The Effect of MBCP block as carrier of rhBMP-2 in combination with e-PTFE membrane on Bone formation in Rat calvarial Defects.

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감사의 글

먼저 오늘에 이르기 까지 지도해주시고 이끌어 주셨던 존경하는 조규성 교수님께 깊은 감사를 드립니다. 그리고 많은 관심과 격려를 해주신 김종관 교수님, 채중규 교수님, 최성호 교수님, 특히 물심 양면으로 애써 주신 김창성 교수님께도 진심으로 감사 드립니다.

연구 내내 많은 도움을 준 정성원 선생님,조직학 조성원 교수님 그리고 치주과 교실원 여러분께도 고마움을 전합니다.

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다시 한번 모든 분들께 감사의 말씀을 올립니다.

마지막으로 오늘에 이르기까지 기회와 여건을 주신 주님께 감사 드립니다.

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저자 씀

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Abstract

The Effect of MBCP block as carrier of rhBMP-2 in combination with e-PTFE membrane on Bone formation in Rat calvarial Defects

Objectives : This study evaluated the osteogenic potential of rhBMP-2 delivered with MBCP block/e-PTFE membrane upon 2weeks and 8weeks wound healing phase in a critical sized rat calvarial defect model.

Material and Methods: Eight-millimeter critical sized calvarial defects were created surgically in 28 male Sprague-Dawley rats. The animals were divided into 2 groups containing 14 animals each. The defects were treated with rhBMP-2/MBCP block and rhBMP-2/MBCP/e-PTFE membrane. MBCP block(8x3 mm)was used as a carrier of rhBMP-2. The e-PTFE membrane was used to cover rhBMP-2/MBCP block. The rat were euthanized at 2 and 8weeks after surgery for histologic and histomorphometric analyses.

Results: The level of Bone formation was significantly higher in defects of both group at 8 weeks than 2 weeks($P<0.05$). A comparison of the rhBMP-2/MBCP block, there was no additional effect in rhBMP-2/MBCP block/e-PTFE membrane. At

8weeks, membrane group showed more even bone formation in the top of the MBCP block.

Conclusions: These results suggest that use of rhBMP-2/MBCP block combined with e-PTFE membrane may achieve bone augmentation, but compared to rhBMP-2/MBCP block(without membrane) there was no significant difference in membrane groups.

Key Words : rhBMP-2, MBCP block, e-PTFE membrane, augmentation, carrier.

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Ⅰ. Introduction

Patients with an edentulous area can show significant ridge resorption and insufficient volume for implant or prosthodontic treatment. The augmentation of resorbed alveolar bone can improve retention and stability of removable denture and enable the implant of a dental prosthesis. A precondition for implant treatment in such patients is appropriate augmentation of alveolar bone to ensure a predictable result and good esthetics.

Autogenous bone is currently the most preferred material used for this purpose. However, this type of graft has some limitation and problem in its application.

The bone morphogenetic protein(BMP) has been expected to be a substitute for autogenous bone[3,4,8]. BMP is the most promising osteoinductive protein for bone regeneration. Recombinant human BMP(rhBMP) stimulates osteoblast differentiation in various cells in vitro, and induces ectopic bone formation in vivo[3]. Among the BMPs, rhBMP-2 has strong in vivo bone-inducing ability[6,7,8,14,15,19,25]. However, implantation of BMPs alone does not induce bone formation because the local retention of rhBMP is quite short. Therefore, the use of carrier system is essential for delivering rhBMP-2 to retain rhBMP-2 for the period of time needed for bone formation[1,6,17,24,34,36,38].

In our previous study, we tried to search for excellent carriers of rhBMP-2 such as absorbable collagen sponge(ACS)[6], ß− tricalcium phosphate(ß-TCP)[19,38], the fibro-fibronectin sealing system[11] and their combinations. According to studies by Nery et al[29], a mixture of 60% hydroxyapatite(HA) and 40%ß-TCP constitues the ideal mixture for using macroporous biphasic calcium phosphate(MBCP) as a bone substitue[2,23]. MBCP™ has the required porous form for inonic change, rhBMP diffusion, and retention of osteogenic cells. In addition, HA provides sufficient mechanical properties and such strength to maintain the bone augmented space. Lee

et al[23] reported that the possibility of using MBCP blocks as a carrier of rhBMP-2 and bone regenerative effect of rhBMP-2/MBCP blocks in rat calvarial defect model[33].

