The Effect of Heparin with rhBMP-2 on Differentiation of Mesenchymal Stem Cells in to Osteoblasts

Hwan Jong Jeong1), Sung Bin Lim1), Chin Hyung Chung1), Ki Seok Hong1), Gyeong Ju Park2)*

Department of Periodontology, School of Dentistry1), Department of Oral Histology, School of Predentistry, Dankook University2)

The regeneration of periodontium is the goal of periodontal therapy. Many periodontologists try to achieve this goal by using guided tissue regeneration (GTR) method. However, these procedures always include several disadvantages. Recombinant human bone morphogenetic protein-2 (rhBMP-2) stimulated ectopic bone formation when it was implanted in rat muscles with insoluble bone matrix by differentiating muscle cells into chondrocytes and osteoblasts. The purpose of this study was to evaluate the osteoinductive potential of the mixture of rhBMP-2 (5 μg/ml) and heparin (0.25 or 25 μg/ml) at the critically sized rabbit calvarial defects. And this study aimed to reveal that heparin also acts to enhance the bone forming activity of rhBMP-2. The 12 rabbits (4-month-old; NewZealand White) were used in the present study, 5 μg/ml of rhBMP-2 and 0, 0.25 or 25 μg/ml of heparin were mixed and blotted into anorganic bovine bone and filled cranial defects. The animals were sacrificed following a time schedule (1, 3, and 6 weeks). Sections were made in 7 μm thicknesses, stained with H&E and Masson’s trichrome method, and examined under a light microscope. The differences among each obtained percent value were evaluated by one-way analysis of variance. A p value of p<0.05 was considered statistically significant and an ANOVA test was performed to verify significant differences. To adjust for multiple comparisons when one-way analysis of variance showed a significant difference between groups (p<0.05), Scheffe’s post hoc test was used to identify which group differences accounted for the significant p-value. In control group, osteoinduction was not outstanding, however, in experimental groups, osteoinduction was significantly outstanding, and as the concentration of heparin mixed with rhBMP-2 increased, osteoinduction was increased. Mixtures of rhBMP-2 and heparin affect bone formation at initial bone formation, but that effect disappeared following a time lapse.

Key word: RhBMP-2, Heparine, Bone, Osteoinduction
and risk of vertical transmission of prions, and thus new procedures in bone regeneration have been pursued\textsuperscript{1-7}).

Bone morphogenetic proteins (BMPs) are regarded as members of the transforming growth factor–β (TGF–β) superfamily owing to the characteristic features in their amino acid sequences\textsuperscript{9}). In the 1960s, Urist demonstrated that demineralized bone matrix could induce the formation of new cartilage and bone tissues in ectopic sites, and this phenomenon was known as the bone induction principle\textsuperscript{8}). In 1988, Wozney et al.\textsuperscript{10}) identified the genetic sequence for BMP. Up to this point, 20 BMPs have been identified, Among these BMPs, BMP–2, –4, –5, –6, and –7, have been shown to have significant osteoinductive activity\textsuperscript{11-17)}. Several studies have reported that BMP has the potential to induce new bone formation, cementum regeneration, and reconstruction of connective tissue attachment in animal models with periodontal defects\textsuperscript{18-22}).

Wang et al.\textsuperscript{23}) found that recombinant human bone morphogenetic protein (rhBMP–2) stimulated ectopic bone formation when it was implanted in rat muscles with insoluble bone matrix by differentiating muscle cells into chondrocytes and osteoblasts.

On the other hand, Zhao et al.\textsuperscript{26}) found that heparin stimulates the ectopic bone–forming activity of BMP–2 in vivo and proposed a novel mechanism for the effects of heparin on the BMP signaling pathway in target cells. They also suggested that heparin enhances BMP–induced osteoblast differentiation in C2C12 myoblasts in vitro.

