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Original Article

Space-making effect for new bone formation by suppressing scar contraction of mucosal epithelium of rat tooth extraction wound using diode laser and CO_2 laser treatment

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Abstract *Background/purpose:* As an extraction wound closes, the mucosal epithelium of the tooth extraction wound impedes the space for new bone formation by invading into the extraction socket. Thus, the height of the alveolar crest decreases, causing significant depression of the alveolar mucosa. In this study, we created a rat tooth extraction model and examined the effects of laser irradiation by CO_2 and diode on the dynamics of myofibroblast expression through α-SMA, and TGF-β1.

Materials and methods: After tooth extraction of five-week-old male Wistar rats, they were divided into two laser treatment groups (CO_2 laser or diode laser was irradiated into tooth extraction socket) and non-laser treatment group (control group). Surrounding tissues, including the extraction socket, were removed at 3, 5, 7, and 21 days after tooth extraction and the expression of α-SMA and TGF-β1 was verified using immunohistological techniques (6 animals in each group and each period, 72 animals in total).

Abbreviations: FDA, the US Food and Drug Administration; HILT, high-intensity laser therapy; PBMT, photobiomodulation therapy.

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Results: α-SMA-positive cells and TGF-β1-positive areas were significantly lower in the two laser treatment groups than in the control group. Particularly, the diode group almost had no TGF-β1-positive areas on the 21st day when healing after tooth extraction was deemed to be completed.
Conclusion: Both CO_2 and diode laser irradiation of tooth extraction wounds decreases α-SMA-positive cells and TGF-β1-positive areas. Further, it causes a decrease in myofibroblast expression and suppresses the invasion of mucosal epithelium into the extraction socket. Therefore, laser irradiation may exert a space-making effect for new bone formation and also contribute to socket preservation.

Introduction

Tooth extraction can decrease the height of the alveolar crest. Therefore, socket preservation is important to maintain the height of the alveolar crest while promoting wound healing after tooth extraction.

One of the guidelines for dental laser treatment proposed by the US Food and Drug Administration (FDA) is the Coagulation of Extraction Sites, which clearly recommends the use of CO_2 laser and diode laser as devices for this treatment.[1] The difference of CO_2 laser and diode laser is due to its characteristic of wavelength. CO_2 laser is a long wavelength that could mainly be absorbed on shallow layer of irradiated tissue. On contrary, diode laser is a short wavelength that could reach into deep layer. However, those characteristic is different, both of them has a enough healing effect on irradiated tissue. In recent years, clinical and basic reports have revealed that laser irradiation after tooth extraction promotes wound healing.[2–8] However, there are no reports on maintaining the height of the alveolar crest.

In our previous reports, histopathological verification was conducted on the effect of laser irradiation on alveolar bone formation in a rat tooth extraction model using two types of laser devices recommended by the FDA and reported that the bone level of the extraction socket was maintained.[9–11]

During the healing process, a normal tooth extraction wound is accompanied by the invasion of the mucosal epithelium, otherwise known as scar contraction. In large injuries and healing of surgical wounds, fibroblasts in granulation tissue typically differentiate into myofibroblasts with the contraction characteristics of the smooth muscle through the expression of TGF-β1. It is crucial for the wound surface to close immediately.[12–16] However, the expression of myofibroblast causes scar tissue formation and scar contraction on the wound surface, resulting in esthetic issues as well as dysfunction.[14–18] When scar contraction occurs in the extraction wound, the space for new bone formation in the extraction socket alveolar bone is reduced. Therefore, the suppression of scar contraction is important for socket preservation.

In recent years, studies on the effect of laser on scarred skin tissue have reported a decrease in the expression of myofibroblast and TGF-β1, a decrease in scar tissue formation and contraction, and suppression of excess collagen fiber production.[19–24] Fukuoka et al. reported that CO_2 laser irradiation on wounds of upper lip had no esthetic issues or dysfunction.[25] However, there are very few reports on the dynamics of the oral mucosa after laser irradiation for scarring,[19] and there are no reports on the mucosal epithelium of a tooth extraction wound.

Therefore, in this study, we created a rat tooth extraction model and examined the effect of CO_2 laser and diode laser irradiation on the invasion of the mucosal epithelium of the tooth extraction wound into the extraction socket, focusing on myofibroblast expression marker α-SMA and the dynamics of TGF-β1 related to myofibroblast differentiation.

