Early healing processes in guided bone regeneration using cross-linked type-I collagen membrane at rabbit calvarial defect

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감사의 글

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그리고, 언제나 변함없는 사랑으로 옆에서 큰 도움이 되어준 평생의 반려자, 남편과 사랑하는 아들 지호와 곧 세상에 나오게 될 둘째 아들에게도 고마움과 사랑하는 마음을 전합니다.

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ABSTRACT

Early healing processes in guided bone regeneration using cross-linked type-I collagen membrane at rabbit calvarial defect

The aim of this study was to evaluate early healing processes in guided bone regeneration using a cross-linked type-I collagen membrane of 1-ethyl-3-(3- dimethyl aminopropyl) carbodiimide (EDC) at rabbit calvarial defects. Eight male New Zealand rabbits were used and four circular calvarial defects were created. Each of the four defects was filled with different graft materials: 1) control group, 2) membrane group 3) bone augmentation group, and 4) bone augmentation and membrane group. The animals were sacrificed following two and four weeks of healing periods. Between two healing periods, collagen membrane was resorbed 28.5% and maintained its original shape and marginal integrity. The collagen membrane group resulted in significantly better defect closure compared to control group ($p<0.05$). The augmented area was significantly higher in bone graft material applied groups ($p<0.05$). There was no statistical difference in new bone formation between all groups at all healing periods, but vascularization was seemed to be promoted and more new bone formation was observed in superficial layer in collagen membrane applied groups.

Key Words : Bone regeneration, Collagen, Cross linking, Membrane

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I. Introduction

There are cases when bone quantity is insufficient in implant surgery and prosthetic treatment due to alveolar bone loss. The guided bone regeneration is widely used for bone generation. Unlike bone augmentation, guided bone regeneration cover top of bone defect with barrier membrane. Guided bone regeneration is known to have a better effect at bone generation than bone augmentation does.[1)] Barrier membrane used in guided bone regeneration maintains space and prevents blood clot falling off from bone defect. Barrier membrane helps bone formation as connective tissue which proliferate rapidly than bone tissue is stopped from ingrowing.[2)] Therefore, effective

barrier membrane is important to successful guided bone regeneration.[3)]

Barrier membrane can be categorized into resorbable and non-resorbable. When this method was first introduced, non-resorbable barrier membrane was mainly used. Non-resorbable barrier membrane stops ingrowth of cell and has an advantage in maintaining space. It also showed successful result clinically.[4)] However, when exposure of barrier membrane is high and barrier membrane is exposed, there are disadvantages as early removal of barrier membrane is unavoidable due to inflammatory reaction and secondary surgery for barrier membrane removal is a must.[5)]

Recently resorbable barrier membrane is developed and used in order to complement the limits of non-resorbable barrier membrane.[6-8)] As resorbable barrier membrane does not require additional surgery for removal of barrier membrane, it has benefits of simplifying implant surgery, saving cost and reducing patient's inconvenience.[9)] Collagen, a material frequently used for resorbable barrier membrane, is highly biocompatible. It merely causes immune response and has characteristics of accelerating wound healing by inducing emigration of fibroblast.[10)] Unlike other resorbable barrier membrane, collagen does not cause inflammatory reaction on neighboring tissues[11)] and it is known to help bone formation by accelerating vascularization inside bone defect.[12)] In long- term follow up study that compared amount of bone generation in resorbable vs. non-resorbable collagen barrier membrane, the results in both cases were successful and there was no significant difference.[13)] The resorbable collagen barrier membrane complemented

weakness of non-resorbable barrier membrane and successfully showed clinical result.

