A Thesis
For the Degree of Master of Veterinary Medicine

# Radiographic Comparison of the Osteogenic Effects of Bone Morphogenetic Protein-2, Platelet-rich Plasma and Platelet-rich Fibrin in Canine Bone Defect Model

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JEJU NATIONAL UNIVERSITY

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(Supervised by professor Joo Myoung Lee)

A thesis submitted in partial fulfillment of the requirement
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This thesis has been examined and approved.

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## Abstract

Bone defect is an important clinical injury commonly happens in fracture, tumor, or infection. Recently, bone morphogenetic protein-2 (BMP-2), platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) came into the spotlight for bone defect patients. The purpose of this study is to compare the osteogenic effects of BMP-2, PRP, and PRF more precisely by excluding all the other effect of growth factors from the bone marrow with

radiographs and computed tomography (CT), and to evaluate their practicality in clinical practice. Two healthy mongrel dogs were used in this study. In the lateral diaphysis of humerus and femur of each dog, four bone defects (2.5 ㎜ in diameter, 2 ㎜ in depth) were made at intervals of 10 ㎜ without invasion of the bone marrow cavity. BMP-2, PRP, PRF, and saline (control group) were applied randomly using absorbable collagen sponge as a scaffold (n = 8 for each group). Osteogenic effects were evaluated with radiography and CT until the 8$^{th}$ week after the surgery. BMP-2 showed significantly higher result than that of PRP and PRF in 4$^{th}$ to 7$^{th}$ week in radiographic score while 4$^{th}$ and 6$^{th}$ week in CT value. On 8$^{th}$ week, CT values of the BMP-2 were significantly higher than that of the control group, while not showing much difference compared to PRP and PRF. The results found out that BMP-2 showed strong osteogenic effect for a longer period of time, while PRP and PRF were only effective on early bone-healing stage. In conclusion, BMP-2 is more effective than PRP or PRF in treating bone defect patients and it may possibly complement the limitations of the autograft.

**Key words**: bone morphogenetic protein (BMP-2), platelet-rich plasma (PRP), platelet-rich fibrin (PRF), growth factor, computed tomography (CT), radiography, dog

# INTRODUCTION

Bone defect is an important clinical injury commonly happens in fracture, tumor, or infection. Bone reconstruction is required to recover bone function. For bone defect patients, bone grafts including autograft, allograft, xenograft, and ceramic are commonly used. Autograft is to take the bone from the donor site of the patient and transplant it to the site requiring treatment (47). Autogenous bone graft is recommended as a gold standard because it is excellent in osteoinduction and osteoconduction with no immune rejection. However, there can be several complications associated with additional surgery on the donor site. In addition, the quality of the grafted bone is affected by the morbidity of the donor site and there is a limitation to the obtainable amount (4, 33, 55). To overcome these disadvantages, recent studies are focusing on methods for treating bone defect patients using several growth factors (24, 27, 41, 50). The growth factors promoting bone healing include transforming growth factor-beta (TGF-$\beta$), bone morphogenetic protein (BMP), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF), which are involved in stem cell proliferation and differentiation, angiogenesis, and matrix synthesis (47). As a way of using these growth factors, bone morphogenetic protein-2 (BMP-2), platelet-rich plasma (PRP) and

platelet-rich fibrin (PRF) have come in to attention in recent studies (14, 18, 21, 32, 36, 44).

In 1965, Urist reported ectopic bone induction occurs when a decalcified bone is inserted into the muscle of a rat (49). Later in 1971, Urist et al. found osteoinductive substance that exists in the bone matrix, and named it BMP (48). BMP belongs to transforming growth factor superfamily. BMP-2 and BMP-7 have been proven to have osteoinductive effect in previous studies including dogs (8, 43). In particular, BMP-2 is known to excel in the formation of new bone and has the greatest effect on the proliferation, differentiation and migration of osteoprogenitor cells (5, 29).

Platelets contain a variety of growth factors that enhance bone regeneration, such as TGF-$\beta$, VEGF, FGF, PDGF, and IGF. These growth factors are released into damaged tissues and promote cell proliferation, cell differentiation, cell migration, angiogenesis and matrix synthesis (2, 15, 39, 40). Therefore, PRP with high concentration of platelet can further improve bone healing by increasing the supply of growth factors for the proliferation and differentiation of osteoblasts (28).

Recent studies have suggested PRF as a way to obtain a concentrated growth factor from autologous blood, which is done simply without the use of anticoagulant. PRF is made in the process of natural coagulation because it does not add anticoagulants during the production process. This naturally formed fibrin network during centrifugation has thin and flexible

characteristics. These fibrin networks contain large amounts of growth factors and release growth factors slowly (6, 7, 10, 11, 12).

