

Comparison of laser and ozone treatments on oral mucositis in an experimental model

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Abstract Oral mucositis (OM) induces severe pain and limits fundamental life behaviors such as eating, drinking, and talking for patients receiving chemotherapy or radiotherapy. In addition, through opportunistic microorganisms, OM frequently leads to systemic infection which then leads to prolonged hospitalization. Severe lesions often adversely affect curative effects in cancer cases. Therefore, the control of OM is important for oral health quality of life and prognosis. Low-level laser therapy (LLLT) and ozone may be useful to accelerate wound healing. In this study, 24 Sprague-Dawley rats were divided into three groups as control, ozone, and laser groups. All groups received 5-fluorouracil intraperitoneally and trauma to the mouth pouch with a needle. After the formation of OM in the mouth, the control group had no treatment; the ozone group was administered ozone, and the laser group, LLLT. Then, all groups were sacrificed and basic fibroblast growth factor (bFGF), transforming growth factor (TGF- β), and platelet-derived growth factor (PDGF) were evaluated in all groups. LLLT was determined to be statistically significantly more effective than ozone on FGF and PDGF. However, in respect of TGF- β , no statistically significant difference was observed between the groups. In conclusion, within the limitations of this study, LLLT is more effective than ozone. However, further studies on this subject are required.

Keywords Ozone · Laser · Mucositis · Treatment · Histology · Dentistry · Healing

Introduction

Cancer treatment with chemotherapy or radiotherapy or both is becoming an increasingly more effective treatment modality. However, these treatments are associated with some side effects such as severe ulceration (mucositis) and fungal infection of the mouth (oral candidiasis, thrush). Almost every patient receiving chemotherapy or radiotherapy experiences increasing oral mucositis (OM) with the progress of treatment. These lesions not only cause pain, mouth and throat soreness but also adversely affect the curative effects on the cancer due to lead infection. Various oral functions such as mastication, swallowing and speaking are affected due to OM. Therefore, the control of oral mucositis is important for oral health quality of life and prognosis [1].

Various treatment options for oral mucositis have been used such as cryotherapy, laser treatment and the construction of a radiation area to protect the oral tissues during irradiation. A range of mouthwash options are used because of the anti-inflammatory, anesthetic, analgesic, antipyretic, and antimicrobial properties. In addition, systemically administered pharmacological agents, such as pentoxifylline, thalidomide, and simvastatin, have been shown to correlate with the development and severity of all the complications reported in transplantation patients. Clinical trials have reported that these drugs reduce the frequency and severity of major complications, including oral mucositis. Despite these treatment options, there is still a need for other cost-effective modalities to prevent mucositis [2].

Low-level laser therapy (LLLT), which has a beneficial effect during the inflammation, proliferation, and maturation

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phases of the wound healing process, is a safe adjunct treatment for OM [1]. It has been suggested in literature that LLLT can ameliorate oral mucositis in adult patients receiving dose chemotherapy [3]. Moreover, medical ozone is described as three atom molecules of oxygen known as O_3 and ozone therapy has been proven safe to use in medical treatment because of antimicrobial, disinfectant, and healing properties [4]. In addition, small doses of ozone can activate biochemical mechanisms and reactivate the antioxidant system. Diseases that can be treated with ozone include infected wounds, circulatory disorders, geriatric conditions, macular degeneration, viral diseases, rheumatism/arthritis, and cancer [5]. Although ozone treatment has substantial effects, there has been no study in literature about the influence of ozone on OM and a comparison with LLLT. The aim of the current study was to determine the effects of laser and ozone on OM and to compare those results.

Material and methods

Sample

The experimental protocol was approved by the Institutional Review Board and Animal Use Committee of the Bezmialem Vakif University (protocol=2015/35). 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 was based on previously published articles and used to cause ulcerative mucositis [3, 6]. A total of 24 Sprague-Dawley rats, each weighing 250–300 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. This technique has been repeatedly used to develop an ulcerative mucositis which is similar to human oral mucositis [3]. After developing oral mucositis (Fig. 1), the rats were divided into three groups as follows:

Group 1 (control group) ($n = 8$) received no laser and ozone treatment,

Group 2 ($n = 8$) received LLLT for 5 days after oral mucositis appeared and,

Group 3 ($n = 8$) received ozone treatment for 5 days after oral mucositis appeared.