However, intensity of resorbable collagen barrier membrane is low. Resorbable collagen barrier membrane has a weak point that it is quickly absorbed by bacteria that exist in periodontal tissue and macrophage and polymorphonuclear leucocyte. This character can cause lack of space creation and maintenance for bone formation. Limited bone generation in big defect can happen as well.[14] So, in order to slow down resorption rate of collagen barrier membrane, various methods (such as ultraviolet radiation, glutaraldehyde and diphenylphosphoryl-azide) that cross-linked collagen are actively researched and developed.[15] However, in most of cross-linking, there is a weak point that biocompatibility is low due to cytotoxicity of product generated in cross-linking.[16] The method of using 1-ethyl-3-(3- dimethyl aminopropyl) carbodiimide (EDC), one of method that cross-linked collagen barrier membrane, effectively cross-linked collagen, improves ability of structure maintenance and show low cytotoxicity.[17]

In this study, we used EDC and transplanted cross-linked collagen membrane to rabbit calvarial defects, then histomorphometric observed bone regeneration effect and process.

II. Materials and Methods

1. Materials

1) Animals

In this study, eight New Zealand white male rabbits (9~20 months old, 3.0~3.5Kg) were used. Animal selection, management, surgical protocol, and preparation followed
routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea (certification #10-0385).

2) Materials

The transplant material used for bone defect is biphasic calcium phosphate (Osteon®, Genoss. Co. Ltd; Suwon, Korea). The barrier membrane used is Rapiderm tape® (Dalim Tissen Co. Ltd; Seoul, Korea). Bone graft material (Osteon®) is biphasic hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). Its ratio of HA to TCP is 7:3 and particle size is 0.5~1.0mm. Collagen membrane (Rapiderm tape®) is a resorbable membrane that were cross-linked type-1 collagen by 1-ethyl-3-(dimethyl aminopropul) carbodiimide (EDC). (Figure 1)

2. Methods

1) Experimental Groups

Four circular calvarial defects were created to the animal and the following categories were made.

(1) Control Group: Bone defect is filled with blood clot only

(2) Membrane Group: Bone defect is filled with blood clot after covering with Rapidem tape®

(3) Bone Augmentation Group: Bone defect is transplanted by Osteon®

(4) Bone Augmentation and Membrane Group: Bone defect is transplanted by Osteon® and covering with Rapidem tape®

2) Surgical procedure

The animals were put under general anesthesia with Ketamine hydrochloride (Ketalar®, Yuhan Co., Seoul, Korea), xylazine(Rumpun®, Bayer Korea Ltd., Seoul, Korea). The surgical area was disinfected with povidone iodine and local anesthesia with 2% lidocaine(LidocaineHCl, Huons, Seoul, Korea) was administered. The frontal bone was incised from front to back. The skull was exposed by elevation of flap including periosteum. Four circular calvarial defects (each 8mm diameter) were

created with caution that dura mater is not damaged by using 8mm trephine bur on the exposed skull area. The experimental materials were applied to the bone defects for each experimental group mentioned above. The scalp was sutured with 4-0 Monosyn® (Glyconate absorbable monofilam-ent, B-Braun, Aesculap,Inc., PA, USA). Periosteum and subcutis were sutured with absorptive suture. For one week after the surgery, intramuscular injection was executed with antibiotic Gentamicin (5mg/kg). One week after the surgery, animals was removed suture.

3. Evaluation

1) Clinical Observation

Two and four weeks after the surgery, unusal of surgical area, inflammation, exposure of grafting material, abnormality were observed with naked eyes.

2) Histologic Observation

Two and four weeks after the surgery, tissue was acquired after the rabbits were sacrificed by intravenous injections of Phenobarbital (100mg/kg). The tissue was then fixed with 10% formalin solution and embedded with paraffin. The specimen was cut in 7μm thickness, dyed with hematoxylin-eosin (H-E), and then observed with optical microscope in a magnifying power of 40 and 200

3) Histomorphometric Observation

The followings were observed and calculated with 3D image analysis program ((Image-Pro Plus, Media cybernetics, Silver Spring, Maryland, USA) (Figure 2)

(1) Defect closure (%): Distance ratio of inter-defect margin and inter-newbone margin

(2) Residual particle (mm^2): Area of bone graft material remaining in bone defect

(3) Total augmented area (mm^2): Total area sum of new-bone, residual particle, connective tissue, adipose tissue and blood vessel in bone defect

