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ORIGINAL ARTICLE

Influence of carbon dioxide laser irradiation on the healing process of extraction sockets

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Abstract

Objective. To clarify the healing-promoting effects of carbon dioxide laser irradiation in high and low reactive-level laser therapies (HLLT and LLLT, respectively) on extraction sockets after tooth extraction. ***Material and methods.*** Forty-two 5-week-old male Wistar rats were divided into laser irradiation and non-irradiation (control) groups and compared. The laser-irradiation group underwent HLLT immediately after tooth extraction and then LLLT 1 day post-extraction. Tissue was excised 6 h and 3, 7, or 21 days after extraction and histopathologically investigated. The alveolar crest height was measured osteomorphometrically 21 days post-extraction, and granulation tissue in the extraction socket surface layer was immunohistologically investigated using anti-α-smooth muscle actin (anti-α-SMA) antibody 3 and 7 days post-extraction. ***Results.*** Many osteoclasts appeared and active bone resorption was noted in the irradiation group 3 days after extraction compared to the controls. On Day 7, new bone formation started around the extraction socket in the control group, but from the superficial to over the middle layer of the socket in the irradiation group. On Day 21, a concavity existed in the alveolar crest region in the controls, whereas this region was flat, with no concavity, in the irradiation group. On osteomorphometry, the alveolar crest height was significantly higher in the irradiation (0.7791 ± 0.0122) than the control (0.6516 ± 0.0181) group ($P < 0.05$). On immunostaining, many α-SMA-positive cells were noted in the control group, but very few in the irradiation group. ***Conclusion.*** Laser-irradiated extraction wound healing showed characteristics different from those of the normal healing process, suggesting a favorable healing-promoting effect.

Key Words: *High reactive-level laser therapy, low reactive-level laser therapy, scar, α-smooth muscle actin, socket preservation*

Introduction

Implant therapy has recently been increasingly applied in addition to dentures in clinical prosthetic treatment. For the long-term maintenance and functioning of prostheses, the alveolar bone condition is very important. The alveolar crest height is often lowered after surgical tooth extraction performed as pretreatment before prosthetic treatment. Accordingly, tooth extraction is performed not only to remove teeth judged as non-conservable due to periodontal disease, apical lesions and inflammatory tissue, such as unfavorable granulation tissue, but also as pretreatment before prosthetic treatment, similarly to vestibular extension and frenectomy, being the first step which determines the success or failure and outcome of subsequent prosthetic treatment of the defective region. Many studies on maximizing alveolar bone preservation while attempting to shorten the extraction wound-healing period, called socket preservation, have been performed. Caplanis et al. [1] investigated the promotion of bone growth by employing autologous bone grafting in the extraction socket and guided bone regeneration (GBR), immediate implant treatment, and a combination of these techniques. The usefulness of laser irradiation in clinical cases has recently been reported [2,3], and effective laser-irradiation conditions for extraction wound healing have also been investigated.

Lasers are roughly divided into tissue surface-absorbed and tissue-permeating types. Of lasers for

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dental use, the former include carbon dioxide (CO_2) and Er:YAG lasers, and the latter include Nd:YAG and semiconductor lasers. The CO_2 laser has a long oscillation wavelength (10.6 μm) within the far-infrared spectrum; it is likely to be absorbed by water, and its energy is mostly absorbed on the surface [4,5]. Laser irradiation methods are divided into high- [high reactive-level laser therapy (HLLT)] and low-power [low reactive-level laser therapy (LLLT)] irradiations. HLLT irradiation is 'surgical laser therapy' with direct ablative, and mostly photothermal, effects on tissue [6–8]. LLLT irradiation is 'sub-ablative laser therapy'. Biomodulation-associated resolution of inflammation and pain relief, and a wound healing-promoting effect have been reported, but none of the studies clarified the biological mechanism [9–14].

