Degradation properties of a bi-layered cross-linked collagen membrane for localized bone regeneration: In vitro and in vivo study

Jin-Young Park

The Graduate School

Yonsei University

Department of Dentistry

Degradation properties of a bi-layered cross-linked collagen membrane for localized bone regeneration: In vitro and in vivo study

Directed by Professor Seong-Ho Choi

The Doctoral Dissertion
submitted to the Department of Dentistry,
the Graduate school of Yonsei University
in partial fulfillment of the requirements for the degree of
Ph.D. in Dental Science

Jin-Young Park

December 2017

연세대학교
YONSEI UNIVERSITY

This certifies that the Doctoral Dissertation
of Jin-Young Park is approved.

Thesis Supervisor : Seong-Ho Choi

Ui-Won Jung

Jung-Seok Lee

Sung-Tae Kim

Dong-Woon Lee

The Graduate School
Yonsei University
December 2017

감사의 글

영국에서 Newcastle University 치과대학을 졸업한 후 1년간 National Health Service (NHS)에 근무하면서 치과의사로써 여러 방면에서 부족함을 느꼈습니다. 이어서 연세대학교 치의학대학원에 들어오면서 박사과정과 치주과 전공의 과정을 통해 세계적인 수준의 학문를 배우게되었고 그에 대한 자부심도 가질 수 있게 되었습니다.

이 과정에서 저를 전폭적으로 지원해주시고 지도해주신 최성호 교수님께 깊은 감사를 드립니다. 그리고 좋은 교육 환경을 마련해주신 채중규 교수님, 조규성 교수님, 김창성 교수님, 정의원 교수님, 이중석 교수님, 차재국 교수님께 감사드립니다. 바쁘신 와중에도 심사를 맡아주신 김성태 교수님과 이동운 교수님께도 감사드립니다. 또한 3년간의 길고도 짧은 전문의 수련기간동안 제 곁을 지켜준 든든한 동기들과 선후배님들에게도 고마운 마음을 전합니다. 마지막으로 제가 오늘 날 이자리에 있도록 버팀목이 되어주신 부모님과 가족, 사랑합니다.

-저자 씀-

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

Degradation properties of a bi-layered cross-linked collagen membrane for localized bone regeneration: *In vitro* and *in vivo* study

Jin-Young Park, B.D.S

Department of Dentistry
The Graduate School, Yonsei University

(Directed by Professor Seong-Ho Choi, D.D.S., M.S.D., PhD.)

Purpose: (i) To evaluate the biologic properties of a bi-layered EDC-cross-linked collagen membrane (CCM) *in vitro.* (ii) To assess the efficacy of CCM for localized bone regeneration *in vivo*.

Methods: Biodegradation of CCM compared to a non-cross-linked collagen membrane (NCM) was assessed *in vitro. In vivo*, twelve male New Zealand White rabbits were used. Four calvarial, circular defects (diameter 8mm) were created in each animal. The sites were randomly allocated to i) CCM + biphasic calcium phosphate (BCP)

(CCM-BCP group), ii) CCM alone (CCM), iii) BCP alone (BCP) and, iv) negative control (control). Animals were sacrificed at 2 (n = 6) and 8 weeks (*n* = 6). Outcome measures included: micro CT analysis (total augmented volume (TAV), new bone volume (NBV)) and histomorphometry (total augmented area (TAA), newly formed bone (NBA), remaining membrane thickness (RMT)).

Results: CCM was more resistant to degradation than NCM. Micro CT analysis showed CCM-BCP (196.43±25.30 mm^3) and BCP (206.23±39.13 mm^3) groups had significantly (P<0.01) larger TAV than the control (149.72 ± 12.28 mm^3) after 8 weeks. Histomorphometrically, CCM-BCP group (17.75 ± 5.97 mm^2) had significantly (P<0.01) greater TAA compared to the CCM group (7.74 ± 2.25 mm^2) and the control (8.13 ± 1.81 mm^2) after 8 weeks. After 8 weeks, RMT was reduced by 67%.

Conclusion: BCP is required for space maintenance in regeneration of a localized bone defect. The dense non-porous layer of the CCM was maintained after 8 weeks, whereas the porous layer was replaced by new bone or dense collagenous tissue.

Key words: Bone regeneration; Biodegradation; Collagen; Hydroxyapatite-beta tricalcium phosphate

Degradation properties of a bi-layered cross-linked collagen membrane for localized bone regeneration: *In vitro* and *in vivo* study

Jin-Young Park, B.D.S

Department of Dentistry
The Graduate School, Yonsei University

(Directed by Professor Seong-Ho Choi, D.D.S., M.S.D., PhD.)

I. INTRODUCTION

Guided bone regeneration (GBR) is a well-established procedure to regenerate an adequate ridge width for implant placement. Moreover, according to a recent study, the number of implants placed in conjunction with GBR is increasing [1]. This serves as an indicator for the predictability of the procedure and the awareness of the clinicians to place implants in a restoration-driven position. The biologic principle of GBR is to place a barrier membrane over an osseous defect, in order to exclude the overlying epithelium

and connective tissues, and to create a secluded space for bone formation [2-4]. Characteristics of an ideal barrier membrane include biocompatibility, cell occlusiveness, space maintenance, tissue integration and ease of handling.

Numerous non-resorbable and resorbable barrier membranes have been introduced in the past. Clinically, the use of resorbable membranes for GBR of peri-implant defects resulted in stable long-term peri-implant marginal bone levels [5-7]. However, due to a lack of stiffness, resorbable membranes need to be combined with a membrane-supporting (bone substitute) material to maintain the defect space [8]. A rapid and sometimes unpredictable degradation rate is considered a further drawback of these membranes. This may result in an early loss of the barrier function prior to complete defect healing.

In order to prolong the barrier function, collagen membranes have been applied pre-clinically and clinically, in the cross-linked form [9], or in double layers [10-12]. A previous study demonstrated good biocompatibility and enhanced regeneration of calvarial defects for a chemically cross-linked collagen membrane (CCM) [13]. Recently, this barrier membrane was modified into a bi-layer form consisting of a dense, non-porous collagen film layer facing the connective tissues, and a porous collagen sponge layer facing the defect. It is considered that this new structure will result in enhanced bone regeneration by improving cell-occlusivity and extending the barrier function.

The aim of the present study was (i) to characterize and evaluate the biocompatibility and degradation of CCM *in vitro*, and (ii) to assess the efficacy of CCM for localized bone regeneration *in vivo*.