(4) New bone (mm^2): area of new bone in bone defect

(5) Remnant membrane (mm^2): Area of barrier membrane remaining in bone defect

4) Statistical Analysis

Measurement values were processed by SAS(Statistical Analysis Software) and significance difference in each group were evaluated using two-way analysis of variance(ANOVA)

III. Result

1. Clinical Observation

During healing process, no significant inflammation, abnormality and complication were found in all the animals

2. Histologic Observation

1) Control Group

In the observation made 2 weeks after the surgery, wedge-shaped new bone was created at the edge of bone defect. Most of new bone was immature bone and most of bone defect was filled with loose connective tissue.

In the observation made 4 weeks after the surgery, more new bone was found and it had more mature bone structure compared to the 2-week observation. The new bone formation was created mostly at the edge of bone defect. In some tissues, island-shaped new bone formation was found at the center of bone defect. (Figure 3)

2) Membrane Group

In the observation made 2 weeks after the surgery, barrier membrane was well maintained though it was partially subsided down at the center of bone defect.

Around barrier membrane, infiltration of blood vessel and erythrocyte was increased. New bone formation was partially observed along bone defect margin and barrier membrane. The boundary between new bone and original bone was clear. The new bone was immature bone similar to the 2-week observation result of the control group.

In the observation made 4 weeks after the surgery, barrier membrane was absorbed and the thickness was more reduced than the 2-week observation. In overall, it maintained relatively uniform thickness and shape. Infiltration of erythrocyte was more reduced than the 2-week observation and it had mature bone structure. (Figure 4)

3) Bone Augmentation Group

In the observation made 2 weeks after the surgery, new bone formation was partially found near bone defect marginal area and bone graft material. The new bone formed at bone defect marginal area was wedge-shaped. The new bone formed near bone graft materials was adjacent to the graft materials. The new bone seems to infiltrate into bone graft material. along with the absorption of graft material.

In the observation made 4 weeks after the surgery, amount of new bone increased and absorption of bone graft material occurred more than the 2-week observation result. In some tissue specimen, loose connective tissue was infiltrated as surface of bone defect was subsided. new bone was not formed around bone graft materials. (Figure 5)

4) Bone Augmentation and Membrane Group

In the observation made 2 weeks after the surgery, outward shape and location of barrier membrane was well maintained. Infiltration of blood vessel and erthrocyte around barrier membrane was increased. New bone formation was similar to the 2-week observation result of the control group.

In the observation made 4 weeks after the surgery, more infiltration was found compared to the 2-week observation. Relatively uniform thickness and shape were maintained and infiltration of erythrocyte was reduced. New bone formation was similar to the 4-week observation result of bone augmentation group. However, unlike bone augmentation group, new bone formation near bone graft material was found similar to other areas in bone defect surface. (Figure 6)

3. Histomorphometric Observation

The 2-week and 4-week observation results (such as defect closure, residual particle, remnant membrane analysis) are written in Table 1. (Table 1) Defect closure was measured in the control group and the membrane group. In both group, more bone coverage was found in the 4-week analysis than the 2-week ($p<0.05$). In the 4-week observation, defect closure was higher in the membrane group than the control group ($p<0.05$). There was no difference in residual particle area in all experimental groups. Residual particle area was similar in the 2-week and 4-week observation. Area of remnant membrane in the 2-week observation was statistically less than the 4-

week. There was 32% decrease in the membrane group, while 25% decrease in the bone augmentation and membrane group. There was statistically no meaningful difference between the two experimental groups. Data and analysis of total augmented area and new bone are written in Table 2. (Table 2) There was more increase of total augmented area in the groups using bone graft material (Bone Augmentation Group, Bone Augmentation and Membrane Group) than the groups without bone graft materials (Control Group, Membrane Group). So, there was a meaningful difference statistically ($p<0.05$). In membrane group and bone augmentation and membrane group, the 2-week observation result showed more formation of new bone than the 4-week and it was statistically meaningful ($p<0.05$). In the membrane group, the largest number of new bone formation was found. (The second largest is the control group). The bone augmentation group showed the least number of new bone formation, but there was no meaningful difference between all the experimental groups.

