# Use of self-setting α-tricalcium phosphate for maxillary sinus augmentation in rabbit

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**Abstract**

**Purpose:** The purpose of this study was to histologically and immuno-histochemically evaluate tissue changes in the maxillary sinus after bone screw implantation and maxillary sinus augmentation using self-setting α-tricalcium phosphate (α-TCP; BIOPEX®-R) in rabbit.

**Study design:** Adult male Japanese white rabbits (n=15, 12-16 weeks, 2.5-3.0 kg) were used. The sinus lift was made from the nasal bone of a rabbit. The bone screws (Dual top auto-screw®) were implanted into the nasal bone, after BIOPEX®-R was implanted in the left elevated space (operated side) an atelocollagen sponge (ACS: Teruplug®) was implanted in the right elevated space (control side). The rabbits were sacrificed at 4, 12 and 24 weeks postoperatively, and formalin-fixed specimens were embedded in acrylic resin. The specimens were stained with hematoxylin and eosin. For immune-histochemical analysis, the specimens were treated with bone morphogenetic protein-2 (BMP-2) antibodies. Finally, these were evaluated microscopically.

**Results:** Tight bonding without fibrous tissue continued between the bone screw and BIOPEX®-R, and the rigidity of bone screw in the nasal bone kept for 24 weeks in all cases. The area of new bone formation increased gradually on both sides, however

there was no significant difference between both sides at 4, 12 and 24 weeks. The number of BMP-2 stained cells on the experimental side was significantly larger than that on control side after 4 weeks (P=0.0361).

**Conclusion**: This study suggested the usefulness of self-setting α-tricalcium phosphate (BIOPEX® -R) to keep the rigidity of implanted bone screws from an early period, and the result of BMP-2 expression suggested that BIOPEX® -R could have bone conductive activity in the maxillary sinus augmentation.

## Introduction

Since, the maxillary sinus floor elevation was introduced by Taum and Boyne (Boyne & James 1980; Taum 1986), the use of autogenous bone grafts in sinus augmentation has been considered to be the " gold standard" because of their excellent survival with loaded implants and the degree of functionality they afford (Kent & Block 1989; Raghoebar 1993; Neukam 1994; Nishibori 1994). However, harvesting autogenous bone is generally associated with several limitations, including morbidity, infection, pain and blood loss. To avoid the limitations associated with autogenous sources of bone, an array of alternatives, including allograft, xenograft and synthetic materials, has been explored. However, allografts and xenografts are susceptible to immunorejection and carry the risk of disease transmission (Moore et al. 2001; Simon et al. 2002; Ueda et al. 2005). Some synthetic materials have limited potential for osteoconduction. Using them alone has usually failed to achieve the bone volume expected (Wiltfang et al. 2002; Engelke et al. 2003).

Bovine hydroxyapatite is frequently used as grafting material in sinus lift procedures because of its specific surface that resembles that of cancellous bone, complete deproteinization of the inorganic component and thus absent antigenicity (Wetzel et al.

1995; Haaset al. 1998). 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. Especially, β-TCP was used as scaffold in combination with hyaluronic acid or bone marrow stromal cells in the sinus lift procedure (Schwarts et al. 2006; Jiang et al. 2009).

On the other hand, Monma et al. (1988) have originally developed a self-setting cement-type calcium phosphate material consisting of α-TCP, dicalcium phosphate dibasic (DCPD) and tetracalcium phosphate monoxide (TeCP). According to their extensive studies, this cement-type material could be refined, demonstrating better biocompatibility and direct integration to bone without any participation of peripheral soft tissue (Kurashina et al. 1997a, 1997b; Kurashina et al. 1998; Yamamoto et al. 1998). As it is free of the infiltration with time of residual monomers of methacrylate resin, which has long been used for orthopedic treatment, this self-setting cement came to be rapidly highlighted for clinical use in Japan. In previous reports related to maxillary sinus augmentation, alternative granular types of materials were mainly used as scaffold. However, there was no report regarding the use of self-setting cement for the maxillary sinus lift procedure.

Therefore, an experimental study is necessary to assess the possibility of the use of

self-setting cement in the sinus lift surgery.

The purpose of this study was to histologically and immuno-histochemically evaluate the change in surrounding bone screws and self-setting α-TCP after sinus augmentation surgery in rabbit.

## Materials and Methods

The experimental protocol was approved by the Institutional Committee for Animal Care, Kanazawa University. Fifteen male Japanese white rabbits (12-16 weeks, 2.5-3.0 kg) were used in this study.

### *Surgical procedure*

The entire procedure was performed under sterile conditions. First, the animals were anesthetized with sodium pentobarbital (25 mg/kg) by injection into the lateral ear vein. After the hair in the nasal region was shaved, 1.8 ml of 2% lidocaine containing 1:80,000 epinephrine was administered to the surgical site. The surgical method by Asai et al. (2002) and Xu et al. (2003 and 2004) was modified and the operation was performed. Both the nasal bone and nasoincisional suture line were exposed via a

perpendicular incision. With the use of a fissure bur, two nasal bone windows were outlined. A surgical defect (5mm diameter) was made with a fissure bur using continuous saline irrigation (Fig.1A). The antral membrane was carefully stripped off posteriorly to make space for implantation of materials. The titanium bone screw (length 8 mm, diameter 2 mm, Dual top auto-screw®, Jeil Medical Co, Seoul, Korea) was implanted at 3 mm posteriorly from the surgical defect on each side （Fig. 1B and C). BIOPEX®-R (Pentax Co, Tokyo, Japan) was implanted in the left elevated space (operated side) (Fig.1 D) and 3×3×10 mm atelocollagen sponge (ACS: Teruplug®, Terumo Co, Tokyo, Japan ) was implanted in the right elevated space (control side) . After the rigidity of the implanted screw and self-setting of the implanted cement were recognized, a suture was placed between the periosterum and the skin.

*Histological examination*

The rabbits were sacrificed at 4, 12 and 24 weeks postoperatively and the specimens were collected for hematoxylin and eosin staining and also for immunno-histochemical analysis. After fixation with 10% phosphate-buffered formalin, the specimens with the titanium mesh were dehydrated in ethanol and technovit 7200VCL (Kultzer and Co., GmbH, Wehreim, Germany) and then embedded in acrylic

resin. The embedded blocks were trimmed by a cutter and ground by abrasive paper. Thereafter, the sections were further ground to a final thickness of about 30 μm. Finally, the specimens were stained with hematoxylin and eosin and examined under the microscope. The region of implanted bone screw surface and the region of antral membrane were mainly observed (Fig. 2A). The measurement area was located between the lateral surface of the implanted screw and the elevated antral membrane (Fig.2B). The new bone and BIOPEX®-R areas were measured with an image software (Image-Pro®, Media cybernetics, Silver Spring, MD,USA) and then the ratio of new bone formation (%) was calculated.

