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Original Article

Er:YAG laser suppresses pro-inflammatory cytokines expression and inflammasome in human periodontal ligament fibroblasts with Porphyromonas gingivalis-lipopolysaccharide stimulation

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KEYWORDS
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periodontal ligament fibroblasts

Abstract *Background/purpose:* Periodontitis is an inflammatory condition of the tooth-supporting structures triggered by the host's immune response towards the bacterial deposits around the teeth. It is well acknowledged that pro-inflammatory interleukin (IL)-6, IL-8, MCP-1 as well as the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, are the key modulators in the activation of this response. Erbium-doped yttrium-aluminium-garnet (Er:YAG) laser, a solid-state crystal laser have been commonly used in the treatment of periodontal diseases. However, little is understood about the molecular mechanism of the Er:YAG laser, especially in targeting the host immune response brought on by periodontal pathogens. Hence, the current study focused on the protective effects of Er:YAG laser on periodontitis in-vitro in terms of pro-inflammatory cytokines, chemokines and NLRP3 inflammasome expressions. *Materials and methods:* Human periodontal ligament fibroblast (PDLFs) were first stimulated with lipopolysaccharides (LPS) from *P. gingivalis* (Pg-LPS) to simulate periodontitis. Cells were then irradiated with Er:YAG laser of ascending energy densities (3.6–6.3 J/cm^2), followed by cell proliferation and wound healing assay. Next, the effects of Er:YAG laser on the expressions of IL-6, IL-8, MCP-1, NLRP3, and cleaved GSDMD were examined.

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Results: Pg-LPS was found to reduce cell's proliferation rate and wound healing ability in PDLFs and these were rescued by Er:YAG laser irradiation. In addition, LPS stimuli resulted in a marked upregulation in the secretion of IL-6, IL-8 and MCP-1 as well as the mRNA and protein expression of NLRP3 and cleaved-GSDMD protein whereas Er:YAG laser suppressed the elicited phenomena.
Conclusion: To our knowledge, this is the first study to look into the laser's implication on the NLRP3 inflammasome in periodontitis models. Our study reveals a crucial role of Er:YAG laser in ameliorating periodontitis in-vitro through the modulation of IL-6, IL-8, MCP-1 and the NLRP3 inflammasome and highlights that the control of the NLRP3 inflammasome may become a potential approach for periodontitis.

Introduction

Chronic periodontitis is a common oral disease that affects almost half of the global population, as stated by the World Health Organization (WHO).[1] Periodontitis is a local inflammation of the tissues surrounding the teeth and can lead to the degradation of underlying connective tissues and alveolar bone.[2] Periodontitis mainly occurs as a result of the host immune response towards bacterial deposition surrounding the teeth and if left ignored, can lead to tooth loss. Porphyromonas gingivalis (*P. gingivalis*), a main causative pathogen of periodontitis, is capable of producing lipopolysaccharide (LPS), which can elicit exaggerated immune system via the modulation of cytokines and chemokines as well as nucleotide-binding domain, leucine-rich—containing family, pyrin domain—containing-3 (NLRP3) inflammasome.[3]

LPS is known to trigger the generation of various cytokines, particularly interleukin (IL) —6 And IL-8 among periodontal ligament cells via the TLR2 and/or TLR4 signaling.[4] Individuals with periodontitis have been found to have greater expression of IL-6[5,6] and IL-8[7–9] in their gingival crevicular fluid (GCF) and saliva compared to healthy individuals. IL-6 is primarily involved in the instigation and acute phase of periodontitis.[10] It has also been implicated in bone homeostasis, where it increases the activity of the receptor activator of receptor activator of nuclear factor kappa-B ligand (RANKL) in osteoblasts, and results in the osteoclasts differentiation and bone degradation.[11,12] As for IL-8, it is a powerful chemoattractant cytokine as well as an activator of neutrophils, which is commonly released from various oral and immune cells in the gingival crevice.[13,14] Activated neutrophils extensively eradicate invading microbes via phagocytosis; however, an overabundance of them can cause intracellular toxicity. This would release oxygen radicals, which further impairs activated macrophages and produces additional reactive oxygen species.[15,16]

In addition, monocyte chemoattractant protein-1 (MCP-1) has also been associated with the breakdown of periodontal tissues.[17] Its expression is highly upregulated when induced with LPS from P. gingivalis.[18] As its name suggests, MCP-1 is a potent mediator in the recruitment and activation of monocyte,[19] which is responsible for the escalation of inflammatory responses in chronic periodontitis.[20] Patients with periodontitis were shown to exhibit higher expression of MCP-1 in their GCF compared to healthy individuals, moreover, the expression of this chemokine is correlated positively with the severity of disease.[20–23] Thus, targeting these cytokines and chemokine maybe a great potential in the amelioration of periodontitis.

