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Original article

Bone Regeneration in Cranial Bone of Hamster By Short Pulse CO_2 Laser

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Abstract: Purpose: The purpose of this study is to evaluate bone augmentation by short-pulse CO_2 laser irradiation to the cranial bone of hamster using histological and immunohistochemical analyses. Materials and Methods: Sixty-four 10-week-old male Syrian Golden Hamsters were the animals used. After the cranial bone was exposed under local and general anesthesia, laser was irradiated directly to the cranial bone under various conditions. The hamsters were sacrificed after 1, 2, 3 and 4 weeks, and new bone formation was evaluated immuno-histologically. Osteocalcin positive cells in each area were calculated to determine the percentages. Results: Group D showed a more significant difference in osteocalcin-positive cell ratio than group A postoperatively at 1, 2 and 3 weeks. However, there were no significant differences at 4 weeks. Group D showed a significant difference in new bone area ratio more than group A postoperatively at 2, 3 and 4 weeks. However, there were no significant differences at 1 week. Conclusion: This study suggested that high output was a more suitable condition than low output for promoting bone augmentation.

Key Words: short-pulse CO_2 laser, Osteocalcin, Bone augmentation, Syrian Golden Hamsters

INTRODUCTION

The placement of a dental implant in the maxillary molar region is difficult when there is a shortage of bone mass. In such cases, bone augmentation of the maxillary sinus floor is required. Since the introduction of maxillary sinus floor elevation by Taum and Boyne[1,2], the use of autogenous bone grafts in sinus augmentation has been considered to be the "gold standard" because of these grafts' excellent survival with loaded implants and the degree of functionality they confer[3–6]. Other graft materials, such as allograft, xenograft, and synthetic materials, have been explored. However, harvesting autogenous bone is generally associated with several complications, including invasion of the host side, pain, and blood loss. Furthermore, allografts and xenografts are susceptible to immunorejection and carry a risk of disease transmission[7–9]. Furthermore, some synthetic materials have limited potential for osteoconduction, and using them alone has usually failed to achieve the bone volume expected[10,11].

Laser-induced bone therapy (LIBT) was

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recently introduced as a method for accelerating the formation of new bone and is now being used in dental surgeries[12]. The use of lasers is now common practice in medical and dental treatments. The clinical applications of lasers are largely divided into low-reactive-level laser therapy (LLLT) and high-reactive-level laser therapy (HLLT). LLLT provides photobiological and photochemical effects. Pretel *et al.*[13] studied the effects of LLLT on bone repair in rats through defects created in the mandible. They observed an advanced tissue response in the animals treated by LLLT compared to the non-treated controls and attributed this result to the modulation of the initial inflammatory response, promoting new bone matrix formation.

Reports of HLLT have been increasing in recent years[12,14,15]. However, HLLT-induced thermal damage results in extensive cell-mediated resorption of bone or sequestration of dead bone,16 thus severely limiting the use of HLLT on bone. To improve the damage induced by the heat generated in HLLT, a new laser device has been developed at the Faculty of Engineering at the University of Yamanashi. Like HLLT, this new technique involves a high level of reactivity, but it induces no thermal damage on target, as the generation of heat is suppressed by shortening the wavelength of the laser.

In the present study, we examined whether or not bone regeneration is possible under conditions of a high output to achieve carbonization and whether or not the bone regeneration amount increases with an increasing output.

MATERIALS and METHODS

The experimental protocol was approved by the Institutional Committee for Animal Care, University of Yamanashi, in accordance with the principles of the ARRIVE guidelines (Approval number: A-25-6).

Experimental animals

Sixty-four male Syrian Golden hamsters (10 weeks old, weighing an average of 80 g) were fed regular standard hamster food and water and housed one animal per cage in a room.

Laser system

In this experiment, a longitudinal excited CO_2 laser that produces a short laser pulse with a circular beam and a low divergence angle (i.e. non-thermal damage) was used[17–19]. The laser pulse had a spike pulse width of 185 ns, a pulse tail length of 87.1 μs, a spike pulse energy of 2.1 mJ, and a total pulse energy of 87.4 mJ. The laser beam was focused using a ZnSe lens with a focal length of 2.5 inches and maintained a 0 mm delivery tip-to-target surface distance. The spot diameter was 4 mm on the target surface. Therefore, the fluence was 0.7 J/cm^2 in a single shot (Fig. 1).

