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ORIGINAL ARTICLE

The effect of low-level laser irradiation on hyperglycemia-induced inflammation in human gingival fibroblasts

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Abstract

Hyperglycemia-induced inflammation can greatly increase the risk of periodontal disease in people with diabetes. Low-level laser irradiation (LLLI) has been used for wound healing and anti-inflammation in many cases, and LLLI is known to inhibit the lipopolysaccharide (LPS)-stimulated inflammatory response. However, the therapeutic effect of LLLI in diabetes patients with periodontitis remains unknown. In this study, we cultured human gingival fibroblasts (HGFs) in high-glucose medium (35 mM) to mimic a hyperglycemic environment, and then measured the anti-inflammatory effect of LLLI by assessing the expression of pro-inflammatory genes including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 by quantitative real-time polymerase chain reaction. The results demonstrated no significant inflammatory response in HGFs cultured in mannitol medium and in those treated only with LLLI. However, HGFs cultured only in high-glucose medium showed significantly higher expression of pro-inflammatory cytokine than in those treated together with LLLI. We then observed that LLLI reduced the expression of pro-inflammatory cytokines in HGFs cultured in high-glucose medium by modulating cAMP signaling. We also investigated whether antioxidant (vitamin C) treatment reduced the inflammatory effect of oxidative stress in HGFs cultured in high-glucose medium but found no additive effect upon co-treatment with LLLI, suggesting that LLLI may activate cAMP signaling, but not reactive oxygen species (ROS) signaling, to reduce the high glucose-induced inflammation. In conclusion, LLLI may have an anti-inflammatory effect on HGFs in a high glucose environment and may benefit the treatment of periodontal disease in diabetes patients.

Keywords Low level laser irradiation · Hyperglycemia · Reactive oxygen species · Pro-inflammatory cytokines · Vitamin C

Introduction

Patients with Type 2 diabetes (T2D) compared to those without this disease have two to five times the risk of periodontal diseases [1, 2]. These diseases have a higher prevalence and greater severity in this diabetes population [3]. Periodontal diseases are inflammatory diseases that can damage soft and hard tissue if left untreated [1, 2]. High blood glucose levels have been found to change the bacterial flora and decrease immunity in patients with diabetes, making them more

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susceptible to periodontal disease [4], and glucose levels in crevicular fluid of periodontal pockets is reported to increase bacterial proliferation and inflammation [5]. Thus, effective control of hyperglycemia can reduce the risk of periodontal disease in patients with diabetes [6].

Hyperglycemia increases oxidative stress resulting from an imbalance between free-radical-generating and free-radical-scavenging systems [7, 8]. The free radicals induce oxidative stress leading to oxidative damage in living cells and tissues. Free radicals might induce gene expression and DNA oxidation or they might induce the secretion of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α), further increasing the production of free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) [9, 10]. Therefore, high glucose levels have been found to increase ROS production and expression of pro-inflammatory cytokines in different cell types [11–13].

Low-level laser irradiation (LLLI), an application that uses red and near infra-red light to change the cell function and boost the cell metabolism, has previously been found able to inhibit the production of pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated human gingival fibroblasts (HGFs) in healthy cells [14, 15]. In addition to reducing inflammatory responses, it also improves wound healing and enhances tissue regeneration in healthy patients [16–18]. However, it is unclear what effect LLLI would have when treating periodontitis of diabetes patients [19].

One of our previous studies showed that LLLI reduced the secretion of IL-1β-, IL-6-, IL-8-, and TNF-α-induced by LPS challenge [20]. In the present study, we investigated the effect of LLLI on the hyperglycemic-induced inflammatory response in HGFs and sought to identify which pathway it might achieve this effect. We first cultured HGFs in a high-glucose medium to mimic a hyperglycemic environment and measured the expressions of pro-inflammatory cytokines. We then measured these biomarkers of inflammation in HGFs treated and untreated with LLLI. To further elucidate possible crosstalk between LLLI and ROS signaling pathways, we measured inflammation response in LLLI-treated HGFs added with either the adenylyl cyclase inhibitor or antioxidant vitamin C.