In their study, they designed MBCP only group as control group and MBCP/rhBMP-2 group as experimental group. They reported that the new bone area of rhBMP-2/MBCP block group was significantly greater than the MBCP block group at both 2 and 8 weeks. However, they showed a limitation in the new bone area in the rat calvarial defect[23].

Previous researches have reported the limited potential for bone augmentation following guided bone regeneration(GBR) in horizontal defects[34]. Moreover, some studies have indicated that the use of occlusive expanded polytetrafluoroethylene membrane(e-PTFE membrane)device for GBR in conjunction with rhBMP-2 technologies may decelerate new bone formation[44].

The aim of this study was to evaluate and compare the osteogenic potentials of rhBMP-2/MBCP block to enhancing GBR using e-PTFE membrane.

Ⅱ. MATERIALS & METHODS

1. Animals

28 male Sprague-Dawley rats weighing 200-300 g were used (table1). Animals were maintained in plastic cages in a room with 12 h-day/night cycles, an ambient temperature of 21^{o}C, and ad libitum access to water and a standard laboratory pellet diet. Animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

Table-1 Experimental design

	2weeks	8weeks
rhBMP-2/ MBCP block	7rats	7rats
rhBMP-2/ MBCP block/ e-PTFE Membrane	7rats	7rats

2. rhBMP-2 implant construction

The rhBMP-2* was diluted to a concentration of 0.025mg/ml. For the rhBMP-2/ MBCP block implant, a sterile MBCP block[Ω] (8X3mm) was loaded with 0.5ml of the rhBMP-2 solution (100 micron litter per 1 block). Following a 5-minute binding time, the implant was prepared to fit the defect.

3. Surgical procedure

The animals were generally anaesthetized by an intramuscular injection of ketamine hydrochloride[θ] at 5mg kg^{-1} body weight. During surgery, routine infiltration anaesthesia[δ] was used at the surgical site. The surgical site was shaved and scrubbed with iodine. An incision was made in the sagittal plane across the cranium. A full thickness flap including periosteum was reflected, exposing the calvarial bone. Then, a standardized, round, transosseous defect 8 mm in diameter was created similarly on the cranium with the use of a saline cooled trephine drill** in the same manner as described by Schmitz and Hollinger[33]. Then, each animal received one of two experimental conditions: the rhBMP-2/MBCP block[Ω] or the rhBMP-2/ MBCP block/ e-PTFE membrane[£]. e-PTFE membrane(about 10mm in diameter) was placed to

* R&D Systems Inc., Minneapolis, MN,U.S.A.
[Ω] Biomatlante Inc, France
[θ] Ketalar®, Yuhan Co., Seoul, Korea
[δ] 2% lidocaine, 1:100,000 epinephrine, Kwangmyung Pharm., Seoul, Korea
** 3i, Palm Beach Gardens, FL,USA

cover the rhBMP-2/MBCP block and there was no additional device for fixation of e-PTFE membrane. The periosteum and skin were closed and sutured for primary closure with 4-0 coated Vicryl sutures[∂].

4. Histological and histomorphometrical procedures

The animals were anesthetized and euthanized at 2 and 8 weeks post surgery. Block sections including the surgical sites were removed. The samples were placed immediately into vials and fixed in 10% neutral buffered formalin solution for 10 days. All samples were decalcified in EDTA-HCl for 7 days, and embedded in paraffin. Three μm thick coronal sections through the center of the augmented area were stained with hematoxylin-eosin(H-E). After conventional microscopic examination, computer-assisted histometric measurements of the newly formed bone were obtained using an automated image analysis system[ƒ] coupled with a video camera on a light microscope[Σ]. The Sections were examined at 10x magnification. A digitizer was used to trace the detect outline versus new bone formation, and the percentage of bone fill was determined. The following histomorphometric parameters

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[∂] Polyglactin 910, braided absorbable suture, Ethicon, Johnson & Johnson Int., Edinburgh, UK
[£] Gore-Tex, W.L. Gore & Associates
[ƒ] Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA
[Σ] Olympus BX50, Olympus Optical Co., Tokyo, Japan

were measured for each sections.

