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MS Dissertation

Recombinant human BMP-2 enhances osteogenesis of demineralized bone matrix

Graduate School of Chosun University

Department of Bio New Drug Development

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This is certify that this thesis has successfully met the dissertation requirements for the degree of Master of Science of the Graduate School of Chosun University

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탈회된 골기질과 인간 재조합 골형성 단백질 BMP-2 의 병합이 신생골 형성에 미치는 효과

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탈무기질화 골기질(demineralized bone matrix: DBM)은 골전도(osteoconductive)효과와 소량의 골형성 단백질이 함유되어 있어 골유도(osteoinductive)효과를 가진 골이식재로 골형성 위한 수술에 사용되고 있다. 그러나 골형성 유도효과가 미미하다는 보고들이 있다. 정제된 인간 골형성 단백질(rh-bone morphogenic protein-2, BMP-2)는 동물실험에서 양호한 골형성 효과를 보이며 미국 FDA 를 통과하여 현재 치과임상분야에서 사용되고 있다. BMP-2 는 수용성이기 때문에 국소적인 골결손 부위에 주로 사용되고 있는 교원질 운반자는 매우 고가이다. 현재까지 유양동 삭개술후 유양동 골형성을 위해 수산화칼슘, 세라믹은 보고가 되었으나, 탈무기질화 골기질에 BMP-2 를 젤라틴 운반자와 함께 결합한

방법은 아직까지 보고되지 않았다. 본 연구의 목적은 BMP-2 를 젤라틴 운반자와 함께 결합한 탈무기질화 골기질을 사용하여 흰쥐의 유양동에 이식한 후 골형성 효과를 관찰하고자 하였다.

탈무기질화 골기질과 BMP-2 를 젤라틴 운반자에 결합된 탈무기질화 골기질의 표면을 실체현미경과 주사전자현미경으로 관찰하였다. 흰쥐 21 마리를 세 군으로 나누어 생리식염완충액을 젤라틴 운반자와 함께 결합한 탈무기질화 골기질 (대조군), BMP-2 (0.075 mg/mL) 를 젤라틴 운반자와 함께 결합한 탈무기질화 골기질 (실험군 I), BMP-2 (0.375 mg/mL) 를 젤라틴 운반자와 함께 결합한 탈무기질화 골기질 (실험군 II)을 흰쥐 양측 유양동에 이식하였다. 골 형성의 이식기간에 따른 변화를 보기 위해 칼슘에 결합하는 형광색소 calcein blue 를 이식 4 주, oxytetracycline 을 이식 8 주, alizarin red 를 이식 10 주째 복강 주사하였다. 12 주에 유양동을 적출하여 *in vivo* CT 를 시행한 후 비탈회 조직을 처리하여 광학현미경과 공주사 현미경으로 골형성을 비교 관찰하였다.

실체 현미경 및 주사 전자현미경 소견상 탈무기질화 골기질은 3 차원적인 다공성 구조를 지니고 있었으며 BMP-2 (0.375 mg/mL) 를 젤라틴 운반자와 함께 결합한 탈무기질화 골기질의

미세표면은 표면이 코팅되어 있었으며 젤라틴으로 인해 다공성이 감소된 소견을 보였다. CT 소견상 실험군 II 에서 현저한 골형성을 나타내었으며 실험군 I 도 양호한 골형성을 나타내었으나 대조군에서 골형성이 미미하였다. 광학현미경에서 실험군 II 에서는 신생골 형성이 전반적으로 잘 형성되어 있었으며 실험군 I 에서는 골전도가 잘 형성되었고 신생골형성은 일부 진행되고 있었다. 하지만 대조군에서 미미한 효과를 보이고 있었다. 공주사 현미경상 실험군 I 에서는 형광색소 3 가지가 이식기간별로 신생골 형성을 나타내는 표지를 보이고 있었으나 대조군에서는 한가지 색만 관찰되어 미미한 골형성 효과를 보였다.

본 실험결과를 토대로 BMP-2 를 젤라틴 운반자와 함께 결합한 탈무기질화 골기질은 유양동 이식에 효과적인 골형성을 보일 것으로 사료된다.

