Lasers Med Sci
DOI 10.1007/s10103-013-1416-0

ORIGINAL ARTICLE

Controlling periodontal bone levels with multiple LED irradiations

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Received: 25 March 2013 / Accepted: 29 July 2013

Abstract Because a single exposure to light-emitting diode (LED) irradiation at 660 nm only demonstrated a 3-day biostimulatory effect in recovering periodontal bone level (PBL), this study sought to evaluate whether the periodontal effect could be extended through the use of multiple LED irradiations. Experimental periodontitis was developed unilaterally in 48 Sprague–Dawley rats after the placement of a silk ligature plus *Porphyromonas gingivalis* lipopolysaccharide injections. The animals were divided into four groups (no irradiation, a single irradiation, or two or three irradiations per week) and exposed to LED light irradiation at a wavelength of 660±25 nm and energy density of 10 J/cm^2 after debridement and detoxification. The animals were euthanized after 7 or 14 days, and the effect of irradiation was evaluated using micro-computed tomography and histology. By day 7, PBL was significantly reduced ($p<0.05$), with significantly reduced inflammation ($p<0.05$) and gingival hyperplasia ($p<0.001$), in the animals receiving three irradiations per week. At day 14, the reduction in gingival hyperplasia was still significant ($p<0.05$), and collagen matrix deposition and realignment appeared to be accelerated in the animals receiving three irradiations per week, despite a lack of significant difference in PBL. The treatment regimen receiving three LED light irradiations per week apparently extended the effects in reducing PBL and inflammation to 7 days. The inclusion of additional inflammation control measures or the addition of bioactive signals to mediate the repairing process is necessary to maintain long-term periodontal stability.

Keywords Periodontitis · Bone regeneration · Histology · Laser therapy lower level

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Introduction

A recent nationwide survey found that periodontitis affects more than 50 % of Americans above the age of 30 years [1] and contributes to tooth loss in adulthood [2]. Periodontitis is the consequence of local bacterial-induced inflammation and is characterized by the destruction of tooth-supporting structures, including the alveolar bone, periodontal ligament, and cementum. The mechanical elimination of bacteria has been considered the gold standard for eliminating inflammation in the periodontium. However, 2 to 4 weeks are usually required to initiate tissue repair [3]. Thus, accelerating the healing process through the use of noninvasive devices appears to be a promising approach to reduce the risk of infection and postoperative symptoms (e.g., pain and sensitivity).

Low-level laser treatment (LLLT) was firstly introduced as an innovative treatment modality in 1968 [4]. With an energy density of 0–100 J/cm^2, temperature change can be neglected so that the tissue damage can be minimized [5]. Studies have demonstrated that wavelengths of 635–670 or 800–850 nm were able to stimulate mitochondrial activity to reduce inflammation, facilitate tissue repair, and promote periodontal attachment [6–10]. However, the beneficial effects appeared to be inconsistent, particularly at long-term clinical follow-up studies [11–14], implying that the LLLT protocol was still not ideal. Recent researches have indicated that light-emitting diode (LED), operating in several wavelengths, has beneficial effects and similar mechanisms as the laser's application [15]. Our group utilized 660 nm LEDs to perform a one-time irradiation of rat gingival tissue, demonstrating only a temporary

Published online: 11 August 2013

resolution of periodontal breakdown in a previous study [16]. Whether the treatment efficacy could be improved or extended by increasing the irradiation frequency has not yet been confirmed.

The aim of this study was to compare the effects of a single LED irradiation with the effects of multiple LED irradiations on the periodontium. Micro-computed tomography (micro-CT) and descriptive histology were used to evaluate the effects of the different treatment regimens on periodontal bone level (PBL).

Materials and methods

Animal model and study design

All of the animal procedures followed the guidelines and approved protocol 032/10 from the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore. Forty-eight male Sprague–Dawley rats were housed in pairs with food and distilled water ad libitum. To induce experimental periodontitis, a 4–0 silk ligature was inserted into the gingival sulcus of the maxillary second molar (M2) of one randomly selected side of each rat for 2 weeks, with additional 10 μl (1.0 mg/ml) interpapillary injection of *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS; from InvivoGen Inc., San Diego, CA, USA) three times per week (periodontitis-induced side). No treatment was delivered to M2 on the other side of the mouth (periodontitis-free side). The ligature was removed after 2 weeks, and manual debridement was immediately performed using a dental explorer. Care was taken to avoid damaging the root surface, and 0.12 % chlorhexidine was topically applied. Supragingival plaque was removed by meticulously applying cotton rolls on the tooth surface twice per week until sacrifice.

