



## Research article

## Octacalcium phosphate collagen composite (OCP/Col) enhance bone regeneration in a rat model of skull defect with dural defect



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## ABSTRACT

Cranial bone defects are a major issue in the field of neurosurgery, and improper management of such defects can cause cosmetic issues as well as more serious infections and inflammation. Several strategies exist to manage these defects clinically, but most rely on synthetic materials that are prone to complications; thus, a bone regenerative approach would be superior. We tested a material (octacalcium phosphate collagen composite [OCP/Col]) that is known to enhance bone regeneration in a skull defect model in rats. Using a critical-sized rat skull defect model, OCP/Col was implanted in rats with an intact dura or with a partial defect of the dura. The results were compared with those in a no-treatment group over the course of 12 weeks using computed tomographic and histological analysis. OCP/Col enhanced bone regeneration, regardless of whether there was a defect of the dura. OCP/Col can be used to treat skull defects, even when the dura is injured or removed surgically, via bone regeneration with enhanced resorption of OCP/Col, thus limiting the risk of infection greatly.

## 1. Introduction

Cranial bone defects are a major issue in the field of neurosurgery. Whether they are due to trauma, surgical craniotomy or craniectomy, or birth defects, they need to be addressed properly to achieve proper cosmetic appearance of the skull as well as functional protection of the brain [1,2]. In the clinical setting, several strategies exist for addressing skull bone defects, taking into consideration the size, shape, and location of the defect and skull growth in children. Strategies include the use of titanium mesh or ceramic implants and the use of synthetic bone materials, which are usually reserved for smaller defects, such as hydroxyapatite (HA)  $\alpha$ - or  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) and calcium phosphate-based cements [2, 3, 4, 5, 6, 7]. However, even though these options are used ubiquitously in the neurosurgical field, they are

not without drawbacks, the best known of which is infection, but also including pain, poor cosmetic appearance, and inferior bone regenerative abilities [4,8].

Given the limitations of these methods, a strategy aiming toward promoting bone regeneration would be the ideal approach. In this study, we used an absorbable bone regenerative material, octacalcium phosphate (OCP), which is reported to be a precursor of the initial mineral crystals of biological apatite in bone [9]. When implanted into the cranial subperiosteal region, OCP is converted to apatite and induces bone regeneration [10,11]. It has higher bone regenerative ability and higher resorption *in vivo* than HA or  $\beta$ -TCP [12]. However, despite the apparent superiority of its bone regenerative ability, OCP has not gained popularity for treatment of cranial bone defects due to its poor molding ability and brittleness, which are consequences of its intrinsic crystal structure

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[9]. To overcome this issue, our group developed a scaffold that combined synthetic OCP and porcine collagen sponge (OCP/Col) [9]. OCP/Col was shown to enhance bone regeneration better than OCP alone [9]. Moreover, OCP/Col was shown to reconstruct a critical-sized skull bone defect in rats and dogs [13,14].

These reports show that OCP/Col is a promising material for repair of cranial bone defects. However, the reports only showed regeneration in the presence of intact dura mater, which is not always the case in the clinical setting, especially in trauma cases or burr-hole surgery. Thus, to expand the applications of OCP/Col and further explore its value as a bone-regenerating material, we evaluated the effect of OCP/Col in rats with a critical-sized bone defect with or without intact dura mater.

## 2. Materials and methods

### 2.1. Animals

Eight-week-old male Sprague–Dawley rats were treated in accordance with the code of ethics of the World Medical Association and Tohoku University guidelines based on the International Guiding Principles for Biomedical Research Involving Animals. The animal protocols were approved by Tohoku University's administrative panel on laboratory animal care.

### 2.2. Experimental groups

The rats were assigned to three groups: group I, OCP/Col implantation without dural defect; group II, OCP/Col implantation with dural defect; and group III, no OCP/Col implantation with dural defect as a control. The total number of animals was 12.

