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Effect of photobiomodulation on inflammatory cytokines produced by HaCaT keratinocytes

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ABSTRACT

Objective: to evaluate the effects of the red and near-infrared wavelength lasers in isolated and simultaneous way on the modulation of inflammatory cytokines produced by human keratinocytes (HaCaT) challenged by cytokines of human monocytes stimulated by lipopolysaccharide from *Escherichia coli*.
Design: HaCaT cells was previously exposed to the laser with wavelengths red (660 nm), near-infrared (808 nm). Then, HaCat cells were stimulated with the supernatant of lipopolysaccharide-challenged peripheral blood cells. The cytokines expressed by HaCat cells were measured using multiplex CBA assay.
Results: HaCaT cells increased the production of inflammatory cytokines when stimulated with infrared laser compared to the control group (IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL -12p70, IL -17A, IL-23, IL-33), the red laser group (IFN-γ and IL-23) and the group of two lasers used simultaneously (IFN-α2, IFN-γ, IL-6 and IL-8, IL-17A, IL-18 and IL-23) ($p < 0.05$). The red laser also stimulated an increase in the expression of IFN-α2 by HaCaT cells in relation to the control group ($p < 0.05$).
Conclusion: Infrared laser, with an energy density of 5 J/cm^2, appear to be able to modulate inflammatory cytokines produced by HaCaT cells challenged by human monocyte cytokines.

1. Introduction

Photobiomodulation therapy uses low doses of radiation in the visible red or near-infrared wavelengths to promote therapeutic effects given evidence of its anti-inflammatory action, assistance in tissue remodeling, and potential alteration of the levels of various cytokines and inflammatory mediators.[1] The mechanism of the photobimodulation is associated with activation of endogenous chromophores eliciting photophysical and photochemical events involving several biological pathways that provide favorable clinical therapeutic results, such as wound healing, regeneration, and immune responses mediates by processes of inflammation.[2] In clinical practice, there are protocols with specific guidelines for preventing effects of oncological therapy, such as oral mucositis and other inflammations of the oral mucosa, using photobiomodulation therapy.[3] However, there is variation in the parameters presented by the various randomized clinical trials used in photobiomodulation therapy for the management of oral mucositis in cancer patients.[4] Therefore, studies that evaluate the mechanisms of photobiomodulation in oral mucosa cells can help improve these protocols.

HaCaT (Human Adult Low-Calcium High-Temperature) cells is a keratinocytes line with capacity to differentiate and proliferate in vitro. Keratinocytes are cells that cover the oral mucosa and are damaged in mucosal ulcerations. Some studies have been evaluated the effects of photobiomodulation in HaCaT lines. It was observed that photobiomodulation therapy could increase ATP production in 2 h, resulting in increase of the cellular metabolism after 24 h associated with cell migration.[5] In another study, keratinocytes showed fast maturation and rapid migration to sites of injury, especially after light irradiation at a length of 660 nm.[2] In cytokines expression, the effect of

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photobiomodulation therapy was evaluated in human periodontal ligament fibroblasts stimulated with lipopolysaccharide (LPS). Fibroblasts increase the expression of proinflammatory cytokines when challenge with LPS, and when irradiated with a laser, considerably decrease the expression of the cytokines IL-1β, TNF-α, IL-6, and IL-8 compared with the non-irradiated group.[6] However, it was not observed studies that evaluated photobiomodulation therapy's effects on the expression of cytokines in keratinocytes challenged by LPS, and the effects of combined red and near-infrared lasers in these cells.

Despite those promising results, efficient photobiomodulation therapy depends on the use of appropriate parameters, a topic that remains under investigation.[7,8] The use of both wavelengths simultaneously to treat temporomandibular disorders and psoriasis has been showed preliminary results, but no studies in the literature have addressed the effects of combining red and infrared wavelengths to treat ulcerations lesions such as oral mucositis.[9,10] Most studies in the literature have primarily addressed using lasers with red wavelengths to modulate inflammation, whereas using infrared has been less evaluated.[11] With the development of new devices that emit both wavelengths simultaneously, the need for studies to elucidate the effect of such joint irradiation is evident.

