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ORIGINAL ARTICLE

In vitro effect of low-level laser therapy on the proliferative, apoptosis modulation, and oxi-inflammatory markers of premature-senescent hydrogen peroxide-induced dermal fibroblasts

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Abstract

Skin aging is a complex biological process induced by intrinsic and extrinsic factors which is characterized by clinical and cellular changes, especially dermal fibroblasts. It is possible that, some procedures, such as low-level laser therapy (LLLT), could decelerate this process. To test this hypothesis, this study evaluated the in vitro LLLT on dermal fibroblast cell line (HFF-1) with premature senescence H_2O_2-induced. HFF-1 cells were cultured in standardized conditions, and initially H_2O_2 exposed at different concentrations. Fibroblasts were also just exposed at different LLLT (660 nm) doses. From these curves, the lowest H_2O_2 concentration that induced indicators of premature senescence and the lowest LLLT doses that triggered fibroblast proliferation were used in all assays. Cellular mortality, proliferation, and the levels of oxidative, inflammatory cytokines, apoptotic markers, and of two growth signaling molecules (FGF-1 and KGF) were compared among treatments. The H_2O_2 at 50 μM concentration induced some fibroblast senescence markers and for LLLT, the best dose for treatment was 4 J ($p < 0.001$). The interaction between H_2O_2 at 50 μM and LLLT at 4 J showed partially reversion of the higher levels of DNA oxidation, CASP 3, CASP 8, IL-1B, IL-6, and INFy induced by H_2O_2 exposure. LLLT also trigger increase of IL-10 anti-inflammatory cytokine, FGF-1 and KGF levels. Cellular proliferation was also improved when fibroblasts treated with H_2O_2 were exposed to LLLT ($p < 0.001$). These results suggest that in fibroblast with some senescence characteristics H_2O_2-induced, the LLLT presented an important protective and proliferative action, reverting partially or totally negative effects triggering by H_2O_2.

Keywords Skin · Aging · Fibroblast · Low-level laser

Introduction

Most skin-aging modification is directly related to fibroblasts that have several functions being responsible to secretion and organization of dermal extracellular matrix (ECM). During aging process, fibroblast presents replicative senescence and alterations in its cytoskeleton structure and in the fibbers secretion. In fact, when fibroblasts are young present a high adherence been responsible to spreading of these cells that exert mechanical force on the surrounding ECM. However, in senescent fibroblasts, skin is a complex outermost organ of the body, organized mainly by epidermal keratinocytes and dermal fibroblasts. During life period, skin accumulates biological changes recognized as skin-aged phenotype that includes wrinkled, sagging, enlarged facial pore size, and generally less elastic and resilient than its youthful counterpart. However, as consequence of interaction between intrinsic

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genetic and extrinsic environmental factors, the skin aging process is very heterogeneous and represents a challenge to dermatologists [1]. The interaction with ECM is greatly interrupted mainly in consequence of matrix disorganization related to collagen fibril deposition [2].

Causal mechanisms of skin aging are associated with decreasing in the mitochondrial efficiency, with consequent elevation of reactive oxygen species (ROS) levels, especially superoxide anion and hydrogen peroxide (H_2O_2). In fact, the generation of high ROS levels is not followed by concomitant increase of enzymatic antioxidant levels and can be potentialized or minimized by external sources including environmental and lifestyle factors [3, 4]. Low-level laser therapy (LLLT) is a clinical procedure generally used as skin biostimulator that could be a potential tool that could decelerate fibroblast aging [5]. However, complementary investigations are necessary to confirm this hypothesis.

The term laser stands for "amplification of light by stimulated emission of radiation". In addition, the term low level refers to the category with therapeutic actions while the high-intensity lasers have destructive actions: cutting, vaporizing, ablating, and/or coagulating a biological tissue. This resource deals with parameters like wavelength, intensity expressed in watts (W), energy expressed in joules (J), the use of different techniques (such as punctual, sweep, intravascular), and the application on acupuncture points [6].

In this context, we conducted here a protocol using a commercial human foreskin fibroblast (HFF-1) cells from normal human newborn foreskin. In in vitro standardized conditions, HFF-1 presents a finite lifespan similar to that observed in senescent fibroblasts that can be obtained by subsequent cell culture passages or by exposure to high levels of ROS molecules. For this reason, HFF-1 fibroblasts have been used by prior studies to test chemical molecules that could revert some fibroblast senescence characteristics [7–9]. Moreover, previous studies also described that H_2O_2 exposure is able to trigger premature in vitro fibroblast senescence [10, 11].

Two integrated in vitro protocols were performed here: (1) potential senescence modulation of LLLT exposure on HFF-1 premature-senescent cells. Main senescent markers studied here included cell mortality rate also investigating if this process could be associated with increase of DNA oxidation levels and subsequent apoptosis events evaluated by protein and gene expression modulation of caspases (CASP 1, 3, and 8). The LLLT effects on oxidative metabolism were studied by quantification of some oxidative and antioxidant molecules, as well as on cytokine levels associated with proinflammatory (interleukins IL-1β, IL-6; tumor necrosis factor alpha, TNF-α; interferon gamma, IFN-γ) and anti-inflammatory response (IL-10) [12]. Therefore, the aim of the present investigation was to analyze potential LLLT influence on senescence markers triggered on HFF-1 fibroblast cells by H_2O_2 exposure.

Methods

Cell culture conditions and general experimental design

HFF-1 cells procured from American Type Culture Collection USA (ATCC® SCRC-1041™) were obtained from Rio de Janeiro Cell Bank to perform all experiments. HFF-1 cell lines were maintained in the growth medium DMEM supplemented with 15% fetal bovine serum (FBS), supplemented with antibiotics penicillin (100 U/ mL) and streptomycin (100 U/mL). The growth conditions of cell lines were 37 °C, 5% CO_2, and 95% humidity. These cells were cultured for several passages, and each passage started with approximately 1×10^5. Proliferative rate was always evaluated in 72 h cell cultures. In the 28th passage, fibroblast in our lab was observed decreasing by approximately 30% on proliferative rate in comparison with younger cultures. Cyto-modifications in the monolayer structure were also observed indicating senescence characteristics similar to previously described in the study performed by McFarland and Holliday with HFF-1 cells [7].