### 2.3. Preparation of collagen and composite of OCP/Col discs

OCP and OCP/Col discs were prepared as previously described [9, 15]. Briefly, OCP was prepared by direct precipitation [10], and collagen was prepared from NMP collagen PS (Nippon Meat Packers, Tsukuba, Ibaraki, Japan), a lyophilized powder of pepsin-digested atelo-collagen isolated from porcine dermis. The sieved granules (particle size, 300–500  $\mu\text{m}$ ) of OCP were added to the concentrated collagen and mixed; the percentage weight of OCP in OCP/Col was 77%. The OCP/Col mixture was lyophilized, and the discs were molded (9-mm diameter, 1.5-mm thickness). The molded OCP/Col underwent dehydro-thermal treatment (150  $^{\circ}\text{C}$ , 24 h) in a vacuum drying oven and was then sterilized using gamma-ray irradiation (15 kGy). Under micro-computed tomography (CT) standardized conditions (60 kV, 10 mA, 11 s), the OCP/Col discs showed no radio-opacity before implantation.

### 2.4. Surgical procedure

The rats were anesthetized with 1.5% isoflurane in 30% oxygen and 70% nitrous oxide using a face mask and were allowed to breathe spontaneously. The rectal temperature during surgical procedures was maintained at  $37 \pm 0.5$   $^{\circ}\text{C}$  using a feedback-regulated heating pad (BWT-100, Bio Research Center, Nagoya, Japan).

The head area was disinfected, and the scalp was shaved and cut with surgical scissors to expose the skull. The periosteum of the calvarium was ablated, and a full-thickness standardized trephine defect, 10 mm in diameter, was made in the calvarium under continuous saline buffer irrigation. Extreme care was exercised to avoid injury to the superior sagittal sinus and dura mater. In the group with dural defect, a  $5 \times 3$  mm piece of unilateral dura mater was carefully resected without injuring the cerebrum. For OCP/Col implantation, the discs were implanted into the trephine defect. After the defects were treated, the ablated periosteum was repositioned and sutured, followed by skin closure.

### 2.5. Micro-CT examination

Morphological and quantitative image analysis of newly formed bone was performed using micro-CT (Scan Xmate-E090; Comscantecno Co., Ltd., Kanagawa, Japan) under standardized conditions (90 kV, 110 mA) immediately after surgery and at 1, 2, 3, 4, 6, 8, 10, and 12 weeks after surgery. In the three-dimensional analysis, the newly formed bone area was analyzed by calculating the mean Hounsfield unit (HU) value of the OCP/Col implant. The measurements were repeated five times, and the means were calculated and used for further downstream analysis.

### 2.6. Tissue preparation and histological analyses

Twelve weeks after surgery, the rats were euthanized by an overdose of isoflurane and then transcardially perfused with cold saline, followed by 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. The implants were resected along with the surrounding bone and tissue and fixed with 4% paraformaldehyde in phosphate-buffered saline. The samples were then decalcified with 10% ethylenediaminetetraacetic acid, embedded in paraffin, and cut in the coronal plane at 3- $\mu\text{m}$  thickness. For morphological evaluation, the sections were stained with hematoxylin and eosin. To confirm the presence of osteoclasts, the sections were stained using a tartrate-resistant acid phosphate (TRAP)/alkaline phosphatase (ALP) stain kit (294-67001, Wako, Japan) without double staining by ALP. To evaluate angiogenesis, the sections were incubated with a rabbit anti-CD34 antibody (1:200, ab81289, Abcam) at 4  $^{\circ}\text{C}$  overnight. Immunohistochemistry was performed with the avidin–biotin technique, and nuclei were counterstained with hematoxylin.

### 2.7. Data analysis

All values were given as mean  $\pm$  SD. Statistical significance was assessed with repeated-measures analysis of variance, followed by Bonferroni's post-hoc comparisons test or Student's *t*-test, as appropriate. Values of  $p < 0.05$  were considered to indicate statistical significance. Graph Pad Prism 5.03 (Graph Pad Software, La Jolla, CA, USA) was used for all statistical analyses.