Materials and methods

The experiment, which used rats as experimental animals, was carried out in accordance with the ARRIVE Guidelines based on the Osaka Dental University Animal Experiment Guidelines (approval number: 18-01008). Seventy-two 5-week-old Wistar male rats (body weight 130—150 g) were used for the study. As for the rearing environment, each cage contained three rats, where solid feed and tap water were available for free intake. The rearing room was maintained at a room temperature of 24 ± 2 °C and at a humidity of 50 ± 5%. A light—dark cycle was repeated every 12 h. The experimental group that received laser irradiation after tooth extraction of the maxillary first molar was further divided into two groups: the diode group, and the CO_2 group. However, the term "laser treatment group" will be used to collectively refer to the diode group and CO_2 group in this study. A control group did not receive laser irradiation after tooth extraction. The rats were observed at 3, 5, 7, and 21 days after tooth extraction. Verification was conducted in groups of six at each period accordingly.

The surgical procedure, laser devices, and their respective irradiation conditions were implemented similarly to our previous studies.[12–14] In the experimental method, after general anesthesia with isoflurane inhalation, the first molar on the left side of the maxilla was extracted using a root elevator and mosquito forceps for rats. First molar was selected for experiments because extraction could performed completely compared with second molar that could avoid fracture of tooth or destruction of alveolar bone in our

preliminary experiments. After extraction, compression hemostasis with a dry cotton ball was performed in the control group. Moreover, the laser treatment groups did not undergo compression hemostasis immediately after extraction; instead, they received high-intensity laser therapy (HILT) to form artificial scabs by coagulation and carbonization of the blood surface layer once the blood reached the height of the extraction socket mucosa. Furthermore, the scab was welded to the mucosa around the tooth extraction wound to prevent it from falling. The day after tooth extraction, the control group underwent disinfection with diamitol. After the same disinfection process, the laser treatment groups were subjected to photobiomodulation therapy (PBMT), which was expected to promote wound healing (Table 1).

Immunohistochemical examination

Anti-human-alpha-smooth muscle actin monoclonal antibody (Clone1A4 N1584; DAKO Japan Inc., Tokyo, Japan) (α-SMA), which is an expression marker of myofibroblast, and transforming growth factor-β1 antibody (Santa Cruz Biotechnology, Inc, Dallas, TX, USA) (TGF-β1), which is involved in myofibroblast differentiation and apoptosis, were used as the primary antibodies for immunostaining. Regarding the preparation of specimens, after euthanizing the rats by anesthetic overdose on each observation day after tooth extraction, the surrounding tissue, including the extraction socket, was excised and fixed with 4% paraformaldehyde for 48 h. The sample size was specified square shape region (10 mm, each side) that contains the extraction socket and whole of second molar.

Then, the specimens were decalcified with 10% EDTA solution for 3 weeks, dehydrated by immersing the specimens in a series of alcohol solutions of increasing concentrations, and embedding them in paraffin. Furthermore, a sagittal continuous sliced specimen with a thickness of 4 μm was prepared using a microtome. The sliced specimens were deparaffinized, hydrophilized, and subjected to antigen activation treatment by microwave irradiation for 5 min. In order to suppress endogenous peroxidase, blocking reagents (DAKO Japan Inc) were allowed to act for 5 min. The specimens were washed with distilled water and immersed in Tris hydrochloric acid buffer (Wako Co. Ltd., Osaka, Japan) for 5 min. Following this, the specimens were allowed to react with the primary antibodies, which underwent 50-fold dilution at room temperature for 1 h. The specimens were washed and allowed to react with peroxidase-labeled streptavidin (Wako Co. Ltd.) for 10 min. They were then counterstained with Mayer's hematoxylin stain solution and observed under an optical microscope.

Measurement of the number of α-SMA-positive cells and TGF-β1 expression areas

Fig. 1 shows a schematic diagram of the laser irradiation site after tooth extraction. The number of α-SMA positive cells was measured in the area of granulation tissue formation on the surface of the extraction socket or the lamina propria in the tissue collected from the same site. The number of α-SMA-positive cells was measured within the scope of 150 μm in the vertical and horizontal directions at three locations with clear staining. The ratio of TGF-β1-positive areas per unit area was first calculated and then the average value of each group was calculated accordingly. A digital microscope (VZ-9000; KEYENCE CO., Ltd., Osaka, Japan) was used for the measurement. After scanning the image, a measurement was performed using Scion-image (Scion Corporation, Frederick, MD, USA). The evaluation was conducted by one co-author in a blind test. The measured values were shown as mean ± standard deviation, and a statistically significant difference was determined by Fisher's exact test (STATISTICA; StatSoft, Tulsa, OK, USA) with a significance level of less than 5% ($p < 0.05$).