Because photobiomodulation therapy appears to play a modulatory role in several cytokines, we aimed to evaluate the effect of photobiomodulation on the expression of inflammatory cytokines by human keratinocytes (HaCaT) using an in vitro culture system and a multiplex Cytometric Bead Array (CBA) assay. Furthermore, irradiation with two wavelengths—660 nm (i.e., red laser) and 808 nm (i.e., near-infrared laser)—in isolated and simultaneous way was evaluated.

2. Material and methods

2.1. Obtaining peripheral blood mononuclear cells and stimulating them with lipopolysaccharide (LPS)

The study was approved by the Research Ethics Committee of the University (CAAE: 96696318.8.0000.5137). Three healthy women 31, 34, and 39 years old were invited to participate in the study and after signing the informed consent form, they were included. Exclusion criteria were immunosuppression, systemic infectious disease, chronic alcoholism, autoimmune disease, chronic inflammatory disease, and the use of antibiotics, chemotherapy, antineoplastic drugs, immunosuppressive drugs, or anti-inflammatory drugs in the past 30 d.

Peripheral blood mononuclear cells contain monocytes that are the main cells of innate immunity involved in the production of cytokines capable of modulating the inflammatory process and respond to microbial stimuli such as LPS. Approximately 20 ml of blood was collected from each participant in heparinized tubes (Becton Dickinson Vacutainer®, City, ST, USA). Purification of peripheral blood mononuclear cells (PBMC) was performed as described previously.[12] Blood was diluted in phosphate-buffered saline (PBS) in a 1:1 ratio. Each 20 ml of diluted blood was then carefully applied over 10 ml of Ficoll-Paque (GE Healthcare, Sigma-Aldrich, St. Louis, MO, USA) gradient. The mixture was centrifuged at 20 °C at 200×*g* for 40 min, and PBMC were collected at the interface between the plasma and the Ficoll. Cells were washed three times by centrifugation with PBS and resuspended in Roswell Park Memorial Institute complete medium (RPMI 1640; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Trypan blue (0.4 %) was added to the cells in a 1:1 ratio for counting in the Countess™ Automated Cell Counter (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Afterward, the peripheral blood mononuclear cells were plated at a cell density of 5×10^5 cells per well in 96-well U-bottomed microplates. A day after plating, cells were challenged with LPS from *Escherichia coli* at a concentration of 5 ng/ml of medium for 1 h (Sigma-Aldrich, Saint Louis, MO 63103, USA). Cells were centrifuged, washed in PBS, and incubated for 3 h in new RPMI medium. Cytokine-conditioned supernatant was collected to be added to HaCaT cells (Fig. 1A and B). To ensure that cytokines produced by mononuclear cells were not removed in the first cell wash, cytokine-conditioned supernatant was measured and the data presented in Table 1 (Supplementary Material).

2.2. HaCaT cells culture

Human keratinocytes cells of the immortalized HaCaT lineage (Cell Line Service 300,493) were commercially obtained and expanded in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum (Gibco/Invitrogen, Grand Island, NY, USA), 1 % penicillin, 1 % streptomycin, and 0.2 % fungizone and incubated in a 95 % humidified atmosphere containing 5 % CO_2 at 37 °C. Cells were seeded at a density of 1000 cells per well in 96-well microplates in duplicate and monitored daily until reaching 80%–90 % of confluence.

2.3. Photobiomodulation therapy

Photobiomodulation therapy was performed with an indium–gallium–aluminum phosphide semiconductor diode laser (DMC, São Carlos, Brazil), with a red wavelength of 660 nm and near-infrared wavelength of 808 nm, an output power of 20 mW, and a beam cross-sectional area (i.e., spot) of 0.028 cm^2 (i.e., 0.6 mm in diameter). The power density was 0.71 W/cm^2, while the energy density was 5 J/cm^2 (7 s, 0.14 J). Prior to the laser's use, power stability was tested using an optical power meter (LaserCheck, Coherent Inc., Santa Clara, CA, USA). Irradiation on HaCaT lineage keratinocytes was performed by contact in the center of each well of the plate at two different times: after 18 h and 1 h of stimulation with peripheral blood mononuclear cells cytokines.

