Expression of bone morphogenetic protein 2 and fibroblast growth factor 2 during bone regeneration using different implant materials as an onlay bone graft in rabbit mandibles

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Expression of bone morphogenetic protein-2 and fibroblast growth factor-2 during bone regeneration using different implant materials as an onlay bone graft in rabbit mandibles.

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Abstract

Purpose: The purpose of this study was to histologically and immunohistochemically evaluate bone regeneration using three different implant materials in rabbit mandibles and also to compare the bone regenerative capability of these materials in an animal model.

Study design: Adult male Japanese white rabbits (n=48, 12-16 weeks, 2.5-3.0 kg) were divided into four groups, consisting of twelve animals each. The implant materials were β -tricalcium phosphate (β -TCP), autologous bone derived from the radius, and recombinant human bone morphogenetic protein-2 (rhBMP-2) with polylactic acid/polyglycolic acid copolymer and gelatin sponge (PGS) complex. After incising along the inferior border of mandible, the materials were implanted as onlay grafts and covered by titanium mesh with screws. No material was implanted into the control group. The rabbits were sacrificed at 2, 4, 8, 12 and 24 weeks postoperatively, and formalin-fixed specimens containing titanium mesh were embedded in acrylic resin. The specimens were stained with hematoxylin and eosin. For immunohistochemical analysis, the specimens were treated with BMP-2 (bone morphogenetic protein-2) and FGF-2 (fibroblast growth factor-2) antibodies. Finally, these were evaluated microscopically.

Results: The autologous bone induced substantially more new bone formation compared to β -TCP at 4 weeks postoperatively. However, rhBMP-2/PGS induced new bone formation at 8 weeks postoperatively. No growth of bony tissue was observed in the control group at any period. In the autologous bone and rhBMP-2/PGS groups, both BMP-2 and FGF-2 appeared specifically at the operated sites at early stages, but expression of BMP-2 and FGF-2 was observed later in the β -TCP group than in other experimental groups.

Conclusion: This study suggested that autologous bone as well as rhBMP-2/PGS implants induced expression of both BMP-2 and FGF-2 specifically at the operated sites even at early stages.

Introduction

Restoration of bone is often required after dental and oral surgical procedures. Treatment of cysts, tumors, and fractures of the jaw can result in bone defects. Such defects must be repaired with bone grafts or bone substitutes to ensure good structural and functional outcomes¹. In the recent years, bone grafts and bone substitutes underneath barrier membranes have been increasingly utilized to optimize the treatment outcome of bone reconstructive therapy for defects in the alveolar process. Conventionally, fresh autologous bone grafts are used to repair oral and maxillofacial bone defects. However, the need for a second surgery at the donor site and the limited availability of bone has led to the development of various alternative materials to autologous bone grafts such as β -TCP and BMPs.

β -tricalcium phosphate (β -TCP) is a calcium phosphate type material that promotes osteogenesis and is also replaced by bony tissue through a process of dissolution and absorption, finally achieving a normal bone structure^{2,3}. However, there are no reports about the use of β -TCP as an onlay bone graft material in mandibles.

Bone morphogenetic proteins (BMPs) are active bone-inducing factors that act on immature mesenchymal cells, including osteoblasts, resulting in osteogenesis. To

date, several types of BMPs have been isolated by molecular cloning and recombinant BMP molecules have been synthesized⁴. Recombinant human BMP-2 (rhBMP-2) is a molecule that has strong bone-inducing capability in vivo⁵. However, to optimize delivery at the bone repair site, rhBMP-2 requires a suitable carrier. Recently, a synthetic polymer, polylactic acid/polyglycolic acid copolymer and gelatin sponge (PGS), that acts as an effective carrier for rhBMP-2 has been developed⁶.

Basic fibroblast growth factor (FGF-2) is a component of the bone matrix that plays a role in regulating bone remodeling⁷. Recent reports have shown that the addition of exogenous FGF-2 to a fracture site or bone defect during the early healing stage accelerates fracture repair and bone formation⁸. However, no experimental studies have been performed to investigate the expression of FGF-2 during bone regeneration. Moreover, the association between the expression of rh-BMP-2 and FGF-2 in mandibular regeneration has not been previously examined.

