Bone healing of critical-sized nasal defects in rabbits by statins in two different carriers

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Running title: Bone healing by statins in two different carriers

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Abstract

Objectives: To evaluate bone healing following implantation of a statin with two different carriers in rabbit nasal bone using histological and immunohistochemical methods**.**

Materials and methods: Twenty adult male Japanese white rabbits (age: 12–16 weeks, weight: 2.5–3.0 kg) were used in this study. Five bone circular defects (5 mm in diameter) per rabbit were created in the nasal bone while preserving the nasal membrane. In the experimental groups, 2.5 mg/ml simvastatin dissolved in 0.2 ml water with hydrogel was implanted in one group, 2.5 mg/ml simvastatin dissolved in 0.2 ml water with an atelocollagen sponge (ACS) in the second group with, only the hydrogel in the third group and only an ACS in the fourth group. No material was implanted in the control group. 4 animals were killed in each period, at 1-, 2-, 4-, 8- and 12 weeks postoperatively. The parts that had been operated on were removed and prepared for histological assessment. The expression of BMP-2 and the bone ration was evaluated using histological and immunohistochemical methods.

Results: No significant differences were observed between the simvastatin with hydrogel group and the simvastatin with ACS group at 1, 2, 4, 8 and 12 weeks postoperatively regarding expression of BMP-2, although the number of cells that

stained positive for BMP-2 in both of the implanted groups increased significantly at 2 and 4 weeks postoperatively in comparison with the control group ($P<0.0001$). For new bone area ratio, there were no significant differences between the simvastatin with hydrogel groups and the simvastatin with ACS group after 2, 4, 8 and 12 weeks, although these groups showed higher value than control group ($P<0.0001$).

Conclusion: This study suggests that both the simvastatin with hydrogel and simvastatin with ACS implants showed similar BMP-2 expression and new bone formation, and there were no significant differences between the two carriers.

Introduction

Statins are specific, competitive inhibitors of 3-hydroxy-2-methyl-glutaryl coenzyme A (HMG CoA) reductase, and include naturally occurring lovastatin, chemically modified simvastatin and pravastatin and the synthetically derived atorvastatin, fluvastatin, and cerivastatin (Henwood & Heel 1988; Kishida et al. 1991; Todd & Goa 1990). All of these agents are widely used to lower cholesterol, and they provide an important and effective approach towards the treatment of hyperlipidemia and arteriosclerosis (Hunninghake 1998). However, statins also appear to modulate bone formation, inflammation, and angiogenesis. The suggestion that statins can increase bone formation has provided an exciting new direction for research as well as providing a greater understanding of the biological importance of cholesterol synthetic pathways. Simvastatin, mavastatin, and lovastatin have been shown to stimulate bone formation (Mundy et al. 1999).

Statins (Simvastatin in particular) can promote osteoblast viability and differentiation via membrane-bound Rss/Smad/Erk/BMP-2 pathway (Chen et al. 2010). Bone morphogenetic proteins (BMPs) are active bone-inducing factors that act on immature mesenchymal cells, including osteoblasts, resulting in osteogenesis (Wozeny

et al. 1988). The evaluation of the BMP-2 expression is most adequate way to investigate ability of bone healing and regeneration. Si et al (1997) found that the BMP-2 signal was greatest at the stage of intramembranous formation of bone and early chondrogenesis, suggested that BMP-2 mediates the differentiation of meshenchymal cells into osteoblasts and chondroblasts. BMP-2 expression and new bone formation have been reported in our previous studies (Marukawa etal. 2005, Alam et al. 2007, Alam et al. 2009, Marukawa etal. 2010). Therefore, BMP-2 expression was chosen in this study. To develop more effective bone graft materials with enhanced osteogenic properties, alloplastic materials are combined with osteoinductive substances such as BMPs and statin (Yamomoto et al. 2003; Tanigo et al. 2010). Numerous carriers were shown to be compatible with the osteoinductive activity (Uludag 1998). Among them, collagen-based carriers are being used in clinical settings. Wong *et al.* (2005) demonstrated that purified fibrillar collagen is a good delivery vehicle for statins and is osteoinductive when grafted into skull defects.

Medgel® (gelatin hydrogel) is a gelatin-based hydrogel for the sustained release of the drug. It was developed on the basis of the research results of Tabata *et al.* (Tabata et al. 1994; Yamamoto et al. 2000; Ozeki et al. 2001; Yamamoto et al. 2003; Tanigo et al. 2010). However, it has not yet been approved for clinical use in humans.

Gelatin is a denatured form of collagen, which is the most abundant component of extracellular matrix in body tissue. The material itself and the product when degraded are both biocompatible. The advantages of gelatin are the ease of chemical modification and the commercial availability of materials with different physicochemical properties. Tabata *et al.* have prepared hydrogels with different biodegradabilities from gelatin or the derivatives and succeeded in augmenting the therapeutic activities of water-soluble drugs, such as growth factors, chemokine, plasmid DNA, anti-tumor drug, and siRNA (Fukunaka et al. 2002; Konishi et al. 2003; Matsumoto et al. 2006; Nakamura et al. 2008; Kimura & Tabata 2010). Thus, since this material could be expected to regenerate new bone, this was selected in this study.

Teruplug® (ACS) was developed as a form of sponge by combining fibrillar atelo-collagen with gelatin, to minimize antigenicity, which is cross-linked by heat treatment for biocompatibility (Koide et al. 1993). It features a sponge block design and is shaped for easy insertion in the extraction wound. It consists of between 85 and 95% of collagen type I and between 5 to 15% of collagen type III. The raw material for the collagen is derived from bovine skin. This material can be used for humans, and it is inserted in the extraction wound where alveolar bone is exposed. It protects wounds and promotes the formation of granulation. This material has adequate residual character of

the volume, so that it can have space maintain mechanical properties and surrounding cell can infiltrate (Matsui 2008). It is suggested that this can induce injury healing rapidly and new bone formation. We have used this material in both clinical and experimental studies, and satisfactory results have been obtained. Therefore, this material was selected in this study for use in a clinic setting.

The purpose of this study was to evaluate bone healing following implantation of a statin with two different carriers (atelocollagen sponge (ACS) and hydrogel) in rabbit nasal bone using histological and immunohistochemical methods**.**

Materials and Methods

The experimental protocol was approved by the Institutional Committee for Animal Care, Kanazawa University.

Experimental animals

Twenty white, male Japanese rabbits (12-16 weeks, 2.5-3.0 kg) were used in this experiment.

Surgical procedure

The whole procedure was performed under sterile conditions. First, the animals were anesthetized with sodium pentobarbital (25 mg/kg), injected into the lateral ear vein. Then the hair on the nasal bone was 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 line were exposed via a perpendicular incision. With the use of a fissure bur, four nasal bone windows were outlined. A surgical defect (5mm in diameter) was made with a fissure bur using continuous saline irrigation. Small leads of mechanical pencil were implanted beside the defects as markers to distinguish the defect area from periphery bone at later period. Great care was taken to avoid injuring the nasal membrane**.** Five bone defects per rabbit were created in the nasal bone while preserving the nasal membrane (Fig. 1). In the experimental groups, one group was implanted with 2.5 mg/ml simvastatin (Wako Junyaku Inc., Osaka, Japan) dissolved in 0.2 ml water with hydrogel (Medgel®, Medgel Co. Kyoto, Japan). Gelatin hydrogel incorporating simvastatin was dropped onto freeze-dried Medgel® and left overnight at 4 ºC. The second group was implanted with 2.5 mg/ml simvastatin dissolved in 0.2 ml water with an atelocollagen sponge (ACS) (Teruplug®, Terumo Co. Tokyo, Japan), in the third group only the hydrogel was implanted and the forth group only ACS was implanted.

No material was implanted in the control group. In short, an animal included 5 defects (groups). 4 animals were sacrificed in each period, at 1-, 2-, 4-, 8- and 12 weeks postoperative. Bone defect in this study is defined as a critical defect in size. This size has been reported to prevent spontaneous healing during animal's lifetime.[9]

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Immunohistochemical examination

The specimens were fixed with 10% buffered formaldehyde overnight at 4°C, and demineralized with 14% EDTA for 4 weeks. The specimens were dehydrated with a graded ethanol series, cleared with xylene and embedded in paraffin. Five-mm multiple sections were cut parallel to coronal plane of the head at the center region in each defects and mounted on gelatin-coated glass slides. Firstly, the prepared sections were stained with hematoxyline and eosin (HE). The new bone area ratio was measured with an image software (Scion image, Scion corporation ML, USA) (Fig.2). The measurement was performed 5 times by an author (A.M.) to confirm the reproducibility of the scores and mean value was used as results.

