Osteoinduction of recombinant human bone morphogenetic protein-2 coated biphasic calcium phosphate in rabbit maxillary sinus augmentation: a histometric analysis

Joo-Young Kwon

Department of Dental Science

The Graduate School, Yonsei University

Osteoinduction of recombinant human bone morphogenetic protein-2 coated biphasic calcium phosphate in rabbit maxillary sinus augmentation: a histometric analysis

Directed by Professor : Ui-Won Jung

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Thesis Supervisor : Ui-Won Jung

Seong-Ho Choi

Dong-Won Lee

The Graduate School

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감사의 글

본 논문이 완성되기까지 부족한 저를 항상 격려해 주시고 사랑과 관심으로 이끌어 주신 정의원 교수님께 깊은 감사를 드립니다. 그리고 바쁘신 와중에도 심사를 맡아주시고 많은 조언과 격려로 지도해 주신 최성호 교수님, 이동원 교수님과 따뜻한 관심으로 지켜봐 주신 채중규 교수님, 조규성 교수님, 김창성 교수님, 논문의 연구와 작성에 아낌없는 조언과 도움을 주신 박정철 교수님, 이중석 교수님께도 감사드립니다.

연구 내내 많은 도움을 주신 최연아 선생님, 이규안 선생님, 조아란 선생님을 비롯한 치주과 의국원들께도 감사의 말씀 전합니다.

늘 조건 없는 사랑을 주시고 말없이 저를 믿어 주시는 사랑하는 부모님과 동생에게 진정으로 사랑과 고마움의 마음을 전합니다. 마지막으로, 논문의 많은 부분을 감수해주고, 항상 저의 기쁨과 슬픔을 함께 해준 제 아내에게 큰 고마움을 표시합니다. 모든 분들께 진심으로 감사드립니다.

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저자 씀

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Department of Dentistry

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Recombinant human bone morphogenetic protein-2 (rhBMP-2) has been used in sinus augmentation but suitable carriers that retain certain amounts of BMPs and space providing properties were required. Biphasic calcium phosphate (BCP) have been suitable candidates for a rhBMP-2 delivery system because of their osteoconductive properties, space-providing properties, biocompatibility and structurally similarity to human bone tissue. The objective of this study was to evaluate the osteoinductive potential of low concentration of rhBMP-2 coated BCP (LCB) in a rabbit maxillary sinus model.

Bilateral, circular windows were prepared at the sinus area of normal skulls in eight New Zealand white rabbits using 6-mm diameter trephine bur. The trephined bony disk was carefully removed and the membrane was elevated and one side received LCB while the other side received BCP with saline. The histologic and histometric analysis was performed after 2 weeks.

Clinical healing was generally uneventful. The total amount of new bone formation revealed no significant difference between groups, but when divided into 3 sectional areas (window / center / membrane) there were some difference. In the rhBMP-2 group, newly formed bone was greater at the membrane areas, however in the control group, window areas represented greater new bone formation.

Within the limitation of this study, LCB was capable of promoting new bone formation in the membrane area relatively and induced chemotaxis in the early healing stage.

Key Words: Bone morphogenetic protein; biphasic calcium phosphate; sinus augmentation; carrier; osteoinduction

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Joo-Young Kwon, D.D.S.

Department of Dental Science
Graduate School, Yonsei University
(Directed by Prof. Ui-Won Jung, D.D.S., M.S.D., PhD.)

I. Introduction

Alveolar bone resorption in the maxillary posterior area and pneumatization of the maxillary sinus often causes difficulties in successful implant placement [1]. These problems have been solved by various surgical procedures such as sinus augmentation to increase bone height and volume to promote the stability of implants [2]. This procedure is based on the elevation of the Schneiderian membrane from the floor of the maxillary sinus and the placement of a bone graft, such as autogenous bone, allograft, alloplast or a combination of these materials [3]. However, the choice of the bone graft material is still under discussion. Autogenous bone has been reported to

have the highest regeneration ability [4] and is able to provide appropriate bone quality when used for sinus elevation [5]. Nevertheless, there are some difficulties when applied in common clinical situations because of the need for additional surgical sites, the risk of complications and limited bone volume availability. Moreover autogenous bone graft could not withstand the sinus pressure for long periods of time, and started to lose their density and height during the first several weeks [4].