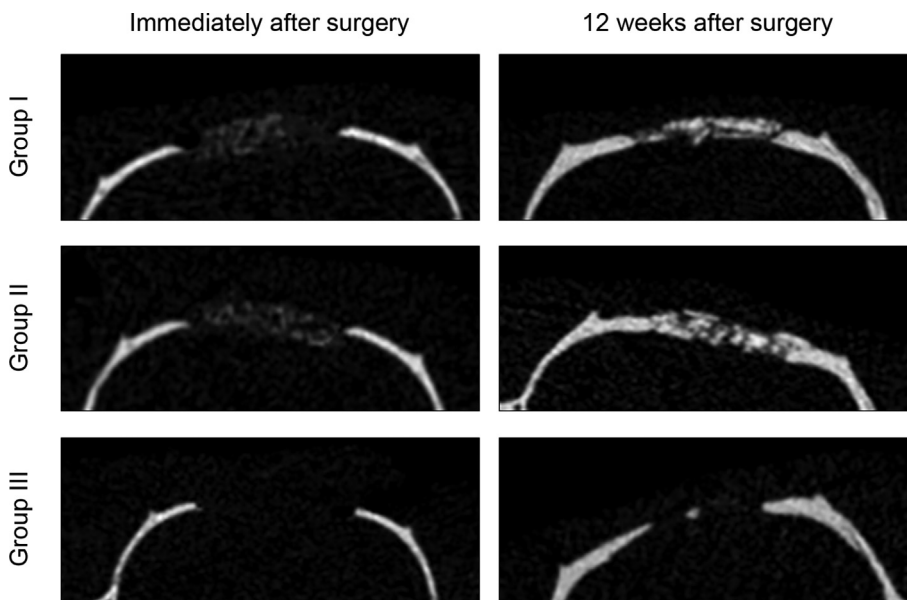
## 3. Results

### 3.1. Macroscopic analysis

Macroscopic analysis of the bone defect after 12 weeks of implantation with an operative microscope showed bonelike hard tissue covering the bone defect in the OCP/Col-treated groups (groups I and II). There were no differences in consistency or appearance of the bonelike tissue between group I (intact dura) and II (dural defect). In contrast, no hard structure was observed in group III (no OCP/Col implantation with dural defect). There were no formed adhesions between OCP/Col and the brain surface, and the OCP/Col fragment was smoothly detachable from the brain surface in all rats. No apparent inflammatory tissue changes or signs of infection were observed in any of the rats.

### 3.2. Morphometric analysis by micro-CT

To monitor the bone regenerative capacity of OCP/Col, we performed serial micro-CT analysis up to 12 weeks after implantation. We first compared the CT appearance of the implant immediately after surgery and after 12 weeks. In the OCP/Col-treated rats (groups I and II), the OCP/Col was visible as a faint white structure immediately after implantation; a similar structure was not recognizable in group III. Twelve weeks after surgery, the area where OCP/Col was implanted had turned into a high-density structure in both groups I and II. In group III, a faint, thin bone structure was visible, but it was not as dense as that seen in groups I and II. There was no visible difference between rats with and without removal of the dura mater (Figure 1).



**Figure 1.** Coronal micro-computed tomography images of rats immediately after surgery and after 12 weeks. Immediately after surgery, groups I and II show a faint white structure, which is the octacalcium phosphate collagen composite (OCP/Col) disc, while group III does not have such a structure. At 12 weeks after surgery, groups I (with dura intact) and II (without dura) both show a dense, white bonelike structure filling the bone defect gap, while group III (no OCP/Col implantation) shows minimal bonelike structure.

The HU value of the OCP/Col disc itself was below 500. The HU value of the treated area increased in a time-dependent manner and reached 1500 after 12 weeks in the OCP/Col-treated groups (groups I and II). It was significantly greater than that in the no OCP/Col treatment group (group III), which had an average of 466 HU (Figure 1). There was no significant difference in HU value between group I (intact dura) and group II (dural defect) (Figure 2). In contrast, no up-regulation of HU was observed in group III (no OCP/Col implantation).

