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Effects of Irradiation by Carbon Dioxide Laser Equipped With a Water Spray Function on Bone Formation in Rat Tibiae

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Abstract. *Background/Aim: Irradiation of tissue with carbon dioxide (CO_2) laser shows a characteristic thermal effect that causes vaporization of tissue in the target region. However, the thermal effect in places other than the target region induces tissue damage. Two methods are used: high reactive-level laser therapy (HLLT), aimed at surgical treatment, and low reactive-level laser therapy (LLLT), aimed at cell and tissue activation. In both, vaporization of tissue is induced by thermal damage. A water spray function may ameliorate thermal damage from CO_2 laser irradiation. In this study, we irradiated CO_2 laser on rat tibiae with or without a water spray function and examined the effects of this technique on bone metabolism. Materials and Methods: Bone defects were created in rat tibiae by dental bur in a Bur group and by laser in laser irradiation groups with (Spray group) and without (Air group) water spray function. At 1 week postoperatively, histological analyses of tibiae were performed using hematoxylin and eosin staining, immunohistochemical staining (IHC) with anti-sclerostin antibody, and 3-dimensional (3D) observation using micro-computed tomography. Results: Histological findings and 3D observation confirmed induction of new bone formation following laser irradiation in both the Air and Spray groups. No bone formation was seen in the Bur group. IHC revealed that the activity of osteocytes in the region of irradiated cortical bone was markedly impaired in the Air group, but osteocyte impairment was ameliorated in the Spray group and absent in the Bur group. Conclusion: The water spray function appears effective in reducing thermal damage to tissues irradiated by CO_2 laser. CO_2 lasers with water spray function may be useful in bone regeneration therapy.*

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Key Words: CO_2 laser, water spray function, LLLT, sclerostin, bone formation.

A CO_2 laser reduces the amount of bacteria and other pathogens in the surgical field and laser effects also result in reduced postoperative discomfort. These are the reasons why lasers have been widely used in surgical treatments (1). Laser irradiation causes two types of reactions in tissues, depending on the level of energy irradiated (2, 3): Tissues that receive high-energy irradiation undergo vaporization and formation of a carbonized layer, and this method of high reactive-level laser therapy (HLLT) is usually applied to cut and remove tissues in place of a scalpel. Conversely, tissue exposed to low-energy irradiation becomes activated and shows increase in cell proliferation, differentiation and dilation of blood vessels. This laser use is called low reactive-level laser therapy (LLLT) and is applied for accelerating tissue healing and in regenerative therapy (4-9). Generally, an LLLT layer is present around the HLLT layer in irradiated tissues (2). Given the presence of such contradictory effects from lasers, protocols need to be developed for irradiation methods that can specifically and effectively apply LLLT from lasers to tissue regeneration therapy.

Lasers can be divided into various types depending on the wavelength and power source. Carbon dioxide (CO_2) laser (wavelength, 10.6 μm) is a gas laser that is well absorbed by water and tissue surfaces (1). For these reasons, CO_2 lasers have been widely used in both medical and dental fields. Particularly