It is known that ventilation of the human maxillary sinus is accomplished by air change with the nasal cavity through the sinus ostium [6]. It is suggested that in rabbits with ostial occlusion, a fully formed bone mass was seen after 3 weeks, while in rabbits without ostial occlusion, space was almost completely replaced by a normal sinus airspace after 3 weeks [7]. A barometric pressure occurs within the maxillary sinus and moves the maxillary sinus membrane and it could affect the augmented bone structure [8].

Various synthetic materials have been developed for use in the maxillary sinus augmentation. More recently, growth factors such as recombinant human bone morphogenetic protein 2 (rhBMP-2) has been used in sinus augmentation [8]. Lee et al. suggest that rhBMP-2/absorbable collagen sponge(ACS) induced bone of significantly greater quality compared with the iliac crest autogenous bone graft [9]. However, in our previous study, when rhBMP-2-loaded ACS was applied to the rabbit maxillary sinus model, it showed enhanced osteoinductive potential, but it could not maintain the augmented volume in the sinus [10]. It is assumed that for successful

results, suitable carriers to provide certain amounts of BMPs at graft sites and space providing properties are required [11].

BCP which is a specific ratio of HA and TCP, has remarkable osteoconductive properties and space-providing properties [12, 13]. Moreover, BCP is not only chemically and structurally similar to human bone tissue, but also has proven biocompatibility [14] and it is suggested that BCP may produce predictable results when used as a grafting material for sinus floor augmentation [15]. When rhBMP-2 is loaded onto BCP moistened with a diluted solution of rhBMP-2, inaccurate dose, uncontrolled flow could be caused. Uludag et al. suggested that when biphasic calcium phosphate (BCP) was soaked in rhBMP-2, up to 75% of the rhBMP-2 could be lost from the site within an hour [16]. The combination of ACS and BCP tended to retain a higher fraction of rhBMP-2 in the immediate postimplantation period than the BCP alone, but the retention of rhBMP-2 decreased afterwards (during day 7 and day 36) [16]. Overall, the retention kinetics of rhBMP-2 exhibited by the BCP group and the combination group of ACS and BCP at the implant site were similar [16]. For these concerns, coating method was used for loading rhBMP-2 onto BCP by using a lyophilization protocol.

The minimum dose of BMP necessary to induce consistent bone formation is higher in nonhuman primates than in rodents [17]. The major Food and Drug Association approved the 1.5 mg/ml concentration used for initial human clinical trials based on this nonhuman primate data and this is the currently approved concentration for human use [18, 19]. In recent studies, 1.5 mg/ml rhBMP-2 coated BCP

was used in rabbits sinus model and adverse results were achieved. In this study, low concentration of rhBMP-2 coated BCP (LCB) which was 10 times lower than previous studies was used. The present study was designed to evaluate LCB in rabbit sinus augmentation model.

Ⅱ. Materials & Methods

1. Animals

Eight Male New Zealand white rabbits weighing 2.5–3.0 kg were included in the study. Animals were maintained in separate cages under standard laboratory conditions with ad libitum access to water and a standard laboratory pellet diet. Animal selection and care, the surgical protocol, and the preparation procedures were certified by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