These fibroblast cultures were exposed to curve concentration of H_2O_2 in order to determine better concentration that triggered effect on cytotoxicity, apoptotic, oxi-inflammatory, and proliferative parameters, which could be potentially modulated by different LLLT conditions. The H_2O_2 concentrations used in the curve were 1, 10, 25, 50, and 100 μM. The H_2O_2 exposure was performed as follows: Culture medium containing 1×10^5 fibroblast cells was transferred by 96-well plates and different H_2O_2 concentrations dissolved in phosphate-buffer solution (PBS) were added in each well for 2 h. Further, plates were centrifuged for 15 min at 252×*g* and PBS washed. Fresh culture medium was then added, and cells were cultured during 72 h, before the variables analyzed here are quantified. Similar procedure was performed when H_2O_2 premature senescent fibroblasts were exposed to LLLT. All procedures were independently performed in triplicate and performed in 72-h cell cultures.

LLLT conditions

Premature senescent fibroblasts H_2O_2 induced were laser irradiated until 2 h after cell to be H_2O_2 exposure using equipment Endophoton LLT 0107 KLD® (Biosystems Electronic Equipment Ltda—Brazilian Industry, Amparo, São Paulo, Brazil). Details of LLLT exposure are presented in Table 1. Cells not exposed to H_2O_2 were also irradiated as a laser control group. A laser (660 nm) was used as the irradiation source with 35 mW output power and 16 Hz frequency in punctual wave mode. The delivered dose for each irradiated set was 3, 4, 5, 6, and 8 J/cm^2 with respective exposure times 10 s, 14 s, 16 s, 20 s, and 28 s. The irradiation process was achieved at room temperature (18–25 °C). For all the

Table 1 Characteristics of low-level laser therapy (LLLT) exposure of HFF-1 fibroblast cell cultures

Parameters	Specifications
Wavelength	660 nm
Energy density	3, 4, 5, 6, and 8 J/cm^2
Power density	35 mW
Time	10 s, 14 s, 16 s, 20 s, and 28 s
Bean area	0.035 cm^2
Beam diameter	0.21 cm^2
Frequency	16 Hz
Emission mode	Pulsed
Number of points	8
Area of the laser application	9.6 cm^2
Contact	No contact—distance of 35 mm

treatments, the distance between the laser and area of irradiation was of 35 mm.

Cytotoxicity assays

The analysis of treatment effects on premature senescent-fibroblast mortality was performed using dye trypan exclusion and double-strand (ds) DNA fragment levels in the culture medium, which is an indicator of cellular mortality determined by a fast fluorimetric assay using the Quant-iT™ dye PicoGreen® (Invitrogen, Life Technologies). This is a fluorescent and stable reagent, with a high affinity for dsDNA, thus enabling the assessment of cellular integrity, assuming that the presence of free DNA in the medium is indicative of cell death due to membrane disruption. The dsDNA protocol was performed as previously described by Costa et al. [13]. Briefly, 20 μL of cell culture samples of each well was transferred by a black 96-well plate and added 10× microliters of DNA Picogreen previously diluted in Tris-EDTA (TE) buffer. A darkness incubation was conducted by 10 min before fluorimetry reading. The fluorimetric analysis recorded at room temperature was performed using a Spectra Max M2/M2 Multimode Plate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at an excitation of 480 nm and an emission of 520 m.

Cell cycle analysis by flow cytometry

Premature senescent-fibroblast cellular proliferation was performed by flow cytometry where the follow different cell cycle phases were determined (G0/G1, S, andG2/mitosis phases) using the similar protocol described by Azzolin et al. [14] that use propidium iodide (PI) reagent that is able to intercalate to the DNA to determine different cell cycle phases. Briefly, sample treatments were incubated with 500 μL of a propidium iodide (PI) PBS solution and incubated for 40 min at 37 °C. Subsequent to incubation period, cells were trypsinized, washed with PBS, and resuspended in 70% ethanol that has been stored at − 20 °C overnight until flow cytometry analysis is performed. To analyze, the solution containing cells were centrifuged and washed once with PBS, resuspended in 500 μL PI solution during x time. This procedure was repeated washing the cells with 1 mL PBS. Finally, cells were centrifuged and resuspended in 500 μL PBS for flow cytometry analysis.

Immunoassay protocols

Protein quantification of the following markers was determined: DNA 8-deoxyguanosine and cytokines involved in inflammatory response interleukin IL-1β, IL-6, IL-10 tumoral necrosis factor alfa (TNF-α), and interferon gama (IFN-γ). The quantification was performed using an immunoassay kit from a Quantikine Elisa kit obtained from R&D Systems (Minneapolis, MN, USA), which is able to quantify cytokines in cell culture supernates. The growth factor fibroblast (FGF), keratinocytes (KGF), and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) quantification was determined using an Elisa kit obtained from MyBioSource (San Diego, CA, USA), which is able to quantify in cell culture supernates. The assays were performed according to the manufacturer's instructions.

The assays were performed according to the manufacturer's instructions. Briefly, a 50-μL RD1W diluent was added in each well. Subsequently, 100 μL of standard control for our sample was added per well, which were covered with the adhesive strip and incubated for 1.5 h at room temperature. After this process, the solution present in each well was aspirated and washed twice for three washes. The antiserum of molecule markers analyzed here was added to each well. The plates were covered with a new adhesive strip and again incubated for 30 min at room temperature. The aspiration/wash step was repeated, and 100 μL t molecules conjugate of each marker tested here was added to each well and incubated for 30 min at room temperature. The aspiration/wash step was repeated, and 200 mL of substrate solution was added to each well and incubated for 20 min at room temperature. After these procedures, 50 μL of a stop solution was added to each well and the optical density was determined within 30 min using a microplate reader set to 450 nm.