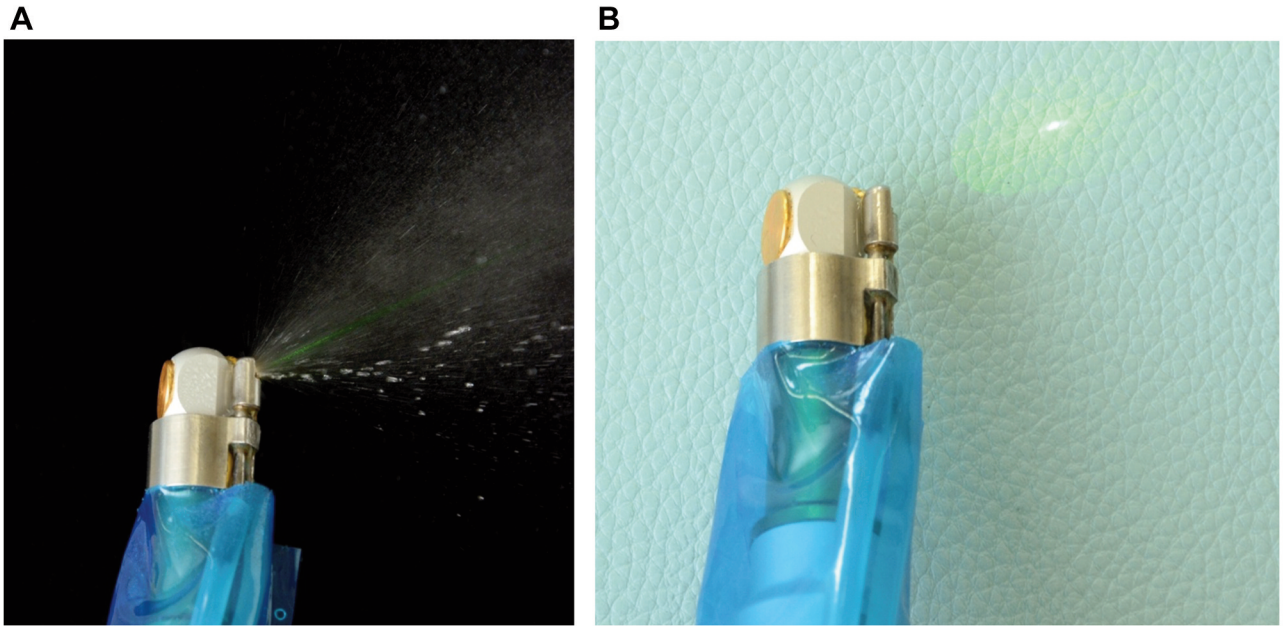

Figure 1. *Laser device with water spray function. The water spray is seen emanating from the apex of the device (A). Laser device without water spray (B).*

in dental treatments, HLLT has been used in surgical treatments (1) of the gingiva (10) and for dental implants (11). We recently demonstrated that LLLT can induce bone formation and proposed the use of CO_2 lasers for bone regeneration therapy as a new method of laser-induced bone therapy (3).

The CO_2 laser beam is converged by the lens in the hand piece for irradiation of tissues (1). A key feature of CO_2 lasers is vaporization of target tissues by thermal damage; formation of a carbonized layer is unavoidable with the use of CO_2 lasers. This thermal activity can be either beneficial or damaging to tissues surrounding the target region. To develop the methods of CO_2 laser irradiation for tissue regeneration, unnecessary thermal activity needs to be suppressed. The present study investigated a CO_2 laser hand piece equipped with a water spray function, and irradiated the surfaces of rat tibiae with or without concomitant water spray. The aim of this study was to develop a CO_2 laser irradiation method for tissue regeneration, particularly of bone tissue. We undertook morphological analyses of bone tissue irradiated by CO_2 laser, and compared tissue reactions and damage to the tibia with and without the water spray function, to confirm the utility of this function on bone regeneration after CO_2 laser irradiation.

Materials and Methods

Experimental design. Experiments were carried out with the approval of the animal Ethics Committee of the Meikai University (approval no. A2130). Fifteen ten-week-old, female Sprague-Dawley (SD) rats were used in the experiment. Three experimental groups were created. In the Bur group, the tibial surface was scratched for a length of about 1 cm with a dental bur to a depth of approximately 0.2 mm. In the Air and Spray groups, tibiae were irradiated with a CO_2 laser (power, 0.5 J) and the laser spot was moved at a speed of about 1 cm/s on the tibial surface for 1 s, either using the water spray function with physiological saline solution (Spray group) or with air cooling alone (Air group). Total energy irradiated to bone tissue was 238.8 J/cm^2, based on the methods of Naka and Yokose (3). Each group comprised 5 animals, with one tibia scratched or irradiated in each animal. The CO_2 laser equipment used in the study was provided by Yoshida Co. (Neos, Tokyo, Japan). The operation was performed under nasal inhalation anesthesia, maintaining the concentration of isoflurane at 2.0% (1.0 l/min). Seven days postoperatively, the tibiae were removed and analyzed.