1) Augmented area (mm^2) was measured including new bone, the residual biomaterials, mineralized bone, fatty marrow and fibrovascular tissue.
2) New bone area (mm^2) was determined by the newly formed bone area within the total augmented area.
3) Bone density was calculated as follows: Bone density (%) = (New bone area / Augmented area) x 100

5. Statistical Analysis

Histomorphometric recordings from the samples were used to calculate group median and range. The Wilcoxon two sample test was used for stastistical analysis. Differences were regarded as statistically significant when $P<0.05$.

Ⅲ. Results

1. Clinical observation

Wound healing was generally uneventful and there was no macroscopic signs of infection. However, there was evidence of membrane movement in the e-PTFE group.

2. Histologic observation

1) rhBMP-2/MBCP group

At 2 weeks, new bone formation was observed in the bottom of the MBCP block. Bone formation was significant enhanced at 8weeks. The new bone appeared more lamellar at 8weeks than at 2weeks. A large number of osteocytes, osteblasts, and osteoclast were observed in the area of new bone formation. The incremental lines, fatty marrow and concentric ring of the Haversian system were also observed in these area. The pattern of newly formed bone moved from outside to the inside of the defect.

2) rhBMP-2/MBCP/e-PTFE membrane group

There was no difference appearance in the membrane compared to rhBMP-2/MBCP block only. At 8weeks, there was significantly more new bone formation was more enhanced and an even bone formation pattern under membrane was observed. In the

rhBMP-2/MBCP group, the bone formation pattern was irregular at the top of MBCP block.

The new bone formation pattern was similar in the central and base parts (bottom of defect) in the both groups.

3. Histomorphometric analysis

Table2,3 and 4 show the results of histomorphometric analysis.

Total augmented area and new bone area were no significant difference in both groups. Regarding the bone density, there were statistically significant differences between 2week and 8week results in both groups($P<0.05$). However, there were no significant differences between the two groups (with membrane/without membrane).

Table-2. Total augmented area (median range ;mm^2,n=7)

	2weeks		8weeks	
MBCP+rhBMP-2	13.45	7.21	19.69	10.7
MBCP+rhBMP-2/e-PTFE	18.57	7.31	17.93	12.64

Table-3. New bone area (median range,mm^2,n=7)

	2weeks		8weeks	
MBCP+rhBMP-2	1.43	0.53	4.52	3.86
MBCP+rhBMP-2/e-PTFE	3.03	3.84	4.88	7.85

Table-4. Bone density (median range, %n=7)

	2weeks		8weeks	
MBCP+rhBMP-2	10.69	6.05	25.06	15.14$^{\oint}$
MBCP+rhBMP-2/e-PTFE	14.27	14.75	26.35	40.72$^{\#}$

$\oint$: Statistically significant difference when compared to 2 weeks (P<0.05)

#: Statistically significant difference when compared to 2 weeks (P<0.05)

Ⅳ. Discussion

The purpose of this study was to evaluate the bone regenerative effects of e-PTFE membrane following implantation of rhBMP-2/MBCP in a critical-size rat calvarial defect model. Eight millimeter diameter standridized, circular, transosseus defects [33]created on the cranium of rats were implanted with rhBMP-2/BMCP or rhBMP-2/MBCP/e-PTFE membrane, and healing was evaluated histologically, histomorphometrically after 2- and 8-week healing intervals. Bone formation was significantly enhanced in defects of both groups after 8weeks compared with that observed at 2 weeks. However, there was no significant difference between membrane group and rhBMP-2/MBCP without membrane group. This shows that rhBMP-2/MBCP induced new bone formation in the augmented defect and placement of e-PTFE membrane had no additional effect in new bone formation.