The purpose of this study was to histologically and immunohistochemically evaluate bone regeneration using β -TCP, autologous bone and rhBMP-2 with PGS implant materials in rabbit mandibles and also to compare the bone regenerative capability of these materials in an animal model.

Materials and Methods

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

Experimental animals

Forty-eight male Japanese white rabbits (12-16 weeks, 2.5-3.0 kg) were divided into three experimental and one control group, consisting of twelve animals each.

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 submandibular region was shaved, 1.8 ml of 2% lidocaine containing 1:80,000 epinephrine was administered to the surgical site. A 25 mm horizontal skin incision was made over the submandibular region to expose the margin of the left mandibular body. The skin, muscles and periosteum were incised along the

inferior border of the mandible and subperiosteal space to accommodate the implant on the buccal aspect of the ramus (Fig. 1).

Implant materials

The implant materials were β -TCP, autologous bone and rhBMP-2 with PGS (rhBMP-2/PGS). These materials were implanted as onlay grafts, covered by titanium mesh (3×3×10 mm cube), and secured with titanium screws. The grafts were secured to the lateral aspect of the mandible with a single titanium screw that was 7mm in length and 1.5 mm in diameter. β -TCP (Olympus Optical, Tokyo, Japan) was porous in the form of a 3×3×10 mm cube, with an average weight of 0.07 g. Autologous bone was derived from the radius bone. For this, the right radius was exposed via a skin incision and reflexion of a facial periosteal flap. A bone segment (length 2.5 cm) was osteotomized from the radius using a diamond drill under copious saline irrigation. The bone graft was preserved in a moist gauze while preparing the recipient site. The rhBMP-2 and PGS were provided by Yamanouchi Pharmaceutical Co, Ltd, Japan. The rhBMP-2 was suspended in a buffer (pH 4.5) consisting of 5 mM L-glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80 (Difco Laboratories, Detroit, MI, USA). It was kept at a temperature of -80°C until needed, when it was thawed at room temperature. The PGS was cut into 5×5×10

mm³ blocks. In the rhBMP-2/PGS implants, 5 µg rh-BMP-2 was combined with 250 mm³ of PGS. This mixture was lyophilized before it was implanted into the animals along the inferior border of mandible. No material was implanted in the control group. In control group, titanium mesh was used in same size as experimental group, but no material was implanted.

Specimen collection

The rabbits were sacrificed at 2, 4, 8, 12 and 24 weeks postoperatively and the specimens containing the titanium mesh were collected for hematoxylin and eosin staining and also for immunohistochemical analysis.

Histological examination

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 cutter and ground by abrasive paper. Thereafter, the sections were further ground to a final thickness of about 10 µm. Finally, the specimens were stained with hematoxylin and eosin and examined under microscope.

Immunohistochemical staining

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 (Yamanouchi Seiyaku Inc., Tokyo, Japan) and anti-FGF-2 antibodies (Takeda Chemical Industries, Osaka, Japan) were used. Sections were incubated overnight with these primary antibodies at 4°C 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 was located between the superior point and the most inferior point of titanium mesh curve and in coronal section (Fig.2). The number of stained cells per voluntary 1000 cells in this area was counted by hand using high magnification photomicrograph ($\times 100$).

Statistical Analysis

Data of all the implanted materials were statistically analyzed with Stat View 4.5 (ABACUS Concepts, Inc., Berkeley, CA, USA). Differences between groups were analyzed by non-paired comparison using Scheffe's F test. Time-dependent changes were examined by analysis of variance (ANOVA). 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 postoperatively, the animals could move and leap without any notable pain or limitation.

HISTOLOGICAL EVALUATION

In the β -TCP group, chondrocytes appeared 2 weeks after the operation. From 4 weeks, active osteoblasts appeared with the remaining β -TCP (Fig. 3) and by 8 weeks, woven bone was formed. From 12 weeks, mature bone formation was observed and by

24 weeks, mature lamellar bone was clearly stained with eosin.