Then, they were treated successively with 0.3% tween 20 (Tokyo chemical industry Co.LTD, Tokyo, Japan) in phosphate buffered saline (PBS) for one hour for cell

permeabilization, and then with 0.3% hydrogen peroxide in methanol for 10 min to inhibit intrinsic peroxides activity. They were then incubated overnight at room temperature with antibody BMP-2 (Wako, Osaka, Japan) at a 1: 100 dilution in PBS. After washing with PBS, the sites of the immunoreactions were visualized by incubating the sections successively with biotinylated anti rabbit IgG antibody at a 1: 200 dilution for 1hr, horseradish peroxides –conjugated streptavidin (Dako Japan, Tokyo, Japan) at a 1: 300 dilution for 1 hr, and 0.01% diaminobenzidine tetrahydrochloride in the presence of 0.02% hydrogen peroxide in 50 mM Tris-HCL, pH 7.5 for 10 min.. The sections counterstained with hematoxylin were observed under on Olympus BX 50 microscope (Olympus; 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 observation area was determined at the center region of the bone defect area (material implanted area) (Fig.2). The number of BMP-2 stained cells per voluntary 1000 cells in this area was counted manually using a high magnification photomicrograph (×100). The measurements were performed 5 times by an author (A.M.) to confirm the reproducibility of the scores, mean value was used as results.

Statistical Analysis

Data of all the implanted materials were statistically analyzed with Stat View 4.5 (ABACUS Concepts, Inc., Berkeley, CA, USA) and Dr. SPSSII (SPSS Japan Inc., Tokyo, Japan). Time-dependent changes were examined by analysis of variance (repeated measure ANOVA), after test for assumption of normality in each group were made. Differences between groups were analyzed by non-paired comparison using Scheffe's F test. Differences were considered significant at $P < 0.05$.

Results

Healing progressed uneventfully in all the animals and no postoperative complications were noted during the 12 week observation period. After resting for 3-6 days postoperatively, the animals could move and leap without any notable pain or limitation.

Histological examination

Simvastatin with hydrogel

The hydrogel structure was absorbed remarkably, and fibrous tissue including osteoblasts was partially found in the region 1 week later. Fibrous tissue was observed

after 2 and 4 weeks and was still present after 8 and 12 weeks. Hydrogel could be observed after 4 weeks, but it could not be identified after 12 weeks. New bone formation was observed partially after 2 weeks (bone area ratio: mean 14.8%) and it continued to increase until 12 weeks postoperative (Figs. 3,4,5,7,8 and9).

Simvastatin with ACS

ACS structure was absorbed remarkably, and fibrous tissue including osteoblast was partially found in the region 1 week later. Fibrous tissue was observed after 2 and 4 weeks and was still present after 8 and 12 weeks. ACS could be observed after 4 weeks, but it could not be identified after 12 weeks. New bone formation was observed partially after 2 weeks (bone area ratio: 14%) and it continued to increase until 12 weeks postoperative (Figs. 3,4,5,7,8 and9).

Hydrogel alone

Hydrogel was absorbed remarkably after 1 and 2 weeks, but formation of fibrous tissue including osteoblast was slower than simvastatin with two carriers. The remaining hydrogel structure existed partially until 8 weeks postoperative. Fibrous tissue could be observed after 4, 8 and 12 weeks. New bone was partially observed after 2 weeks (bone

area ratio: mean 0.5%), and it increased after more than 4 weeks (Figs. 3,4,5,7,8 and9).

ACS alone

ACS was absorbed remarkably after 1 and 2 weeks, but formation of fibrous tissue including osteoblast was slower than simvastatin with two carriers. The remaining of the ACS structure existed partially until 8 weeks postoperative. Fibrous tissue could be observed after 4, 8 and 12 weeks. New bone was partially observed after 2 weeks (bone area ratio: mean 0.8%), and it increased after more than 4 weeks (Figs. 3,4,5,7,8 and9).

Control (no material)

The blood clot was still present after 1 week. Fibrous tissue could be observed after 2 weeks, and it filled in the defect after 4 weeks. New bone could be found slightly after 4 weeks (bone area ratio: mean 0.8%), and it increased after more than 8 weeks (Figs. 3,4,5,7,8 and9).

Epithelial tissue except for nasal membrane and muscular tissue were not found in all observation area of defects.

Statistical analysis

Number of BMP-2 stained cells

For BMP-2 antibody, these time-course changes showed statistically significant differences by ANOVA (between subjects; $F=60.666$, $df=4$, $P<0.0001$; within subjects; $F=14.098$, $df=16$, $P<0.0001$). After 2 weeks, the simvastatin with hydrogel group and the simvastatin with ACS group showed maximum values for the number of BMP-2 stained cells (Fig.5). However, there were no significant differences between both groups after 2, 4, 8 and 12 weeks. Similarly, there were no significant differences between the hydrogel alone group and the ACS alone group after 2, 4, 8 and 12 weeks. After 1 week, the simvastatin with ACS group was significantly larger than the control group ($P=0.0150$), however there were no significant differences between other groups. After 2 weeks, the simvastatin with hydrogel group showed a significantly higher value than the hydrogel alone group ($P=0.0011$) and the control group ($P=0.0002$). Similarly, the simvastatin with ACS group also showed a significantly higher value than the ACS alone group ($P=0.0029$) and the control group ($P=0.0008$). However, there were no significant differences between the hydrogel alone group and the control group, and between the ACS alone group and the control group. After 4 weeks, simvastatin with

ACS group significantly showed higher value than the control group (P=0.0199). However, there were no significant differences between the other groups. After 8 weeks, there were no significant differences in all groups. After 12 weeks, the control group showed a higher value than the other groups (*vs.* the hydrogel alone group; P=0.0160, *vs.* the ACS alone group; P=0.089, *vs.* the simvastatin with hydrogel group; P=0.0248, *vs.* the simvastatin with ACS group; P=0.0439). However, there were no significant differences between the groups regarding implanted materials (Fig. 10, Table 1 and 3).

Bone area ratio

For new bone area ratio, these time-course changes showed statistically significant differences by ANOVA (between subjects; F=12792.687, df=4, P<0.0001; within subjects; F=247.645, df=16, P<0.0001). There were no significant differences between the simvastatin with hydrogel groups and the simvastatin with ACS group after 2, 4, 8 and 12 weeks. Similarly, there were no significant differences between the hydrogel alone group and the ACS alone group after 2, 4, 8 and 12 weeks. The simvastatin with hydrogel group and the simvastatin with ACS group were significantly larger than hydrogel alone group and ACS alone group after 2, 4, 8 and 12 weeks (P<0.0001). The control group was significantly smaller than any other groups after 2, 4,

8 and 12 weeks ($P<0.0001$) (Fig. 11, Table 2 and 4).

Discussion

In recent years, interest has been shown in the potential effects of statins that appear to be different from those well-known on serum cholesterol. Among these, the possible effect of statins on bone tissue has received particular attention (McFarlane et al. 2002). Mundy *et al.* (1999) first reported that simvastatin stimulated *in vivo* bone formation in rodents and increased new bone volume in cultures from mouse calvaria. Recently, several reports about the positive effect of statins on bone tissue have been confirmed both *in vitro* and *in vivo* (Maeda et al. 2001; Ohnaka et al. 2001; Montagnani et al.2003; Uzzan et al. 2007).

HMG-CoA reductase is one of the rate-limiting enzymes within the mevalonate pathway, through which cholesterol is biosynthesized. This enzyme is effectively inhibited by statins causing a reduction in blood-cholesterol levels. Other products of the mevalonate pathway are also important for the prenylation of some kinds of small GTPases. Since small prenylated GTPases are important both for activating osteoclasts and inhibiting the synthesis of BMP-2, statins inhibit the prenylation of

small GTPases and, as a result, they can increase bone mass systemically (Rogers 2000).[20]

In this study we evaluated the bone regeneration process using two different carriers and we compared the bone regenerative capability of these materials histologically and immunohistochemically in an animal model. Regarding use of the experimental model, the region of nasal bone of rabbit was comparatively flat and wide, so that bone defects with equal size could be made easily. Therefore, it was possible to compare the 5 bone defects with similar condition in an rabbit. Furthermore, our previous study suggested that 5 mm diameter defects in the rabbit nasal bone were adequate size to observe the bone regeneration. It was considered that this animal model and the location and dimension of the defect were valid. Hydrogel and ACS of the same size were used as carriers in all groups except in the control group so that the same amount of space would be available for bone regeneration.