2. Preparation of ErhBMP-2-loaded BCP and study design

For control, BCP (Bio-c^{TM}; Cowell medi; Busan, Korea) was used as a carrier in the present study. This carrier comprises HA/-TCP at a ratio of 30: 70. The BCP lyophilized ErhBMP-2 (CowellBMP^{TM}; Cowell medi; Busan, Korea), was prepared for the experimental group. E. coli–expressed rhBMP-2 solution (0.67 mL in 0.15 mg/mL buffer) was pipetted into an ampule containing 1 g of the BCP granules and lyophilized in a freezer drier (Shinil, Co, Korea). The solution was frozen by placing the ampule on precooled shelves and was cooled down to –43°C. The formulations were maintained at this temperature for 3 hours, after which they were dried in a

condenser at –40°C (primary drying) and kept in a pressure chamber at 5 m Torr for 2 hours. Secondary drying was performed on a shelf using the following sequence: –20°C for 4 hours, –10°C for 4 hours, 0°C for 2 hours, and 20°C for 20 hours. The chamber pressure was constant throughout the procedure.

For the experimental group, 150mg of BCP lyophilized ErhBMP-2 was mixed with saline and inserted into the maxillary sinus. For the control group, 150 mg of BCP was mixed with saline, and then inserted into the opposite maxillary sinus. For randomization, experimental group was located right or left side alternatively.

3. Experimental procedures

The animals were anesthetized with an intramuscular injection, using a mixture of Ketamine hydrochloride (KetalarVR^{TM}, Yuhan Co, Seoul, Korea) and Xylazine (RumpunVR^{TM}, BayerKorea Ltd, Seoul, Korea). Surgical sites were shaved and draped with alcohol and povidone iodine. Surgical sites were locally anesthetized with 2% lidocaine. An incision was made along the sagittal midline from the frontal bone to the occipital bone and a full-thickness flap including the skin and periosteum was elevated laterally. Bilateral, standardized, circular windows were prepared at the sites determined previously using the normal skulls. Initially, a 6-mm-diameter trephine bur (Neobiotech, Seoul, Korea) was gently used in reverse mode to avoid damaging the underlying membrane. Drilling was stopped when the grayish

membrane was visible through the thinned bone. The trephined bony disk was carefully removed from the nasal bone and the sinus membrane. The sinus membrane was then elevated to form a pouch to receive the grafting materials (Fig 1). For the reference point, a pin (Dentium, seoul, korea) was located at midline between two windows. After the graft material was implanted, the periosteum was positioned over the window on each side. The skin/periosteum flap was then sutured layer by layer with 4-0 Glyconate absorbable monofilament (Monosyn^{TM}, B-Braun, Aesculap, Center Valley, PA, USA), which was removed after 7 days. The animals were allowed to heal for 2 weeks postoperatively, after which euthanasia was performed by overdosing them with anesthesia.

4. Histologic and histometric analysis

The sections were decalcified in 5% formic acid for 14 days and then embedded in paraffin. Serial sections of 5 μm thickness were coronally cut along the center of the augmented sinus. The two most-central sections were chosen and stained with hematoxylin and eosin stain and Masson's trichrome. The histologic slides were observed and digitally captured under a light microscope (BX50, Olympus, Tokyo, Japan). The total augmented area (TAA), newly formed bone area (NB), residual graft particle (RG), fibrovascular tissue (FV) area were recorded. Each slide were divided

into 3 section areas of interest (window/ center / membrane, 1.3 x 1.3 mm^2) and analyzed as a ratio of each component.

5. Statistical analysis

The statistical analysis was performed using a statistical software program (SPSS 12.0, SPSS, Chicago, IL, USA). The Kruskal –Wallis test was used to compare differences among the window/center/ membrane group. The post hoc Bonferroni test was used to analyze the difference between the groups (P <0.05). Man-Whitney U test was used to compare differences between the experimental and control groups. The data are presented as mean ± Standard deviation (SD) values.

Ⅲ. Results

Clinical Observation

All the animals were healthy throughout the entire follow-up and no major complications occurred during the observation time. Healing process after sinus graft procedure was uneventful, even though small tears (<1mm) have occurred in 1 of the 16 sinuses on the control side. None of the animals had influential complications such as infection, maxillary sinusitis.