The ability of rhBMP-2 to stimulate bone formation has been demonstrated in previous studies[6,7,8,14,23,,28]. However, carrier system is necessary to delivery, retention, and release of BMP at a regeneration site due to its rapid diffusion[37]. Ahns et [1]al. reported that effect of absorbable collagen sponge(ACS)[36] as a carrier system for delivery of rhBMP. However,one of the problem with the biodegradable materials currently available is that they do not have a sufficient amount space maintaining properties for vertical bone augmentation. A previous study demonstrated that the

bone regenerative effect of rhBMP-2 delivered with MBCP in rat calvarial defect models[23]. The MBCP block is considered to be an effective carrier system of rhBMP-2 and the possibility of space maintaining properties for GBR. This carrier system has good properties for delivering rhBMP because MBCP blocks consist of a mixture of 60% HA and 40% ß-TCP[29]. ß-TCP is porous and can entrap rhBMP within its micropores, thereby allowing the intrinsically diffusion of rhBMP[16]. However, there are some limitations in the application of ß−TCP. ß-TCP is difficult to manipulate and maintain the appropriate space due to its rapid resorption[19]. By adding HA[20], the MBCP blocks have macropore, and have mechanical stability.

In this study, rat defect model[33]had some compressive force, which was considered suitable for evaluating space maintenance for vertical bone augmentation.

The requirement for the successful incorporation of a bone graft in the defect is stability of the graft material. Another precondition is to prevent ingrowth of suprabony connective tissue into the defect area. The use of a barrier membrane to cover and retain graft material might satisfy both prerequisites. The placement of a biocompatible membrane of e-PTFE membrane or some type of biodegradable barrier over bone defects might help guide bone regeneration[40].

However, even though membrane techniques were reported to be benefical[34], there are applications where this GBR has limited or no benefit. Zellin et al[44]. reported that the use of occlusive e-PTFE device for GBR in combined with rhBMP-2

decreased new bone formation, and that membrane placement had a negative effect on the osteoinductive capacity of rhBMP.

In contrast, Lundgren et al[26]. demonstrated increasing bone augmentation when using a bioresorbable barrier(Guidor® Matrix Barrier) in the rabbit. They used autogenous particulate bone graft and evaluated the effect of membrane.

Wikesjö et al[40]. reported that bone formation was significantly enhanced in the defects receiving rhBMP-2/ACS using the space-providing, macro-porous e-PTFE device. This device was fixed to alveolar bone using medical-grade stainless-steel tacks. In this study, there was no device for fixation of e-PTFE membrane. This was because it is difficult to add the fixation device due to of limitation in rat calvarial defect.

Zellin et al[44]. reported that one benefit of using membrane in conjunction with implantation of growth-stimulatory factors is that it can keep the implanted material in place. In addition, they reported that membrane placement restricts the boundaries of osteogenesis for a desired anatomical contour[37].

In this study, the membrane group at 8weeks showed enhanced bone formation and more even bone formation beneath membrane in the top of MBCP block. However, the results were similar to the rhBMP-2/MBCP block only. This suggest that MBCP block is suitable for carrier of rhBMP-2 and might be effective in maintaining the space for GBR. In addition, under limited conditions, using e-PTFE

membrane, there was no additional benefit in the rat calvarial defect. Further studies will be needed to evaluate the osteogenic effect of rhBMP/MBCP using a biodegradable membrane.

V. Conclusion

These results suggest that use of rhBMP-2/MBCP block combined with e-PTFE membrane may achieve bone augmentation, but compared to rhBMP-2/MBCP block(without membrane) there was no significant difference in membrane groups. The MBCP blocks may be considered effective carrier system of rhBMP-2.

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Legends

Figure1. Representative photomicrograph of rhBMP-2/MBCP block group at 2 weeks. At 2 weeks, New bone formation was observed in the bottom of MBCP block and adjacent to the margins of the defect (x10).