1. INTRODUCTION

Bone regeneration is a complex and long lasting process. The critical sized defects do not heal spontaneously and their complete regeneration can only be ensured by the implantation of materials such as bone grafts or bone substitutes. The aim of cholesteatoma surgery is to obtain a safe, dry, and self-cleaning ear. Unfortunately, the development of a problematic mastoid cavity is a complication of this treatment in more than 10 % of cases, even when the surgical technique is meticulous [1, 2].

The purpose of mastoid obliteration is to replace the open mastoid cavity with a material that becomes viable and is free of infection or cholesteatoma. Various materials, both biological and synthetic, have been used as a bone substitute for mastoid obliteration [3]. Autogenous graft (bone pate or bone chip) has been considered the gold standard for bone graft because it poses no risk of rejection and is naturally osteoinductive. However, bone pate collection is not easy to perform in recurrent cholesteatoma. Allograft material has been used to avoid the complications of donor site morbidity but it has increased risks of infection and rejection and poor osteoconductive properties. Synthetic bone graft substitutes are osteoconductive agents that consist of hydroxyapatite, β-tricalcium phosphate, calcium sulfate, or a combination of these minerals [4]. Demineralized bone matrix (DBM) is

derived from human cortical bone and is prepared by removing the minerals, allowing the organic and protein constituents to remain. It contains bone morphogenic protein (BMP), which is a potent osteoinductive glycoprotein, and collagen, which is an osteoconductive matrix. DBM has been successfully used in orthopedic and craniofacial applications [5, 6], and an animal study was performed to evaluate the use of this product for mastoid obliteration [7, 8]. However, the DBM content in commercially available preparations is highly variable. The mean content of BMP is expressed as pictograms per gram [9]. The osteoinductive effect of DBM is controversial and limited effects of DBM have been reported [10, 11]. BMP-2 is a member of the BMP subgroup of the transforming growth factor-β super family. BMP -2 stimulates healing of bone defects [12, 13], and therefore, has great potential for clinical applications. However, BMP disperses away too rapidly when topically injected into the target tissue because it unexpectedly diffuses into the blood stream or nearby tissues. Hence, its continued action is diminished and bone is produced in the nearby tissues instead of forming locally [14].

Therefore, the use of a scaffold for rhBMP-2 is important. Collagen has been widely applied in tissue engineering applications and to some extent in delivery systems as a scaffold [15]. Recently, gelatin sponge has been been used to effectively deliver proteins or genes [16, 17]. Moreover, gelatin

sponge is less expensive than atelocollagen. There are several commercially available gelatin based carriers for drug delivery that are being applied in tissue engineering applications [18, 19]. The most commonly used ones are Gelfoam®, which is a sterile and workable surgical sponge prepared from a specially treated and purified gelatin solution, and it is used as a haemostatic device. The effect of BMP-2 on osteogenesis in mastoid obliteration has not yet been fully investigated. Only two papers were found in a search of English Medline [20, 21]. Nishizaki et al. used the BMP-2/collagen composites for mastoid obliteration [20]. Kim et al used commercial BMP-2 coated hydroxyapatite granule for mastoid obliteration [21]. DBM is more effective than hydroxyapatite because it is both osteoinductive and osteoconductive. To date, the synergistic effects of using rhBMP-2 combined with DBM for mastoid obliteration have not been reported on. The purpose of this study was to evaluate the enhancing osteogenesis of rhBMP-2 loaded DBM using a gelatin sponge in the mastoid obliteration model.

2. MATERIALS AND METHODS

2.1. Materials

Sprague-Dawley rats (male) were purchased from Samtako (SAMTAKO

BIOKOREA Co. Osan, Korea). Demineralized bone matrix (Korea Bone Bank Co., Ltd., Seoul, Korea), rhBMP-2 (CowellMedi Co. Ltd, Pusan, Korea), and gelatin sponge were obtained (Spongostan standard, Ethicon Korea Co, Ltd., Seoul, Korea). Calcein blue, alizarin red (Sigma Aldrich Co, Seoul Korea), and oxytetracycline (Pfzer Korea, Seoul, Korea) were used.

2.2. Gross and stereomicroscopic observation of DBM and, BMP-2 soaked gelatin hydrogel loaded DBM

The surfaces of the DBM, gelatin sponge, and BMP-2 soaked gelatin hydrogel were observed by stereomicroscopy.