II. MATERIALS AND METHODS

1. Materials

Bilayered cross-linked collagen membrane (CCM)

The bilayered chemically cross-linked type-I collagen membrane (CCM) (Dalim Tissen co., Ltd., Seoul, Korea) is derived from porcine skin and is composed of type I collagen. The membrane features two layers: a compact non-porous film layer designed to prevent infiltration of soft connective tissue cells into the membrane-protected space; and (ii) a porous sponge layer, facing the bone defect and consisting of loosely arranged collagen fibers. The porous layer is intended to stabilize the blood clot and to enhance bone formation.

Native collagen membrane (NCM)

The native collagen membrane (NCM) (Biogide, Geistlich Biomaterials, Wolhusen, Switzerland) is derived from porcine dermis and is composed of type I and III collagen. The membrane has two layers and been characterized in numerous studies [11].

Synthetic bone substitute

The biphasic calcium phosphate (BCP; Osteon^{TM} III, Genoss, Seoul, Korea) consists of 60% hydroxyapatite and 40% β-tricalcium phosphate (TCP). The bone graft particles were 0.5-1.0 mm in size with interconnected macro and micro pores.

2. Experiments

In vitro

Tensile strength test

Dry samples of CCM and NCM of 30 x 5 mm in size were compared for tensile strength. The membranes were placed on a universal tensile machine and stretched at 10 mm/min until the point of breakage. The ultimate strength at the point of breakage was recorded three times, from which an average was deduced.

Enzymatic degradation assay

In vitro degradation test was performed using bacterial collagenase from Clostridium histolyticum (Sigma, Type I, 0.5-5.0 units/mg, 85.3 unit/ml). Collagenase was dissolved in 50 mM TES buffer (50 mM Tris, 5 mM EDTA, 2.5% sucrose) with 0.36 mM $CaCl_2$, pH 7.4. Samples of CCM and NCM were weighed to obtain an initial dry weight (Wi) and immersed in 5 ml TES buffer containing 0.1 mg of collagenase for 4 h at 37 ℃. The remaining samples were collected, lyophilized, and weighed to obtain the dry weight after enzymatic degradation (Wd). The proportion of the remaining weight of the samples was defined as: remaining weight (%) = Wd/Wi X 100.

Cytotoxicity test

CCM, positive (latex film) and negative (polyethylene film) controls were extracted in 0.9% sodium chloride at 37 ℃ for 72 h. L-929, a mouse fibroblast cell line, was

cultured for 48 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37 ℃ in a 5% CO2 humidified incubator. The extracts were used to replace the maintenance medium of the cell culture. All cultures were incubated for 24 h, under the same growth condition as described above. After the incubation, each culture was stained with tetrazolium dye solution and lysed with dimethyl sulfoxide. Absorbance was measured at 570 nm with an automatic microplate reader. The relative cell viability was expressed as the percentage of the optical densities in the test medium to the fresh medium.

In vivo

Animals

Twelve male New Zealand White rabbits weighing 2.8-3.2 kg and aged 16-20 weeks were used in this study. The sample size was determined from a previous study using the same experimental model. The animals were housed in separate cages under standard laboratory conditions with ad libitum access to water and a standard laboratory pellet diet. Animal selection and care, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee (Yonsei Medical Center, Seoul, Korea; approval number 2015-0072). The ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines were referred to for the design and implementation of this study [14].

Surgical procedures (Figure 1)

The surgical procedures were decribed in detail in a previous publication [15]. In brief, an incision was made along the midline of the cranium from the frontal bone to the occipital bone in order to expose the calvarium. A full-thickness flap was elevated. Under copious saline irrigation, four standardized round defects, each 8 mm in diameter, were created using a trephine bur. The resected bone windows were removed carefully to avoid injury to the underlying dura mater and brain tissue. The defects were randomly assigned to the following four treatment modalities:

- CCM-BCP: cross-linked collagen membrane plus biphasic calcium phosphate
- CCM: cross-linked collagen membrane plus blood clot
- BCP group: biphasic calcium phosphate only
- Control group: filled with blood clot only

In group CCM-BCP, the BCP particles were placed into the defect, then the CCM covered the entire site. In group CCM, blood was allowed to coagulate inside the defect, which was covered by the CCM. In group BCP, only the BCP particles were packed into the defect. In control, only the blood clot occupied the defect.

For the groups containing BCP, the amount of BCP was standardized at 0.07-0.08 g to completely fill each defect by application of gentle pressure with a surgical instrument. For the groups covered by CCM, the membrane was cut to the size of 10 x 10 mm to cover the entire perimeter of defect.

Subsequently, the flaps were repositioned and sutured. The sutures were removed one

week postoperatively. The animals were euthanized at 2 weeks (n = 6) or 8 weeks (n = 6) and specimens harvested.

Outcome measures

Micro-computed tomography analysis

All harvested specimens were fixed in 10% formalin for 10 days and scanned with a micro-computed tomography (μCT) system (Sky-Scan 1173, SkyScan, Aartselaar, Belgium) at a resolution of 13.85 μm (130 kV, 60 μA). The scanned data set was processed in DICOM (Digital Imaging and Communications in Medicine) format, and the area of interest was reconstructed with On-Demand 3-dimensional (3D) software (Cybermed, Seoul, Korea). The overall dimensional topography of the calvaria and the grafted sites were visualized in 3D reconstructed images. The ROI_{MC} for the total augmented volume was defined by the outline of the circular defect margin, laterally; connective tissue border, superiorly; and the dura mater, inferiorly. For detailed analysis of augmented volume, all sectioned images were processed with NRecon 1.6.9.8 reconstruction software (SkyScan). 2240 x 2240 pixel images were acquired for creation of cross-sections and analysis of bone formation. Radiopaque areas were distinguished from the total augmented area according to specific 8-bit threshold grayscale values. The gray-scale values were set from 120 to 255 for bone substitute and 72 to 120 for newly formed bone. Areas with grayscale values lower than 72 were considered as fibrovascular tissue. Within the ROI_{MC}, the following parameters were measured:

- Total augmented volume (TAV; mm^3): entire augmented volume.
- New bone volume (NBV; mm^3): volumetric measurement of the newly formed bone within the defect.
- Residual graft volume (RGV; mm^3): volumetric measurement of the remaining graft particles.

Histologic and histomorphometric analysis

The fixed specimens were decalcified in 5% formic acid for 14 days and then embedded in paraffin. Serial 5μm thick sections were cut through the central portion of each experimental site. Only sections located at the middle of the defects were selected, and stained with hematoxylin and eosin and Masson trichrome. The histologic slides were observed and digitally captured under a light microscope (DM LB, Leica Microsystems, Wetzlar, Germany) equipped with a camera (BX50, Olympus, Tokyo, Japan). After the conventional microscopic examination assessing the various tissues (bone, bone substitute material, non-mineralized tissue, membrane), computer-assisted histometric measurements in the calvarial defect were performed using an automated image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). The ROI_H was defined by the margins of the original bone, laterally; the periosteum or the CCM, superiorly; and the dura mater, inferiorly. The following parameters were measured within the ROI_H:

- Total augmented area (TAA): This consists of the sum of the area of new bone, residual particles, fibrovascular tissues within the ROI_H.

