The effect of enamel matrix derivative (EMD) in combination with deproteinized bovine bone material (DBBM) on the early wound healing of rabbit calvarial defects

법랑기질 단백질 유도체와 혼합된 이종골 이식재가 토끼 두개골 결손부의 초기 치유에 미치는 영향

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치주치료의 가장 중요한 목적은 상실된 치주조직의 형태적, 기능적 재건이다. 법랑기질 단백질 유도체(enamel matrix derivative: EMD)는 치주 병소에 사용시 상피세포의 증식을 억제하며 치주인대 및 백악아세포를 활성화시켜 무세포성 백악질 및 치주인대와 골조직의 생성을 유도한다고 보고되고 있다. 또한 법랑기질 단백질 유도체는 골모세포의 증식 및 분화를 촉진시키며 alkaline phosphatase의 활성 및 mineralized nodule의 형성을 촉진시킨다고 보고되고 있다. 이에 본 연구에서는 토끼 두개골 결손부에 법랑기질 단백질 유도체와 이종골 이식재를 이식한 후 골밀도를 방사선학적으로 분석하고, 신생골 형성 및 주변 조직 반응을 조직학적으로 관찰, 평가하고자 하였다.

토끼 두개골에 6 mm trephine bur(외경 8 mm)를 이용하여 경뇌막에 손상을 주지 않도록 하면서 4개의 결손부를 형성하였다. 아무것도 이식하지 않은 군을 음성 대조군으로, 이종골 이식재 (Bio-Oss®, Geistlich, Wolhusen, Switzerland)을 이식한 군을 양성 대조군으로 설정하였다. 법랑기질 단백질 유도체 (Emdogain®, Biora, Inc., Sweden)만 이식한 군과 법랑기질 단백질 유도체와 이종골 이식재를 혼합하여 이식한 군을 실험군으로 설정하였다. 각각의 재료를 이식한 후 비흡수성 차폐막 (Tefgen®, Lifecore Biomedical, Inc., U.S.A.)을 위치시키고 흡수성 봉합사로 일차 봉합을 시행하였다. 각 군당 술 후 1, 2, 4주의 치유기간을 설정하였다. 동물을 희

생시킨 후 두개골을 절제하여 먼저 방사선학적인 골밀도 측정을 시행한 후 10% formalin에 고정한 후 통법에 따라 조직표본을 제작하여 광학현미경으로 관찰하였다.

1. 방사선학적인 평가에서 1, 2, 4주에 대조군과 법랑기질 단백질 유도체만 이식한 군과 비교해 이종골 이식재만 이식한 군과 이종골 이식재에 법랑기질 단백질 유도체를 이식한 군에서 더 큰 골의 밀도를 보이고 있었다 ($P<0.01$). 하지만, 동일한 시기에 대조군과 법랑기질 단백질 유도체만 이식한 군과의 차이는 발견할 수 없었으며 ($P>0.05$), 이종골 이식재만 이식한 군과 이종골 이식재에 법랑기질 단백질 유도체를 이식한 군의 차이 또한 발견할 수 없었다 ($P>0.05$).
2. 조직학적인 평가에서 1, 2, 4주에 대조군과 법랑기질 단백질 유도체만 이식한 군과 비교해 이종골 이식재만 이식한 군과 이종골 이식재에 법랑기질 단백질 유도체를 이식한 군에서 골의 형성이 더 진행됨을 알 수 있었다. 법랑기질 단백질 유도체만 이식한 군이 대조군보다 2주에서 더 많은 신생골을 볼 수 있었으며, 이종골 이식재에 법랑기질 단백질 유도체를 이식한 군이 이종골 이식재만 이식한 군보다 1, 2주에서 더 많은 신생골을 관찰할 수 있었다.

이상의 결과에서 법랑기질 단백질 유도체는 토끼 두개골 결손부 치유단계에서 초기 골 형성을 촉진하는 것으로 사료되며 골 이식시에 법랑기질 단백질 유도체를 적용하는 것은 유용한 술식으로 사료된다.

I . Introduction

Implant insertion according to planned prosthetic position often causes the problem of an insufficient amount of host bone at the recipient site. The long-term prognosis of dental implants is adversely affected by an inadequate bone volume[1]. Several methods have been described for the regeneration of lost alveolar bone: First, the principle of osteoconduction is executed by the use of filling materials that serve as a scaffold for new bone growth[2].; Second, the principle of osteoinduction uses appropriate growth factors with the aim of inducing differentiation of mesenchymal stem cells to osteoblasts[1,3].

Grafting materials for osteoconduction served as a scaffold for the ingrowth of capillaries, perivascular tissue, osteoprogenitor cells from the recipient beds[2] and maintained mechanical stability and volume during the initial healing[4-6]. Grafting materials are autogenous bone, synthetic materials, allografts, and xenografts. Autogenous bone is considered the "gold standard" for grafting oral bony defects[7,8].