Results

Immunohistological analysis and measurement results of anti-αSMA antibodies

The immunohistological stained images of the anti-α-SMA antibodies are shown in Fig. 2A–L, and the measurement

Table 1 Irradiation conditions of HILT and PBMT of CO2 laser and diode laser.

	CO2 laser		Diode laser	
	HILT	PBMT	HILT	PBMT
Power	1.0 W	1.0 W	1.0 W	0.3 W
Irradiation mode	Continuous wave	∑mode[a]	Continuous wave	CP-1 mode
Pulse width	–	0.0008 s	–	0.0001 s
Pulse interval	–	0.03 s	–	0.0002 s
Pulse frequency	–	32.5 Hz	–	3333 Hz
J/sec	–	0.052 J/s[b]	–	0.1 J/s
Air	None	None	–	–
Irradiation time	30 s	15 s	27 s	7 s
Energy	27 J	0.7 J	27 J	0.7 J
Blood contact	Noncontact	Slight contact	Contact	Slight contact

HILT: high-intensity laser therapy. PBMT: photobiomodulation therapy.
[a] ∑-mode uses an ultrashort pulse width to increase peak power during irradiation, thereby enabling photobiomodulation.
[b] Calculated using the joule conversion table for Panalas CO5∑.

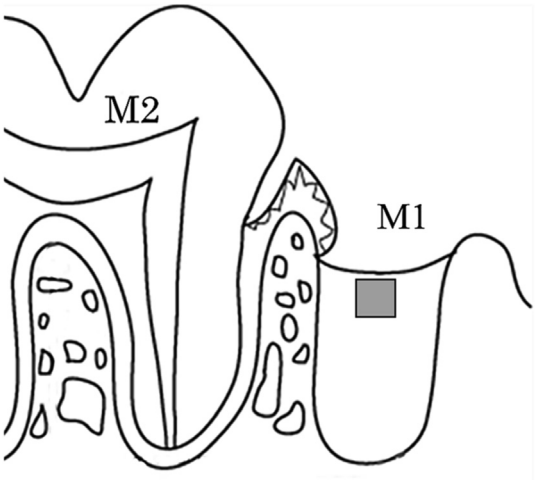

Figure 1 Morphological measurement methods. The measurement area was 150 × 150 μm in length and width, respectively, in the superficial granulation tissue formation or the intrinsic mucosal layer of the extraction socket of the first molar (M1: first molar, M2: second molar).

results of the number of positive cells are shown in Fig. 2M. Expression of α-SMA-positive cells was recorded throughout the observation period in the control group. The peak number of positive cells was on day 5, and then it showed a decreasing trend on day 7 and 21. The number of positive cells on day 21 was almost similar to that of day 3 (Fig. 2A—D). Similarly, the number of positive cells in both the CO_2 group (Fig. 2E—H) and diode group (Fig. 2I—L) peaked on day 5 and showed a decreasing trend thereafter. In the early stage of healing on day 3, the number of α-SMA-positive cells was significantly lower in the laser treatment groups (CO_2 group: 16.65 ± 8.79; Diode group: 3.36 ± 1.56) than in the control group (38.28 ± 10.51). In particular, there was only a small number of α-SMA positive cells in the diode group. On days 5 and 7, the number of α-SMA-positive cells in each group increased at least 2 to 3 times as compared to day 3; however, contrary to that of the control group, the number of positive cells in the laser treatment group was smaller. The number of positive cells was significantly lower in the diode group on day 5 and in the laser treatment groups on day 7. On day 21, there was no significant difference between the control group (41.28 ± 8.28) and the CO_2 group (35.35 ± 4.19); however, in the diode group (24.67 ± 8.25), this number was significantly lesser.