Caspase-1, caspase-3, and caspase-8 activities were determined by assay kits, fluorimetric (Biovision, Inc., Milpitas, CA, USA). The fluorescence intensity of caspase-1, caspase-3, and caspase-8 were recorded at wavelength of 400 nm for excitation, and at wavelength of 505 nm for emission. The activity of caspase was calculated as fluorescence intensity (FI)/min/ml = ΔFlt/ (t × v), where ΔFlt = difference in fluorescence intensity between time zero and time t minutes, t = reaction time in min and v = volume of sample in mL.

The sensitivity and detection range of each marker were respectively DNA 8-deoxyguanosine (0.9–57 pg/mL), IL-1 β (12.5–800 pg/mL), IL-6 (3.1–300 pg/mL), TNF-α (5.6–1.000 pg/mL), INFγ (15.6–1.000 pg/mL), IL-10 (7.8–500 pg/mL), FGF (0.78–50 ng/mL) and KGF (25–1600 ng/mL), SOD (0,1–209 U/L), CAT (3.12–200 U/L), GPX (15.6 U/L-1000 U/L), CASP 1 (1.8–32.0 ng/mL), CASP 3 (3.6 -42 ng/mL), and CASP 8 (7.0–60 ng/mL).

Statistical analysis

In order to compare modifications triggered by H_2O_2 and laser exposure, all data were transformed into percentages in relation to the negative control group and presented as media ± standard deviation (SD) to perform statistical comparisons using GraphPad Prism software, version 7.0 (2017). Results were statistically analyzed by one-way or two-way analysis of variance (ANOVA) followed by a *post-hoc Tukey's test* according presumption of variables added in the analysis. Results with a $p \leq 0.05$ were considered statistically significant.

Results

Initially, H_20_2 curve concentration was performed in order to determine the best concentration that triggers several indicators of fibroblast senescence. As expected, H_2O_2 increased fibroblast mortality rate in a concentration-dependent way ($p \leq 0.001$). This event was followed by increase of DNA oxidation and three apoptosis CASP1, 3, and 8 marker levels (Fig. 1a) ($p \leq 0.001$). From ≥ 25 μM H_2O_2 exposure, it was observed increase in the levels of antioxidant enzymes (Fig. 1b) and proinflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ ($p \leq 0.001$). Moreover, from this concentration, lower H_2O_2 effects were observed in the levels of IL-10, an anti-inflammatory cytokine (Fig. 1c) ($p \leq 0.001$). The whole of these results allowed the choice of H_2O_2 at 50 μM concentration to perform complementary studies involving LLLT effect on fibroblast senescence markers. Histological analysis of cell culture exposure to H_2O_2 at 50 μM concentration confirmed its "senescent effect on these fibroblasts" (Fig. 1d) ($p \leq 0.001$).

A complementary analysis was performed to define the LLLT range exposure that could affect positively and negatively fibroblast cultures until LLLT exposure. The levels of cell mortality, DNA oxidation, and all apoptosis markers investigated here (CASP 1, 3, and 8) increased significantly when fibroblasts were LLLT-exposed ≥ 5 J doses (Fig. 2a) ($p \leq 0.01$). Analysis of antioxidant enzymes of fibroblast LLLT-exposed showed an increase of GPX levels from ≥ 4 J doses and SOD and CAT levels from ≥ 6 J (Fig. 2b) ($p \leq 0.001$). LLLT exposure also modulated cytokine levels mainly in higher doses (6 and 8 J) that presented higher levels of proinflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ) and lower levels of IL-10, an anti-inflammatory cytokine. It is important to point out that fibroblast LLLT-exposed at 4 J doses presented significant increase of IL-1β levels, but this effect was slight. Other cytokines were not affected by LLLT at 4 J doses (Fig. 2c) ($p \leq 0.001$).

From these results, interaction between H_2O_2 at 50 μM and LLLT at 4 J doses on fibroblast mortality, DNA damage, apoptosis, antioxidant enzymes, and inflammatory cytokines was analyzed. Fibroblast culture pre-exposed to H_2O_2 and further exposed to LLLT at 4 J presented partial reversion of cell mortality when compared with cultures just pre-exposed to H_2O_2 (Fig. 3a) ($p \leq 0.01$). The higher levels of DNA oxidation triggered by H_2O_2 exposure were also partially reverted when cells were subsequently LLL-T 4 J exposed (Fig. 3b) ($p \leq 0.001$), and similar results were also found to CASP 3 and CASP 8 levels (Fig. 3c, d) ($p \leq 0.001$).

The levels of all antioxidant enzymes decreased significantly in fibroblasts H_2O_2 plus LLL-T 4 J exposed when compared to cells just H_2O_2 exposed and also in comparison with not-treated control cells (Fig. 4a) ($p \leq 0.001$). Cytokine levels were also modulated by interaction between H_2O_2 and LLL-T 4 J fibroblast-exposed. However, whereas higher levels of IL-1B, IL-6, and INFy were partially reverted, treatment with H_2O_2 plus LLL-T 4 J presented similar TNF-α levels than control group. This same treatment was able to increase partially IL-10 levels when compared to control group (Fig. 4b) ($p \leq 0.01$).

A complementary analysis was performed among these treatments to evaluate potential effect on fibroblast proliferative state evaluated by cell cycle phases (Fig. 5a) ($p \leq 0.01$). The G1 can be considered a stationary phase and S and G2/mitosis as proliferative cell cycle phases. Analysis showed higher frequency of cells in S plus G2 phases in cells pre-exposed to H_2O_2 and subsequently treated with LLLT 4 J (Fig. 5b) ($p \leq 0.01$). Fibroblast just H_2O_2 exposed showed significant decrease in the FGF-1 and KGF levels than control group. On the other hand, just LLLT-exposed did not change the FGF-1 levels than control group whereas the interaction between H_2O_2 and LLLT triggered higher levels of KGF molecule that is an important skin proliferative factor (Fig. 5c) ($p \leq 0.01$).