One of the xenogenic materials, deproteinized bovine bone materials (Bio-Oss®, Geistlich, Wolhusen/Switzerland) showed good clinical success and proven osteoconductive properties[9-11]. Electron microscopic evaluation shows that this material has a structural configuration similar to human bone. Its compressive strength and modulus of elasticity are also similar to the values for human bone[12,13]. A number of studies have shown that the bone mineral particles were completely encapsulated in newly formed bone when they were used in combination with Guided bone regeneration for the reconstruction of bone defects around dental implants in dogs[11] or under nonpermeable silicone domes on the skull of rats[14].

Enamel matrix proteins are known to have important biologic roles in the formation of acellular cementum, periodontal ligament, and alveolar bone during tooth development[15-19]. Based on this concept, a recently-developed porcine enamel matrix derivative (EMD) compound (Emdogain®, Biora, Inc., Sweden) has been suggested to encourage periodontal tissue regeneration such as activation of biosynthesis of cementum, periodontal ligament, and alveolar bone[20-24].

The bone tissue might also be influenced by EMD according to the observations reported at the histologic[16], clinical[20,21] and cellular levels[27,28]. It has reported that EMD could effect early stages of osteogenic maturation by stimulating bone cell proliferation[28]. When used in combination with demineralized freeze-dried bone allograft (DFDBA) for heterotopic bone formation, it seemed to enhance the bone induction potential of DFDBA[29]. In vivo studies have demonstrated that EMD increase new trabeculae formation in rat long bone repair model[30]. EMD also has promoted repair of circular defects in rat parietal bone. More mineralized tissues were formed in the defects applied with EMD compared to carrier alone[31]. Osteoinductive proteins require a carrier material, which serve as a delivery system and as a scaffold for cellular ingrowth[32].

Enamel matrix derivative is already used clinically[25,26] however, little is known about bone formation effect of EMD. The aim of the present investigation was to evaluate the effect of enamel matrix derivative (EMD) in combination with deproteinized bovine bone materials (DBBM) on the early wound healing of rabbit calvarial defects.

Ⅱ. Material and Methods

A. Surgical protocol

Nine New Zealand white male rabbits between 2.8 and 4kg were included in this randomized, blinded, prospective study. Each rabbit was anesthetized with Ketamine Hcl (5mg/kg) and Xylazine Hcl (1.5ml/kg). The fur was shaved over the cranium, which was prepared and draped in a sterile fashion. An incision was made to the bony cranium and the periosteum was reflected. By means of a trephine bur (external diameter : 8 mm). Four critical-sized defects (critical size being 15 mm wide) could not be created in the rabbit cranium because it is too small. Four non-critical sized 8 mm defects were created with copious irrigation. The four calvarial defects were randomly grafted with DBBM, DBBM with EMD, EMD alone, and no graft as a control. The Four defects were covered with nonresorbable PTFE membrane (Tefgen®, Lifecore Biomedical, Inc, U.S.A.). The wound was closed with resorbable suture materials, and the rabbits were extubated and allowed to recover. At the end of the surgical procedure, all animals received a single intramuscular injection of Gentamicin (0.1ml/kg)

Rabbits were killed using phentobarbital (100mg/kg) intravenously at 1, 2, and 4 weeks. There were 3 rabbits in each group. the entire cranium was removed with a reciprocating saw, without encroaching on the grafted areas.

B. Evaluation

1) Radiographic evaluation

Radiographs were taken of the rabbit calvaria in its entirety before histologic sections were performed. A aluminum step-wedge was used in each radiograph for comparison. The radiographs were scanned and images were analyzed with a ImageJ 1.31v software on a IBM computer.

2) Histologic evaluation

The rabbit calvarias were fixed in 4% paraform-aldehyde, and decalcified in hydrochloric acid decalcifying solution (Fisher Scientific, Tustin, CA) at 4℃ for 2-4 weeks. It was embedded in paraffin and cut into 6㎛ thickness. The sections were stained with H&E and observed by optical microscope.

3) Statistical methods

Numerical data was presented as mean plus one standard deviation. One way analysis of variance (ANOVA) with fisher's Tukey test was used for multiple comparisons to compare with the control. The probability level of $P < 0.05$ was regarded as statistically significant.

Ⅳ. Results

A. Radiographic evaluation

Figure 1 demonstrates the bone density as determined radiographically. There was a significant increase in bone density of DBBM group as compared with control and EMD group at 1, 2 and 4 weeks ($P<0.01$). The results showed a significant increase in bone density when DBBM with EMD group were compared with control and EMD group at 1, 2, and 4 weeks ($P<0.01$). However significant increase was not seen at all time when control group was compared with EMD group ($P>0.05$). There was also no significant difference between DBBM and DBBM with EMD group at 1, 2, and 4 weeks ($P>0.05$).

Table 1. The four groups randomly grafted at the calvarial defects.