- New bone area (NBA): Area of newly formed bone within the ROI_H.
- Residual particle area (RPA): Area of the remaining bone graft particles within the ROI_H.
- Remaining membrane height (RMH): Height of the CCM measured at 3 different points of the defect; left, middle and right.

3. Statistical analysis

The statistical analysis was performed using a commercially available software program (IBM SPSS Statistics 23). Micro-CT measurements and histomorphometric records were summarized in terms of the mean and standard deviation (SD) values. The relationship between the measured parameters and each of the factors was examined using the Kruskal-Wallis test (for factors with more than two levels) and Mann-Whitney-U test (for factors with two levels). Wilcoxon Rank test was used to compare the same experimental group between the two time periods (2 and 8 weeks). Statistical significance was considered when $P<0.05$.

III. RESULTS

1. In vitro

SEM imaging (Figure 2)

The thickness of CCM was measured at 350 μm, the dense non-porous layer was 25 μm, and the spongy porous layer was 325 μm. The pore size of the spongy layer was approximately 20 μm. Thin sheets of collagen structures were arranged in a lattice shape to form the porous layer of CCM. The dense portion had a smooth surface with no pores. The surface of the dense film layer appeared smooth even at a very high magnification (x1000).

Tensile strength test

The results showed that the CCM (5.22 ± 0.1 N/mm^2) and NCM (5.1 ± 0.1 N/mm^2) had similar mechanical strengths.

Enzymatic degradation assay

After 4 h of degradation by bacterial collagenase, the remaining sample weight of CCM (43.09 ± 0.05%) was significantly greater than that of NCM (13.46 ± 0.08%).

Cytotoxicity test

The relative cell viability was comparable between CCM (99.74 ± 2.7%) and the negative control (polyethylene, 100 ± 2.1%). These values were significantly greater than that of the positive control (latex, 2.15 ± 0.38%)

2. In vivo

Although 3 of the animals had slight tearing of the dura mater during surgery, postoperative complications such as swelling and bleeding were at low levels and no signs of infection or abnormal appearances could be detected. All animals remained healthy throughout the entire experimental period. Wound healing was uneventful.

Micro CT analysis

The results of the micro CT analysis are summarized in Table 1. In the 3D reconstructed view, BCP particles were well-contained by the CCM in the CCM-BCP group, whereas in the BCP group, scattering of the BCP particles was frequently seen (Figure 3). Cross-sectional view of each group at both time points are represented in Figure 4.

At 2 weeks, the CCM-BCP group showed the highest total augmented volume (TAV) (222.32 ± 80.41 mm^2), but there was no significant difference compared to controls (P=0.369). The CCM-BCP group (34.37 ± 11.04 mm^3) also demonstrated the highest new bone volume (NBV) when compared to the control (6.7 ± 6.46 mm^3; P=0.000) and the

CCM group (13.80 ± 9.79 mm^3, *P*=0.004). NBV was significantly greater *(P*=0.006*)* in the BCP group (26.91 ± 5.71 mm^3) than in the control group (6.7 ± 6.46 mm^3).

At 8 weeks, CCM-BCP and BCP groups had significantly higher TAV than the control group. However, in terms of the NBV, there was no significant difference between the four groups. The remaining material volume (RMV) showed a slight decrease from 2 weeks (CCM-BCP group, 17.77 ± 3.41 mm^3; BCP group, 15.06 ± 3.29 mm^3) to 8 weeks (CCM-BCP group, 17.38 ± 6.42 mm^3; BCP group, 13.63 ± 5.06 mm^3).

Descriptive histology

CCM-BCP group

At 2 weeks, the defect spaces were well-augmented and BCP particles well-contained below the CCM membrane (Figure 5A). The CCM maintained an unfragmented barrier. Callus-like woven bone was found in abundance, and started to proliferate from the periphery of the defects. At 8 weeks, although all defects were not completely repaired, woven bone was replaced by dense lamellar bone. Lamellar bone had a tendency to form clusters around the BCP particles (Figure 6A). Evidence of intramembranous neo-ossification was seen in most of the specimens. In one of the specimens in particular, the porous layer of CCM was completely replaced by a sheet of mature lamellar bone, and covered the cranial part of the defect (Figure 6A).

CCM group

At 2 weeks, membranes maintained the original thickness and shape (Figure 5B). The dense film layer could be distinguished from the porous sponge layer (Figure7). Newly formed bone was found extending from the native bone.

At 8 weeks, in the majority of the specimens, almost complete closure of defect was seen (Figure 6B). In general, the porous layer was mostly resorbed, whereas the dense layer remained. In two of the specimens, formation of blood vessels could be seen within the dense collagen film layer (Figure 7).

BCP group

At 2 weeks, direct apposition of new bone onto the porous structure of the BCP particles could be observed (Figure 5C). At 8 weeks, new bone formation seemed to occur in small clusters around BCP particles as focal points, rather than forming a continuous layer from the periphery (Figure 6C). Less particles occupied the defect compared to the CCM-BCP group.

Control

At 2 weeks, minimal new bone formation was observed (Figure 5D). In general, the defect spaces were filled with underlying brain tissues. At 8 weeks, new bone formation originating from the periphery reached further into the center of the defect. In part lamellar bone formation was observed (Figure 6D).

Histomorphometrical analysis

The results of the micro histomorphometrical analysis are summarized in Table 2. At 2 weeks, the CCM-BCP group had the highest total augmented area (TAA) (15.78±4.63 mm^2), when compared to the control (8.13 ± 1.81 mm^2) and the CCM group (7.74 ± 2.25 mm^2) (*P*=0.009, 0.002 respectively). CCM group also showed significantly higher TAA compared to the control group (*P*=0.009).

At 8 weeks, CCM-BCP group (17.75±5.97 mm^2) had a significantly higher TAA compared to the CCM (9.23 ± 2.91 mm^2) and control groups (3.7±0.76 mm^2) (*P*=0.004, 0.004), but not to the BCP group (13.93 ± 2.29 mm^2) (*P*=0.792).

Measurements for the area of newly formed bone (NBA) revealed significantly higher values for CCM-BCP (2.15±0.99 mm^2) and CCM (2.43±0.29 mm^2) compared to the control group (1.12±1.92mm^2) (*P*=0.004, 0.03 respectively) at 2 weeks.

