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ORIGINAL ARTICLE

Comparison of the efficacy of low-level laser therapy and photodynamic therapy on oral mucositis in rats

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Abstract

Cancer treatment with chemotherapy or radiotherapy is associated with some side effects including in the oral cavity. One of the more significant oral complications is oral mucositis (OM) which induces severe pain and limits fundamental life behaviors such as eating, drinking, and talking. Although advancements in cancer treatment improved the survival rate, severe OM and opportunistic infection affect treatment adversely. Therefore, the control of OM is important for oral health quality of life and prognosis. Low-level laser therapy (LLLT) and photodynamic therapy (PT) are noninvasive methods that reduce inflammation and pain during wound healing. The aim of this study is to evaluate immunohistochemical and histological examination of the OM region of the PT comparing LLLT. In this study, 24 Sprague-Dawley rats were divided into three groups as control, LLLT, and PT groups. All groups received 5-fluorouracil intraperitoneally and a linear trauma to the mouth pouch with a needle. After the formation of OM in the mouth, the control group had no treatment; the LLLT group was administered LLLT, and the PT group had LLLT after indocyanine green application. Then all groups were sacrificed, and histological analyses and protein level detection of basic fibroblast growth factor (bFGF), transforming growth factor (TGF-β), and platelet-derived growth factor (PDGF-BB) were evaluated in all groups. PT was determined to be more statistically significantly than LLLT with bFGF and PDGF-BB. However, regarding TGF-β, no statistically significant difference was observed between the groups. Within the limitations of this study, indocyanine green may accelerate the LLLT effect. However, further studies on this subject are required.

Keywords Oral mucositis · Indocyanine green · Low-level laser therapy · Photodynamic therapy

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Introduction

Oral mucositis (OM) refers to inflamed erosive or ulcerative lesions of the oral mucosa. It is an acute, painful, and dose-limiting condition that is caused by damage to the oral epithelium [1]. Although the frequency of OM differs in literature, it is known that almost every patient receives a high dose of radiotherapy (RT) to the head and neck region, especially in the oral cavity area [2]. This rate is 30–60% in patients with RT in the head and neck region [3]. The incidence of OM detection in patients receiving standard chemotherapy is 40%, while the frequency in patients receiving a high dose of chemotherapy is up to 76% [4].

Daily life function of patients is directly affected by OM. These complications include nutrition, speech difficulty resulting in dehydration, weight loss, pain and infection, alteration in taste, anorexia, and cachexia. Moreover, OM has a higher risk of septicemia in patients with neutropenia [5].

There is no consensus in the literature about a single agent for prophylaxis or treatment of OM [5–9]. Antimicrobial, anesthetic, analgesic or natural agents, ozone, low-level laser therapy (LLLT), and cryotherapy are among these methods. In our previous study, we found that LLLT is more effective than ozone although ozone treatment has also substantial effects [8]. LLLT consists of the application of light with the purpose of promoting tissue repair, decreasing inflammation, and producing analgesia, usually using a low-power light source (laser or LED) [10]. LLLT has traditionally employed red (600–700 nm) and near-infrared (NIR, 780–1100 nm) wavelengths [11]. The mechanism of LLLT is proposed to rest on photon absorption by cytochrome c oxidase, the terminal enzyme in the mitochondrial respiratory chain that catalyzes the reduction of oxygen for energy metabolism [12, 13]. However, besides metabolic effects on tissues, LLLT may generate non-negligible thermal effects due to laser heating on the tissue [14]. Application of LLLT has a beneficial effect on OM recovery with biostimulatory feature during the inflammation, proliferation, and maturation phases of the wound healing process [15, 16]. It has been shown that photodynamic therapy (PT) which is described in the literature as the interaction between a light source of a specific wavelength and a photosensitizer in the presence of oxygen can also ameliorate wound healing [17, 18]. Cruz et al. suggested that PT can be safely used in animals with infected OM [7]. One of the most commonly used photosensitizer is methylene blue. Because of the lower toxicity, indocyanine green (ICG) can be chosen in PT instead of methylene blue [17, 19–21].