Immunohistological analysis and measurement results of anti TGF-β1-antibodies

The immunohistological stained images of the anti-TGF-β1 antibody are shown in Fig. 3A—L, and the measurement results of the positive areas are shown in Fig. 3M. In the control group, a TGF-β1-positive area was observed throughout the observation period. The positive areas peaked on day 7 and decreased on day 21 (Fig. 3A—D). The CO_2 group showed an increasing trend toward day 21 (Fig. 3E—H). In the diode group, there was an increasing trend on days 5 and 7; however, almost no positive areas were noted on day 21 (Fig. 3I—L). On day 3, positive areas tended to be smaller in the CO_2 group (6.81 ± 2.36%), and these were significantly smaller in the diode group (3.08 ± 1.13%) than those of the control group (9.11 ± 3.30). On day 5, the CO_2 group was 13.39 ± 5.34%, the diode group was 6.43 ± 2.50%, and the control group was 25.84 ± 9.45%. Compared with the control group, the positive areas were approximately 50% lesser in the CO_2 group and approximately 75% lesser in the diode group. The results on day 7 were almost the same as on day 5. On day 21, the positive areas of the control group (19.22 ± 6.53%) and CO_2 group (15.81 ± 3.35%) were similar, while the expression in the diode group (0.58 ± 0.42%) almost disappeared, thus showing a significant difference.

Discussion

Based on previous reports stating that irradiation with CO_2 laser or diode laser suppresses scar tissue formation, this study examined the dynamics of myofibroblast through α-SMA-positive cells as well as the TGF-β1-positive areas involved in myofibroblast differentiation. As a result, the number of α-SMA-positive cells and the TGF-β1-positive areas tended to decrease significantly in the laser treatment groups compared with the control group at all periods after tooth extraction (Figs. 2 and 3).

In clinical practice, Tawfic and Makboul evaluated scar tissue after laser irradiation with a fractional CO_2 laser and reported that the improvement of the scar was caused by decreasing the number of myofibroblast with a significant decrease in TGF-β1 expression.[26,27] It has also been reported that the diode laser demonstrated the same effects.[28] Basic research has reported that only a small number of myofibroblasts appear when CO_2 laser or diode laser is used after creating a wound surface using a scalpel on the back of the tongue and the skin at the back of rats.[22,23] Although laser irradiation is expected to become the first-line treatment for reducing scar tissue formation, there further clarification is needed in establishing evidence-based medicine.[29]

On the basis of this study and our previous reports,[9—11] we proposed the model of socket preservation after tooth extraction in each laser device summarized in Fig. 4. Our previous reports have revealed that active bone remodeling in shallow layer of extraction socket is occurred early after CO_2 laser irradiation that could prevent invasion of the mucosal epithelium (Fig. 4B).[9—11] Moreover, results in this study showed fewer α-SMA-positive cells and TGF-β1-positive areas in the CO_2 group than in the control group. Accordingly, it can be deduced that the reduction of myofibroblast expression by CO_2 laser irradiation reduces the invasion of the mucosa into the extraction socket.

In the diode group, α-SMA-positive cells and TGF-β1-positive areas were significantly lower than in the CO_2 group. In our previous reports, after diode laser irradiation, the bone level of the extraction socket was maintained even without bridging osteogenesis, which was also found in the α-SMA-positive cells during the wound healing process in this study. We believe that this is because the reduced invasion of the mucosa into the extraction socket, which is

