between the PC, LBPF, PBM, LBPF + PBM, and PBM + LBPF groups. Quantitative expression analysis of the enzyme MMP-9 (**f**). **** indicates significant differences ($p < 0.0001$) between the CTRL and PC groups and also the PC and PBM + LBPF groups. Values are represented as means ± SDs. Results were analyzed by one-way ANOVA and Tukey post-test for comparison between groups using Graph Pad Prism® 7.0 software

of application may have influenced the response to the treatment of this group. It is known that UVR can directly damage cutaneous biomolecules that are rich in chromophores, inducing the production of ROS, acting on the cells and components of the ECM [24]. Apparently, the application of the PBM after the UVR session may have provoked an oxidizing response due to the amount of energy deposited in the tissue in a short period of time, being further potentiated by a probable synergism of the LBPF with the PBM.

Taken together, our results indicate that the treatments produced different responses if applied singly or associated with LBPF, and that the order of their application is important. Photoprotective effects against epidermal thickening and low collagen density were found with the associated treatment of LBPF followed by PBM, despite no changes were found in the MMP levels.

This study has some limitations, including the use of a single concentration of LBPF, as well as a unique dose of PBM. The use of different LBPF concentrations and PBM doses in the assays would help to find the ideal conditions to decrease the UVR-induced photodamage in hairless mice skin. In fact, there is a lack of references in the literature regarding PBM parameters to be applied in human skin targeting photorejuvenation. Yet, this work can bring contributions to the current state of the noninvasive therapies against photoaging and, as such, open up new possibilities for the effective and safe application of these treatments in the human skin.

Author's contributions LMG: conception and design, data acquisition, data analyses and interpretation, review of the final version; CRT: data acquisition, review of the final version; EMF: data interpretation, review of the final version; LRSA: data interpretation, review of the final version; JBF: data interpretation, review of the final version; NAP: conception and design, data analyses and interpretation, review of the final version; MRC: conception and design, data acquisition, funding acquisition, data analyses and interpretation, review of the final version

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Data availability The dataset supporting the results will be available upon request.

Compliance with ethical standards

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

Ethics approval This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments (information excluded for the sake of animosity).

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