2.3. Scanning electron microscopic observation of surface of DBM and gelatin-BMP-2 hydrogel loaded DBM

The morphology of the DBM, gelatin sponge, gelatin sponge-BMP loaded DBM were observed by a S-4700 field emission scanning electron microscope (FE-SEM, Hitachi,Tokyo, Japan). Before FE-SEM observation, the materials were sputter-coated with gold. Sample preparation and measurements were performed in accordance with the manufacturer's instructions.

2.4. Surgery

All animal experiments were performed in accordance with the local ethical committee at Chosun University. Fourteen male Spargue-Dawley outbred rats (age 12 weeks) with normal eardrums and Preyer reflexes were used for the experiment and they were housed separately in sterile cages. The animals were divided into experimental and control groups. The rats were anesthetized with an intramuscular injection of zolletil and xylazine. Lidocaine 1 % with 1/100,000 epinephrine was injected into the soft tissue over the tympanic bulla and then a retroauricular incision was made. The bulla was exposed and a hole was created by drilling. After removing the mucoperiosteum of the bulla by using a microelevator with alligator forceps, bulla obliteration was done using the rhBMP-2 (0.075 mg/mL) soaked gelatin sponge loaded DBM in the experimental group I (n = 7) and the rhBMP-2 (0.375 mg/mL) soaked gelatin sponge loaded DBM in the experimental group II. In the control group (n = 7), the bulla was obliterated using the PBS soaked gelatin sponge loaded DBM. The wounds were then closed. Ciprofloxacin was injected intramuscularly for prevention of infection.

2.5. Fluorescent bone labeling

The animals were administered fluorescent bone labels for the qualitative evaluation of sequential bone formation. To assess the active mineralization of new bone formation, each group received calcein blue intravenously at 4 weeks, oxytetracycline hydrochloride at 8 weeks, and alizarin red at 10 weeks (Sigma-Aldrich Chemical Co. St Louis, USA).

2.6. In vivo microCT

The animals of each group were sacrificed 12 weeks post-surgery. Temporal bone computed tomography (CT) images were obtained using a NFR-MXSCAN-G90 whole body CT scanner (General Electric, Fairfield, CT, USA).

2.7. Histological evaluation

The bullae were harvested in each group. They were fixed in a formaldehyde solution (10 %, 48 h). Samples were embedded in Technovit 7200 VLC

(Kultzer & Co, Wehrhein, Germany), a glycol methacrylate solution. After polymerization, the samples were processed using the sawing and grinding technique. After examination of the fluorescent labeling by using confocal microscopy (Leica, Wetzlar, Germany), the specimens were stained with hematoxylin and eosin. Histomorphometric analysis of the middle portion of each group was evaluated by a PC-based image analysis system (Image Inside, Focus Technology, Daejon, Korea). All values are presented as the mean ± SD. Significance was set at 5 % and p values were adjusted for multiple comparisons.

3. RESULTS

3.1. Gross and stereomicroscopic findings

Yellow colored porous three dimensional appearance of DBM and the rhBMP-2 soaked gelatin sponge covering the DBM is noted (Fig. 1, Fig. 2)

3.2. SEM findings

SEM showed an irregular shape, surface roughness, and porous structure. BMP-2 loaded DBM showed the presence of gelatin coated particles and

reduced porous structure (Fig. 3A, B, C)

3.3. CT findings

No adverse events were observed during the healing interval. The CT of the bulla cavity 12 weeks after obliteration shows the state of bone regeneration. Radio-opaque regions are clearly visible at the bulla in the rats that received gelatin-BMP2 hydrogels incorporating bound DBM (Fig. 4A). The size of the radio-opaque regions clearly showed that the largest amount of bone had formed at the bulla in the experimental group, group II, compared to group II. However, limited bone formation in the control group was noted (Fig. 4C).

3.4. Light microscopic findings

Histological observations showed that a significant amount of new bone was formed in group I. Trabecular bone with marrow tissue was visible. New bone formations of DBM in group I were incomplete but diffuse osteoconduction in the spaces and partial osteoinduction were observed. In contrast to groups I and II, no remarkable osteoconduction was observed in the control group. No new bone formation was visually detectable in the

control group. Histomorphometric analysis showed that there were significant differences between each group ($p = 0.124$, group I vs. control, group II vs. control $p = 0.252$, group I vs. II, $p = 0.042$) (Fig. 6).