Several carriers with suitable characteristics have been developed (Ueki et al. 2003, Inoda et al. 2004; Alam et al. 2009). Gelatin hydrogel is a gelatin-based hydrogel for the sustained release of the drug. . It was developed on the basis of the research results of Tabata *et al.* (Tabata et al. 1994; Yamamoto et al. 2000; Ozeki et al. 2001; Yamamoto et al. 2003; Tanigo et al. 2010). ACS was developed as a form of

sponge by combining fibrillar atelo-collagen with gelatin, to minimize antigenicity, which is cross-linked by heat treatment for biocompatibility (Koide et al. 1993). An ideal carrier should not only be resorbed and nonimmunogenic, but it should also provide a three-dimensional structure as a scaffold for new bone formation. In this study, Hydrogel and ACS were selected as carriers. Hydrogel is used solely for experimental studies and cannot be used for human treatment yet. On the other hand, ACS can be administered to humans in a clinical setting, because it was approved by ministry of health, labour and wealth in Japan.

Simvastatin is one of many water-insoluble drugs. Therefore, Tanigo *et al.* (2010) reported that simvastatin could be made water soluble by gelatin grafted with L-lactic acid oligomer and mixed with gelatin, followed by chemical cross linking to obtain gelatin hydrogels incorporating water-soluble simvastatin. However, Mundy *et al.* (1999) first reported that statins stimulated bone formation in rodents *in vivo* and that they increased the volume of new bone in cultures from mouse calvarium. The enhancement of bone formation by statins is associated with an increase in the expression of BMP-2 through the activation of the gene promoter. Wong *et al.* (2005) also reported that a pro-drug statin (Zocor®) dissolved in water mixed with absorbable collagen sponge can induce BMP expression in surrounding tissues directly, and they

found that following implantation of simvastatin, new bone was observed at 5 days post-operative. In this study, there were no findings to suggest the induction of inflammation in any of the specimens at 1 week post-operative. Osteoblasts and fibroblasts were clearly observed at 2 weeks post-operative, BMP expression was maximum and the formation of new bone was partially observed in both simvastatin groups. Significant bone formation was found in the study of Tanigo.*et al.* (2010), when the gelatin hydrogel incorporating statin-micelles was used. Further development of the carrier may make the effective bone formation possible.

Local –dose statin application causes considerable soft tissue inflammation. Stein *et al.* (2005) applied a single dose of 0.1, 0.5, 1.0, 1.5, or 2.2 mg of simvastatin and doses ranging from 0.5 mg to 2.2 mg reduced inflammation to a more clinically acceptable level without sacrificing bone-growth potential. Lee *et al.* (2008) confirm that 0.5 mg of the statin produced the best bone growth/inflammation ratio. In this study, similarly, 2.5 mg/ml simvastatin dissolved in 0.2 ml water was used with two different carries

Osteogenesis induced by osteoblastic cells, is characterized by sequential events involving cell proliferation, followed by the expression of markers of osteoblast phenotypes and the synthesis, deposition, and mineralization of a collagenous matrix

(Bellows et al. 1986; Maniatopoulos et al. 1988). Bone formation depends mainly on the number of osteoblastic cells rather than the activity of the osteoblasts (Marie 1995). The recruitment of osteoblastic cells plays a crucial role in osteogenesis. This suggests that new bone formation might be seen over a longer post-operative time period than in the current study.

In the present study, most hydrogel and ACS structures containing simvastatin could not be seen at 8 weeks post-implantation but could be seen partially in the hydrogel alone group and ACS alone group. This suggested there was no significant histological difference between the two carriers regarding absorption of the carrier structure.

The change in BMP-2 expression over time showed significant differences when the two simvastatin groups were compared with the control group ($P < 0.0001$). Furthermore, when data of the multiple comparison analysis with Scheffe's F test at each period were added, our statistical analysis suggested that BMP-2 was expressed more intensely in both groups at 2 weeks post-operative than at the other post-operative times. Only the simvastatin with ACS group showed higher values than the control group at 1 and 4 weeks postoperative, although there was no significant difference between the simvastatin with hydrogel and the control groups. This result might be

due to the difference in drug release or the properties of the two carries. At 12 weeks, the control group showed a higher value than the other groups. This suggested that though slow, there was new bone forming ability even when no material was implanted. However, the sample size in each post-operative period was very small and the data varied notably such that the significant differences obtained with Scheffe's F test could not be considered as accurate. Therefore, further examination using a larger sample size is required.

New bone area ratio showed maximum value at 12 weeks postoperative in all the groups. New bone formation in two groups that used simvastatin was recognized at an earlier stage than the two different carriers alone. Furthermore, use of these carriers could induce new bone formation much more than the control group. This proved that the carriers preserved the defect space and act as scaffolds for osteoblast and vascular proliferation. However, if progress was observed for more than 24 weeks, bone regeneration would have been complete in all the groups.

Actually, in the current model is impossible to identify whether or not the observed osteoblast were "host" osteoblast proliferating from the margins of the defect or the product of differentiation process of mesenchymal cells coming from other sources as result of the osteoinductive properties of statins. Therefore, number of

BMP-2 stained cell was counted at the center region of the defects. However, the fact that high number of BMP-2 stained cells was observed at somehow later stages in the control groups supported that osteoinductive ability is not necessarily induced by statin.

This immunohistochemical studies demonstrated that BMP-2 was strongly expressed in the connective tissue and periosteum following the implantation of the statin in the rabbit nasal bone. In addition, BMP-2 expression at every postoperative week of tissue examination was similar in the sites where statin with hydrogel and statin with ACS were used. We conclude that statin with hydrogel and statin with ACS produced similar BMP-2 expression and osteoinductive activity. In short, there was no significant difference between hydrogel and ACS, when the statin was used to regenerate new bone. In the future, the hydrogel will be more useful carrier in term of drug release by improvement fore-mentioned, although it can not be used clinically now. It was difficult to compare between the hydrogel and the ACS in the material ptoperties and resorption simply. However, when we used the statin with carrier clinically now, the ACS can induce similar new bone formation as the carrier. The results of this study can be useful to assert that early introduction of the use of statin in clinical treatment is beneficial for bone regeneration.

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Legends

FIGURE 1. Intra-operative finding. (A) Bone defect was made at the nasal region. (B) Diagram of the superior view of rabbit skull. Showing the sites of 5 surgically –created bone defects (1) simvastatin with hydrogel, (2) simvastatin with ACS (3) hydrogel alone (4) ACS alone and (5) Control(no material) that were implanted within the defects.

FIGURE 2. The coronal section of the nasal region with implanted materials. The yellow colored area indicates the area of observation.

FIGURE 3. Histological images of total area after 2 week. (1) simvastatin with hydrogel, (2) simvastatin with ACS, (3) hydrogel alone, (4) ACS alone and (5) Control, (hematoxylin-eosin staining)

FIGURE 4. Photomicrographs after 1 weeks. (1) simvastatin with hydrogel, (2)simvastatin with ACS, (3) hydrogel alone, (4) ACS alone and (5) Control, (hematoxylin-eosin staining, original magnification ×400), M：mother bone.

FIGURE 5. Photomicrographs after 2 weeks. (1) simvastatin with hydrogel, (2)simvastatin with ACS, (3) hydrogel alone, (4) ACS alone and (5) Control, (hematoxylin-eosin staining, original magnification ×400), M : mother bone. NB : new bone.

FIGURE 6. Photomicrographs after 2 weeks. (1) simvastatin with hydrogel, (2) simvastatin with ACS, Red arrows show BMP-2 stained cells. (Immunohistochemical staining, original magnification × 100(upper photographs), × 600(lower photographs)), M：mother bone. NB：new bone.

FIGURE 7. Photomicrographs after 4 weeks. (1) simvastatin with hydrogel, (2) simvastatin with ACS, (3) hydrogel alone, (4) ACS alone and (5) Control, (hematoxylin-eosin staining, original magnification ×400), M : mother bone. NB : new bone.

FIGURE 8. Photomicrographs after 8 weeks. (1) simvastatin with hydrogel, (2) simvastatin with ACS, (3) hydrogel alone, (4)ACS alone and (5) Control,

(hematoxylin-eosin staining, original magnification × 400), M : mother bone. NB : new bone.

FIGURE 9. Photomicrographs after 12 weeks. (1) simvastatin with hydrogel, (2) simvastatin with ACS, (3) hydrogel alone, (4) ACS alone and (5) Control, (hematoxylin-eosin staining, original magnification × 400), M : mother bone. NB : new bone.