In this study, we aim to compare the effects of PT and LLLT immunohistochemically and histologically on experimental OM.

Materials and methods

Sample

This study was performed in accordance with the National Institutes of Health guidelines on animal care and with the approval of the Ethics Committee of Bezmialem Vakif University (protocol = 2017/11). The study was conducted in accordance with the accepted guidelines for the care and use of laboratory animals in research. The protocol for ulcerative mucositis, suggested by Sonis et al. and improved by Leitão et al., was based on previously published articles and used to cause ulcerative mucositis [6, 7, 22, 23]. A total of 27 Sprague-Dawley rats, each weighing around 180–270 g, were intraperitoneally injected with 100 mg/kg of 5-fluorouracil (5-FU) on the first day and 65 mg/kg of 5-FU on the third day. The tip of a 21-gauge needle was used in order to develop a superficial scratch on the right cheek pouch mucosa on days 3 and 5. The animals were weighed daily and were fed in powder form to improve food intake. After the appearance of OM (Fig. 1a, b), the rats were divided into three groups: group 1 (control group) ($n = 8$) received no LLLT or PT, group 2 ($n = 8$) received LLLT for 5 days (Fig. 2a–c) after oral mucositis development, and group 3 ($n = 8$) received PT for 5 days (Fig. 3a, b) after oral mucositis development.

Low-level laser therapy

The OM occurred on the sixth day of the study and the LLLT was started 1 day later (on the seventh day) and lasted for 5 days. LLLT was applied with a diode laser Cheese (810 nm, Idealdent, Türkiye), about 2-mm distance from the ulcerated mucosa (continuous mode, 0.08 cm^2 focal spot size, and application time 5 s, energy density 0.3 W × 5 s/0.08 cm^2 = 18.75 J/cm^2 for 5 days). The device was used according to the manufacturer's instructions.

Photodynamic therapy

Photodynamic therapy was started on the seventh day of this study and lasted for 5 days. ICG (Periogreen, Elexxion) solution was prepared at 1 mg/ml [24]. This solution can be preserved for 6 h. Laser application was the same as that of the LLLT group of this study.

Thirty minutes after completion of the last laser application, the rats were sacrificed under anesthesia. Excisional biopsy was taken from the site of the wound and divided into two equal parts. One part was snap freezed, and the frozen samples were used for future western blot analyses. The other part of tissues was fixed with 10% formalin solution for histopathologic examination.

Preparation of total protein extracts

Approximately 100-μg cheek pouch mucosa tissues were used for total protein extraction. Protein extraction was performed by 1x Cell Lysis Buffer according to the protocol provided by the Cell Signaling Company (Cell Signaling, MA, USA). All buffers contained a protease inhibitor mixture (Cell Signaling, MA, USA). The suspension was frozen at − 80 °C overnight, and the extract was centrifuged for 15 min at 15,000×*g*. The supernatant was collected and kept at − 80 °C. The Coomassie (Bradford) Protein Assay Kit (Biorad, PA, USA) and standard bovine γ-globulin were used for quantification of the concentrations.

Western blot analysis

Fifty micrograms of total protein extract was separated on 4–12% gradient SDS-polyacrylamide gels at 120 V for 1 h 20 m at room temperature and transferred to 0.2-μm PVDF membranes (Biorad, PA, USA) in 1× Transfer Buffer which contains 20%