The U.S. Food and Drug Administration (FDA) specified laser application as the "coagulation of extraction sites" [15], but the objective evaluation of its efficacy in humans has been impossible because of the diversity of clinical cases, such as the presence or absence of underlying and periodontal diseases, or for various other reasons. There have been some reports in which extraction sockets were irradiated with a tissue-permeating laser and the influence on the healing process investigated [2,3,12–14]: scar tissue irradiated with a laser was reduced and disappeared in clinical cases [5] and an inhibitory effect of laser irradiation of human extraction sockets on the cicatrization of granulation tissue has been suggested [14]. The dynamics of myofibroblasts [expressing α-smooth muscle actin (α-SMA)] involved in cicatrization [16–18] are also of interest. We have immunohistologically investigated the usefulness of irradiation using a tissue surface-absorbed CO_2 laser with regard to the promotion of extraction wound healing by observing the pathological healing process, morphometry of alveolar bone, and myofibroblast dynamics, and obtained interesting findings.

In this study, we histopathologically and immunohistologically verified the effect of CO_2 laser irradiation on the extraction socket healing process in rats, particularly regarding trabecular changes and the appearance of myofibroblasts, which are reportedly involved in cicatrization.

Material and methods

Animals, tooth extraction, and laser irradiation

Male Wistar rats (age 5 weeks, body weight 130–150 g) were used. Animals were maintained in cages with three animals per cage and given free access to pellets/powdered feed (CLEA Rodent Diet CE-2; Clea Japan Inc., Tokyo, Japan) and drinking (tap) water throughout the study period. The animal room was controlled at 24 ± 2°C and 50% ± 5% humidity on a 12-h lighting cycle.

The animals were divided into two groups: a non-laser irradiation (control) group and a CO_2 laser irradiation (experimental) group. The animals were observed for 6 h and 3, 7, or 21 days after tooth extraction. Three animals were allocated to observation for 6 h and six animals each to the other durations in each group (42 animals in total). Animals were fed powdered feed for 3 days after tooth extraction and then pellets. The study protocol was established in accordance with the animal experiment guidelines of Fukuoka Dental College (No. 08019).

The operative procedures applied to the groups were as follows.

Non-irradiation (control) group. General anesthesia was induced by the intraperitoneal injection of 0.1–0.12 ml of pentobarbital sodium (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) diluted to 64.8 mg/ml with physiological saline, and the upper left first molar was extracted while avoiding injuring the alveolar bone using a (prototype) elevator exclusive to rats and mosquito forceps, followed by hemostasis by pressing the wound with a dry tampon. The wound was disinfected with 0.025 w/v% Germitol (Japan Pharmacopoeia benzalkonium chloride solution; NIKKO Pharmaceutical Co., Ltd., Gifu, Japan) 1 day after extraction.

CO_2 laser irradiation group. The tooth was extracted as described above. Compression hemostasis was not applied immediately after extraction, but HLLT irradiation was applied to coagulate blood and prevent the loss of blood clots following the clinical procedure. The wound was disinfected with 0.025 w/v% Germitol 1 day after extraction, and treated with LLLT after wiping off the disinfectant solution with a dry tampon. The animals were managed similarly to the control group after extraction.

Laser device and irradiation conditions

A CO_2 laser using a CO_2 oscillator (PanalasCO5Σ; Panasonic Shikoku Electronics Co., Ltd., Osaka, Japan) and a laser tip with an inner diameter of 0.15 cm (Taper 1A; transmittance 90%) were used. This laser device employs no guiding light, unlike the HeNe laser.

The irradiation conditions were as follows. The LLLT irradiation conditions were investigated in a preliminary experiment in which the rat gingiva was irradiated with a 1.0-W, Σ-mode laser for 15, 30, and 45 s to adjust the energy density to 40, 80, and 120 J/cm^2, respectively, and the course was

followed for 6 h. Since the epithelium was not disrupted at 40 J/cm^2, this density was adopted for LLLT.

HLLT irradiation was performed without the laser tip contacting the scar, so as to avoid touching blood clots, under the following conditions: 1.0 W; continuous wave mode; ≈30 s; non-air; ≈152 J/cm^2.