FIGURE 10. The labeling index of BMP-2 positive cells. The time-course of changes in all the groups showed significant differences with ANOVA ($P<0.0001$). SM: simvastatin with hydrogel group, ST: simvastatin with ACS group, NM: hydrogel alone group, NT: ACS alone group, Control: no implantation group.

FIGURE 11. The ratio of new bone. The time-course of changes in all the groups showed significant differences with ANOVA ($P<0.0001$). SM: simvastatin with hydrogel group, ST: simvastatin with ACS group, NM: hydrogel alone group, NT: ACS alone group, Control: no implantation group.

TABLE 1. The labeling index of BMP-2 positive cells. SM: simvastatin with hydrogel group, ST: simvastatin with ACS group, NM: hydrogel alone group, NT: ACS alone group, Control: no implantation group. SD shows standard deviation.

TABLE 2. The ratio of new bone. SM: simvastatin with hydrogel group, ST: simvastatin with ACS group, NM: hydrogel alone group, NT: ACS alone group, Control: no implantation group. SD shows standard deviation.

TABLE 3. Results (P-value) of comparisons between groups in the labeling index of BMP-2 positive cells. SM: simvastatin with hydrogel group, ST: simvastatin with ACS group, NM: hydrogel alone group, NT: ACS alone group, Control: no implantation group. S shows significant difference at $P<0.05$. NS shows no significant difference.

TABLE 4. Results (P-value) of comparisons between groups in the ratio of new bone. SM: simvastatin with hydrogel group, ST: simvastatin with ACS group, NM: hydrogel alone group, NT: ACS alone group, Control: no implantation group. S shows significant difference at $P<0.05$. NS shows no significant difference.