Histochemical staining. Tibiae were fixed in 10% neutral formalin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for 48 h and decalcified in 0.5 mol/L EDTA (pH 7.5; Fujifilm Wako Pure Chemical Corporation) for 14 days at 4˚C. After decalcification, specimens were dehydrated in ethanol series and embedded in paraffin for serial sections. After deparaffinizing, some sections were stained with hematoxylin and eosin (HE) and others were used for immunohistochemical staining with anti-sclerostin antibody (PAC864Ra01; Cloud-Clone Corp, TX, USA) for 2 h at room temperature. Negative control sections were stained with non-immune serum instead of primary antibody. After reacting with the primary antibody, sections were washed with Tris-buffered saline (Takara, Kyoto, Japan). Sections were reacted with biotinylated goat immunoglobulin G and avidin peroxidase (Histofine kit; Nichirei,

Tokyo, Japan). Finally, sections were reacted with DAB solution (Histofine DAB kit; Nichirei) according to the instructions from the manufacturer.

Micro-computed tomography (micro-CT) and 3-dimensional (3D) analysis. Micro-CT was performed using ScanXmate-RX (ComscanTecno, Kanagawa, Japan). The scanner was set at a voltage of 27 kV and a current of 250 mA.

Reconstruction of 3D images was performed using Thermo Scientific Amira software (Thermo Fisher Scientific, Waltham, MA, USA). The sample area selected for scanning was a 5-mm length of the tibial diaphysis, originating 3 mm above the junction of the tibia and fibula and extending to the proximal metaphysis. Regions of interest were defined by drawing newly formed bone tissue connected to the inner surface of the cortical bone directly under each region treated with the dental bur or by laser irradiation.

Results

Figure 1 shows the laser device equipped with the water spray function. Saline spray was released from the apex of the laser device.

Figure 2 shows the surface of tibiae from the Bur group (A), Air group (B), and Spray group (C). A groove is apparent in the tibial surface of the Bur group (A) and a carbonized layer is seen in the surface of tibiae from the Air (B) and Spray (C) groups. However, the carbonized layer was much more prominent in the Air group than in the Spray group.

Figure 3 shows HE-stained sections from the Bur group (A and D), Air group (B and E), and Spray group (C and F). Newly formed bone tissue (asterisks in E and F) was recognized right below the laser-irradiated region, with no differences in the histological structure of newly formed bone tissue between the Air and Spray groups. No new bone formation was seen in the Bur group. The carbonized layer was seen on the surface of cortical bone irradiated with the laser. The carbonized layer was thicker in the Air group (B and E) than in the Spray group (C and F).

Figure 4 shows immunohistochemical staining for anti-sclerostin antibody in rat tibiae from the Bur (A and D), Air (B and E), and Spray (C and F) groups. Upper and lower panels show low and high magnifications, respectively. Osteocytes expressing sclerostin (arrows in D) could been seen entirely within cortical bone, with no reaction to antibody in the lacunae of osteocytes in cortical bone in the Air group (E). Conversely, osteocytes located in the deep layer close to the bone marrow space expressed sclerostin (arrows in F), but expression was barely seen in osteocyte lacunae in the vicinity of the irradiated cortical bone surface in the Spray group. No specific reactions were seen in negative control sections (data not shown).

Figure 5 shows the 3D structure of newly formed bone tissue (blue color) on the surface of cortical bone on the bone marrow side. Bone formation induced by laser irradiation could be recognized in both the Air and Spray groups, but not in the Bur group according to HE staining.