Surgical procedure

The whole procedure was performed under sterile conditions. First, the animals were anesthetized with sodium pentobarbital (25 mg/kg) injected into the abdominal cavity. The hair on the nasal bone was then shaved. Next, 1.8 ml of 2% lidocaine containing 1:80,000 epinephrine was administered into the operating site. Both the nasal bone and nasoincisinal suture lines were exposed via a perpendicular incision. The musculature and periosteum were reflected, exposing the cranial bone. The cranial bone was irradiated directly under each condition (Fig. 2). In this study, we did not intentionally make a bone defect for the purpose of observing bone augmentation. Small pieces of mechanical pencil lead were implanted beside the defects as

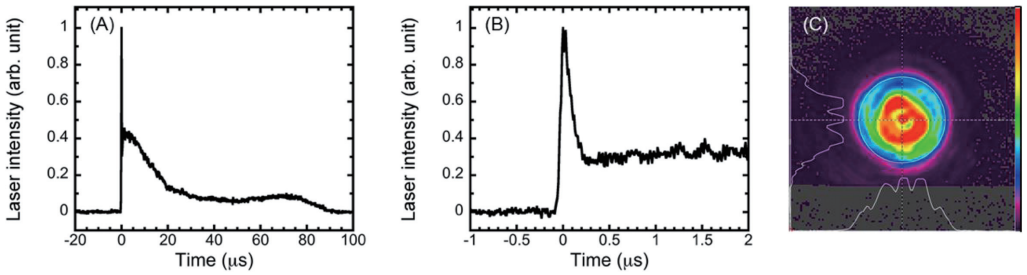

Fig. 1. (A) Overall laser pulse waveform. (B) Magnified time-scale view of spike pulse. (C) Laser beam profile.

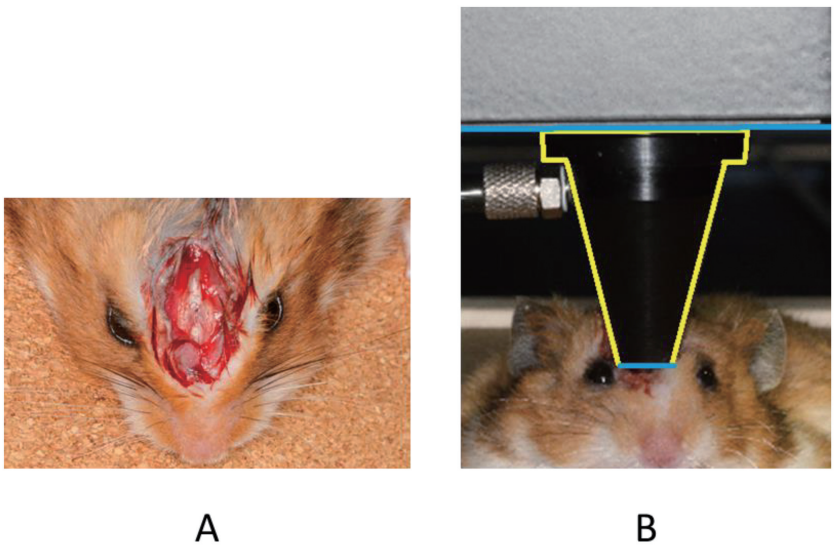

Fig. 2. Intra-operative finding. (A) Exposing the skull. (B) Application of LIBT at the skull.

markers to later distinguish the irradiated area from the periphery bone. The animals were classified into 4 groups according to the dose of irradiation: Group A: 88.2 J/cm^2 (N=16), Group B: 220.5 J/cm^2 (N=16), Group C: 441 J/cm^2 (N=16), and Group D: 661.5 J/cm^2 (N=16). Soft tissue and skin incisions were closed with 4-0 silk-interrupted sutures. Four animals in each group were sacrificed at 1, 2, 3 and 4 weeks postoperative. In addition, a control group was established in which the cranial bone was exposed and closed with no irradiation.