Histologic and histometirc findings

In the histologic analysis, the first parameter quantified was new bone area, which was calculated as a square millimeter of the grafted region. The experimental groups and control groups showed early initiation of bone formation and remodeling (Fig 2). The new bone area of the experimental group was 0.21 ± 0.09 mm^2, which was not significantly different compared to the control group (Table 1). The grafts created an increase in the quantity of FV (experimental group 42.6%, control group 37.3%). When the composition of the experimental group and control group were compared, FV and TAA had slightly increased in the experimental group but there

were no significant differences. The augmented space was convex, and more newly formed trabeculae were found close to the parent bony wall and lifted membrane.

Each slide was divided into 3 section areas of interest (window/ center / membrane) and the compositions of each area were analyzed (Fig 3, Fig 4). In both experimental and control groups, a minimal amount of new bone was detected at the center area significantly (Table 2). Thin and loose connective tissue filled the space. In the control groups, window areas represented greater new bone formation evident at the periphery of the defect margin. In the experimental groups, defects exhibited bone ingrowth into the BCP particles, and newly formed woven bone was deposited in close contact with the membrane. Although there were 36% more bone formation at the membrane area in the experimental group than the control group, no statistically significant difference was observed. Fibrovascular tissue was greater at the window and center area in the experimental group and when compared to the control group, statistically significant difference was observed at the center area.

IV. Discussion

Maxillary sinus augmentation induces bone formation by promoting osteoconduction from surrounding adjacent bone [20, 21] and is dependent on the revascularization and osteoblast recruitment [21]. Despite the proven clinical usefulness of the sinus floor elevation procedure, an extended healing period is required for osseointegration of dental implants [7]. BMP-2 has been considered a sufficient factor for the acceleration of bone regeneration [22] but they also induce adverse clinical effects, including cyst-like bone formation and significant soft tissue swelling [23]. In this study, there was no significant difference between the composition of the experimental group and the control group, but FV and TAA were slightly increased in the experimental group. From these results, it can be assumed that swelling may be caused by rhBMP-2 and it could impede the stability of the bone graft material.

It is controversial that the periosteum of sinus membrane has an effect on new bone formation. Sharawy et al. suggested that the periosteal portion of this membrane is not similar to the periosteum covering the cortical plates of the maxillary or mandibular residual ridges and jaws [24]. The minimal presence of osteoblasts may account for the enlargement of the antrum after tooth loss [25]. In this study, the control group revealed 78.72% more new bone formation in the window area than the membrane area. The present study showed that bone formation started from the

resident bone, both from the sinus walls as well as the septa in the control group. This is in agreement with previous experimental studies performed in rabbit models [10,26] . But Lundgren suggested that theoretically a source of bone-forming cells is the periosteum of the lifted sinus membrane [26]. Gruber et al. showed that cells derived from the porcine sinus associated mucosa express STRO-1, a marker of osteoprogenitors, and responds to BMP-6 and BMP-7 [27]. In this study, experimental groups revealed 18.52% more new bone formation at the membrane area and it could be suggested that this is a result of the osteogenic potential of the sinus membrane. However, few studies have demonstrated direct osteogenic capacity in human sinus membrane either in vitro or in vivo. Exact characterization of the sinus-derived osteoprogenitors and explanation of their role in sinus augmentation should be studied further.

The use of rabbits as a model for maxillary sinus elevation has been well-documented. It was suggested that rabbits have the same ventilation with air exchanges through the nasal cavity and a well-defined ostium opening to their nasal cavities as humans [28]. As air pressure causes movement of the maxillary sinus membrane, it could affect the graft material and bone healing process [10, 29]. Under these conditions, the graft material is considered to be an important factor of the success or failure [30]. It was suggested that autogenous bone graft could not withstand sinus pressures for long periods of time, and start to lose their density and height during the first several weeks [5]. Johansson suggested that the absorption rate using