Materials and methods

Cell culture and collection

After obtaining informed consent from all patients and approval from Kaohsiung Medical University Hospital ethics committee (KMUH-IRB-20110031), we collected healthy marginal gingival tissues from patients receiving surgical extraction of impacted third molars. HGFs were isolated and 2×10^5 cells per well were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibson, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin-G, and 50 μg/ml streptomycin in a humidified incubator at 37 °C with 5% CO^2. The medium was changed every other day. Cells were grouped into two parts: the first part was further divided into six groups for determining the effect of low-level laser irradiation as follows: (1) control group, (2) osmotic control group containing mannitol at a concentration of 35 mM, (3) high glucose group containing glucose at a concentration of 35 mM, (4) laser-irradiated group, (5) high glucose with laser irradiated group, and (6) high glucose with laser irradiation and the adenylyl cyclase inhibitor SQ22536 (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 100 μmol/L. The second part of cells was divided into four groups for determining the crosstalk between LLLI and ROS: (1) control group, (2) high glucose group containing glucose at a concentration of 35 mM, (3) high glucose with the antioxidant vitamin C at a concentration of 0.1 mM, and (4) high glucose with the antioxidant and laser irradiation. The cells in all groups were collected at 24 h of treatment for RNA extraction.

Low-level laser irradiation

A gallium-aluminum-arsenide red laser with *Powell* lenses to produce a flat top beam profile (wavelength 660 nm diode laser, Transverse Industries Co., Ltd., Taipei, Taiwan) was used as the laser source, output set at 70 mW. The distance between the bottom of culture plate and laser source was 3 cm, the irradiated area 3.8 cm^2. Cells were irradiated once for 528 s in a continuous mode, receiving a total of 8 J/cm^2 of laser energy density. All irradiated experiments were performed on a clean bench at room temperature. The control groups were processed under the same conditions but were not subjected to laser irradiation.

MTT and LDH assay

The MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is based on living cell conversion of MTT into formazan crystals and is used to detect mitochondrial activity and cell viability. Briefly, normal control HGFs, osmotic control HGFs, and high-glucose HGFs were seeded onto a 96-well plate for 24 h and incubated with 5 mg/mL MTT for 4 h. Cell reaction was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Model no. 680, Bio-Rad) at 595 nm wavelength. Lactate dehydrogenase (LDH), an oxidoreductase present in a wide variety of organisms, catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+ [21]. LDH leakage was

measured to access cytotoxicity using a Cytotoxicity Detection kit (Roche, Germany) following the manufacturer's directions. The absorbance values were measured using an ELISA reader set at 490 nm wavelength.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNAs were extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. After purification, total mRNA was reverse transcribed into single-strand cDNAs and then quantitative real-time PCR was performed in a Bio-Rad iQ5 real-time detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The reactions were performed in a mixture containing cDNA, primers for each gene, and iQ™ SYBR-Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). The sequences of forward and reverse primers for GAPDH, TNF-α, IL1-β, IL-6, and IL-8 are listed in Table 1. The relative mRNA expression levels were calculated from the threshold cycle (Ct) value of each PCR product and normalized to the housekeeping gene GAPDH using the comparative Ct method.

Statistical analysis

All the data are expressed as mean ± SD from three to six independently performed experiments. Statistical significance was analyzed using the one-way analysis of variance test (ANOVA). A two-sided Student's *t* test was used to determine the differences between various treatment groups. A probability value of 0.05 or less was considered significant. All statistical operations were performed using SPSS Version 20 (IBM, Chicago, IL, USA).

Table 1 Primer sequence

Gene	Primer sequence
GAPDH	Forward: 5′-CAATGACCCCTTCATTGACC-3′
	Reverse: 5′-TTGATTTTGGAGGGATCTCGAT-3′
TNF-α	Forward: 5′-CTCGAACCCCGAGTGACAAG-3′
	Reverse: 5′-TGAGGTACAGGCCCTCTGAT-3′
IL1-β	Forward: 5′-AAACAACCTGAACCTTCCAAAGA-3′
	Reverse: 5′-GCAAGTCTCCTGATTGAATCCA-3′
IL-6	Forward: 5′-CCTGACCCAACCACAAATGC-3′
	Reverse: 5′-ATCTGAGGTGCCCATGCTAC-3′
IL-8	Forward: 5′-CAGGAATTGAATGGGTTTGC-3′
	Reverse: 5′-AAACCAAGGCACAGTGGAAC-3′

Results

Cell viability and cytotoxicity

Cell viability was analyzed by microscope (×100) and MTT assay. Cytotoxicity of controls, osmotic controls, and high-glucose HGFs were analyzed by LDH leakage assay. As seen in Fig. 1, we found no difference in morphological changes in any of the groups. No differences were found in MTT and LDH leakage assay results for controls, osmotic controls, and high-glucose HGFs at 24 h (Fig. 2a, b). Together, these results show no short-term reduction in cell viability or cytotoxicity.