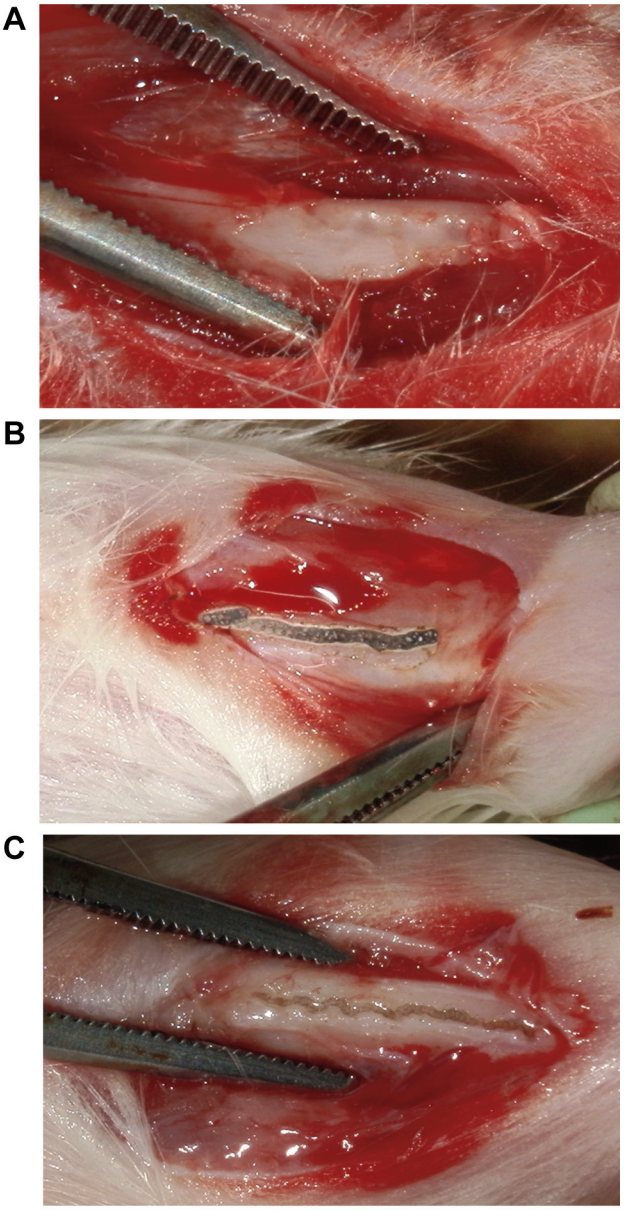

Figure 2. *Tibial surface in the Bur group (A), Air group (B), and Spray group (C). A scratched groove is seen on the tibial surface in the Bur group (A), and carbonized layers are seen on the tibial surface in both the Air (B) and Spray (C) groups.*

Discussion

Bone tissue shows splendid adaptability to mechanical stress, as revealed by Wollf's law (12). Our prior results on the effects of CO_2 laser irradiation on sclerostin expression in osteocytes showed that the effect of CO_2 laser irradiation on bone formation was the same as that of mechanical stress (13). CO_2 laser irradiation has been confirmed to induce bone formation,