The prepared sections were deacrylated in 2-methoxyethyl acetate, inhibited by endogenous peroxidase with 0.3% hydrogen peroxide and blocked in 10% normal serum prior to staining. For immunostaining, commercially available monoclonal anti-BMP-2 antibodies (Dako North America, Inc, CA, USA) were used. Sections were incubated overnight with these primary antibodies at 4ºC in a humidified chamber. A biotinylated goat anti-mouse IgG antibody (Wako Junyaku Inc., Osaka, Japan) was used as the secondary antibody. The Vectastain-Elite ABC kit detection system, DAB revelation kit and DAB enhancing solution (Wako Junyaku Inc., Osaka, Japan) were used to complete the immunostaining. Finally, a light Meyer's hematoxylin counter

stain was applied. 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 located between the elevated antral membrane and BIOPEX®-R far from the implanted screw (Fig.2B). BMP-2 labeling index (%) was determined as the number of BMP-2 stained cells per voluntary 1000 cells in this area that were counted by hand using a high magnification photomicrograph (×400).

*Statistical Analysis*

Data of all the implanted materials were statistically analyzed with Stat View 4.5 (ABACUS Concepts, Inc., Berkeley, CA, USA). Differences between the groups were analyzed by Mann-Whitney U's test. Kruskal –Wallis test was used for the comparisons between the groups at 4, 12 and 24 weeks. Differences were considered significant at $P < 0.05$.

**Results**

Healing progressed uneventfully in all animals and no postoperative complications were noted during the 24-week observation period. After resting for 3-6

days postoperative, the animals could move and leap without any notable pain or limitation.

*Antral membrane region*

After 4 weeks, fibrous tissue or cell components including osteoblasts were recognized on both the experimental and control sides. The area occupied by fibrous tissue on the control side seemed to be comparatively larger than that on the experimental side. New bone formation was barely recognized between the BIOPEX®-R and the antral membrane was accompanied by absorption of the BIOPEX®-R by osteoclasts on the experimental side but not on the control side (Fig.3). On the experimental side, new bone seemed to have been derived not only from the mother bone of the nasal bone, but also the antral membrane.

After 12 weeks, fibrous tissue was found on both the experimental and control sides. Although the elevated space on the control side decreased, that on the experimental side was maintained. New bone formation was first found on the control side. On the experimental side, absorption of surrounding BIOPEX®-R derived from osteoclasts was observed. New bone was also observed in the pores of BIOPEX®-R. (Fig. 4 and 5).

Fibrous tissue between new bone and BIOPEX®-R was not found at all. New bone seems to have been derived from only the mother bone of nasal bone on the control side.

After 24 weeks, new bone formation increased gradually on both sides. On the experimental side, absorption of BIOPEX®-R also increased (Fig. 6).

*Implanted bone screw region*

After 4 weeks, fibrous tissue or cell components including osteoblasts were recognized on the control side. However, on the experimental side, fibrous tissue was not observed and complete bonding between the bone screw surface and BIOPEX was established.

After 12 weeks, fibrous tissue was found on the control sides. New bone formation was first seen on the control side. On the experimental side, absorption of surrounding BIOPEX®-R was not observed and complete bonding between the bone screw surface and BIOPEX®-R remained tight.

After 24 weeks, new bone formation increased gradually on the control side. On the experimental side, absorption of BIOPEX®-R was not found and few new bone

formation areas were observed at the region a little far from the bone screw in the pores of BIOPEX®-R. The bonding between the bone screw surface and BIOPEX®-R remained tight and bonding between the replaced new bone and screw was partially observed (Fig. 7).

*New bone area*

There were no significant differences between the experimental side and control side in the rate of new bone area after 4, 12 and 24 weeks. On both sides, there were significant differences at 4, 12 and 24 weeks in the rate of new bone area ( the experimental side, 4 vs 12 weeks: P=0.0090, 4 vs 24 weeks: P=0.0090, 12 va 24 weeks: P=0.0474)( control side, 4 vs 12 weeks: P=0.0090, 4 vs 24 weeks: P=0.0090, 12 va 24 weeks: P=0.0090) and new bone increased time-dependently (the experimental side: P=0.0037, control side: P=0.0019)(Fig. 8, Table 1).

*BMP-2 labeling index*

The BMP-2 labeling index after 24 weeks was significantly larger than that after 12 weeks on the control side (P=0.0361), but the time-course change was not significant in the experimental side. The BMP-2 labeling index on the experimental side was significantly larger than that on the control side after 4 (P=0.0361). However there was no significant difference between the 2 sides (Fig. 9, Table 2).

**Discussion**

Some models for maxillary sinus elevation using rabbits have been reported and they have played an important role to understand the histological change after the surgery for the use of various grafted materials. As air pressure causes movement of the maxillary sinus membrane, the grafted material in the sinus is subjected continuously to this air pressure, which will ultimately affect the augmented bone healing process and structure (Garey et al. 1991; Asai et al. 2002). In the study of Sun et al.(2007), both the group that used a tissue engineered bMSCs (bone marrow stromal cells) with OsteoBone® and the group that used OsteoBone® alone showed a convex segmented space, which suggested that the grafted material can withstand sinus air pressure and maintain the augmented space. Both groups showed increased bone area. Lamellar bone

structure in histological sections was observed at 8 weeks after surgery. Jiang et al. (2009) concluded that bMSCs modified with the AdBMP-2 (Adenovirus with BMP-2) gene can promote new bone formation and maturation in the rabbit maxillary sinus.

To achieve osseous healing, a grafted material needs to serve as a scaffold for the migration, attachment, and subsequent differentiation of osteoprogenitor cells from the recipient bed (Burchardt 1983; Yang et al. 1996). Various types of biocompatible graft materials have been developed in consideration of bone regeneration and augmentation as well as further procedures such as dental implantation (Simon et al. 1994; Becker et al. 1996; Piatteli et al. 1997). Autogenous bone graft has drawbacks such as the requirement of a second operation, which is sometimes accompanied by broad invasion, and a restricted source of materials. Alloplastic materials composed of inorganic substances such as tricalcium phosphate (TCP) or hydroxyapatite show biocompatibility and osteoconductivity (Leadley et al. 1991; Hollinger et al. 1996), which are the major reasons why this type of material has been widely employed for hard tissue repair (Oreamuno et al. 1990; Steven et al. 1991; Guillemin et al. 1993).