The mRNA expression levels of inflammatory cytokines

Cells were cultured in different conditions, and the mRNA expressions of IL-1β, IL-6, IL-8, and TNF-α were analyzed by RT-PCR. Interestingly, as can be seen in Fig. 3, the four pro-inflammatory cytokines showed similar expression patterns. No obvious changes were found in any of the pro-inflammatory cytokines in the HGFs cultured in mannitol or only LLLI-treated, compared to the control. Significant increases in these pro-inflammatory cytokine mRNA expressions were found in the high glucose group but when the same group was treated with LLLI, the levels of mRNA expressions were decreased significantly.

To determine if the inhibition of mRNA expression of the high glucose-induced pro-inflammatory cytokines from LLLI occurred through the cAMP signaling pathway. To find out, we added the pharmacological inhibitor of adenylyl cyclase (SQ22536) to high-glucose cells treated with LLLI. The cytokines were significantly elevated in the cAMP-inhibited group, suggesting that LLLI exerted its effect through the cAMP signaling pathway (Fig. 3).

In another experiment, we wanted to determine whether LLLI altered ROS production in high-glucose medium cultured HGFs. To find out, the cells were incubated with vitamin C to inhibit high glucose-induced production of ROS. As can be seen in Fig. 4, both LLLI treatment and vitamin C treatment significantly decreased the mRNA expression levels of the pro-inflammatory cytokines in high-glucose HGFs. However, the combined use of LLLI and vitamin C did not provide any additive effect or synergistic effect, suggesting that while LLLI exerted its effect though the cAMP pathway, it may not work though ROS signaling (Fig. 4).

Discussion

This study found that LLLI could be used to control hyperglycemia-induced inflammatory responses in high-

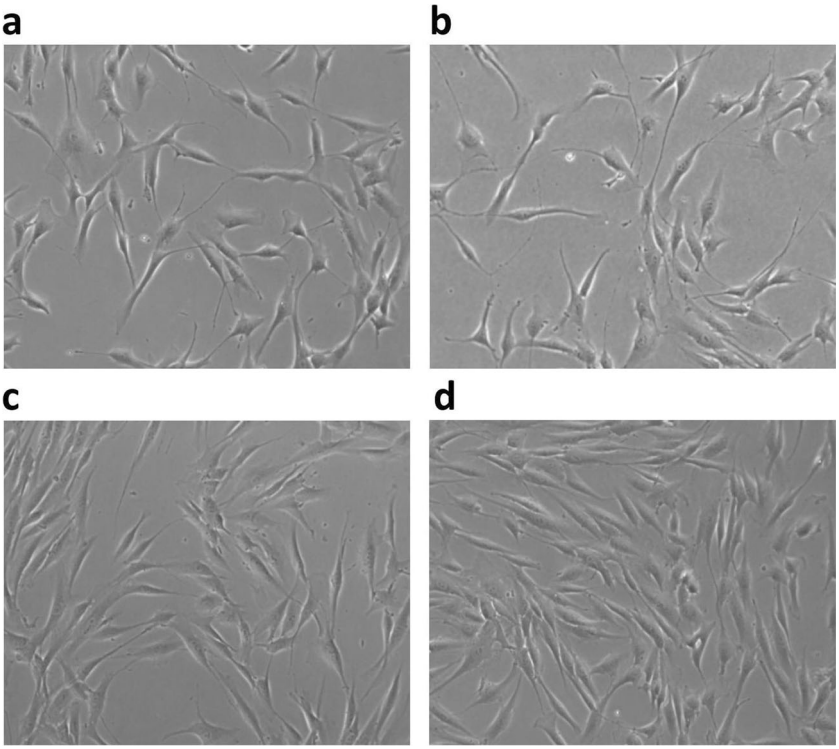

Fig. 1 Photomicrographs of HGFs in different condition culture medium (× 100). No morphological changes of HGFs were investigated in any of groups. **a** Control group. **b** Osmotic control group. **c** High glucose group. **d** LLLI+ High glucose group