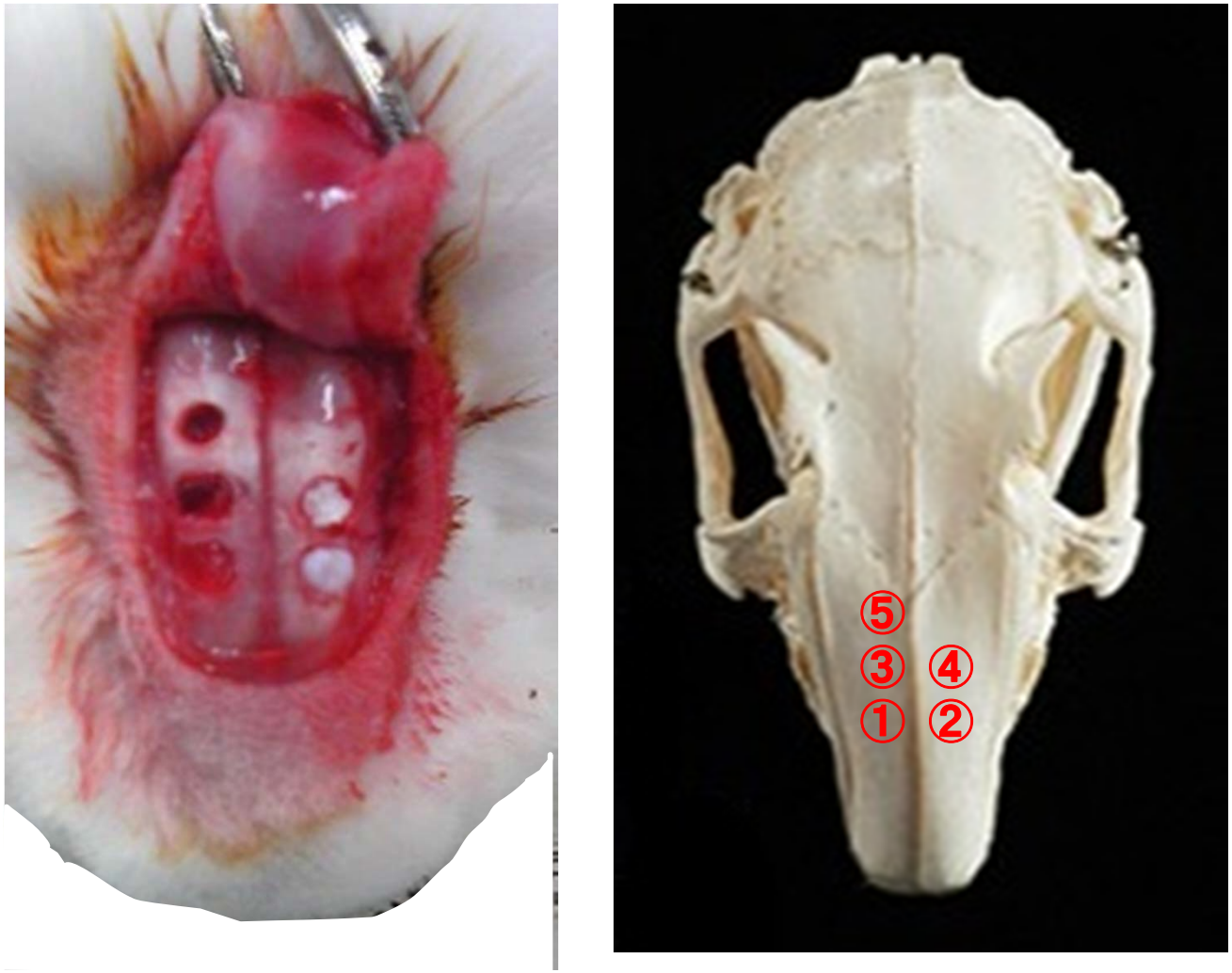


Fig.1

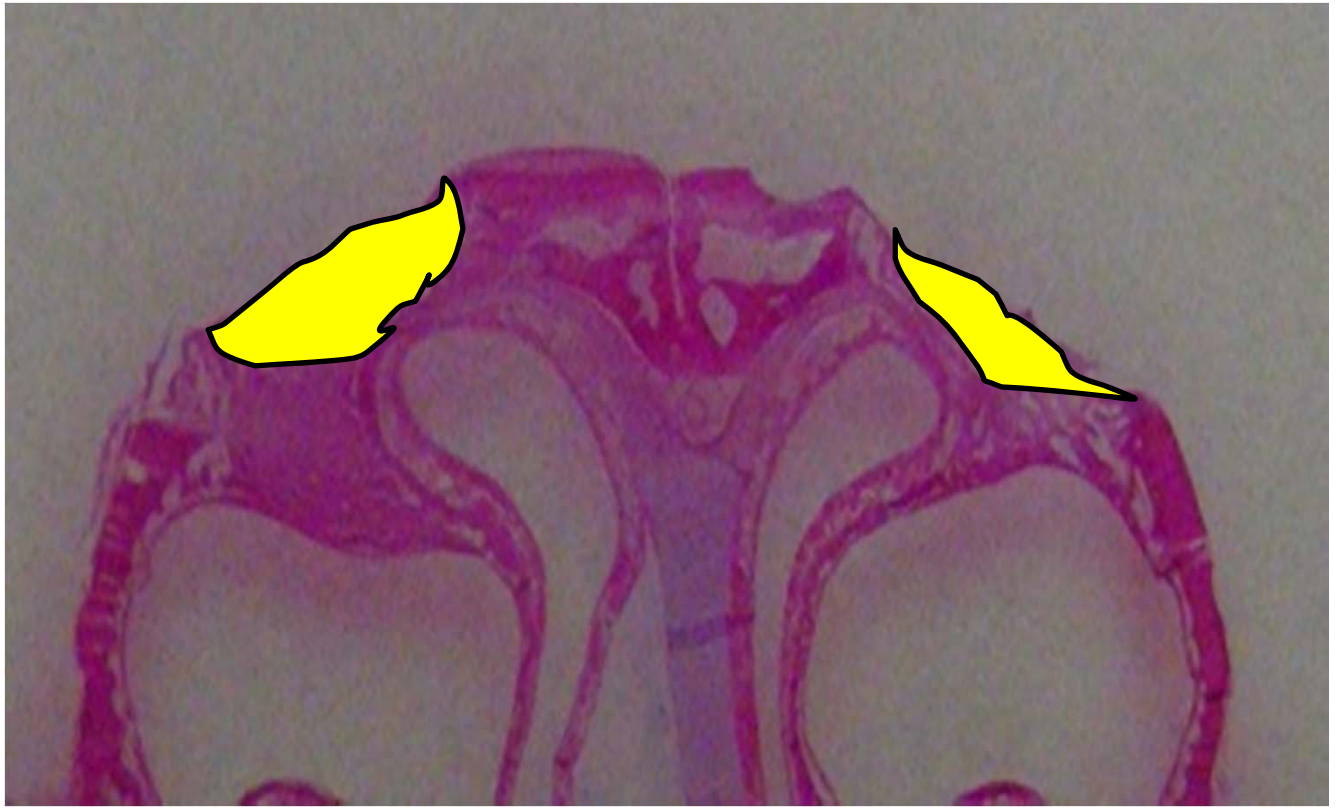


Fig.2

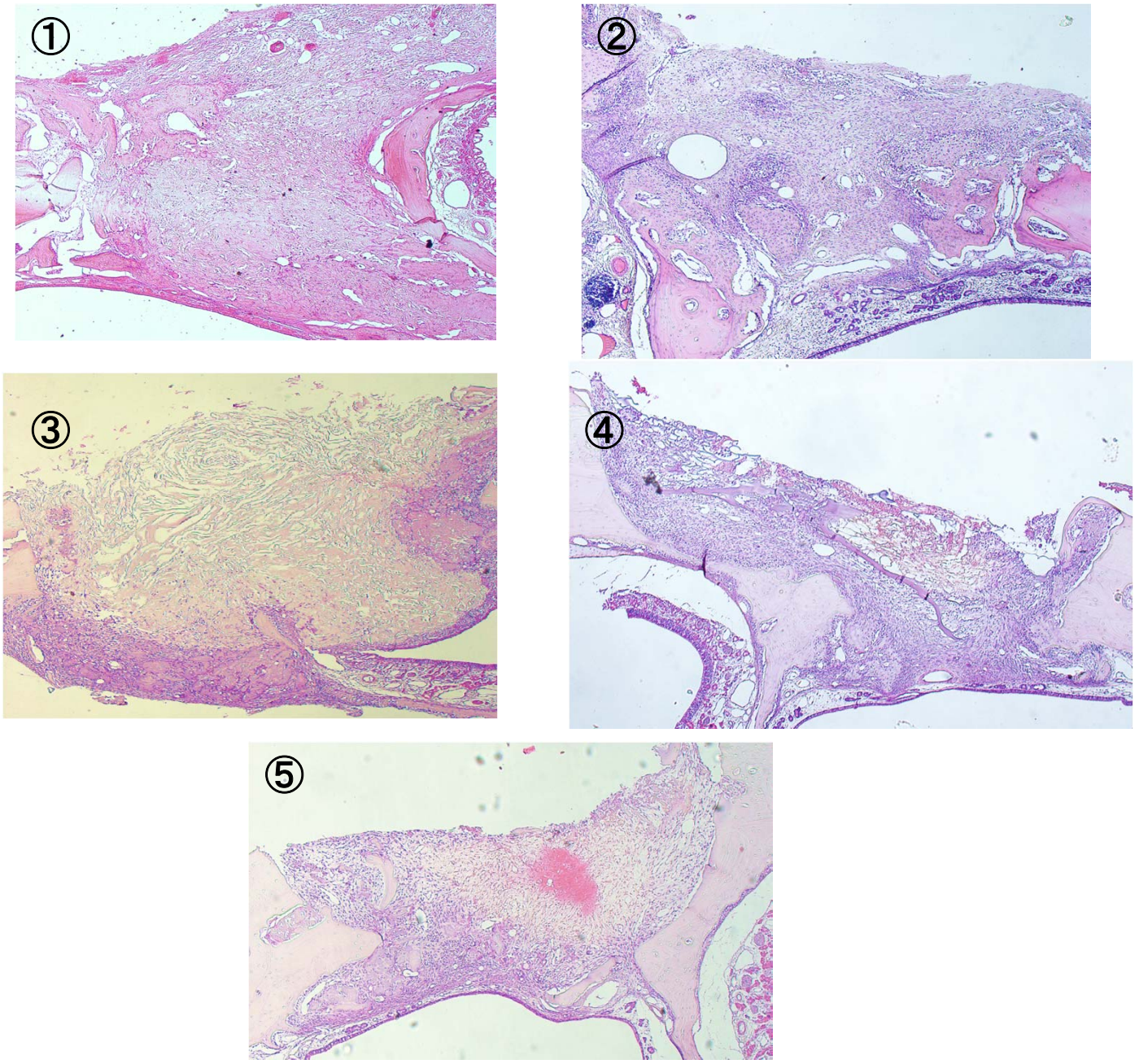


Fig.3