### 3.3. Histological analysis

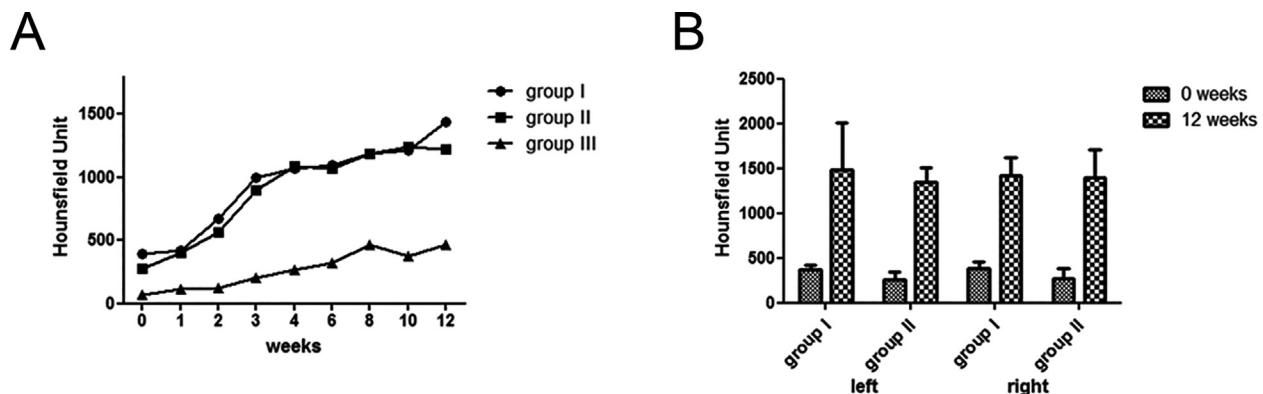
Next, we performed histological analysis of the regenerated area to assess the extent of bone regeneration. Hematoxylin and eosin staining of the newly formed bone filling the defect did not show a substantial difference between groups I and II 12 weeks after OCP/Col implantation. Newly formed bone was observed in the calvarial defects implanted with OCP/Col. Some OCP granules within the OCP/Col disc were surrounded by polynuclear giant cells, flat mononuclear cells, and osteoids, but inflammatory cell infiltration was not observed at the implanted site (Figure 3). In contrast, bone regeneration was detectable only at the

margin of the defect in the non-OCP/Col-treated rats, and it was much thinner than that in rats implanted with OCP/Col (Figure 3).

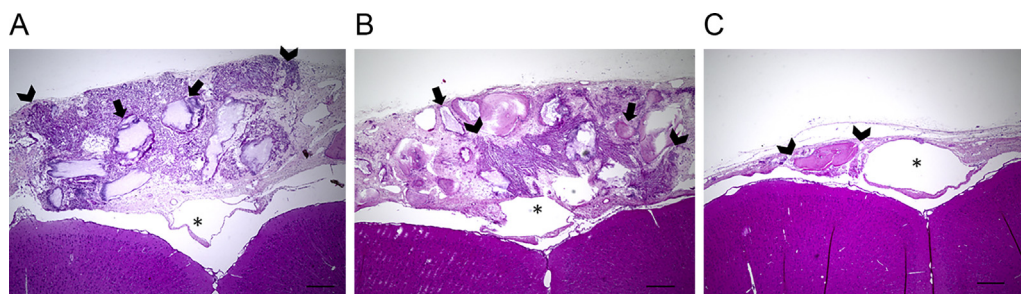
TRAP staining revealed osteoclasts surrounding the newly formed bone and residual OCP/Col particles 12 weeks after surgery. More osteoclasts were detectable in groups I and II than in group III (Figure 4). In contrast, CD34 staining showed positively stained microvessels in all groups, with no significant differences between groups with and without OCP/Col treatment (Figure 5).