Immunohistochemical examinations

The specimens were fixed in 10% buffered formaldehyde overnight at 4°C and demineralized with 14% EDTA for 4 weeks. The specimens were dehydrated with a graded series of ethanol washes, cleared with xylene, and embedded in paraffin. Multiple 5-μm-thick sections were cut parallel to the coronal plane of the head at the center region and mounted on gelatin-coated glass slides. First, the prepared sections were stained with hematoxylin and eosin (HE). The new bone area ratio was measured with an

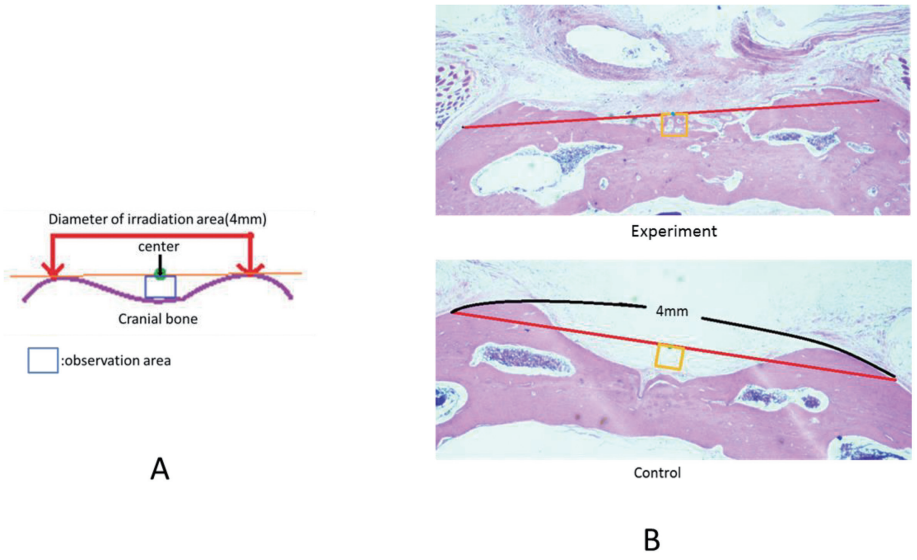

Fig. 3. (A) Schematic drawing showing the area of observation. The area below the center of the irradiation area of the control group was compared with the new bone area in the observation area, which was 0.25 mm square in the diameter of the irradiation area. (B) The concave of control is natural anatomy. Measurement of the photomacrograph stained with hematoxylin-eosin.

imaging software program (Image J; the Research Services Branch, National Institute of Mental Health, Bethesda, ML, USA). The measurement was performed five times by an author (A.K.) to confirm the reproducibility of the scores, and the mean value was used as the result.

The sections were treated successively with 0.3% Tween 20 (Tokyo Chemical Industry, Co., Ltd., Tokyo, Japan) in phosphate-buffered saline (PBS) for 1 h for cell permeabilization, and then with 0.3% hydrogen peroxide in methanol for 10 min to inhibit intrinsic peroxide activity. For immunohistochemical staining of osteocalcin (OCN) synthesis, paraffin-embedded tissue sections were prepared for antigen retrieval by enzymatic treatment. Following enzymatic antigen retrieval, the sections were treated with anti-OC produced in mouse (Monoclonal Anti-Rat Osteocalcin: Clone D-8G; Takara BIO M185). The sections were incubated with primary antibodies overnight at 4 °C. For OCN staining, DAB Kit (TaKaRa DAB Substrate [MK210]) was used. For immunofluorescence staining of OCN the approaches outlined above were applied except for visualization; secondary antibodies conjugated to One-step Polymer-HRP (BioGenex, Hk595-50K) were used.

The sections counterstained with hematoxylin were observed under a Nikon ECLIPSE Ci microscope (Nikon, Tokyo, Japan). The sections were then dehydrated in alcohol and mounted for light microscopy to count the number of positively stained active cells in the regeneration site. The area of observation was set as the center region of the irradiated area. The area below the center of the irradiation area of the control group was compared with the new bone area in the observation area, which was a 0.25-mm square in the diameter of the irradiation area (Fig. 3). The number of OCN-stained

cells per voluntary 1000 cells in this area was counted manually using a high-magnification photomicrograph (×400). The measurements were performed five times by one author (A. K.) to confirm the reproducibility of the scores, and the mean value was used as the result.

Statistical analyses

Data of all the measurement values were analyzed statistically with the Dr. SPSSII software program (SPSS Japan Inc., Tokyo, Japan). Time-dependent changes were examined by a repeated measure analysis of variance (ANOVA) after testing for the assumption of normality in each group. Differences between groups were analyzed by a non-paired comparison using Bonferroni's test. Differences were considered significant at $P < 0.05$.

Results

Healing progressed uneventfully in all the hamsters, and no postoperative complications were noted during the four-week observation period. After resting for 3.5 h postoperatively, the animals were able to move and leap without any notable pain or limitations.