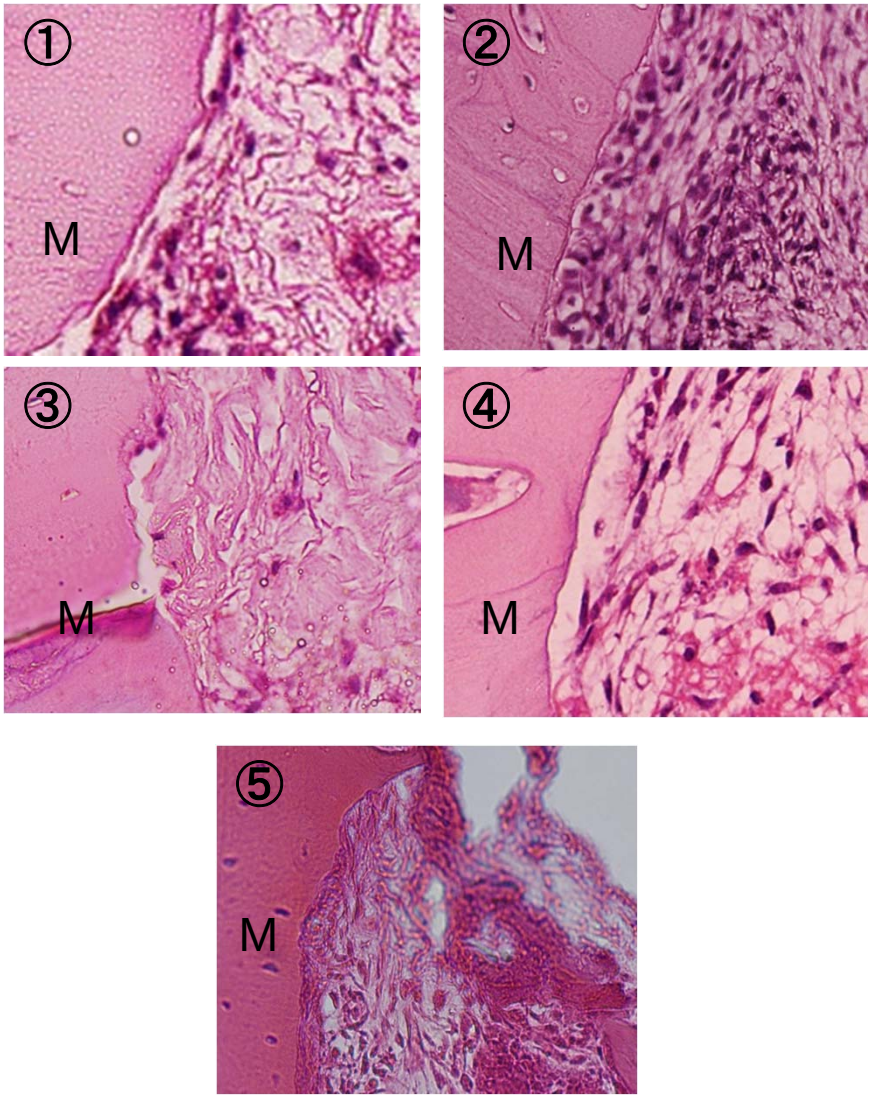


Fig.4

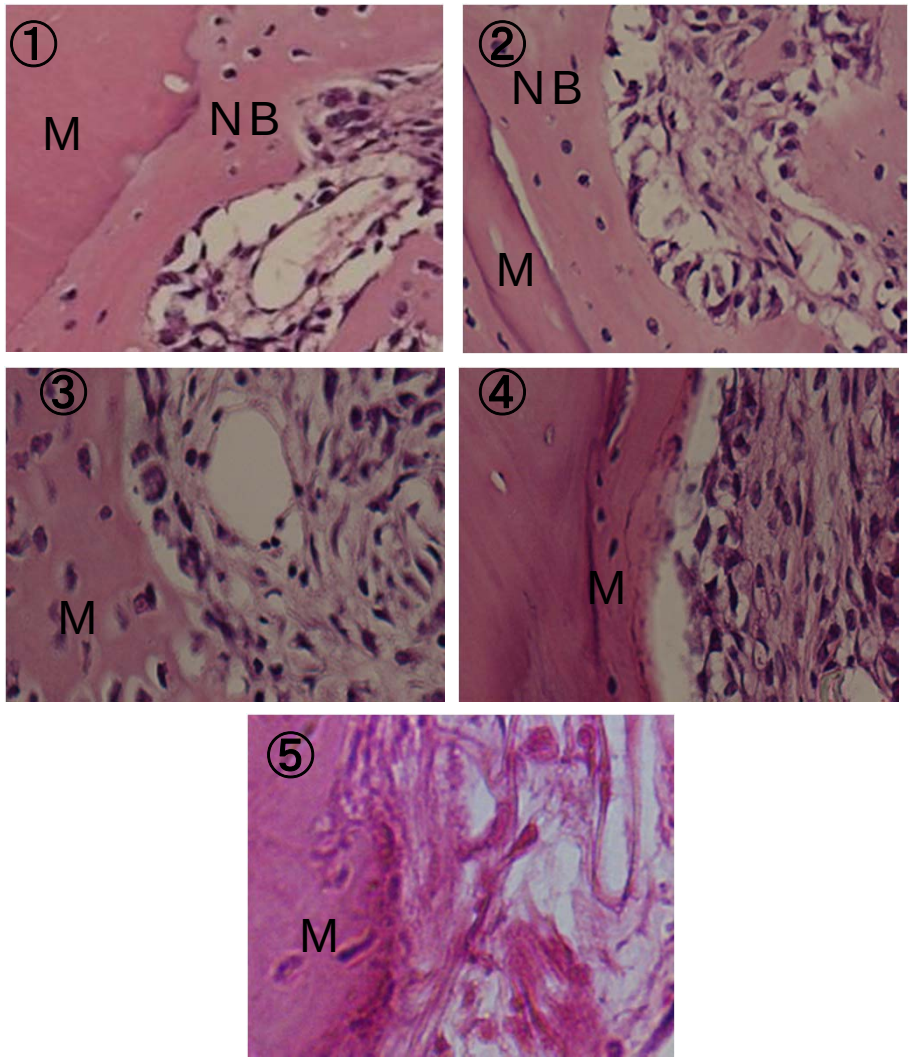


Fig.5

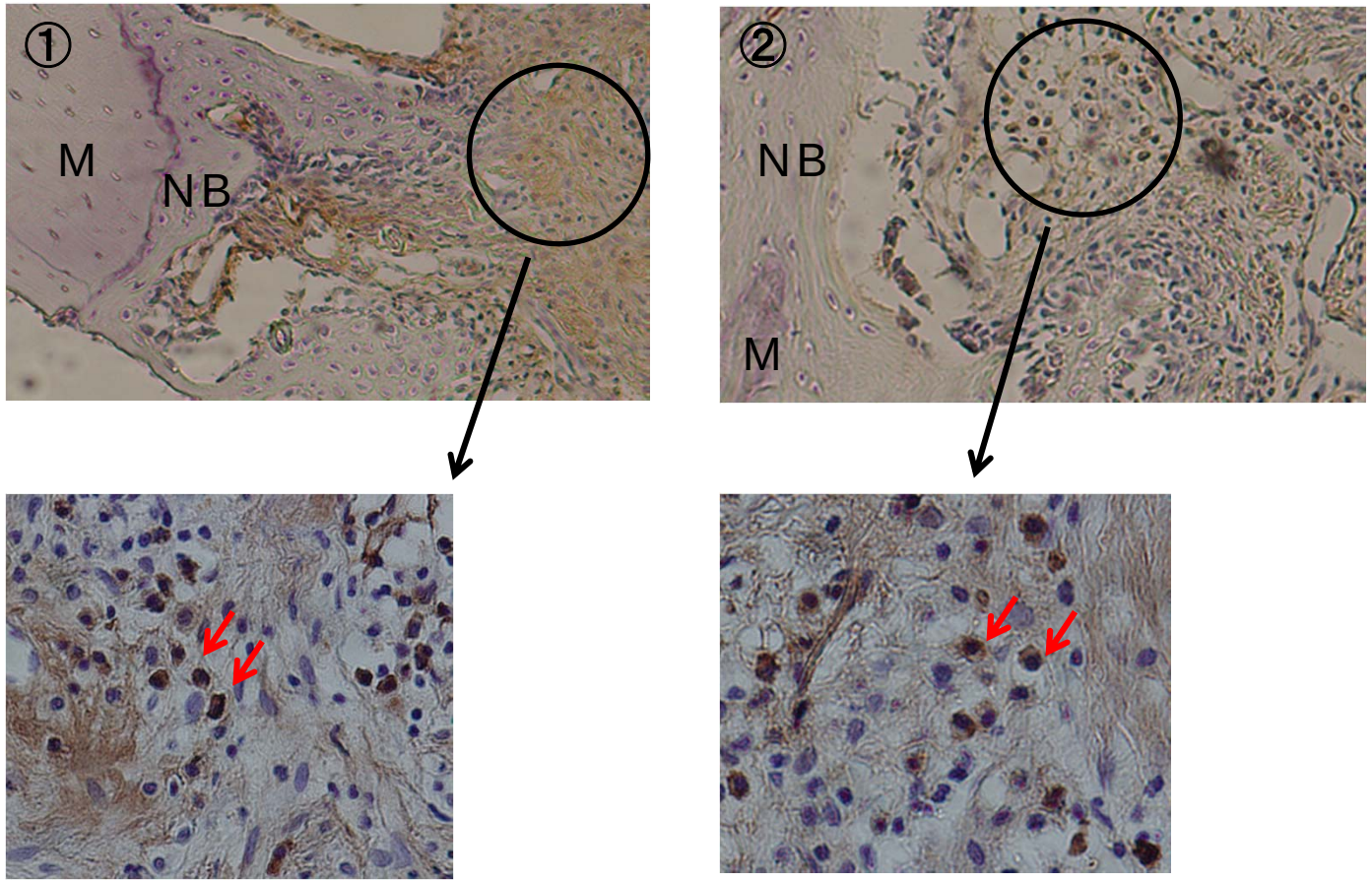
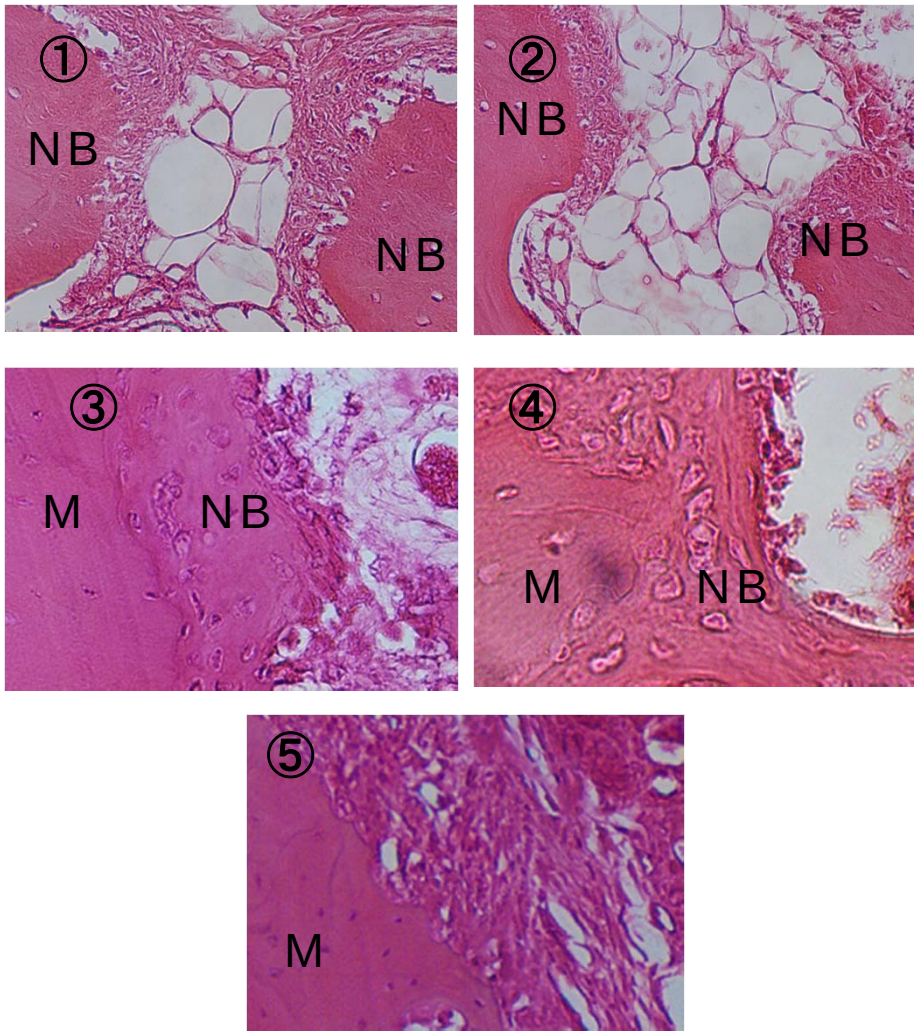