The height of the membranes (RMH) in the CCM-BCP and CCM groups decreased between 2 weeks (0.28 ± 0.55 mm) and 8 weeks (0.18 ± 0.12 mm).

IV. DISCUSSION

This study revealed that the combined use of a membrane and bone substitute particles (CCM-BCP) resulted in the highest total augmented area based on histomorphometry. In vitro, CCM demonstrated a significantly higher resistance to degradation compared to a native collagen membrane. In vivo data, indicated, however, that the membrane underwent degradation process predominantly of the spongeous layer and the dense layer remained up to 8 weeks.

The CCM-BCP group in this study showed the greatest augmented area, although the difference between the CCM-BCP and BCP groups did not reach a statistical significance. Nonetheless, micro CT images showed that there was less scattering of the bone substitute particles in the CCM-BCP group compared to BCP group. These results indicate that bone substitute is needed to gain graft volume, and CCM may help to stabilize the graft by preventing scattering of the particles. It is well known that hydrated collagen membrane lacks structural rigidity, therefore, bone substitute material is required to support the membrane [16]. In a systematic review, the combined approach of a particulated bone substitute and collagen membrane was the most widely used method for guided bone regeneration in localized bone defects [17]. A study in the rat calvarial defect model utilized different combinations of native collagen membrane, deproteinized bovine bone mineral (DBBM), and enamel matrix derivative [18] for GBR. The results after 4 months revealed that the presence of barrier membrane increased the

rate of complete defect closure, however, the regenerated bone had a concave appearance due to membrane collapse. The use of DBBM resulted in increased thickness of calvaria but DBBM particles were consistently encapsulated by loose fibrous tissue even when membrane was used. Likewise, in the current study, the use of BCP did not result in significant increase in NBA/NBV after 8 weeks, which confirms that its role is confined to support and provision of space under membrane. Furthermore, the use of a membrane only (CCM group) was not able to maintain space, and the TAV was significantly less compared to the bone grafted groups (BCP, CCM-BCP groups). When interpreting this result, it should be noted that the membrane was placed only over the top of the defect, which cannot prevent the underlying brain tissue from invading the defect space.

Sustained barrier function for up to 6 months [3,4,12,19] and space maintenance [18] are reported to be key elements for successful GBR. This study revealed that CCM was more resistant to degradation than a native collagen membrane *in vitro*. *In vivo*, the dense film layer of CCM remained intact up to 8 weeks, whereas the porous sponge layer was substituted by newly formed bone or dense collagenous tissue. Similar attempts have been made previously to improve the bio-durability of collagen membranes. A study in the rabbit calvaria also applied a porcine-derived native collagen membrane (NCM) in a double layer for onlay graft [10]. They reported that a single layer disappeared completely after 4 months, whereas a double layer sustained barrier function after 6 months and produced better bone mineralization density. In

another study in rat calvarial defect model, NCM was also applied in double layers [11]. They found remaining collagen area was greater in the double layer than the single layer. The NCM itself is a bi-layered membrane consisting of a compact layer and a porous layer, which are designed to serve the same purposes as the CCM in this study. Considering that a single layer of NCM underwent 30% reduction in thickness over a 5 week period (between 4 and 9 weeks), CCM might be more susceptible to degradation *in vivo* (67% reduction in thickness between 2 and 8 weeks). Nevertheless, only the porous sponge layer of CCM appeared to undergo biodegradation, whereas the dense film layer remained intact. The slow degradation of the dense layer appears to be accompanied by limited tissue integration, and is observed by the split separating the membrane from adjacent connective tissue. This separation could have been enhanced as a result of histologic processing and weak attachment of the connective tissue to the surface. The dense film layer may be speculated to retain its cell-occlusivity, as it appears to maintain the continuous outline histologically.

Although the bone filler in this study was able to maintain the graft volume after 8 weeks, there has not been a significant increase in new bone. This could be explained by the slow rate of graft degradation and large amount of residual particles still occupying the defect after 8 weeks. β-TCP is known to undergo fast resorption and replacement by new bone, whereas HA is more resilient and provides osteoconductivity. The HA: β-TCP ratio of 60:40 was utilized in this study, and the rate of degradation was comparable to the reports of other studies [15,20,21]. Materials with slow degradation rate can be more suitable

for challenging defect sites that takes longer time to regenerate, for example, onlay grafts and vertical augmentation. However, in self-containing defects such as the present study, a greater β-TCP content may be preferred.

Biocompatibility of the crosslinked collagen membrane has been a cause for concern in the recent literature. Certain crosslinking agents such as glutaraldehyde are known to evoke tissue reaction as a byproduct of enzymatic degradation [22,23]. A previous clinical trial demonstrated more frequent incidence of membrane exposure with cross-linked collagen membranes [24], and transmembraneous angiogenesis, which is supposedly beneficial for osteogenesis, was prevented until 24-weeks in an enzymatically cross-linked membrane [25]. In addition, an increase in the degree of cross-linking was inversely related to tissue integration in a study in rats [26]. The collagen membrane in the present study was chemically cross-linked using EDC, which has been shown to be safe to use *in vitro* [27,28] and in animal studies [13]. In contrast to other chemical agents such as glutaraldehyde or polypeptide, EDC does not remain as a part of the cross-linkage after degradation but simply change to water-soluble urea derivatives that have very low cytotoxicity [28]. *In vitro* analysis of the present study showed high cellular compatibility, and no complications in terms of membrane exposure and abnormal tissue reaction were observed *in vivo*. Transmembraneous angiogenesis, which has been reported to be hindered in cross-linked collagen membranes, showed to take place at 8 weeks in the dense film layer of the CCM. Furthermore, it is noteworthy that in the 8-week specimens, intra-membrane neo-ossification could be observed in the porous layer of the CCM. In

one of the specimens, the porous layer was nearly completely replaced by a sheet of new bone, which suggests that the current membrane has excellent tissue integration and biocompatibility. These results propose that the EDC-crosslinked collagen membrane has the potential for enhanced performance with a better structural design than the current one. An interesting suggestion in a study using NCM [11] was that the main pattern of resorption was internal and not external, which can be related to the transmembraneous angiogenesis of NCM [25]. A second layer of cross-linked collagen membrane on top of NCM may compensate for the disadvantage of each membrane, while enhancing the longevity of barrier function.