Histological examinations

After one week, groups A and B showed little formation of fibrous tissue, including osteoblasts, but significant formation was noted in groups C and D (Fig. 4).

After two weeks, group A showed little

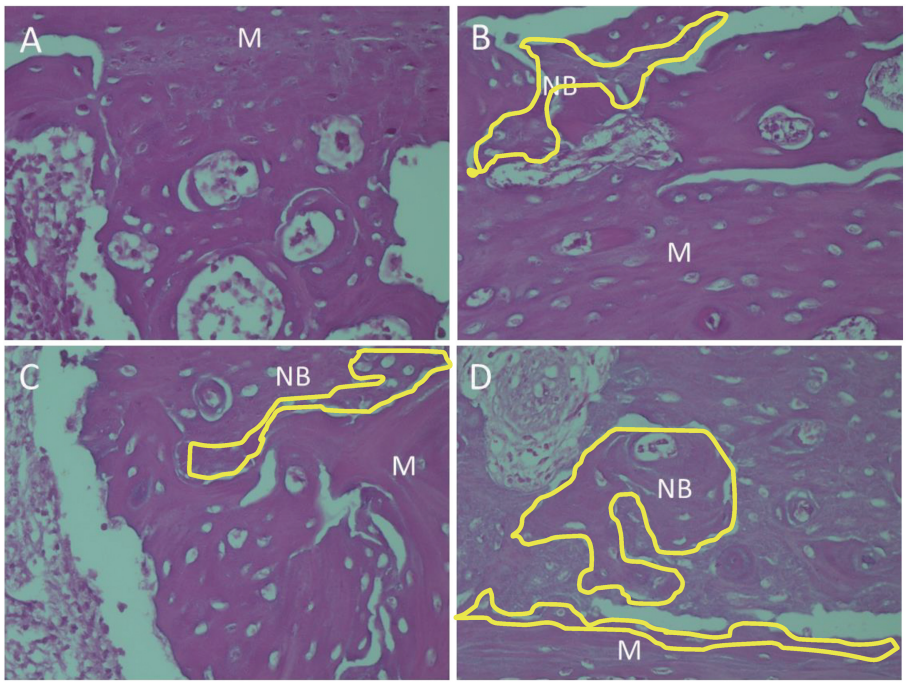

Fig. 4. Photomicrographs after 1 week. (A) Group A: 88.2 J / cm^2, (B) Group B: 220.5 J / cm^2, (C) Group C: 441 J / cm^2, (D) Group D: 661.5 J / cm^2 , (hematoxylin-eosin staining, original magnification ×400), M: mother bone, NB(Range surrounded by yellow line): new bone.