IV. Discussion

In this study, we used 8mm-defect in rabbit's cranium to evaluate the guided bone regeneration of cross-linked collagen membrane. Rabbit's cranium is composed of appropriate amount of marrow, so it is widely used in study evaluating bone regeneration with new materials.[18-21] Critical size of bone defect differs per study, but it is usually 10~15mm diameter.[19-21] The 8mm diameter defect used in this study is smaller than the critical size for study evaluating bone regeneration ability,[22] but it is appropriate size for evaluating guided bone regeneration.[23] Therefore, the 8mm defect model is useful in comparing and evaluating reaction of bone generation and early healing process caused by bone graft materials.[22] In this study, the 8mm defect model is used for evaluating bone generation in the 2-week and 4-week observation during healing process.

The purpose of this study is to evaluate bone generation ability of newly developed cross-linked collagen membrane. We used the EDC to cross-link collagen membrane. The EDC's role is to connect carboxyl group and primary amine. When EDC and carboxyl cause reactions, O-acylisourea is formed and it reacts with primary amine, resulting in cross-linking. However, intermediate of O-acylisourea is very unstable, causing low efficiency in cross-linking. In order to improve efficiency of cross-linking, N-hydroxysulfosuccinimide (Sulfo-NHS) is added to transform unstable O-acylisourea to stable NHS-ester. EDC which is line of water soluble carbodiimide has a merit of low cytotoxicity unlike other cross-linking methods. The EDC does not

become a part of linkage unlike widely-used cross-link methods such as glutaraldehyde and polyepoxides. The EDC becomes water soluble urea derivative with low cytotoxicity, so it is easily removed by washing.[17, 24-27)]

In this study, absorption rate of barrier membrane was 28.5% after 4-week healing process. In overall, absorption was uniform and outward shape was maintained. Role of barrier membrane was effectively performed as new bone was formed from upper side of bone defect area. According to Ofer Moses's study,[28)] using rat's calvarial defect, collagen membrane that is not cross-linked is absorbed 69% after 2 weeks. Compared to that result, EDC cross-linked collagen membrane has relatively slow absorption speed and can function as barrier membrane for a long time.

In histological observation after 2-week healing, increase in infiltration of blood vessel and red blood cell around barrier membrane can be found when barrier membrane was applied. In 2008 Schwarz and etc, [12)] said that collagen membrane accelerates vascularization, thereby creating favorable environment for bone regeneration. It is in accord with histological observation of this study.

The collagen membrane used in this study accelerates vascularization at initial healing process. Its cross-linking maintains structural stability for a longer time than existing collagen membrane. So, it is advantageous in bone regeneration. According to this study, new bone formation was weak and bone defect was mostly filled with fibrotic tissue and adipose tissue in the control group. On the other hand, new bone was formed from upper part of bone defect in the membrane group. The membrane prevented neighboring soft tissue from infiltration into defect, thereby accelerating

new bone formation. In order to precisely compare/evaluate bone regeneration effect of EDC cross-linked collagen membrane, an additional experiment may be required to compare with existing non-cross-linked collagen membrane.

One major weak point [29] of resorbable membrane is decrease of ability of maintaining space as mechanical intensity is reduced along with absorption process. Method of supporting barrier membrane with bone graft material under membrane is recommended, as intensity of resorbable membrane is low compared to non-resorbable membrane.[18] When bone graft material and resorbable membrane are used together for guided bone regeneration, it is known to have similar effect of bone generation compared to the case of using non-resorbable membrane.[1, 30, 31] In this study, total augmented area in the membrane group showed statistically no meaningful difference compared to the group in which bone augmentation and membrane were both used. Using only barrier membrane does not guarantee maintaining space, so bone augmentation should be used along with membrane when guide bone regeneration is executed with resorbable membrane. Total augmented area and new bone formation was statistically not different in the membrane group and the bone augmentation and membrane group. However, according to histological observation, when only bone graft material is used infiltration of soft tissue and scratch of defect surface were found. Also bone generation around bone graft material was impeded, unlike the observation result of bone augmentation and membrane group. Therefore, when barrier membrane is not used, hindrance of bone generation

occurs by soft tissue infiltration on defect surface. More bone generation can be expected when barrier membrane is used