glucose HGFs. It exerted its effect via cAMP signaling, not ROS signaling. Hyperglycemia, one feature of diabetes, has been found to increase inflammation (IL-6 and IL-8 levels) in HGFs in patients with periodontitis [2, 22, 23]. We found similar increased expressions of inflammatory cytokines in our HGFs cultured in a high-glucose medium, compared to those cultured in mannitol. This finding is in agreement with that of Jiang et al. who reported that high glucose stimulated TNF-α and IL-1β secretion by inducing TLR2 through PKC-α and PKC-δ in HGFs [24]. Wu et al. also documented high glucose-induced secretion of pro-inflammatory cytokines such as IL-6, TNF-α, and CCL2 and promoted the production of IL-10 [25]. Together, their findings and ours show that hyperglycemia can induce inflammation in HGFs.

Hyperosmolarity, another biophysical factor occurring in diabetic hyperglycemia, may influence cell fate in physiological and pathological conditions [26]. According to Liu et al., osmotic pressure does not induce extra inflammatory responses with the same oscillation as periodic high glucose [27]. Our study cultured HGFs in a mannitol medium (a hyperosmotic environment) to create osmotic controls. In these controls, we found reduced cell viability but no significant difference between osmotic controls and normal controls with regard to mRNA expression of pro-inflammatory cytokines (IL1-β, IL-6, IL-8, and TNF-α), indicating that the hyperosmotic condition did not induce the inflammatory responses in the HGFs we studied.

LLLI has been found able to reduce inflammatory responses in a dose-dependent manner in in vitro and in vivo

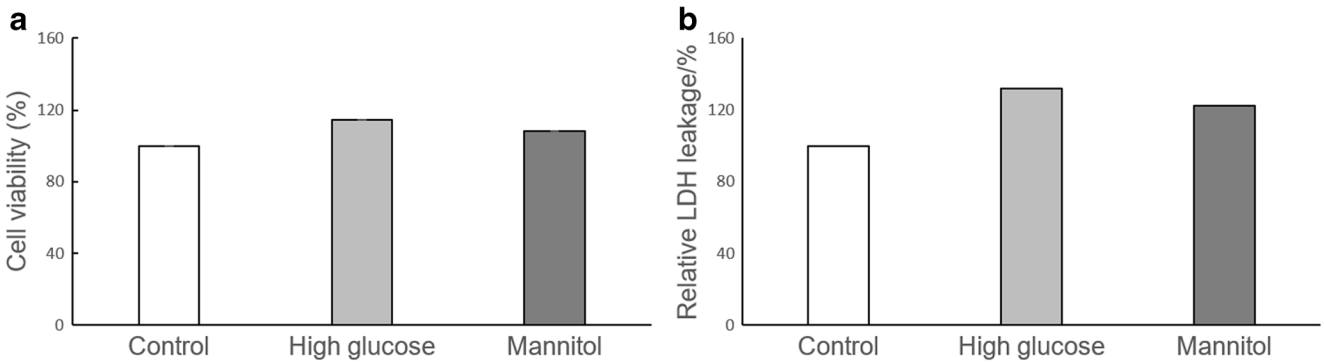

Fig. 2 High glucose did not induce cytotoxic effect nor reduce the viability of HGFs. Cell viability and cytotoxicity were investigated by **a** MTT assay and **b** LDH leakage assay at 24 h. The data are shown as the mean ± SD. $N = 6$ (N numbers of independent experiments)