Moreover, potential effect on cellular proliferation and in the modulation of two fibroblast growth factors (FGF-1 and KGF) was also evaluated in this treatment.

Discussion

LLLT is a clinical procedure candidate to use in deceleration of fibroblast's aging processes [5]. Therefore, this investigation tried to contribute to the test of this hypothesis by an in vitro analysis involving foreskin fibroblasts exposed at

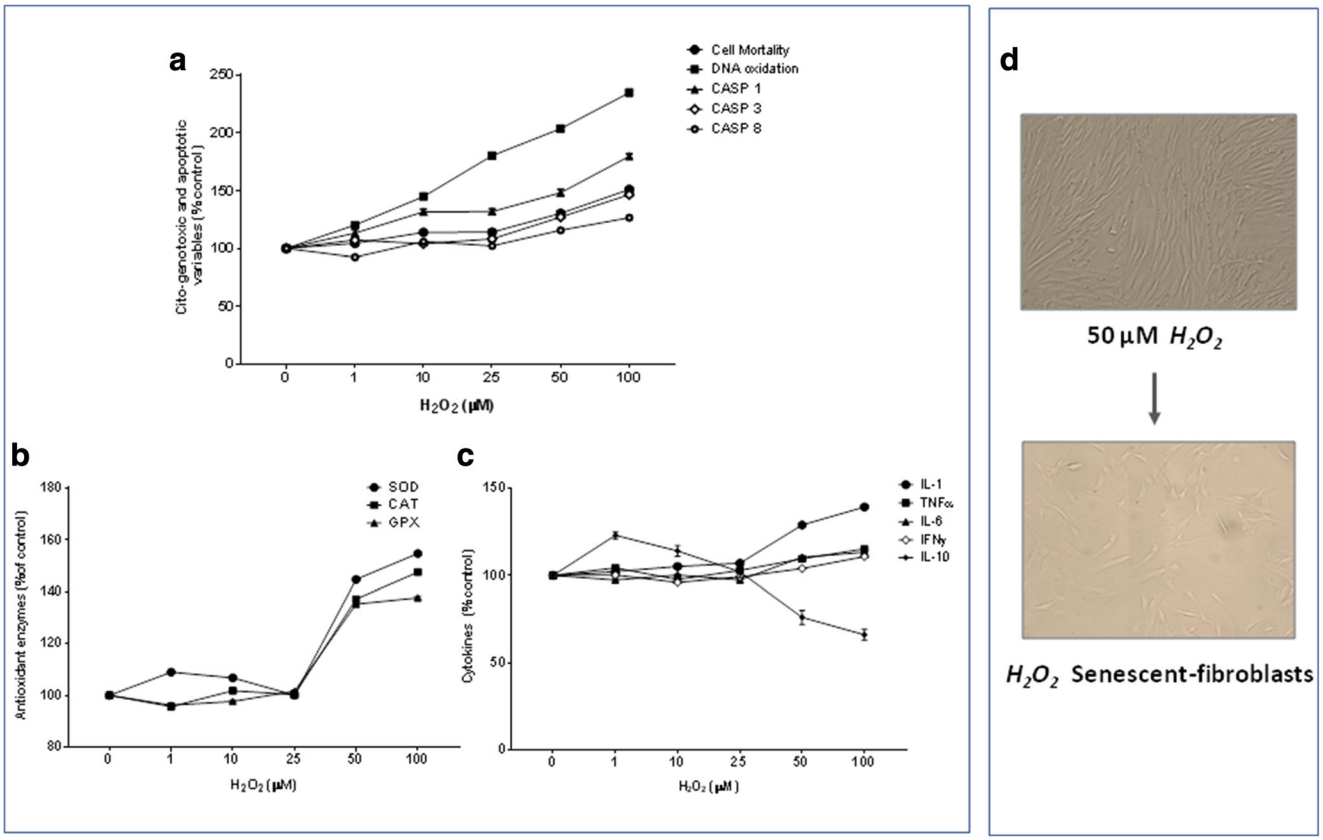

Fig. 1 Effect of different H_2O_2 series on markers of cytotoxicity and apoptosis (**a**), antioxidant enzymes (**b**), cytokines (**c**), and histological (**d**) analysis of fibroblasts

different LLLT doses with and without acceleration of aging markers obtained from cultures temporally H_2O_2-supplementation. In general, LLLT exposure caused positive and negative effects on fibroblasts dependent of range dose. LLLT at ≤ 4 J doses did not affect cell mortality, DNA oxidation, and apoptotic markers (CASP 1, 3, and 8) when compared to control cells. On the other hand, higher LLLT doses triggered increase in the levels of citogenotoxic markers and antioxidant enzyme. Moreover, higher LLLT dose leads to increase of proinflammatory cytokine supernatant concentrations indicating occurrence of a proinflammatory state in these cells. However, interaction between H_2O_2 at 50 μM and 4 J LLLT suggested that this treatment was able to minimize fibroblast senescent markers studied here. Despite methodological constrains related to in vitro studies, these results may be considered relevant in relation to the question of the LLLT effects on fibroblast aging needing to be discussed more thoroughly.

Before we discuss the potential LLLT effect on HFF-1 fibroblast, it is important to comment about the main variables to choose to do this evaluation. Senescence replication is an intrinsic process that disrupts the proliferative state of these cells. Despite it being an endogenous phenomenon, evidence showed that this process could be accelerated by extrinsic factors, such as irradiation exposure [15, 16].Therefore, a canonical analysis when cellular senescence is studied is respect to proliferative capacity that can be observed from cell cycle analysis.