A large number of extracellular molecules were reported to regulate positively and negatively the biological activities of BMPs in vivo and in vitro\textsuperscript{27,28}). Sulfated polysaccharides such as heparin and heparan sulfate are macromolecules associated with the cell surface and the extracellular matrix\textsuperscript{29-33)}. These polysaccharides have been shown to interact directly with a number of growth factors, including BMPs, via highly negative charged polysaccharide chains. Indeed, heparin–affinity chromatography was used to purify the BMP activity from extracts prepared from demineralized bone matrix\textsuperscript{34,35}). Heparin enhances the anticoagulation and the growth promoting activities of antithrombin III and FGFs, respectively\textsuperscript{36-39}), and the anticoagulation activity of heparin is widely used clinically.

Takada T. et al.\textsuperscript{40}) reported that sulfated polysaccharides including heparin, he–aran sulfate and dextran sulfate, but not desulfated heparin, enhance the osteoblast differentiation induced by homodimers and heterodimers of BMPs. The biological activity of BMP–2 was prolonged in the presence of heparin.

Nevertheless, investigators have reported bone formation effects for any graft material by calculating the amount of bone formation around the graft material (peripheral bone formation of graft materials) and therefore determined the amount of newly formed bone containing not only osteoconductive bone formation but also osteoinductive bone formation. Thus the measurement of peripheral bone formation around the grafted materials were not accurate for the measurement of osteoinductive activity of a certain material\textsuperscript{25}). The purpose of this study was to evaluate the osteoinductive potential of the mixture of rhBMP–2 and heparin at the critically sized rabbit calvarial defects. And this study aimed to reveal that heparin also acts to enhance the bone forming activity of rhBMP–2.

II. Materials and Methods

1. Animals

The present animal investigation was evaluated, and approved by the Animal Ethics Committee of the University of Dankook, Korea and strictly followed
its regulations.

The 12 rabbits (4-month-old; New Zealand White) were used in the present study. The animals were kept in a specially-designed room for experimental animals and were fed a standard laboratory diet.

2. BMP, heparin and anorganic bone

Purified rhBMP-2 was obtained from Cowel Medi Co., Ltd (Seoul, Korea).

Purified heparin for injection was obtained from ChoongWae Pharma Co., Ltd (Seoul, Korea). And anorganic bovine bone (OCS-B⃝) was obtained from NIBEC (Seoul, Korea, porous type, 0.2~1.0 mm pore size).

RhBMP-2 (5 μg/ml), heparin (0, 0.25 or 25 μg/ml) and distilled water were mixed and blotted into anorganic bovine bone. And the mixtures were freeze dried (lyophilized) and kept at −60℃ before the surgical grafting procedure.

3. Surgical procedure

The animals were anaesthetized by an intramuscular injection (2 ml each) of ketamine hydrochloride (Ketamine, Yu-han Co., Seoul, Korea) and Rompun (Rompun, Bayer Korea Co.). Routine lidocain infiltration anesthesia was used at the surgical site. An incision was made in the sagittal plane across the cranium, and a full thickness flap was made, exposing the calvarial bone, 3−standardized, circular, transosseous defects, 8 mm in diameter, were created on the cranium using a saline-cooled trephine drill (Neo biotech, Korea) until durameter was exposed. After removing the trephined bone chips, rh-BMP2 and heparin−treated anorganic bovine bone was applied to each defect.

In each rabbit, the first defects were filled with 5 μg/ml of rhBMP−2 and 0 μg/ml of heparin mixture blotted into an anorganic bovine bone, the second defects were filled with 5 μg/ml of rhBMP−2 and 0.25 μg/ml of heparin mixture blotted into anorganic bovine bone, and the third defects were filled with 5 μg/ml of rhBMP−2 and 25 μg/ml of heparin mixture blotted into an anorganic bovine bone.

Finally, the periosteum and skin were then closed and sutured with 4−0 coated sutures (4−0 Ethicon Coated VICRYL⃝, Johnson & Johnson).