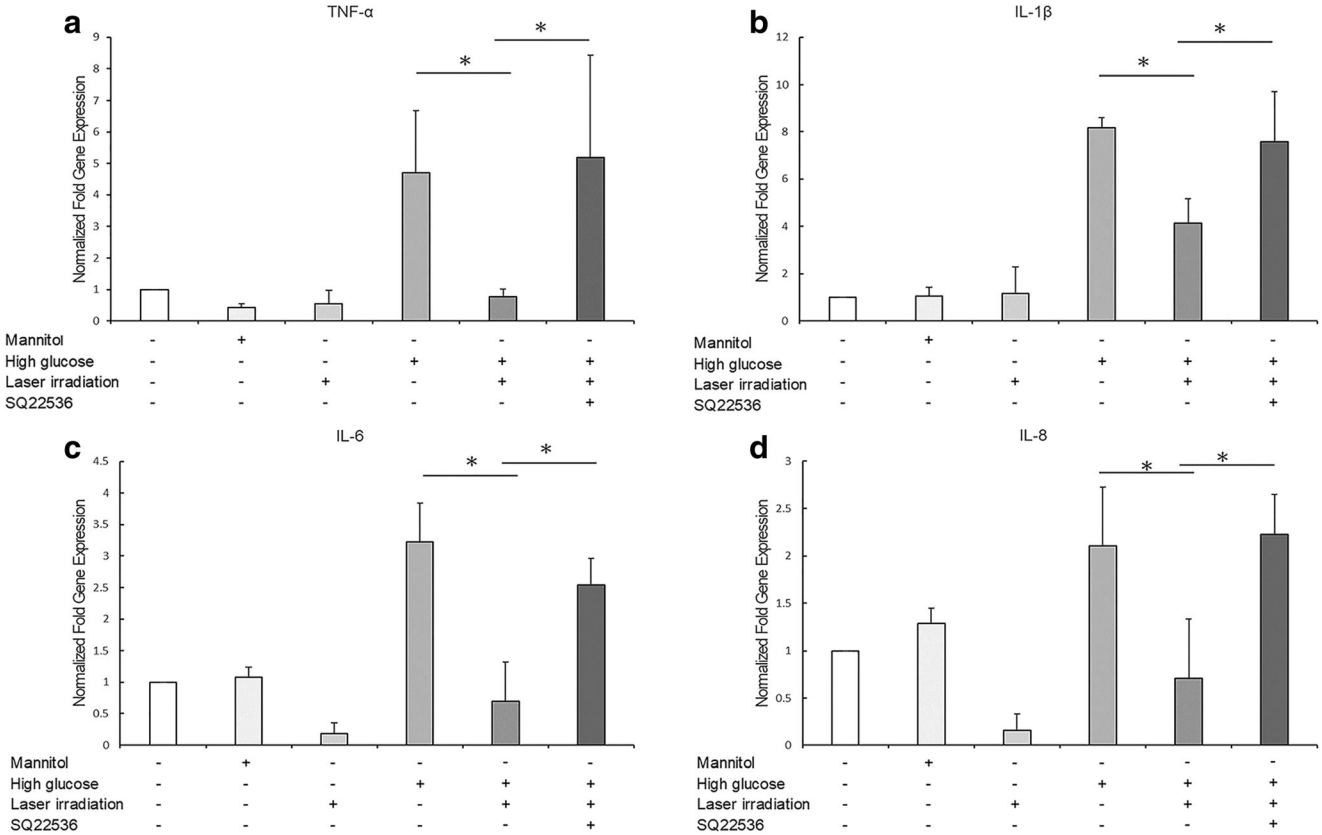

Fig. 3 LLLI had the anti-inflammatory effect on high glucose cultured HGFs, and a cAMP inhibitor (SQ22536) hindered the anti-inflammatory effect of LLLI. Real-time RT-PCR was performed to determine the mRNA levels of a TNF-α, b IL1-β, c IL-6, and d IL-8 at 24 h. The results were analyzed with the $2-\Delta CT$ method based on the control. The data are shown as the mean ± SD. $N = 6$ (N numbers of independent experiments). $^{*}p < 0.05$

studies using laser energy units ranging from 3 to 7.7 J/cm^2 [28–30]. Previously, we performed a series of studies showing that LLLI (GaAlAs laser, 660 nm) could effectively suppress the inflammatory response in human adipose-derived stem cells at an optimal dose of 8 J/cm^2 [20, 31]. Laser irradiation has previously been found by several studies to reduce the expression of pro-inflammatory cytokines under hyperglycemic conditions [32–34]. Consistent with previous studies, our current experiments found the LLLI effectively suppressed the mRNA expression levels of pro-inflammatory cytokines (IL1-β, IL-6, IL-8, and TNF-α) in high-glucose HGFs.

In an additional experiment, we observed that the anti-inflammatory effect of LLLI was decreased in high-glucose cells after we added the cAMP inhibitor SQ22536 to stop the activation of the cAMP pathway. This phenomenon is similar that was reported by one of our previous studies in which we found that the cAMP inhibitor SQ22536 hindered the anti-inflammatory effect of LLLI on inflammation induced by LPS in hPDLCs [31]. Considering all these findings together, we hypothesize that LLLI suppresses inflammation in high-glucose HGFs by modulating the activity level of cAMP, downregulating nuclear factor kappa B (NF-κB) transcriptional activity [20, 31]. This hypothesis needs to be tested in future studies.