V. Conclusion

In this study, we used rabbit's calvarial defect to evaluate bone generation effect of newly-developed collagen membrane. The cross-linked collagen membrane used in this study has slower absorption rate than existing non-cross-linked membrane. It effectively helps bone generation on the inside and surface of defect area as it functions as a barrier membrane for a long time. This is thought to be due to 1) accelerated vascularization under collagen membrane and 2) prevention infiltration of soft tissue by collgen membrane. However, it should be used with bone graft material in order to achieve effective bone generation, as it lacks ability of maintaining space.

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TABLES

Table 1. Defect closure, residual particle and residual membrane at 2 and 4 weeks.

	Parameters	Control	Membrane	Bone graft Material	Bone graft material with membrane
2 weeks (n = 4)	Defect closure(%)	27.8±8,83	32.88±7.07		
	Residual particle(mm^2)			10.28±1.94	9.9±1.06
	Remnant Membrane(mm^2)		11.44±1.05		11.00±1.04
4 weeks (n = 4)	Defect closure(%)	44.18±1.89¥	73.32±7.75*¥		
	Residual particle(mm^2)			6.97±2.41	8.24±1.51
	Remnant Membrane(mm^2)		7.76±1.17¥		7.51±2.31¥

*: Significant statistically difference from control group

¥: Significant statistically difference from the same experimental group at 2 weeks

Table 2. Augmented area and new bone at 2 and 4 weeks.

	Parameters(mm^2)	Control	Membrane	Bone graft material	Bone graft material with membrane
2 weeks (n = 4)	Augmented area	8.27±1.8	11.3±5.16	24.93±5.2*¶	22.92±5.44*¶
	New bone	1.86±0.84	2.38±0.55	1.39±0.59	1.57±0.70
4 weeks (n = 4)	Augmented area	13.38±2.80	17.87±4.39	31.52±5.29*¶	28.26±4.11*¶
	New bone	5.76±2.93	5.79±1.48§	3.24±1.16	4.21±1.15§

*: Significant statiscally difference from control group

¶: Significant statiscally difference from membrane group

§: Significant statiscally difference from the same experimental group ant 2 weeks

FIGURE LEGENDS

Figure 1. Scanning electron microscopic images of Rapiderm tape®

Figure 2. Schematic diagram of histometric analysis.

Figure 3. Transversal histologic section of control group at 2 weeks (A, B) and 4 weeks (C, D). Arrow head: defect margin, NB: new bone, OC: osteocyte, OB: osteoblast (H&E stain; original magnification: X40 [A, C], X200 [B, D]

Figure 4. Transversal histologic section of membrane group at 2 weeks (A, B) and 4 weeks (C, D). Arrow head: defect margin, NB: new bone, CM: collagen membrane, BV: blood vessel (H&E stain; original magnification: X40 [A, C], X200 [B, D]

Figure 5. Transversal histologic section of bone graft material group at 2 weeks (A, B) and 4 weeks (C, D). Arrow head: defect margin, NB: new bone, RM: residual material, LC: loose connective tissue (H&E stain; original magnification: X40 [A, C], X200 [B, D]

Figure 6. Transversal histologic section of membrane with bone graft material group at 2 weeks (A, B) and 4 weeks (C, D). Arrow head: defect margin, NB: new bone, CM: collagen membrane, RM: residual material (H&E stain; original magnification: X40 [A, C], X200 [B, D]