Figure2. Representative photomicrograph of rhBMP-2/MBCP block at 8 weeks. At 8 weeks, more bone formation was observed in the base area comparing to 2 weeks (x10)

Figure3. Representative photomicrograph of rhBMP-2/MBCP block/e-PTFE membrane group at 2 weeks. New bone formation were observed in the bottom of MBCP block(x10).

Figure4. Representative photomicrograph of rhBMP-2/MBCP block/e-PTFE membrane group at 8 weeks. In the upside of augmented area, a lot of new bone was observed, and the appearance of the new bone was more lamellar at 8 weeks(x10).

Figure5. Representative photomicrographs of rhBMP-2/MBCP block/e-PTFE membrane group at 8 weeks (base of MBCP block, x200). A lot of osteocyte, centric rings of Haversian system, incremental line and fatty marrow were observed.

Figure6. Representative photomicrographs of rhBMP-2/MBCP block/e-PTFE membrane group at 8 weeks (middle of MBCP block, x200). .

Figure7. Representative photomicrographs of rhBMP-2/MBCP block/e-PTFE membrane group at 8 weeks (Top of MBCP block, x200). A lot of new bone was observed in top of MBCP block and more even bone formation beneath membrane.

Figure8. Representative photomicrographs of rhBMP-2/MBCP block group at 8 weeks (Top of MBCP block, x200). A lot of new bone was observed in top of MBCP block.Bone formation pattern was irregular.