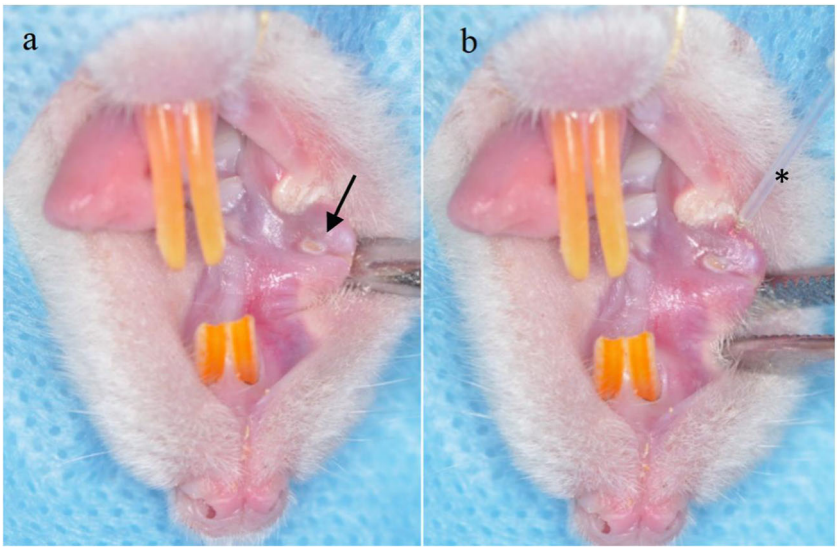

Fig. 1 **a** OM (arrows). **b** LLLT application (asterisk)

MeOH at 25 V for 10 min. The membranes were blocked with TBST solution containing 5% nonfat milk at + 4 °C overnight. The primary antibodies were rabbit polyclonal anti-PDGF antibody (5 μg/ml, Abcam), anti-FGF antibody (5 μg/ml, Abcam), and anti- TGF-β antibody (1:1000 dilution, Cell Signaling), and the secondary antibody was an anti-rabbit IgG, HRP-linked antibody (1:2000 dilution, Cell Signaling). The membranes were washed five times for 5 min each between antibody incubations with TBST. The blots were developed using the ECL detection kit (Advansta, USA).

Histopathological examination

All tissues harvested were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. One- to three-micrometer-thick sections were obtained with a microtome (Leica, 1–3 μ feather A 35, Germany) and stained with hematoxylin and eosin (H&E) for assessing inflammatory response, muscular atrophy, ulceration, and granulation tissue [6, 7, 25, 26]. Images were captured and examined with a microscope (Olympus Bx 51). The histological findings were blindly checked by a single pathologist three different times with a 1-month interval and reviewed in random orders to minimize learning bias. Data were recorded on a subjective grade of 1 to 4 (1 = same, 2 = mild change, 3 = moderate change, 4 = severe change) [27]. And then, the average of each parameter was calculated.

Statistics

According to the power calculation from the results of our previous study, a minimum sample size is determined as 9 ($\alpha = .05$, 80% power, Cohen's d 1.41067). The Kolmogorov-Smirnov test was used for distributional adequacy. The Kruskal-Wallis test was used to identify significant differences within experiments, and Dunn's multiple comparison post-test was used to assess the significance of differences between experimental groups with $p \leq$

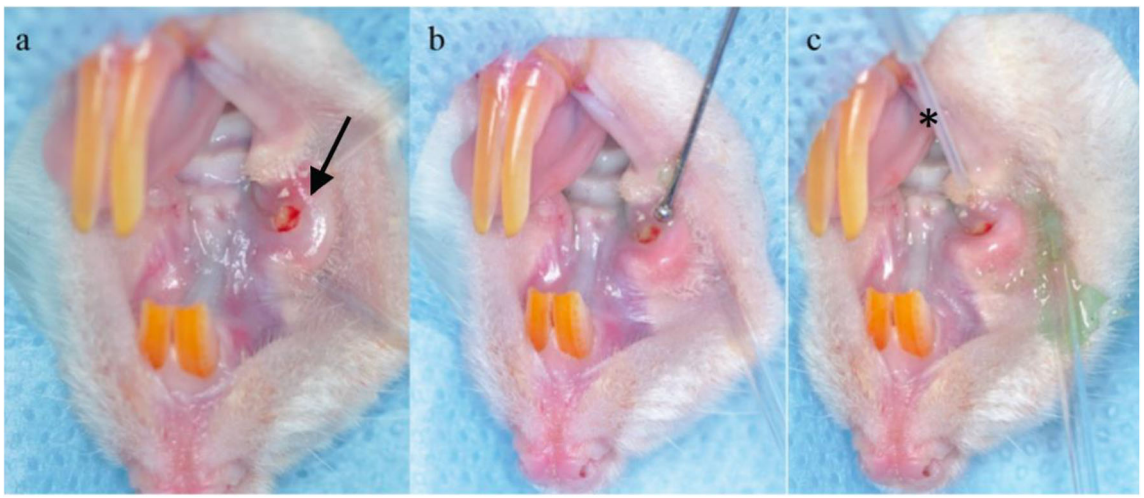

Fig. 2 **a** OM (arrows). **b** ICG application. **c** Laser application (asterisk)

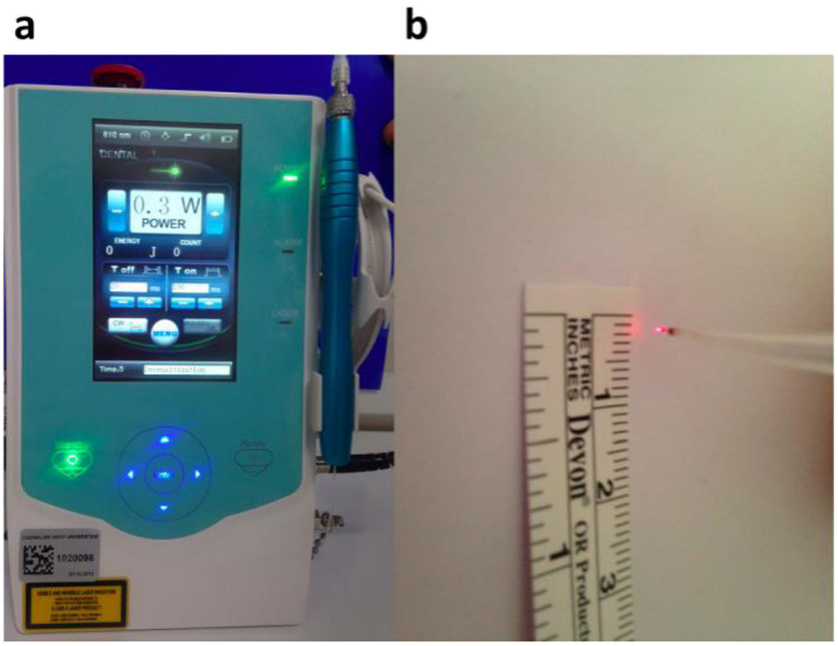

Fig. 3 **a** Laser device and application mode. **b** Laser probe and diameter of the light calculation (0.3 W × 5 s/ 0.08 cm^2 = 18.75 J/cm^2)

0.05 considered as significant (IBM SPSS 20.0 (SPSS Inc., Chicago, IL).

Results

On the 11th day of this experiment, two rats (one from the control group and the other from the LLLT group) died before sacrification because of aggressive OM.

Western blot results

The two dead animals were not included in the analysis because protein extraction could not be managed. Western blot analysis was applied to total protein extracts from cheek pouch mucosa isolated from 25 animals: eight from the control group, eight from the LLLT group, and nine from the PT samples. In all 25 samples, bFGF, PDGF-BB, and TGF-β expression were detected as can be seen in the western analyses shown in Fig. 4. Changes in TGF-β expression were not statistically significant between all groups (Kruskal-Wallis, $p = 0.4925$). PDGF-BB (Kruskal-Wallis, $p = 0.0169$) and bFGF (Kruskal-Wallis, $p = 0.0175$) significantly increased compared to the control group. In Dunn's test for PDGF-BB and bFGF, the LLLT and PT groups were compared. Both PDGF-BB (Dunn's test, $p = 0.0161$) and bFGF (Dunn's test, $p = 0.0308$) values were significant in PT (Fig. 4).

Histopathological analyses

Since the biopsy material was divided into two parts, four samples of the control group remained unfortunately superficial. As a result, histological evaluation was performed on 23 rats (five controls, nine LLLT, and nine PT).

It is seen in all groups that the areas where inflammatory infiltration is more common increase in ulceration of the surface epithelium, and inflammatory granulation tissue around the ulceration was detected. In cases without ulceration in the epithelium, the inflammatory response was between mild and moderate. In these cases, wound healing (re-epithelization) is thought to have improved. However, no statistically significant differences were found in histopathological examinations between treatment and control groups (Fig. 5).

According to inflammatory response scores, in the control group, inflammatory infiltration was mild in one, moderate in two, and severe mixed type in two cases. In the LLLT group, there was mild inflammation in five cases, moderate in one case, and severe in three cases, whereas the PT group had mild inflammation in all three cases with severe inflammatory infiltration in three. In general, inflammatory infiltration was observed in mild to severe changes in all groups. Scores 2, 3, and 4 are shown from the samples scored in Fig. 5. Score 1 was not observed because inflammation was seen in all cases. There was no statistically significant difference between groups in terms of inflammatory response ($p = 0.664$).

In histologic examination, musculoskeletal atrophy was observed in four cases in the control group and ten cases in the treatment groups. There was no significant difference between the groups in the statistical examination ($p = 0.461$).

There was widespread ulceration which varies slightly in the control group. There are two cases in the LLLT group and four cases in the PT group. There was a mild to moderate ulceration in all the remaining treatment groups. It has been thought that re-epithelization occurs in cases where the surface

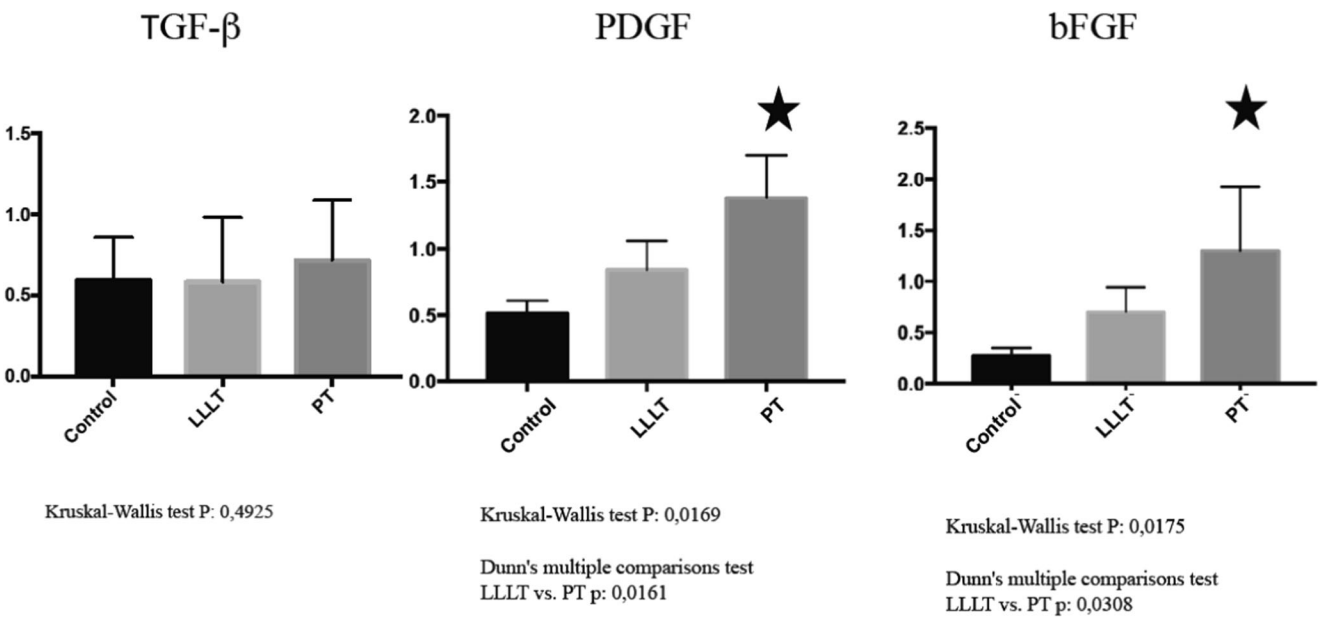

Fig. 4 Statistical analysis of TGF-β, PDGF-BB, and bFGF results is shown on the graph. Expression of TGF-β was not statistically significant among groups. PDGF-BB and bFGF were significantly increased compared to the control group. Both PDGF-BB and bFGF expression values were statistically significant in the PT group

epithelium is not completely ulcerated and the epithelium is regularly detected. Although the development of re-epithelization was more prominent in the treatment groups than the control group, no statistically significant difference was found between the groups ($p = 0.320$).

The development of granulation tissue is not observed in two cases in the LLLT and PT groups but it is present in mild to severe rates in all other cases. Statistically, there was no significant difference between groups ($p = 0.483$). Scores 1, 2, 3, and 4 are shown from the samples scored in Fig. 5.

Daily weights

The weights of the rats were measured daily. No statistically significant difference was found between the groups.

Discussion

Photodynamic therapy is used in antimicrobial therapy when the diode laser is administered in high doses [24, 28]. There are a limited number of studies showing that growth factors are stimulated by PT in lower doses. In this study, the aim was to apply the laser dose lower than PT for antimicrobial treatment to compare the feasibility of LLLT and PT on wound healing. Souza et al. revealed the increase of TGF in the gingival fluid and suggested the beneficial effect of inflammation may be due to the administration of LLLT during PT [29]. Thus, further work is needed to assess each treatment separately. In this study, immunohistochemical and histological examination of the OM region of the PT comparing LLLT were evaluated.

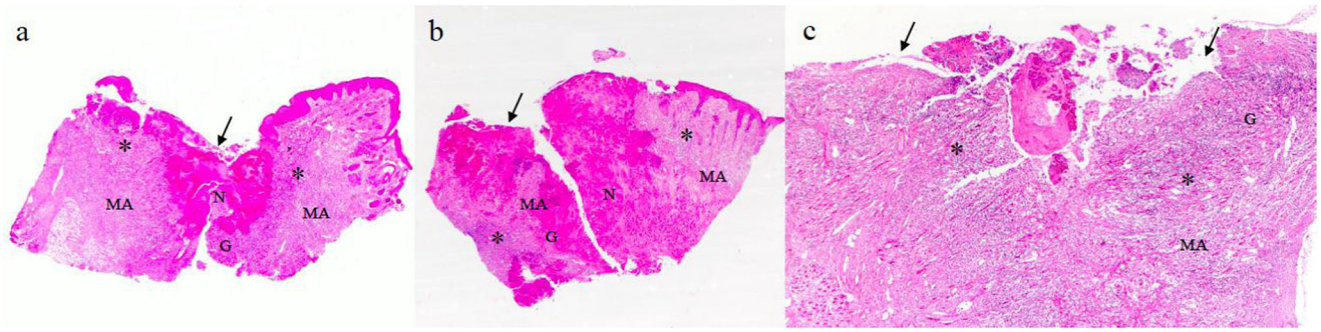

Fig. 5 Light microscope image of experimentally generated OM. **a** Control group. **b** LLLT group. **c** FDT group. Arrows indicate ulceration, (*) inflammation, N necrosis, G granulation tissue development, MA muscular atrophy. **a** Sample showing moderate ulceration, severe inflammation and musculoskeletal atrophy, and ulceration necrosis in the control group, H&E × 20. **b** LLLT group showing severe ulceration and inflammation, moderate granulation tissue, and muscular atrophy, H&E × 20. **c** Sample showing severe ulceration and inflammation, moderate granulation tissue, and muscular atrophy in the FDT group, H&E × 200

Mucositis is a common side effect of CT and RT that involves any part of the digestive tract. Naidu et al. reported that 30–60% of all patients with radiotherapy (RT) in the head and neck region have OM [3]. Scully et al. and Gautam et al. reported in the literature that OM is seen in 75–100% of patients receiving RT from the head and neck region in different studies [30, 31]. The incidence of OM detection in patients receiving standard chemotherapy is 40%, while the frequency in patients receiving a high dose of chemotherapy is up to 76% [4]. In addition, OMs usually occurred 3 to 5 days after the application of CT. According to evidence-based clinical practice guidelines of MASCC/ISOO, the key component of reducing OM score due to RT or CT is patient education and practices about multidisciplinary oral care protocols [32].

The protocol used in this study for the experimental OM is described by Sonis et al. [22] and improved by Leitão et al. [23]. Sonis et al. applied a single dose RT of 35–40 Gy and formed a linear trauma to the mucosa [22]. Similar to our study, experimental OM may be created by introducing 5-FU instead of RT. Leitão et al. applied intraperitoneally 60 and 40 mg/kg 5-FU, respectively, at the first and third days [23]. Yoshino et al. applied 60 mg/kg 5-FU on the first and third day [33]. However, in our pilot study, we could not create experimental OMs with these lower doses. We used higher doses on days 1 and 3 with 100 and 65 mg/kg, respectively. [6]

A longer wound healing process increases the severity of OMs and nutrition. In this study, two rats (one from control and one from the LLLT group) died on the day of euthanasia and all rats lost weight. We used powder feed in all groups to improve nutrition of the rats. Despite the weight lost, Cruz et al. did not observe any difference in water and food intake of rats in all groups. They weighed experimental animals on days 5, 7, and 10. On the 10th day, the mean weight of the experimental group of rats was 30 g less than the control group and they found this difference to be statistically significant. However, on the fifth and seventh days, no significant differences were found [7]. Yoshino et al. weighed the rats on days 0, 1, 2, 3, 4, 7, 9, 11, 14, and 16. Weight loss was observed in all 5-FU injection groups [33]. In this study, weights of rats were measured daily. Moreover, weight loss was seen in all groups and there was no statistically significant difference on days 1, 5, 7, and 10. Moreover, weight loss was also observed in the period between the beginning of the administration of 5-FU and the formation of OM. This shows feeding is not the only problem in OMs.

Er: YAG, Nd: YAG, and diode can be used for LLLT. The wavelength of the Er: YAG laser is 2940 nm, and the wavelength of the Nd: YAG laser is 1064 nm. There are devices with various wavelengths as a diode laser, and the most commonly used devices in the literature are the wavelengths between 780 and 910 nm [34, 35]. In this study, diode lasers at a wavelength of 810 nm were used because the range of maximum light absorption of the ICG solution was 800–900 nm.

LLLT is reported to facilitate healing in soft tissue lesions such as recurrent aphthous stomatitis, OM, and oropharyngeal ulcers. Biostimulation with LLLT during a prodromal phase of aphthous ulcer and recurrent herpetic lesions interrupts lesion formation, accelerates the healing period, and decreases the frequency of recurrence. Franca et al. [6] and Cruz et al. [7] applied LLLT before formation of OM in experimental animals to evaluate the preventive effects of LLLT in their study. In this study, LLLT and PT were started on the seventh day after OM was observed in order to evaluate the effects of the treatments.

During LLLT, laser application with appropriate output parameters, wavelength, and power density stimulates proliferation of fibroblasts [36]. Pourzarandian et al. reported decreased fibroblast proliferation with Er: YAG laser between 1.68 and 5 J/cm^2 energy value [34]. In the literature, there are dosages of laser between 0.99 and 120 J/cm^2 as a prophylactic or symptom-reducing therapy in OM [9]. Antimicrobial efficacy is increased when the treatments are applied with higher doses [7]. However, in studies investigating the stimulation and healing factors of healing, the laser is administered at lower doses [8]. AlGhamdi et al. stated that low-level laser application increases the proliferation in many cell types in the 0.5–4.0 J/cm^2 energy range and in the 600–700-nm visible light spectrum [37]. Pereira et al. reported that the optimal dose for cell development was 3 and 4 J/cm^2 with Ga-As diode laser at 904 nm. They also emphasized that 5 J/cm2 LLLT is not effective for procollagen synthesis and cell development [38]. However, Bensadoun et al. stimulated cell proliferation with 5 J/cm^2 energy density [35]. They stated that the optimum dose for prophylaxis is 2–3 J/cm^2 and that the therapeutic effect cannot be achieved below 4 J/cm^2 [39]. In this study, we applied laser in continuous mode while the distance was 2 mm from the tissue and energy dose of 18.75 J/cm^2 in both LLLT and PT [40].

Continuous irradiance with laser or light-emitting diodes over a period of time at a particular region of interest would result in an accumulated thermal effect and thus lead to an increase of skin or local temperature at the stimulated region. Such a thermal effect could lead to local increases of blood flow and tissue oxygenation, which could confound the association or interplay between metabolic and hemodynamic effects induced by LLLT [14]. Most of the high-power lasers, however, can cause thermal damage if used at exaggerated power settings or long irradiation times. This does not seem to be the case for low-power lasers [41]. In this study thermal effect of the laser could not be assessed.

Phototherapy with LLLT has prophylactic and therapeutic effects and reduces pain on OM. Although in vitro cell studies help to identify the potential mechanism of LLLT, the complex wound healing mechanism has not been totally explained [35, 42]. Studies on the effect of LLLT on different animal models on wound healing have been investigated in vivo [7, 24].

Topaloglu et al. emphasized that ICG is sensitive around 800 nm light and structural defects may be seen because of sunlight during the preparation. In their study on an abrasion wound model on rats, they compared the applied dose of ICG. Among 0.5, 1, and 2 mg/ml of ICG, there was no statistically significant difference. According to the results, they recommended 0.5 mg/ml to reduce the toxic effect [24]. In clinical studies, administered dose of ICG can be higher. This may be related to saliva that may decrease penetration of the solution to the tissue. In this study, ICG solution with a dose of 1 mg/ml was used as a photosensitizing agent and the ICG solution was kept in the dark area during the preparation.

Souza et al. reported PT as an alternative to surgical treatment because of increased TGF-β in gingival fluid after administration of PT with energy dose of 0.06 J/cm^2 [29]. De Moraes et al. applied PT with energy dose of 7 J/cm^2 and decreased the release of a tumor necrotizing factor in the gingival fluid and cell apoptosis. In this case, the supported reduction of tissue destruction and improvement in healing without surgical curettage is possible. Mendoza-Garcia et al. investigated the healing effect of PT on the wound healing model and applied the dose of 20 J/cm^2 with a radiometer [43]. They demonstrated that PT increased reepithelization and tissue remodeling. In our study, we found that bFGF and PDGF-BB levels were higher in both the LLLT and PT groups compared to the control group and the increase in bFGF and PDGF-BB levels in the PT group was statistically significant compared to that in the LLLT group. Therefore, we found that PT stimulated healing more than LLLT. The energy density we used in our study was reported by Mendoza-Garcia et al. and at a low dose of 18.75 J/cm^2.

Safavi et al. examined the effect of LLLT on the periodontal tissues with IL-1β, TNF-α, interferon (IFN-IF), PDGF, TGF-β, and bFGF levels. They found the increase in PDGF and TGF-β was statistically significant. Moreover, they suggested that PDGF, TGF-β, and bFGF are among the most important growth factors of periodontal tissues, and therefore, healing processes may be promoted by laser irradiation via increased production of these growth factors [44]. It is reported that LLLT accelerates cell proliferation, fibroblast growth, and wound healing by increasing levels of PDGF-BB and bFGF [8]. In this study, we also found an increase in PDGF-BB and bFGF levels with LLLT and the effects of PT on increase in these growth factors were statistically significant compared to those of LLLT.

In this study, the toxicity of ICG could not be assessed because 5-FU was also toxic. ICG toxicity and thermal effects of laser may be investigated in further studies. One of the limitations of the present study is the lack of equal number of animals in studied groups. In "Materials and methods", stereology and histomorphometry should be improved. There is no consensus of the energy dose of the laser application and a single agent for healing, and the number of clinical trials should be increased.

Conclusion

In conclusion, the effects of PT and LLLT were compared and PT caused more PDGF-BB and bFGF stimulation. More studies are needed on growth factors especially after PT.

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Compliance with ethical standards

Ethical approval The experimental protocol was approved by the Institutional Review Board and Animal Use Committee of the Bezmialem Vakif University (protocol = 2017/11).

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

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