FIGURES

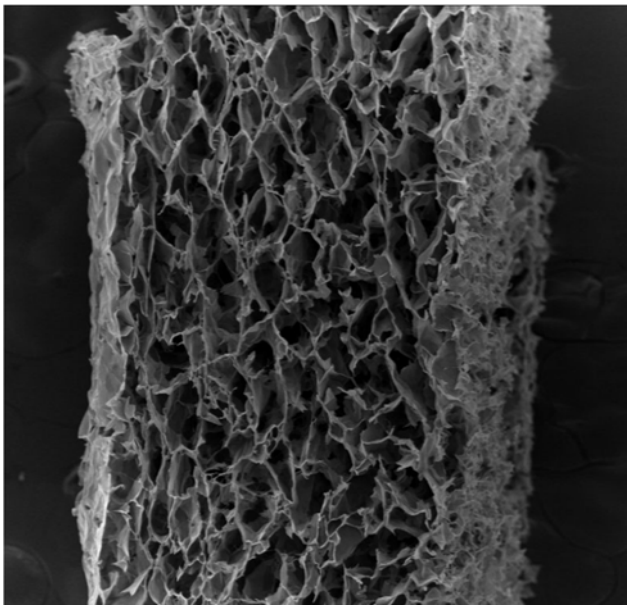

Figure 1.

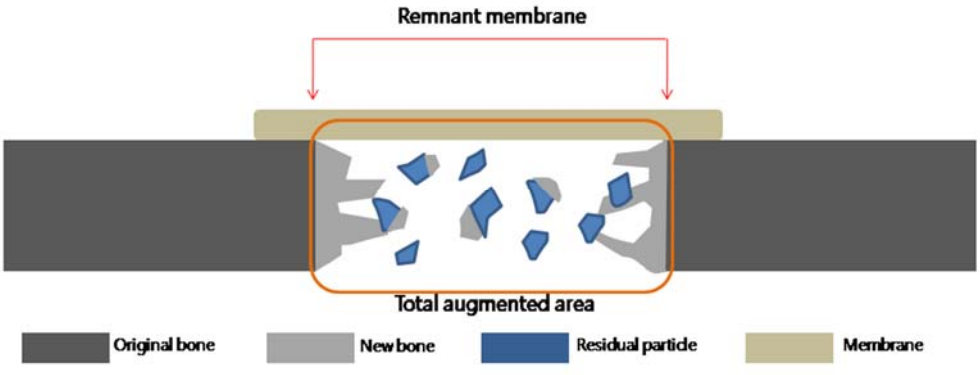

Figure 2.

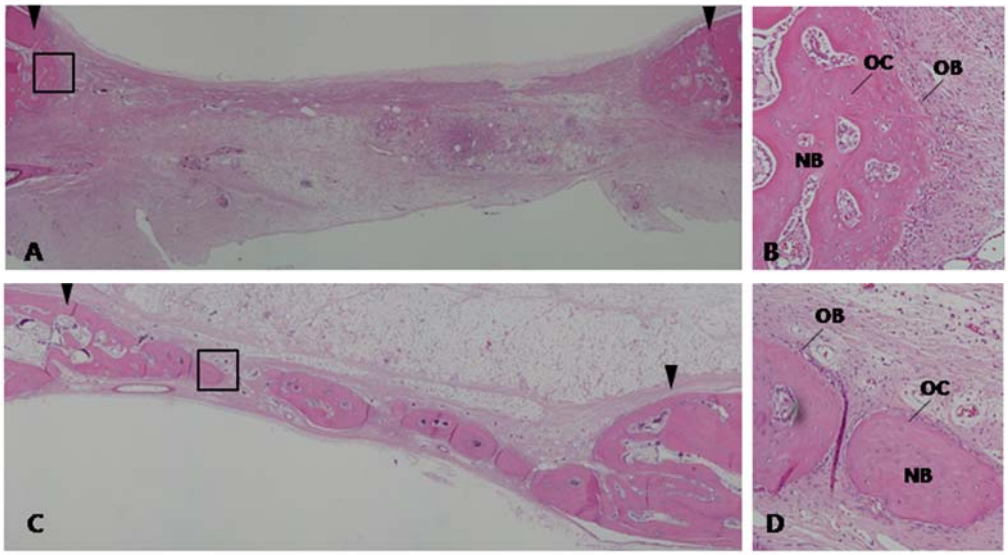

Figure 3.