Fig.6



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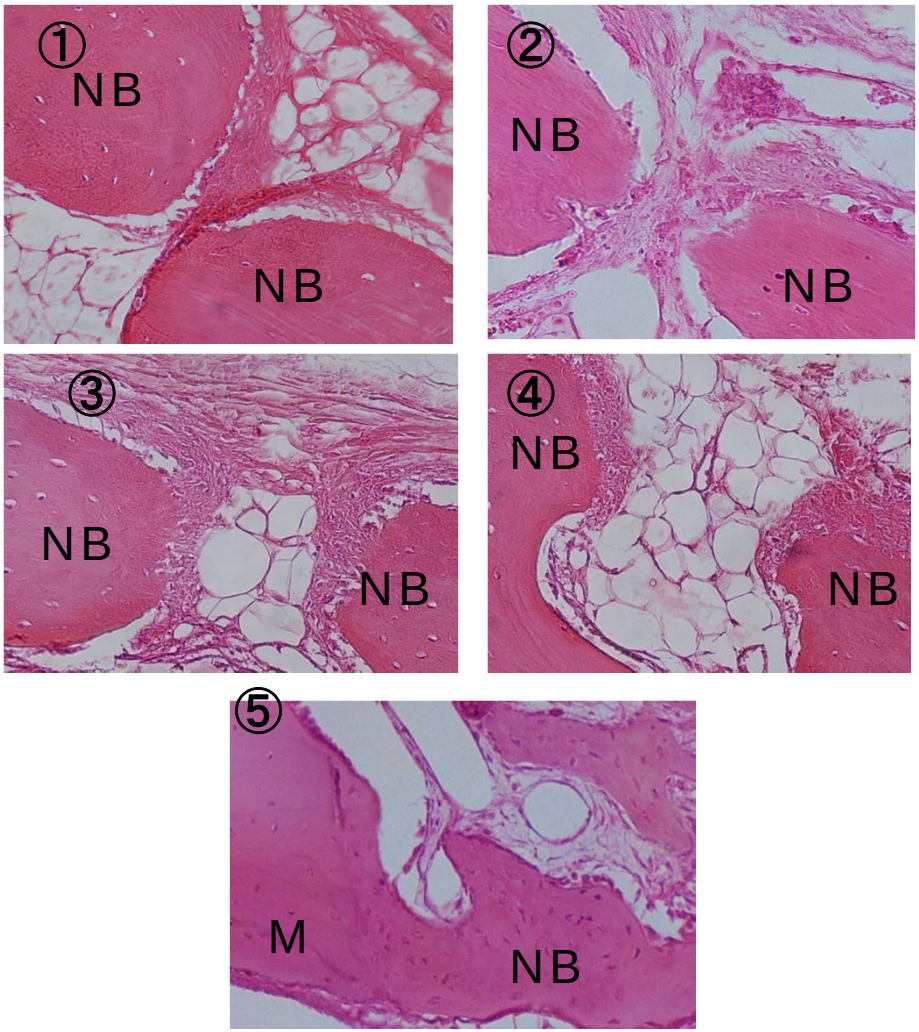