autogenous bone in sinus augmentation was 47%, 6 months after surgery [31]. Xu et al. suggested that the positive air pressure within the sinus might have a role in osteoclastic activation and cause absorption of newly formed bone in rabbit sinus model and ten weeks after grafting, there was thin continuous cortical bone under the elevated sinus membrane [30]. In this study, the experimental and control group both showed the convex augmented space, which was suggested that the BCP can withstand sinus air pressure and maintain the augmented space. Especially in experimental groups, newly formed trabeculae were mainly found close to the raised membrane and it built a partial shell adjacent to the membrane. We could assume that this shell might withstand the sinus pressure and retain density and height during the first several weeks. But when applied clinically, relatively less bone formation occured at other sites compared to the newly formed bone adjacent to the membrane, which impeded the initial stability of implant. Further studies to clarify the role of rhBMP-2 inducing bone formation at the sinus membrane and the advantages and disadvantages of rhBMP-2 are needed

V. Conclusion

Within the limitations of this study, the following conclusions were drawn.

1. FV and TAA slightly increased in the experimental group but there was no significant difference compared to the control group.
2. In both experimental and control groups, a minimal amount of new bone was detected at the center area significantly.
3. In the control groups, window areas represented the greatest new bone formation whereas in the experimental group, the membrane area represented the greatest new bone formation. But there was no statistically significant difference between the two groups.
4. In the center area, more FV was observed in the experimental group with significant difference compared to the control group.

It can be concluded that LCB may promote new bone formation in the membrane area and induce chemotaxis in the early healing stage.

LEGENDS

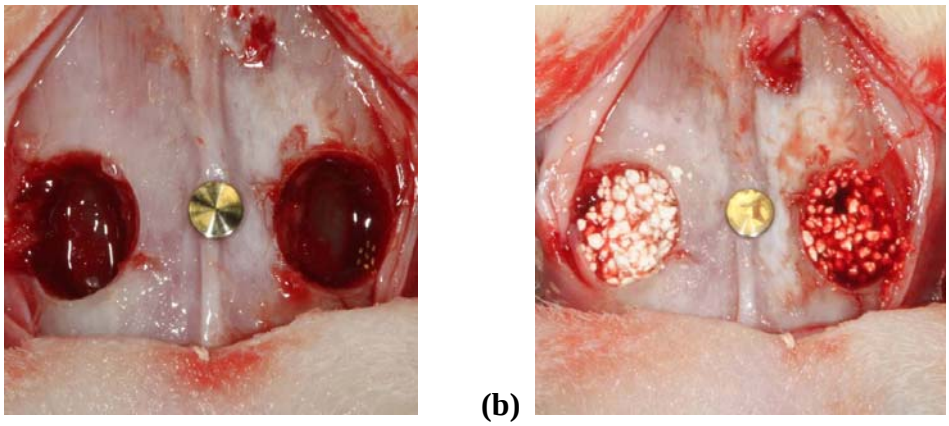
Figure 1. Clinical photograph of window preparation. (a) Windows were prepared bilaterally using a 6mm trephine bur. (b) Graft materials were inserted into each sinuses. For the experimental group, 150mg of BCP lyophilized ErhBMP-2 was mixed with saline and inserted into the maxillary sinus. For the control group, 150 mg of BCP was mixed with saline, and then inserted into the opposite maxillary sinus.

Figure 2. Histologic findings. (a) is experimental side and (b) is control side. Arrow heads indicate surgically created window sites. Newly formed bone and a cortical layer beneath the Schneiderian membrane were found more distinct at experimental side. Total volume of augmented space was more greater at experimental side. Masson trichrome staining, scale bar : 1mm

Figure 3. Histologic findings of the experimental group (a) Groups were divided into 3 section areas (b) window (c) center (d) membrane. Newly formed woven bone was deposited in close contact with the membrane. Minimal amount of new bone was detected at the center area. Masson trichrome staining, scale bar : 200 μm, (NB: New bone, RG: Residual graft, FV: Fibrovascular tissue)