Many previous studies have reported the effect of bone healing of BMP-2, PRP and PRF. However, no studies have compared BMP-2, PRP, and PRF at the same time in the canine bone defect model. Also, there is no study excluding the osteogenic effect of the growth factor in the bone marrow in canine bone defect model.

The purpose of this study is to compare the osteogenic effects of BMP-2, PRP, and PRF more precisely by excluding all the other effects of growth factors from the bone marrow with radiographs and computed tomography (CT), and to evaluate their practicality in clinical practice.

# MATERIALS AND METHODS

## Animal

Two healthy 2-year-old male mongrel dogs weighing 30 ㎏ and 40 ㎏ were used in this study. In prior to the experiment, the dogs were checked for physical conditions through physical examination, thoracic and abdominal radiography, complete blood count and blood chemistry examination. The experimental dogs received standard care, food and water under veterinary supervision during the experiment.

This experiment was approved by the Animal Care and Use Committee JEJU NATIONAL UNIVERSITY (Approval no. 2018 - 0027).

## PRP Preparation

PRP was prepared based on 'double centrifugation method' established in previous studies (45). 22 ㎖ blood was collected from the jugular vein using a syringe and 2 ㎖ of blood was used for the complete blood count. 10 ㎖ of blood was divided into five 3.2% sodium citrate tubes (VACUETTE® TUBE, Greiner Bio-One, Thailand). Then, the blood was transferred to a 15 ㎖ conical centrifuge tube and ran through primary centrifugation at 1000 × g for 5 minutes at room temperature (Combi-514R, Hanil, Korea). From the bottom, red blood cell layer, the buffy coat layer, and plasma were formed. Plasma and buffy coat layers were collected by pipette and transferred to

another empty 15 ㎖ conical centrifuge tube. Secondary centrifugation was performed at 1500 × g for 15 minutes. The upper layer, platelet-poor plasma (PPP, Acellular plasma) was discarded and PRP in the lower layer was collected with a pipette. The number of platelets in PRP was counted using an automated hematology analyzer (MEK-6450K, Nihon kohden, Japan).

### PRF Preparation

The remaining 10 ㎖ of blood was transferred to a 15 ㎖ conical centrifuge tube without anticoagulant and centrifuged at 400 × g for 10 minutes (10). From the bottom, red blood cell layer, PRF, and platelet-poor plasma (PPP, Acellular plasma) were made. After stabilizing for 5 minutes, upper part of the red blood cell layer and PRF were collected. PPP was used to count the number of platelets by using an automated hematology analyzer to indirectly confirm that platelets were well captured in PRF.

### BMP-2 Preparation

Recombinant human bone morphogenetic protein-2 (rhBMP-2, Cowellmedi, Korea) was used as BMP-2. Sterile distilled water was added 1 hour before use and reconstituted to 1500 ㎍/㎖. Then, using a 5 X buffer solution (glutamic acid 25 mM, sodium chloride 25 mM, glycine 12.5%, sorbitol 2.5%), it was diluted to 125 ㎍/㎖. 10 ㎕(1.25 ㎍ of BMP-2) of this

diluted solution was applied in each bone defect, which is the dose (100 ㎍/㎤) established in previous study (20).

## Absorbable Collagen Sponge Preparation

On a clean bench, an absorbable collagen sponge (CollaTape®, Zimmer, USA) that is used as a scaffold was prepared in advance in the size (2 ㎜ × 4 ㎜) for use in bone defects.

## Surgical Procedures

Antibiotics (Cefazolin sodium, Chongkundang Pharm, Korea, 22 ㎎/㎏, IV) was administered 30 minutes before surgery to prevent infection. The induction of anesthesia was performed with Alfaxalone (Alfaxan®, Jurox, Australia, 3 ㎎/㎏, IV) and maintained with isoflurane (Ifran Liq, Hana Pharm, Korea, 1.5% ~ 2.0%) with 100% oxygen by endotracheal intubation. During operation, oxygen saturation and vital sign were continuously monitored.

The dog was placed in lateral recumbency. The lateral skin of the humerus and femur was shaved and disinfected with 10% povidone iodine and 70% ethanol. The skin of the surgical site was incised and the lateral diaphysis of each humerus and femur was exposed with minimal damage to the soft tissues. Then, using a drill (Cordless driver CD3, Stryker, USA) on the cortex and ensuring not to reach the bone-marrow cavity, four bone

defects (2.5 ㎜ in diameter, 2 ㎜ in depth) were created at 10 ㎜ apart from one another. BMP-2, PRP, PRF and saline (control group) were randomly applied by using scaffold absorbable collagen sponge in each bone defect (n = 8 for each group) (Figure 1). And then muscle, subcutaneous tissue, and skin were closed.