2.4. Determination of the viability of HaCaT cells

After the supernatant for cytokine analysis was collected, HaCaT cells, plated in 96-well microplates, were incubated with methyl tetrazole formazan (MTT, 5 mg/ml; (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 20 % (v/v) in DMEM culture medium (Eagle's medium modified by Dulbecco, Gibco) for 3 h at 37 °C in a humidified atmosphere containing 5 % CO_2 in order to evaluate mitochondrial activity. The formazan precipitates were dissolved by adding 100 μl of 10 % (w/v) sodium dodecyl sulfate solution (Sigma-Aldrich, St. Louis, MO, USA) overnight, and, after homogenization, optical density was read at 570 nm on a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA). The experiments were performed with three cell donors in duplicate, and cells grown under ideal conditions (without stimulation) were considered to be the controls. Data were recorded as relative units and compared with the control group.

2.5. Detection of cytokine concentration

Multiple cytokines (i.e., IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33) were measured simultaneously by flow cytometry using CBA assay. Bead-based multiplex LEGENDplex™ analysis (13-plex LEGENDplex™ 1 Human Inflammation Panel, BioLegend, San Jose, CA, USA) was applied following the manufacturer's instructions, and acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Data from three donors are shown. The experiments were performed in duplicate. Quantitative results were generated using the LEGENDplex™ data analysis program (https://legendplex.qognit.com/user/login?next=home).

2.6. Statistical analysis

The production of cytokines was compared between the experimental groups G1 (i.e., HaCaT cells stimulated by the supernatant of