Group	n	Graft materials	membrane
control	3	no graft	PTFE (Tefgen®)
EMD	3	Emdogain®	PTFE (Tefgen®)
DBBM	3	Bio-Oss®	PTFE (Tefgen®)
DBBM with EMD	3	Bio-Oss® with Emdogain®	PTFE (Tefgen®)

Table 2. the bone density as determined radiographically

	1 week	2 week	4 week
control	0.15±0.06	0.28±0.06	0.37±0.12
EMD	0.17±0.07	0.31±0.07	0.41±0.09
DBBM	0.91±0.08*	1.05±0.17*	1.30±0.20*
DBBM + EMD	0.87±0.11*	1.16±0.06*	1.33±0.22*

mean ± SD (gram/square inch) analyzed by a ImageJ 1.31v software

statistical analysis : one-way ANOVA with fisher's Tukey test ; $P<0.05$

* : Significantly different from corresponding control (P<0.05)

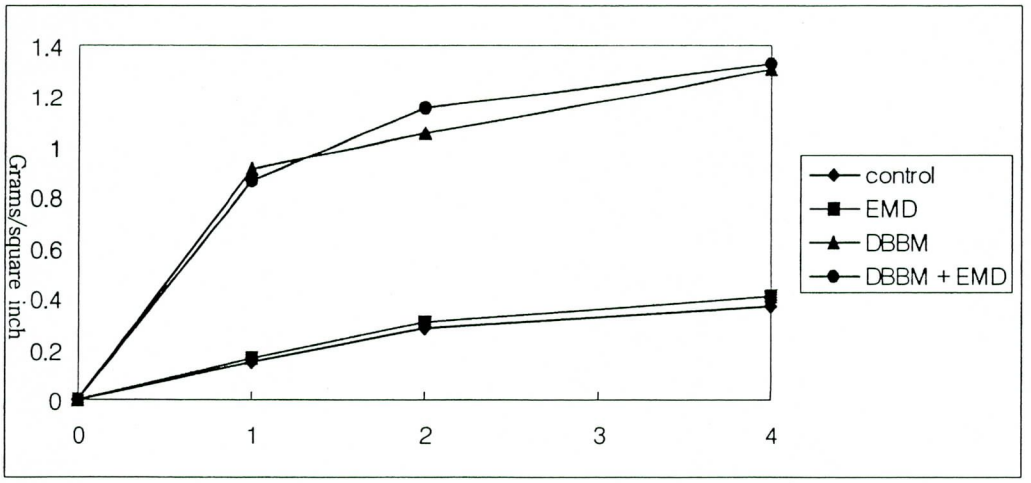

Fig 1. Amount of bone fill determined radiographically over the 4 weeks study period.

B. Histologic evaluation

In all specimens, the defects were completely closed by the PTFE membrane and all group showed an increase in bone formation over time (Figs. 4, 6, 9). The perforated areas were filled with granular tissue (Fig. 4a,b). There was active osteoblastic activity and immature bone formation at the border of the defect in control and EMD group, it was similar histologically between control and EMD group (Fig. 5a,b). A slightly increase in osteoblastic and osteoid layers were seen for DBBM group compared with control group (Fig. 4a,c). Figure 4 c, d showed that the original thickness of bone at the defect site was maintained. There were osteoblastic and osteoid layers at the border of the defect and around deproteinized bovine bone material particles at DBBM and DBBM with EMD group, more osteoblastic and osteoid layers of DBBM with EMD group were seen than that of DBBM group (Fig. 4c,d, 5c,d).

Newly formed bone was seen at the border of the defect at control and EMD group and it extended further toward the central area of the defect (Fig. 6a,c). Newly formed bone within perforations in EMD group was more evident than that in control group at 2 weeks (Fig. 7a,b). There was newly formed bone at the border of the defect and around deproteinized bovine bone material particles in DBBM and DBBM with EMD group (Fig. 6c.d). There was slightly increase of newly formed bone at DBBM with EMD group as compared with DBBM group (Fig 7c.d).

All group showed an increase in bone formation at 4 weeks as compared with 1, 2 weeks (Fig. 8a,b,c,d). There was no difference at newly formed bone when control group was compared with EMD group (Fig. 8a,b) and DBBM group was compared with DBBM with EMD group (Fig. 8c.d). The individual particles of the bovine bone material were clearly identifiable and they were found to be

surrounded by varying amounts of newly formed bone without being encapsulated by loose fibrous connective tissue in DBBM and DBBM with EMD group at all time.

Newly formed bone was not seen at the center of the defect in control group (Fig 9a). But there was newly formed bone around deproteinized bovine bone material particles at the center of the defect in DBBM group (Fig 9b).

V. Discussion

The present study has demonstrated the application of EMD in combination with deproteinized bovine bone materials results in the complete healing of defects in the calvaria of rabbits.