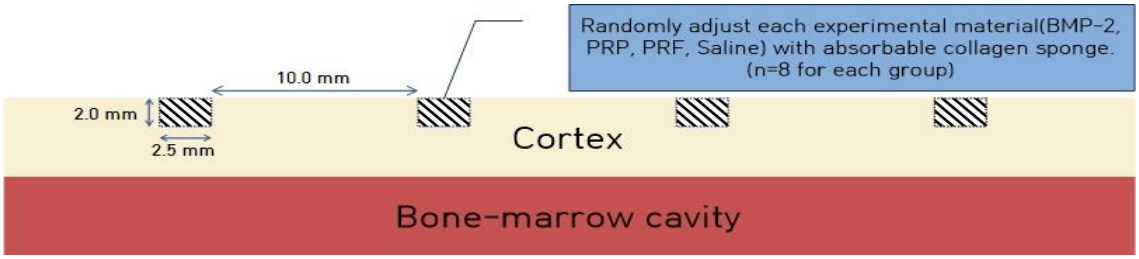


**Figure 1.** In the lateral diaphysis of humerus and femur, four bone defects (2.5 ㎜ in diameter, 2 ㎜ in depth) were made at intervals of 10 ㎜ without invasion of bone marrow cavity. BMP-2, PRP, PRF, and saline (control group) were applied randomly using absorbable collagen sponge as a scaffold (n = 8 for each group).

## Postoperative Care

After surgery, analgesics (Tramadol, Zipan Cap, Ilsung Pharm, Korea, 2 ㎎/㎏, PO, tid) and antibiotics (Cephalexin, Falexin Cap, Dongwha Pharm, Korea, 22 ㎎/㎏, PO, tid) were administered for 7 days to prevent the

infection and to control the pain. The surgical site was disinfected with 10% povidone iodine twice daily, and skin sutures were removed 14 days after the surgery.

## Radiographic Evaluation

Anterio-Posterior view radiographs (REX-525R, Listem, Korea) of humerus (90 kVp, 12.8 mAs, 40-inch film-focus distance) and femur (95 kVp, 16 mAs, 40-inch film-focus distance) were obtained weekly for 8 weeks after the surgery. Radiographs were taken 5 times repeatedly for each bone defect, and three veterinarians who were not informed about the experiment scored the healing degree on the bone defect area (0: none, 1: <20%, 2: 20%~39%, 3: 40%~59%, 4: 60%~79%, 5: 80%~100%).

## Computed Tomographic Evaluation

On $4^{th}$, $6^{th}$, $8^{th}$ week after the surgery, CT was performed after sedation of the experimental dogs with medetomidine (Domitor®, Pfizer, USA, 0.05 ㎖/kg, IV). The CT scan (Somatom Emotion 16, Siemens, Germany) was performed at 0.75 ㎜ interval and the three-dimensional image was reconstructed using CT software (Syngo CT, Siemens, Germany). The region of interest (2.0 ㎜ in width, 1.5 ㎜ in height) was set at the center of the bone defect and the CT value (Hounsfield Unit: HU) was obtained. Each bone defect was measured 3 times and the results were averaged. The HU

is a relative value of air as −1000, distilled water as 0, and human compact bone as +1000.

## Statistical Analysis

Statistical analysis was performed with SPSS software (SPSS22, IBM, USA). Radiographic and CT assessments were analyzed by one-way ANOVA. Post-hoc analysis was performed using Tamhane T2 test for radiography and Tukey's test for CT to identify significant differences between groups ($P < 0.05$ was considered statistically significant).

# RESULTS

## Platelet Counting

Each platelet counts of PRP were 1447 × $10^3$ platelets/㎕ and 1356 × $10^3$ platelets/㎕, and were 5.02 times and 5.94 times higher than whole blood, respectively. PPP platelet counts of the upper layer of PRF were measured as 2 × $10^3$ platelets/㎕, 4 × $10^3$ platelets/㎕. These results indirectly confirmed that platelets were well captured in PRF.

## Radiographic Evaluation

The results of the radiographic evaluation are shown in Figure 2 and 3. The mean values of all evaluation intervals were higher in the order of BMP-2, PRF, PRP and control group.