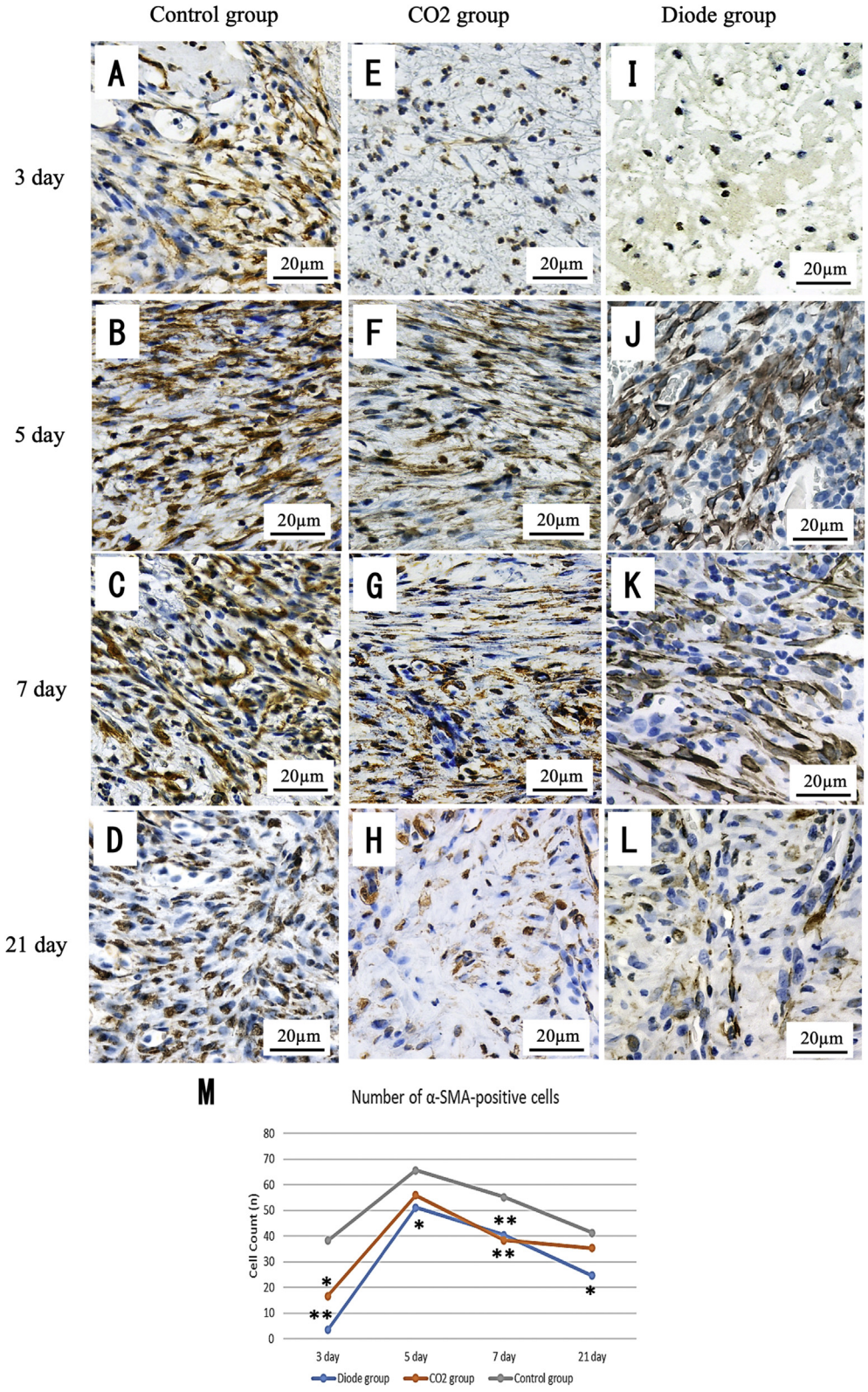

Figure 2 Anti-α-SMA immunostaining of the extraction socket and measurement of α-SMA-positive cells. Original magnification ×400. (A–D) Control group; (E–H) CO_2 group; (I–L) diode group. (M) Measurement of a-SMA-positive cells. The number of α-SMA-positive cells in this area was measured and means were obtained (*$p < 0.01$; **$p < 0.001$, compared to the control group).

