Original

Comparison of Surface Morphology and Healing in Rat Calvaria Bone Defects between Ultrasonic Surgical Method and Rotary Cutting Method

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Abstract: The aim of this study was to investigate the influence of ultrasonic surgical method (piezoelectric surgery) on invasions of bone structure in early stages and the subsequent healing process of bone defects of rat calvaria as compared with that of rotary cutting using an appropriate rotation speed. Thirteen-week-old male Sprague-Dawley rats were used in this study. Bone defects were prepared in rat calvaria using either an ultrasonic surgical instrument with an ultrasonic insert tip (Ui) or a handpiece with a round bur (Rb) as a rotary cutting instrument (control). Morphological evaluations of the cutting surfaces in the bone defects were assessed by scanning electron microscope (SEM) and confocal laser microscope (CLM) observation. The degree of invasions of the bone structure in the early stages after bone defects were made was assessed by the ratio of empty osteocyte lacunae relative to the total number of osteocytes. The process of bone healing was analyzed by histological observation, immunohistochemical staining with proliferating cell nuclear antigen (PCNA) and osteocalcin (OC), and ratio of PCNA-positive cells. Many fine, parallel streaky cutting traces were observed in the Ui groove surfaces, whereas the Rb groove surfaces were flat and no parallel cutting traces were observed. The arithmetic average roughness of the Ui group (10.06 ± 1.96 µm) was significantly higher than that of the Rb group (2.52 ± 0.16 µm) (p < 0.05). During the entire observation period, no differences in histological observation and immunohistochemical staining were observed between the Ui and Rb groups. No significant differences were recognized in the ratio of empty lacunae and PCNA-positive cells between the Ui and Rb groups. In the present study, there were no appreciable differences in the invasions of bone structure in the early stages and the subsequent healing process of the bone defects of rat calvaria between ultrasonic surgical method and rotary cutting method.

Key words: Ultrasonic surgical method, Rotary cutting method, Osteogenesis, Animal models

Introduction

In the rotary cutting technique, burs and drills are conventionally used for cutting the bone tissue. As the bone tissue is cut away with these rotary cutting instruments, the surrounding soft tissue is sometimes injured through contact with the instruments¹. In addition, extreme care is required in the oral maxillofacial area, where structures such as vessels, nerves, and mucosa are concentrated in a small area. In these types of surgeries, the use of an ultrasonic surgical instrument, which causes minimal injury to soft tissue²-⁴, is recommended.

An ultrasonic surgical technique employing a piezoelectric element with strong output, modulated frequency, and controlled tip vibration range, i.e., piezoelectric surgical device, was first reported for bone surgery (ridge expansion technique) by Vercellotti T⁵, which then began to be widely used in dentistry.

Such applications have become more advanced in operations involving osteotomy in craniofacial surgery⁶,⁷, surgical orthodontics⁸, and sinus floor augmentation⁹. In implant treatment, where important soft tissues such as nerves, blood vessels, and mucosa are involved, Gruber RM et al. reported in a pilot clinical study that postoperative neurosensory disturbances of the inferior alveolar nerve were reduced when an ultrasonic surgical instrument was used in sagittal split ramus osteotomy⁹. Vercellotti T et al. reported that when an ultrasonic surgical instrument was used in sinus floor augmentation, which is a technically demanding operation for removing the extremely thin Schneiderian membrane from the maxillary bone, no perforation was found in the Schneiderian membrane in 95 % of the cases⁹. Also, Wallace SS et al. reported that when performing sinus floor augmentation with only rotary cutting instruments, perforation of
the Schneiderian membrane occurred in 30% of the cases, whereas when using ultrasonic surgical apparatus, it only occurred in 7% of the cases6. From these reports, the usefulness of ultrasonic surgical instruments is clear in the case of surgeries having a high risk of soft tissue injury. On the other hand, in surgery involving bone tissue, direct use of ultrasonic surgical instruments depends on the usability of the instrument or the operative method of surgery6,9.

For histopathological examination, a few reports are available that compare a rotary cutting instrument10,11 with an oscillating saw12. However, the circumstances of these reports are unclear in that, for example, the number of rotations of the rotary cutting instrument is not described10 or the number of rotations used appears to be too invasive of the bone10. Thus, the degree of bone invasiveness of ultrasonic surgical instruments or their influence on the healing process is still not clear.

Because invasion of bones largely determines the prognosis of implant treatment, the influence of bone cutting devices on bone tissue has been studied for a long time13. Reingewirtz Y et al.14 had determined the number of rotations of rotary cutting instruments that produces minimum invasion of bone; the number was strictly defined by implant manufacturer (Institut Straumann AG, Basel, Switzerland).