In the past decade, the use of barrier mambrane became a clinically well-documented and succeessful procedure[33,34]. The placement of a rigid, memebrane-like barrier created a secluded space adjacent to a bone surface. The barrier impeded cells originating from the surrounding soft tissues to invade the created space that becomes gradually filled with newly formed bone[35,36], which is stable on a long term basis[37]. The placement of barrier membranes - separating undesired tissue from the secluded wound space into which only cells with the potential of forming bone - are necessary to predictably close bone defects[38]. When comparing bioabsorbable and non-resorbable membranes, better results with the non-resorbable membranes[39] have been reported. Donos[38] reported that bioabsorbable membrane had no occlusive properties that long enough to allow bone formation within the defects. In this study, he showed that there were deproteinized bovine bone material particles encapsulated by loose fibrous connective tissue in the center of the rat calvarial defect covered by bioabsorbable membranes. In the present study, when non- resorbable membrane was placed to create a secluded space between the bony edges, the defects were completely closed at all group. There were deproteinized bovine bone material particles not encapsulated by loose fibrous connective tissue, but surrounded by newly formed bone at the center of the defect in DBBM and DBBM with EMD group. It supported that the placement of non-resorbable membranes are necessary to completely close the

defects.

It has been reported that collapse of the barrier membranes into the bone defect compromises the amount of newly formed bone by eliminating the space, which is necessary for the bone to form[35,40]. The mambranes used in GBR are often supported by mambranes-supporting materials including allografts, xenografts, and synthetic materials. Khalid[41] reported that the inorganic bovine bone materials posseessed the best potential of osteoconductive grafting material, followed by the biograss crystals and the hydroxyapatite particles respectively. In this study, he showed that the bovine bone materials showed significant increase in newly formed bone when compared to the no graft as a control. The collapse of the membrane could be avoided by using autogenous bone[42,43] or Bovine bone material[44], such as the one used in this study. In the present study, there was more newly formed bone area in the DBBM group than in control group at 1, 2 and 4 weeks. Indeed the use of bovine bone materials for the space maintenance under the membrane resulted in maintenance of the original thickness of bone at the calvarial defect.

Enamel matrix derivative has been developed as a clinical treatment to promote periodontal regeneration. It is derived from embryonal enamel of porcine origin, based on the high degree of homology between porcine and human enamel proteins[16,45,46]. Amelogenins comprise 90% of the proteins present in EMD, the remaining 10% are proline-rich non-amelogenins, including tuftelin, tuft protein, and various serum proteins, as well as, ameloblastin[47] and amelin[48].

In periodontal ligament (PDL) fibroblasts, EMD stimulates their migration, proliferation, differentiation, and enhances the expression of alkaline phosphatase activity, mineral nodule formation, and increases the autocrine release of cytokines such as transforming growth factor-β, IL-6 and platelet-derived growth factor BB[27,49,50]. In gingival fibroblasts, EMD stimulates their

proliferation[51]. however, little is known about bone formation effect of EMD.

Concerning the biological effects of EMD on osteoblastic mesenchymal cells, EMD has been reported to stimulate the proliferation of preosteoblatic 2T9, OCT-1 and MC3T3-E1 cells, and enhance the differentiation of osteoblast-like osteosarcoma MG-63 cells[49,50,52,53]. Enamel matrix derivative up-regulated osteopontin mRNA level and slightly enhanced BSP(bone sialoprotein) transcripts in these cell llines[53]. In primary mouse osteoblasts, EMD enhanced the gene expression of collagen Iα, interleukin-6 (IL-6) and prostaglandin G/H synthase 2[54]. Schwar[52] reported that EMD enhanced the Alkaline phosphatase activity of human-osteoblast cells. It has been reported that EMD increased the mineral nodule formation in mouse osteoblastic cells[55]. These results suggest that in osteoblastic and preosteoblastic cells EMD promotes proliferation, differentiation and enhances alkaline phosphatase activity and mineral nodule formation.

It has been reported that EMD is not osteoinductive but osteopromotive in vivo rat study[29]. Kawana and cowoker[30] reported that EMD exhibited osteopromotive effects on bone and medullary regeneration during wound healing of injured femurs and increased the initial trabecular bone formation. The trabecular bone area formed in EMD-applied femurs was significantly greater than that in PGA-applied control at 7 days. Sawae and cowoker[31] reported that EMD exhibited osteopromotive effects on bone regeneration during wound healing in rat parietal bone defects. at both 7 and 14 days post-operation, Mineralized tissue volume in EMD-applied bones was greater than that in PGA-applied control. In the present study DBBM and DBBM with EMD group showed a significant increase in newly formed bone when compared to control and EMD group at all time. There was a significant difference in histological analysis at 2 weeks when control was compared with

EMD, there was a significant increase in newly formed bone surrounding bovine bone material at DBBM with EMD group as compared with DBBM group at 1, 2 weeks.