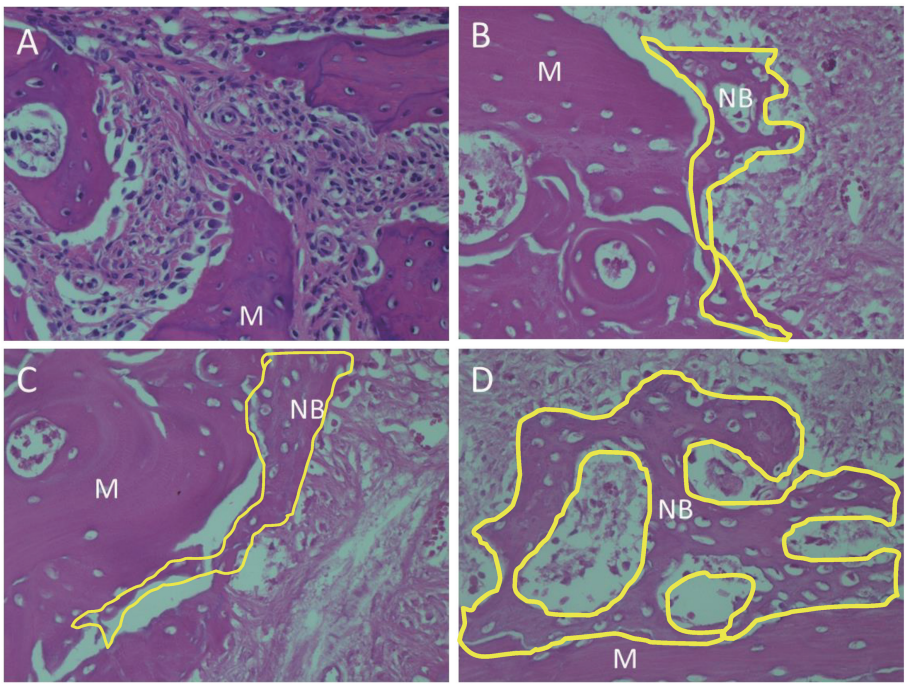

Fig. 5. Photomicrographs after 2 weeks. (A) Group A: 88.2 J / cm^2, (B) Group B: 220.5 J / cm^2, (C) Group C: 441 J / cm^2, (D) Group D: 661.5 J / cm^2, (hematoxylin-eosin staining, original magnification ×400), M: mother bone, NB(Range surrounded by yellow line): new bone.

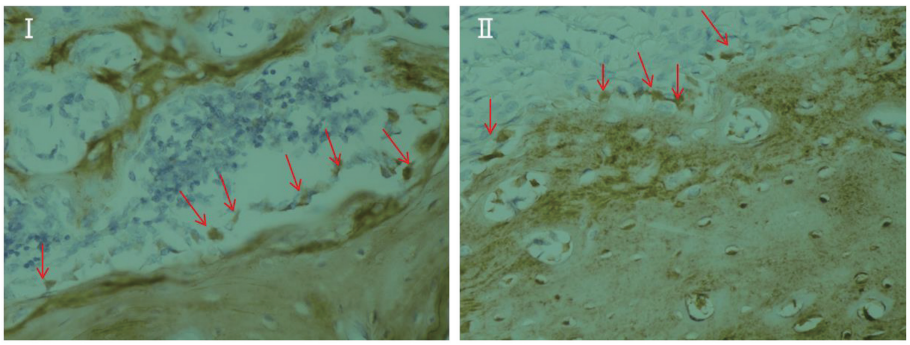

Fig. 6. Photomicrographs after 2 week. (I) Group D (immunohistochemical staining, original magnification ×400), (II) Group C (immunohistochemical staining, original magnification ×400). Red arrows show osteocalcin stained cells.

formation of fibrous tissue, including osteoblasts, just as after one week. However, groups B, C, and D showed extensive new bone formation compared to that seen after one week. Among the groups, the amount of regenerated bone in group D increased most sharply compared with that after one week (Figs. 5, 6).

After three weeks, group A still showed little

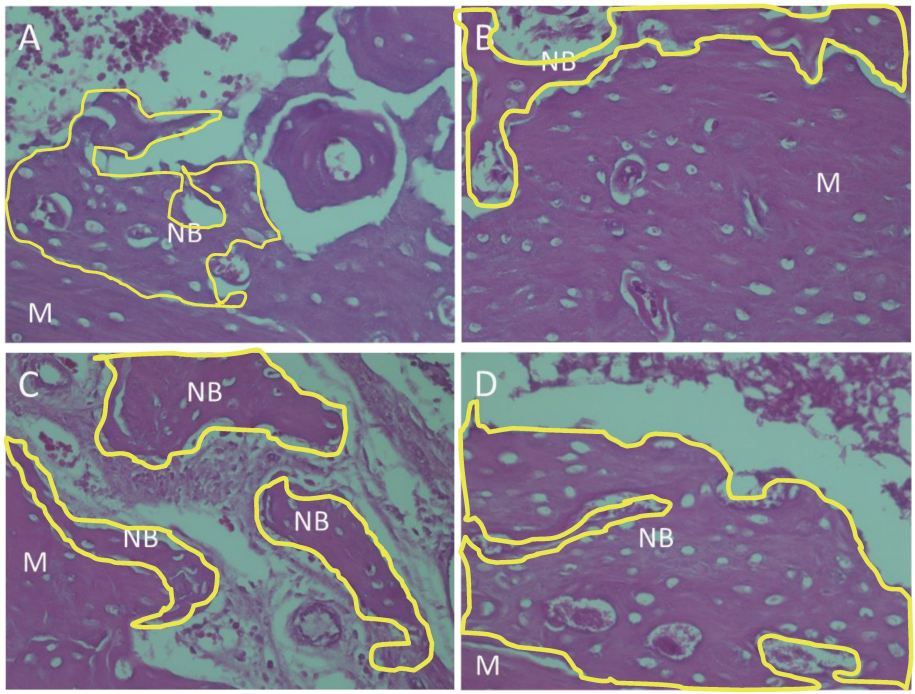

Fig. 7. Photomicrographs after 3 weeks. (A) Group A: 88.2 J / cm^2, (B) Group B: 220.5 J / cm^2, (C) Group C: 441 J / cm^2, (D) Group D: 661.5 J / cm^2, (hematoxylin-eosin staining, original magnification ×400), M: mother bone, NB(Range surrounded by yellow line): new bone.

formation of fibrous tissue, including osteoblasts, just as after one and two weeks. Group B showed bone augmentation over time just as after two weeks, and the difference in the bone augmentation between groups B and C had almost been completely eliminated. In group D, significant bone augmentation as well as an increased density were observed (Fig. 7).