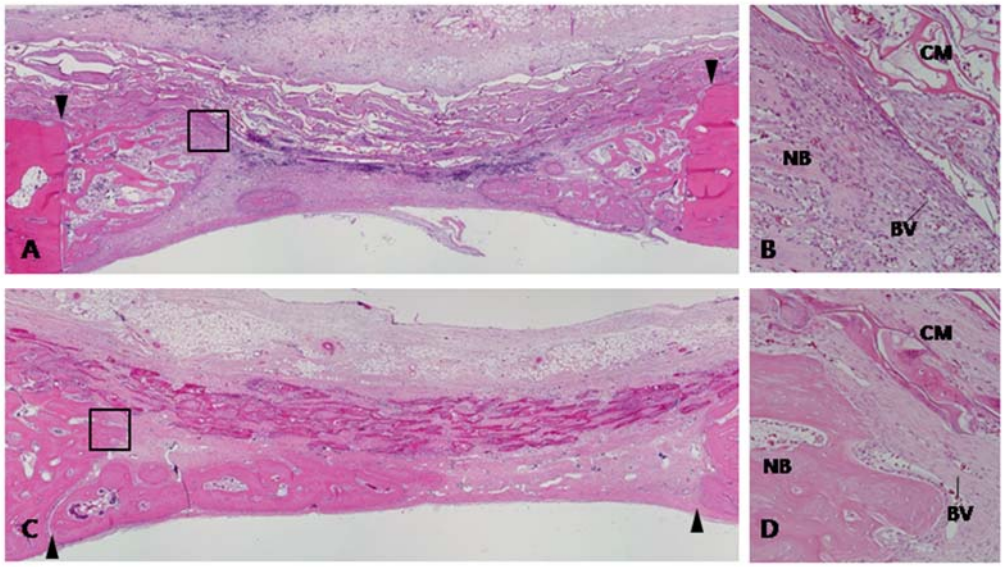

Figure 4.

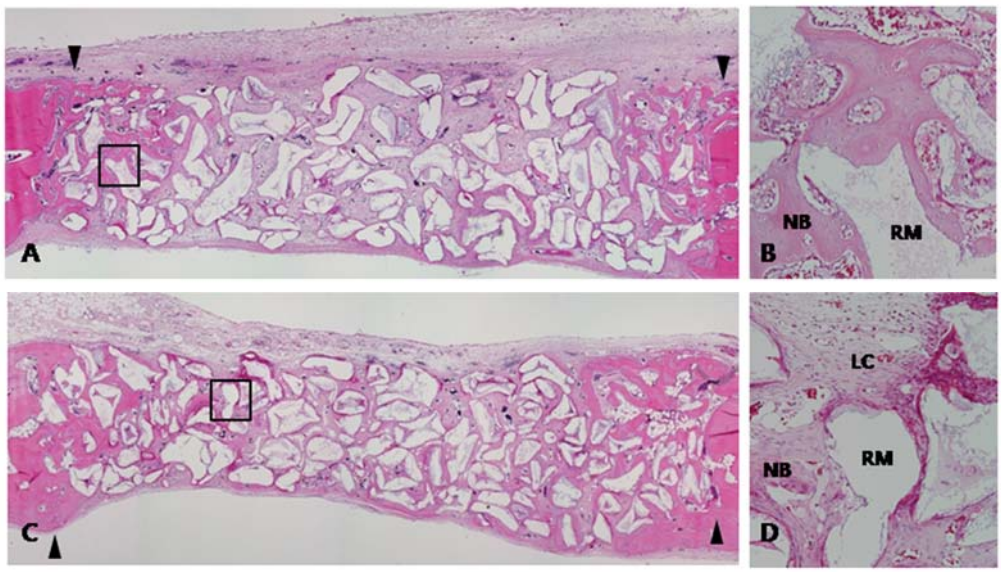

Figure 5.