In the study of Jiang et al. (2009), the augmented space in all groups using β-TCP as scaffold showed a convex shape, and the statistical results proved that these groups maintained their augmented height over 8 weeks in the rabbit model. In our previous

study on onlay graft using rabbit mandible (Alam et al. 2007), in the immuno-histochemical examination after treatment with BMP-2 antibody, when the autologous group was compared with the control and β-TCP groups, the cell numbers increased significantly, and there was no significant difference between the control and β-TCP in terms of cell number at 2, 4 and 8 weeks. This suggested that bone inductive activity of β-TCP could be low. However, the β-TCP still remained and kept the volume at 8 weeks postoperative. There was also a clinical study that showed slow degradation of β-TCP in sinus augmentation (Zijderveld et al. 2005). However, in still other sinus elevation studies, faster absorption was reported using β-TCP (Horch et al. 2006), which might indicate delayed oral function restoration for dental implants. Different findings from these studies might suggest differences between animal models and humans, or differences in the methods.

α-TCP is predominant during bone resorption in the early stage of bone healing (Rajda et al. 1977; Nery et al. 1978; Nagase et al. 1989; Wada et al. 1989); this may lead to technical difficulties in both the filling of particles to fit the bone defects and their delivery to form the desired shape (Desjarding 1985; Propper 1985; Wittkampf 1988; Wittkampf 1989; Mirtchi et al. 1990). In the study of Yuan et al. (2000), they found that porous β-TCP ceramic could induce bone formation in soft tissue in dogs, but porous

α-TCP ceramic could not. They concluded that no bone formation in α-TCP might have resulted from its higher resorbability or more accurately, due to its rapid dissolution. The rapid dissolution of α-TCP also affected its cell-mediated resorption. However, cement type calcium phosphate material in paste form processes self-setting property in situ, thereby solving these technical problems (Mirtchi et al. 1991; Koshino et al. 1995; Miyamoto et al. 1995, 1997). The characteristic properties of the self setting materials work to prevent intervention for undesirable connective tissues or dispersion of the calcium phosphate particles in to the surrounding tissues. A cement-type calcium phosphate material with a self-setting property could be accurately fit on the surface of bone defects (Kent et al. 1983; Munting et al. 1993; Comuzzi et al. 2002), as was made evident by the study. This is further supported by a previous study using contact radiomicrogram where the tight attachment between rabbit bone surfaces and this material provided proof that BIOPEX® is inert in tissue fluid and maintains its strength in vivo for long periods (Kurashina et al. 1997; Kurashina et al. 1998). In the study of Hao et al. (2004) using rabbit femoral cortical bone, a range of osteoclasts accumulated on the surface of the BIOPEX®, whilst many osteoblasts were localized on the surface opposing the BIOPEX® from days 5 to 10. However, remnants of the BIOPEX® particles were present in the new born with a profile of compact bone on days 30 and 40.

The BIOPEX® was so hard that the osteoclasts could not resorb rapidly.

In this study, the absorption and bone formation of BIOPEX® was clearly observed at the region of the antral membrane after 12 weeks. However, complete bonding between the bone screw surface and BIOPEX® kept and no fibrous tissue could be seen even at 24 weeks postoperative. This suggested that bone formation and absorption of BIOPEX® could occur at the region close to the antral membrane and mother bone, but it could not occur at the contact surface between the screw and BIOPEX, because no cell component is present. In other words, bone replacement of BIOPEX could begin slowly from the contact surface to the antral membrane. The number of BMP-2 stained cell in the experimental side was significantly larger than that in the control side after 4 weeks. This suggested that BIOPEX® could have bone formation activity from an early stage after surgery. The relatively slow deposition of bone matrices appears to induce adequate bone formation temporarily in parallel to physiological mineralization speed, which results in compact bone rather than immature woven bone. This appears important for building new bone with sufficient strength and rigidity (Hao et al. 2004). However, it was not known whether the BIOPEX® could replace new bone tissue completely. Therefore, further examination and long time follow-up are needed.

On the other hand, Comuzzi et al. (2001) stated that injectable calcium phosphate cement showed excellent clinical handling property combined with superior bone behavior and the degradation rate was very slow in the femoral bone of the goat. Therefore, they concluded that this current characteristic could hamper the final clinical applicability of the material as gap filler for peri-implant. In both of their studies and our current study, the finding that surrounding cement of the implanted screw replaced new bone completely was not recognized because of short follow-up period. When granules of bone substitute or cancellous bone are used in sinus augmentation, the fixture implanted in a thin posterior maxillary alveolar bone cannot bear occlusion in the early period. In this study, there was no fibrous tissue between the bone screw surface and BIOPEX® -R, the BIOPEX® -R was directly replaced by new compact bone, keeping the tight bonding to the screw surface. Therefore, bone formation in slow construction, rather than prompt bone formation, appears to provide a rigid bone suitable for implantation.

In conclusion, this study suggested the usefulness of self-setting α-tricalcium phosphate (BIOPEX® -R) to keep the rigidity of implanted bone screws from an early period, and the resultant BMP-2 expression suggested that BIOPEX® -R could have bone conductive activity in maxillary sinus augmentation.

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Legends:

**FIGURE 1.** (A) Bone defect at the nasal region from the superior view. (B) Surgically–created bone defects and implanted bone screws.

(C) Bone screw (Dual top auto-screw®, Jeil Medical Co, Seoul, Korea). (D) Self-setting α-TCP (BIOPEX®-R, Pentax Co, Tokyo, Japan).

**FIGURE 2.** (A) Schematic drawing of an implanted screw and BIOPEX®-R. New bone was measured in the blue colored part, (B) Whole histological image of the implanted screw and BIOPEX®-R (HE staining). Histological findings were observed in the black squares. N; Nasal cavity. S; Sinus cavity. Se; Nasal septum.BI; BIOPEX®-R

**FIGURE 3.** Photomicrographs after 4 weeks. (A) Experimental side (HE staining, original magnification × 40), (B) Experimental side (BMP-2 staining, original magnification ×400), (C) Control side (HE staining, original magnification ×40), (D) Control side (BMP-2 staining, original magnification ×40), N: Nasal cavity, Se: Septum, I : Implanted screw, Bi: BIOPEX-R, F: Fibrous tissue, Nb: Newly formed bone, P: Positive stained cells. Black bur shows 200 μm.