Next, LPS has been demonstrated to promote NLRP3 inflammasome, which participate in the secretion proinflammatory cytokines such as IL-1β and IL-6 and triggering of migratory damage among oral fibroblasts.[24–27] This inflammasome comprises of NLRP3, pro-caspase-1 (procasp1), and apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC).[28] When this inflammasome is assembled and activated, it induces the cleavage of the gasdermin D (GSDMD) to form a pore and release the cytoplasmic pro-inflammatory cytokines.[29] In addition, it has been discovered that NLRP3 inflammasome enhances osteoclastogenesis by upregulating RANKL synthesis and decreasing osteoprotegerin (OPG) levels.[30] Thus, it was not surprising to find high levels of NLRP3 inflammasome expressed in GCF, serum and saliva in patients with periodontitis.[31–33] Considering this, the NLRP3 inflammasome could be viewed as an inflammatory biomarker in periodontitis and as such, serve as a therapeutic target for periodontitis.

Meanwhile, Erbium-doped yttrium-aluminum-garnet (Er:YAG) laser, a solid-state crystal laser which operates in the infrared spectrum of 2940 nm, is acknowledged for its application in periodontal treatment.[34–38] Improvements had been observed in the clinical parameters of patients with chronic periodontitis when Er:YAG laser was used as an aid to scaling and root planing.[39,40] However, the molecular mechanism of Er:YAG laser, particularly in terms of its ability to target the host immune response triggered by the periodontal pathogen, is not well characterized. Considering that periodontal ligament fibroblasts (PDLFs) is among the most plentiful cell lines in the periodontium, we

focused on the inhibitory effects of Er:YAG laser on the lipopolysaccharides (LPS)-stimulated PDLFs, with regard to the productions of pro-inflammatory cytokines, chemokine and NLRP3 inflammasome expression.

Materials and methods

Periodontal ligament fibroblast (PDLFs) extraction and culture condition

In accordance to Institutional Review Board-approved norms of Chung Shan Medical University Hospital, the extraction of PDLFs were performed. PDLFs were isolated from extracted premolars from 2 healthy individuals, whom their consent were taken. Extraction of PDLFs were done at the middle 1/3rd of the root to avoid contamination of possible gingival fibroblasts and epithelial root sheath cells. Dulbecco's modified Eagle's medium of 10 % fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin were used to keep PDLFs nourished.

Laser irradiation

Er:YAG laser (Erwin AdvErlTM, wavelength 2940 nm, pulse width 250 μs, J. Morita Mfg, Kyoto, Japan) with a 2940 nm emission wavelength was used to deliver the irradiation. Withdrawal of the medium of the cells was first done to expose the monolayer to the irradiation. At a height of 15 or 20 cm, the laser was pointed perpendicularly at the culture dishes, with no covering sleeve or contact point for the hand piece. Based on the selected energy densities (3.6, 4.2, and 6.3 J/cm^2), the laser parameters were adjusted accordingly based on the previous studies.[41] Tracing of the laser irradiation area were done using thermal-hypersensitive paper to make sure the irradiation covers targeted area of a 35 mm-cell culture dish.

Cell proliferation assay

1x10^4 cells/well of PDLFs were seeded in the 96-well plates and they were induced with various LPS concentrations followed by laser irradiation at 24th hour. After irradiation, the cells were further cultured at 37 °C for 48 h. This was followed by the addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and further cultured for 3 h. The MTT formazan was then dissolved in DMSO and measured spectrophotometrically at 570 nm. Each group's optical density measurements were calculated as a percentage of the control.

Wound healing assay

Cells were sowed into a 12-well culture dish and left to grow till 80 % confluence, followed by the denudation across the monolayer at the central of the well with a sterile 1000 μL pipette tip. Cells were given a further 48 h to grow before capturing images. The displacement towards the wound region in the cells will be recorded under a microscope at 0 and 48 h.