Fig.8

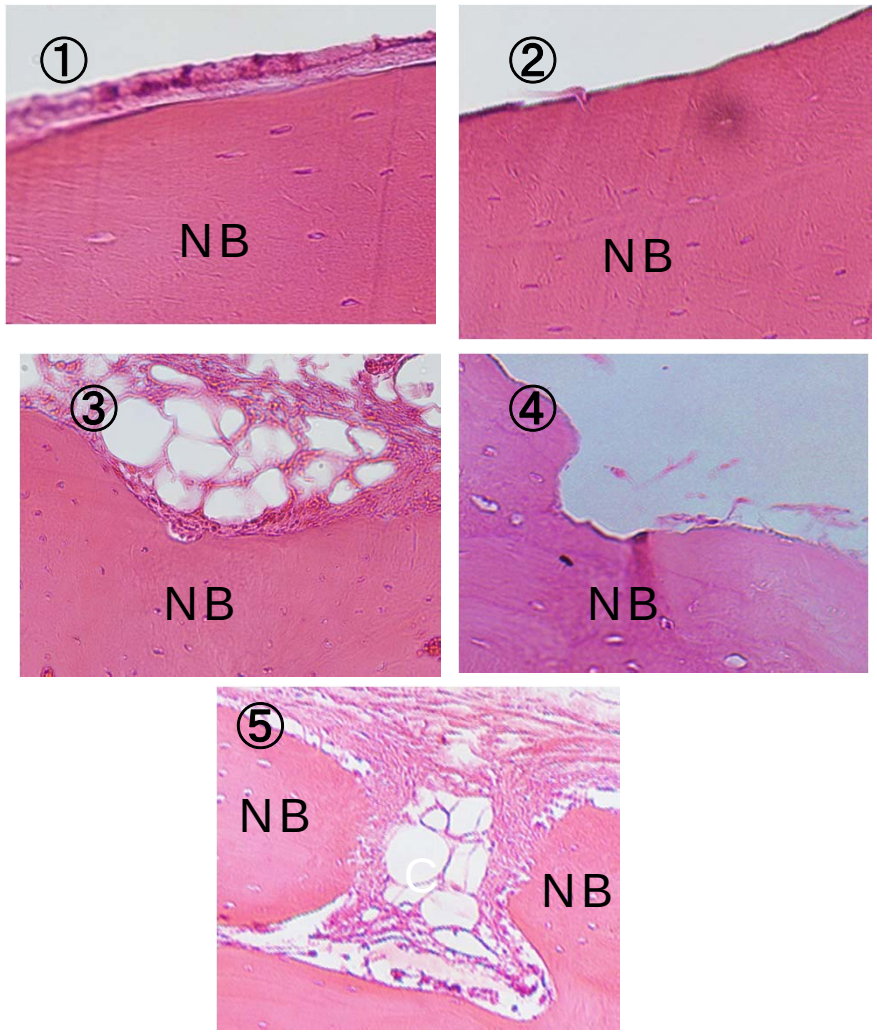

Fig.9

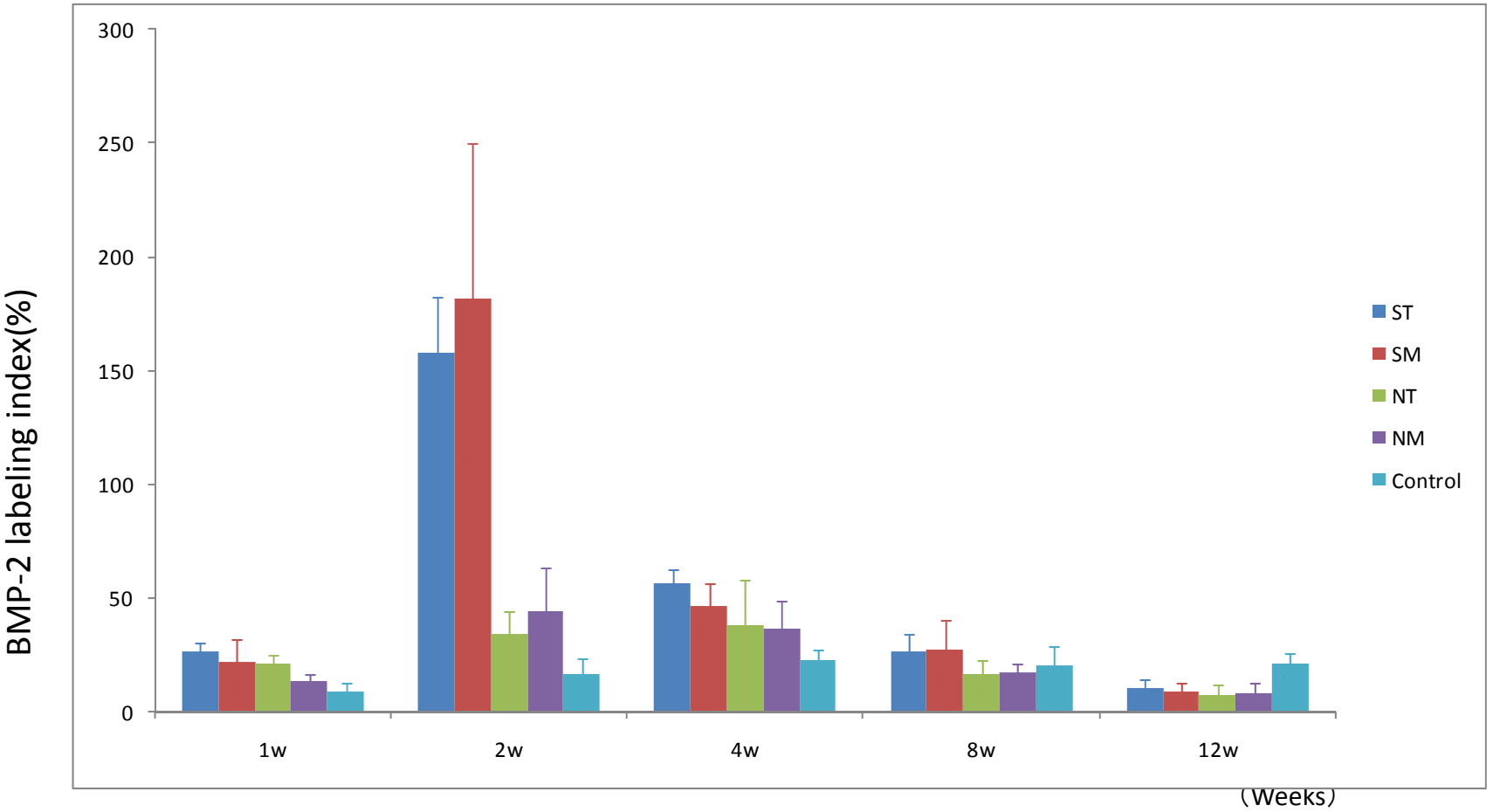
BMP-2 labeling index(%)
300
250
200
150
100
50
0
1w
2w
4w
8w
12w
(Weeks)
ST
SM
NT
NM
Control

Fig.10

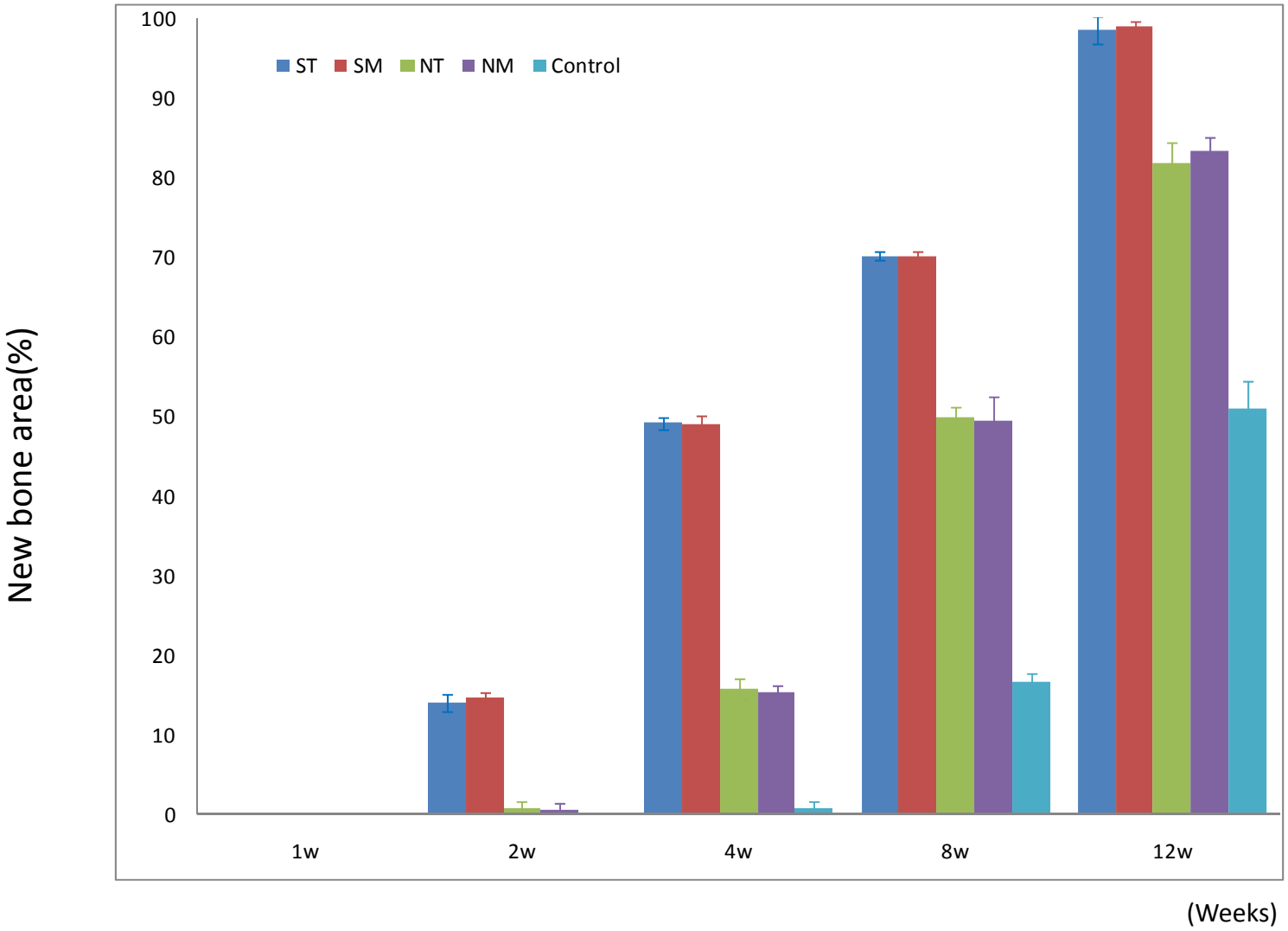
New bone area(%)
100
90
80
70
60
50
40
30
20
10
0
ST
SM
NT
NM
Control
1w
2w
4w
8w
12w
(Weeks)

Fig.11

	1w		2w		4w		8w		12w	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ST	26.3	4.6	157.8	24.8	56.8	5.9	26.5	7.7	10.3	4.1
SM	21.8	10.4	182.0	68.3	46.3	10.6	27.3	13.3	9.3	3.6
NT	21.3	4.0	34.3	10.4	38.3	20.0	16.8	6.2	7.5	4.2
NM	13.5	3.1	44.3	19.3	36.8	11.8	17.8	3.8	8.5	4.5
Control	9.0	3.7	16.5	7.4	22.8	5.0	20.3	8.9	21.0	4.8

Table.1

	1w		2w		4w		8w		12w	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ST	0.0	0.0	14.0	1.1	49.1	0.8	70.1	0.6	98.5	1.7
SM	0.0	0.0	14.8	0.5	49.1	1.0	70.0	0.7	99.0	0.7
NT	0.0	0.0	0.8	1.0	15.7	1.2	49.7	1.5	81.8	2.5
NM	0.0	0.0	0.5	1.0	15.4	0.7	49.4	3.0	83.3	1.7
Control	0.0	0.0	0.0	0.0	0.8	1.0	16.6	1.0	50.8	3.6

Table.2

BMP-2	1w	2w	4w	8w	12w
ST vs SM	NS	NS	NS	NS	NS
NT vs NM	NS	NS	NS	NS	NS
ST vs NT	NS	0.0029S	NS	NS	NS
SM vs NM	NS	0.0011S	NS	NS	NS
ST vs Control	0.015S	0.0008S	0.0199S	NS	0.0439S
SM vs Control	NS	0.0002S	NS	NS	0.0248S
NT vs Control	NS	NS	NS	NS	0.0089S
NM vs Control	NS	NS	NS	NS	0.0160S

Table.3

Bone area ratio	1W	2w	4w	8w	12w
ST vs SM		NS	NS	NS	NS
NT vs NM		NS	NS	NS	NS
ST vs NT		<0.0001S	<0.0001S	<0.0001S	<0.0001S
SM vs NM		<0.0001S	<0.0001S	<0.0001S	<0.0001S
ST vs Control		<0.0001S	<0.0001S	<0.0001S	<0.0001S
SM vs Control		<0.0001S	<0.0001S	<0.0001S	<0.0001S
NT vs Control		NS	<0.0001S	<0.0001S	<0.0001S
NM vs Control		NS	<0.0001S	<0.0001S	<0.0001S

Table.4