Corneline and cowoker[56] reported that EMD implanted in 8 mm bone defects of rabbit tibia bone is fully resorbed after 4 to 8 weeks and dose not adversely affect bone formation and regeneration. Other investigations have shown controversial evidence on the inductive properties of the enamel matrix proteins[29]. Donos and cowoker[42] reported that the predictability of bone formation in critical-size defects of rat calvaria depends mainly on the presence or absence of barrier membranes(GBR). The combined use with deproteinized bovine bone mineral and/or enamel matrix proteins did not significantly enhance the potential for complete healing provided by the GBR procedure at 4 months. EMD failed to show any significant benefit in promoting new bone formation around titanium implants in a rabbit model[57] and dog model[58]. In the present study, there was no significant difference histologically at 4 weeks when EMD group was compared with control group and when DBBM with EMD group was compared with DBBM group.

These inconsistent results may be due to the different experimental systems and different animal employed in their experiments[42]. Experimental system has been described; First, defect site : tibia, femur, clavaria; Sencond, defect size : critical size defect and non-critical size defect; Third, evaluation period : long term data and short term data. The bioactive effects of EMD on bone wound healing and mineralized tissue formation depend on the local osseous environment where EMD has been applied[31]. These results suggest that EMD may positively influence initial bone wound healing, but EMD does not significant enhance the complete bone healing.

Digital subtraction radiography with aluminum step-wedge calibration showed

a significant increase in bone density when DBBM, DBBM with EMD were compared to control and EMD group at all time. But radiographic assessment did not show any significant difference between DBBM and DBBM with EMD. The clinical significance of these data is difficult to determine because any radiopaque bone grafting material will look more dense on a radiograph.

In conclusion, this study has clearly demonstrated that the addition of DBBM with EMD in the rabbit cranial defect model was shown to be potentially beneficial at early wound healing. Deproteinized bovine bone materials had the good osteoconductive properties and served as a space maintainer successfully. Further studies are needed to evaluate the potential benefits of EMD in combination with various grafting materials such as autogenous bone, allograft, and alloplast graft. In vivo studies of EMD also are needed for the long term evaluation in wound healing.

V. Conclusion

The true periodontal regeneration is morphologic and functional reconstruction of periodontal tissue. It has been reported that EMD (Emdogain®, Biora, Inc., Sweden) inhibited epithelial proliferation and encouraged periodontal tissue regeneration such as activation of biosynthesis of acellular cementum, periodontal ligament, and alveolar bone. In osteoblastic and preosteoblastic cells EMD promoted proliferation, differentiation and enhances alkaline phosphatase activity and mineral nodule formation. This study was to evaluate the effect of enamel matrix derivative (EMD) in combination with deproteinized bovine bone material (DBBM) in the early wound healing of rabbit calvarial defects.

Nine New Zealand white male rabbits between 2.8 and 4kg were included in this randomized, blinded, prospective study. Four non-critical sized 8 mm defects were created by a trephine bur (external diameter : 8 mm). The four calvarial defects were randomly grafted with DBBM (Bio-Oss®, Geistlich, Wolhusen, Switzerland), DBBM with EMD, EMD alone, and no graft as a control. The Four defects were covered with nonresorbable PTFE membrane (Tefgen®, Lifecore Biomedical, Inc., U.S.A.). The wound was closed with resorbable suture materials. Rabbits were killed using phentobarbital (100mg/kg) intravenously at 1, 2 and 4 weeks. There were 3 rabbits in each group. the entire cranium was removed with a reciprocating saw, without encroaching on the grafted areas.

The results were as follows :

1. In radiographic evaluation, the results showed a significant increase in bone density when DBBM and DBBM with EMD group were compared with control

and EMD group at 1, 2, and 4 weeks ($P<0.01$). However significant increase was not seen at all time when control group was compared with EMD group ($P>0.05$). There was also no significant difference between DBBM and DBBM with EMD group at 1, 2, and 4 weeks ($P>0.05$).

2. In histological evaluation, DBBM and DBBM with EMD group showed a significant increase in newly formed bone when compared to control and EMD group at 1, 2 and 4 weeks. Newly formed bone within perforations in EMD group was more evident than that in control group at 2 weeks, A slightly increase in newly formed bone was seen for DBBM with EMD group compared with DBBM group at 1, 2 weeks.

In conclusion, this study has demonstrated that the addition of DBBM with EMD in the rabbit calvarial defect model was shown to be potentially beneficial at early wound healing. EMD might positively influence the early bone wound healing.

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Fig. 4, 5

(A). A light micrograph of control group at 1 week postoperatively

: The perforated areas were filled with loose fibrous tissue. There were osteoblastic and osteoid layers from cortical bone margin.

(B). A light micrograph of EMD group at 1 week postoperatively

: The perforated areas were filled with loose fibrous tissue. There were osteoblastic and osteoid layers from cortical bone margin.

(C). A light micrograph of DBBM group at 1 week postoperatively

: There were osteoblastic and osteoid layers at the border of the defect and around deproteinized bovine bone material particles.