**FIGURE 4.** Photomicrographs after 12 weeks. (A) Experimental side (HE staining, original magnification × 40), (B) Experimental side (BMP-2 staining, original magnification ×400), (C) Control side (HE staining, original magnification ×40), (D) Control side (BMP-2 staining, original magnification ×40), N: Nasal cavity, Se: Septum, I : Implanted screw, Bi: BIOPEX-R, F: Fibrous tissue, Nb: Newly formed bone, P: Positive stained cells. Black bur shows 200 μm.

**FIGURE 5.** Photomicrographs of experimental side after 12 weeks. (A) HE staining, original magnification ×100, (B) HE staining, original magnification ×400. Bi: BIOPEX-R, Nb: Newly formed bone, M:Antral membrane.

**FIGURE 6.** Photomicrographs after 4 weeks. (A) Experimental side (HE staining, original magnification × 40), (B) Experimental side (BMP-2 staining, original magnification ×40), (C) Control side (HE staining, original magnification ×40), (D) Control side (BMP-2 staining, original magnification ×400), N: Nasal cavity, Se: Septum, I : Implanted screw, Bi: BIOPEX-R, F: Fibrous tissue, Nb: Newly formed bone, P: Positive stained cells. Black bur shows 200 μm.

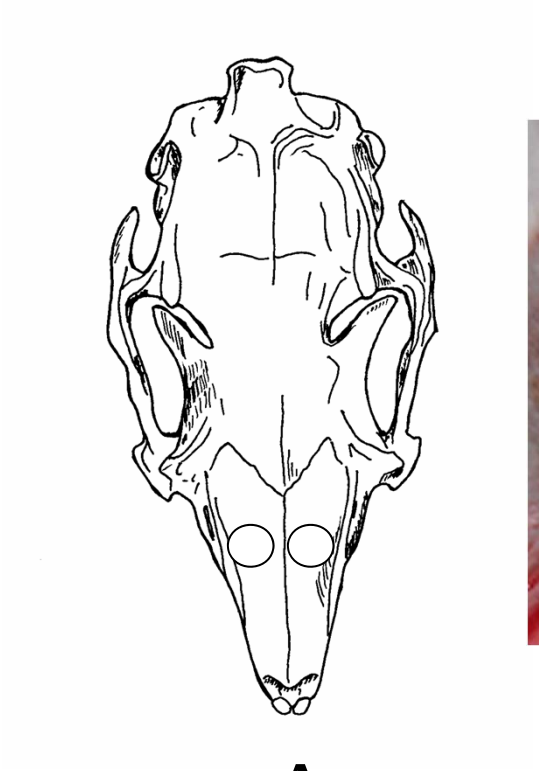
**FIGURE 7.** Photomicrographs of experimental side after 24 weeks. HE staining, original magnification ×100, Bi: BIOPEX-R, Nb: Newly formed bone, I; Implanted screw.

**FIGURE 8.** The ratio of new bone area. * and ** indicate a significant difference at $P<0.05$.

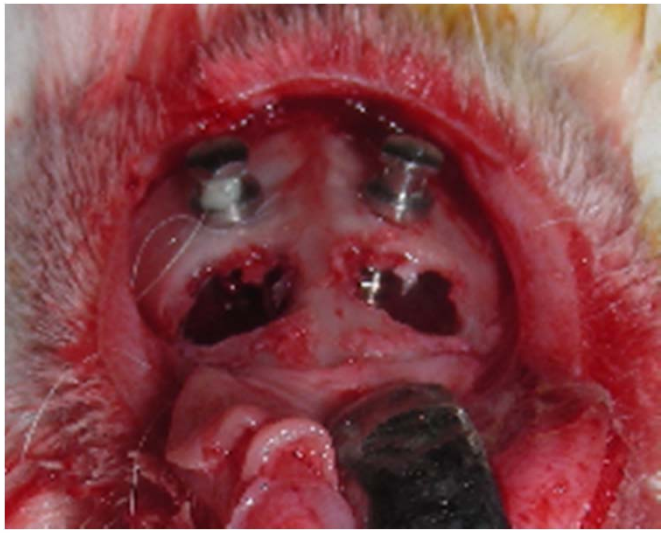
**FIGURE 9.** BMP-2 labeling index. * and ** indicate significant difference at $P<0.05$.