3.5. Confocal microscopic findings

Confocal microscopic findings revealed that three distinct colors which corresponded to sequential osteogenesis were observed in the group II. However, poor sequential osteogenesis was observed in the control group.

4. DISCUSSION

The use of autogenous material remains the gold standard for use in bone repair due to the fact that there is little chance of immune rejection and because of its innate osteoconductive, osteoinductive and osteogenic potential. For mastoid obliteration, it is not easy to collect the bone pate sufficiently in revision surgery. Numerous alternatives to autologous bone graft are available. Common sources of bone graft materials include allogenic bone, synthetic calcium phosphate salts, coralline materials and bioactive glass [22]. It is well known that structural bone allografts frequently fail to heal and remodel, and result in many complications [23]. These poor long-term results of the structural bone allograft have led to the exploration of new

alternative using tissue engineering technology. Human DBM, which has become a very common bone graft substitute, has shown the ability to aid in new bone formation in many different clinical settings include long bone defects, craniofacial reconstruction and spinal fusion [24, 25]. Recombinant human BMP- 2 (rhBMP-2) is a potential activation factor for bone repair, when combined with a matrix like collagen or decalcified bone. Although BMP implantation without a carrier induces bone formation, BMP is relatively soluble and therefore requires the use of a carrier [26]. The carrier has the important role of delivering BMP-2 to the implant site, retaining it locally at an effective concentration and providing an environment compatible for obliteration [27]. Many common carriers including the absorbable type I collagen sponge, and synthetic polymers (polylactic acid, polyglycolic acid) have been investigated [28]. Atelocollagen has commonly been used as a carrier. In the present study, gelatin sponge (Gelfoam®) was used for BMP-2 delivery. Gelfoam® is a water-insoluble, haemostatic device prepared from purified porcine skin gelatin. It is a widely used material for hemostasis. Gelatin sponge is also used as a scaffold for the controlled release of growth factors [29]. BMP-2 has the significant properties of inducing differentiation of undifferentiated mesenchymal cells into osteogenic cells and promoting the proliferation of these cells. In the present study, the combination of DBM and BMP-2 (0.375

mg/mL, group II) exhibited a strong synergistic effect on osteogenesis, whereas the PBS soaked gelatin sponge and DBM did not. We also demonstrated that the incorporation of DBM could be enhanced if it is loaded by an rhBMP-2 soaked gelatin sponge. In the present study, sequential osteogenesis was observed by confocal microscopy. As a marker of bone regeneration, the fluorochrome-labeled bone surface differed between each groups. Bone seeking fluorochromes provide a useful tool for analyzing changes of bone morphology. The presence of the fluorochromes indicates the site, time and amount of bone deposition, and adds to the information contained in the bone specimens [30]. In the present study, examination by confocal microscopy showed that more intense and homogenous bands of integrated fluorochromes were present in the group II compared to the control group**.** From these results, rhBMP-2 activated DBM for the enhancement of bone regeneration. These results might provide a basis for the clinical application of BMP-2 in mastoid obliteration.

5. SUMMARY AND CONCULSIONS

Demineralized bone matrix (DBM) has been successfully used in orthopedic and craniofacial applications. The osteoinductive effect of DBM is controversial and the limited effect of DBM has been reported. Recombinant

human bone morphogenic protein (BMP) -2 stimulates the healing of bone defects; however, BMP disperses away too rapidly when topically injected into the target tissue because it unexpectedly diffuses into the blood stream or nearby tissues. Therefore, the use of a scaffold for rhBMP-2 is important. To date, there has been no report on the synergistic effects of using rhBMP-2 combined with DBM for mastoid obliteration. The purpose of this study is to evaluate the enhanced osteogenesis of rhBMP-2 loaded DBM using a gelatin sponge in the mastoid obliteration model.

The surface of the DBM and the rhBMP-2 soaked gelatin sponge loaded DBM was observed by scanning electron microscopy (SEM). The bulla was exposed and a hole was created by drilling. After removing the mucoperiosteum of the bulla by using a microelevator with alligator forceps, bulla obliteration was done using the rhBMP-2 (0.075 mg/mL) soaked gelatin sponge loaded DBM in the experimental group I (n = 7) and the rhBMP-2 (0.375 mg/mL) soaked gelatin sponge loaded DBM in the experimental group II. In the control group (n = 7), the bullae were obliterated using the PBS soaked gelatin sponge loaded DBM. To assess the active mineralization of new bone formation, each group received intravenous calcein blue at 4 weeks, oxytetracycline hydrochloride at 8 weeks, and alizarin red at 10 weeks. The animals of each group were sacrificed 12 weeks post-surgery. Osteogenesis was evaluated by *In vivo* CT and histological observation.