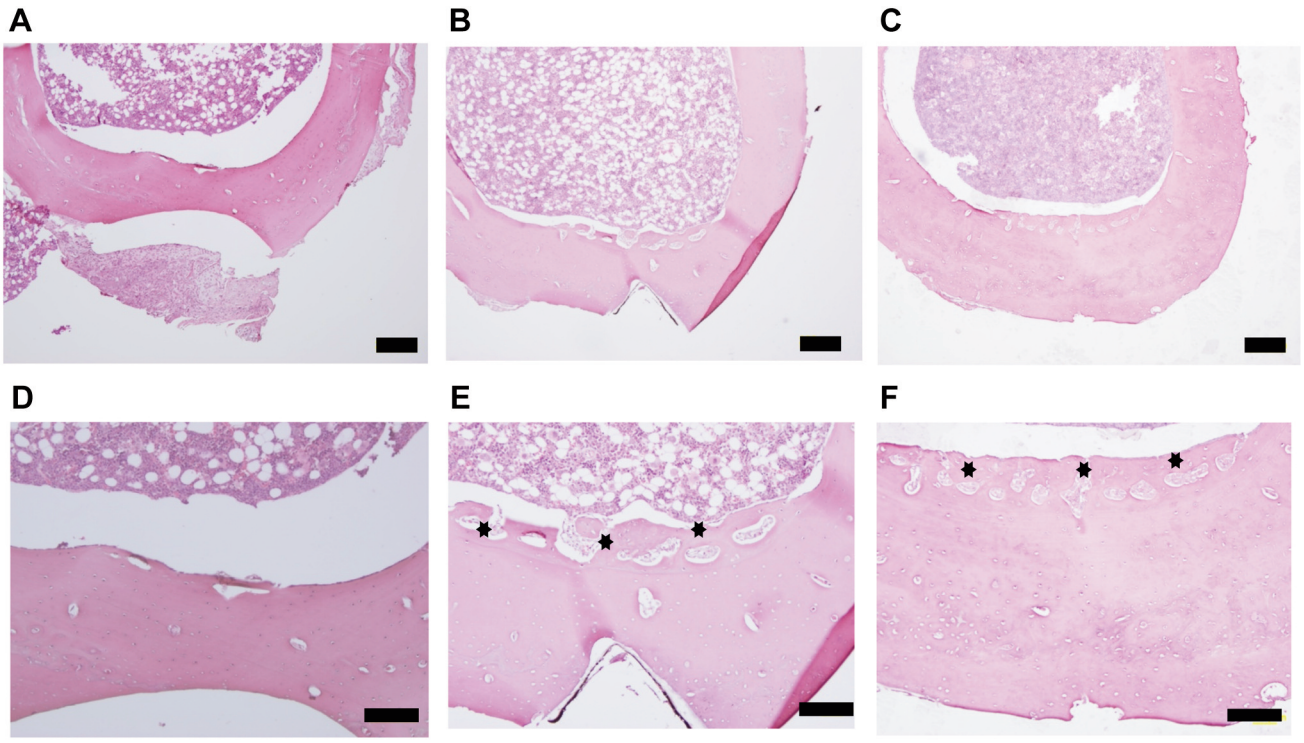

Figure 3. *Hematoxylin and eosin (H&E) stained sections from the Bur group (A and D), Air group (B and E), and Spray group (C and F). Newly formed bone tissue (asterisks) is recognized right below the laser-irradiated regions (B, C, E and F). Bars: A-C, 200 μm; D-F, 100 μm.*

whereas damage caused by a dental bur did not induce bone formation despite the bone surface showing the same bone defects as that irradiated with CO_2 laser. This indicates that bone defects caused by dental bur or laser irradiation have different properties in terms of bone metabolism. LLLT by CO_2 laser irradiation may offer advantages that are applicable to regeneration therapy for bone tissue.

Irradiation with CO_2 laser shows a characteristic thermal effect that causes vaporization of tissue in the target region (1), but the thermal effect in places other than the target region induces tissue damage. The water spray function may be useful to compensate for that flaw. As CO_2 laser wave length is well known to be absorbed by water (1), we expected that this would represent an obstacle to cooling systems for CO_2 lasers. However, the present results demonstrated that the laser beam could reach the bone surface and vaporize the bone matrix with a small carbonized layer. This indicates that the cooling system could be effective for cooling tissue irradiated with CO_2 laser without disturbing the original nature of the laser. In fact, histological examinations in this study revealed that osteocyte lacunae located across the entire area of laser-irradiated cortical bone were empty, but osteocytes in the deep region of irradiated cortical bone remained alive and showed sclerostin expression in the Spray group. These results confirm that the water spray function protected not only histological structures, but also cellular activity from the thermal damage caused by laser irradiation. To the best of our knowledge, this is the first report to show that CO_2 laser irradiation with water spray function can induce new bone formation.