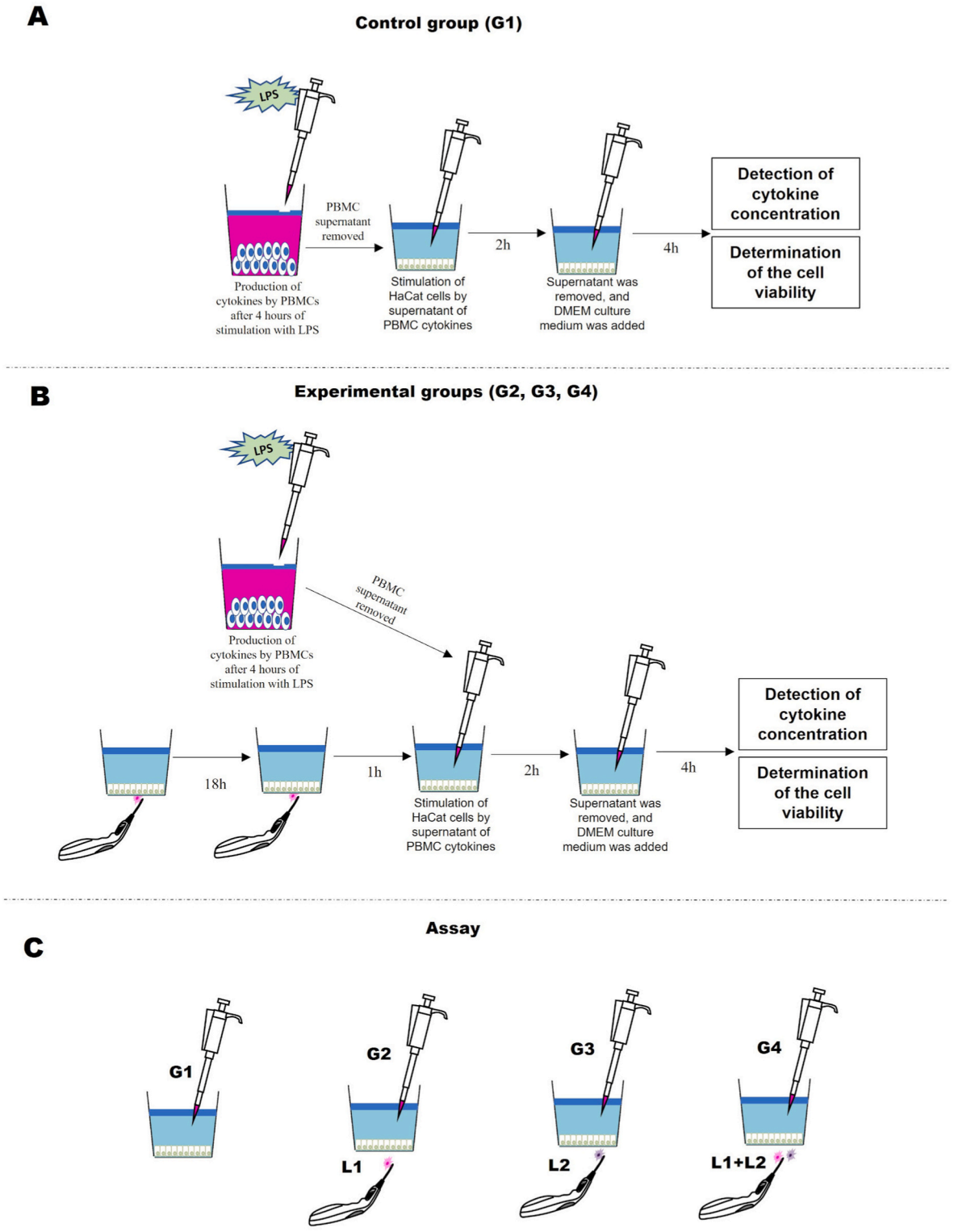

Fig. 1. Timeline of the experimental procedures, and experimental groups of the assay. (A) Assay of the control group (G1): The supernatant of peripheral blood mononuclear cell cytokines was collected to stimulate HaCaT cell cytokines. (B) Assay of the experimental groups with laser (G2, G3, and G4): The effect of photobiomodulation on cytokine production by HaCaT keratinocytes stimulated by peripheral blood mononuclear cells cytokines was evaluated. (C) Flowchart to illustrate the experimental procedures. PBMC, peripheral blood mononuclear cells. LPS, lipopolysaccharide. DMEM, Dulbecco's Modified Eagle Medium. L1, red laser. L2, near-infrared laser.

peripheral blood mononuclear cells cytokines without laser), G2 (i.e., HaCaT cells stimulated by supernatant of peripheral blood mononuclear cells cytokines and red laser), G3 (i.e., HaCaT cells stimulated by supernatant of peripheral blood mononuclear cells cytokines and near-infrared laser), and G4 (i.e., HaCaT cells stimulated by supernatant of peripheral blood mononuclear cells cytokines, red and near-infrared lasers simultaneous way) after 6 h (Fig. 1). The Kolmogorov–Smirnov normality test was used to verify the distribution of data. Next, to

identify differences in cell viability in the concentration of cytokines between the different treatments, the one-way ANOVA with repetition was used, followed by Tukey's post hoc test for comparison between pairs. All analyses were performed at a significance level of 5 % ($p < 0.05$) using the GraphPad Prism v.9 Software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. HaCaT cell viability

Viability analysis showed no significant changes when HaCaT cells were stimulated by cytokines from peripheral blood mononuclear cells associated or not with red laser, infrared laser and two lasers simultaneously, with an energy density of 5 J/cm2 (Fig. 2).

3.2. Cytokines production by HaCat and peripheral blood mononuclear cells

Three hours after changing the culture medium containing bacterial LPS (i.e., 4 h after stimulation), cytokines production by peripheral blood mononuclear cells was measured (Table 1- Supplementary Material). 100 µl of supernatant of these cytokines were added to HaCaT keratinocyte culture wells containing 100 µl of medium. After 2 h, the plated cells were washed twice with PBS and incubated again with the culture medium. After 6 h of cytokine stimulation (i.e., 4 h of incubation with the new culture medium), the cytokine concentration was measured again to reflect the results of new productions (Table 1 Supplementary Material).

3.3. Effects of photobiomodulation on cytokine production by HaCaT cells

The effects of photobiomodulation on cytokine production by HaCaT cells stimulated by human monocyte cytokines are shown in Figs. 3 and 4.