Western blot assay

The Western blot test was carried out in accordance to the procedure formerly described.[42] Primary antibodies used included anti-human NLRP3 (sc-134306; Santa Cruz Biotechnology, Dallas, Texas, USA) and anti–GAPDH (AB2302, Millipore, Burlington, MA, USA) was used as loading control. Following blocking, the membranes were incubated with indicated primary antibodies fsollowed by corresponding secondary antibodies. The immunoreactive bands were generated with an ECL-plus chemiluminescence substrate (PerkinElmer, Waltham, Massachusetts, USA) and taken with ImageQuant LAS 4000 Mini (GE Healthcare, Piscataway, New Jersey, USA). Each densitometric value was expressed as the mean ± standard deviation.

Enzyme-linked immunosorbent assay (ELISA)

In LPS-stimulated cells of 24 h, cells were given different dosage of irradiation and their IL-6, IL-8 and MCP-1 concentrations were measured after 48 h by ELISA kits (R&D Systems, Minneapolis, MN, USA). The absorbance was measured with a 450 nm filter on a microplate reader (MRX; Dynatech Laboratories, Chantilly, VA, USA). Each individual model was evaluated three times.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR for the evaluation of NLRP3 expression: Utilizing Trizol reagent and following the manufacturer's instructions, total RNA were extracted from cells (Invitrogen Life Technologies, Carlsbad, CA, USA). The Superscript III first-strand synthesis technology for RT-PCR were used to reverse-transcribe mRNAs for qRT-PCRs (Invitrogen Life Technologies). ABI StepOneTM Real-Time PCR Systems will be used to perform qRT-PCR experiments on the resultant cDNAs (Applied Biosystems, Carlsbad, CA, USA). The expression of NLRP3 were measured while using GADPH as an internal check. NLRP3 primers were designed: (forward) 5′-CCCCGTAATCAACGGGACAA-3′, and (reverse) 5′-CCTTCCACTCACCCCACTTC-3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were designed: (forward) 5′-CTGGTGGCTGGCTCAGAAAA-3′ and (reverse) 5′-GGAGATTCAGTGTGGTGGGG-3'.

Statistical analysis

Repetition of each experiment was carried out three times. The data was expressed as mean ± standard deviation. For multiple comparisons, a one-way ANOVA was employed, while for comparative analysis between two groups, a Student's t-test was utilized. Statistical significance was considered as $P < 0.05$.

Results

In Fig. 1A, MTT test was employed to investigate the cell proliferation rate in periodontal ligament fibroblasts (PDLFs) when subjected to LPS of *P. gingivalis*. It was

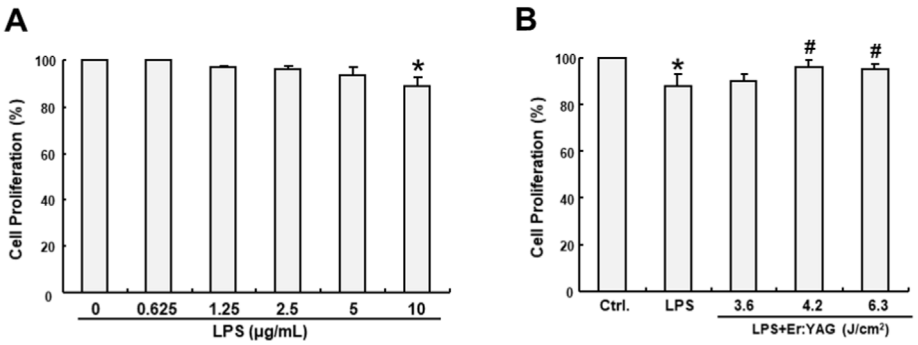

Fig. 1 Er:YAG laser restored the reduced cell proliferation rate in LPS-induced PDLFs. (A) LPS at 10 μg/mL had an impairment on the cell proliferation in PDLFs and (B) Er:YAG laser at higher dosages restored it, although it was not significant. Data was expressed in mean ± standard deviation. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to LPS group.

observed that cells proliferation rate in PDLFs was slightly decreased at the higher dosages of LPS. The cell proliferation rates were dramatically recovered after Er:YAG laser irradiation of 4.2 and 6.3 μg/mL were given to LPS-induced cells (Fig. 1B). Next, the findings also revealed that LPS impaired the wound healing tremendously and this was rescued by Er:YAG laser irradiation in a dose-dependent manner (Fig. 2). In addition, LPS stimuli resulted in a marked upregulation in the secretion of IL-6 (Fig. 3), IL-8 (Fig. 4), and MCP-1 (Fig. 5) respectively. When these cells were subjected to Er:YAG laser intervention, it was demonstrated that these phenomena were reversed considerably. While Er:YAG laser of higher energy densities were more superior in inhibiting these cytokines and chemokines, the effects of 4.2 and 6.3 μg/mL groups were equivalent. In Fig. 6, we explored the impacts of LPS on the activation of NLRP3 inflammasome and how Er:YAG laser mediates them. When the cells were cultured with LPS, the expression of NLRP3 mRNA and protein were found increased (Fig. 6). Er:YAG laser with the energy density of 3.6 μg/mL seems to have minimal influence on NLRP3 marker while 4.2 and 6.3 μg/mL groups managed to suppress the elicited events.