(D). A light micrograph of DBBM with EMD group at 1 week postoperatively

: There were osteoblastic and osteoid layers at the border of the defect and around deproteinized bovine bone material particles.

Fig. 6, 7

(A). A light micrograph of control group at 2 week postoperatively

: The perforated areas were filled with dense fibrous tissue. There was formation of new bone from cortical bone margin.

(B). A light micrograph of EMD group at 2 week postoperatively

: The perforated areas were filled with dense fibrous tissue. There was formation of new bone from cortical bone margin.

(C). A light micrograph of DBBM group at 2 week postoperatively

: There was formation of new bone from cortical bone margin. Osteoprogenitor cells and preosteoblasts were seen on the periphery of the graft materials.

(D). A light micrograph of DBBM with EMD group at 2 week postoperatively

: There was formation of new bone from cortical bone margin. The graft materials have been incorporated into the newly formed bone matrix.

Fig. 8

(A). A light micrograph of control group at 4 week postoperatively

: The perforated areas were filled with dense fibrous tissue. There was formation of new bone from cortical bone margin.

(B). A light micrograph of EMD group at 4 week postoperatively

: The perforated areas were filled with dense fibrous tissue. There was formation of new bone from cortical bone margin.

(C). A light micrograph of DBBM group at 4 week postoperatively

: There was formation of new bone from cortical bone margin. The graft materials have been incorporated into the newly formed bone matrix.

(D). A light micrograph of DBBM with EMD group at 4 week postoperatively

: There was formation of new bone from cortical bone margin. The graft materials have been incorporated into the newly formed bone matrix and were resorbed during the remodeling process.

Fig 9.

(A). A light micrograph of control group at 4 week postoperatively

: There was not formation of new bone in the center of the perforated areas.

(B). A light micrograph of DBBM group at 4 week postoperatively

: There was newly formed bone around deproteinized bovine bone material particles in the center of the perforated areas.

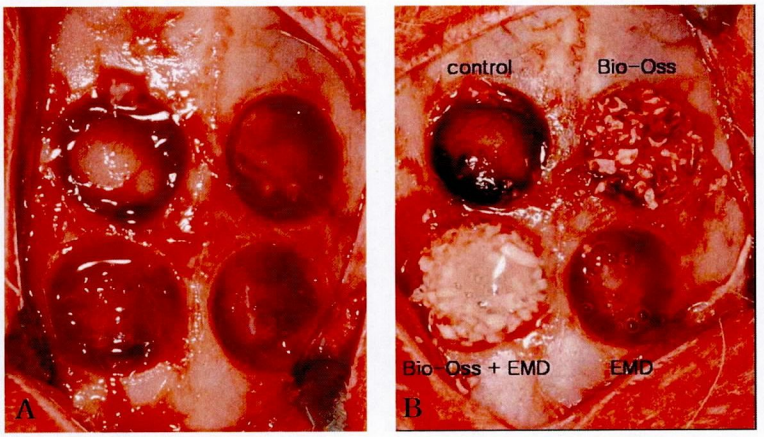

Fig. 2. Photographs of the surgical sites

A, Rabbit calvaria with surgical sites prepared.

B, Rabbit calvaria with surgical sites grafted

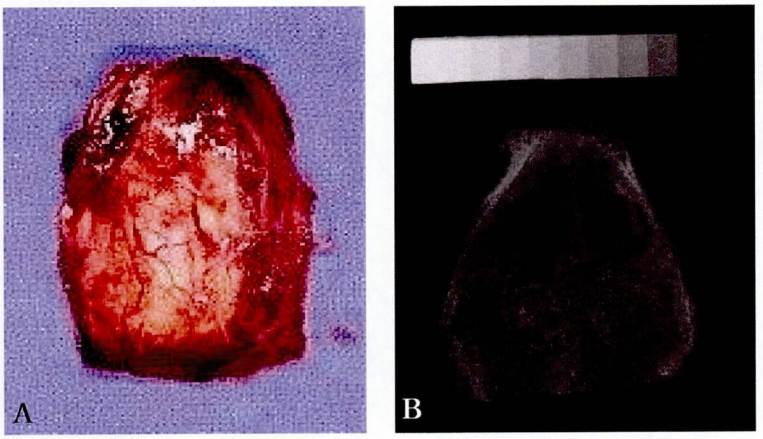

Fig. 3. Gross and radiographic examination of surgical site

A, Rabbit calvaria harvested after 1, 2 and 4 weeks of healing.

B, Radiograph of a rabbit calvaria with aluminium step wedge.