**Table 1.** The ratio of new bone area. Bold number indicates median.

**Table2.** BMP-2 labeling index. Bold number indicates median.

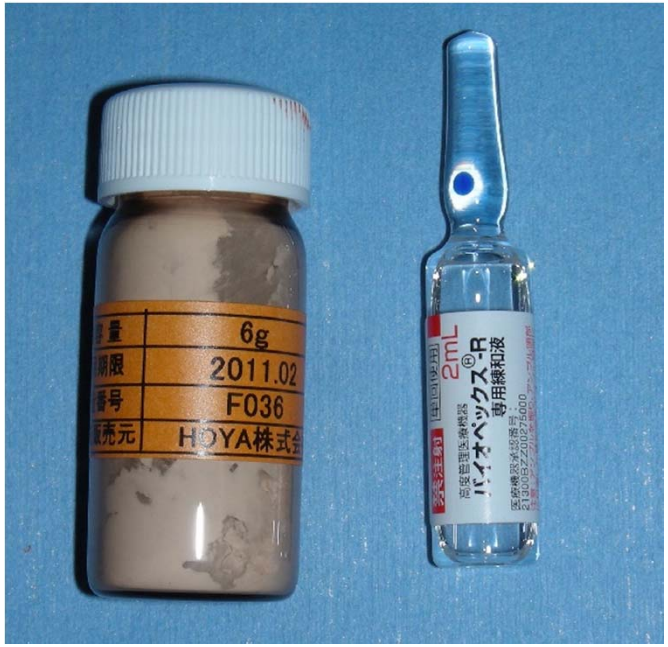


A



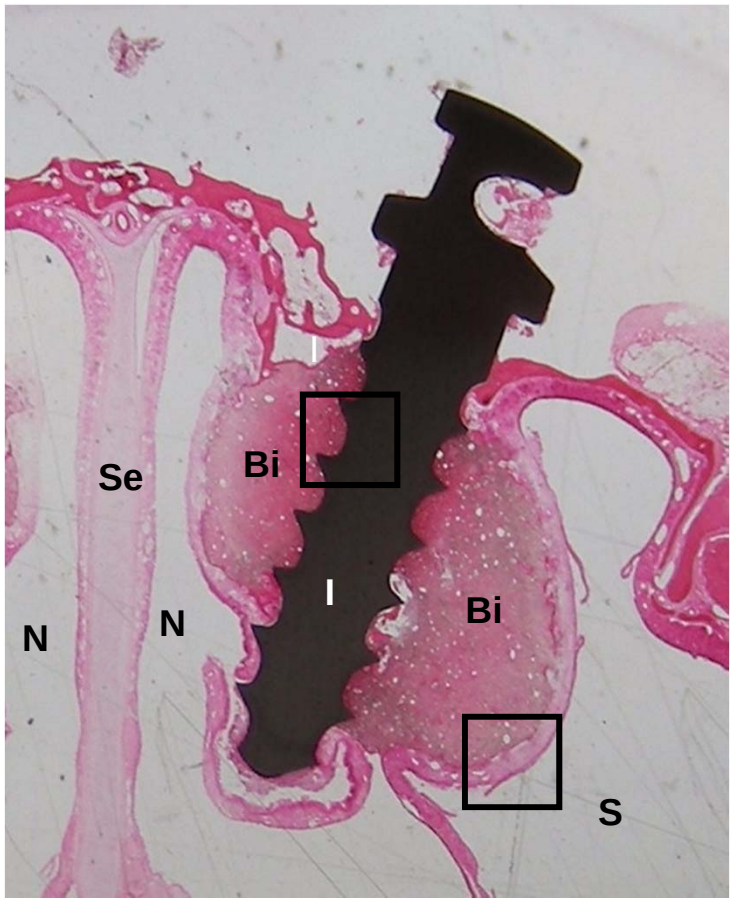
B

C




D

Fig. 1

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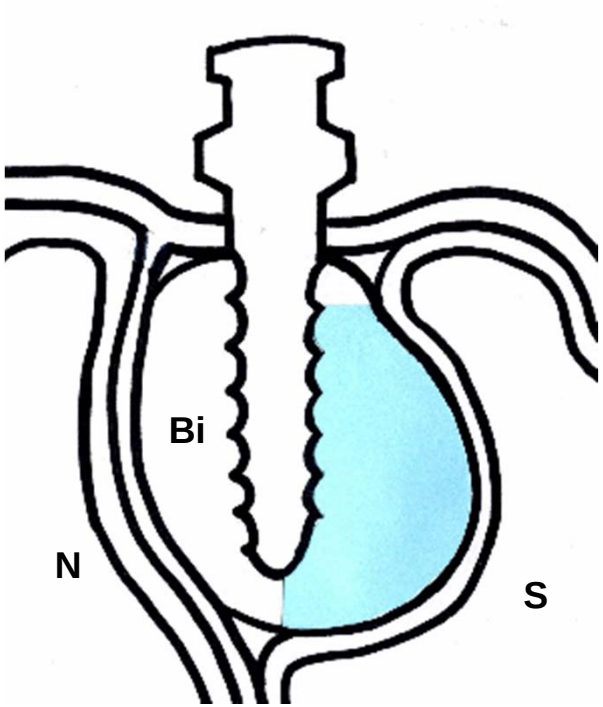
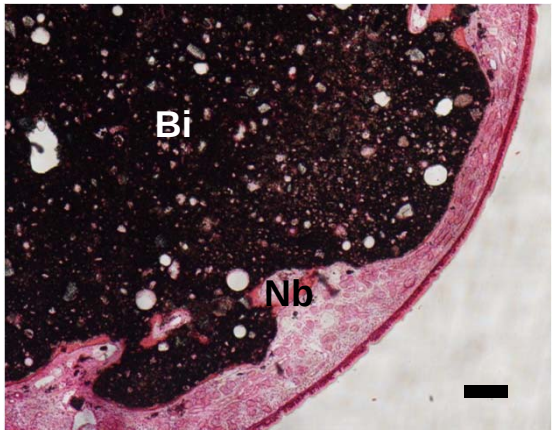
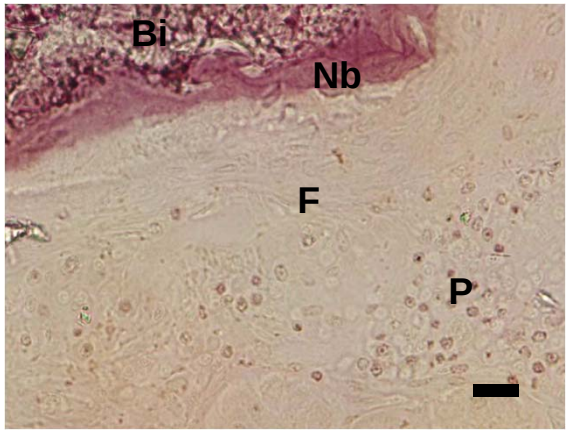