There are some limitations to this study. Firstly, there was no *in vivo* comparison between CCM and NCM, although the two materials were compared *in vitro*. Secondly, the sample size was too small to reach rigid statistical power. The sample size of the current study was based on previous experiments carried out on the same experimental model. However, sample size calculations based on the results from this study revealed that ideally, 30 animals should be used to reach a statistical value. Thirdly, in the CCM group, the membrane was applied over the top of the defect, but the brain tissue below often bulged into the defect space, especially if the dura mater was torn during preparation of defect. Therefore, ideally, the membrane should be placed on both sides of the defect to properly assess the space maintaining ability.

V. CONCLUSION

In conclusion, CCM has better resistance to enzymatic degradation than natural collagen membrane, *in vitro*. *In vivo*, the presence of bone substitute particles enhances the regeneration of a localized bone defect by maintenance of defect space. The dense non-porous layer of the CCM was maintained after 8 weeks, whereas the porous layer was replaced by new bone or dense collagenous tissue.

REFERENCE

1. Brugger OE, Bornstein MM, Kuchler U, Janner SF, Chappuis V, Buser D. Implant therapy in a surgical specialty clinic: an analysis of patients, indications, surgical procedures, risk factors, and early failures. Int J Oral Maxillofac Implants 2015;30(1):151-60.
2. Hammerle CH, Karring T. Guided bone regeneration at oral implant sites. Periodontol 2000 1998;17:151-75.
3. Buser D, Dula K, Hess D, Hirt HP, Belser UC. Localized ridge augmentation with autografts and barrier membranes. Periodontol 2000 1999;19:151-63.
4. Schenk RK, Buser D, Hardwick WR, Dahlin C. Healing pattern of bone regeneration in membrane-protected defects: a histologic study in the canine mandible. Int J Oral Maxillofac Implants 1994;9(1):13-29.
5. Zitzmann NU, Scharer P, Marinello CP. Long-term results of implants treated with guided bone regeneration: a 5-year prospective study. Int J Oral Maxillofac Implants 2001;16(3):355-66.
6. Zitzmann NU, Naef R, Scharer P. Resorbable versus nonresorbable membranes in combination with Bio-Oss for guided bone regeneration. Int J Oral Maxillofac Implants 1997;12(6):844-52.
7. Jung RE, Fenner N, Hammerle CH, Zitzmann NU. Long-term outcome of implants placed with guided bone regeneration (GBR) using resorbable and non-

resorbable membranes after 12-14 years. Clin Oral Implants Res 2013;24(10):1065-73.

8. Strietzel FP, Khongkhunthian P, Khattiya R, Patchanee P, Reichart PA. Healing pattern of bone defects covered by different membrane types--a histologic study in the porcine mandible. J Biomed Mater Res B Appl Biomater 2006;78(1):35-46.
9. Tal H, Kozlovsky A, Artzi Z, Nemcovsky CE, Moses O. Long-term bio-degradation of cross-linked and non-cross-linked collagen barriers in human guided bone regeneration. Clin Oral Implants Res 2008;19(3):295-302.
10. Kim SH, Kim DY, Kim KH, Ku Y, Rhyu IC, Lee YM. The efficacy of a double-layer collagen membrane technique for overlaying block grafts in a rabbit calvarium model. Clin Oral Implants Res 2009;20(10):1124-32.
11. Kozlovsky A, Aboodi G, Moses O, Tal H, Artzi Z, Weinreb M, Nemcovsky CE. Bio-degradation of a resorbable collagen membrane (Bio-Gide) applied in a double-layer technique in rats. Clin Oral Implants Res 2009;20(10):1116-23.
12. von Arx T, Buser D. Horizontal ridge augmentation using autogenous block grafts and the guided bone regeneration technique with collagen membranes: a clinical study with 42 patients. Clin Oral Implants Res 2006;17(4):359-66.
13. Park JY, Jung IH, Kim YK, Lim HC, Lee JS, Jung UW, Choi SH. Guided bone regeneration using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-cross-linked type-I collagen membrane with biphasic calcium phosphate at rabbit calvarial defects. Biomater Res 2015;19:15.

14. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 2010;8(6):e1000412.

15. Yang C, Unursaikhan O, Lee JS, Jung UW, Kim CS, Choi SH. Osteoconductivity and biodegradation of synthetic bone substitutes with different tricalcium phosphate contents in rabbits. J Biomed Mater Res B Appl Biomater 2014;102(1):80-8.

16. Schliephake H, Tavassol F, Gelinsky M, Dard M, Sewing A, Pompe W. Use of a mineralized collagen membrane to enhance repair of calvarial defects in rats. Clin Oral Implants Res 2004;15(1):112-8.

17. Sanz-Sanchez I, Ortiz-Vigon A, Sanz-Martin I, Figuero E, Sanz M. Effectiveness of Lateral Bone Augmentation on the Alveolar Crest Dimension: A Systematic Review and Meta-analysis. J Dent Res 2015;94(9 Suppl):128S-42S.

18. Donos N, Lang NP, Karoussis IK, Bosshardt D, Tonetti M, Kostopoulos L. Effect of GBR in combination with deproteinized bovine bone mineral and/or enamel matrix proteins on the healing of critical-size defects. Clin Oral Implants Res 2004;15(1):101-11.

19. Hutmacher DW, Kirsch A, Ackermann KL, Hurzeler MB. A tissue engineered cell-occlusive device for hard tissue regeneration--a preliminary report. Int J Periodontics Restorative Dent 2001;21(1):49-59.

20. Jensen SS, Bornstein MM, Dard M, Bosshardt DD, Buser D. Comparative study

of biphasic calcium phosphates with different HA/TCP ratios in mandibular bone defects. A long-term histomorphometric study in minipigs. J Biomed Mater Res B Appl Biomater 2009;90(1):171-81.

21. Lim HC, Zhang ML, Lee JS, Jung UW, Choi SH. Effect of different hydroxyapatite:beta-tricalcium phosphate ratios on the osteoconductivity of biphasic calcium phosphate in the rabbit sinus model. Int J Oral Maxillofac Implants 2015;30(1):65-72.

22. Speer DP, Chvapil M, Eskelson CD, Ulreich J. Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. J Biomed Mater Res 1980;14(6):753-64.

23. Rothamel D, Schwarz F, Sculean A, Herten M, Scherbaum W, Becker J. Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. Clin Oral Implants Res 2004;15(4):443-9.

24. Tal H, Kozlovsky A, Artzi Z, Nemcovsky CE, Moses O. Cross-linked and non-cross-linked collagen barrier membranes disintegrate following surgical exposure to the oral environment: a histological study in the cat. Clin Oral Implants Res 2008;19(8):760-6.

25. Schwarz F, Rothamel D, Herten M, Sager M, Becker J. Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat. Clin Oral Implants Res 2006;17(4):403-9.