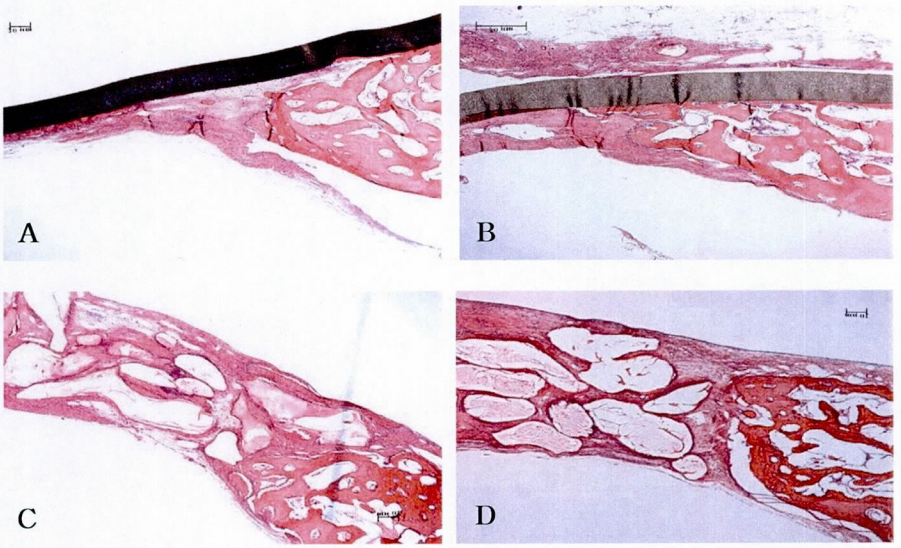

Fig. 4. Light micrographs at 1 week postoperatively; control (A), EMD (B), DBBM (C), and DBBM with EMD (D). Haematoxylin and eosin staining, Magnification × 40

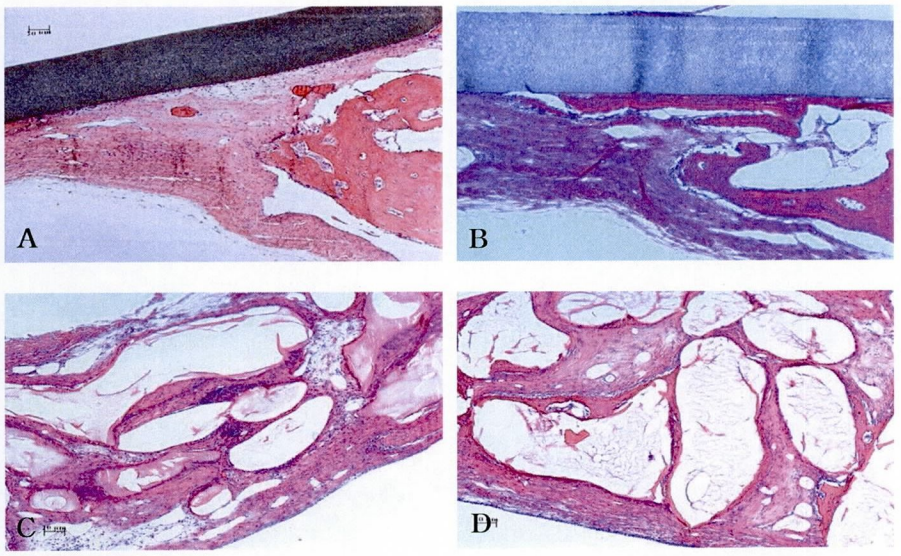

Fig. 5. Light micrographs at 1 week postoperatively; control (A), EMD (B), DBBM (C), and DBBM with EMD (D). Haematoxylin and eosin staining, Magnification × 100

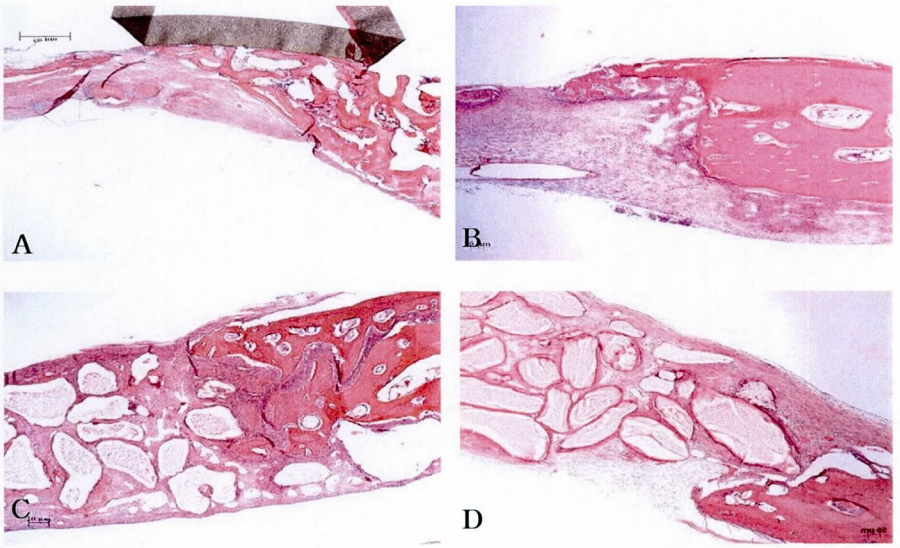

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Fig. 6. Light micrographs at 2 weeks postoperatively; control (A), EMD (B), DBBM (C), and DBBM with EMD (D). Haematoxylin and eosin staining, Magnification × 40