LLLT irradiation was performed with the laser tip contacting the scar of the superficial layer of the extraction wound under the following conditions: 1.0 W; Σ-mode; ≈15 s; non-air; ≈40 J/cm^2.

Σ-mode is capable of low-power laser irradiation by adjusting the pulse width to an ultra-short time and elevating the peak power on irradiation (pulse time 0.0008 s; pulse interval 0.03 s; 1 cycle = 0.0308 s; peak power = power twice that of main body setting). For example, the lowest mean power setting of 1.0 W was generated by 32.47 pulses of 1.6 mJ within 1 s.

Observation by light microscopy

The animals were sacrificed by overdose anesthesia 6 h or 3, 7, or 21 days after extraction, and the extraction socket, including the surrounding tissue, was excised and fixed in 4% paraformaldehyde for 48 h following the standard method [19–21]. The specimens were decalcified in 10% EDTA solution at 4°C for 3 weeks, dehydrated in a graded alcohol series, and embedded in paraffin. Serial sagittal sections with a 4-μm thickness were prepared using a microtome (Leica Microsystems Co.), stained with hematoxylin–eosin (H&E), and histopathologically observed.

Immunohistological observation

Thin sections of specimens excised 3 and 7 days after extraction were deparaffinized and hydrated, followed by antigen inactivation by microwave irradiation for 5 min. To inhibit endogenous peroxidase, the sections were treated with blocking reagent (DAKO, Tokyo, Japan) for 5 min, washed with distilled water, and immersed in Tris-hydrochloride buffer (TBS) for 5 min. To specifically observe myofibroblasts, the sections were reacted with the primary antibody, 50-fold diluted anti-human-α-SMA monoclonal antibody (Clone 1A4 N1584; DAKO), for 1 h at room temperature. The sections were then washed with TBS, reacted with peroxidase-labeled streptavidin (LSAB2 Kit; DAKO) for 10 min, washed with TBS, and reacted with diaminobenzidine (DAKO) for brown color development. After washing with distilled water, the sections were counterstained with Mayer's hematoxylin stain solution and observed under a light microscope.

Osteomorphometry

In H&E-stained sections of specimens excised 21 days after extraction when the extraction socket was filled with new bone, suggesting the completion of alveolar bone formation, the height of the new bone on the distal root of the extraction socket from the baseline passing the lower margin of the maxillary lamella [19,20] was measured (Figure 1). The perpendicular distance of the lowest point of the M2 interadicular septum (α) from the baseline and the distance of the lowest point of the new bone on the distal root (β) from the baseline were measured, and β/α (mean±standard error) was calculated as an index to correct for individual differences and distortion produced during the preparation of sections. The images were scanned using a digital microscope, and the distances were measured using software (Scion Image).

Statistical analysis

The measured values are presented as the mean ± standard error, and were analyzed using the Mann–Whitney U-test (STATISTICA; StatSoft Inc., Tulsa, OK). $P < 0.05$ was regarded as significant.

Results

Histopathology

The extraction socket was filled with blood clots 6 h after extraction in both the control and CO_2 laser

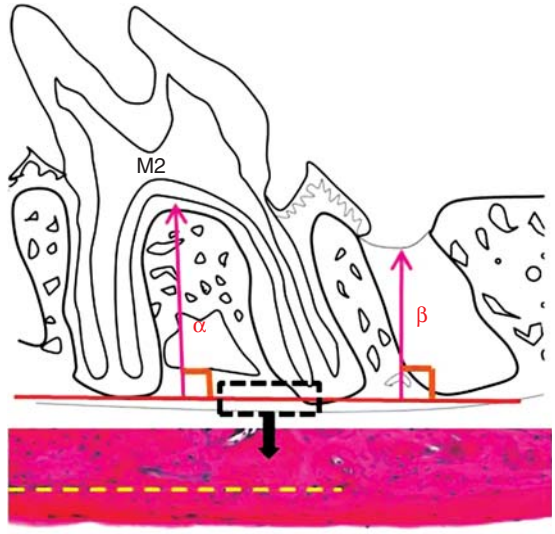

Figure 1. The osteomorphometric method for determining the alveolar bone height on Day 21 after extraction. The rectangle denoted by broken lines indicates the maxillary lamellar line (*broken yellow line*), referred to as the baseline (H&E staining; original magnification ×100).