The animals were weighed daily and were fed in powder form to improve food intake. Throughout the period of the experiment, four rats from the control group died because of aggressive OM on the third and fourth day after ulceration appeared. Thirty minutes after completion of the laser and ozone treatment, the rats were sacrificed under general anesthesia. Excisional biopsy was taken from the site of the wound



Fig. 1 After developing oral mucositis, the rats were divided into three groups

and frozen samples were used for future laboratory procedures and specimens were kept in nitrogen tank.

Laser treatment

LLLT was applied with a 940-nm diode laser (Epic, BioLase) about 2 mm distance from the ulcerated mucosa (continuous mode, 2.8 cm² focal spot size, and application time: 20 s, energy density: $1W \cdot 20s / 2.8 = 7.14 \text{ J/cm}^2$) for 5 days. The device was used according to the manufacturer instructions.

Ozone treatment

Ozone therapy was performed using an ozone generator (Biozonix GmbH, Munich, Germany) with a sterile tissue probe (AL probe), attached to the hand-piece, hand-guided over the ulcerated area like to clinical procedure. It was applied with 80% oxygen for 120 s per day, for 5 days.

Preparation of total protein extracts and western analysis

Cheek pouch mucosa tissues of approximately 100 μg in weight were collected and snap frozen in liquid nitrogen to extract protein [7]. Protein extraction was performed by 2X Cell Lysis Buffer according to the protocol provided by the Cell Signaling Company (Cell Signaling, MA, USA). All buffers contained a protease inhibitor mixture (1x PMSF and PIC, Cell Signaling, MA, USA). The suspension was frozen at -80°C overnight and the extract was centrifuged for 1 min at $15,000 \times g$ and the supernatant was collected and kept at -80°C . The total protein concentrations were measured using the Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific).

For western analyses, 50 μg of total protein extract was separated on 4–10% gradient SDS-polyacrylamide gels and transferred to PVDF membranes (Biorad, USA) in

1x Tris/Glycine/SDS at 120 V for 1 h 20 m at room temperature. The membranes were blocked with TBST wash solution containing 5% nonfat milk. The primary antibodies were rabbit polyclonal anti-PDGF antibody (5 ug/mL, Abcam), anti-FGF antibody (5 ug/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 (5 \times 5 min) each between antibody incubations with TBST. The blots were developed using the ECL detection kit (Advansta, USA).

Statistics

One-way ANOVA with Barlett's test (GraphPad Software, Inc., La Jolla, CA) was used for analysis of the quantitation of the western blot results.

Results

Western analysis was applied to total protein extracts from cheek pouch mucosa isolated from 20 animals; 4 from the control group and 8 from the LLLT and 8 from the Ozone treated samples. In all 20 samples, FGF, PDGF-BB, and TGF- β expression was detected as can be seen in the western analyses shown in Fig. 2. TGF- β was not statistically significant in all the groups as shown in Fig. 2a (one-way Anova, Bartlett's test, $p = 0.2985$). PDGF-BB was slightly increased in the laser treated group (Fig. 2b) (one-way Anova, Bartlett's test, $p < 0.0001$); although, bFGF was also stimulated by ozone, the results of the laser group were statistically significant (Fig. 2c) (one-way Anova, Bartlett's test, $p = 0.0002$).

Discussion

OM is often associated with ulceration in the mucosal tissues of the oral cavity of patients undergoing chemotherapy and radiotherapy. Radiation to the head and neck region may lead to different grades of OM in almost 98–100% of patients [8]. Unfortunately, the healing rate of OM is very slow so the negative impact increases [3]. In the current study, four animals that received 5-FU and induction of OM in the control group died because of malnutrition. This may have been because the control group had no treatment. It was possible to perceive the weight loss in these animals and debilitation causing impairment to the feeding abilities. Cruz et al. [4] observed that even with an improvement in OM severity, the animals in the experimental group continued to lose body weight and their food intake did not increase. This phenomenon was associated first to OM and secondly to feeding impairment. In the current study, the animals continued to lose weight although they were fed in powder form and food intake increased. Feeding was not the only problem. In this study, evaluation was made of the wound healing of OM seen after the chemotherapeutic agent was administered. It was planned to improve OM severity after LLLT and ozone therapy and the effects of the two treatments were compared.