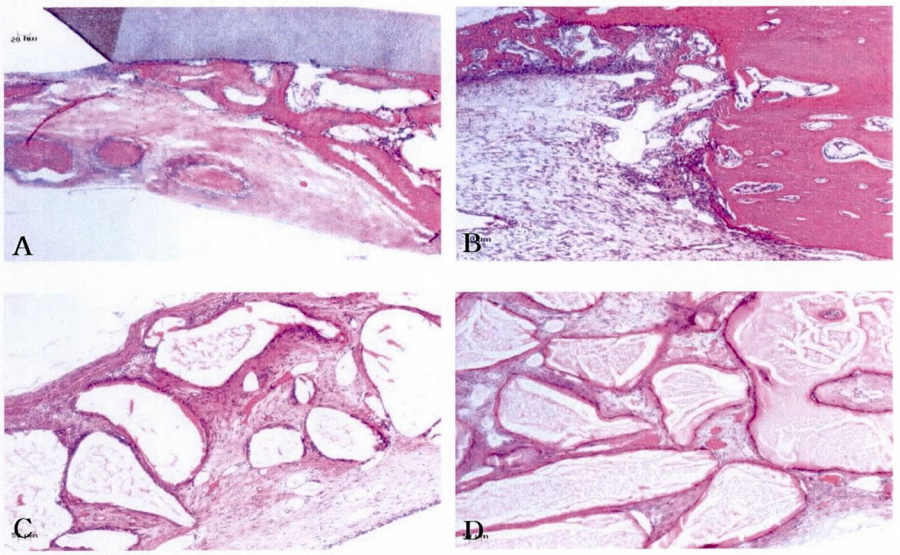

Fig. 7. Light micrographs at 2 weeks postoperatively; control (A), EMD (B), DBBM (C), and DBBM with EMD (D). Haematoxylin and eosin staining, Magnification × 100

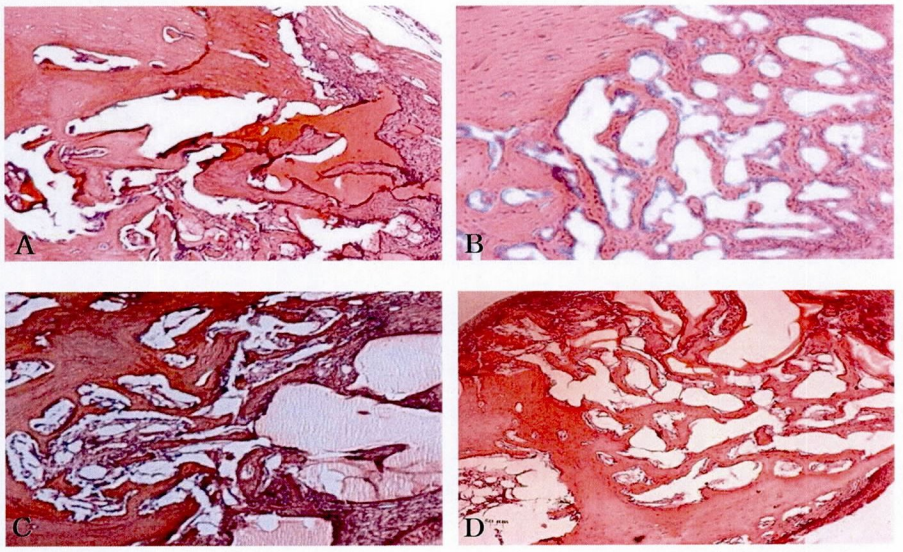

Fig. 8. Light micrographs at 4 weeks postoperatively; control (A), EMD (B), DBBM (C), and DBBM with EMD (D). Haematoxylin and eosin staining, Magnification × 100

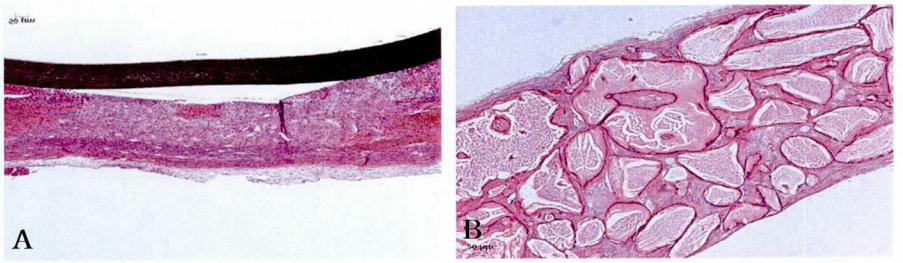

Fig. 9. Light micrographs of the center of the defect at 4 weeks postoperatively; control (A), DBBM (B). Haematoxylin and eosin staining, Magnification × 40