SEM showed an irregular shape, surface roughness, and porous structure. BMP-2 loaded DBM showed gelatin coated particles and reduced porous structure. The size of the radio-opaque regions clearly showed that the largest amount of bone had formed at the bulla in the experimental group II compared to group I. However, limited bone formation in the control group was noted. New bone formation in group I was incomplete but diffuse osteoconduction in the spaces and partial osteoinduction were observed. In contrast to group I and II, no remarkable osteoconduction was observed in the control group. Histomorphometric analysis showed that there were significant differences between each group. Confocal microscopic findings revealed that three distinct colors which corresponded to sequential osteogenesis were observed in the group II. However, poor sequential osteogenesis was observed in the control group.

From the results, rhBMP-2 activated DBM for the enhancement of bone regeneration. These results might provide a basis for the clinical application of BMP-2 in mastoid obliteration.

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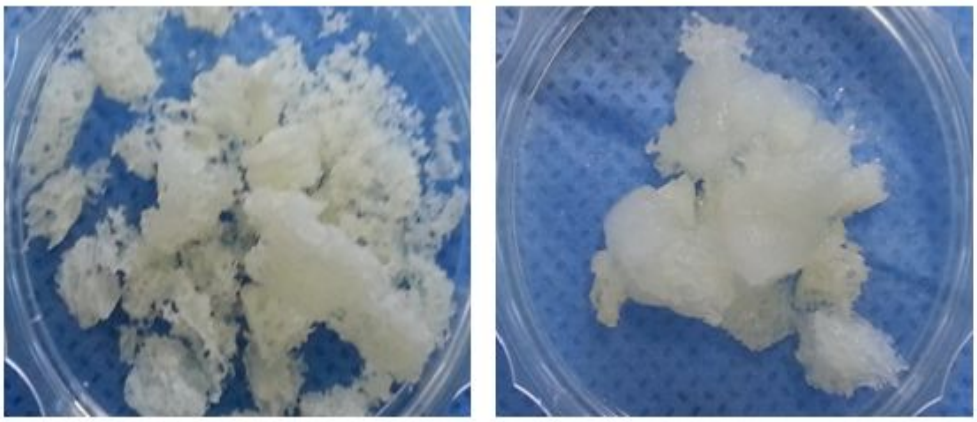
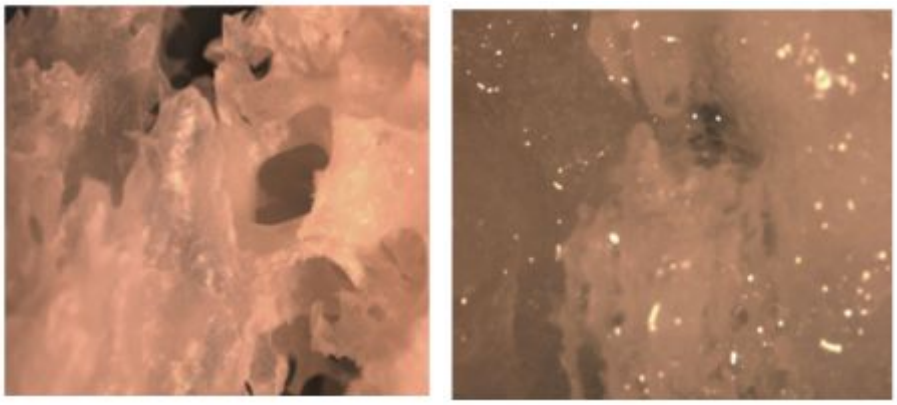
FIGURE LEGENDS



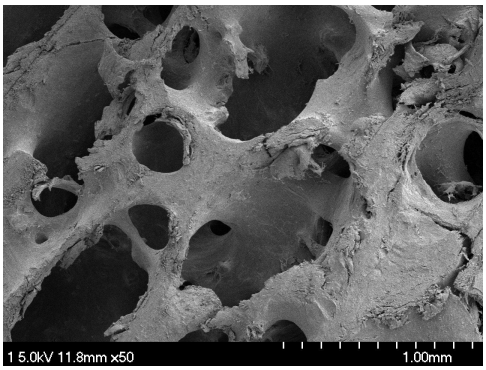
(A) (B)

Fig. 1. Gross appearance of demineralized bone matrix (A), BMP-2 soaked gelatin hydrogel loaded demineralized bone matrix.