The increase in the cellular mortality is also expected in senescent than younger cell cultures. The higher mortality induction of older cells is directly related to increase in the levels of DNA damage. During all time of cellular life, the DNA molecule is subject to oxidative damage from metabolic byproducts such as reactive oxygen species (ROS). Cells present an effective system of control of DNA damage including endogenous antioxidant enzymatic mechanism and a DNA-repair system. However, cells undergoing senescence trend to decrease the efficiency of ATP production by mitochondria and subsequently to increase ROS production, in special superoxide anion [3]. Therefore, analysis of oxidative and antioxidant markers can be used to compare younger and older fibroblast cells. Again, extrinsic factors can induce prooxidative states accelerating senescence events in young cell cultures. This process is well described when cells are exposed to molecules such as H_2O_2, and this effect can be prevented or reverted by some protector agent [17].

Furthermore, it is important to point out that if the rate of DNA damage exceeds the capacity of the cell to repair it, the

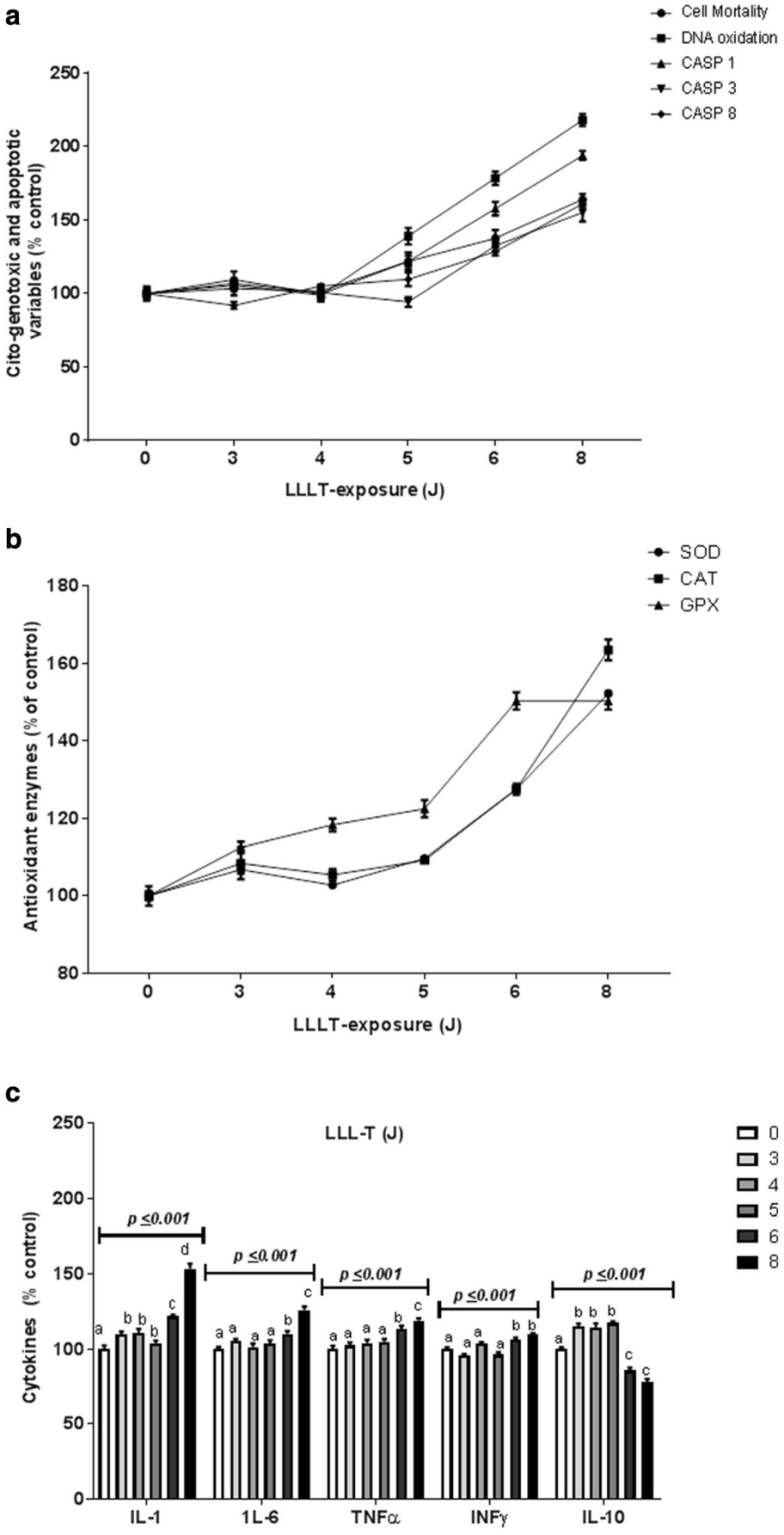

Fig. 2 Effect of different doses of LLLT on markers of citogenotoxic and apoptotic (**a**), antioxidant enzymes (**b**), and cytokines (**c**) in fibroblasts

accumulation of errors can overwhelm the cell. These cumulative DNA errors can lead to mutations and potentially to cancer [18, 19]. In order to control the negative effect of extensive DNA damage, cells present an intrinsic mechanism of apoptosis based in the detection of DNA damage by p53 protein. The intrinsic apoptosis is triggered by downregulation of Bcl-2 and overexpression of the Bax c that triggers change in the mitochondrial membrane realizing cytochrome c from into this organelle to cytoplasm. Elevation in the cytochrome c levels is able to trigger caspase apoptotic pathway including CASP 8, a regulator protein, and CASP3 and CASP 1, which are executive proteins of this. Extrinsic agents also can bind with external membrane receptors named "dead receptors" and trigger directly apoptosis pathway. Therefore, cellular H_2O_2 exposure can trigger both, extrinsic and intrinsic apoptotic events, in fibroblast cells. For this reason, we studied here the LLLT effects on CASP modulation [16].