After four weeks, group A still showed little formation of fibrous tissue, including osteoblasts, just as after one, two, and three weeks. Group B showed bone augmentation over time, but there was no marked difference from that noted after three weeks, and the difference in the bone augmentation between groups B and C had almost been completely eliminated. In group D, significant bone augmentation as well as an increased density were observed (Fig. 8).

Bone area ratio

With regard to the changes in the bone area ratio over time, there were significant differences between and within subjects (between subjects: $F = 10.797$, df = 3, $P = 0.0001$; within subjects: $F = 2.258$, df = 3, $P = 0.0983$). There were no significant differences among the groups after one week. Group D showed a significantly larger bone area ratio than group A after 2 weeks ($P = 0.0068$), 3 weeks ($P = 0.0052$), and 4 weeks ($P = 0.0031$). However, there were no significant differences among the other groups after 2, 3, or 4 weeks (Fig. 9).

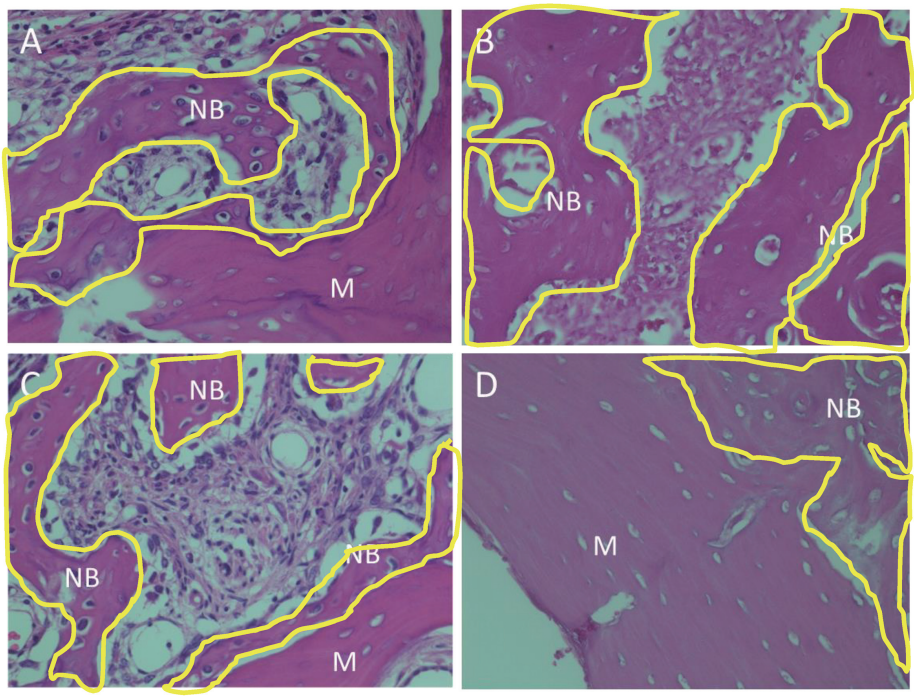

Fig. 8. Photomicrographs after 4 weeks. (A) Group A: 88.2 J / cm^2, (B) Group B: 220.5 J / cm^2, (C) Group C: 441 J / cm^2, (D) Group D: 661.5 J / cm^2, (hematoxylin-eosin staining, original magnification ×400), M: mother bone, NB(Range surrounded by yellow line): new bone.