In the autologous bone group at 2 weeks, the operated surface was covered with fibrous tissue. From 4 weeks, more osteoblast cells became active (Fig. 4). After 8 weeks, bone resorption and new bone formation were observed at the recipient site in the contact area to the graft. After 12 weeks, bone remodeling activity and new bone was noted at the graft. After 24 weeks, the resorption had continued.

In the RhBMP-2/PGS group at 2 weeks, the operated area was partially covered with fibrous tissue. At 4 weeks, the operated area was not restored completely and residual PGS was still visible (Fig. 5). However, osteoblasts and fibroblasts were observed under the implant. After 8 weeks, trabeculae of new bone extended towards the internal surface of the operated area, which was still thin and primitive. At 12 weeks, the lamellar structure had developed well in the newly formed bone and the trabecular bone was thicker than it was at 8 weeks. Remodeling and consolidation of the new bone were also observed within the operated area. At 24 weeks, mature bone was clearly observed.

There was little new bone formation at 24 weeks in the control group.

IMMUNOHISTOCHEMICAL ANALYSIS

β -TCP bone grafting treated with BMP-2 antibody

At 2 weeks, a few positive stained cells were observed. After 4 weeks, there were positive stained cells and a few bone forming cells (Fig. 6A). After 8 weeks, there was slight new bone formation. At 12 and 24 weeks, bone formation was more clearly observed, but the number of positive stained cells decreased gradually with time (Fig. 6B).

β -TCP bone grafting treated with FGF-2 antibody

The results were nearly the same as those after treatment with BMP-2 antibody (Figs. 6C, D).

Autologous bone grafting treated with BMP- 2 antibody

At 2 weeks, positive stained cells were observed. After 4 weeks, there were more positive stained cells accompanied by new bone formation with preservation of the graft bone (Fig. 7A). New bone formation was observed over time, which increased at 8, 12 and 24 weeks. On the contrary, the positive stained cells decreased with time (Fig. 7B).

Autologous bone grafting treated with FGF-2 antibody

Results were the same as in case of staining with BMP-2. We could see both positive stained cells and new bone formation at 4 weeks (Fig. 7C). But the number of positive stained cells decreased gradually at 8 weeks and reached a minimum at 24 weeks (Fig. 7D)

rhBMP-2/PGS bone grafting treated with BMP-2 antibody

At 2 weeks, positive stained cells were observed. At 4 weeks, there were many positive stained cells (Fig. 8A). New bone formation also increased gradually and reached a peak at 24 weeks (Fig. 8B), but the number of positive stained cells markedly decreased and reached the minimal level by this time.

rhBMP-2/PGS bone grafting treated with FGF-2 antibody

The results were nearly the same as those after treatment with BMP-2 antibody (Figs. 8C, D).

Control group treated with BMP-2 and FGF-2 antibodies

In both cases, fewer positive stained cells were observed compared to the

experimental groups. However, we observed fibrous tissue in the earlier period, whereas little new bone formation was observed later. (Figs. 9A, B, C, D).

Statistical measurements

All results of the statistical measurements are presented in Table 1 and Fig 10,11.

For BMP-2 antibody, the time-course changes showed statistically significant differences by repeated measure ANOVA (between subjects; $F=28.222$, $df=12$, $P<0.0001$; within subjects; $F=120.132$, $df=4$, $P<0.0001$).

In the immunohistochemical examination after treatment with BMP-2 antibody, when autologous group was compared with control and β -TCP groups, the cell numbers significantly increased after 2, 4 and 8 weeks ($P<0.05$). However, when autologous group was compared with only rhBMP-2/PGS group, cell numbers were found significantly high only at 4 weeks ($P<0.05$) (no positive cells were found at 2 and 8 weeks). Similarly, when rhBMP-2/PGS was compared with control and β -TCP, the cell numbers were significantly higher at 2, 4 and 8 weeks ($P<0.05$). But there was no significant difference between control and β -TCP in cell number for 2, 4 and 8 weeks. However, in all groups, no positive cells were detected at 12 and 24 weeks (Fig. 10 and Table 1).