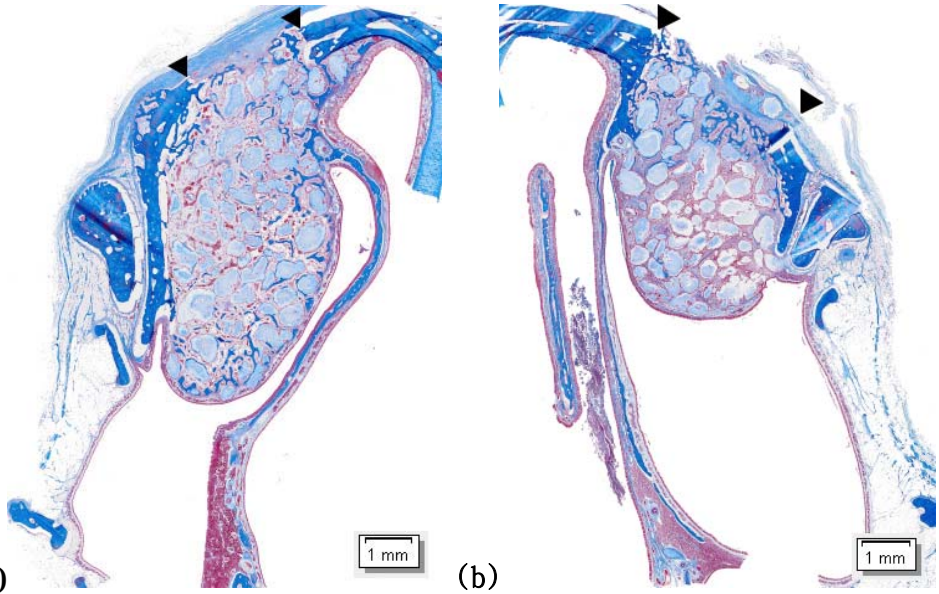
Figure 4. Histologic findings of the control group (a) Groups were divided into 3 section areas (b) window (c) center (d) membrane. Window areas represented greater new bone formation evident at the periphery of the defect margin. Masson trichrome staining, scale bar: 200 μm
(NB: New bone, RG: Residual graft, FV: Fibrovascular tissue)

Figures



(a) **(b)**

Figure 1.



(a) (b)

Figure 2.