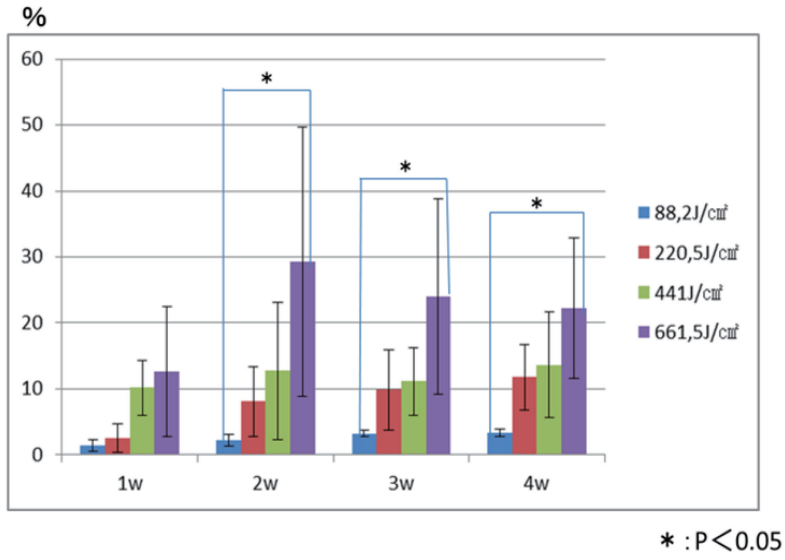

Fig. 9. The ratio of new bone area. The time-course of changes in all the groups showed significant differences with ANOVA (P=0.0001). *Significant difference; P<0.05.

Number of OCN-stained cells

A repeated measure ANOVA revealed statistically significant differences in the time-course changes (between subjects; $F = 10.032$, df = 3, $P = 0.0014$; within subjects; $F = 69.403$, df = 3, $P < 0.0001$). After 1 week, group C showed a significantly larger number of stained cells than group A ($P = 0.0004$), as did group D ($P = 0.0025$). Group D also showed a significantly larger number of stained cells than group A after 2 weeks ($P = 0.0034$). After 3 weeks, group B showed a significantly larger number of stained cells than group A ($P = 0.0022$), as did group D ($P = 0.0005$). After 4 weeks, group B showed a significantly larger number of stained cells than group A ($P = 0.0006$), as did group C ($P = 0.0001$), and group B also showed a significantly larger number of stained cells than group D ($P = 0.0034$), as did group C ($P = 0.0006$) (Fig. 10).

Discussion

Bone augmentation is a good procedure when placing a dental implant in the maxillary molar region, which has a shortage of bone mass. In such cases, we have used bone grafts to elevate the maxillary sinus floor. To date, the materials that have been used for bone grafts include autogenous bone grafts, allograft, xenograft, and synthetic materials. Bovine hydroxyapatite is a synthetic material frequently used as a grafting material in sinus lift procedures[20,21]. In addition, β-tricalcium phosphate (β-TCP) was one of the earliest calcium phosphate compounds to be used as a bone graft substitute due to its good osteoconductivity, biocompatibility, and sufficient mechanical stress[22,23]. However, allografts and xenografts are susceptible to immunorejection and carry a risk of disease transmission[7–9]. Furthermore, harvesting autogenous bone is generally associated with several complications,

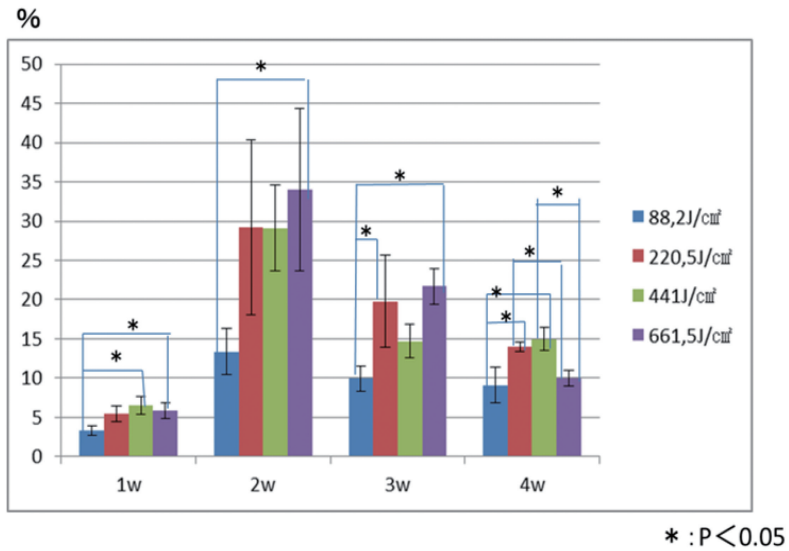

Fig. 10. The number of osteocalcin positive cells. The time-course of changes in all the groups showed significant differences with ANOVA (P=0.0014). *Significant difference; P<0.05.

including invasion of the host site, pain, and blood loss. Furthermore, some synthetic materials have limited potential for osteoconduction. We have therefore been left with little choice but to explore whether or not bone augmentation can be performed without a transplant. Several therapies have been evaluated for their potential to enhance bone regeneration, including ozone therapy, platelet therapy, and low-intensity pulsed ultrasound (LIPUS)[24–26]. LIBT is another such therapy.