Body aging processes have been closely related with existence of chronic inflammatory states that present an imbalance in the production of proinflammatory and anti-inflammatory cytokines. Generally, tissue inflammatory states are considered as a process triggered just by leucocytes, in special resident macrophages. However, fibroblast seems also to contribute with inflammatory processes These are key components of the stroma and are strongly involved in maintenance of extracellular matrix and tissue function. Probably due to its plastic function in the tissues, fibroblasts are able to trigger immune response. For example, these cells possess Toll-like receptors allowing them to respond to pathogen and damage-related signals by producing proinflammatory mediators such as IL-6, PGE2, and GM-CSF and can produce a range of chemokines such as CXCL12, CXCL13, and CXCL8 which attract B and T lymphocytes, monocytes, and neutrophils to sites of inflammation [20]. Fibroblast also produces proinflammatory cytokines that can also be modulated by anti-inflammatory agents [21], and prooxidant molecules, such as H_2O_2 and UV radiation, can trigger gene and protein expression of proinflammatory cytokines by fibroblast cells [22]. These initial considerations justify the variables analyzed here in order to evaluate potential LLLT effects on senescence fibroblast markers.

Moreover, in the present discussion, it is important to point out that laser stimulation is a procedure widely used for clinical applications due to its properties that did not cause iatrogenic malignancies, including LLLT therapy [23–25]. However, in the present investigation, we observed that higher LLLT doses triggered citogenotoxic effect including apoptosis induction. These results were expected since it is well

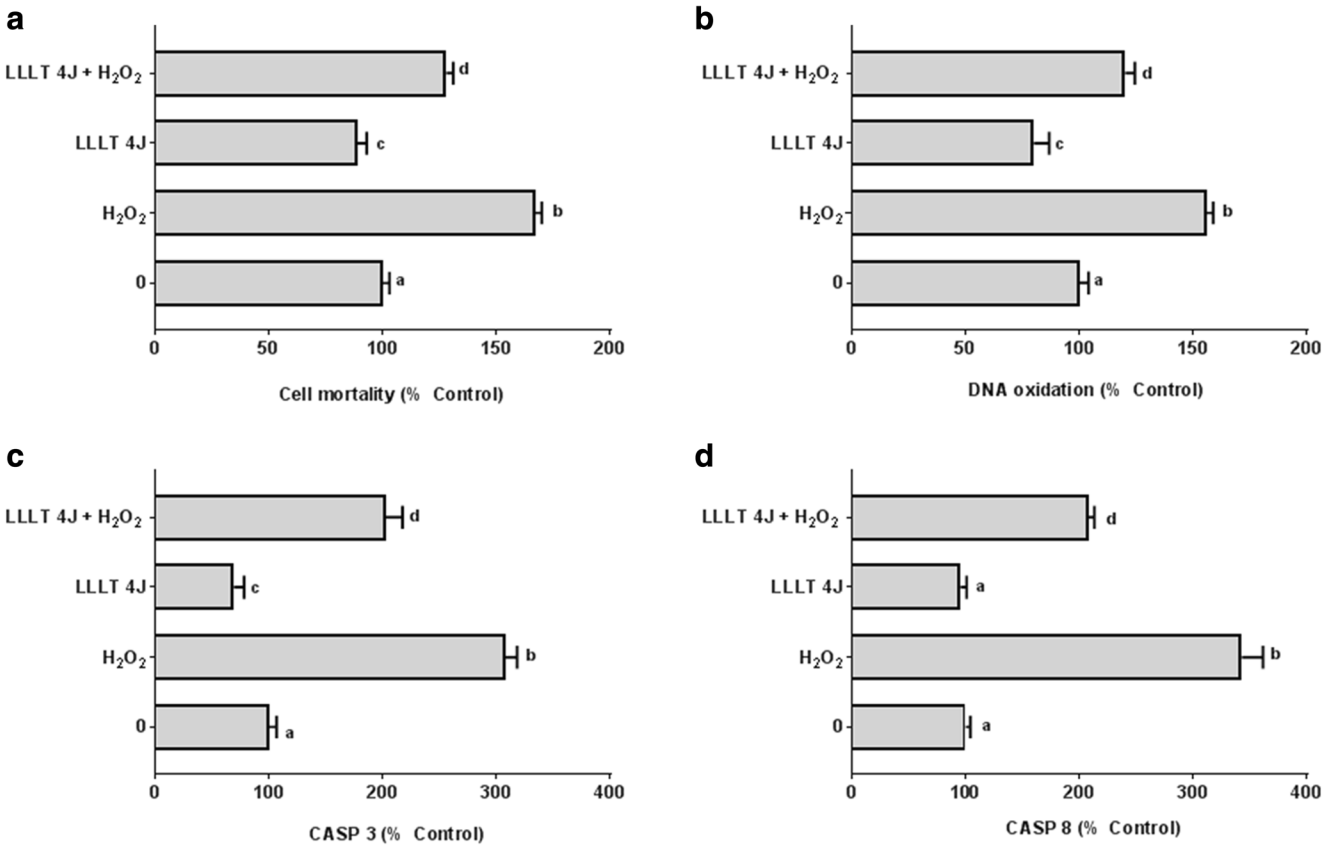

Fig. 3 Comparison of changes in the levels of cell mortality (**a**), DNA oxidation (**b**), and caspase 3 (**c**) and caspase 8 (**d**) markers in fibroblast and treated with LLLT 4 J + H_2O_2, LLLT 4 J, H_2O_2, incubated for 72 h