The radiographic score of control group was 0.8 ± 0.76 in $4^{th}$ week, 1.6 ± 0.78 in $5^{th}$ week, 2.5 ± 1.10 in $6^{th}$ week, 3.8 ± 0.90 in $7^{th}$ week. The radiographic score of PRP was 1.6 ± 1.06 in $4^{th}$ week, 2.3 ± 1.01 in $5^{th}$ week, 3.0 ± 0.78 in $6^{th}$ week, 3.9 ± 0.74 in $7^{th}$ week. The radiographic score of PRF was 1.7 ± 1.13 in $4^{th}$ week, 2.5 ± 0.93 in $5^{th}$ week, 3.2 ± 0.92 in $6^{th}$ week, 3.9 ± 0.78 in $7^{th}$ week. The radiographic score of BMP-2 was 2.5 ± 0.83 in $4^{th}$ week, 3.2 ± 0.78 in $5^{th}$ week, 4.0 ± 0.81 in $6^{th}$ week, 4.5 ± 0.59 in $7^{th}$ week.

Radiographic scores were significantly higher in all experimental groups

than in control group on 4th to 5th week, and in the case of BMP-2, it showed significantly higher results than PRP and PRF. In 6th to 7th week, BMP-2 showed significantly higher results than that of other groups, while PRP and PRF did not show a significant difference compared to the control group.

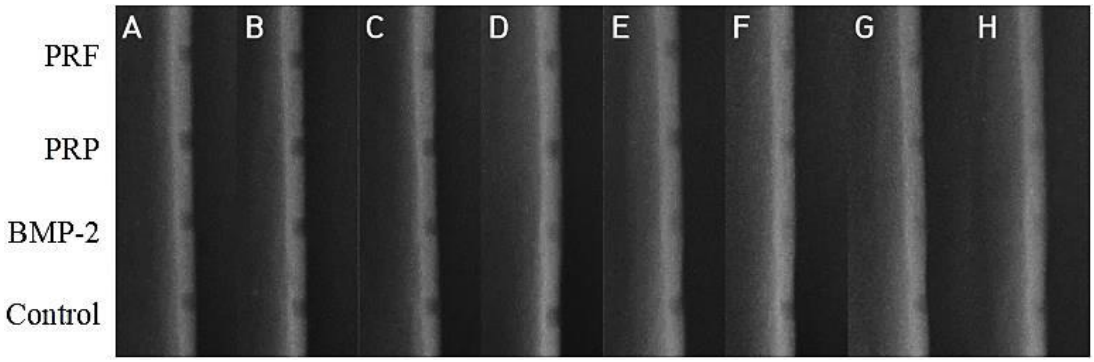


**Figure 2.** Anterio-Posterior view radiographs of the defects at (A)1st week, (B)2nd week, (C)3rd week, (D)4th week, (E)5th week, (F)6th week, (G)7th week, (H)8th week after the surgery. Radiographs were taken 5 times repeatedly for each bone defect, and three veterinarians who were not informed about the experiment scored the healing degree on the bone defect area (0: none, 1: <20%, 2: 20%~39%, 3: 40%~59%, 4: 60%~79%, 5: 80%~100%).