Discussion

Periodontitis is primarily caused by the host's immunological response to bacterial biofilm around the tooth. It is acknowledged that lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*P. gingivalis*), the main causative pathogen of periodontitis,[43] provokes the host immune response[3] through the mediation of cytokines, chemokines, and NLRP3 inflammasome.[4,24–27] Meanwhile, Er:YAG laser, a solid-state crystal laser that operates in the infrared spectrum of 2940 nm, is renowned for its potential in treating periodontal diseases.[34–37] In patients with periodontitis or peri-implantitis, this laser therapy has demonstrated improvements in clinical parameters such as pocket depth (PD) and clinical attachment loss (CAL).[44,45] However, there is a lack of information on the molecular basis of this laser's impact on periodontal tissues,

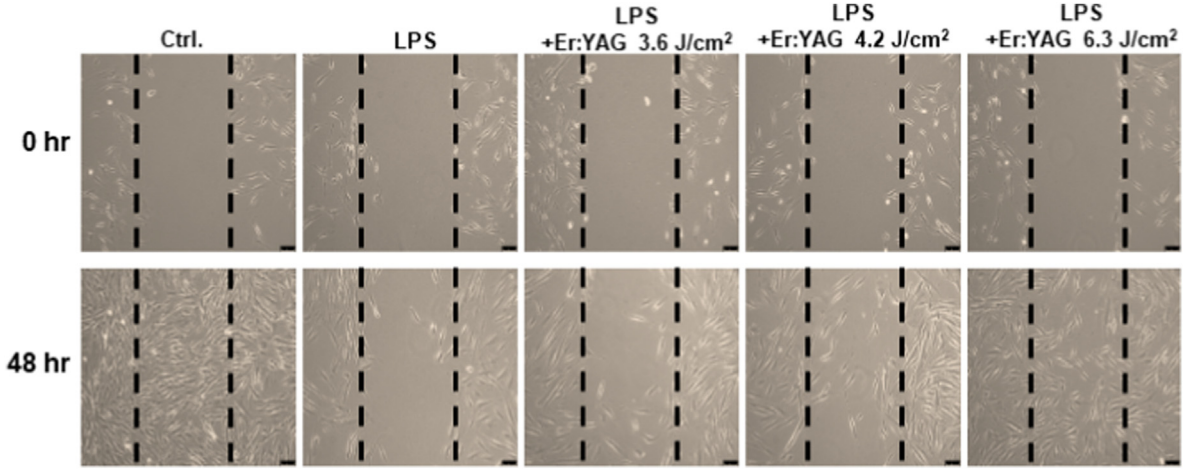

Fig. 2 Er:YAG laser improved the wound healing trait in PDLFs stimulated with AGEs/LPS. The LPS-induced poor wound healing was reversed in PDLFs after treated with Er:YAG laser irradiation of higher energy densities. Data was expressed in mean ± standard deviation. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to LPS group.

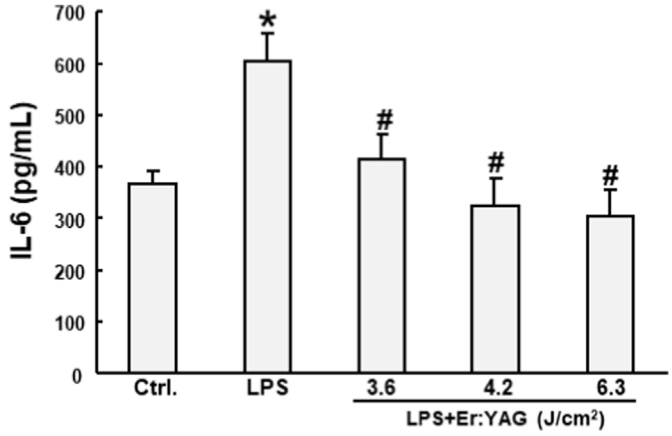

Fig. 3 Er:YAG laser suppressed IL-6 secretion in LPS-induced PDLFs. The elicited IL-6 secretion in LPS-stimulated PDLFs was downregulated significantly following Er:YAG laser treatment. The higher energy densities of Er:YAG laser have more superior effect compared to 3.6 μg/mL. Data was expressed in mean ± standard deviation. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to LPS group.