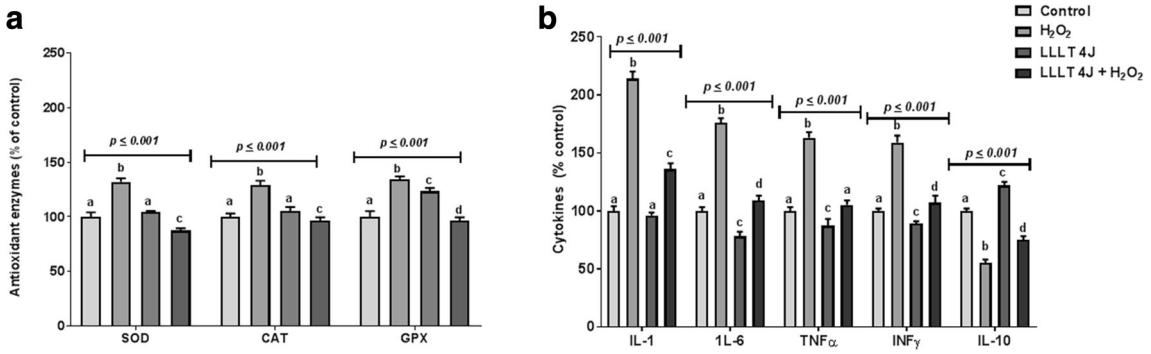

Fig. 4 Comparison of changes in the levels of antioxidant enzymes (**a**) cytokines in fibroblast and treated with LLLT 4 J + H_2O_2, LLLT 4 J, H_2O_2, incubated for 72 h

established that biological response to LLLT stimulation is dependent of several parameters including wavelength, irradiance, time, pulse, light coherence, polarization, and others [6].

These results allowed us to infer best LLLT dose-exposure that could have some beneficial effects in the reversal of changes caused by temporary fibroblast exposure to H_2O_2. In fact, H_2O_2 is an important biological molecule because low and controlled concentrations are a regulatory signaling molecule that has several actions including regulation of proliferative states of some cells. However, when H_2O_2 is present in high concentrations, such as at 50 μM, its molecule triggered cellular events, especially in fibroblast cells that are closely related do senescence characteristics. For this reason,

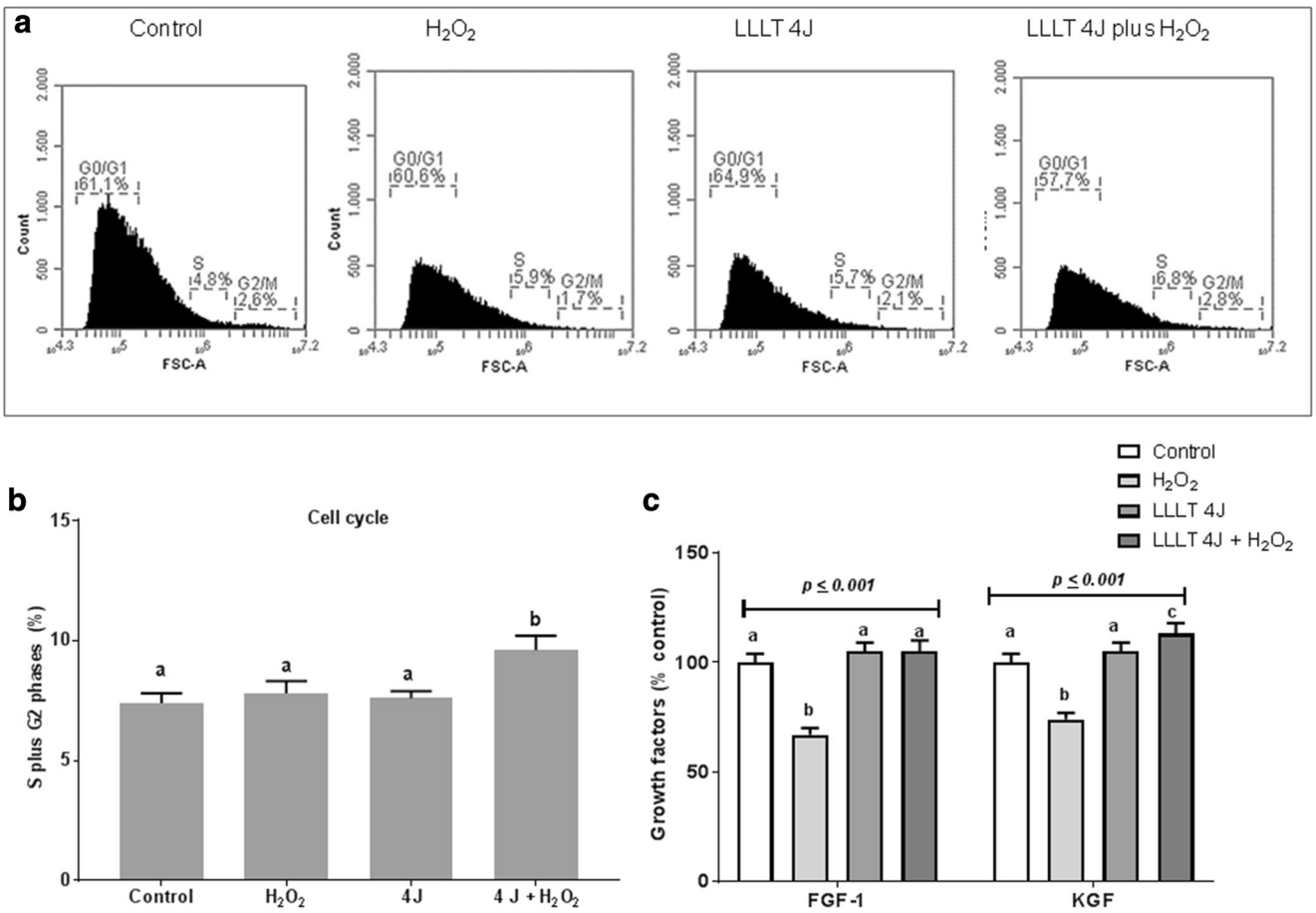

Fig. 5 Comparison of cell cycle progression in fibroblast treated with LLLT 4j + H2O2, LLLT 4j, H_2O_2 cultured for 72 h. **a** Representative flow cytometry analysis graphs for each treatment. G1 = gap 1 phase, G2 = gap 2 phase, and S = synthesis phase. **b** Representative de S plus G2 phases for each treatment, and the graphic (**c**) indicates the effects of different treatments in fibroblasts and keratinocyte grow factors

fibroblast exposure to H_2O_2, as used here, is considered a good in vitro model to understand mechanisms and identify regulatory molecules related to replicative senescence and other aging characteristics [26–28].