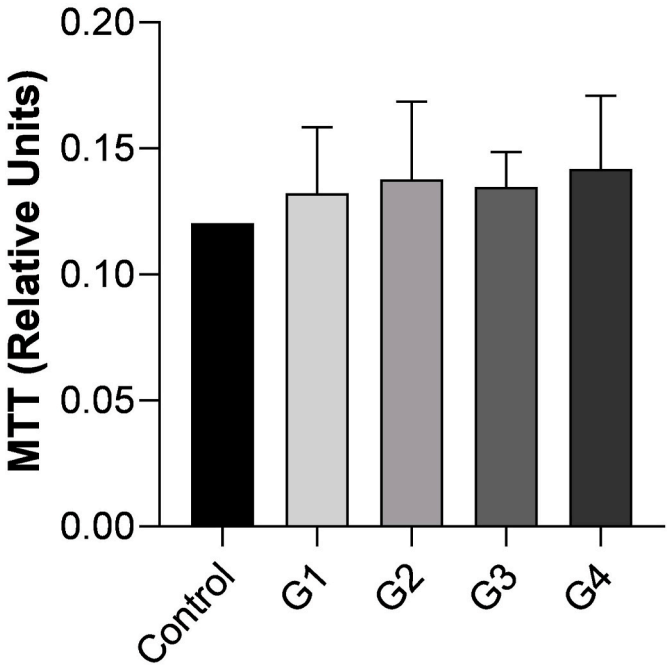

Fig. 2. Evaluation of the viability of HaCaT cells with the MTT method after stimulation with cytokines (i.e., G1), cytokines and the red laser (i.e., G2), cytokines and the near-infrared laser (i.e., G3), and cytokines and both lasers (i.e., G4) in relation to the control (without stimulation). The experiments were performed in biological triplicate and experimental duplicate. Error bars indicate the standard error of the mean.

3.3.1. Cells when stimulated by the red laser (wavelengths—660 nm)

The red laser's effect on HaCaT cells was observed with increased expression of the IFN-α2 cytokine in relation to control group ($p < 0.05$) (Fig. 4B). Moreover, the production of IFN-γ (Fig. 3C) and IL-23 by the HaCaT cells decrease when stimulated by the red laser compared with cells stimulated by near-infrared laser ($p < 0.05$) (Fig. 4C).

3.3.2. Cells when stimulated by the near-infrared laser (wavelengths—808 nm)

The near-infrared laser's effects on HaCaT cells were observed with increased cytokine production for IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, and IL-12p70 (Fig. 3B–I, respectively), IL-17A, IL-18, IL-23, and IL-33 (Fig. 4A–D, respectively) compared to control group ($p < 0.05$).

3.3.3. Cells when stimulated by the red and near-infrared laser simultaneous way

The production of IFN-α2, IFN-γ, IL-6, and IL-8 (Fig. 3B, C, 3F and 3G, respectively), IL-17A, IL-18, and IL-23 (Fig. 4A–C, respectively) by HaCaT cells decreased when stimulated by red and near-infrared lasers used simultaneous way compared with cells stimulated by infrared laser isolated ($p < 0.05$).

4. Discussion

Given its distinct biological effects, photobiomodulation therapy has been shown to be a promising therapeutic option in cases of tissue repair.[1] Keratinocytes are cells that play an important role in the repair and immune defense of the skin and mucous membranes, namely by secreting growth factors, cytokines, and chemokines.[13] In our study, an in vitro model was used in which keratinocytes of the HaCaT lineage were stimulated by cytokines produced by human peripheral blood mononuclear cells, simulating a local inflammatory condition. The objective was to evaluate the cytokine expression profile of HaCaT keratinocytes after an inflammatory stimulus and to what extent photobiomodulation therapy can affect cytokine production in these conditions. It has been demonstrated that red and near-infrared lasers used alone or simultaneously at doses of 5.0 J/cm2 appear to be capable of modulating different inflammatory cytokines produced by HaCaT cells when challenged by cytokines from human monocytes. Considering the more significant effects observed in the present study for the infrared laser in relation to the red laser on the expression of cytokines by keratinocytes, research on *in vivo* models may help to better understand these results and assist in the development of future clinical protocols for the treatment of injuries or defects that affect keratinocyte function.

In our study, the production of proinflammatory cytokines IFN-α2, IFN-γ, TNF-α, IL-6, IL-8, IL-12p70, IL-17A, IL-23 and IL-33, along with anti-inflammatory cytokine IL-10 and the MCP-1/CCL-2 chemokine by HaCaT cells irradiated with near-infrared wavelength laser significantly increased compared with non-irradiated cells under the same inflammatory conditions. It is possible that the low-dose infrared laser exerted a stimulatory effect on proinflammatory cytokines in human keratinocytes, which would corroborate other published findings.[14,15] However, those results contradict data obtained by Baroni et al. (2018), who observed the inhibitory effect of a low-dose infrared laser on proinflammatory cytokines (i.e., IL-8 and TNF-α) in keratinocytes with infected *Candida albicans*. Those differences can be explained by the use of different experimental models. In our study, keratinocytes were stimulated by cytokines produced by monocytes challenged with bacterial LPS, without coming into direct contact with the microorganism.