In the present study, CO_2 laser irradiation in both the Air and Spray groups induced bone formation as LLLT in cortical bone, as revealed by histological features and 3D analysis. We have already reported that CO_2 laser irradiation with air cooling could induce bone formation in the bone marrow cavity (3). Several studies have reported similar results (14, 15). Importantly, new bone formation was recognized in tibiae from both the Air and Spray groups, but was more prominent in the Spray group. This indicates that CO_2 laser irradiation with the water spray function reduced thermal damage to tissue and induced new bone formation. Such findings suggest that LLLT with CO_2 laser combined with water spray function may provide a powerful tool for bone regeneration therapy. In addition, the results provide evidence supporting for the clinical application of the CO_2 laser in bone regeneration therapy (16, 17).

Sclerostin is a secreted glycoprotein involved in bone metabolism. Sclerostin binds to the Wnt co-receptor Lrp5/6 to

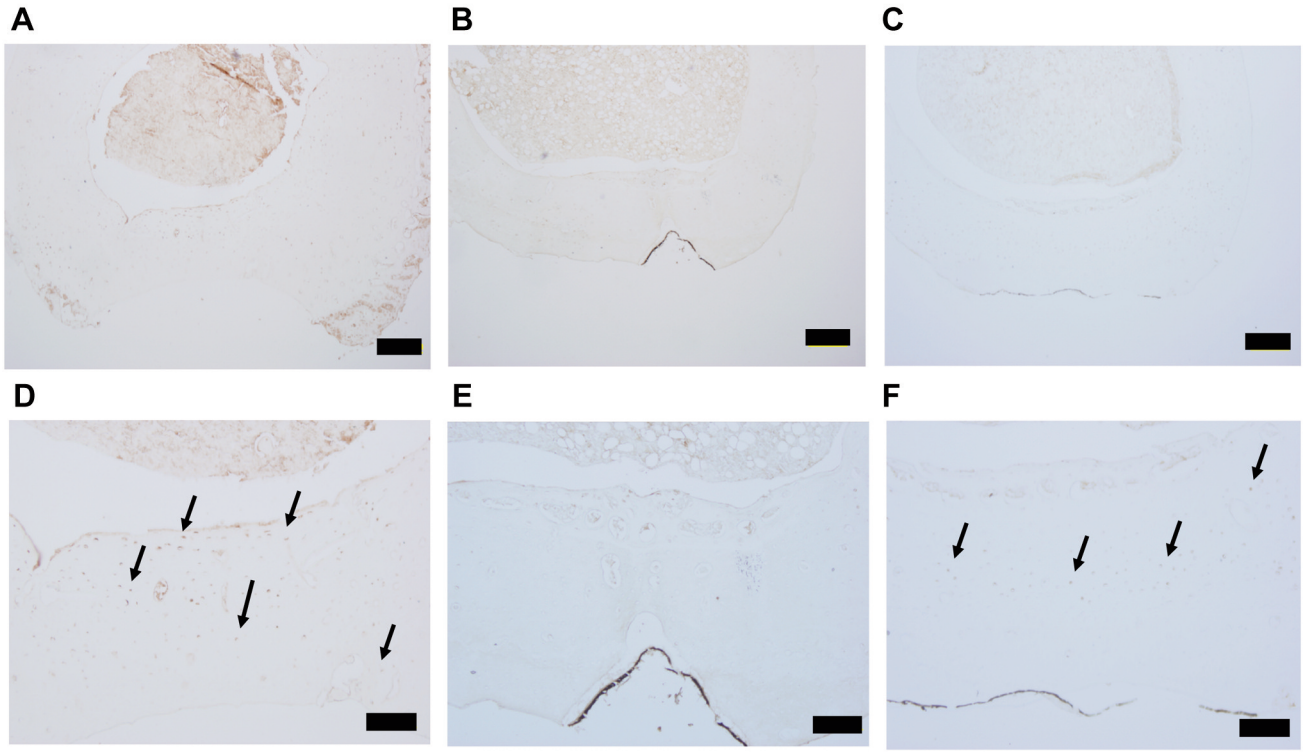

Figure 4. *Immunohistochemical staining of sections from the Bur group (A and D), Air group (B and E), and Spray group (C and F). Bars: A-C, 200 μm; D-F, 100 μm. Arrows indicate osteocytes expressing sclerostin.*