Accordingly, when comparing ultrasonic surgical instruments with rotary cutting instruments, it is necessary to use the specific number of rotations in a rotary cutting instrument that is minimally invasive of the bone.

The aim of this study was to investigate the influence of ultrasonic surgical instruments (piezoelectric surgery) on invasions of bone structure in the early stages and the subsequent healing process of bone defects of rat calvaria as compared with that of rotary cutting instruments under appropriate rotation speeds.

Materials and Methods

Experimental device

The following experimental devices and conditions for making bone defects were used in this study, as shown in Fig. 1.

1) Ultrasonic surgical method (Ui); experimental group

Ultrasonic surgical instrument (Surgery Falcon, Osada Electric, Tokyo, Japan, Fig. 1a) with an ultrasonic insert tip ø1.2 mm (ST84, Osada Electric, Tokyo, Japan, Fig. 1b) at a frequency of 28.8 ± 0.2 kHz and power of 12 W.

2) Rotary cutting method (Rb); control group

Handpiece (CYCLON Z, Sea Force, Tokyo, Japan, Fig. 1c) with a round bur ø1.2 mm (1648190, Dentsply Maillefer, Ballaigues, Switzerland, Fig. 1d) at a rotational speed of 800 rpm, recommended by the implant manufacture.

Experimental animals

Thirteen-week-old male Sprague-Dawley rats (Sankyo Labo Service, Tokyo, Japan) were used (n = 86, details shown in Fig. 3). The rats were allowed food and water ad libitum and maintained on a 12h light/dark cycle (lights on from 8:00 to 20:00) at 23 ± 1°C with 60 ± 10% humidity during the experiment. All animal experiments in this study were conducted in accordance with the Tokyo Dental College Guidelines for Animal Experimentation (Approval number: 263001; Approval date 1/4/2014).

Operating procedure for making bone defects

Fig. 2 shows the operating procedure for creating the bone defects in this study. Eighty rats were placed under general anesthesia with an intraperitoneal administration of sodium pentobarbital (Somnopentyl® 0.8 µl/g, Kyoritsu Seiyaku, Tokyo,
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For surgery, the dorsal area of the calvaria of the rats was shaved thoroughly and disinfected with ethanol, and then a linear sagittal skin incision was made in the skull. Subsequently, full thickness epidermal, hypodermal, and periosteal flaps were then elevated, and 4 grooves (about 1.2 mm wide, about 0.5 mm deep, and 4 mm long) were prepared in the dorsal area of the calvarial bone according to the previous report\textsuperscript{15}. The operating time for making one groove was 4 min and 3 min for the Ui and Rb groups, respectively. The load was approximately 5 N on both the Ui and Rb groups during the operation. The operation was carried out under continuous saline solution irrigation (25 ml/min). The flaps were then closed by suturing.

All animals survived and recovered quickly from surgery. The rats appeared to be in good health throughout the test periods. The experimental protocol of this study is shown in Fig. 3.

**Morphological evaluation of cutting surface in bone defects**

Six rats were used for morphological evaluation of the cutting surface. The rats were divided into 3 for the Ui and Rb groups. The animals were euthanized with an overdose of sodium pentobarbital. The calvarial bone area including the 4 grooves and the surrounding soft tissues were removed. These specimens were washed with sterile saline and distilled water, after which they were subjected to a dehydration process consisting of a series of ethanol aqueous solutions and 2-methyl-2-propanol replacement.

After sputter coating with Au-Pd, the surface topography of the grooves were examined and photographed with a scanning electron microscope (SEM) (SU6600, Hitachi, Tokyo, Japan) ( Experiment A described in Fig. 3).

The arithmetic average roughness was quantitatively analyzed using a confocal laser microscope (CLM) (OLS4000 CLM, Lect; Olympus, Tokyo, Japan). For CLM analysis of both groups, 2 grooves of 4 were randomly selected from one specimen. Values were expressed as the mean of the measurements of six grooves. Each CLM analysis was performed over an area of 259 μm × 259 μm with a 50× lens to determine the values of surface roughness (Experiment A described in Fig. 3).