Laser-induced bone therapy (LIBT) was recently introduced as a method for accelerating the formation of new bone. Bone healing is a complex and lengthy process involving inflammation, bone formation, and bone remodeling; however, the use of devices such as lasers may accelerate the healing process.

HLLT is known to produce a photobiodestructive reaction (PAR) inducing cellular vaporization, whereas LLLT generates a photobioactive reaction stimulating cellular proliferation and differentiation[27]. There are many reports supporting the benefits of LLLT for inducing bone formation[28–35].

Bone tissue subjected to laser irradiation has been found to demonstrate accelerated bone formation. While several types of lasers are available, for the present study, we focused on CO_2 lasers. CO_2 lasers have a wavelength of 10.6 μm, which falls within the specific absorption spectrum for calcium hydroxyapatite (9.0 to 11.0 μm)[36]. A previous report using LLLT found that, when laser irradiation was performed on cortical bone, only a CO_2 laser was able to induce new bone formation in the marrow cavity[37]. Furthermore, bone formation induced by CO_2 laser has been widely reported[37-39,40,41]. In addition, a previous report in which HLLT was used as well as another report by Naka *et al.* stated that the use of laser irradiation was effective for promoting bone formation[12].

However, despite its beneficial effects, laser irradiation has been shown to induce not only damage to the cortex but also inflammatory reactions and degeneration of the bone marrow. Thermal damage is considered to be the cause of these adverse effects. Continuous-wave (CW) or long-pulse (i.e. pulse width of several dozen microns) CO_2 lasers induce carbonization through the generation of heat[18,41]. Carbonate generated by heat from HLLT may adversely affect the bone augmentation. However, a short-pulse CO_2 laser does not induce carbonization.

There have been no reports of bone augmentation in experiments using HLLT setups that did not induce carbonization by thermal damage. Furthermore, the optimum irradiation conditions remain unknown. Therefore, CO_2 lasers producing short laser pulses that do not have any thermal influence on the irradiated area are required. As such, a new CO_2 laser device that does not inflict thermal damage was developed[17–19]. In the present study, we evaluated the bone augmentation under varying energy settings ranging from a high to a low output. We explored whether or not adjusting the laser reactive level influenced the formation of cranial bone in hamsters without carbonization. We used immunohistochemical and histological methods to compare the bone augmentation in various experimental groups, each of which had been exposed to a different reactive level of irradiation.

Investigating the bone formation and resorption processes involves the identification of products that are synthesized by osteoblasts and osteoclasts[32]. Noncollagenous proteins found in the organic matrix of bone tissue (osteocalcin) are commonly used as bone mineralization markers. Mineralization can be achieved only when proteins that have an affinity for calcium (such as OCN) promote mineral deposition.

In this study, osteoblasts and fibroblasts were clearly observed at two weeks postoperatively, and the OCN expression was maximized.

The new bone area ratio showed small in group A which being similar to the total irradiation energy of LLLT. The effects of bone augmentation were hardly observed under the irradiation conditions of group A. These results suggest that LLLT may need heat a few influences to the bone augmentation. In contrast, the new bone area ratio showed most in group D which being similar to the total irradiation energy of HLLT. This indicated that the bone augmentation was significantly increased in HLLT without any induction of thermal damage.

There were no significant differences in the new bone area ratio among the groups after one week. However, the OCN values in groups C and D were significantly different from those in group A. Furthermore, the new bone area ratio in Group D was significantly higher than that in group A after two weeks. These results suggest that the bone regeneration peaked after two weeks in this study. It was impossible to confirm the bone formation macroscopically. For clinical applications, studies should conduct further investigations into the most favorable conditions for laser therapy. In addition, the effects of short-pulse CO_2 laser irradiation should be compared with those of LLLT in the future.

Conclusion

The present findings indicated that high output without thermal damage has a greater bone augmentation effect than low-output approaches.

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