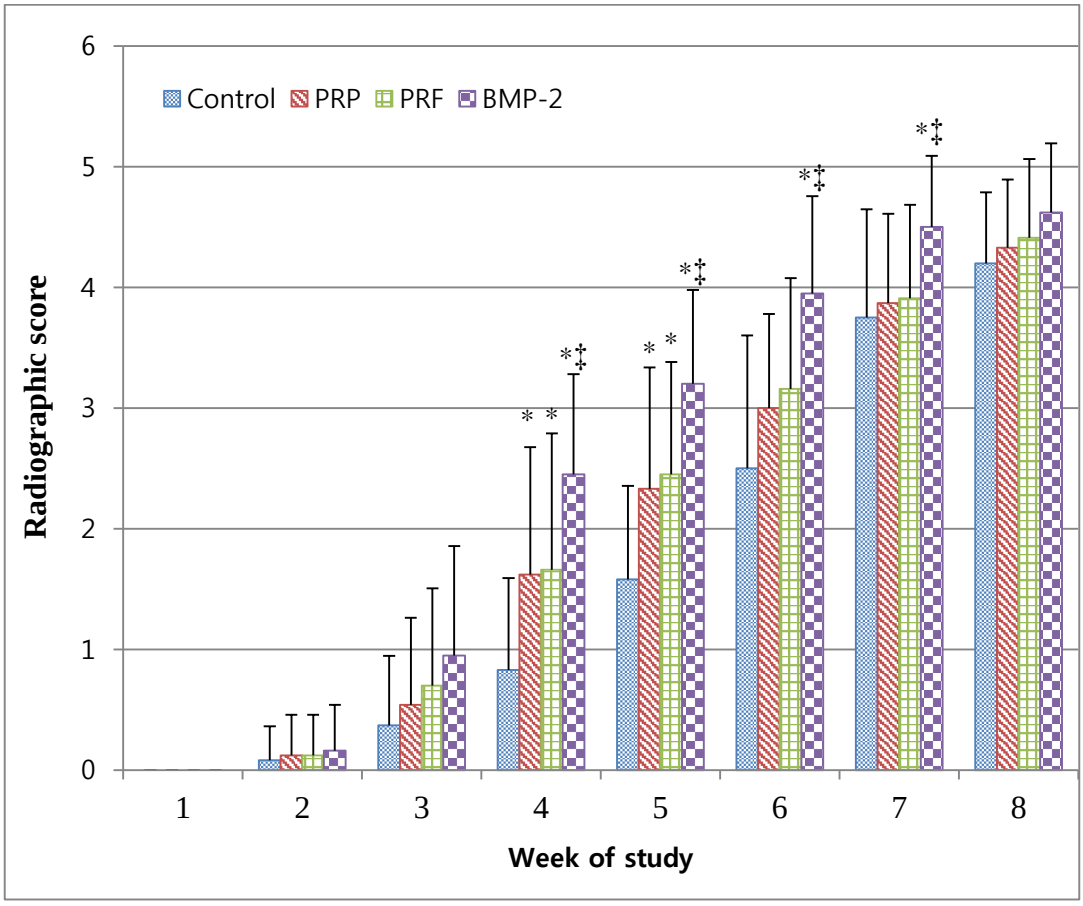


**Figure 3.** Radiographic score of healing degree on the bone defect area (Mean ± SD). Compared to the other groups, BMP-2 showed significantly higher results in 4th to 7th week. PRP and PRF showed significantly higher results than the control group only in 4th to 5th week.

* Significantly higher than control group ($P < 0.05$).

‡ Significantly higher than other experimental groups ($P < 0.05$).

## Computed Tomographic Evaluation

The results of the CT evaluation are shown in Figure 4 and 5. The mean values of all evaluation intervals were higher in the order of BMP-2, PRF, PRP and control group.

The CT value of control group was 73 ± 48,2 in 4th week, 291 ± 147.8 in 6th week, 689 ± 72.7. The CT value of PRP was 197 ± 105.6 in 4th week, 451 ± 139.0 in 6th week, 785 ± 96.9 in 8th week. The CT value of PRF was 214 ± 108.2 in 4th week, 480 ± 166.0 in 6th week, 805 ± 113.9 in 8th week. The CT value of BMP-2 was 387 ± 78.2 in 4th week, 744 ± 106.6 in 6th week, 916 ± 134.4 in 8th week.

CT values were significantly higher in all experimental groups than in control group at 4th week, and in the case of BMP-2 showed significantly higher results than PRP and PRF. In the 6th week, BMP-2 showed significantly higher result than other group, and PRP and PRF did not show a significant difference compared to the control group. In the 8th week, only BMP-2 showed significantly higher result than that of the control group, while there was no significant difference in BMP-2, PRP, and PRF groups.