B

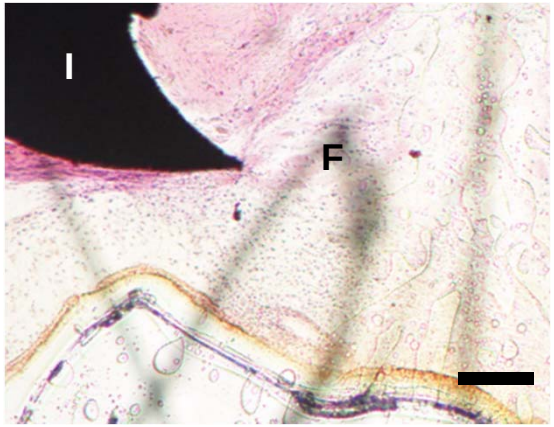
Fig. 2




A




B

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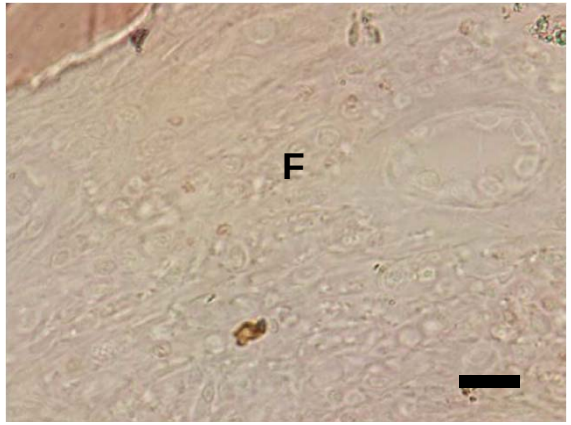
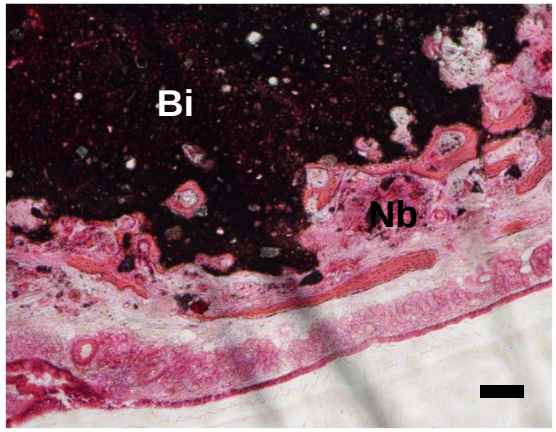
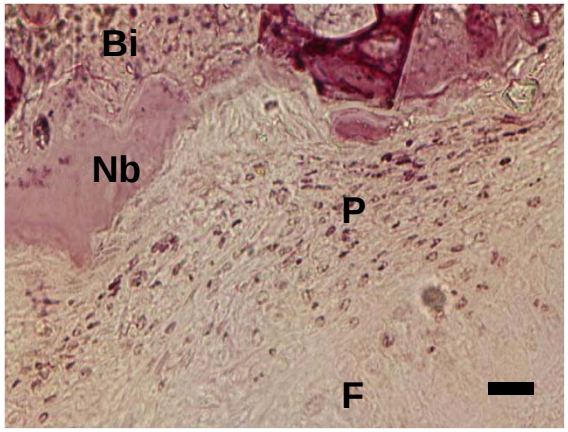



D

Fig. 3

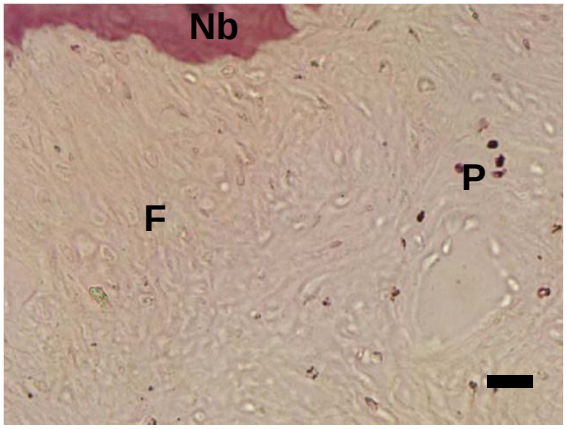



A



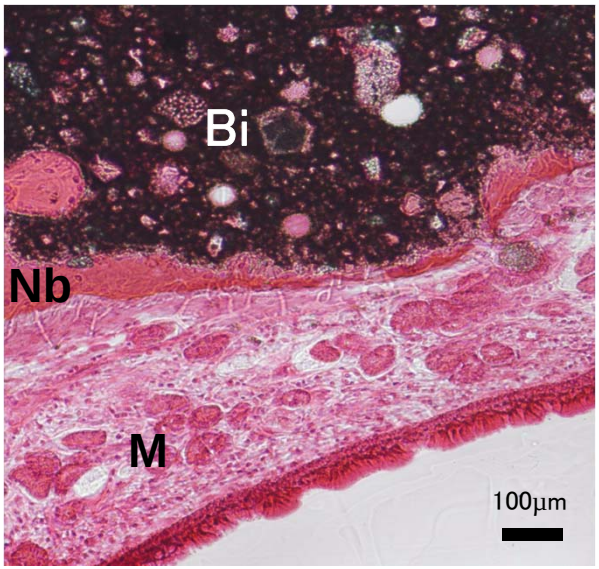

B


C




D

Fig. 4

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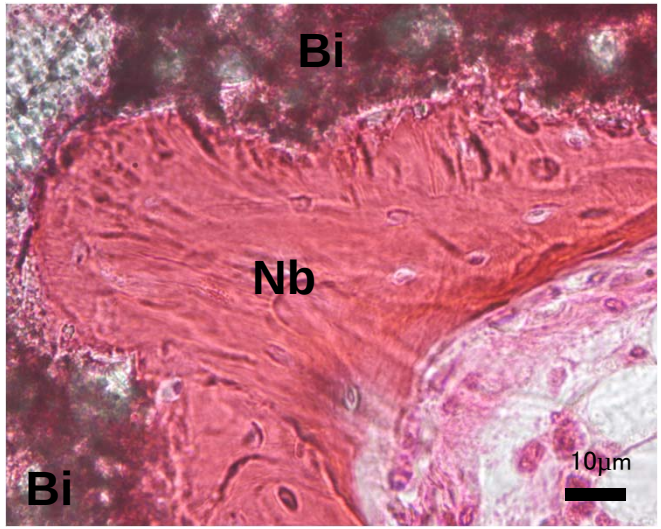
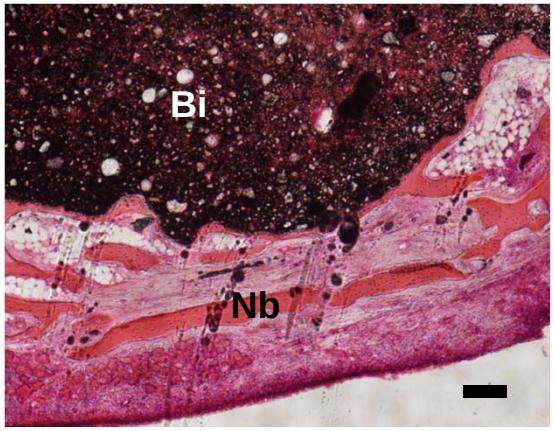