Cytokine IL-33 is an inflammatory cytokine overexpressed in keratinocytes among patients with atopic dermatitis, as are IL-23, IL-17A, and IL-18, which are produced by human keratinocytes under inflammatory conditions.[16–18] To date, studies evaluating the effects of red and

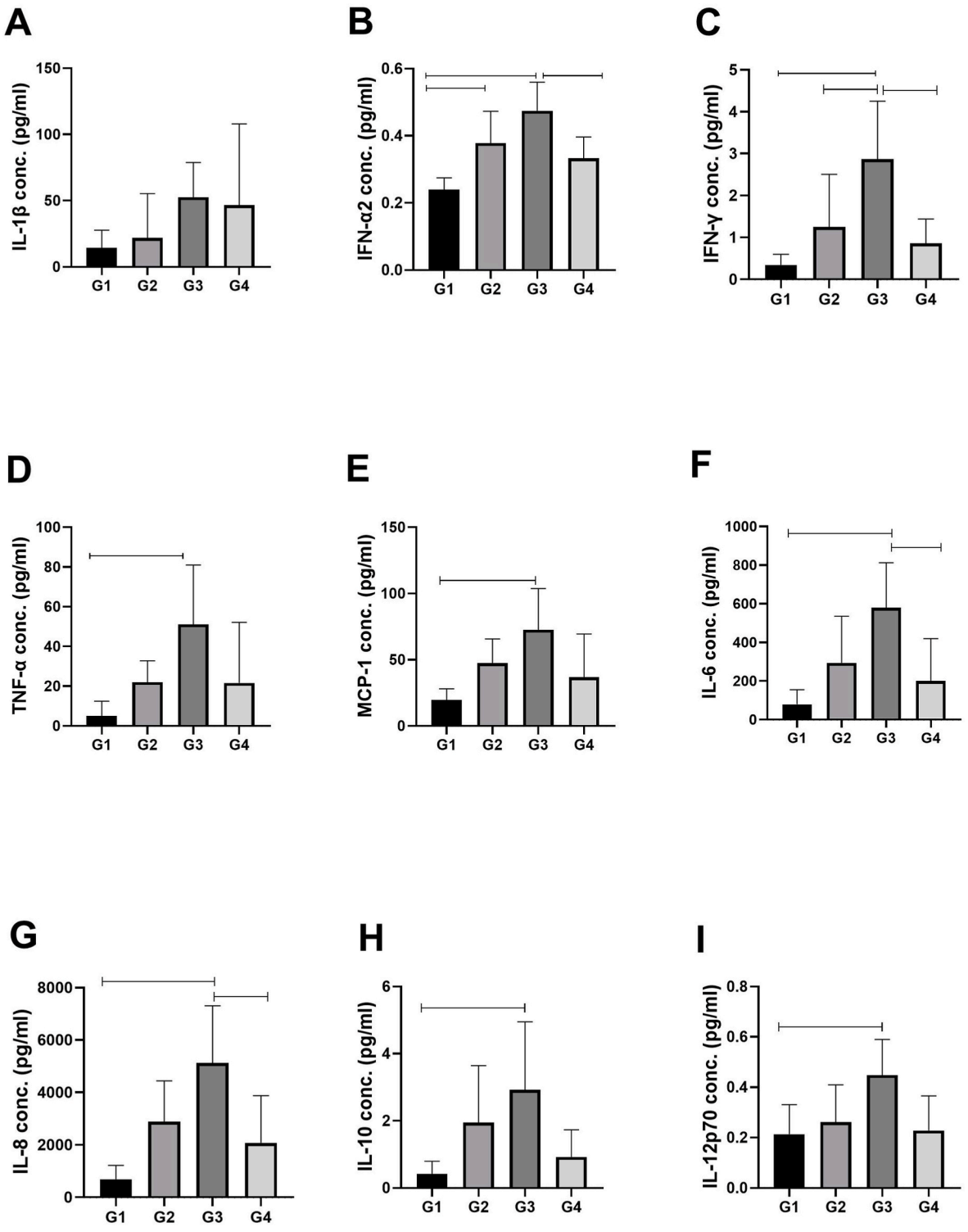

Fig. 3. - Comparison of the production of cytokines (A) IL-1β, (B) IFN-α2, (C) IFN-γ, (D) TNF-α, (E) MCP-1, (F) IL-6, (G) IL-8, (H) IL-10, and (I) IL-12p70 after 6 h of incubation in culture medium containing cytokines produced by peripheral blood mononuclear cells in the control group and groups treated with lasers, namely G1 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and without laser), G2 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and the red laser), G3 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and the infrared laser), and G4 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and both lasers). Data from three donors are shown. The experiments were performed in duplicate. Connecting lines represents a significant difference ($p < 0,05$) between the experimental groups (one-way ANOVA with repeated measures, by Tukey's post hoc test).