Light-emitting diode (LED) irradiation was initiated 1 day after debridement and detoxification, and the animals were divided into four groups: (1) no LED irradiation (non-irradiated control), (2) a single exposure to LED irradiation, (3) LED irradiation twice per week until the sacrifice of animals, and (4) LED irradiation three times per week until the sacrifice of animals. The animals were euthanized 7 or 14 days after LED treatment (n=6/group/time point).

LED device

LED irradiation was delivered using a customized LED device as previously described [16]. Four diodes (2×1 mm^2) were arranged (2×2) on a plate at 1 mm intervals mesiodistally and 5 mm intervals buccolingually. The device was placed over the palate of the rats and settled on the occlusal surface of the maxillary molars, leaving 0.5 mm clearance between the diodes and the palatal aspect of the maxillary molars. Each diode consistently emitted 660±25 nm and 3.5 mW/cm^2 visible red lights directly on the gingival tissue. Based on the dosimetric calculation from the output of 4 diodes (equaling to 14 mW/cm^2), the irradiation procedure lasted 12 min and achieved an energy density of 10 J/cm^2 within the tested region.

Micro-computed tomography and histologic assessments

The harvested maxillae were examined using a Shimadzu SMX-100CT X-ray CT scanner (Shimadzu Corp., Tokyo, Japan) with a final effective pixel size of 19.54 μm. The image was then reoriented based on the following criteria: (1) in the transverse plane, the crowns of the first molar (M1) to the third molar (M3) were centrally and vertically positioned; (2) in the sagittal plane, the occlusal surfaces of M2 and M3 were aligned horizontally; and (3) in the frontal plane, the occlusal surface of M2 was aligned horizontally.

PBL was defined as the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at the palatal aspect of M2. This measurement was obtained slice-by-slice from the distal mid-root margin of M1 to the mesial mid-root margin of M3 by using a customized MATLAB (MathWorks®, Natick, MA, USA) algorithm as previously described [17].

The specimens were decalcified with 12.5 % ethylene diaminetetraacetic acid and embedded in paraffin, after which two cross-sectional planes (one plane from the mid-mesiopalatal root region and one plane from the mid-distopalatal root region) of M2 were selected for observation. The sections were stained with hematoxylin and eosin, and images were acquired with a digital image acquisition system (Leica Microsystems GmbH, Wetzlar, Germany).

Inflammatory cells was quantified in five randomly selected areas within palatal gingival connective tissue in each slide under the magnification of ×400, and the result was presented as the fraction of inflammatory cells within the total cell count. The palatal gingival epithelium was divided into ten equivalent intervals under the magnification of ×100, and thickness was measured at the middle of each interval and defined as the distance from the inner surface of stratum basale to the outer surface of stratum corneum.

Statistical analysis

One-way ANOVA followed by Tukey's post hoc test was used to compare the differences between the LED-irradiated groups and non-irradiated controls or the periodontitis-free side of the specimens without LED irradiation. The data are presented as the mean ± standard deviation (SD) of the measurements, and p value less than 0.05 was considered statistically significant.

Results

General condition and gross observations

All of the animals recovered well from the anesthesia and interventions. In the periodontitis-induced side, gingival redness and swelling, as well as the hypermobility of the tooth, were noted at all of the ligature-placed sites by 3 days, and spontaneous gingival bleeding could be observed at most sites in the first 3–7 days but subsided at 10 days. The gingival redness and swelling gradually subsided after ligature removal, whereas tooth hypermobility was still present until the date of sacrifice. There was no detectable difference with regard to the gross gingival patterns between the non-irradiated control and LED-irradiated specimens in any of the observation time point.

Reductions in periodontal bone level

PBL was defined as the CEJ–ABC distance as previously described [16]. The average PBL at M2 of the periodontitis-free side without LED irradiation was 346.10±67.41 μm at day 7 and 382.69±55.23 μm at day 14 (Fig. 1). There was no significant difference between the non-LED and LED-irradiated specimens in the periodontitis-free side (data not shown).

In the periodontitis-induced side, PBL was significantly higher than the periodontitis-free side at both time points in the specimens without LED irradiation ($p<0.001$, Fig. 1). PBL appeared to decrease with LED irradiation at day 7, and the group receiving irradiation three times per week exhibited a significant reduction in PBL when compared to the non-irradiated controls ($p<0.05$). PBL in the non-irradiated control group was not obviously changed at day 14, whereas PBL tended to increase in all of the LED-irradiated groups. There was no significant difference between any of the LED-irradiated groups and the non-irradiated control at day 14.