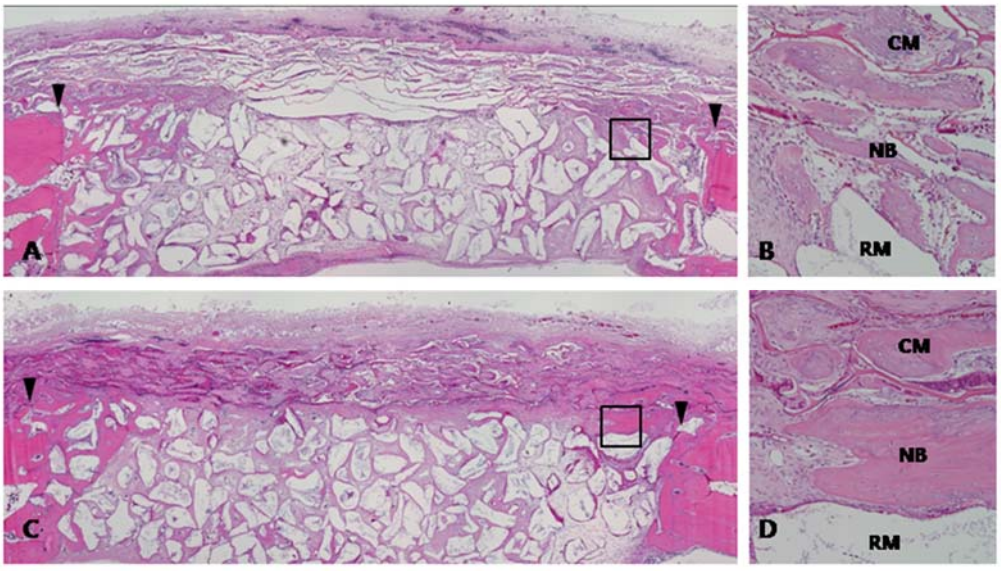

Figure 6.

국문요약

토끼 두개골 결손부에서 가교화된 제 1형 콜라겐 차폐막의 초기 골형성 효과

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정 은 주

본 연구의 목적은 토끼 두개골 결손부에서 1-ethyl-3-(3- dimethyl aminopropyl) carbodiimide (EDC)를 이용하여 가교화된 콜라겐 차폐막을 골유도재생술과 함께 시행할 때 초기 치유 과정을 평가하는 것이다. 8마리 토끼(New Zealand white male rabbit)가 사용되었고 두개골에 원형의 골결손부를 4개 형성하였다. 4개의 결손부는 각각 다른 골이식재로 채워졌다.

1) 대조군 (Control Group)
2) 차폐막군 (Membrane group)
3) 골이식재군 (Bone Augmentation Group)
4) 골이식재, 차폐막군 (Bone Augmentation and Membrane Group)

실험 동물은 2주, 4주의 치유과정을 거친 후 희생시켰고, 각각의 치유 기간 동안

차폐막의 흡수 정도는 28.5%였다. 또한 본래의 외형과 변연부 모양도 잘 유지되었다. 콜라겐 차폐막을 적용한 군이 대조군보다 통계적으로 유의하게 결손부 피개율이 높았다 ($p<0.05$). 조직 증대 면적은 골이식재를 적용한 군에서 골이식재를 사용하지 않은 군들보다 통계적으로 유의하게 높은 결과를 보였다 ($p<0.05$). 신생골 형성은 실험군간, 실험기간간 통계적으로 유의할 만한 차이는 없었다. 하지만 콜라겐 차폐막을 적용한 군에서 차폐막 표면에 신생골 형성이 증가되었고 혈관화도 더 촉진된 것으로 보인다.

핵심되는 말 : 골재생, 콜라겐, 가교화, 차폐막