An NSAID, diclofenac (MINO−V⃝, Re yon pharm., 0.2 ml, I.M.) was administered immediately post−surgery and redosed twice daily for 3 days.

An aminoglycoside group antibiotics, Amicacin sulfate (AMIKTAM⃝, Kun wha pharm., 0.2 ml, I.M.) was administered immediately post−surgery and redosed twice daily for 7 days.

The animals (12 rabbits) were divided into three groups and each were allowed to heal for 1−(four rabbits), 3−(four rabbits), or 6−(four rabbits) weeks. And then the animals were anaesthetized by an intramuscular injection (2 ml each) of ketamine hydrochloride (Ketamine, Yu−han Co., Seoul, Korea) and Rompun (Rompun, Bayer Korea Co.). After anaesthesia the animals were put to death by strangling.

4. Preparation of specimens and analysis of tissues

Harvested bony tissues were fixed in 10% formaline and decalcified with 5% nitric acid. Sections were made in 7 μm thicknesses, stained with H&E and Masson’s trichrome method, and examined under a light microscope. Photographs of each stained section (well prepared 2 sections per one defect: total 6 specimens on each rabbit) under x50, 100, or 200 magnification. Images were measured about the total area of bone formation in the inner area of graft material’s particle pore and the total area of graft materials using IPTK version 5.0 software at ×50 (Reindeer Co., USA). We used photographs of each stained section only under 50x
magnification for measurements. Values of the areas of bones in the inner area of graft material’s particle pore were divided by values of areas of graft materials.

5. Statistical analysis
The difference among each obtained percent values were evaluated by one–way analysis of variance (ANOVA).

A p-value of \( p < 0.05 \) was considered statistically significant. When ANOVA showed a significant difference between groups (\( p < 0.05 \)), Scheffe post hoc test was used to identify which group differences accounted for the significant \( p \) -value. An ANOVA test was performed to verify significant differences. These analyses were conducted using SPSS version 13 (SPSS Inc, USA).

III. Results

1. Clinical findings
The experimental subjects recovered well after surgery and no signs of infection were found at any time during the postoperative periods.

2. Descriptive histologic analysis

1) The first group (rhBMP−2 only)
After 1 week collagen fibers were visible around multiporous graft material. A very small number of lymphocytes and many blood capillaries appeared around the graft materials (Fig. 1A). Cuboidal cells with prominent nuclei that were presumed to be mesenchymal cells were found at the intimate contact area between graft materials and connective tissues. This means osteoconduction activity by graft materials. However, no changes were developed in the inner area of graft material’s particle pore (Fig. 1B).

After 3 weeks, newly formed collagen fibers had increased in connective tissue around the graft materials, but few changes were found in graft materials (Fig. 2A, B).

After 6 weeks, newly formed collagen fibers and newly synthesized bones were more formed around the graft materials (It means that osteoconductive activity broke out around the graft materials.). Some changes developed in the inner area of graft material’s particle pore, but the structure was not clear. This means the osteoinduction activity is not clear (Fig. 3A). A more magnified picture showed that newly formed normal bone have osteocytes and lacunae in the newly formed peripheral bones (Figure 3B).

2) The second group (rhBMP−2 and 0.25 \( \mu g/ml \) heparin)
After 1 week, the amount of newly formed peripheral bone were very small, but clear change had occurred in the inner area of graft material’s particle pore (Fig. 1C). More magnified pictures of internal alterations showed newly formed ring−shaped collagen bundle deposition in graft materials that did not have osteocytes or lacunae. In addition, inner areas of ring contained unidentified cells that were presumed to be mesenchymal cells (Fig. 1D).

After 3 weeks, the amount of newly formed peripheral bone increased more than 1 week and the amount of newly formed collagen fiber in the inner area of graft material’s particle pore increased more than 1 week (Fig. 2C).