Figure 1

Figure 2

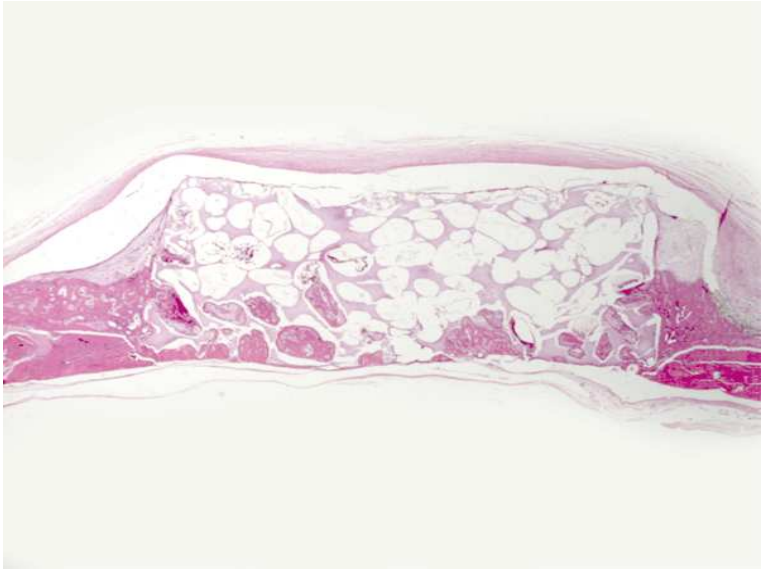

Figure 3

Figure 4

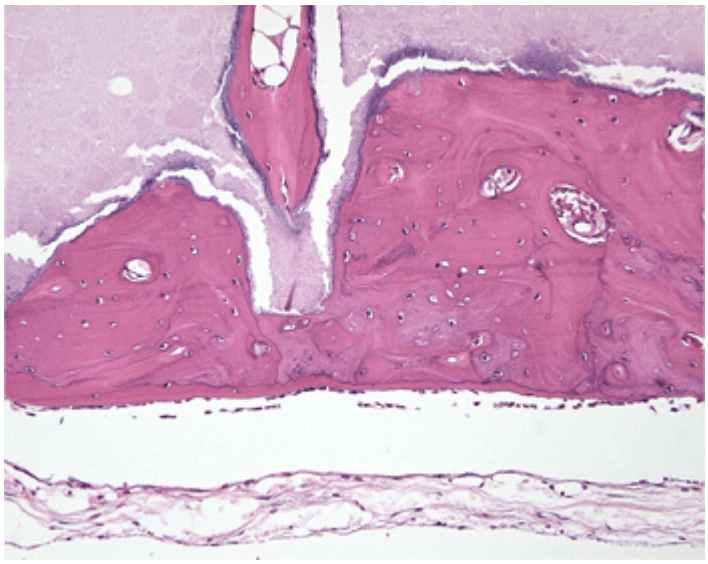

Figure 5

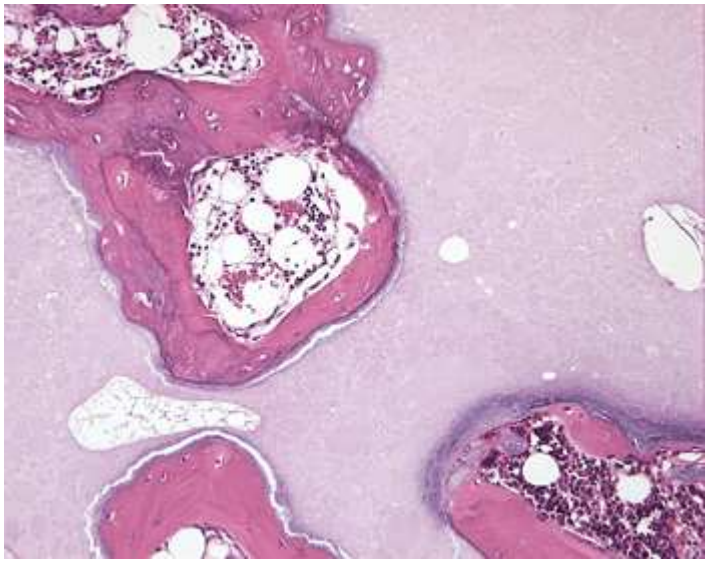

Figure 6

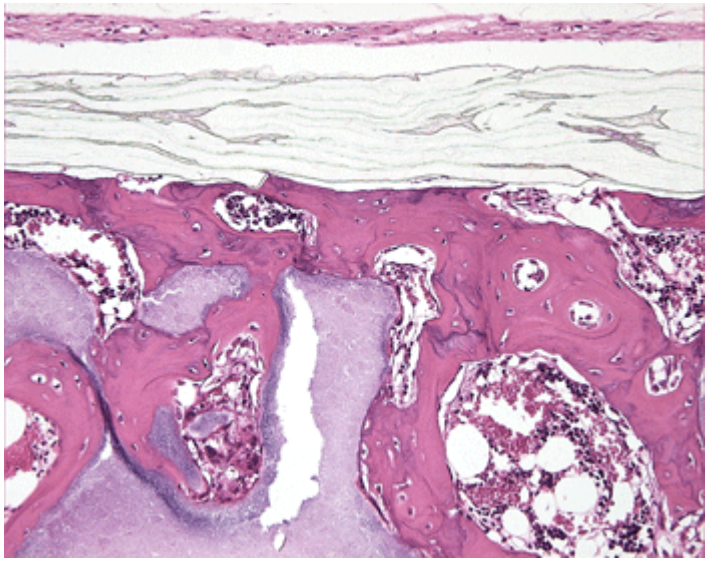

Figure 7

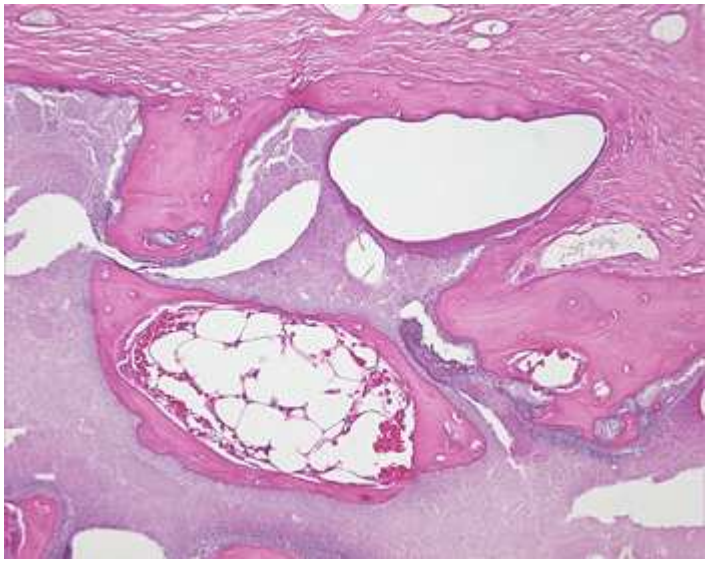

Figure 8

국문요약

백서 두개골 결손부에서 bone morphogenetic protein-2의 전달체로서 macroporous biphasic calcium phosphate-block의 골재생효과와 비흡수성 차폐막의 영향에 관한 연구

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신 철 우

임플란트 및 치과보철 치료 시 임상가는 심하게 흡수된 치조골에 직면하게 되며 이로 인해 치조골재생술 및 치조골 증대술에 대한 필요가 증가된다. 골이식재 중 자가골이 가장 우수한 재료임에도 불구하고, 여러가지 제한점을 가지며, 이에 대한 대체재로 골 형성 유도 단백질(bone morphogenetic protein, BMP)이 연구되고 있다. 이 골 형성 유도 단백질은 성장이나 골 형성 과정에서 중요한 역할을 한다고 입증 되었고 그것의 운반체에 대한 연구가 이뤄져 왔다. 수직압이 존재하는 곳에서는 공간유지능력이 있는 운반체가 필요하게 된다. macroporous biphasic calcium phosphate Block (MBCP block)은 공간유지능력이 뛰어나며 강한 수직압을 견딜 수 있는 골대체물질이다. 또한, 수직적 골증대술시 흡수성