B

**Fig. 5**

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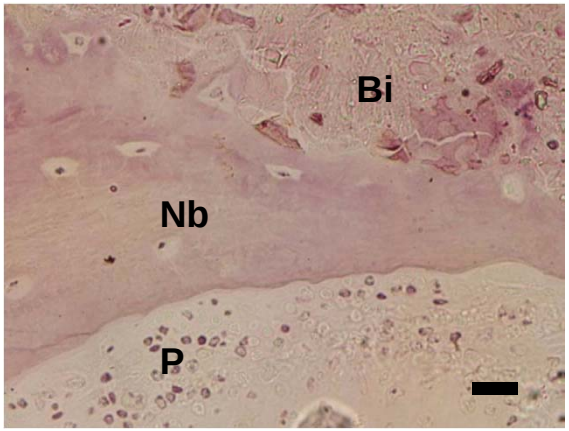
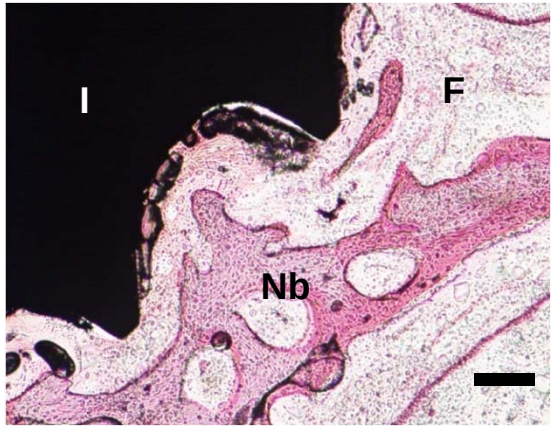



B

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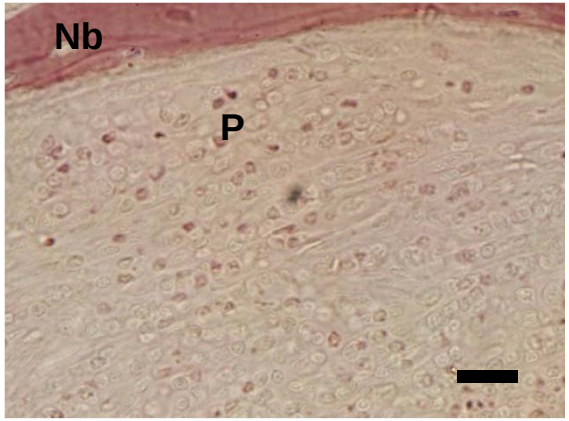