## 4. Discussion

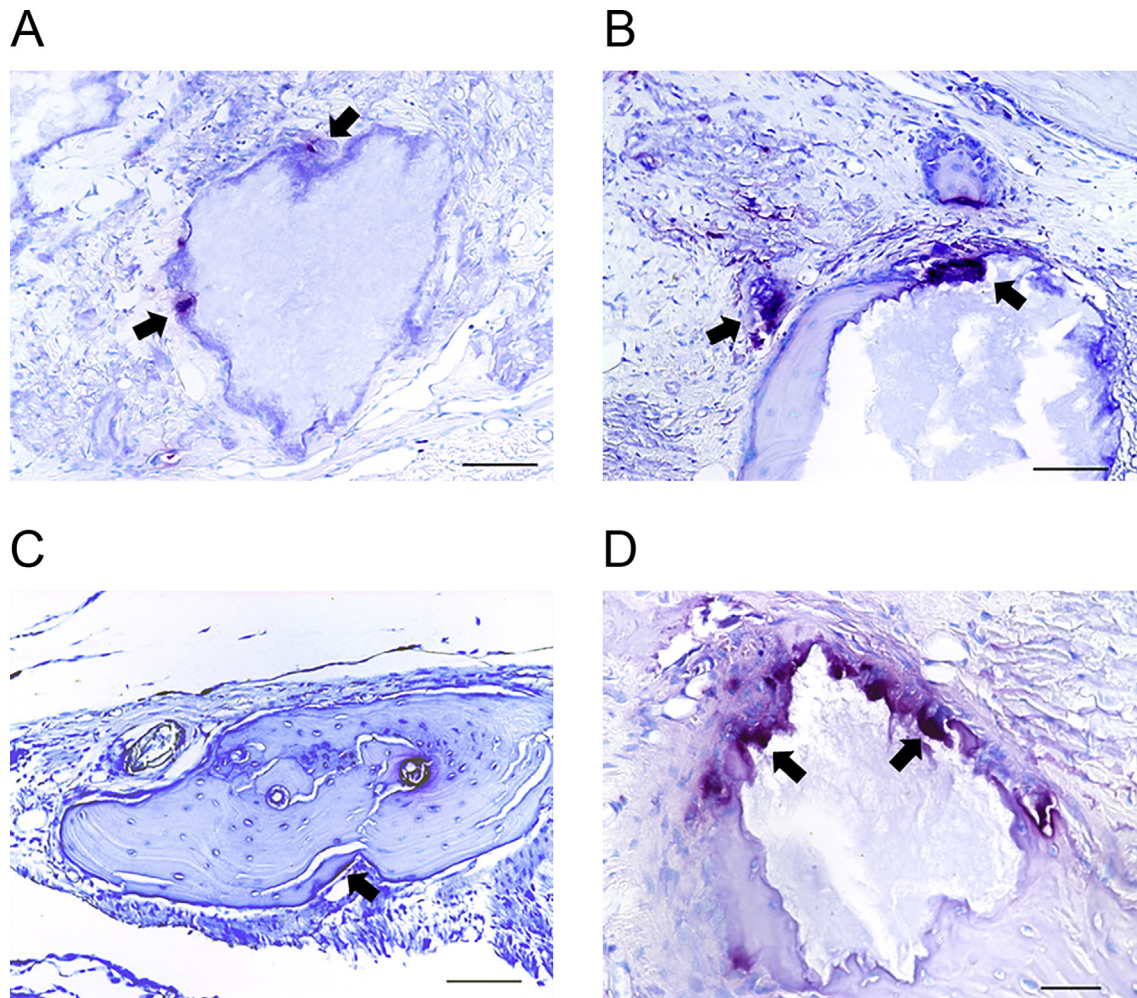
In this report we demonstrated the bone regenerative capacity of OCP/Col in treating calvarial bone defects in rats; most importantly, we demonstrated this capacity in the absence of dura. The presence of dura was previously shown to be of utmost importance in promoting bone regeneration [16, 17, 18], and its value as a promoter of bone regeneration was shown to be superior to that of the periosteum [16]. Thus, the absence of dura in situations that require management of bone defects, such as in severe trauma or after treatment of chronic subdural hemorrhage with burr-hole craniectomy [19], would create a major issue for



**Figure 2.** Analysis of bone density observed with micro-computed tomography using Hounsfield units (HU). (A) In both groups I and II, there is an increase in HU value with time, whereas group III shows no significant changes over time. The changes in both groups I and II were statistically significant compared with group III.  $p < 0.01$ , group I vs. III.  $p < 0.01$ , group II vs. III. (B) HU units acquired from both right and left sides of the skull defect were compared immediately after and 12 weeks after implantation of octacalcium phosphate collagen composite (OCP/Col) in groups I and II. Significant changes were observed in both groups 12 weeks after implantation.  $p < 0.0005$ . However, there were no significant differences between the left and right sides.



**Figure 3.** Hematoxylin and eosin stain of the decalcified tissues extracted from the bone defects after 12 weeks. Thick tissue was observed with newly formed bone, osteoid, and residual particles in group I (A) and group II (B). In contrast, group III (C) had only a thin layer of tissue and a small amount of newly formed bone. Arrowheads: newly formed bone or osteoid. Arrows: residual particles of octacalcium phosphate collagen composite (OCP/Col). \*Superior sagittal sinus. Bar = 300  $\mu$ m.