26. Thoma DS, Villar CC, Cochran DL, Hammerle CH, Jung RE. Tissue integration of collagen-based matrices: an experimental study in mice. Clin Oral Implants Res 2012;23(12):1333-9.

27. Hafemann B, Ghofrani K, Gattner HG, Stieve H, Pallua N. Cross-linking by 1-ethyl-3- (3-dimethylaminopropyl)-carbodiimide (EDC) of a collagen/elastin membrane meant to be used as a dermal substitute: effects on physical, biochemical and biological features in vitro. J Mater Sci Mater Med 2001;12(5):437-46.

28. Park SN, Park JC, Kim HO, Song MJ, Suh H. Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. Biomaterials 2002;23(4):1205-12.

TABLES

Table 1. Results from the micro CT analysis.

		TAV	NBV	RMV
2 weeks	CCM-BCP	222.32 ± 80.41	*†34.37 ± 11.04	17.77 ± 3.41
	CCM	186.55 ± 66.9	13.80 ± 9.79	
	BCP	186.33 ± 38.32	*26.91 ± 5.71	15.06 ± 3.29
	Control	151.68 ± 29.91	6.7 ± 6.46	
8 weeks	CCM-BCP	*196.43 ± 25.30	38.43 ± 7.22	17.38 ± 6.42
	CCM	166.02 ± 30.38	23.73 ± 5.4	
	BCP	*206.23 ± 39.13	34.61 ± 13.96	13.63 ± 5.06
	Control	149.72 ± 12.28	22.83 ± 10.56	

Values are presented as mean±standard deviation

* Statistically significant difference compared to the control group.

† Statistically significant difference compared to the CCM group.

TAV=total augmented volume; NBV=new bone volume; RMV=remaining material volume.

Table 2. Results from the histomorphometric analysis.

		TAA	NBA	RPA	RMH
2 weeks	CCM-BCP	*†15.78 ± 4.63	2.15 ± 0.99	3.44±0.88	0.58 ± 0.28
	CCM	*9.23 ± 2.91	2.69 ± 2.07		0.52 ± 0.21
	BCP	*13.93 ± 2.29	1.27 ± 0.58	3.52±0.5	
	Control	3.7 ± 0.76	1.12 ± 1.92		
8 weeks	CCM-BCP	*†17.75 ± 5.97	3.79 ± 2.62	3.49±0.85	¶0.14 ± 0.13
	CCM	7.74 ± 2.25	2.43 ± 0.29		¶0.17 ± 0.13
	BCP	*15.09 ± 2.44	3.92 ± 1.25	3.44±0.68	
	Control	8.13 ± 1.81	2.76 ± 1.28		

Values are presented as mean±standard deviation.

* Statistically significant difference compared to the control group.

† Statistically significant difference compared to the CCM group.

¶ Statistically significant difference compared to the corresponding groups at 2 weeks.

TAV=total augmented volume; NBV=new bone volume; RMV=remaining material volume.

FIGURES

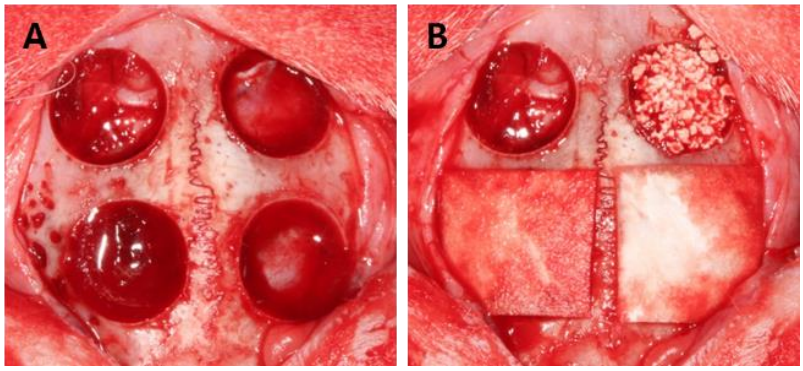

Figure 1. Experimental design in the rabbit calvarium.

(A) Four circular defects 8 mm in diameter were prepared using trephine bur. (B) Each defect was assigned to an experimental group. Clockwise from top left; negative control, BCP, CCM-BCP and CCM.

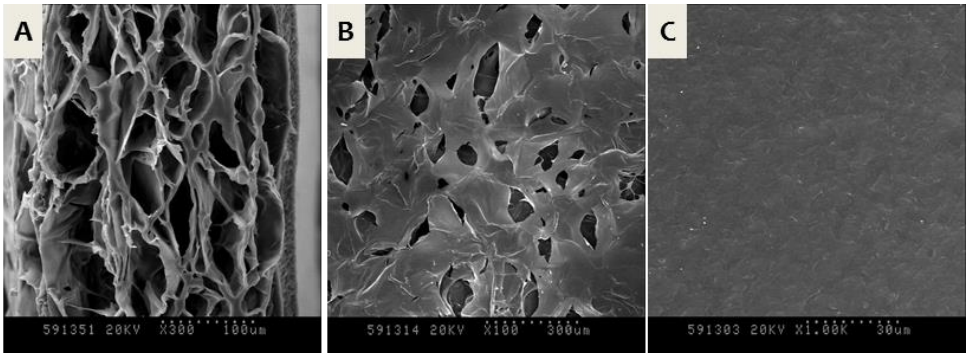

Figure 2. SEM images of the bilayer EDC-cross-linked type-I collagen membrane (CCM).

(A) Cross-sectional view of the CCM showing the porous sponge layer (left) and the dense film layer (right). (Dotted line=100 μm) (B) The surface of the porous layer. (Dotted line=300 μm). (C) The smooth surface of dense film layer showing no pores (Dotted line=30 μm).

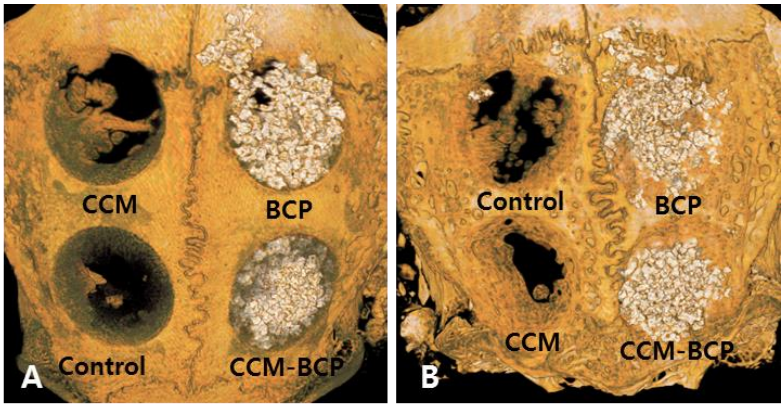

Figure 3. 3-dimensional reconstructed radiographic views of the defects after 2 weeks (A), and 8 weeks of healing (B).