**Histological assessment**

Histological assessment was performed separately for the Ui and Rb groups after 3, 7, 14, 21 days on 10 animals. All animals were euthanized with an overdose of sodium pentobarbital. The calvarial bone area including the 4 grooves with surrounding soft tissues were extracted en bloc. These specimens were fixed with 10% neutral buffered formalin solution for 7 days at room temperature. Subsequently, the specimens were decalcified for 21 days with 10% EDTA (pH 7.0-7.5) at room temperature before being embedded in paraffin. Paraffin sections approximately 5 μm thick were cut and stained with hematoxylin and eosin (H-E staining) according to standard protocols (Experiment B described in Fig. 3). The specimens were morphologically observed using a universal photomicroscope (Axiophot 2, Carl Zeiss, Oberkochen, Germany).

**Ratio of empty osteocyte lacunae**

The ratios of empty osteocyte lacunae along the existing bone were calculated. The existing bone area of 935 μm wide and up to a depth of 50 μm from the lowest point of the bone defect were observed at days 3, 7, and 14 for the Ui and Rb groups (n = 60) (Experiment C described in Fig. 3, dotted area in “a” Fig. 4). Ratio was obtained from the percentage of number of empty lacunae for all lacunae.

**Immunohistochemical staining**

For immunohistochemical staining, the paraffin sections were
deparaffinized with xylene and rehydrated using an ethanol series. The sections were washed in 10 nmol/l with pH 7.4 phosphate buffered saline (PBS); endogenous peroxidase activity was blocked by incubating sections with 0.3 % H$_2$O$_2$ in methanol for 30 min.

For the analysis of proliferating cell nuclear antigen (PCNA) and osteocalcin (OC), the sections were incubated for 12 h at 4 °C with mouse anti-PCNA primary antibody (PC-10, DAKO, Carpinteria, CA, USA) and rabbit anti-osteocalcin (OC; Bioss, MA, USA) at a dilution of 1 : 200 (PCNA) and 1 : 100 (OC). The sections were washed in PBS and then incubated with the secondary antibody, peroxidase-labeled anti-mouse IgG polyclonal antibody (Histofine Simple Stain Rat MAX-PO [MULTI]; Nichirei, Tokyo, Japan) for 30 min and washed with PBS. The sections were stained with 3, 3’-diaminobenzidine (DAB substrate kit, Nichirei, Tokyo, Japan), washed in sterilized water, and counterstained with hematoxylin. The sections were then dehydrated according to established protocol and photographed using a universal photomicroscope (Axiophot 2). PCNA was stained at days 3 and 7. OC was stained at days 14 and 21 (Experiment D, E described in Fig. 3).
quantitative analysis ($n = 40$) (shaded area “b” in Fig. 4). The ratio of PCNA-positive cells was calculated from the percentage of number of PCNA-positive cells for all cells (Experiment D described in Fig. 3) in the Ui and Rb groups at days 3 and 7.

**Statistical analysis**

For statistical analysis of the results at each time point, data were analyzed with Welch’s $t$-test and were considered significant at $p < 0.05$. Data are expressed as means ± standard deviation.

**Results**

*Morphological evaluation of cutting surface in bone defects*

Many fine parallel streaky cutting traces and rough spots were observed along the entire surface of the Ui group cutting groove.

**Figure 6.** Arithmetic average roughness of the Ui and Rb groups (measurement area of $259 \mu m \times 259 \mu m$). Sa of the Ui group was significantly rougher than that of the Rb group. Asterisk indicates $p < 0.05$.

**Figure 7.** Histological analyses of wound healing in Ui (a, c, e, g) and Rb (b, d, f, g) groups at days 3 (a, b), 7 (c, d), 14 (e, f), and 21 (g, h).

At day 3, the cutting bone defect areas of both the Ui (a) and Rb (b) groups were filled with clots and empty osteocyte lacunae were locally observed under the cutting surface. At day 7, newly formed bone including immature osteocytes was recognized in the connective tissue adjacent to the bone cutting surface (c, d). At day 14, newly formed bone with marginally arranged osteoblasts and an immature lamellar structure were observed in the cutting bone defect areas of both the Ui (e) and Rb (f) groups. At day 21, newly formed bone with a further matured trabecular bone-like structure filled the cutting bone defect areas of both the Ui (g) and Rb (h) groups. Scale bar: 200 $\mu m$.

**Ratio of PCNA-positive cells**

At a distance $30 \mu m$ above the lowest point of the bone defect, an area of $175 \mu m \times 231 \mu m$ in the bone defect was subjected to (Fig. 5a). High magnification images showed that the surfaces of the cutting traces were smooth and contained thin, fiber-like structures (Fig. 5c).
Figure 8. Ratio of empty osteocyte lacunae at days 3, 7, and 14. There were no significant differences between the Ui and Rb groups during the observation periods (days 3, 7, and 14) ($p > 0.05$).