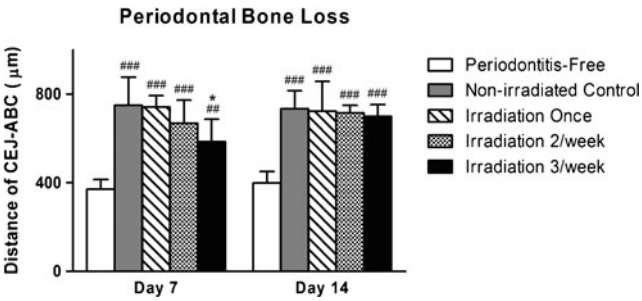

Fig. 1 PBL at days 7 and 14 after LED irradiations. PBL was defined as the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at the palatal aspect of M2. (Compared to the periodontitis-free side of the non-irradiated control: $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$; compared to the periodontitis-induced side of non-irradiated control: $^{*}p<0.05$)

Descriptive histology

In the periodontitis-induced side, at day 7, gingival epithelium was apparently hyperplastic with prominent rete pegs in the non-irradiated controls, with mild to moderate inflammatory cell infiltration in the basal layer and the underlying connective tissue adjacent to the junctional epithelium. Loose and immature collagen matrix was distributed within the lamina propria, and neogenic bone matrix was deposited on the bundle bone crest with a clear reversal line (Fig. 2a). The histologic results were similar in the animals receiving single LED irradiation, while the enlargement of the junctional epithelium and inflammatory cell infiltration in this group appeared to be less prominent than the same measures in the non-irradiated controls (Fig. 2b). The gingival hyperplasia in the animals receiving two or three LED irradiations per week was less substantial with less prominent rete pegs. However, the gingival connective tissue was still mostly occupied with loose and immature collagen fiber matrix (Fig. 2c).

At day 14, gingival hyperplasia appeared to be reduced, and the area occupied by immature collagen matrix was clearly decreased. Vessel formation could be observed at all of the periodontitis-induced sites, regardless of LED irradiations or not (Fig. 2d–f). Therefore, the animals receiving three LED irradiations per week exhibited a relatively thin gingival epithelium, with the collagen matrix appearing relatively well organized and densely arranged within the lamina propria compared to the other groups (Fig. 2f). On the other hand, one or two of the animals in every group still exhibited an enlargement of the junctional epithelium with mild to moderate inflammatory cell infiltration.

Quantitative histologic measurements

In the non-irradiated specimens, inflammatory cells were significantly increased in the periodontitis-induced side compared to periodontitis-free side at day 7 ($p<0.01$) and 14 ($p<0.05$) (Fig. 3a). In the periodontitis-induced side, inflammation appeared to reduced all LED-irradiated specimens, and significant difference to non-irradiated control was noted in the specimens receiving three LED irradiations per week at day 7 ($p<0.05$). However, at day 14, only specimens receiving three LED irradiations per week demonstrated slightly but insignificant reduced inflammation to the non-irradiated controls.

The thickness of gingival epithelium was significantly increased with the induction of periodontitis in the specimens without LED irradiation at days 7 and 14 ($p<0.001$), and the decrease of epithelial thickness at day 14 was noted in the periodontitis-induced side (Fig. 3b). Specimens receiving two and three LED irradiations per week demonstrated significantly reduced epithelial thickness to non-irradiated controls at day 7 ($p<0.001$), and only the ones receiving three LED