Oxidative stress, commonly found in un-controlled hyperglycemia, results from the mitigation of the antioxidant defense system or overproduction of free radicals [7, 8, 35]. The antioxidant defense system reduces tissue damage by removing excess free radicals by increasing intracellular levels of non-enzymatic antioxidants such as vitamins C and E that have been found to scavenge O^{2}– levels in coronary endothelial cells [7, 36]. Intake of a moderate amount of vitamin C has been found to significantly reduce the expression of the inflammatory markers hs-CRP and IL-6 in obese patients with diabetes [37]. Similarly, our results showed that the mRNA expressions of pro-inflammatory cytokines were significantly reduced after adding vitamins C into high-glucose HGFs.

Hyperglycemia-induced elevation of ROS may further activate NF-κB signaling to promote inflammatory processes [38, 39]. The effects of LLLI on ROS formation is interesting. At low levels, LLLI might block the release of ROS and activation of NF-κB to reduce the trauma-induced inflammation [40]. LLLI has been found to suppress the formation of ROS and activation of NF-κB in LPS-treated mesenchymal stem

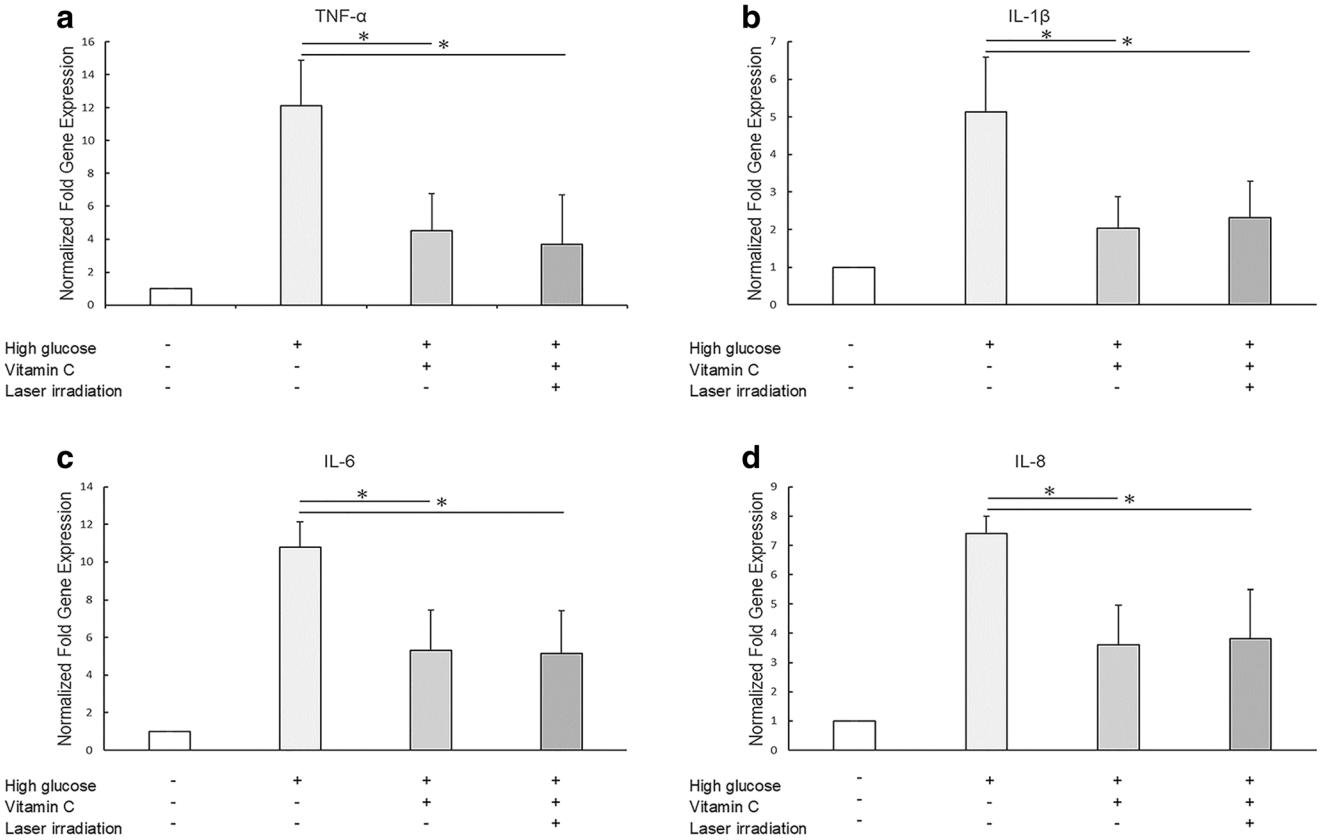

Fig. 4 The co-treatment of LLLI and vitamin C had no additive anti-inflammatory effect on high-glucose cultured HGFs. Real-time RT-PCR was performed to determine the mRNA levels of **a** TNF-α, **b** IL1-β, **c** IL-6, and **d** IL-8 at 24 h. The results were analyzed with the $2-\Delta CT$ method based on the control. The data are shown as the mean ± SD. $N = 3$ (N numbers of independent experiments). $^{*}p < 0.05$

cells [41]. At high levels, LLLI has been found to significantly increase the level of ROS [42]. However, Chen et al. indicated that 810-nm laser irradiation increased the production of ROS and activated the NF-kB signaling in fibroblasts [43]. This kind of controversial results may due to irradiation under different wave length of laser or different cell types may display different response to laser irradiation. In the present study, we found that co-treatment of LLLI and vitamin C did not have an additive effect or synergistic effect on the decrease of mRNA expression levels of the pro-inflammatory