Figure 9. Immunohistochemistry of PCNA at days 3 and 7. At day 3, positive reactive cells were observed in the fibroblast-like cells of both the Ui (a) and Rb (b) groups. At day 7, strong immunoreactions were observed in the osteoblast-like cells of both groups (c, d). Scale bar: 50 µm.

Histological assessment

At day 3 after the bone defect was made, similar to the results shown by the SEM, many fine rough spots were observed along the entire Ui group cutting surface (Fig. 7a), while flat surfaces were observed along the cutting grooves of the Rb group (Fig. 7b). The bone defect areas of both the Ui and Rb groups were filled with clots and empty osteocyte lacunae were locally observed under the cutting surface (Fig. 7a, b).

At day 7, granulation tissue with fibroblasts, lymphocytes, and capillary angiogenesis were observed in the bone defect areas in both the Ui (Fig. 7c) and Rb (Fig. 7d) groups. Furthermore, newly formed bone including immature osteocytes was recognized in the connective tissue adjacent to the cut surface.

At day 14, in both the Ui (Fig. 7e) and Rb (Fig. 7f) groups, newly formed bone with marginally arranged osteoblasts and an immature lamellar structure were observed in the bone defect areas. Capillary dilatation in fibrous connective tissue between the newly formed bone in both groups was recognized.

At day 21, the bone defect areas of both the Ui (Fig. 7g) and Rb (Fig. 7h) groups were filled by newly formed bone with a further matured trabecular bone-like structure. The upper surface of the new bone became flat and the volume its volume had clearly increased.
Ratio of empty osteocyte lacunae

No significant differences ($p > 0.05$) of the ratio of empty osteocyte lacunae in the marginal bone of the defect areas were recognized between the Ui and Rb groups at days 3, 7, and 14 in each group. However, the number of osteocyte disappearances in both groups tended to decrease between days 3 and 7, and to increase between days 7 and 14 ($p > 0.05$) (Fig. 8).

At 14 days after bone defect creation, an immunopositive reaction of OC was recognized in the osteoblasts in the connective tissue of newly formed bone and cells in osteocyte lacunae in both groups (Fig. 11a, b). At day 21, positive reactions were observed only in the osteoblasts of both groups (Fig. 11c, d).
In ultrasonic surgical instruments, the frequency and output appropriate for bone cutting has already been determined, with the majority of devices using a frequency between 24 kHz and 36 kHz. Output is changed according to the voltage of the piezoelectric element. The ultrasonic surgical instrument used in this experiment had 10 possible output settings. As the minimum output setting at which bone cutting was possible, this experiment used a power of 12W, a frequency of 28.8 ± 0.2 kHz, and a water flow rate of 25 ml/min.

For the rotary cutting instrument, effects on bone tissue have been studied for a long time and conditions for use have been examined in detail, particularly in the field of dental implants, because bone damage is largely related to implant osseointegration. Eriksson RA et al. stated that 47 °C for 1 minute was the threshold level for bone survival. Reingewirtz Y et al. showed that when the settings of rotary cutting instruments are 8-20 N and 400-800 rpm, there was little increase in bone temperature. Also, Hillery MT et al. reported that at 400-1200 rpm, the temperature during bone cutting decreased. However, in previous assessments of ultrasonic surgical instruments, the conditions used for controlling the rotary cutting instrument had not yet been established, and there are also reports in which conditions were set to levels that would clearly cause bone damage. Therefore, in this experiment, we set the number of rotations at 800 rpm in the rotary cutting instrument, because it was necessary to have suitable speed that causes little damage to the bone for reference.

**Morphological evaluation of cutting surface in bone defects**

In the SEM findings in this study, the Ui group surface was coarser than that of the Rb group. Even in the quantitative analysis of surface roughness, the Ui group had a significantly higher value than did the Rb group.

High magnification images showed that the surfaces of the cutting traces in the Ui group were smooth and contained thin, fiber-like structures. On the other hand, many small, irregular scaly structures covered the entire Rb group cutting surfaces and a few cracks were observed. This result suggested that the ultrasonic surgical instrument causes minimal injury to soft tissue, because the thin, fiber-like structures remain. The small, irregular structures in the Rb group cutting surfaces seem similar to the previous report by Sasaki KM et al. that bur-drilled surfaces show cracking and roughness.