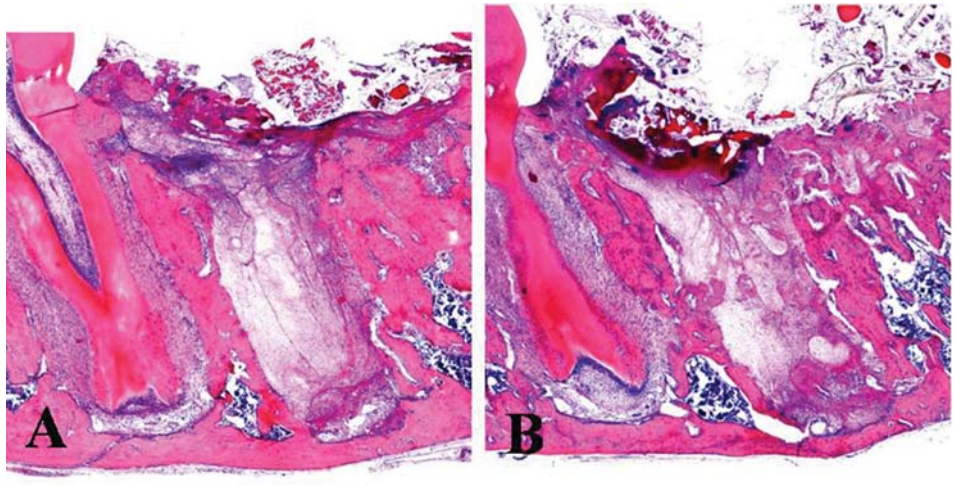

Figure 2. Histopathology 6 h after extraction. H&E staining; original magnification ×40. (A) Control group: the extraction socket was filled with blood clots. (B). CO_2 laser irradiation group: a surface-carbonized layer and blood clots were present in the extraction socket.