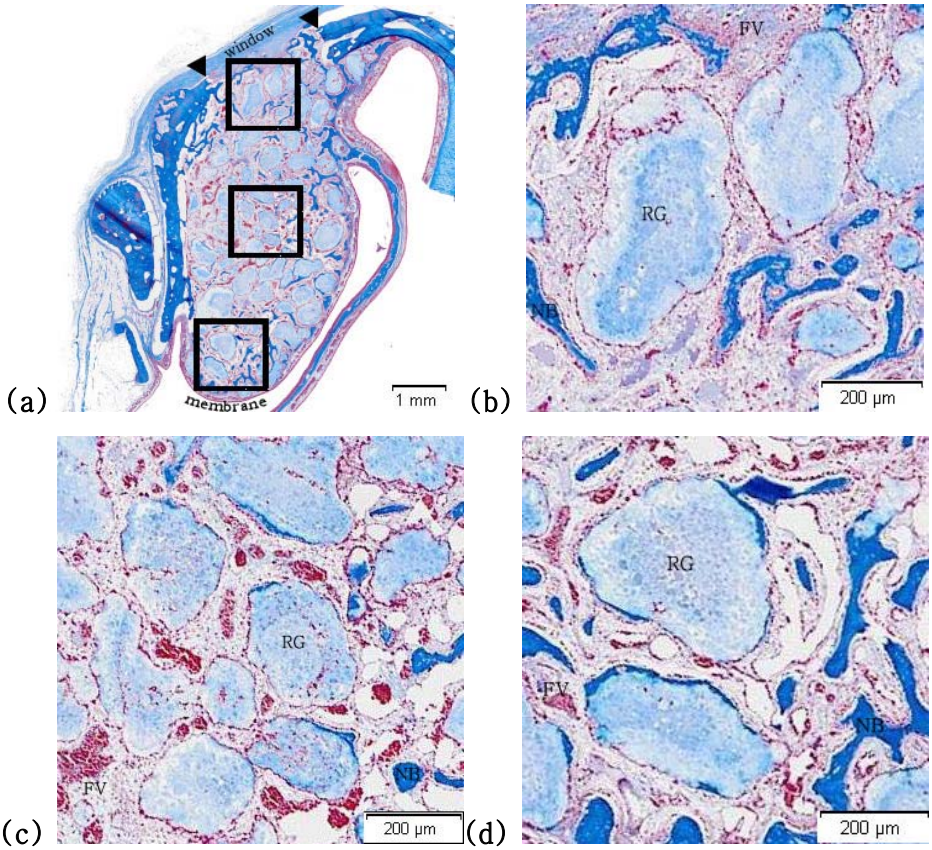

(a)
window
membrane
1 mm
(b)
FV
RG
NB
200 μm
(c)
RG
FV
NB
200 μm
(d)
RG
FV
NB
200 μm

Figure 3.

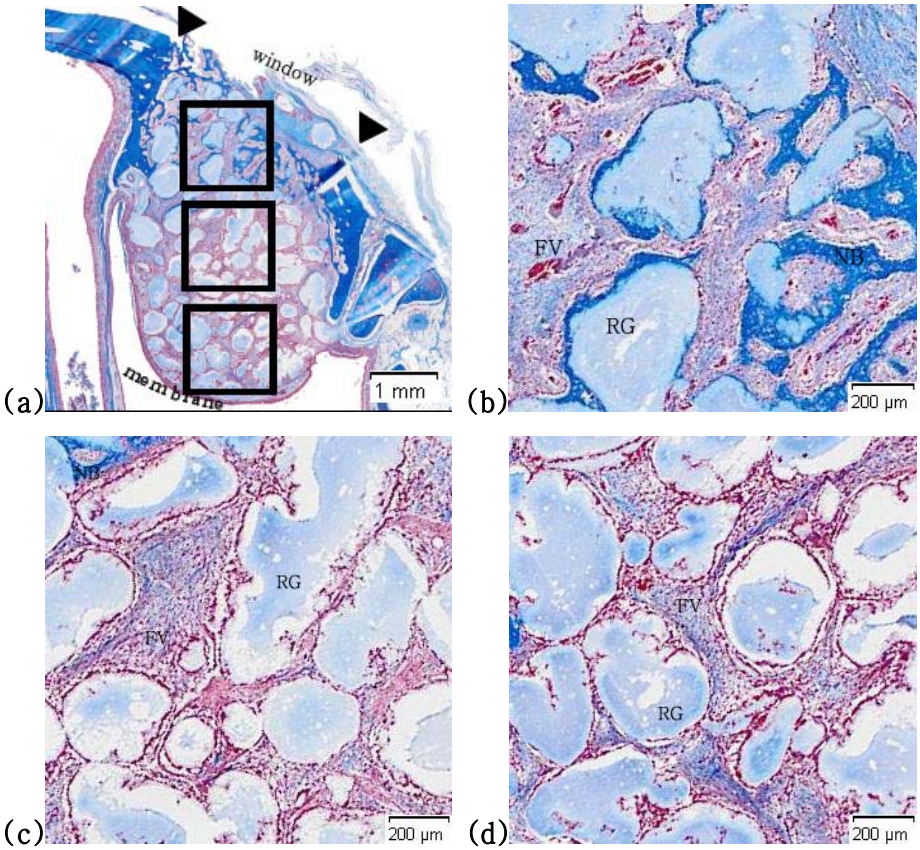

(a)
window
membrane
1 mm
(b)
FV
NB
RG
200 μm
(c)
NB
RG
FV
200 μm
(d)
FV
RG
200 μm

Figure 4.