D

**Fig. 6**

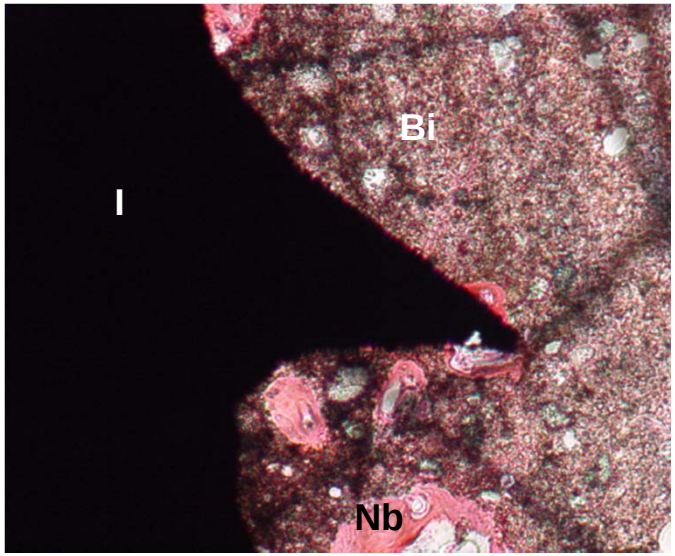


**Fig. 7**

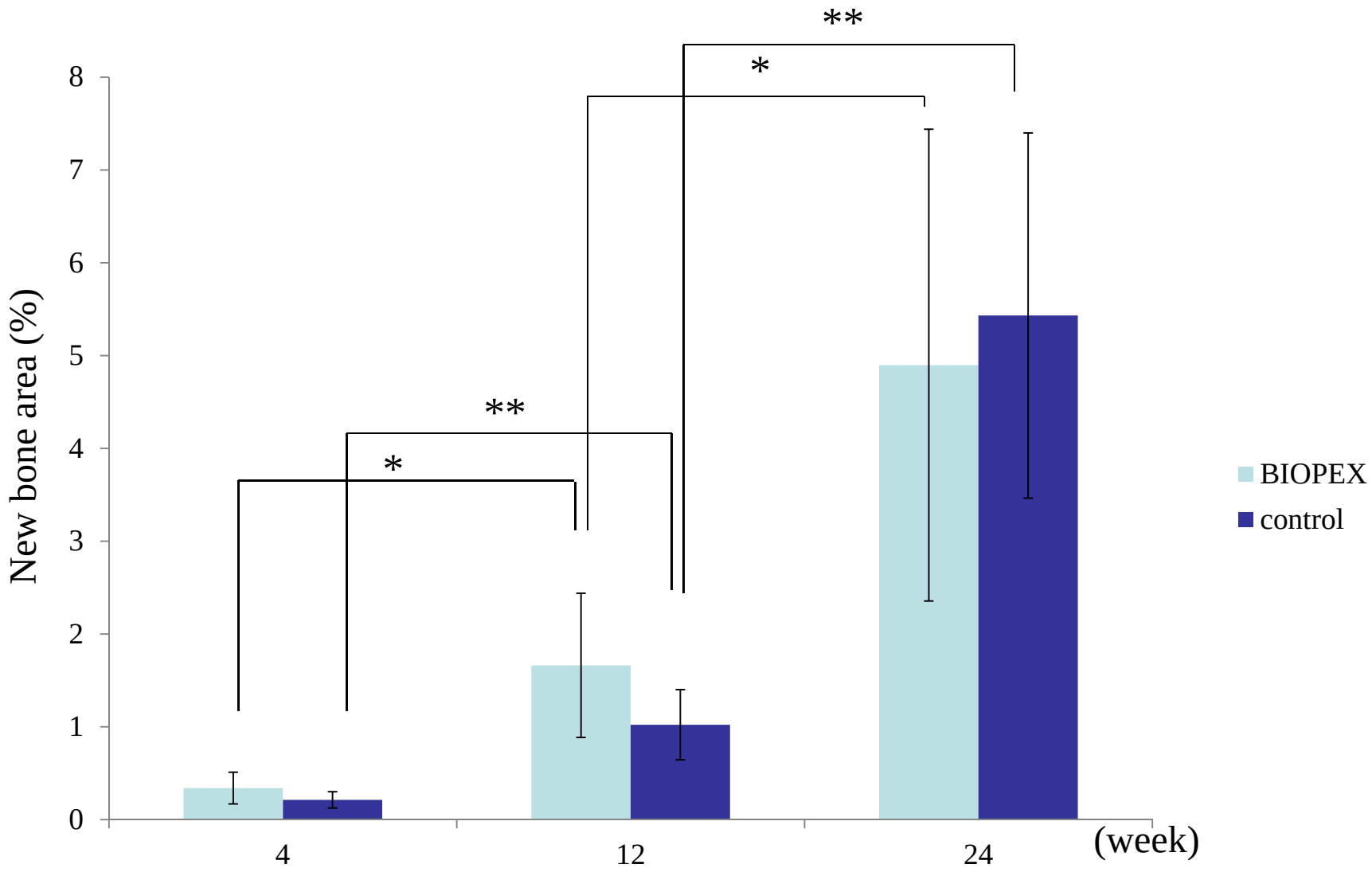


**Fig. 8**

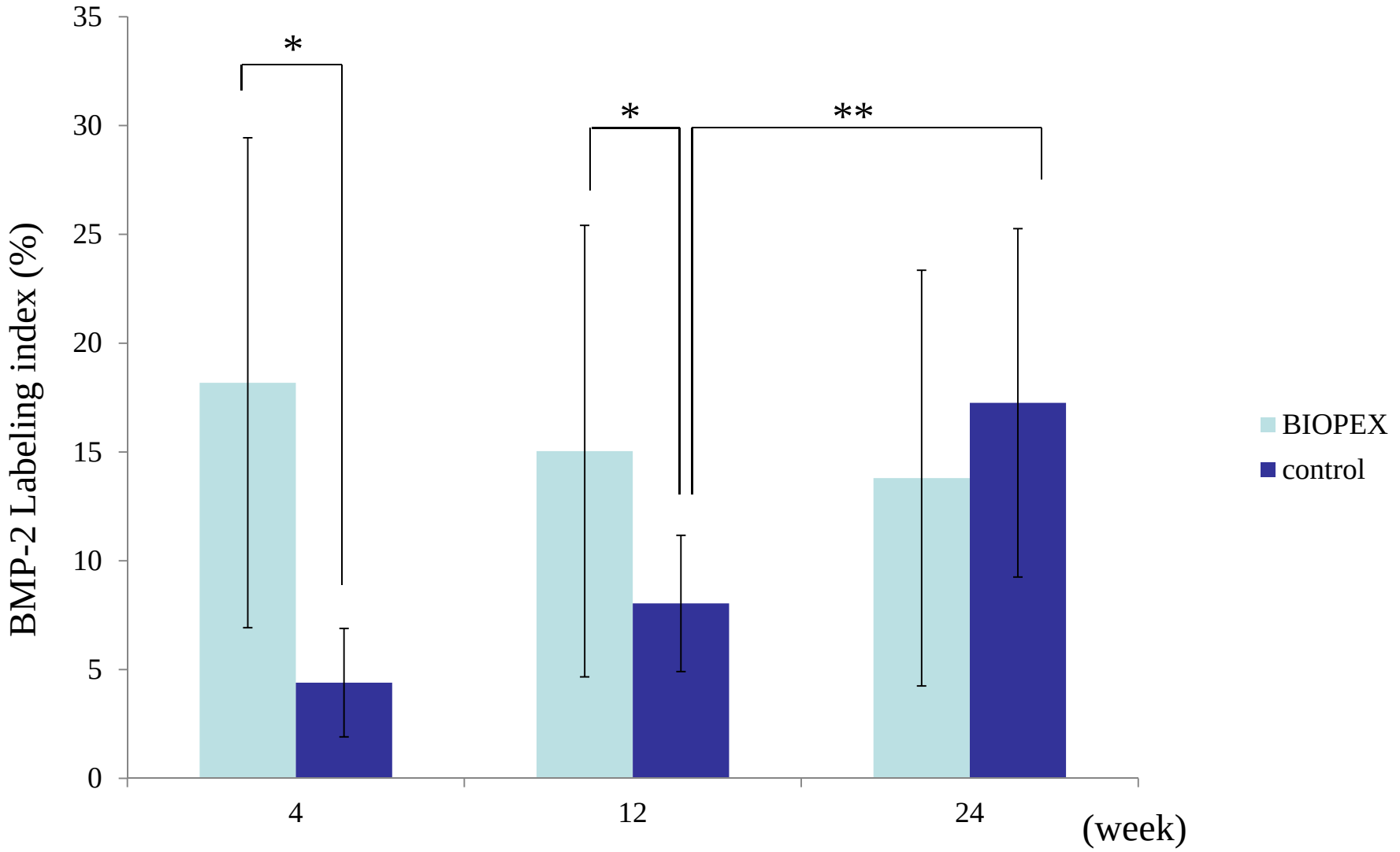
BMP-2 Labeling index (%)
35
30
25
20
15
10
5
0
*
*
**
4
12
24
(week)
BIOPEX
control

**Fig. 9**

| | 4weeks | | 12weeks | | 24weeks | |
|---|---|---|---|---|---|---|
| | Biopex | control | Biopex | control | Biopex | control |
| max. | 0.567 | 0.322 | 2.928 | 1.732 | 7.696 | 8.347 |
| ↕ | 0.528 | 0.298 | 2.061 | 1.002 | 7.253 | 6.238 |
| ↕ | **0.229** | **0.211** | **1.545** | **0.968** | **5.752** | **5.568** |
| ↕ | 0.200 | 0.123 | 1.032 | 0.751 | 2.129 | 5.000 |
| min. | 0.173 | 0.106 | 0.743 | 0.657 | 1.654 | 2.330 |

Table 1

| | 4weeks | | 12weeks | | 24weeks | |
|---|---|---|---|---|---|---|
| | Biopex | control | Biopex | control | Biopex | control |
| max. | 33.8 | 8.7 | 29.9 | 13.0 | 30.1 | 31.0 |
| ↕ | 28.6 | 5.6 | 24.5 | 9.8 | 18.6 | 29.3 |
| ↕ | **15.1** | **3.4** | **11.0** | **7.7** | **8.8** | **23.1** |
| ↕ | 8.7 | 2.2 | 6.6 | 5.5 | 7.1 | 13.2 |
| min. | 4.7 | 2.1 | 3.2 | 4.2 | 4.4 | 11.6 |

Table 2