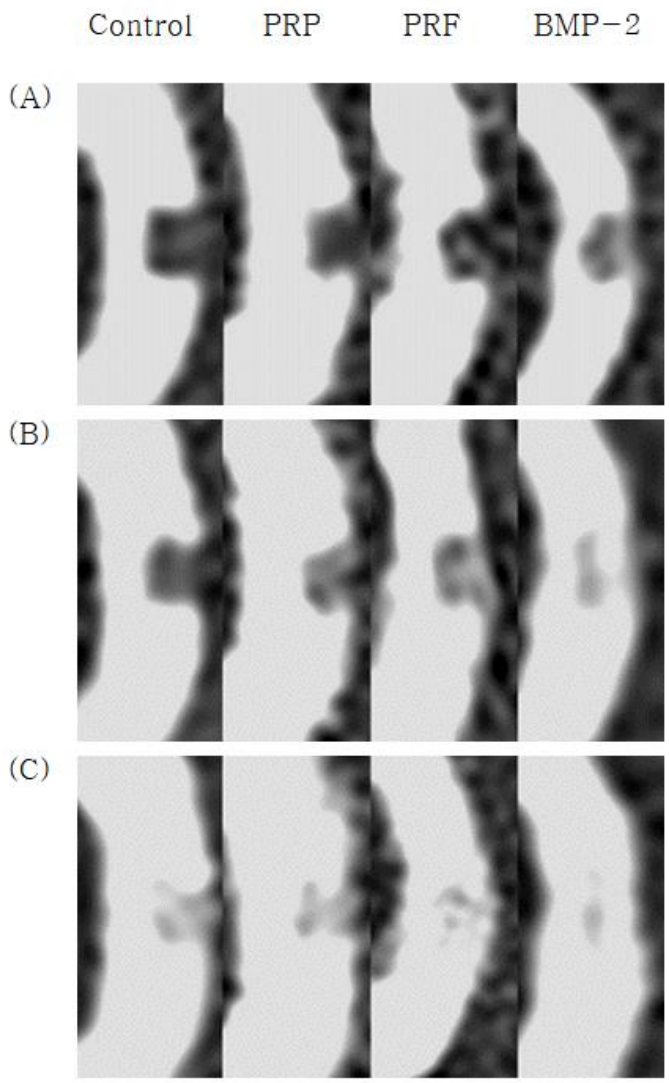


**Figure 4.** CT scan of the defects at (A) 4th week (B) 6th week (C) 8th week after the surgery. The region of interest (2.0 ㎜ in width, 1.5 ㎜ in height) was set at the center of the bone defect and the CT value (Hounsfield Unit: HU) was obtained. The HU is a relative value of air as −1000, distilled water as 0, and human compact bone as +1000.