Scattering of the BCP particles can be observed in the BCP group, whereas BCP is contained within the defect in the CCM-BCP group.

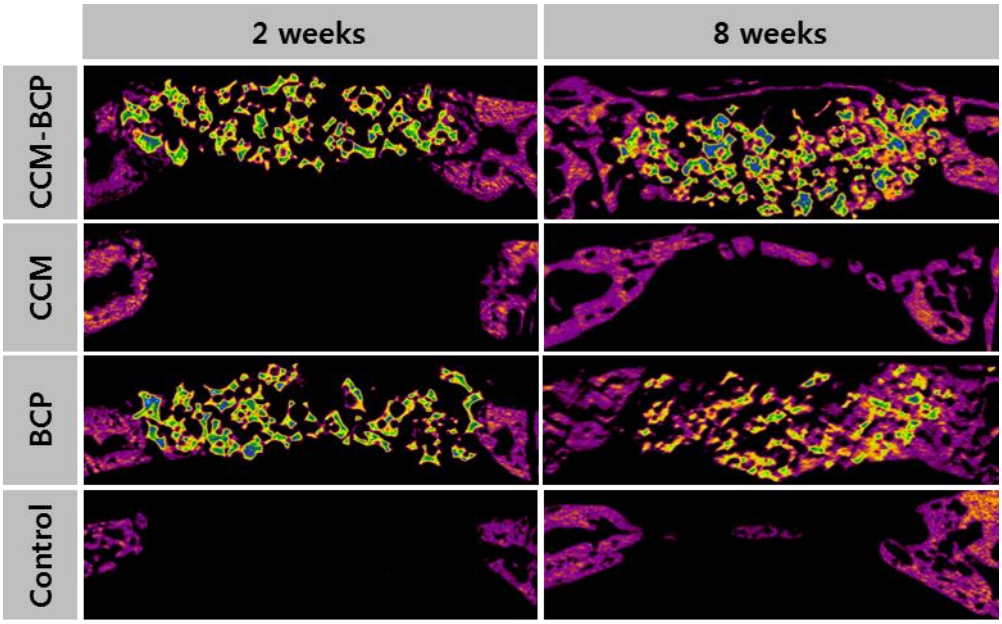

Figure 4. Color coded cross-sectional radiographic view of the defects at 2 and 8 weeks.

Bone is shown in purple, bone substitute particles are shown in yellow-blue. New bone formation occurred mainly at the defect periphery in the control group. The original volume of the cranial bone before defect preparation is maintained with CCM-BCP and BCP groups.

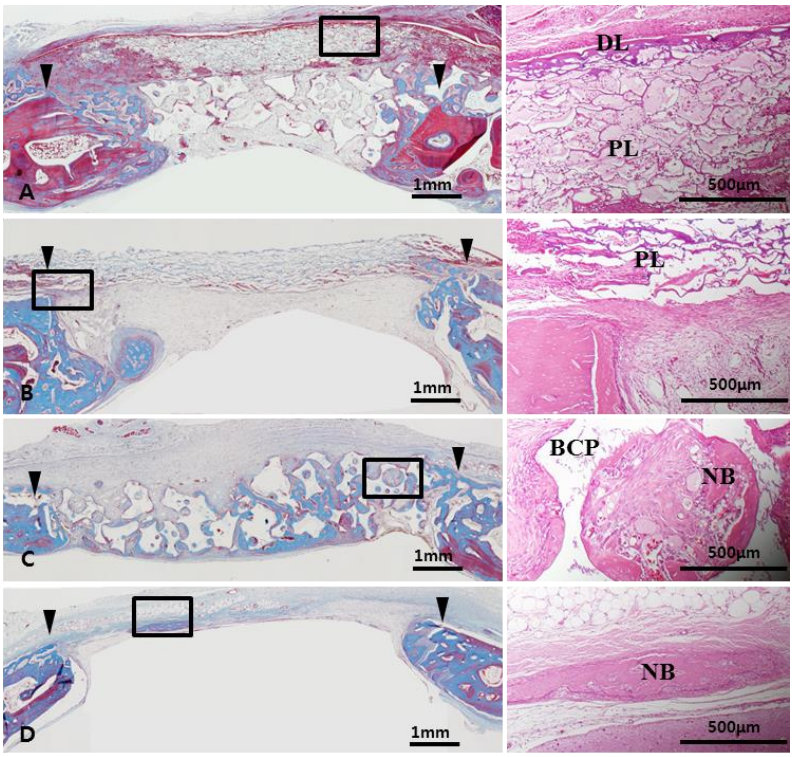

Figure 5. Histological images obtained at 2 weeks.

(A-D) Low magnification views of the groups CCM-BCP, CCM, BCP, and control, respectively (Masson Trichrom, bar=1 mm). (E-H) High magnified views of each highlighted area (H&E, bar=500 μm). (E) CCM appears to maintain the original shape. (F) The porous layer is integrated and adapted to the defect margin without any adverse reactions. (G) The pores of the BCP are beginning to fill with woven bone structures. (H) Woven bone formation in island produced by the overlying periosteum. The defect margins are labeled with arrowheads. NB=new bone, BCP=biphasic calcium phosphate, PL=porous layer, DL=dense layer.