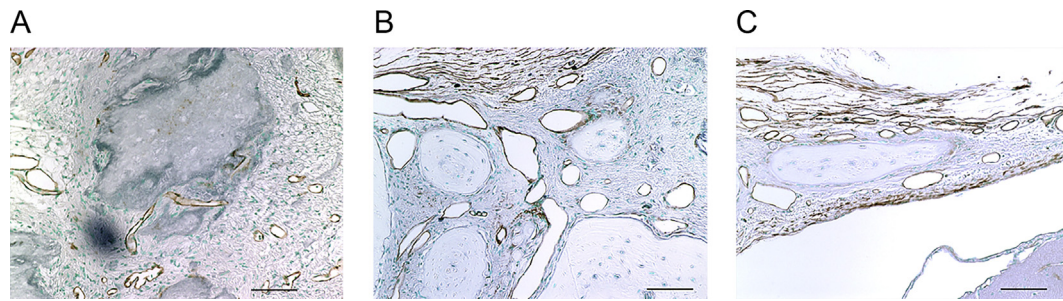
**Figure 4.** Tartrate-resistant acid phosphate (TRAP) staining of the decalcified tissues extracted from the bone defects after 12 weeks. In all groups, TRAP-positive osteoclasts were observed around newly formed bone and residual particles. However, there was a significant difference in bone density between groups I (A) and II (B) on the one hand and group III (C) on the other hand. Arrows: osteoclasts. Bar = 100  $\mu$ m. (D) Higher-magnification image of TRAP staining showed osteoclasts (arrows) surrounding the newly formed bone. Bar = 40  $\mu$ m.

bone regenerative therapies. However, we have shown evidence of significant bone regeneration in the absence of dura with OCP/Col treatment, complete with generation of osteoids, osteoclasts, and microvessels, signifying true bone formation. Although the exact origin of the regenerated bone is yet to be determined, this treatment could be of great value in the aforementioned situations.

OCP/Col was demonstrated to enhance bone regeneration in a number of bone defect models [12, 13, 15, 20, 21], including calvarial and alveolar cleft models. The regenerative ability was shown to stem, at least in part, from the ability of OCP/Col to enhance the differentiation of osteogenic cells [20], and pretreatment of OCP/Col discs with

mesenchymal stem cells before implantation resulted in enhanced osteogenesis and an increased number of osteocytes in the graft [20].

Another important feature of OCP/Col is its enhanced resorption after implantation [21], which, although not 100% complete, is superior to that of  $\beta$ -TCP or HA, both of which are not totally resorbed and thus increase the risk of infection and inflammation after implantation due to the presence of a foreign body in the tissue [22, 23, 24, 25, 26, 27]. The previous studies reported that implantation of OCP/Col enhanced bone regeneration more than implantation of HA or  $\beta$ -TCP [28, 29]. HA is osteoconductive and biocompatible, but it does not form bone and it will not increase the volume of vital bone [30]. Thus, clinical application of



**Figure 5.** Immunohistochemistry staining with anti-CD34 antibody. Positively stained, newly formed microvessels were observed in all groups. (A) Group I, (B) group II, (C) group III. Bar = 100  $\mu$ m.

HA seems to be limited [31]. On the other hand, TCP is available in a resorbable form. As it resorbs, a readily available source of calcium becomes available in sites that have osteogenic potential. Sakai et al., reported that HA or  $\beta$ -TCP remained unchanged, although OCP tended to converted and resorbed through cell-mediated osteoclastic resorption [32].

OCP/Col was proven not only to be effective in preclinical studies, but also to be safe in the clinical setting [13, 33]. Thus, the present study acts as a proof of concept study for the future clinical use of OCP/Col in situations where cranial defects need to be addressed and the dura is either present or absent. Notably, the absence of dura in the rat model was not associated with increased infection after OCP/Col implantation, further supporting the safety of OCP/Col for use in cranial defects.

Because OCP/Col has many desirable properties for bone regeneration, it might be applied to the treatment of skull base or spinal bone defects. However, further study is needed to confirm the effectiveness of OCP/Col for these conditions. Because of the difficulty of using an animal model for skull base defects, animal studies of skull base defects are challenging. For spinal cord defects, further studies are needed to confirm the effectiveness of OCP/Col for defects of load-bearing bone. We might consider a combination therapy of some fixation method with OCP/Col implantation to treat spinal bone defects.

## 5. Conclusion

OCP/Col can be used to treat skull defects, even when the dura is injured or removed surgically, via bone regeneration with enhanced resorption of OCP/Col, thus limiting the risk of infection greatly.

## Declarations

### Author contribution statement

Takashi Sasaki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kuniyasu Niizuma: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Atsushi Kanoke, Keiko Matsui, Shogo Ogita, Tadashi Kawai, Mika Watanabe: Performed the experiments.

Shinji Kamakura: Conceived and designed the experiments; Performed the experiments.

Sherif Rashad: Analyzed and interpreted the data; Wrote the paper.

Hidenori Endo, Tetsu Takahashi: Conceived and designed the experiments.

Teiji Tominaga: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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