infrared lasers on those cytokines, especially on keratinocytes, have not been observed. In our study, we observed that the low-dose infrared laser stimulated the expression of those cytokines in comparison with the control group and groups irradiated by the red laser or both wavelengths. However, additional studies are needed to better understand the effects of infrared lasers, especially at high doses, or on cytokine expression in *in vivo* models.

Except for the expression of the cytokine IFN-α2, no effects of red

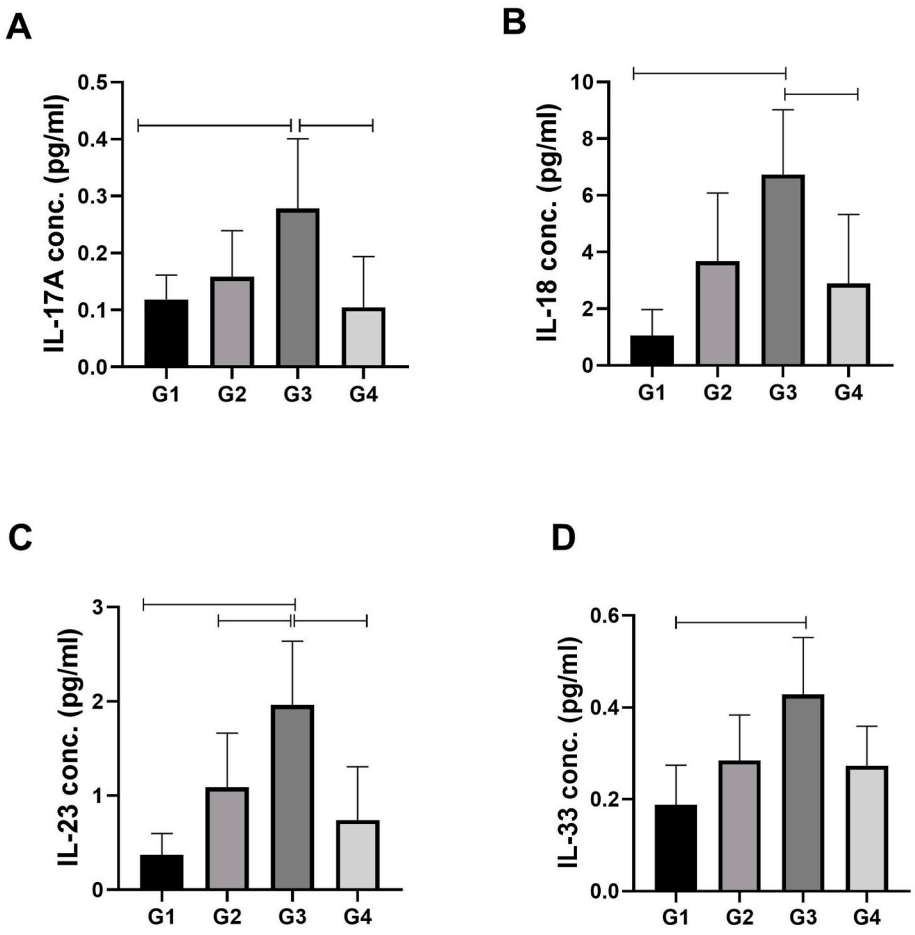

Fig. 4. – Comparison of (A) IL-17A, (B) IL-18, (C) IL-23, and (D) IL-33 cytokine production after 6 h of incubation in culture medium containing cytokines produced by peripheral blood mononuclear cells in the control group and groups treated with laser(s), namely G1 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and without laser), G2 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and the red laser), G3 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and the infrared laser), and G4 (i.e., HaCaT stimulated by peripheral blood mononuclear cells cytokines and both lasers). Data from three donors are shown. The experiments were performed in duplicate. Connecting lines represents a significant difference ($p < 0,05$) between the experimental groups (one-way ANOVA with repeated measures, by Tukey's post hoc test).