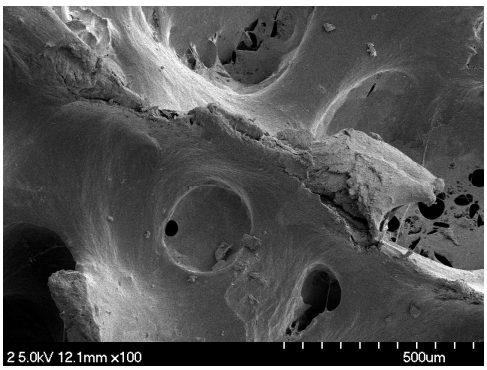


(A) (B)

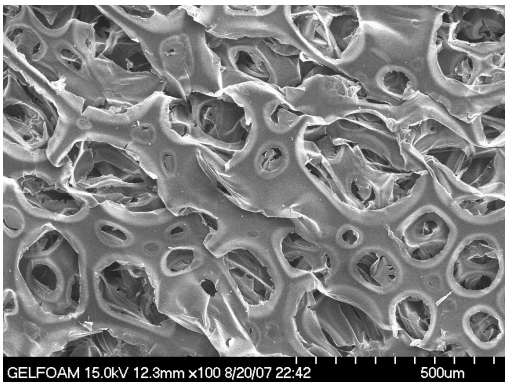
Fig. 2. Stereomicroscopic surface of DBM (A) and BMP-2 soaked gelatin hydrogel loaded DBM (B). x 40



(A)

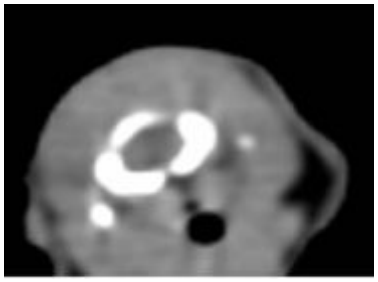


(B)

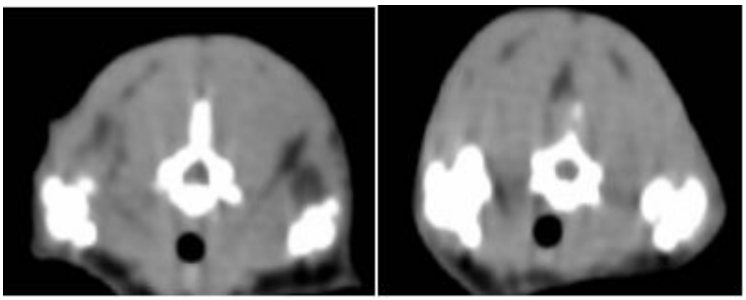


(C)

Fig. 3. Scanning electron microscopic findings of the surface of DBM (A), BMP-2 soaked gelatin loaded DBM (B), and gelatin sponge (C).

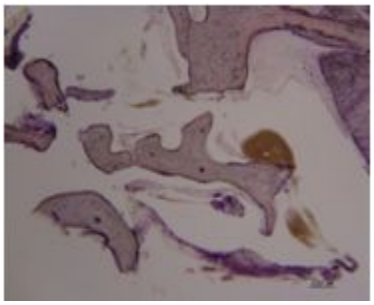


Control



Group I Group II

Fig. 4. The CT findings after treatment of rhBMP-2. Group1, 0.075mg/Ml; Group 2, 0.375mg/Ml. Prominent bilateral osteogenesis in groups I and II.