Results obtained from fibroblast just LLLT-exposed showed that at ≤ 4 J/cm^2 dose occurred significantly, by slight increase in IL-1 levels whereas other proinflammatory cytokines were not modulated by this treatment. On the other hand, lower LLLT doses were able to improve the levels of IL-10, an anti-inflammatory cytokine. Prior evidences showed that LLLT treatment acts on wound-healing process by increase keratinocyte migration [29], also contributing in the extracellular matrix organization by increase of collagen production, as well as other tissue factors such as matrix metalloproteinase-2 (MM-2) [29]. Moreover, LLLT seems to be able to modulate several genes related to skin wound-healing processes. For example, a recent study described that LLLT (1 to 5 J/cm^2) induced in vitro protein synthesis in fibroblasts upregulating the gene expression of vascular endothelial growth factor (VEGF), which is an important molecule that stimulates the formation of blood vessels in the body. In the same study, the authors also observed downregulation of IL-6 gene expression in these cells [25]. An investigation performed by Silveira et al. [30] reported that in rats, LLLT (3 to 5 J/cm^2) is able to prevent proinflammatory state muscle trauma-induced. Results from this investigation also reported that after 2 h, muscle injury caused an increase in several oxidative markers, as well as in antioxidant enzyme levels. The higher antioxidant enzyme levels remained higher in the next 24 h after trauma injury. However, these alterations were prevented by LLLT treatment especially at a 3 J/cm^2 dose.

In fact, when cells were previously H_2O_2 exposed, LLLT action on fibroblast was more pronounced, since this treatment was able to prevent cell mortality, DNA oxidation, and CASP level increase caused by H_2O_2 exposure. Similar to the study performed by Silveira et al. [30], LLLT decreased significantly the levels of the three main antioxidant enzymes evaluated here: SOD, CAT, and GPX. In general, LLLT also reduced, at least partially, the levels of proinflammatory cytokines and increased IL-10 levels. Actually, anti-inflammatory property of LLLT treatment has been described from previous studies, such as performed by Da Ré Guerra et al. [31] also after physical trauma (partial tenotomy). Therefore, the whole of results suggest that LLLT can modulate some apoptotic, oxidative, and inflammatory markers present in fibroblast H_2O_2 aging induced.

A complementary analysis was performed in order to determine if LLLT could also act on proliferative state of fibroblast H_2O_2-exposed fibroblast. Surprisingly, cell cycle analysis in 72-h cultures did not show significant change in the frequency of S and G2 phases in control fibroblasts and fibroblast just H_2O_2 or LLLT-exposed. However, the interaction between H_2O_2 and LLLT changed this result, since in this treatment, there occurred an increase in the frequency of cells that was in S/G2 phases.

As this result could be confused, since just proliferation does not necessarily reflect a desired proliferative state of the fibroblasts, analysis of two important growth factor markers was also performed. The first marker studied here was FGF-1. This molecule is a powerful mitogen involved in the stimulation of DNA synthesis and the proliferation of a wide variety of cell types, including fibroblasts. For this reason, recombinant FGF-1 has been developed and studies, such as Żerańska et al. [32], showed that this molecule strongly stimulated fibroblast and keratinocyte proliferation. In our results, fibroblast just H_2O_2-exposed showed lower FGF-1 levels whereas cells just LLLT-exposed presented similar levels of this molecule than control cells. However, interaction between H_2O_2 and LLLT reverted totally lower FGF-1 levels triggered by H_2O_2, indicating that in some "aging fibroblast states," LLLT could help to stimulate skin cell proliferation. This suggestion is corroborated by a prior investigation that described LLLT property to upregulate the FGF-1 gene expression in the healing of diabetic wounds [33].

The interaction between H_2O_2 and LLLT exposure also increased levels of KGF-1 molecule, which is also member of a growth factor family that present broad mitogenic and cell survival activities. In fact, this is considered a crucial molecule in fibroblast-keratinocyte cross talk. However, in some situations, such fibrotic disorders KGF-1 can be overexpressed in the tissue [34, 35]. However, results found here did not show extensive increase in the KGF-1 levels despite that cells just LLLT treated presented similar concentrations of this molecule than control fibroblasts. Furthermore, despite that interaction between H_2O_2 and LLLT induced a significant increase in KGF-1 levels, this induction was considered slight being less than 20% when compared to the untreated control group.

Finally, it is important to point out that better LLLT effects on fibroblast cells H2O2-exposure can be associated with its biological properties. In fact, any LLLT induces photobiological response that is determined by the absorption of the light energy by a chromophore. From LLLT exposure, two main types of photo acceptors can be stimulated: cytochrome oxidase c and intracellular water. The photon absorption by these two photo acceptors has pleiotropic effect on laser-stimulated cellular metabolism. In this way, LLLT exposure can trigger cellular proliferation and also act by interacting with other proposed primary acceptors. These emergent acceptors include cell membrane molecules such as ion channels and porphyrins and flavoproteins. Moreover, LLLT exposure can act on cellular homeostasis parameters, such as pH, redox state, and redox-sensitive factors [36]. Evidence from literature has suggested that low or moderate ROS levels act as signaling factors in the cellular metabolism and did not present extensive negative effects. It has been demonstrated that LLLT can induce ROS production, and that action could be responsible

to stimulate fibroblast proliferation [37, 38]. Therefore, we consider that there is biological plausibility to explain the whole of results related to LLLT fibroblast exposure described here.

Conclusion

In summary, the whole of results described here suggested that LLLT exposure on fibroblast without any injury is relatively safe and innocuous considering its potential citogenotoxic, oxi-inflammatory, and proliferative effects. On the other hand, in fibroblast H_2O_2-injured that mimetic premature senescent cells characteristics, LLLT presented an important protective and proliferative effect, reverting partially or totally negative effects triggering by H_2O_2. In these terms, it is possible to infer that, in some dose range, LLLT could present some anti-aging properties.

Compliance with ethical standards

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

Ethics approval Since the study used commercial cell lines, it is not necessary to submit to an ethics committee.

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