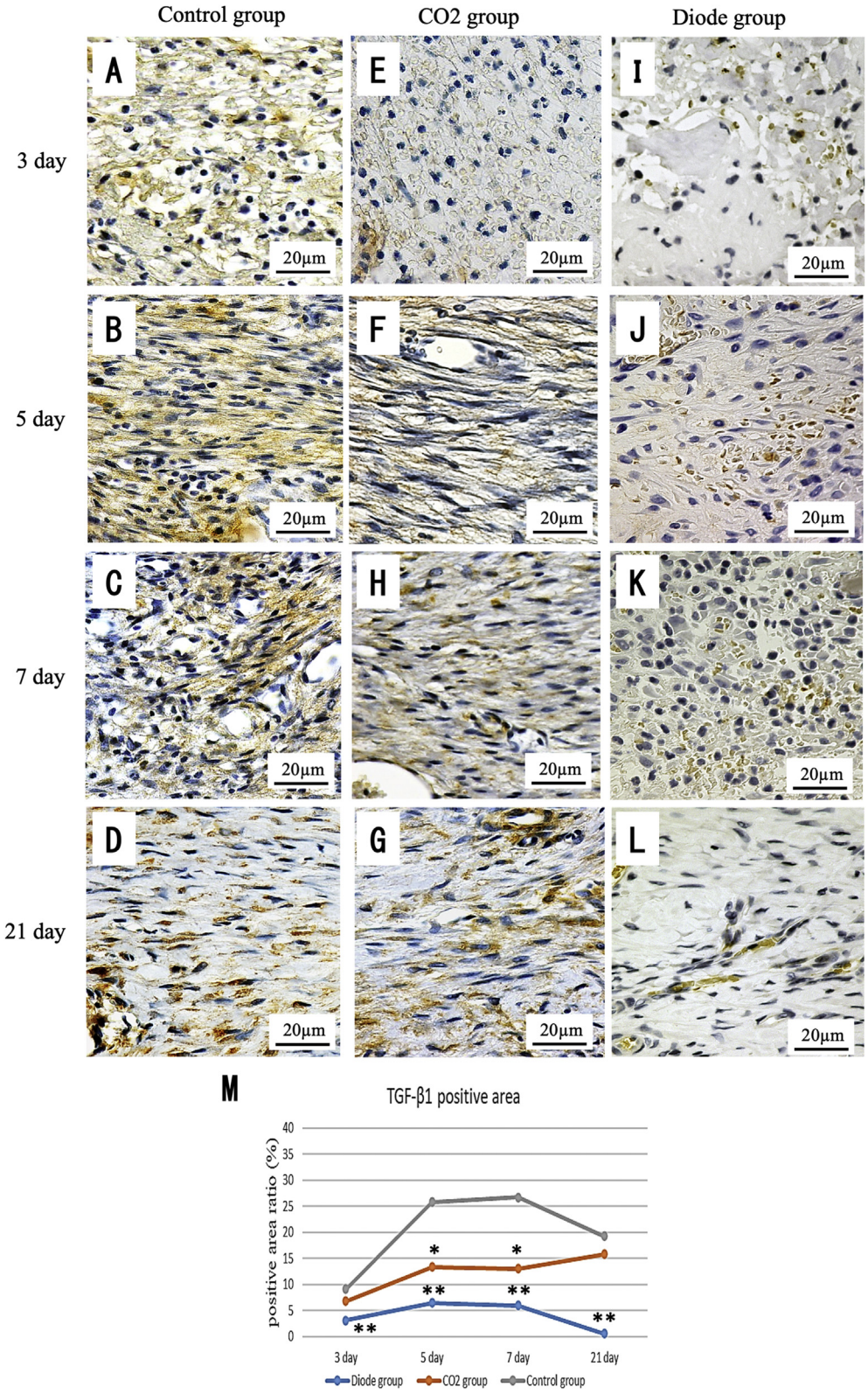

Figure 3 Anti-TGF-β1 immunostaining of the extraction socket and measurement of TGF-β1-positive area. Original magnification ×400. Arrow head indicates TGF-β1 positive area. (A—D) Control group; (E—H) CO2 group (I—L) diode group. TGF-β1-positive area was calculated as the percentage of TGF-β1-positive area per area of the site of measurement (M) (*$p < 0.01$; **$p < 0.001$, compared to the control group).