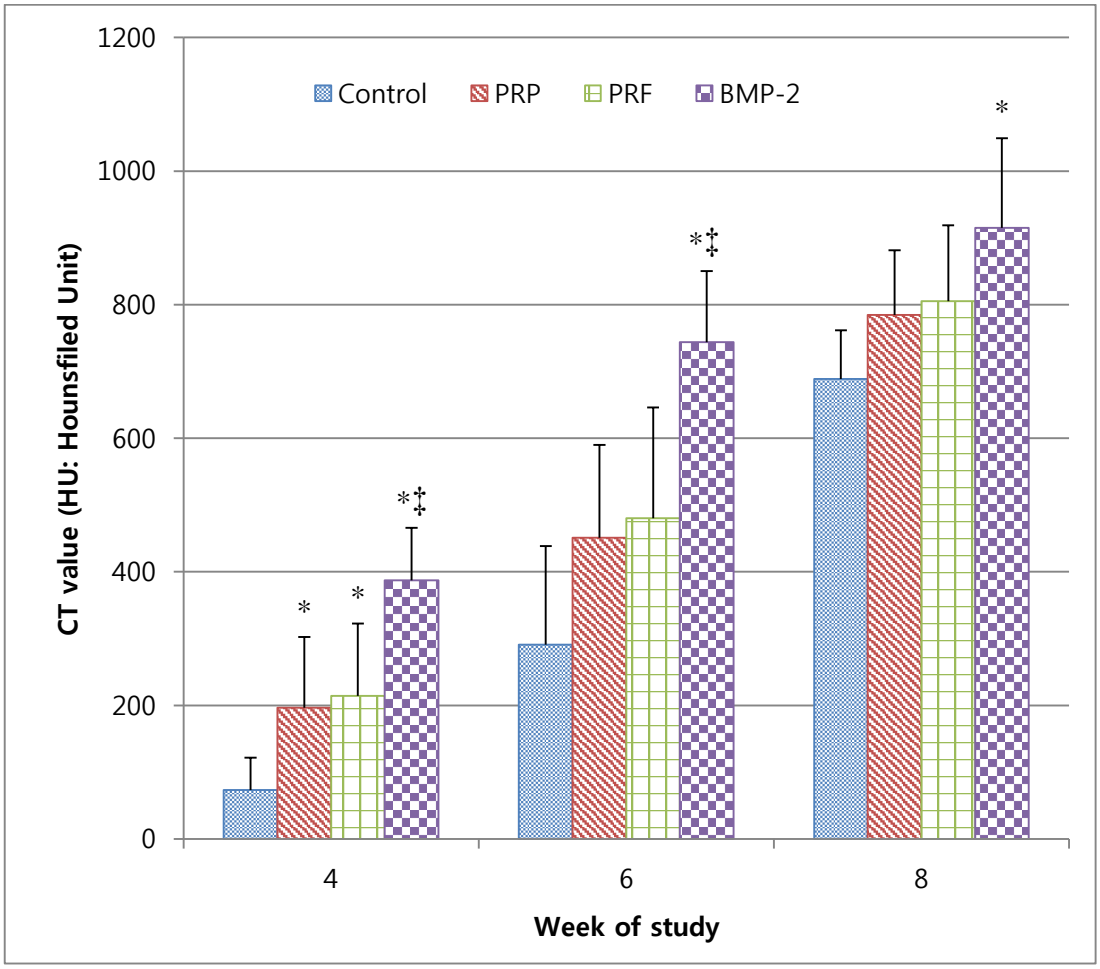


**Figure 5.** CT value on bone defect area (Mean ± SD). Compared to the other groups, BMP-2 showed significantly higher results in 4th and 6th week. In 8th week, while CT value of BMP-2 showed no significant difference with the other experimental groups, the result was significantly higher than the control group. PRP and PRF showed significantly higher results than control group only in 4th week.

* Significantly higher than control group ($P < 0.05$).

‡ Significantly higher than other experimental groups ($P < 0.05$).

# DISCUSSION

Bone defect models in dogs used in previous studies include ulnar segmental defect, radius segmental defect, mandibular defect, peri-implant bone defect, calvarial bone defect and so on (20, 21, 30, 44, 46). However, the bone defect of the present study was made in the cortex without invading the bone marrow cavity so that the effect of the experimental materials might be evaluated accurately, excluding the osteogenic effect of the bone marrow.

BMP-2 is the most effective osteoinductive growth factor that promotes proliferation, differentiation, migration and matrix synthesis of osteoprogenitor cells (9, 17, 35, 56). The osteogenic effect of BMP-2 has been demonstrated in various studies on rats, rabbits, goats and dogs (3, 32, 39, 51, 53). Especially in dogs, when BMP-2 is applied to the tibial bone defect model, the radiographic evaluation result is high after $8^{th}$ week, and limb function recovered more rapidly in previous study (14). Another study also reports that callus formation was observed 2 weeks after BMP-2 application in a femoral nonunion fracture, and union was completed 8 weeks after radiographic evaluation (19). These results are similar to present study of showing significant osteogenic effect from $4^{th}$ week by using radiographic evaluation and CT evaluation when BMP-2 was applied to the bone defect. This suggests that BMP-2 promotes bone regeneration

through osteoinduction from the early stages of healing.

The osteogenic effect of PRP has been demonstrated in many studies (25, 27, 31). However, there have been many reports of no efficacy (36, 37, 52). This is because the efficacy of PRP is affected by the quality of PRP being used. Several commercial products for preparing PRP from human blood have been coming into the market. When human-PRP kit is used with canine blood, the quality of PRP is inconsistent (16). The best canine-PRP can be obtained by primary centrifugation at 1000 × g for 5 min and secondary centrifugation at 1500 × g for 15 min (45). Therefore PRP is prepared by this double centrifugation method to obtain high quality PRP which is concentrated more than 5 times.

When PRP is activated, 70% of the contained growth factor are released within 10 minutes and reaches 100% release after 1 hour. These released growth factors are only effective on the bone defect site for up to 10 days (13, 28). Non-activated PRP shows better wound healing and angiogenic effects in vivo than activated PRP, and the differentiation of fibroblasts is better in vitro (42). For this reason, non-activated PRP was used and let it activate slowly in vivo when applied to the bone defect site in this study.

Recently, PRF is suggested as a simple way of obtaining a concentrated growth factor from autologous blood without using anticoagulant (6, 7, 10, 11, 12). So far, no method of obtaining high quality PRF from canine blood has been studied. The blood was centrifuged at 400 × g for 10 min in

present study, which is the method of preparing PRF from human blood (10). In the case of the canine blood, the fibrin did not form perfectly, so it was stabilized for five more minutes. Then the separated PRF layer was used.

When BMP-2 was applied to bone defect, the result was significantly higher than that of PRP and PRF on $4^{th}$ to $7^{th}$ week in radiographic score and $4^{th}$ and $6^{th}$ week in CT value. But there was no difference in the $8^{th}$ week. These results are similar to previous studies suggesting that BMP-2 significantly promotes bone regeneration till $6^{th}$ week (1, 26, 34). In addition, the results are similar with the previous study in rabbit calvarial defect model. When BMP-2 is applied, wider radiopaque area is shown on $6^{th}$ week in radiological evaluation compared to that of PRP, while there is no significant difference in the $12^{th}$ week (54). These results indicate that BMP-2 promotes early bone healing through strong osteoinduction, and that the effect of BMP-2 is lower in the later stages is due to the short half-life of BMP-2 (9).