For FGF-2 antibody, the time-course changes showed statistically significant differences by repeated measure ANOVA (between subjects; $F=7.965$, $df=12$, $P<0.0001$; within subjects; $F=36.997$, $df=4$, $P<0.0001$).

In the immunohistochemical examination after treatment with FGF-2 antibody, when rh BMP2/ PGS group was compared with the autologous, control and β -TCP groups, the cell numbers significantly increased at 2, 4 and 8 weeks ($P<0.05$). Similarly, when the control group was compared with the autologous and β -TCP groups, cell numbers were found significantly increased only at 4 weeks ($P<0.05$). However, comparison between the autologous and β -TCP groups indicated that no positive cells were detected at any of the periods. In all groups, no significant difference was detected at 12 and 24 weeks (Fig. 11 and Table 1).

Thus, in the cell number stained by both BMP-2 and FGF-2 antibodies, similar results were found at 12 and 24 weeks.

Discussion

The ability of bone grafts to increase osteogenesis is important in maxillofacial surgery, because an increase in healing rate can decrease the time of intermaxillary

fixation. However, only a few graft materials actually increase osteogenesis. This can be done in two ways: (1) by providing viable cells that are either osteoblasts or can differentiate into osteoblasts, and (2) by inducing host tissue to increase the number of osteoblasts. These two effects have been termed osteogenetic and osteoinductive^{9,10} respectively..

In the present study, we evaluated bone regeneration using three different implant materials and also compared the bone regenerative capability of these materials in an animal model histologically and immunohistochemically. In this study, a titanium mesh of same size was used in all groups in order to create same space for bone regeneration under different implant materials. The rigid titanium mesh could maintain the space for bone regeneration under the same dynamic situation. Although, it was a limitation of the model that the cortical border of the mandible was perforated by the mesh retention screws, but it was necessary to fix the experimental materials. However, our observation area was difference from perforation site, so that this study could be evaluated objectively. This study showed that new bone was formed following the implantation of all types of implant materials.

Although we used titanium mesh in our study, calcium phosphate type β -TCP has been used as artificial bone. β -TCP promotes osteogenesis and is replaced by

autologous bone in the course of dissolution and absorption^{3,11,12,13}, finally achieving a normal bone structure. However, Saito et al.¹⁴ reported that the periosteum had osteoinduction capacity while β -TCP had osteoconduction capacity and that better osteogenesis occurred with vascularized periosteum. They also suggested that bone marrow fluid was involved in the promotion of osteoblastic activity, but not in calcification. It has been suggested that newly formed bone infiltrates and binds with the indented part of the dissolved β -TCP; thereby a mechanical bond exists between β -TCP and bone¹². Since no chemical bond is present, the β -TCP gradually dissolves, even when osteogenesis progresses around the β -TCP. However, in our study, regeneration rate was slow and β -TCP remained after the implantation of β -TCP. It has been reported that β -TCP is replaced by bone when it is implanted in bone marrow^{2,3, 14}. It does not remain as a foreign body and is absorbed. At present, calcium phosphate type hydroxyapatite (HA), which is considered to promote osteogenesis, is generally used as artificial bone. However, HA has a disadvantage: it shows almost no absorption and remains in the body for a long time as a foreign substance¹⁵. Therefore, we focused our attention on β -TCP, which is a calcium phosphate type material, and used it in the present study.

Autologous onlay bone grafts have been used in the craniofacial skeleton to repair bony deficiencies in those afflicted with congenital anomalies or in those who

have sustained traumatic injuries. This type of graft is considered the most compatible of the materials available. An experimental rabbit model, in which the radius bone is transplanted onto the mandibular lower border, has been successfully tested for the study of endosseous implants placed into onlay grafts¹⁶. This model also provides the conditions for the study of graft-host bone integration under critical revascularization conditions, since the radius is almost devoid of cancellous bone. Difficulties may arise in the prediction of maintenance of graft volume over time when bone grafts are used for facial contour reconstruction. Therefore, we hypothesize that graft fixation will decrease movement and lead to decreased resorption. John et al.¹⁷ reported that when onlay bone grafts are stabilized, improved results with respect to graft resorption can be expected. The method of autologous bone graft was different from previous reports, so that it was difficult to compare with previous report. Kahnberg et al.¹⁶ stated that after 6 weeks osteoclastic and osteoblastic activity was primarily observed in the graft-recipient contact area. In our study, new bone formation with titanium mesh and screw was observed after 4 weeks. New bone formation using mesh fixation might occur earlier than others.

In vivo, among subperiosteal implantation sites, BMP induces new bone formation in heterotrophic, intermuscular and orthotrophic sites^{18,19}. In vitro, BMP

induces cartilage formation in neonatal muscle tissue^{20,21,22}. It is thought that BMP induces differentiation of prevascular mesenchymal connective tissue cells into bone and cartilage. Various preparations of BMP have been reported to initiate new bone formation when implanted in animals, but there have been few reports of BMP implants in alveolar defects of mandible to induce osteogenesis at intraskeletal sites^{23,24}. The use of BMP at the submandibular region has not been reported so far.

BMPs may initiate all of the molecular mechanisms required for bone induction, including the orderly migration, proliferation and differentiation of osteoprogenitors and undifferentiated mesenchymal cells into functional osteoblasts. New bone formation by bone inductive protein depends on the quantity of the BMPs and the delivery system or carrier. BMP activity is greatest when it is placed with a suitable carrier. Several carriers with suitable characteristics have been developed^{25,26,27}. An ideal carrier is not only bioresorbable and non-immunogenic, but also provides a three dimensional structure as a scaffold for new bone formation. It has been reported previously that the polylactic acid/polyglycolic acid (PLGA) gelatin sponge (PGS) copolymer is an effective carrier for BMPs^{28,29}. So PGS has been successfully used for bone repair with rhBMP-2 in bone defect models of periodontal tissues, mandible and ulna in experimental animals^{30,31,32}. PGS can play an important role perhaps by acting

as a space retainer and shock absorber during the formation of new bone. Hence, we used PGS as a carrier for rhBMP-2. Mao et al.³³ reported that at 12 weeks the composite using rhBMP with chitin, coral or xenogenetic cancellous bone was replaced completely by new bone. In our study, the new bone formation with rhBMP/PGS was observed after 8 weeks earlier than previous reports. This might be due to difference in carrier material and titanium mesh to maintain the regeneration space.

When the results of this study are adapted to human, we have to consider some factors to affect the bone formation. Firstly, there was difference in turn over period between rabbit and human. We did not know whether the follow up period of this study was valid or not. However, on the basis of previous rabbit study, it seemed to be significantly long. There are numerous parameters that can influence the outcome of onlay bone grafts. For example, rigid fixation, degree of graft contact with the recipient surface, properties of the recipient bed and size of bone graft, prevention of bacterial infection are all very relevant. Above these parameters are depend on success or failure examined both of human and animal study for bone regeneration. Because of the above phenomena, the patterns of bone formation in the three groups in our study were different. Our study was the duration follow-up at six months. During these periods bone regeneration was observed better. This study would helpful for clinician.

. Our immunohistochemical studies demonstrated that BMP-2 was strongly expressed in the connective tissue of the gap, after implantation on the rabbit mandible. Bone marrow was also strongly stained at the edge of the osteogenesis site. FGF-2 expression followed a similar pattern to that of BMP-2 specifically at the edge of the osteogenesis site.

In the control group, the tissues that formed were a variable mixture of fibrous tissue formed by mesenchymal cells derived from the open marrow space, but little bone formation was observed,

.
Our study statistical analysis revealed BMP-2 with PGS and autologous group expression levels were higher than in the other groups 2, 4 and 8 weeks after BMP2 and FGF 2 antibody treated. But it decreased both 12 and 24 weeks. However, β -TCP and control group expression level were not well changed at all period after treated with BMP-2 and FGF-2 treated. Hence, we conclude that autologous bone as well as rhBMP-2/PGS implants induced expression of both BMP-2 and FGF-2 specifically at the operated sites in early stages.