After 6 weeks, the amount of peripheral new bone and newly formed collagen fibers in the inner area of graft material’s particle pore were higher than what they had been at 3 weeks (Fig. 3C).
The first group (BMP only)

The second group (BMP +0.25 μg/ml heparin)

The third group (BMP +25 μg/ml heparin)

Fig. 1. Specimen after 1 Weeks
A: MT staining, ×50, B: More magnified picture of A, Cuboidal cells with prominent nucleus(red arrow) is seen at the periphery of porous graft materials (H&E staining, ×200), C, Amount of newly formed peripheral bones were very small, but newly formed bone matrix in the inner area of graft material’s particle pore were showed (MT staining, ×50), D, More magnified picture of C, Showing newly formed ring−shaped collagen bundle deposition in graft materials (red arrow) that did not have osteocytes and lacunae (MT staining, ×200), E, Newly formed vivid bones(red arrow) were showed in graft materials (MT staining, ×50).

Fig. 2. Specimen after 3 Weeks
A: Newly formed collagen fibers(red arrow) were increased in connective tissue around the graft materials, but any changes were not showed in the inner area of graft materials’s particle pore(MT staining, ×50), B: H&E staining, ×50, C: Newly formed collagen fibers in graft material (red arrow) (MT staining, ×50), D: New bone in the inner area of graft materials’s particle pore(MT staining, ×50).

Fig. 3. Specimen after 6 Weeks
A: Newly formed collagen fibers and newly synthesized bones around the graft materials were showed (red arrow), and some changes were developed in the inner area of graft material’s particle pore but the structure of the changes were obscure (MT staining, ×50), B: Newly formed peripheral normal bone that had osteocytes and lacunae (red arrow) were shown (MT staining, ×200), C: Newly formed collagen fibers in the inner area of graft material’s particle pore (red arrow) (MT staining, ×50), D: Newly formed bone marrow exhibited hematopoietic marrow and fat marrow(red arrow) (MT staining, ×50).
Table 1. Amount of bone formation in the inner area of graft material's particle pore (%)

<table>
<thead>
<tr>
<th>Duration-Heparin concentration (μ g/ml)</th>
<th>Mean(%)±SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1W–0.25H (1)</td>
<td>1.157±0.285</td>
<td>0.100</td>
</tr>
<tr>
<td>3W–0.25H (2)</td>
<td>2.391±0.509</td>
<td>0.196</td>
</tr>
<tr>
<td>6W–0.25H (3)</td>
<td>3.572±1.003</td>
<td>0.354</td>
</tr>
<tr>
<td>1W–25H (4)</td>
<td>5.468±0.705</td>
<td>0.249</td>
</tr>
<tr>
<td>3W–25H (5)</td>
<td>6.033±1.046</td>
<td>0.370</td>
</tr>
<tr>
<td>6W–25H (6)</td>
<td>6.785±1.041</td>
<td>0.368</td>
</tr>
</tbody>
</table>

SD: Standaed deviation, SE: Standard error

Table 2. Statistical analysis for difference according duration and heparin concentration

<table>
<thead>
<tr>
<th>Corresponding item 1</th>
<th>Corresponding item 2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1W–0.25H (1)</td>
<td>3W–0.25H (2)</td>
<td>0.509</td>
</tr>
<tr>
<td>1W–0.25H (1)</td>
<td>6W–0.25 (3)</td>
<td>0.002*</td>
</tr>
<tr>
<td>1W–25H (4)</td>
<td>3W–25 (5)</td>
<td>0.991</td>
</tr>
<tr>
<td>1W–25H (4)</td>
<td>6W–25 (6)</td>
<td>0.414</td>
</tr>
<tr>
<td>3W–0.25H (2)</td>
<td>3W–25 (5)</td>
<td>0.000*</td>
</tr>
<tr>
<td>6W–0.25H (3)</td>
<td>6W–25 (6)</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

* Denote statistical significancy with p<0.05

Fig. 4. Amount of bone formation in the inner area of graft material’s particle pore(%) At 0.25 μg/ml heparin concentration the difference of the amount of bone on the inner area of graft material’s particle pore between 1 week and 6 weeks was very significant. And difference of amount of bone in the inner area of graft material’s particle pore at 3 weeks and 6 weeks was very significant. And difference of amount of bone on the inner area of graft material’s particle pore between 0.25 and 25 μg/ml heparin concentration was very significant (P vale: (0.05).