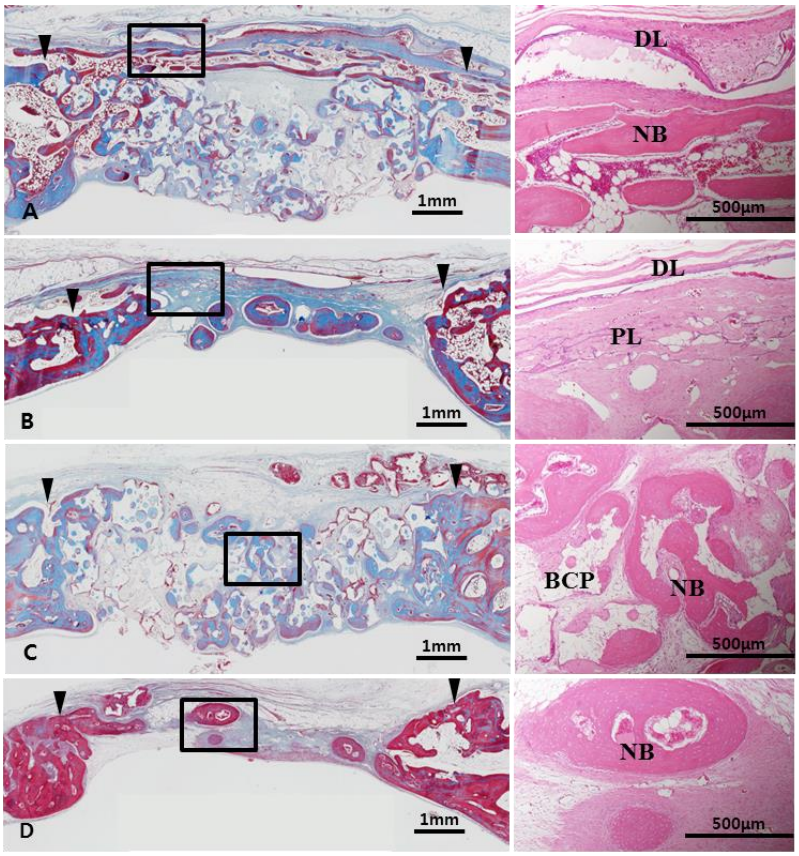

Figure 6. Histological images obtained at 8 weeks.

(A-D) Low magnification views of the groups CCM-BCP, CCM, BCP, and control, respectively (Masson Trichrome, bar=1 mm). (E-H) High magnified views of each highlighted area (H&E, bar=500 μm). (E) The dense layer is remaining after 8 weeks, whereas the porous layer has been replaced by a sheet of new bone. (F) Porous layer shows integration with connective tissue matrix that can be expected to turn into bone

tissue after a longer healing period. The dense layer remains unresorbed and provides protection over the top. (G) BCP particles at the top middle aspect of the defect are surrounded by newly formed bone, which displays good osteoconductivity of the bone substitute material. (H) Bony islands in the control group without much defect closure. The defect margins are labeled with arrowheads. NB=new bone, BCP=biphasic calcium phosphate, PL=porous layer, DL=dense layer.

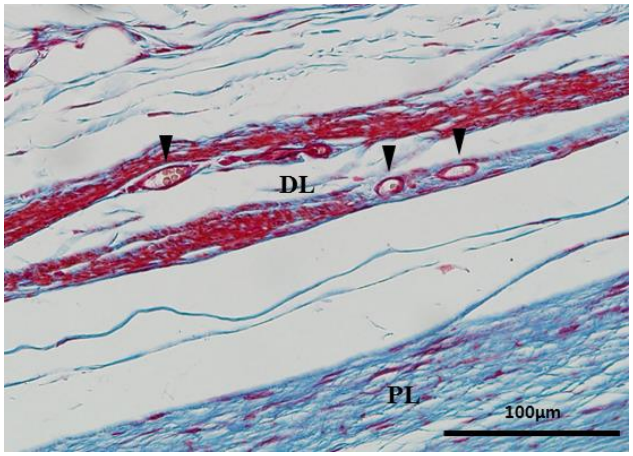

Figure 7. High magnification view of the CCM after 8 weeks, showing the dense layer (DL) and the porous layer (PL).

Penetration of blood vessels (arrows) can be seen within the membrane. (Masson Trichrome, bar=100 μm)

국문요약

국소적 골 재생 술식을 위한 가교화된 이중층 콜라겐 차폐막의 분해 성질: *In vitro* and *in vivo* study

<지도교수 **최 성 호**>
연세대학교 대학원 치의학과
박 진 영

골 재생 술식에 사용되는 이상적인 차폐막은 생체적합성, 세포 차단성, 공간 유지성, 조직 친화성과 같은 다양한 성질을 필요로한다. 따라서 비흡수성과 흡수성 차폐막에 해당되는 다양한 소재의 차폐막들이 과거에 소개되었왔다. 이중 흡수성 콜라겐 차폐막은 생체적합성이 우수하고 제거를 위한 이차 수술이 필요하지 않기 때문에 현재 가장 널리 사용되고 있다. 하지만 콜라겐은 체내에서 빠르고 예측하기 힘든 속도로 분해되어 골 결손부의 완전한 치유가 일어나기 전에 차폐막의 기능이 소멸된다. 차폐막의 기능을 더 오래동안 유지하기 위한 방법으로는 콜라겐의 분해를 지연시키기 위한 콜라겐의 가교화나 막의 이중층 적용을 들 수 있다. 본 연구의 목적은 가교화된 이중층 콜라겐 차폐막을 이용하여 (1) 생체외에서 생체적합성과 흡수속도를 평가하고, (2) 생체내에서 국소적 골 재생 술식을 시행하였을 때 효능을 평가하는 것이다.

생체외에서 효소 분해 실험을 통해 가교화된 콜라겐 차폐막(CCM)과 자연적 콜라겐 차폐막의 분해 속도를 비교하였다. 생체내 실험에는 12마리의 토끼(New Zealand White; 남성)가 사용되었다. 각각의 토끼 두개골에 4개의 직경

8 mm 골 결손부를 형성하여 4개의 군을 적용하였다; (1) CCM과 합성골이식재(BCP)군 (CCM-BCP), (2) CCM만 적용한 군 (CCM), (3) BCP만 적용한 군 (BCP), (4) 비어있는 대조군. 동물들은 2주와 8주에 여섯마리씩 희생되었다. 결과의 계측을 위해 micro CT 분석(총 증강된 부피 (TAV), 신생골 부피 (NBV)) 그리고 조직학적 계측(총 증강된 면적 (TAA), 신생골 면적 (NBA), 잔존 차폐막의 두께 (RMT))을 시행하였다.

생체외에서 CCM은 자연적 콜라겐 차폐막보다 분해에 대해 더 큰 저항력을 가지고 있었다. Micro CT 분석을 통해 개개의 실험 군에 대한 TAV를 계측했을 때, 8주 째에 CCM-BCP군 (196.43±25.30 mm3)과 BCP군(206.23±39.13 mm3)이 대조군(149.72 ± 12.28 mm3)보다 통계적으로 유의차있게 큰 결과를 보였다. 조직학적인 분석을 통해 TAA를 계측했을 때, 8주 째에 CCM-BCP군이 CCM군과 대조군보다 유의차있게 큰 결과를 보였다. 8주 후에 RMT는 67% 가량 감소되었다.

가교화된 이중층 콜라겐 차폐막을 이용한 국소적 골 재생 술식에서 골 이식재의 사용은 공간 유지성을 확보하기 위해 필수적이다. 이중층 차폐막 상부의 고밀도 층은 8주 후에도 유지가 되는 반면 하부의 다공성 층은 흡수되어 신생골이나 dense한 결합조직으로 대체되었다.

핵심되는 말: 골 재생, 생체 흡수, 콜라겐, 합성골