Tables

Table 1. Areas and proportion of each composition of the augmented space at 2 weeks after surgery (mean±Standard deviation, N=8)

(unit = mm^2)

	Experimental group (%)	Control group (%)
NB	0.21±0.09 (7.2%)	0.24±0.12 (9.1%)
RG	1.44±0.31 (49.6%)	1.41±0.32 (53.6%)
FV	1.25±0.95 (43.6%)	0.98±0.32 (37.3%)
TAA	2.90±0.57 (100%)	2.63±0.90 (100%)

*No significant difference compared to control group (P >0.05)

NB : New bone, RG : residual graft, FV : fibrovascular tissue, TAA : Total augmented area

Table 2. Amounts of composition of the augmented area at each section

(mean±SD values) **(unit = %)**

	NB		RG		FV	
	E	C	E	C	E	C
Window	4.4±2.7	6.3±4.2	23.1±8.1	27.7±13.1	72.5±7.2	66.0±10.6
center	0.7±0.8	0.8±0.8	28.8±9.7	41.1±12 .	70.5±10.1	58.1±13.2
membrane	5.5±3.5	4.0±1.9	34.5±6.5	41.5± 6.9	56.0±8.1	60.0±7.8

*Significant different from control group (P <0.05).

†Significant high value from another site(P <0.05).

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국문요약

재조합 제2형 인간 골형성단백질을 코팅한 이상 인산 칼슘을 토끼 상악동 거상술에 적용하였을 때의 골유도능 평가 :조직학적 분석

<지도교수 **정 의 원**>

연세대학교 대학원 치의학과

재조합 제 2 형 인간 골형성단백질은 상악동 거상술에 사용되고 있으며, 특정한 양의 재조합 제 2 형 인간 골형성단백질을 함유할 수 있고, 이식된 형태를 유지할 수 있는 적절한 운반체가 필요하다. 이상 인산칼슘은 골전도성, 공간 유지 능력, 생적합성, 인체와 유사한 구조성 등의 이유로, 재조합 제 2 형 인간 골형성단백질의 적합한 운반체로 여겨져 왔다. 이 연구의 목적은 0.15 mg/ml 의 재조합 제 2 형 인간 골형성단백질을 이상 인산칼슘에 코팅하는 방법을 사용하여 토끼의 상악동 모델에 적용하였을 때, 골 형성능을 평가하는 것이다.

여덟 마리의 뉴질랜드 흰 토끼의 상악동 부위에 6mm 지름의 trephine bur 를 사용하여 양측에 원형의 창을 형성하였다. 상방의 골을 주의하여 분리하고, 상악동 막을 거상한 후, 한쪽에는 재조합 제 2 형 인간

골형성단백질을 코팅한 이상 인산칼슘을 적용하였고, 반대쪽에는 이상 인산 칼슘을 식염수와 혼합하여 적용하였다. 2 주후 조직학적 계측을 시행했을 때 특별한 임상적인 징후 없이 치유가 일어났다. 전체적인 신생골의 양을 측정하였을 때 실험군과 대조군간에 통계적으로 유의한 차이를 나타내지 않았다. 하지만 개창부위, 중앙부위, 상악동 막 부위의 세 영역으로 구분하여 측정하였을 때, 실험군에서는 새로운 골이 상악동 막 부위에서 많이 형성되고 대조군에서는 상대적으로 개창부위에서 많은 양의 골이 형성되는 것을 관찰할 수 있었다. 섬유혈관 조직과 전체 거상된 양은 실험군에서 조금 더 많이 형성된 것으로 나타났지만 유의적인 차이는 없었다.

제한된 범위의 연구지만, 0.15mg/ml 농도의 재조합 제 2 형 인간 골형성단백질을 이상 인산칼슘에 코팅하여 토끼 상악동 거상술에 적용하였을 때, 상악동 막 주변으로 상대적으로 많은 양의 골 형성이 촉진되며 초기 치유기에 화학주성을 가지는 것으로 추정된다..

Key Words: 골형성단백질; 이상 인산칼슘; 상악동 거상술; 운반체; 골유도