3) The third group (rhBMP–2 and 25 μg/ml heparin)

After 1 week, new bone formation was seen in the periphery of graft materials, and newly formed collagen fibers were laid down in the inner area of graft material’s particle pore and newly formed vivid bones were shown in the inner area of graft material’s particle pore (Figure 1E). It means that osteoinductive activity broke out in the inner area of graft material’s particle pore.

After 3 weeks, the amount of peripheral new and new bone in the inner area of graft material’s particle pore were higher than the first week (Fig. 2D).

After 6 weeks, the amount of peripheral new bone and new bone in the inner area of graft material’s particle pore had increased over the third week. Newly formed bone marrow exhibited not only a large amount of hematopoietic marrow but also fat marrow (Fig. 3D).
3. Histomorphometric analysis

Quantitative histomorphometry revealed that bone formation in the inner area of graft material’s particle pore increased with an increasing concentration of heparin (Fig. 4). After 1 week, bone formation in the inner area of graft material’s particle pore amounted to 1,577 ± 0.219% for the second group (rh–BMP2 and 0.25 μg/ml heparin mixture), and 5,468 ± 0.141% for the third group (rh–BMP2 and 25 μg/ml heparin mixture). After 3 weeks, bone formation in the inner area of graft material’s particle pore amounted to 2,391 ± 0.655% for the second group (rhBMP–2 and 0.25 μg/ml heparin mixture), and 6,033 ± 1,046% for the third group (rhBMP–2 and 25 μg/ml heparin mixture). After 6 weeks, bone formation in the inner area of graft material’s particle pore amounted to 3,572 ± 1,003% for the second group (rhBMP–2 and 0.25 μg/ml heparin mixture), and 6,785 ± 1,041% for the third group (rhBMP–2 and 25 μg/ml heparin mixture) (Table 1).

At 0.25 μg/ml heparin concentration the difference of the amount of bone in the inner area of graft material’s particle pore between 1 week and 6 weeks was very significant (p–value: <0.05).

Bone formation in the inner area of graft material’s particle pore at a 25 μg/ml heparin concentration increased following a time lapse, but the increasing amount of bone in the inner area of graft material’s particle pore at each period did not show a statistical significance (p–value: >0.05).

And the difference of amount of bone in the inner area of graft material’s particle pore at 3 weeks and 6 weeks between 0.25 and 25 μg/ml heparin concentration was very significant (p–value: <0.05) (Fig. 4, Table 2).

IV. Discussions

Several studies have reported that BMP has the potential to induce new bone formation, cementum regeneration, and reconstruction of connective tissue attachment in animal models with periodontal defects and that rhBMP–2 stimulated ectopic bone formation\(^{18–23}\).

On the other hand, Zhao et al. found that heparin stimulates the ectopic bone-forming activity of BMP–2 in vivo\(^{26}\).

In Zhao’s study\(^{26}\) they used rhBMP–2 (5 μg/ml) and heparin (0, 0.25, 2.5, 25μg) for ectopic bone formation assay. But in this study, we used heparin of 0, 0.25, 25 μg concentration to clearly identify effect of heparine concentration. In the present study, with increasing concentrations of heparin, overall bone formation in the inner area of graft material’s particle pore also increased during the given periods. However, the comparative ratio of the increasing amount of bone in the inner area of graft material’s particle pore following a time lapse was different for the second group (0.25 μg/ml heparin) than the third group (25 μg/ml heparin) (Figure 4). These results mean that the increasing rates of the bone formation in the inner area of graft material’s particle pore following a time lapse was for the group with a low concentration of heparin showed a higher value than the group in high concentration of heparin. In other words, at the early stage, the amount of induced bone formation is large in quantity with an increasing concentration of heparin, but the rate of additional bone formation comparatively was relatively less at the higher concentration of heparin. Therefore we can conclude that heparin enhanced the bone–forming activity of BMP, and BMP and heparin affect the early stage of bone formation.