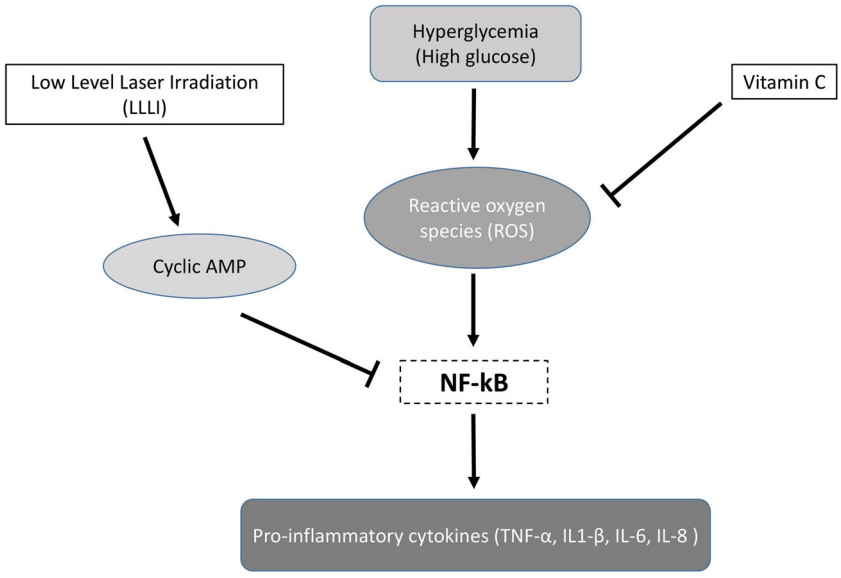

Fig. 5 LLLI and vitamin C both reduced the expression levels of pro-inflammatory cytokines through inhibiting the activity of NF-κB, one via cAMP signaling, and the other via ROS signaling

cytokines. As seen in Fig. 5, LLLI and vitamin C both decreased the expression of the high glucose-induced pro-inflammatory cytokines. They both also reduced the activity of NF-κB, one via cAMP signaling and the other via ROS signaling.

Obradovic et al. performing histologic studies of gingival samples taken from patients with DM and periodontitis found that LLLI reduced inflammatory cells and tissue edema [44, 45]. Our study found that LLLI reduced the expression of the four pro-inflammatory cytokines in high-glucose HGFs. Thus, it provides some insight into the possible mechanisms through which LLLI has this effect on hyperglycemia-induced inflammation.

Conclusion

The present study demonstrates that high glucose significantly induced the production of pro-inflammatory cytokines (IL1-β, IL-6, IL-8, and TNF-α) in HGFs, and LLLI effectively reduced the mRNA expression levels of these pro-inflammatory cytokines at a dose of 8 J/cm^2. LLLI may activate cAMP signaling pathway, but not ROS signaling pathway, in its reduction of high glucose-induced inflammatory reaction. These findings from our in vitro study suggest that LLLI can potentially be used as an effective adjunct therapy in the treatment of periodontitis in patients with diabetes.

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Compliance with ethical standards

Ethical approval This study was independently reviewed and approved by the human subjects ethics board of Kaohsiung Medical University and was conducted in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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