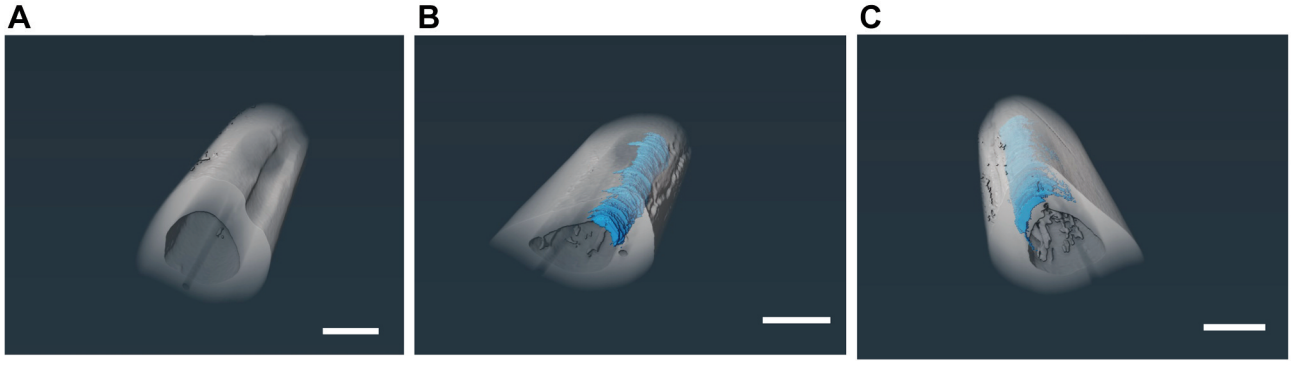

Figure 5. *Three-dimensional analysis of rat tibiae in the Bur group (A), Air group (B), and Spray group (C). Newly formed bone tissue is shown by blue color in tibiae. Bars: A-C, 1 mm.*

inhibit binding to Wnt molecules (18). This glycoprotein is secreted from osteocytes and has inhibitory effects on bone formation (19, 20) by inhibiting the effect of the Wnt canonical pathway. Osteocytes expressing sclerostin thus inhibit new bone formation, representing one reason why no new bone formation was seen in the marrow space of cortical bone in the Bur group. Osteocytes in cortical bone damaged by the dental bur were actively expressing sclerostin. On the other hand, expression of sclerostin in osteocytes in cortical bone were completely suppressed or drastically reduced by CO_2 laser irradiation. This phenomenon is considered to represent a mechanism of bone formation induced by laser irradiation. In fact, Tamplen *et al*. (21) reported that the inhibition of sclerostin induced bone formation in studies using anti-sclerostin antibody.

However, other factors seem to be involved in the induction of bone formation by laser irradiation. No differences in

histological features of new bone formed after laser irradiation were seen between the Air and Spray groups, even though sclerostin expression patterns differed between the Air and Spray groups. Further investigation is needed to clarify these issues.

In conclusion, the present results suggest that a CO_2 laser equipped with a water spray function may be beneficial for reducing thermal damage and could contribute to the development of bone regeneration therapies.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Author's Contributions

Y.I. and S.Y. performed all experiments and wrote the article. Y.K., and A.H. supported the animal experiments. R.I. and N.U. supported micro-CT analysis. U.K. and S.Y. edited and revised the article. S.Y. is corresponding author.

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