laser irradiation on keratinocytes in inflammatory conditions were observed for the evaluated cytokines. These results suggest a lower effect of the red laser when the target cells are keratinocytes, mainly in inflammatory processes. IFN-α2 was initially identified for its antiviral activity and can be expressed by keratinocytes, particularly in the basal layer, primarily in inflammatory processes. Although IFN-β levels have been expressed in keratinocytes after stimulation by ultraviolet light, the literature contains no work evaluating the effects of red light on IFN-α2.[19] Additional studies are needed to better understand the effects of infrared lasers, especially at high doses, or on cytokine expression in *in vivo* models.

It is known that several cytokines and inflammatory mediators present altered levels when irradiated with low-intensity lasers, but few studies have evaluated the effects of photobiomodulation therapy on the expression of cytokines in keratinocytes. A systematic review showed that the main cytokines and growth factors evaluated to date are IL-1α, IL-8, TNF-α, IFN-γ, GM-CSF, IL-6, HB-EGF, TGF-α, VEGF -A, IL-1β and KGF and, in most studies, an increase in the expression of cytokines was observed mainly after irradiation with red and infrared lasers at doses of 0.1–5.0 J/cm2.[11] However, until now, there have been no studies that evaluated the effects on the production of cytokines by keratinocytes after stimulation of two wavelengths simultaneously.

The combination of red and infrared wavelengths was evaluated for the treatment of temporomandibular disorders and psoriasis and showed preliminary results; however, no studies have addressed the effects of associated irradiation for treating ulcerated lesions (e.g., oral mucositis).[9,10,] In our study, the combination of both wavelengths compared with the non-irradiated group affected the expression of cytokines evaluated in keratinocytes under inflammatory conditions. A decrease in the expression of IFN-α2, IFN-γ, IL-6, and IL-8, as well as IL-17A, IL-18, and IL-23, was observed in the group in which irradiation was performed using both wavelengths compared with only the infrared laser, suggesting a biological effect by interaction of two wavelengths associated.

Previous studies have shown that photobiomodulation therapy in the parameters used was not able to affect the cell viability of the HaCaT keratinocyte lineage in short-term cultures.[11] However, it has not been demonstrated whether HaCat cells, when cultured in medium containing molecules secreted by peripheral blood mononuclear cells, show changes in viability. This study demonstrated that the cellular viability of HaCaT keratinocytes stimulated by human monocyte cytokines did not change when irradiated with red or infrared laser or both simultaneously, with an energy density of 5 J/cm^2. That result may indicate a safe energy density, one that does not alter cell viability in keratinocyte cultures cultured in vitro under inflammatory stimuli.

The use of photobiomodulation therapy should be carefully evaluated, and the choice of wavelength to be used should be based not only on the depth of tissue penetration but also the biological activity exerted on irradiated cells.[20] Research indicates that, when inflammation is present, photobiomodulation therapy exerts an anti-inflammatory

action; however, when it is absent, irradiation induces proinflammatory mediators, which can assist in tissue remodeling and cellular function.[2] Even so, other results suggest that the pro- or anti-inflammatory stimulus caused by photobiomodulation therapy may be affected by the stimulated cell line, dose, and wavelength used.[11] The results of our study demonstrate that infrared wavelength (i.e., 880 nm), with an energy density of 5 J/cm2, can modulate inflammatory cytokines by increasing their production by HaCaT keratinocytes, challenged by human monocyte cytokines produced from of an LPS stimulus. However, the limitations of this study must be highlighted such as lack of kinetics of inflammatory cytokines at various times after photoactivation, lack of knowledge of the time required for expression of each cytokine evaluated, the use of other doses of energy and a single cellular type, since the profile of cytokines produced by keratinocytes can change when interacting with other cells in the tissue. More in vitro and, subsequently, *in vivo* studies are needed to better clarify the effect of infrared lasers on cytokine production and whether such stimulatory effects modulate the inflammatory process and may have the potential to benefit patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobcr.2023.12.007.

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