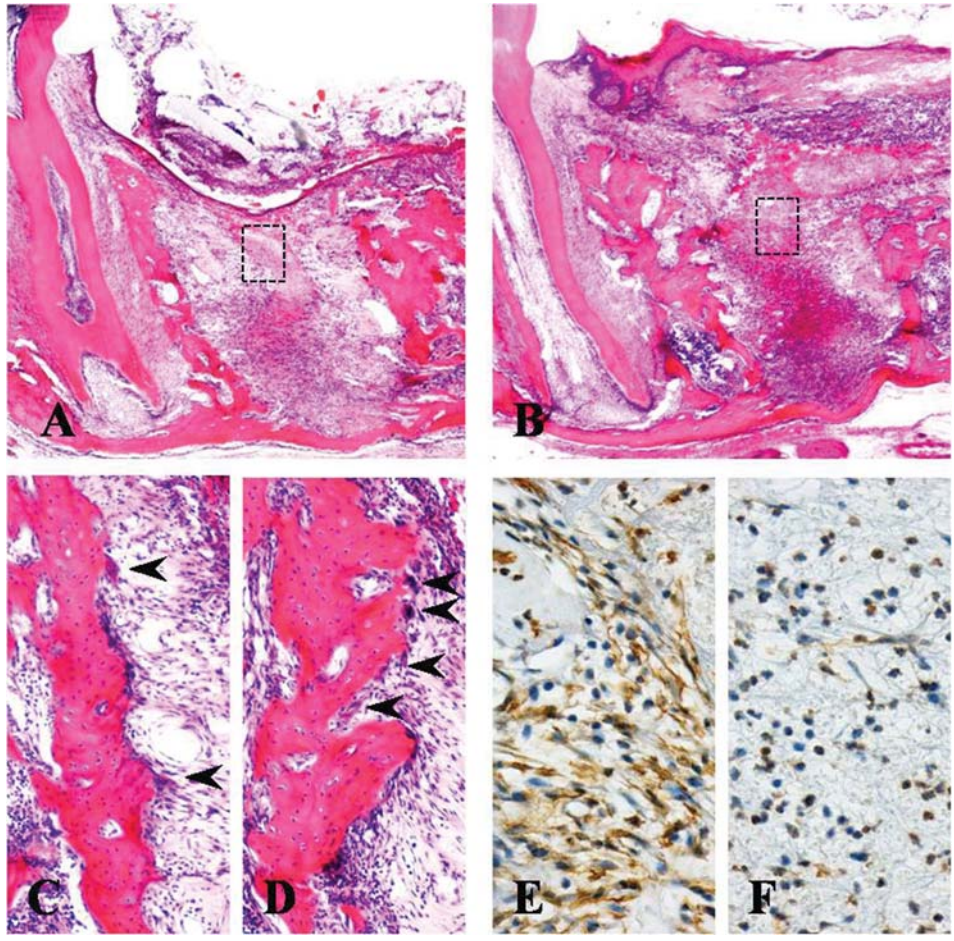

Figure 3. Histopathology and immunostaining of the extraction socket surface layer with anti-α-SMA antibody 3 days after extraction: (A,B) H&E staining, original magnification ×40; (C,D) alveolar bone walls of (A,B), original magnification ×100; (E,F) immunostaining findings in the broken square regions, original magnification ×400. (A) Control group: organization progressed from the region around the extraction socket, and blood clots were present in the center. (B) CO_2 laser irradiation group: the extraction socket was mostly filled with blood clots. (C) The alveolar bone wall in the control group. There were only a few osteoclasts (*arrowheads*). (D) The alveolar bone wall in the CO_2 laser irradiation group: many osteoclasts (*arrowheads*) appeared, showing active bone resorption. (E) Control group: many α-SMA-positive cells were present. (F) CO_2 laser irradiation group: there were almost no α-SMA-positive cells.

irradiation groups, and a carbonized layer was present on the surface in the irradiation group (Figure 2).

Organization had started around the extraction socket on Day 3 after extraction and blood clots were present in the center in the control group. In contrast, in the irradiation group, the extraction socket was mostly filled with blood clots (Figures 3A and 3B). On observation at high magnification, only a small number of osteoclasts were noted in the alveolar bone wall in the extraction socket in the control group, but many osteoclasts were present in the irradiation group, showing active bone resorption (Figures 3C and 3D).

On Day 7, granulation tissue accompanied by mild inflammatory cell infiltration had increased in the surface layer of the extraction socket, and the surface was being covered with regenerated mucosal epithelium extending from the surrounding tissue. Bone resorption by osteoclasts and bone formation which had begun around the extraction socket simultaneously progressed in the control group. In contrast, the carbonized surface layer disappeared, no osteoclasts were noted, and marked new bone formation in a bridging manner was noted in the superficial layer over the middle layer of the extraction socket in the irradiation group (Figures 4A and 4B).

On Day 21, the socket was filled with new bone in both the control and irradiation groups. The alveolar crest was concave in the control group, whereas trabeculae were dense and the alveolar crest was flat, with no concavity, in the irradiation group (Figures 4C and 4D).

Immunohistology

On Day 3, many α-SMA-positive myofibroblasts were present in the surface layer of the extraction socket in the control group, but almost no α-SMA-positive cells were present in the irradiation group (Figures 3E and 3F). On Day 7, many α-SMA-positive cells were present in granulation tissue in the extraction socket surface layer in the control group, but very few α-SMA-positive cells were present in the irradiation group compared to the control group.