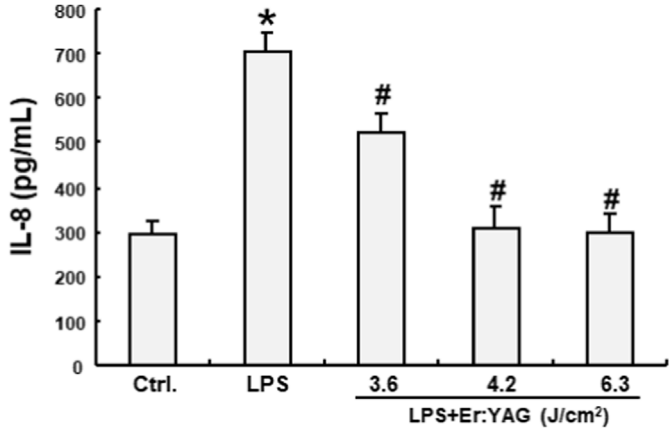

Fig. 4 Er:YAG laser inhibited IL-8 secretion in PDLFs stimulated with LPS. LPS markedly increased the release of IL-8 in PDLFs while Er:YAG laser treatment reversed this phenomenon. The higher energy densities of Er:YAG laser have greater inhibition on IL-8 secretion compared to 3.6 μg/mL. Data was expressed in mean ± standard deviation. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to LPS group.

particularly on the LPS-elicited host response. Thus, the present study hoped to look into the influences of Er:YAG laser on LPS-stimulated PDLFs with respect to pro-inflammatory cytokines, chemokines, and NLRP3 inflammasome expression.

In the present study, periodontal ligament fibroblasts (PDLFs) were exposed to LPS from *P. gingivalis* to imitate periodontitis. LPS at 10 μg/mL was revealed to impair the cell proliferation and wound healing in PDLFs, while Er:YAG laser irradiation of higher energy densities on the cells significantly restored them, which is similar to Lin's study.[41] Apart from PDLFs, Er:YAG laser irradiation has also promoted wound healing in other cell lines, such as gingival fibroblast and osteoblast.[46–48] Considering that the irradiated cells in prior studies demonstrated a boost in the expression of Galectin-7 and showed remarkable cellular phenotypes,[41,49] it is reasonable to assume that Er:YAG laser improves cell proliferation and wound healing in PDLFs via the activation of Galectin-7. Nonetheless, additional investigations will be required to validate this hypothesis.

As previously stated, LPS from *P. gingivalis* triggers the host immune response[3] via the modulation of cytokines, chemokines, and NLRP3 inflammasome.[4,24,25,27] It can be seen that when the cells were challenged with LPS, the secretion levels of IL-6, IL-8 and MCP-1 rose tremendously, which is in line with the findings from previous studies.[4,18] Moreover, patients with periodontitis also presented upregulated expression of these cytokines and chemokines in their GCF and saliva.[5–9,18] When these cells were intervened with laser therapy, all energy densities groups were observed to exert suppression on the elevated IL-6, IL-8, and MCP-1. Furthermore, greater inhibition was seen in the higher two energy densities, 4.2 and 6.3 J/cm^2, but there was no significant difference between these two energy densities.

In this work, it was discovered that LPS markedly stimulated the mRNA and protein expression of NLRP3 as well as

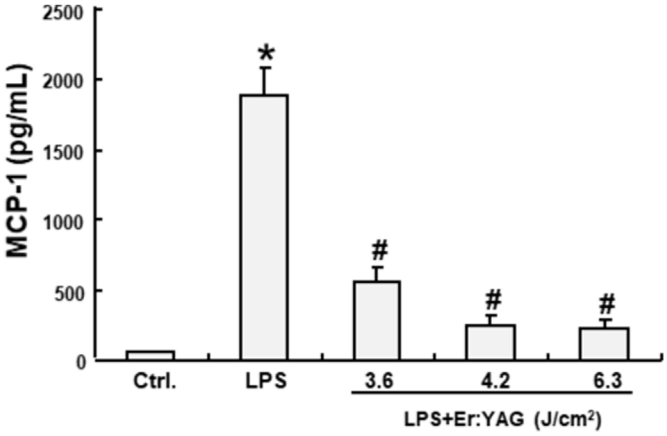

Fig. 5 Er:YAG laser repressed MCP-1 secretion in PDLFs stimulated with LPS. LPS significantly upregulated the release of MCP-1 in PDLFs while Er:YAG laser treatment restored its level. The higher energy densities of Er:YAG laser have more repression on MCP-1 release in comparison to 3.6 μg/mL. Data was expressed in mean ± standard deviation. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to LPS group.