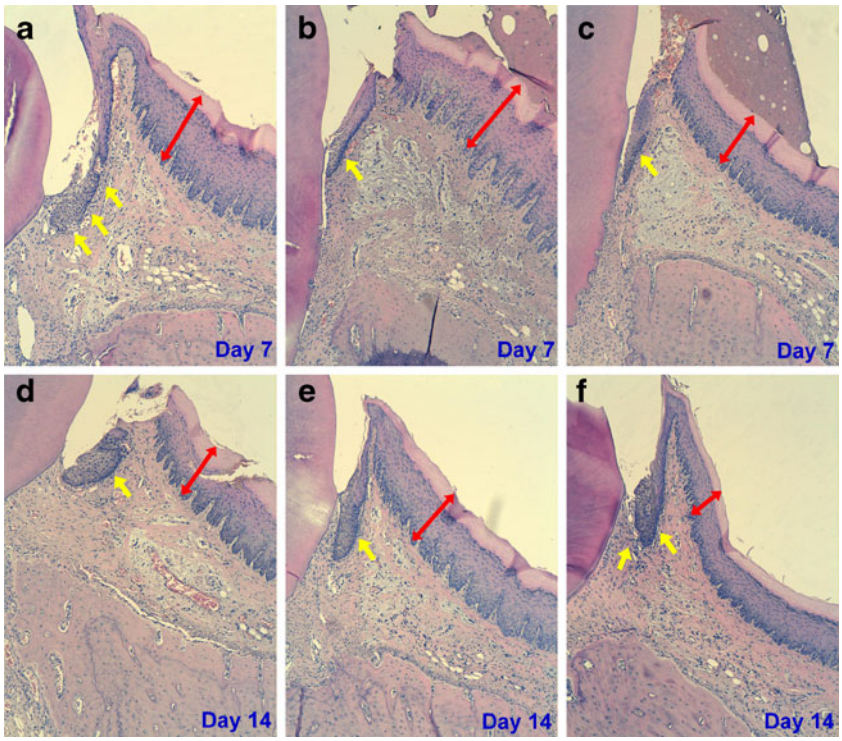

Fig. 2 Descriptive histology at days 7 and 14 (the periodontitis-induced side). **a** A nonLED-irradiated site at day 7. **b** A LED-irradiated site (single treatment) at day 7. **c** A LED-irradiated site (three times per week) at day 7. **d** A nonLED-irradiated site at day 14. **e** A LED-irradiated site (single treatment) at day 14. **f** A LED-irradiated site (three times per week) at day 14. The *red arrows* indicate the thickness of gingival inflammation, and *yellow arrows* refer to the inflammatory cells infiltration adjacent to the junctional epithelium

irradiations demonstrated consistently reduced epithelial thickness to non-irradiated controls at day 14 ($p<0.05$).

Discussion

LLLT, within a wavelength of 635–670 or 800–850 nm and an energy density of 0–100 J/cm^2, has been recognized as a potential mean for managing periodontal diseases because this treatment promotes the viability of periodontal cells [5] and reduces the viability of periodontopathogens and related inflammatory signals [9, 18]. Clinically, LLLT had been shown to reduce dental plaque accumulation and gingival inflammation and temporarily accelerate the reduction of periodontal pocket depth and PBL after nonsurgical debridement [6, 7, 19, 20]. Compared to the laser device, LED irradiation can be safely applied to body surfaces without heat or tissue damage in a reduced cost [21, 22]. Another advantage of LED application is probably the reduction of the exposure time of the irradiation comparing to the laser operating in the same dosage [23]. Because we have already confirmed that a single exposure to 660 nm LED light with an energy density of 10 J/cm^2 facilitated periodontal healing and temporarily reduced the PBL for 3 days [16], in the present study, we subjected gingival tissue to repeated LED irradiation, demonstrating that three LED irradiations per week significantly reduced PBL and extended the duration of gingival hyperplasia resolution to 7 days. However, the irradiated and non-irradiated groups did not exhibit obvious differences in inflammation reduction, and this finding might be due to the reduction in inflammation achieved by debridement and detoxification. Although the biostimulatory action of LLLT is linked to the activation of the mitochondrial photo-acceptor molecule cytochrome c oxidase [24], LLLT may also upregulate the inflammatory

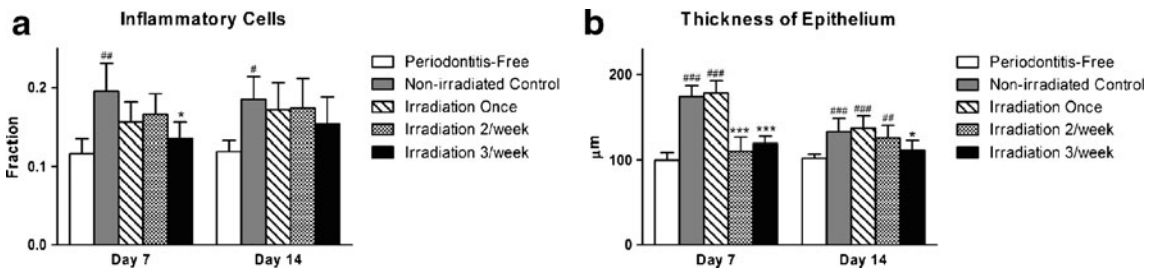

Fig. 3 Quantitative histologic assessments at days 7 and 14 after LED irradiations. **a** The fraction of inflammatory cells with the total cell count. **b** The thickness of gingival epithelium. (The periodontitis-free side of the non-irradiated control: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to the periodontitis-induced side of non-irradiated control: $^{*}p<0.05$, $^{***}p<0.001$)

signaling of leukocytes and other immune cells [25]; therefore, the removal of etiological factors to prevent unwanted inflammatory responses should be considered a prerequisite of the LED light-stimulated periodontal repair process.

The lamina propria of the LED-irradiated animals, especially those animals receiving three irradiations per week, was more mature and better organized than the lamina propria of the non-irradiated controls (Fig. 2d–f). Considering the fact that the early maturation of periodontal wounds was achieved in 1–2 weeks [26], our results indicate that repeated LED irradiation treatments accelerate the maturation of gingival connective tissue. However, the reduction in PBL did not persist at day 14 in the groups receiving LED irradiations (Fig. 1), presumably due to the uncoupling of collagen matrix deposition within the connective tissue and osteogenesis at the alveolar bone crest. LED light irradiation activates cellular functions to facilitate the repairing process by accelerating collagen matrix deposition and realignment [16]. Therefore, osteogenesis requires not only the recruitment of cells but also bioactive signals such as bone morphogenetic proteins (BMPs) to initiate osteogenic differentiation [9]. Although studies indicated that BMPs were upregulated by LLLT in vitro [27, 28], the upregulation of BMPs in vivo was only evident in the later recovery stage [29]. While osteogenesis was accelerated as rapid as matrix deposition in the gingival connective tissue, the reduction of PBL was not prominent at day 14 in the current experimental set-up. On the other hand, due to the periodontitis induced in our study exhibiting significant early inflammation [16] and the difficulty to control oral plaque accumulation of the rats, extended inflammation could still destroy the stromal tissue as well as impede tissue recovery and osteogenesis. Studies have also demonstrated that the cytokines and elevated oxidative stress levels that result from inflammation could retard the differentiation and viability of mesenchymal stem cells [24–26]. In this sense, the strict control of inflammation and exogenous supplementation of bioactive signals may be needed to ensure the long-term maintenance of PBL.

As an effective alternative to the lasers, LED light therapy has been reported as nonsignificant risk by FDA and approved for the use in humans [30, 31]. Nowadays, LED device has been developed to treat skin lesion and reduce pain and erythema after skin treatment [32, 33]. However, to our knowledge, only a few reports demonstrated the use of LED devices in the field of clinical dentistry. By using an extraoral LED device (OsseoPulse®, Biolux Research Ltd., Canada), the stability of dental implants was increased compared to nonLED-irradiated sites [31], and osteogenesis was accelerated with faster resorption of hydroxyapatite grafts in the post-extraction tooth socket [34]. On the other hand, LED has also been suggested as an adjunct to reduce pain and accelerate tooth movement in orthodontics according to a randomized clinical trial of LLLT [35], and a subsequent preclinical study demonstrated that LED light irradiation was able to reduce root resorption during orthodontic tooth movement [36]. Due to the limited clinical evidence of LED-mediated biomodulation available at this time, further investigations to prove the clinical efficiency of LED are warranted.

This study has limitations. The observation window was 2 weeks, and only a single wavelength and energy density was evaluated. Therefore, this experimental model of periodontitis, as well as the healing capability of the animals, might not be relevant to adult periodontitis. For instance, experimental periodontitis was induced in just 2 weeks, whereas periodontitis is considered a slowly progressive disease in humans. Despite the limitations of the study, we concluded that the treatment with three LED irradiations per week at a wavelength of 660 nm and energy density of 10 J/cm^2 apparently extended the effects of LED irradiation in reducing PBL and inflammation to 7 days. However, LED irradiations did not appear to maintain PBL in the long-term, and the additional control of inflammation as well as exogenous supplementation of bioactive signals should be still considered.

Acknowledgments The authors would like to acknowledge JETTS Technology Co., Ltd. (New Taipei, Taiwan) for fabricating the LED device and Man-Jung Kao at the National University of Singapore (NUS) for the assistance in animal care and micro-CT processing. The study was supported by research grants R-221-000-034-133 from NUS and 101R7465 from National Taiwan University.

Conflict of interest The authors declare no financial conflicts of interest.

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