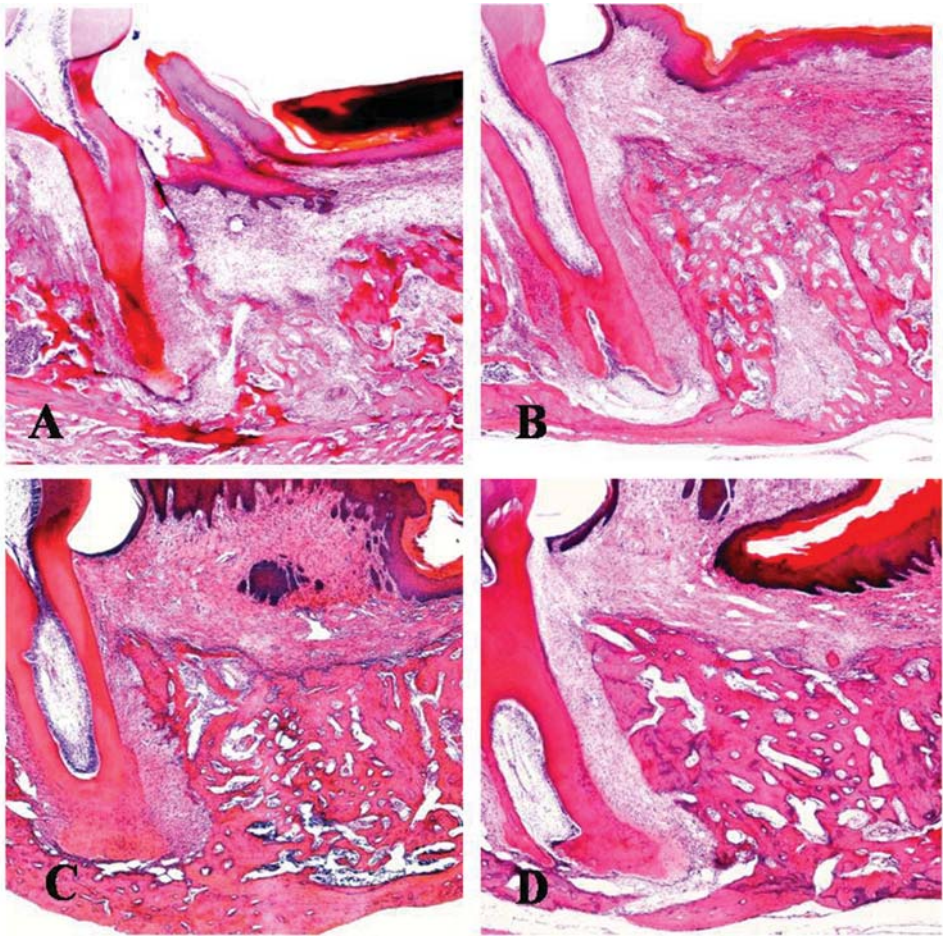

Figure 4. Histopathology 7 and 21 days after extraction. H&E staining; original magnification ×40. (A) Control group on Day 7: bone formation from the region around the extraction socket was noted. (B) CO_2 laser irradiation group on Day 7: Marked new bone formation was noted mainly in the superficial layer over the middle layer of the extraction socket. (C) Control group on Day 21: The extraction socket was filled with new bone, and the alveolar crest was concave. (D) CO_2 laser irradiation group on Day 21: The alveolar crest was flat, without concavity.

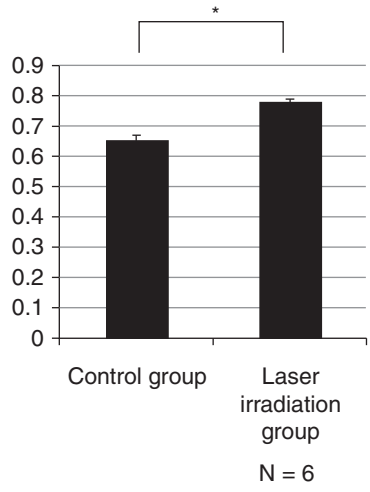

Figure 5. Measurement result of osteomorphometry of the alveolar bone height on Day 21 after extraction. The value was significantly greater in the CO_2 laser irradiation group than in the control group (*$P < 0.05$).