Although in this experiment, the Ui group output and Rb group rotations were set at levels that had little invasive effects on the bones, the Ui group presented a coarser surface than did the Rb group. The reason for this is thought to be the inherent differences in the bone cutting mechanism between the ultrasonic surgical instrument and the rotary cutting instrument. The ultrasonic surgical instrument cuts by vibrating the blade portion. The methods of vibration include vertical, deflective, twisting, combination, etc. and generally, cuts are made by colliding with and crushing the target. In rotating cutting, the blade strikes through the target and cuts it by scooping up pieces. In other words, the Ui group cuts by collision and crushing while the Rb group cuts by scooping with the blade, with the result that the surfaces exposed to a round bur are comparatively flat.

**Invasions of bone structure in early stages after making bone defects**

In this study, no difference between the Ui and Rb groups was found in the bone structure in the early stages after the bone defects were made based on an assessment of the ratio of empty osteocyte lacunae. Ide Y et al. reported a lower amount of empty lacunae in the surrounding area when using an ultrasonic surgical instrument compared with use of a rotary cutting instrument. In this report, the difference in bone invasion is thought to be due to the number of rotations of the rotary cutting instrument and an invasion by the Lindemann bur. In addition, no differences were recognized in cell proliferation properties and differentiation characteristics in vitro, and it was concluded that there were no differences in the degree of bone invasion. There are also reports of bone damage due to the denaturation of proteins, changed metabolism or changed enzyme activity by increased heat, and changes in protoplasma lipids. Accordingly, both the ultrasonic surgical instrument and the rotary cutting instrument used at the settings in this experiment may cause little stress and it was assumed that there was no difference in invasiveness, even though a clear difference in the cutting surface was observed between the Ui and Rb groups.

**Healing process after bone cutting**

When using H-E stain to conduct histological examinations, no differences were seen between the Ui and Rb groups during the entire observation period. To assess the initial healing reactions during wound healing, we performed immunohistochemical studies 3 and 7 days after the procedure using PCNA-positive cell rates, but no significant differences were found between the Ui and Rb groups. Furthermore, in order to assess bone healing in the later stages, we performed OC immunohistochemical studies 14 and 21 days after the procedure, but no clear differences were seen regarding OC expression.

Several previous studies report superior bone healing, such as new bone formation, with the ultrasonic surgical instrument compared with that obtained from rotary cutting instruments. However, the conditions of the rotary cutting instruments used in that report were settings that would easily cause damage to bone and were different from the conditions and settings used in this study. On the other hand, Mouraret S et al., when comparing ultrasonic surgical instruments and a fissure carbide bur, used 800 rpm as the number of rotations for the fissure carbide bur and...
reported that no obvious differences were found in the extent or duration of postoperative inflammation\textsuperscript{3,10}. PCNA-positive cells are used as an indicator of cell proliferation\textsuperscript{24,25} and OC is used as an indicator of osteoblast activity in the later stages of osteogenesis\textsuperscript{26}. In this study, we compared the bone healing process using these two assessment methods, and no difference was found in the healing process. In other words, while there was a difference in the state of the cutting surface created by the two instruments, the invasion of the existing bone was equivalent to that observed at low-invasiveness settings and the subsequent bone healing process was also equivalent.

Given the rough cutting surface observed in this study, ultrasonic bone cutting instruments might be considered disadvantageous to healing, but the actual difference between the two in the bone healing process was minimal, making it clear that bone healing cannot be based on the coarseness of the cutting surface.

**Clinical applications of ultrasonic surgical methods**

The results of this study show that there were no appreciable differences in the effects on bone tissue between methods employing ultrasonic surgery and rotary cutting instruments running at a relatively low speed. Crosetti E et al. reported that operation time was prolonged by 30% when ultrasonic surgical instruments we used as compared with conventional instruments\textsuperscript{28} and that ultrasonic surgical instruments might have some negative features when used for cutting bone. However, ultrasonic surgical instruments cause minimal injury to soft tissues\textsuperscript{2,4,10}, which is their most appealing feature. Furthermore, ultrasonic surgical instruments provide the equivalent invasiveness of bones as that provided by conventional rotary cutting instruments, suggesting that they are highly effective tools for procedures of high difficulty in which bone tissue and soft tissue are in close proximity.

Accordingly, the ultrasonic surgical instrument is useful for implant treatment involving sinus floor augmentation, osteotomy in craniofacial surgery, and surgical orthodontics, where important soft tissues including nerves, blood vessels, and mucosa. However, on the clinical point of view, it is necessary to make improvements such as cutting efficiency, decrease in the usage of saline, and reduction of cost of the ultrasonic surgical instrument.

In conclusion, there were no appreciable differences between ultrasonic surgery and rotary cutting on invasions of bone structure during the early stages as well as the subsequent healing process of bone defects in rat calvaria.

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