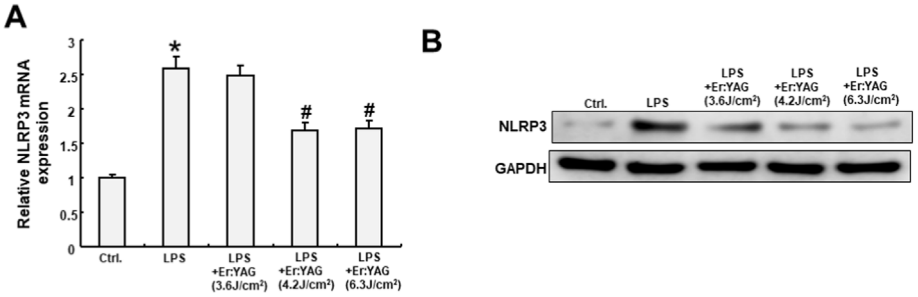

Fig. 6 Er:YAG laser of higher dosage reversed the LPS-induced NLRP3 inflammasome in PDLFs. The expression of NLRP3 mRNA and protein were found increased in LPS group. Er:YAG laser with the energy density of 3.6 μg/mL did not have significant influence on these markers while 4.2 and 6.3 μg/mL groups suppressed the elicited events. Data was expressed in mean ± standard deviation. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to LPS group.

the protein expression of cleaved gasdermin D (GSDMD).[50] Other oral cells, such as PDL stem cells and HGFs, were also observed to have upregulated NLRP3 inflammasome when stimulated with LPS.[51–54] The formation of NLRP3 inflammasome is known to cleave GSDMD, resulting in the formation of a pore that permits the release of pro-inflammatory cytokines such as IL-1β and IL-6.[24–27,29] When treated with laser, the higher energy densities (4.2 and 6.3 J/cm^2) inhibited the evoked events. It is intriguing that the inhibitory effects of this laser on the cleaved GSDMD proteins were significantly stronger than on NLRP3 proteins and given the role of this inflammasome in the LPS-induced inflammation, it would be worthwhile to delve further into the impact of this laser on the upstream pathway of GSDMD protein cleavage. One theory for the inhibitory effect of this laser on NLRP3 inflammasome is that this laser might be capable of eradicating LPS and its subsequent effects because the wavelength of Er:YAG laser approximates to the absorption wavelength of LPS.[55]

In this study, NLRP3 demonstrated impaired cell proliferation and wound healing in PDLFs but interestingly, these phenotypes were inconsistent in different cell types, especially cancer cells.[56] NLRP3 was shown to promote cell proliferation and migration in oral squamous cells and colorectal cancer cells (CRC),[57,58] while inducing cell death in lung cancer[59] and inhibiting proliferation in head and neck squamous cell carcinoma[60] and hepatocellular carcinoma.[61,62] These diverse outcomes maybe attributed to varying levels of its downstream cytokine, IL-1β.[63] High level of IL-1β prompts apoptosis, while lower levels stimulate clonal expansion.[64] In gastric cancer and CRC, NLRP3-driven IL-1β production activates NF-κB and JNK signaling, fostering proliferation.[56,65,66] Conversely, NLRP3 overexpression hinders proliferation and promotes apoptosis in leukemic cells.[67] As for PDLFs, activation of NLRP3 has been found to prompt inflammatory cell death caused by the secretion of pro-inflammatory cytokines, which has also been observed in oral osteoblasts, chondrocytes and macrophages.[68]

Overall, the current findings showed that Er:YAG laser was able to enhance cell proliferation and wound healing in LPS-induced PDLFs through the suppression of IL-6, IL-8, MCP-1, and NLRP3 inflammasome. To our knowledge, this is the first study to look into the influence of Er:YAG laser on the NLRP3 inflammasome in LPS-stimulated oral cells. Our study reveals a crucial role of Er:YAG laser in ameliorating periodontitis through the modulation of pro-inflammatory cytokines and the NLRP3 inflammasome and highlights that

the regulation of the NLRP3 inflammasome may become a potential approach for periodontitis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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