및 비흡수성의 차폐막이 자주 사용된다. 이 연구의 목적은 MBCP block을 골 형성 유도 단백질 (rhBMP-2)의 운반체로 사용하여 백서 두개골 결손부에 적용하였을 때, 골 형성 효과를 확인하고, 차폐막을 MBCP block상방에 위치시켜 차폐막이 골 형성에 미치는 영향을 비교 평가하는 것이었다.

40 마리의 웅성 백서에서 8 ㎜ 지름을 갖는 임계크기의 두개부 결손을 형성하였다. 20 마리씩 2 개의 군으로 나누어 MBCP block 을 운반체로 사용하여 골 형성 단백질(농도 0.025mg/ml rhBMP-2)만 이식한 군, MBCP block 을 운반체로 사용하여 골 형성 단백질(농도 0.025mg/ml rhBMP-2)를 이식한 후 MBCP block 상방에 비흡수성 차폐막(e-PTFEmembrane)을 위치한 군으로 나누어 각각 술 후 2 주와 8 주에 치유 결과를 조직학적, 조직계측학적으로 비교 관찰하였다.

조직계측학적 관찰 결과, 두군 모두에서 8주째가 2주째보다 골 밀도가 유의성있게 증가 하였다 ($P<0.05$). 두 군간의 비교에서는 유의성 있는 차이가 없었다. 총 조직 형성량 (augmented area) 에서도 변화는 없었다. 조직학적 비교에서 단지 차폐막이 사용된 군에서 MBCP block상방의 골 형성이 좀더 연속성 있는 소견을 보였으며, 이에 비해 막을 사용하지 않은 군에서는 좀더 불규칙적인 골 형성 소견을 보였다.

백서 두개골 결손부에서 MBCP block은 골 형성 유도단백질의 운반체로 사용하였을 때 신생골 형성에 유의성 있는 효과가 있을 뿐 아니라

공간유지능력이 우수해서 수직압이 존재하는 골 증대술 (vertical bone augmentation) 시 골 형성 유도단백질의 운반체로 가능성이 있으며, 이때 차폐막은 특이한 부가적 효과는 보이지 않았다.

핵심되는 말: 골형성 유도 단백질, MBCP Block, 차폐막, 골증대술.