LLLT has a positive effect on surgical wound healing, skin flap survival; burn wound healing, and bone healing in nerve tissue healing [9, 10]. There have been studies in literature indicating that ozone treatment reduces oxidative stress, improves wound healing, and increases tissue partial oxygen pressure [11]. Soto et al. [12] applied LLLT to pediatric patients for 22 days and concluded that a combined protocol of intra- and extra-oral application of LLLT reduced the severity of or prevented OM. In vitro data have suggested that LLLT

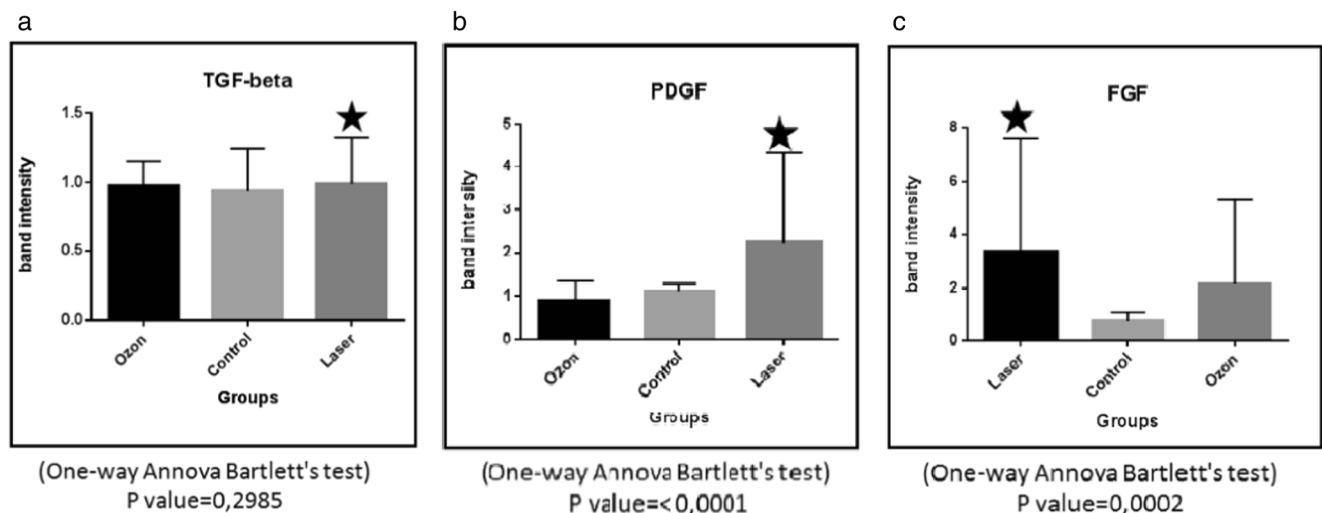


Fig. 2 a (One-way Anova, Bartlett's test, $p = 0.2985$). b (One-way Anova, Bartlett's test, $p < 0.0001$). c (One-way Anova, Bartlett's test, $p = 0.0002$)

stimulates collagen synthesis, keratinocyte cell motility, growth factor release and transforms fibroblasts to myofibroblasts [13].

A great number of growth factors and cytokines are involved in the wound site [14]. Of the growth factors, basic fibroblast growth factor (bFGF), transforming growth factor (TGF- β), and platelet-derived growth factor (PDGF) are anti-inflammatory and the most important in the wound healing process [15]. The bFGF (FGF-2) is a member of the FGF's family and has been shown in several studies to be an essential growth factor for fibroblast and vascular endothelial cell [16]. bFGF is increased in acute wound healing and plays a role in granulation tissue formation, re-epithelization, and tissue remodeling [17]. Although clinical studies have shown that bFGF was not successful in the treatment of diabetic foot ulcers, topical bFGF application has been found to increase the healing of burns and venous ulcers [16, 18, 19]. Moreover, TGF- β and PDGF are both crucial for inflammation, granulation, angiogenesis, connective tissue regeneration, re-epithelization, and remodeling which stimulate macrophages by increasing the secretion of other growth factors [16, 20, 21]. They stimulate collagen production, affect matrix formation, and inhibit metalloproteinase (MMP) activity, which degrades collagen deposition. Levels of PDGF, TGF- β , and FGF are decreased in chronic wounds. Therefore, PDGF-BB (Becaplermin) and bFGF are commonly used in the clinic in current treatments [16]. PDGF-BB has been successfully applied in diabetic and pressure ulcers and is the only FDA approved drug for chronic wound treatment. Usumez et al. [22] reported that diode laser therapy accelerated the wound healing process in oral mucositis by changing the expression of PDGF and FGF genes responsible for the stimulation of cell proliferation and fibroblast growth. In the current study, LLLT was found to increase PDGF-BB and bFGF.

In this study, the western blot technique was used to determine levels of TGF- β , PDGF-BB, and bFGF. It is a widely used technique to detect specific proteins in a sample of tissue and can be used to evaluate wound healing. Previous studies have shown that bFGF, TGF, and PDGF are enough for evaluation of soft tissue healing [22]. Although, TGF- β and PDGF play a role in cell proliferation on soft tissues, they cannot independently induce wound healing without FGF [7]. Fibroblasts have an important role in wound healing and many studies in literature have examined the effect of LLLT on fibroblast cell growth. FGFs are heparin binding types of key proteins in the processes of proliferation and differentiation of many cells and tissues [23]. The growth factors evaluated in these studies have anti-inflammatory effects. The results of this study demonstrated that LLLT increased bFGF quantity more than in the ozone treated groups.

There is no consensus on a single agent to increase the quality of life in cancer patients with OM [24]. Ozone therapy has been reported to enhance blood perfusion units in the first post-operative week and this outcome is also consistent with an improvement in wound healing accompanied by an increase in quality of life and decrease in post-operative pain [25]. Erdemci et al. [5] found that ozone has beneficial effects on wound healing for hard and soft tissue. Small doses of ozone can activate biochemical mechanisms and reactivate the antioxidant system. Histopathological examination has shown that ozone reduces inflammation and edema and is useful in wound healing on both soft and hard tissue [11, 26]. Kazancioglu et al. [27] found that although both ozone and laser therapies had a positive effect on bone formation in rat calvarial defect, ozone therapy was more effective than LLLT. To the best of our knowledge, there has been no study about the effect of ozone therapy in the treatment of oral mucositis, or any study comparing LLLT and ozone therapy in the treatment of oral mucositis.

Although the effects of LLLT are dose-dependent and the irradiation of the dose or energy density is one of the most important parameters in laser therapy, there is no consensus on dose calculation. Photobiomodulation obtained by LLLT is not limited to the area that receives the light beam but also occurs in the surrounding area [1, 22]. The energy density used in this study has been employed in previous studies.

The current study did have some limitations. Firstly, the error bar of the results is high due to the unexpected death of rats in the control group. The cheek tissue samples taken were insufficient for evaluation of both western and histopathological analysis. In the literature, there are several studies which evaluate wound healing solely histopathologically [3, 5]. In these studies, it had been found that bFGF, TGF- β , and PDGF are significant markers of the wound healing process [3, 5, 22]. Also, these growth factors could be stimulated by LLLT [15]. In this study, western analyses were chosen instead of histopathology.

The data of this study suggest that both laser and ozone therapies have positive effects in the treatment of oral mucositis. However, LLLT therapy seems to be more effective than ozone therapy. These results may be related to the duration and dose of the laser and ozone applications. Different laser wavelengths and different duration or dose of ozone application may change the results.

Conclusion

Within the limitations of this study, LLLT was seen to be more effective than ozone treatment on OM. Further studies on this subject, which is a popular topic in the treatment of soft tissue injuries, will provide necessary new insights on how laser and ozone therapy should be applied in cases of OM.

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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 = 2015/35).

Role of funding source Purchasing of chemicals and animals.

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

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