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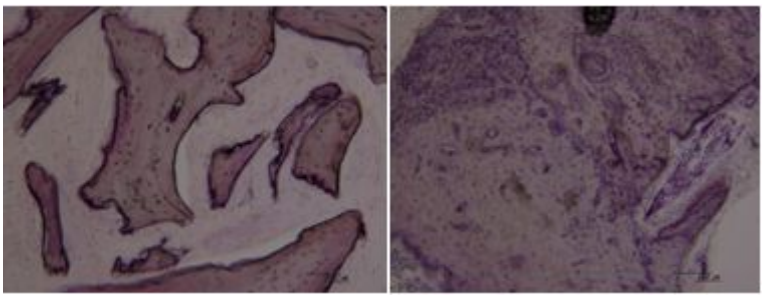


Group I Group II

Fig. 5 Light microscopic findings reveal prominent new bone formation in group I. Diffuse osteoconduction between the DBM particles and the partial osteoinduction of the DBMs are seen. However, poor osteoconduction and osteoinduction are observed in the control group. Others are same as in figure 4.

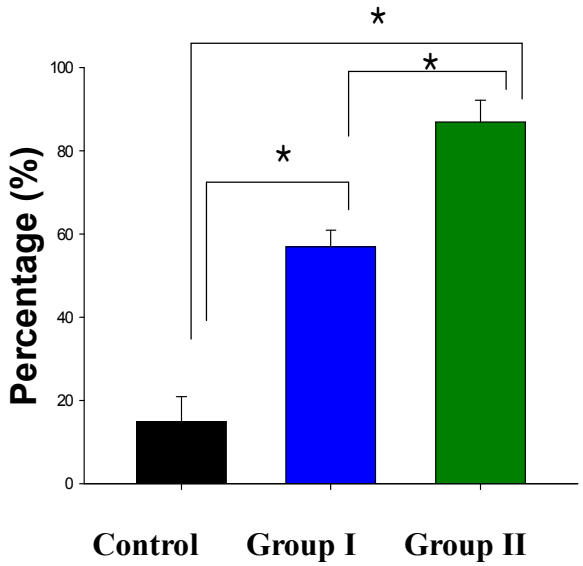
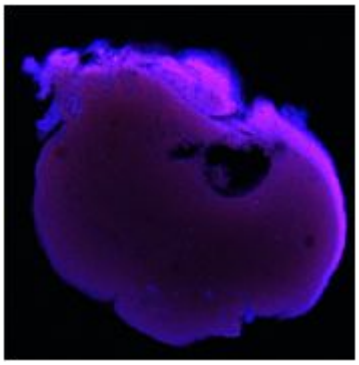


Fig. 6. Histomorphometery reveals the significant differences between each group. $p < 0.05$.



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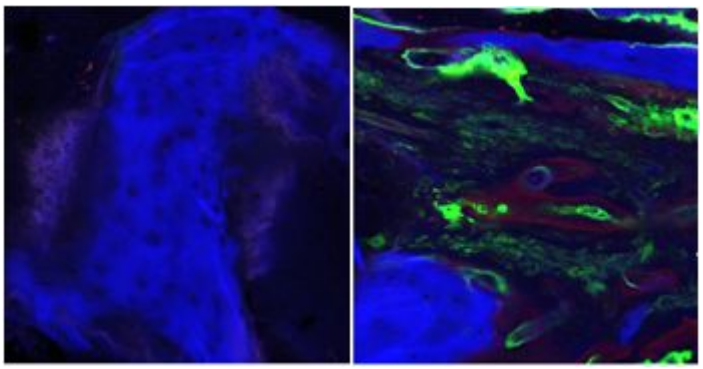

Group I Group II

Fig. 7. Confocal microscopic findings. Good sequential osteogenesis in group II compared to group I but poor osteogenesis in the control. Blue, calcein; green, oxytetracycline; red: alizarin.

ACKNOWLEDGEMENT

논문작성을 무사히 잘 마칠 수 있게 해주신 하느님께 감사 드립니다.
조선대학교와 인연을 맺으면서 감사 드릴 분들이 너무나 많습니다.
먼저 따뜻한 아빠 같으셨던 최철희 지도교수님.
학자의 기본자세란 무엇인지를 저에게 행동으로 가르쳐주셨고,
꼼꼼함과 아주 날카로운 지적으로 좋은 논문이 탄생할 수 있게 해 주셨습니다.
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또한 논문을 심사를 위해 수고해 주신 조훈 교수님과
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그리고 나의 뒤를 지켜주는 가족들
아빠 엄마 그리고 사랑하는 누나 다들 고맙습니다.
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2013년 12월 2일
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장윤석