Some groups have reported independently that
sulfated polysaccharides such as heparin, heparin sulfate, and dextran sulfate enhance osteoblast differentiation induced by BMPs in vitro\textsuperscript{24,25}. In this study, we have demonstrated that heparin enhanced the bone formation induced by rhBMP–2 in vivo as well. This result supports the study of Zhao et al, they reported finding that heparin maintained the concentration of BMP–2 in the culture medium at higher levels and protected BMP–2 from inhibition by noggin, which was induced as part of the negative feedback loop in response to BMP–2. On the basis of the findings they obtained, they proposed a novel mechanism for the stimulatory activity of heparin on BMPs (Fig. 5). In the absence of heparin, BMP activities are negatively regulated by the inhibitory microenvironment for example, prorease or antagonists. Practically, the BMP concentration immediately decreased due to degradation, and BMP antagonists were induced by BMP signaling as part of the negative feedback loop to suppress excess signaling. However, in the presence of heparin, bioactive BMPs remain in the extracellular space for a longer period, and active ligands are protected from suppression by antagonists\textsuperscript{30}.

Bone grafting is possible because bone tissue, unlike most other tissues, has the ability to regenerate completely if provided the space into which to grow. The biologic mechanisms that provide a rationale for bone grafting are osteogenesis including osteoconduction and osteoinduction. Osteoconduction occurs when the bone graft material serves as a scaffold for new bone growth that is perpetuated by the native bone. Osteoblasts from the margin of the defect that is being grafted utilize the bone graft material as a framework upon which to spread and generate new bone. Osteoinduction involves the stimulation of osteoprogenitor cells to differentiate into osteoblasts that then begin new bone formation. This is a phenomenon regularly seen in any type of bone healing process. Osteoinduction implies the recruitment of immature cells and the stimulation of these cells to develop into preosteoblasts. In a bone healing situation such as a fracture, the majority of bone healing is dependent on osteoinduction\textsuperscript{41}.

Fig. 5. Possible mechanism of the effects of heparin on BMP activities

\textbf{A:} In the absence of heparin, BMPs disappear quickly from the culture medium due to degradation, Noggin is also induced by a negative feedback loop to suppress BMP activity. \textbf{B:} In contrast, in the presence of sulfated polysaccharides, BMPs are maintained in the culture medium for longer periods of times, and the protected BMPs present these ligands to receptors even in the presence of noggin. Finally, intracellular signaling of BMPs is continuously activated in the presence of the sulfated polysaccharides.
In this study, we can observe that 6 weeks after grafting of anorganic bone, in the rhBMP-2 and 25 μg/ml heparin mixture, the amount of peripheral new bone and new bone in the inner area of graft material’s particle pore were increased over the 3 weeks. Formed bone marrow exhibited a great deal of hematopoietic marrow but also fat marrow (Figure 3D). Thus, this phenomenon reveals that the rhBMP-2 and heparin mixture has osteoinductive activity. However, new bone formed in the graft materials was thinner than new bone at the surface of graft materials. Thus further study is required concerning bone quality formed by anorganic bone (Fig. 3D).

V. Conclusions

When the graft materials were mixed with only rhBMP-2, osteoinduction was not outstanding. When the materials were mixed with rhBMP-2 and heparin, osteoinduction was outstanding. As the concentration of heparin mixed with rhBMP-2 increased, osteoinduction was increased. Therefore, heparin enhances the osteoinductive potential of rhBMP-2 in vivo. Mixtures of rhBMP-2 and heparin affect bone formation at initial bone formation, but that effect disappeared following a time lapse.

VI. Reference


27. Miyazono K, Kusanagi K, and Inoue H:


