Lasers in Medical Science (2020) 35:1975–1979
https://doi.org/10.1007/s10103-020-02993-5

ORIGINAL ARTICLE

The effect of low-level laser radiation and doxycycline on the levels of osteoprotegerin and receptor activator of nuclear factor kappa-B ligand

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Received: 25 May 2019 / Accepted: 1 March 2020 / Published online: 27 March 2020

Abstract

The present in vitro study was conducted to investigate the effect of low-level laser (LLL) radiation and doxycycline on the levels of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) derived from MG-63 osteosarcoma cell line. MG-63 cells were divided into four groups. In the first group, 2 mg/mL of doxycycline was injected into the cell culture medium. Diode laser (810 nm, 100 mw, 75 s) was radiated to the culture medium of the second group. The third group received both doxycycline and laser radiation. In the fourth group (control), the culture medium was replaced daily, similar to the above three groups. Mentioned interventions were performed once a day for 4 consecutive days. Then, on the sixth day, the levels of OPG and RANKL mediators were measured using real-time polymerase chain reaction by isolating the cells from the samples. OPG expression had the highest to lowest levels in the laser + doxycycline, doxycycline, laser, and control groups, respectively. The level of OPG was significantly different between all the study groups ($p < 0.05$) except in the doxycycline + laser and doxycycline groups ($p = 0.061$). The highest to lowest levels of RANKL was observed in the doxycycline, laser + doxycycline, control, and laser groups, respectively. The RANKL expression was not significantly different between all the study groups ($p > 0.05$). The results of this study revealed that LLL and doxycycline reduced the RANKL/OPG ratio derived from the MG-63 osteosarcoma cell line, which may result in the diminished activity of osteoclasts and osteoclastogenesis.

Keywords OPG · RANKL · Laser · Doxycycline · Bone · Remodeling

Introduction

Under normal physiological conditions, bone remodeling occurs as a result of keeping the balance between the bone formation and resorption. Such conditions can be seen during orthodontic procedures where bone resorption occurs on the side of pressure and where bone formation occurs on the side of stretching [1].

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Accelerating the bone repair following trauma or surgical processes, such as fracture and implant placement, plays a vital role in an effective treatment [2, 3]. This is particularly important for low-density bony areas (thin cortex and trabecular bone) as well as in patients with osteoporosis [4, 5].

In recent years, low-level laser (LLL) has been proposed as adjuvant therapy for bone repair in laboratory studies [6–10]. LLL has been reported to enhance the stability of bone implants [11, 12]. Besides, LLL radiation has analgesic [13], immunomodulatory [14], and antibacterial effects [15], improving its advantages. Different types of laser have various biocellular effects. Accordingly, the results of a kind of radiation may not necessarily be generalized to other radiation settings [16]. It has been well observed that 810 nm diode laser irradiation differentiated human bone marrow mesenchymal and dental pulp stem cells into the osteoblasts [17, 18]. Nevertheless, there is limited evidence about the biocelluar mechanism of 810 nm diode laser on bone remodeling.

Tetracyclines, such as doxycycline (Dox), are widely used for the treatment of infectious diseases. Dox can improve bone formation, which can be considered as a bone repair agent in addition to its antibacterial effects [19]. Dox decreases bone loss by suppressing the osteoclasts [20, 21]. The cellular mechanisms of Dox on bone cells are not well understood [21, 22].

Bone remodeling is regulated by a major system, including the receptor activator of nuclear factor kappa-B (RANK) and its complement (RANKL, receptor activator of nuclear factor kappa-B ligand) as well as osteoprotegerin (OPG) [23]. RANK is expressed by osteoclast progenitor cells and mature osteoclasts. RANKL and OPG are expressed by bone marrow stromal cells, osteoblasts, fibroblasts, and periodontal ligament cells. Attachment of RANKL to RANK leads to the differentiation of the osteoclasts and their survival. OPG, as a soluble receptor for RANKL, prevents this attachment. Thus, OPG acts as a natural inhibitor for differentiation and activation of the osteoclasts [24].

The present in vitro study was designed to investigate the effect of LLL and Dox alone or in combination on the levels of OPG and RANKL. For this purpose, the MG-63 osteosarcoma cell line was used to simulate bone proliferation and remodeling. This cell line has some advantages, including unlimited cell proliferation, hormonal response (vitamin D and PTH) similar to human osteoblast cells [25], and the ability to secrete RANKL and OPG [25, 26].

Materials and methods

This in vitro study was conducted after receiving approval by the Ethics Committee of Royan Institute, Isfahan Province, Iran, in 2018 (code: IR.ACECR.ROYAN.REC.1397.141).

Cell preparation and study groups

The human osteosarcoma cell line (MG-63) was obtained from the cell bank of Royan Institute (Isfahan, Iran).

Frozen MG-63 cells were melted at 96 °C and were transferred to the culture medium container. After that, the culture medium containing DMEM (Biowest, France), FBS (Gibco, Germany), L-glutamine (Biowest, France), and penicillin-streptomycin was added to the cells, then was transferred and kept in an incubator at 37 °C. Once the cells were filled in the culture medium container, they were passaged and moved to a larger culture medium container and were kept at 37 °C in an incubator. After the proliferation of the cells, they were counted based on spectrophotometry and were transferred in equal numbers (2×10^5) into 16 cellular dishes (4 × 4 cm in dimensions) containing the culture medium. The dishes were randomly categorized into four 4-member groups: (1) Dox; (2) diode laser; (3) the Dox + laser; and (4) control.

Two milligrams/milliliter of Dox (Razak.Co, Iran) [27] was injected into the culture medium in the first group once every 24 h after replacing the medium. In the second group, 810 nm diode laser (GIGAA, Wuhan GIGAA Optronics Technology Co., Ltd., China) was radiated once every 24 h for 4 consecutive days after replacing the medium. The radiation setting was as follows: power 100 mw, power density 100 mw/cm^2, energy density 5 J/cm^2, 75 s, and a continuous wave. Generally, the radiation parameters used in the present study were close to other studies conducted on bone healing [28, 29]. In the third group, a combination of Dox and laser was administered concurrently after replacing the medium. Eventually, in the fourth group, only the culture medium was replaced every 24 h for 4 days (control) similar to other groups. After completion of the treatment, the cells were allowed to rest for 48 h, and RNAs of OPG and RANKL genes were extracted in the study groups.

RNA extraction

After administration of the interventions, RNA extraction was performed according to the standard protocol. First, the cells were counted, and 250 μl of Trizol (Trizol Invitrogen, Carlsbad, USA) was added to the cells. Then, the cells were homogenized and were kept in the incubator at room temperature for 5 min. Next, 50 μl of chloroform was added to the cells, and the resulting solution was vigorously shaken for 15 s. Thereafter, it was kept in the incubator for 3 min. The sample was centrifuged for 15 min at 1200*g* at 4 °C, where three phases were formed at each stage so that the top, middle, and bottom phases were RNA, DNA, and protein, respectively. Thereafter, 50 μl of isopropanol (Merck Co., Germany) was added to the top phase and was shaken gently (this material causes RNA sedimentation). After that, incubation was performed for 10 min at room temperature, and centrifugation

was conducted for 10 min at 12,000 rpm at 4 °C. In the next stage, the top phase was discarded, and obtained sediment was placed under the hood for 10 min (liquid evaporation). Next, 30 μl of RNase-free water (Cleaver Scientific, UK) was added to the sample, and the vial was first exposed to room temperature for 15 min, and then was placed inside the incubator again for 15 min. Once these stages were completed, equal concentration of RNA was isolated from each group using spectrophotometer (Nanodrop, BioTek, USA) to prepare cDNA.

cDNA synthesis

Due to the instability of RNA, cDNA was prepared from them. The RNA concentration was measured by Nanodrop device to obtain the RNA level required for cDNA synthesis from each group to initiate the protocol of cDNA synthesis. After that, the RNase-free water value was calculated for the RNA concentration according to the standard table. Next, the desired sample was incubated for 30 min at 37 °C. Once the samples were produced, 2 μl of EDTA (Merck Co., Germany) was added to them, and they were kept at 65 °C for 10 min (EDTA was employed to deactivate DNase enzyme). After 10 min, the following materials were added to the samples.

- Reverse Transcription (2 uL, Thermo Fisher Scientific Co., USA)
- Riblock RNase Inhibitor (2 uL, Thermo Fisher Scientific Co., USA)
- Deoxynucleotide Triphosphates (4 uL, Thermo Fisher Scientific Co., USA)
- Reaction Buffer (8 uL, Thermo Fisher Scientific Co., USA)

Afterward, the sample was incubated at 37 and 85 °C for 15 min and 5 s, respectively. Based on this protocol, cDNA was synthesized and was kept in a fridge at − 20 °C.

SYBR Green and real-time PCR

After cDNA synthesis, RT-PCR reaction mixtures were combined with SYBR Green Master Mix (Takara, Dalian, China). RT-PCR was performed using standard method and specific primers [30]. The primers utilized in this study were as follows [31]:

- OPG, *5′-GCTAACCTCACCTTCGAG-3′* (*forward*) *and 5′-TGATTGGACCTGG TTACC-3′* (*reverse*); RANKL, *5′-AACAGGCCTTTCAA GGAGCTGTGC-3′* (*forward*) *and 5′- AAGAGGACAGACTCACTTTAT GGGG-3′* (*reverse*)

The extent of gene expression in each sample was evaluated quantitatively after RT-PCR using SYBR Green dye. A melting curve measured the fluorescence peaks [-d(RFU)/dT] of the samples.

Statistical analysis

SPSS software Ver.17 was used to analyze the data. The intergroup OPG and RANKL gene expression (fluorescence peaks) were assessed by one-way analysis of variance (ANOVA) and post hoc Fisher's least significant difference (LSD) tests. A p value of < 0.05 was considered as statistically significant.

Results

Table 1 presents the levels of OPG and RANKL gene expression across the groups. Laser + Dox ($p < 0.001$), Dox ($p < 0.001$), and laser groups ($p = 0.042$) had a higher level of OPG gene expression compared to the control group, respectively. Levels of OPG gene expression were significantly higher in the laser + Dox ($p < 0.001$) and Dox groups ($p = 0.03$) than the laser group. The difference between the laser + Dox and Dox groups was not significant ($p = 0.06$).

Laser + Dox and Dox increased, and laser irradiation decreased the levels of RANKL gene expression in comparison with the control group. Laser + Dox group showed a higher level of RANKL gene expression in contrast with the other study groups. The RANKL gene expression was not significantly different between all the study groups ($p = 0.3$).

Discussion

Results of the present study showed that LLL and Dox synergistically caused a significant elevation in the levels of OPG

Table 1 The OPG and RANKL gene expression according to fluorescence peaks in the melting curve

Groups		Mean	SD	Minimum	Maximum
OPG $^{-d(RFU)/dT}$	Control	76.7	25.4	45.8	101.4
	Laser	130.9‡	41	93	197
	Dox	197.9†	66.5	114.8	285.3
	Laser + Dox	249.9†	57.4	184.4	339.4
RANKL$^{-d(RFU)/dT}$	Control	3.2	2.2	2	6
	Laser	0.2	0.1	0.1	0.5
	Dox	8.9	9.1	0.2	19.3
	Laser + Dox	6.9	5.8	0.01	15.4

‡Laser vs. control, laser vs. Dox, $p < 0.05$

† Dox vs. control, Laser + Dox vs. control, laser + Dox vs. laser, $p < 0.001$

compared to the control group. Moreover, Dox, laser, or the combination did not change the expression of RANKL significantly in comparison with the control group. Accordingly, Dox and laser irradiation reduced the RANKL/OPG ratio, which can have an inhibitory effect on the activity of the osteoclast cells.

So far, limited and controversial experimental evidence has been published about the effect of LLL radiation on the levels of mentioned mediators. In an in vivo study, administration of 810 nm diode laser irradiation (100 mw, 75 J/cm^2) has been reported to be associated with a decrease and increase in the RANKL and OPG gene expression [32], which is consistent with the present findings. In contrast, Yamaguchi et al. reported that 810 nm diode laser radiation (100 mw, 54 J, 9 min) on the jaw of mice, under orthodontic treatment increased metalloproteinase-9 (MMP-9), cathepsin K, and alpha$^{(v)}$ beta$^{(3)}$ integrin [33] that could potentially increase RANKL gene expression and osteoclast activity consequently [22, 34]. In vivo environment and a different radiation setting [32, 35] may increase osteoclastic activity after 810 nm diode laser irradiation. Other diode lasers, with wavelengths close to the present study, have shown different results. Fujita et al., in their laboratory study, observed that daily LLL radiation for 1 week (850 mm, 75 mw) during orthodontic treatment resulted in increased expression of RANKL and RANK, but it did not have any significant effect on the level of OPG [36]. Altan et al., in an animal study, investigated 38 Wistar rats that underwent orthodontic treatment and LLL radiation (820 nm, 100 mw) during 3 consecutive days. In an immunohistochemical assessment, the levels of RANKL and OPG did not change significantly in comparison with the control group [37].

Dox prescription has been approved for people older than 8 years old [38]. Dox did not decrease bone strength in the experimental model, and it reduced the severity of bone loss and mechanical weakness against bone fracture force in ovariectomized mice (osteogenic mice) [39]. In other laboratory studies, Dox resulted in a 4–30% increase in bone formation compared to the placebo [40, 41]. Furthermore, the osteoclast/osteoblast cell ratio and the density of inflammatory cells at the site of bone loss decreased by Dox [41]. In clinical observations, Dox also improved bone density without significant side effects [42].

Dox can cause bone repair through various mechanisms. Similar to the present study, in other in vitro and in vivo studies, Dox decreased RANKL [20, 22] and RANKL/OPG ratio [20]. The matrix metalloproteinases (MMPs) play an important role in tissue repair as well as chronic inflammation and healing defect. It has been well observed that an increase in MMP-1,8 expression is associated with a lack of bone repair [43]. MMP-7, 9, 13, 14 have an important role in the activity and migration of the osteoclasts [44]. Tetracyclines are non-selective inhibitors of MMPs, but they mostly inhibit the MMP-8, 9, 13, and to a less extent, they inhibit the MMP-1,3 [45]. It seems that Dox influences RANKL levels by decreasing MMP-9 [22]. Increased Wnt pathway activity and reduced release of Dickkopf-related protein 1 (a Wnt pathway inhibitor) are other osteogenesis-related mechanisms of Dox [41].

LLL radiation and Dox showed a significant synergistic effect on OPG expression. This finding suggests that their combination can have a better inhibitory effect on the osteoclasts. Therefore, using the combination mentioned above may accelerate the bone healing process.

In summary, the findings of this study suggested that Dox and the laser radiation reduce the RANKL/OPG ratio, which can enhance the osteogenesis. This finding can be further investigated in orthopedic, orthodontic, and bone implantation models regarding bone healing in future in vivo studies.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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