Osteomorphometry

On Day 21, the alveolar bone height was significantly higher in the irradiation group (0.7791 ± 0.0122) than in the control (0.6516 ± 0.0181) group ($P < 0.05$) (Figure 5).

Discussion

The biological influence of a laser depends on its wavelength and mode (continuous or pulse waves) and, reportedly, the following interactions occur in the body: (1) thermal effect; (2) photoablation; (3) photodisruption; (4) a photodynamic effect; and (5) biostimulation [4,5,22]. Regarding temperature-related influences on soft tissue, a thermal effect occurs at 37°C to <60°C, protein degeneration, coagulation, and tissue shrinkage occur, a hemostatic effect is exhibited at 60°C to <100°C, tissue evaporates at ≥100°C, and carbonization and the combustion of tissue occur at ≥200°C [4,5]. In an experimental study using rabbits during the bone regeneration process, the implant was inserted into the tibia for electrode connection, and adverse effects were found at 47–50°C for 1 min, while application at 44°C for 1 min had no effect on tissue regeneration [23]. The CO_2 laser wavelength is 10.6 μm, which is longer than those of other lasers, and is likely to be absorbed by water due to its absorption characteristics. When blood clots in the extraction socket are irradiated with a high-power laser in HLLT, the energy is mostly absorbed [4,5]. Accordingly, the heat produced on laser irradiation of an extraction socket is not readily transmitted to the alveolar bone, suggesting that the promotion of blood coagulation by irradiation of the extraction socket is relatively safe. Although no HLLT of the extraction socket with a CO_2 laser has been reported, Romanos & Nentwig [24] transplanted autologous and allogenic bone grafts into bone-defective regions in patients with peri-implant inflammation, covered the region with a bioabsorbable membrane, and irradiated it with a CO_2 laser. They observed that bone regeneration occurred in the bone-defective region and around the implant, suggesting that irradiation not only removed necrotized tissue on the implant surface but also retained blood clots. Deppe et al. [25] performed an experimental study using dogs in which peri-implant inflammation was irradiated with a CO_2 laser, and re-osseointegration was noted in an X-ray photograph and histopathological preparation 4 months after extraction and thereafter. All these studies showed the effects of HLLT with a CO_2 laser, but we applied HLLT to coagulate blood and prevent the loss of blood clots. The irradiation condition of LLLT was set at 40 J/cm^2 based on a preliminary experiment, as described in the *Material and methods*. In previous studies on LLLT, low-power laser application promoted wound healing [7–10] and the differentiation and migration of cultured cells *in vitro*. Many studies on bone-system cells have been performed [26,27]. Stein et al. [26] conducted an *in-vitro* experimental study in which LLLT irradiation of human osteoblast-like cells (SaOS-2 cells) enhanced alkaline phosphatase activity and the expression of osteopontin and type I collagen compared to those in an untreated group, and the effects were exhibited within 72 h. Saracino et al. [27] similarly applied LLLT to human osteoblast-like cells (MG-63 cells) and observed that the expression of growth factors, such as transforming growth factor (TGF)-β and bone morphogenetic protein-4 and -7, was enhanced after 4 days, and calcified deposits were formed 20 days after irradiation. However, irradiation of the extraction socket may not have reached the deep tissue because the CO_2 laser is the surface-absorbed type, and the influence of laser irradiation was likely to be limited to the surface layer of the extraction socket.

Regarding the healing process of extraction wounds, granulation tissue growth promoted organization after blood clot formation in the control group, and bone formation started mainly in the deep layer of the extraction socket 7 days after extraction. In contrast, in the CO_2 laser irradiation group, marked bone resorption by osteoclasts occurred, with organization after blood clot formation occurring mainly in the alveolar crest region 3 days after extraction. On Day 7, almost no alveolar bone resorption was noted, and new bone formation was promoted in the superficial layer over the middle layer of the extraction socket, showing a healing process slightly different from the normal one. Mendes et al. [21] applied sodium hyaluronate, which reportedly exhibits a metabolism-activating effect, such as the promotion