PRP and PRF were significantly different from the control group on $4^{th}$ to $5^{th}$ week in radiography and $4^{th}$ week in CT, and there was no difference thereafter. These results are similar to previous studies that the effects of PRP and PRF are confined to the early stages of bone healing (18, 22, 23, 52). This is because the growth factors released from PRP and PRF have a direct effect on the cells only for 5 to 7 days (27).

No significant difference was found between PRP and PRF in this study. These results are consistent with the previous study (25). However, the results were inconsistent in another study which reported that PRF group had a significantly higher new bone formation rate and bone-implant contact than PRP group in the peri-implant bone defect models of canine (21). This difference seemed to be caused by executing different centrifugation method. While primary centrifugation (2000 × g, 3 min) and secondary centrifugation (5000 × g, 5 min) was used in the previous study, primary centrifugation (1000 × g, 5 min) and the secondary centrifugation (1500 × g, 15 min) was used in this study. And the two studies had a different defect model.

There was no significant difference among BMP-2, PRP, and PRF in radiography and CT evaluation at $8^{th}$ week. This is different from the previous study that compares BMP-2 and PRP in the critical-size calvarial defect in rabbits. Their study reports that radiographic evaluation and histopathological evaluation of BMP-2 shows significant bone regeneration for three months after the experiment, which is significant than PRP (38). The fact that our defect model being not critical-size defect seems to be the cause of this difference because it can heal naturally over time without any treatment. The results of the radiographic score ($4.2 \pm 0.59$), CT value ($689 \pm 72.7$) of the control group in $8^{th}$ week of our study showed that natural healing has actually occurred.

Further studies will need to compare BMP-2, PRP and PRF in a critical-size defect model with large-size samples. In addition, the method of preparing high quality canine-PRF may be also needed to be studied.

# CONCLUSION

The osteogenic effects of BMP-2, PRP and PRF were compared using radiography and CT in the canine bone defect model excluding the bone marrow osteogenic effect. The results show that BMP-2 has strong osteogenic effect for a longer period of time, while PRP and PRF were only effective on early bone-healing stage. In conclusion, BMP-2 is more effective than PRP or PRF in treating bone defect patients and it may possible complement the limitations of the autograft.

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# 개의 골결손 모델에서의 골형성단백질-2 (BMP-2), 혈소판풍부혈장 (PRP), 혈소판풍부섬유소(PRF)의 방사선을 통한 골형성효과 비교

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## 초 록

골결손은 골절, 종양, 감염 등에 의해 흔히 발생하는 임상에서 중요한 손상이다. 골형성단백질-2 (BMP-2), 혈소판풍부혈장 (PRP), 혈소판풍부섬유소 (PRF)는 골결손 환자를 위해 사용되는 방법으로 최근 연구들에서 주목 받고 있다. 본 연구의 목적은 골수에 들어있는 성장인자의 영향을 배제하여 더욱 정확하게 방사선촬영과, 컴퓨터단층촬영 (CT)을 통해 BMP-2, PRP, PRF의 골형성효과를 비교하고, 이들의 임상에서의 실용성을 평가하는 것이다. 두 마리의 건강한 잡종견을 본 연구에서 사용하였다. 각 실험견의 상완과 대퇴의 외측골간피질에 4개의 결손을 골수강을 침범하지 않게 10 ㎜ 간격으로 만들어주었다. 그 후 BMP-2, PRP, PRF, saline (대조군)을 흡수성 콜라겐 스펀지를 비계로 사용하여 무작위로 결손부에 적용하였다 (n = 8). 수술 후 8주까지 방사선촬영과, CT를 통해 골형성효과를

평가하였다. 4 ~ 7째주 방사선촬영 점수와, 4째주 6째주 CT값에서 BMP-2가 PRP, PRF보다 유의적으로 높은 결과를 보여주었다. 8째주에서는 BMP-2의 CT값이 대조군과 비교해 유의적으로 높은 값을 보여주었지만 PRP, PRF는 대조군과 유의적인 차이를 보이지 않았다. 본 연구의 결과 BMP-2는 PRP, PRF보다 더 긴 기간 높은 골형성효과를 보여주었다. 이러한 결과를 통해 골결손환자를 치료하기 위한 방법으로 BMP-2가 PRP, PRF보다 더욱 효과적이며, 자가골이식이 가지고 있는 한계점들을 보안해 줄 수 있을 것으로 판단된다.

**주요어**: 골형성단백질-2 (BMP-2), 혈소판풍부혈장 (PRP), 혈소판풍부섬유소 (PRF), 성장인자, 컴퓨터단층촬영 (CT), 방사선촬영, 개