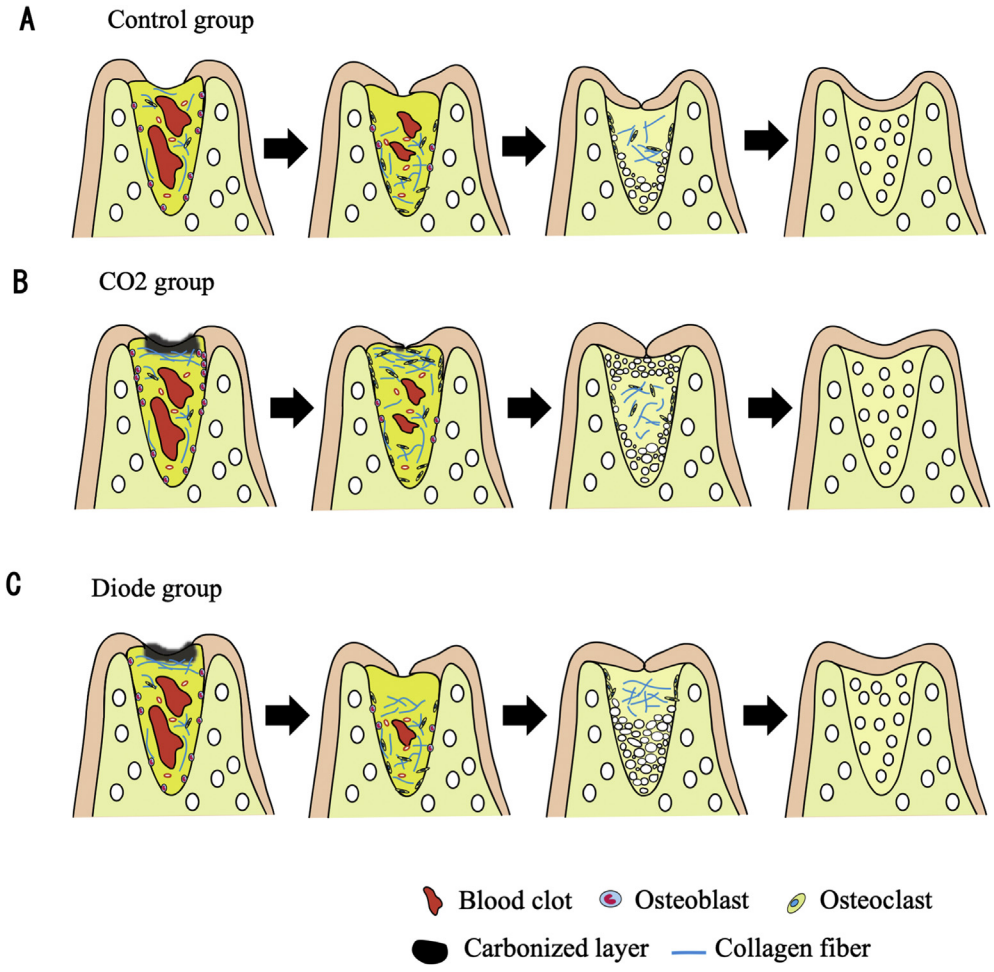

Figure 4 Schematic diagram of the mucosal and alveolar bone healing process after tooth extraction based on this study and previous reports. (A) Control: Fibroblasts in the granulation tissue differentiate into myofibroblast, resulting in scar contraction. This results in the invasion of the mucosal epithelium into the extraction socket, thereby reducing the space for alveolar bone formation, which in turn, reduces the alveolar crest height. (B) CO_2 laser: Myofibroblast expression is suppressed, and mucosal invasion into the extraction socket is reduced. However, myofibroblast is active until the end of healing to close the layer below the epithelium. At the same time, active bone remodeling occurs during the early healing process, and the height of the alveolar crest is maintained by the new formed bone, which contributes to the space-making effect. (C) Diode laser: Myofibroblast expression is greatly suppressed, and mucosal invasion into the extraction socket is reduced. Space-making for bone formation results in alveolar bone neogenesis, which maintains the height of the alveolar crest.

due to an insufficient number of α-SMA-positive cells and TGF-β1-positive areas in the healing process, contributed to space-making for bone formation (Fig. 4C). In addition, in the two types of laser irradiation, coagulation and carbonization of the blood that filled the extraction socket through HILT to form an artificial scab, prevented the invasion of the mucosal epithelium. This finding is consistent with Huebsch's report stating that the location of blood clot formation within the extraction socket affects the height of future new bone formation.[30]

However, there remains careful point for operating laser. We need to insert laser tip into extraction socket for diode laser irradiation. When laser tip contacted carelessly alveolar bone might be invaded by excessive heat with wrong procedure. From that point of view, CO_2 laser is a safer device because that doesn't demand inserting laser tip into extraction socket. On the other hand, wavelength of diode laser is shorter than CO_2 laser that could reach deeper and wider area of irradiated tissue. That would be beneficial in some clinical case. Nevertheless, both of laser devices used in this study are recommended by FDA and defined as safe one that doesn't require any complicated skills for operating.

It was hence suggested that the suppression of scar contraction of the mucosal epithelium of the tooth extraction wound by CO_2 laser or diode laser irradiation contributes to the space-making effect for new bone formation in the extraction socket, thereby leading to subsequent socket preservation.

Declaration of competing interest

The authors declare no conflict of interest in this study.

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