of cell differentiation, proliferation, and migration, to the extraction socket and investigated the healing course in Hotzman rats. They observed active alveolar bone resorption 7 days after extraction, followed by the marked promotion of new bone formation in the hyaluronic acid-treated group compared to the untreated group, and a high density and an unclear boundary between the existing and new bones on Day 21, suggesting that metabolic activation by hyaluronic acid induced favorable bone formation. CO_2 laser irradiation-induced active bone resorption was noted on Day 3 and rapid new bone formation on Day 7, which were similar to the effect of hyaluronic acid application. The surface carbonized layer formation induced by CO_2 laser irradiation sufficiently retained blood clots in the extraction socket, and this may have been an important factor, but LLLT irradiation may have also been a bone formation-promoting factor, similar to the effect of hyaluronic acid. New bone formation was rapidly promoted in the superficial layer over the middle layer in the extraction socket. Although comparison with previous reports is impossible because no similar experimental study has been reported, it was assumed that the healing effect was due to the combination of CO_2 laser HLLT and LLLT.

In association with the CO_2 laser irradiation-induced promotion of alveolar bone formation, we measured the alveolar crest height by osteomorphometry on Day 21 after extraction, when the extraction socket was filled with new bone. Several studies on the osteomorphometric method have been reported. A baseline was established by standardizing the extraction socket based on an approximated line of the alveolar bone wall in the extraction socket and the muscle attachment site of the maxilla in a study involving upper tooth extraction sockets [19], and the baseline was set at the lowest orbital point of the maxillary cortical bone in a study which investigated alveolar bone changes with odontoptosis [20]. In our study, it was difficult to set these baselines because of an irregular alveolar bone wall morphology due to the early occurrence of active alveolar bone resorption and addition and relative changes in the lowest orbital point of the maxilla with growth. Thus, we set the baseline at a lamellar line present on bone assumed not to be altered based on histological observation, i.e. the lamellar line passing the lower margin of the maxillary deep compact bone as described in the *Material and methods*, and the vertical height from the line was measured. A significantly higher alveolar crest height was maintained in the CO_2 laser irradiation group compared to that in the control group. Since it was suggested that new bone was rapidly formed and a high alveolar crest was maintained, CO_2 laser irradiation of the extraction socket may lead to a useful healing morphology for denture stability and the favorable esthetics of bridges and implant therapy, and its clinical application is expected.

Myofibroblasts are a variation of fibroblasts and possess the characteristics of both fibroblasts and smooth muscle cells. They are contractile, and delayed healing due to the marked proliferation of myofibroblasts in large wounds has been reported [16–18]. Fewer α-SMA-positive cells were present in the extraction socket in the irradiation group than in the control group on Days 3 and 7. A comparison of the wound-healing process between incisions made by a scalpel and CO_2 laser irradiation has been reported, and suggested that fewer myofibroblasts appeared in the CO_2 laser irradiation group [16], but no detailed dynamics of myofibroblasts in the extraction socket healing process have been reported, and so many unclear points remain. It has been reported that TGF-β1 expression in the granulation tissue was enhanced in biopsied specimens of human extraction sockets after LLLT, suggesting an influence on cicatrization [14], and ossification progressed rapidly in regions with low α-SMA expression in the bone tissue regeneration process, suggesting a relationship between the state of α-SMA expression and osteoblast differentiation [28]. These reports suggest that the proliferation of α-SMA-positive myofibroblasts leads to cicatrization and subsequently inhibits bone formation. The appearance of fewer α-SMA-positive myofibroblasts in the granulation tissue in the CO_2 laser irradiation group indicated mild cicatrization of the extraction socket, which may have been closely associated with favorable new bone formation and a high alveolar crest.

Conclusions

The rapid bone resorption and new bone formation on the surface layer over the middle layer and the appearance of fewer myofibroblasts in the surface layer may have been the effects of CO_2 laser irradiation. It is suggested that CO_2 laser irradiation, particularly the combination of HLLT and LLLT, promotes the healing of tooth extraction sockets.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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