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Legends:

FIGURE 1. Intra-operative finding: (A) Mandible exposed at the submandibular region,
(B) Fixation of titanium mesh with different materials.

FIGURE 2. Area of immunohistochemical observation (indicated by triangle) located
between the superior point and the most inferior point of titanium mesh curve.

FIGURE 3. β -TCP group histopathological findings at 4 weeks. New bone was formed around the remaining β -TCP (hematoxylin-eosin, original magnification $\times 40$). B: β -TCP, M: mandible, NB: new bone, T: titanium mesh.

FIGURE 4. Autologous bone group histopathological findings at 4 weeks. New bone was formed (hematoxylin-eosin, original magnification $\times 40$). NB: new bone, T: titanium mesh.

FIGURE 5. rhBMP-2/PGS group. Histopathological findings at 4 weeks. There was new bone formation around the remaining PGS (hematoxylin-eosin, original magnification $\times 40$). PGS: polylactic acid/polyglycolic acid copolymer and gelatin sponge.

FIGURE 6. Photomicrographs of β -TCP group. Stained with BMP-2 antibody at 4 weeks (A), and at 24 weeks (B). Stained with FGF-2 antibody at 4 weeks (C), and at 24 weeks (D). (Immunohistochemical staining, original magnification $\times 200$). B: β -TCP, I: implant, NB: new bone, P: positive cell area.

FIGURE 7. Photomicrographs of autologous bone group. Stained with BMP-2 antibody at 4 weeks (A), and at 24 weeks (B). Stained with FGF-2 antibody at 4 weeks (C), and at 24 weeks (D). (Immunohistochemical staining, original magnification $\times 200$). GB: graft bone, NB: new bone, P: positive cell area.

FIGURE 8. Photomicrographs of rhBMP-2/PGS group. Stained with BMP-2 antibody at 4 weeks (A), and at 24 weeks (B). Stained with FGF-2 antibody at 4 weeks (C), and at 24 weeks (D). (Immunohistochemical staining, original magnification $\times 200$). NB: new bone, P: positive cell area.

FIGURE 9. Photomicrographs of control group. Stained with BMP-2 antibody at 2 weeks (A), and at 12 weeks (B). Stained with FGF-2 antibody at 2 weeks (C), and at 12 weeks (D). (Immunohistochemical staining, original magnification $\times 200$). F: fibrous tissue, P: positive cell area.

FIGURE 10. The ratio of BMP-2 positive cells. The time course changes in all groups showed significant differences with ANOVA.

FIGURE 11. The ratio of FGF-2 positive cells. The time course changes in all groups showed significant differences with ANOVA.

Table 1. The results of statistical analysis with Scheffe's F test.

S indicates significant difference at $P < 0.05$, and NS indicates no significant difference.

FIGURE 1

A

B

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

A

B

C

D

FIGURE 8

FIGURE 9

A

B

C

D

FIGURE 10

FIGURE 11

Table 1

BMP2 antibody		Weeks	2	4	8	12	24
Autologous bone	vs	rhBMP/PGS	NS	S	NS	NS	NS
Autologous bone	vs	Control	S	S	S	NS	NS
Autologous bone	vs	β -TCP	S	S	S	NS	NS
rhBMP/PGS	vs	Control	S	S	S	NS	NS
rhBMP/PGS	vs	β -TCP	S	S	S	NS	NS
Control	vs	β -TCP	NS	NS	NS	NS	NS
FGF2 antibody		Weeks	2	4	8	12	24
Autologous bone	vs	rhBMP/PGS	S	S	S	NS	NS
Autologous bone	vs	Control	NS	S	NS	NS	NS
Autologous bone	vs	β -TCP	NS	NS	NS	NS	NS
rhBMP/PGS	vs	Control	S	S	S	NS	NS
rhBMP/PGS	vs	β -TCP	S	S	S	NS	NS
Control	vs	β -TCP	NS	S	NS	NS	NS