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가토 두개골에서 PLGA 나노섬유 차폐막의 골재생 효과

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임플란트 시술 시 상실된 치조골 재생을 위하여 흡수성 차폐막을 이용한 골유도재생술 및 조직유도재생술이 사용된다. 흡수성 차폐막의 재료에는 주로 콜라겐 및 합성 고분자가 사용되는데 이 중 poly (lactic-co-glycolic acid) (PLGA)는 미국 FDA의 승인을 받은 공중합체로써 우수한 생분해성 및 생체 적합성을 가지고 있다. 이러한 특징으로 인하여 PLGA는 여러 의료기기 제조분야에 적용되고 있다.

전기방사법을 이용하여 제작된 나노섬유는 높은 산소투과성과 우수한 생체분해성 및 다공성을 가지고 있어 조골세포의 부착과 증식을 증진시킨다는 연구가 보고되었다. 전기방사 나노섬유에 관한 연구는 세포 수준에서 활발히 이루어지고 있으나 치주조직 및 치조골 재생을 위한 차폐막으로써의 연구는 많이 이루어지지 않은 상태이다. 따라서 본 연구에서는 전기방사를 통해 제조된 소수성 및 친수성 poly (lactic-co-glycolic acid) (PLGA) (lactide:glycolide, 85:15) 나노섬유 차폐막의 골재생능을 토끼 두개골 결손부 모델을 이용하여 평가하고자 한다.

PLGA를 혼합용매인 DMF/THF (80/20 col.%)에 18 wt.%가 되도록 용해한 뒤 고전압 발생장치 (AU-100R6, Matsusada Precision, Shiga, Japan)를 이용하여 PLGA 나노섬유를 제조하였다. 또한 친수화 개질 첨가제인 F127 (Pluronic® F127, Sigma-Aldrich, Missouri, USA)을 첨가하여 친수성 PLGA 나노섬유를 제조하였다. 제조된 PLGA 나노섬유 및 친수성 PLGA 나노섬유를 3겹으로 열압착

하여 차폐막으로 제작하였다. 총 32마리의 토끼 두개골에 4개의 직경 8 ㎜ 골결손부를 형성하였다. 4개의 골결손부는 음성대조군 (no membrane), 양성대조군 (collagen membrane), 실험군 Ⅰ or Ⅲ (bone graft material with PLGA nanofiber membrane or hydrophilic PLGA nanofiber membrane), 실험군 Ⅱ or Ⅳ (PLGA nanofiber membrane or hydrophilic PLGA nanofiber membrane)으로 그룹을 나눈 뒤 각각의 재료를 적용시켰다. 술 후 2주, 6주 뒤 희생하였으며 골결손 모델의 두개골 조직 시편을 이용하여 미세단층촬영을 통해 방사선학적 평가 및 분석을 시행하였다. 또한 조직학적 평가를 위해 Hematoxylin and Eosin (H&E) 염색법과 Masson's trichrome (MT) 염색법을 수행하였다.

방사선학적 평가 및 분석 결과 2주, 6주 후 골이식재와 함께 차폐막을 사용한 실험군에서 가장 많은 양의 신생골이 형성됨을 관찰하였다. 특히 친수성 PLGA 나노섬유 차폐막 실험군에서 6주 후 양성대조군인 콜라겐 차폐막과 동일한 수준의 신생골이 형성됨을 확인하였다. 조직학적 평가 결과 PLGA 나노섬유 차폐막 및 친수성 PLGA 나노섬유 차폐막을 사용한 실험군에서 차폐막 하방에 염증반응이 없음을 확인하였다. 또한 신생골의 형성이 골결손부 경계면에 국한되어 있는 음성대조군과 달리 PLGA 나노섬유 차폐막 및 친수성 PLGA 나노섬유 차폐막은 경계면부터 연속된 신생골 형성이 관찰되며 이는 콜라겐 차폐막 실험군과 비슷한 양상을 보였다.

이러한 연구결과를 바탕으로 전기방사법을 이용하여 제작된 PLGA 나노섬유 차폐막 및 친수성 PLGA 나노섬유 차폐막은 현재 시판되어 사용되고 있는 콜라겐 차폐막과 유사한 수준의 신생골 형성능을 가진 것으로 확인되었다. 따라서 PLGA를 이용한 전기방사 나노섬유는 향후 의료용 차폐막으로써 활용 가치가 높을 것으로 사료된다.

Ⅰ. Introduction

Guided bone regeneration (GBR) is being used with various bone grafting materials and membranes to regenerate the bone structure lost due to periodontitis in implant surgery. GBR is a technique that uses the membrane to maintain the space required for bone regeneration and inhibits the early penetration of epithelial cells and connective tissue cells to induce the proliferation of bone cells [1, 2]. In addition to GBR, the guided tissue regeneration (GTR) was introduced through study by Nyman and Karring [3, 4]. The selection of appropriate bone graft materials and membranes in GBR or GTR is important factor leading to the success of the procedure and the membrane plays a significant role because it directly prevents the penetration of soft tissues and protects regenerating bone [5-7].

The membrane is qualified for sufficient mechanical strength to maintain space, biocompatibility, cellular interception, ease of operation, cell closure and economical efficiency [8-10] and is divided into non-resorbable membrane and resorbable membrane. In the case of non-resorbable membrane, many studies have proven effective [11, 12], but secondary surgery for the removal of membrane is essential and has a weakness of high exposure frequency of membrane, leading to the possibility of inflammation and infection [13]. On the other hand, secondary surgery is unnecessary for resorbable membrane and a use of resorbable membrane has the advantage of reducing the patient's economic and psychological burden [12, 14]. Therefore, various studies have been conducted on resorbable membrane that can complement disadvantages of non-resorbable membrane [15-17].

Collagen, poly (lactic-co-glycolic acid) (PLGA), polyglycolide, etc. are used as materials mainly used for the resorbable membrane. However, collagen has rapid absorption rate that is insufficient time for periodontal tissue regeneration. Therefore, copolymers of glycolide and lactide or synthetic polymers are frequently used. PLGA of copolymers is approved by Food and Drug Administration (FDA) as drugs and biological agents in recognition of

biodegradability and biocompatibility. PLGA is hydrolyzed in the body to release lactic acid and glycolic acid, which are finally decomposed into water (H_2O) and carbon dioxide (CO_2), showing slight cytotoxicity. PLGA also has the ability to control the rate of degradation by changing the ratio of these monomers [18]. This flexibility of decomposition has been applied to medical devices such as surgical suture, bone fixation, drug preparation, tissue engineering [19-21]. The electrospinning for producing microfiber or nanofiber is being actively conducted to develop various membranes using synthetic biodegradable materials such as PLGA.

The nanofiber fabricated using the electrospinning were confirmed through various studies that nanofiber promote osteoblast adhesion and proliferation and increase alkaline phosphatase (ALP) [22, 23]. In addition, nanofiber fabricated using eletrospinning has high oxygen permeability and outstanding biodegradability and porosity [24]. The studies related with bone regeneration of electrospun nanofiber are being conducted at *in vitro* level [25-27], and studies on the regeneration of periodontal tissue and alveolar bone have not been carried out actively. Therefore, this study aims to evaluate effect of the bone regeneration of poly (lactic-co-glycolic acid) (PLGA) (85:15) nanofiber membrane manufactured by electrospinning using rabbit calvarial defects model.

Ⅱ. Materials and Methods

1. Materials and reagents

In this study, poly (lactic-co-glycolic acid) (85:15) (PLGA, Evonik Industries, Essen, Germany) was used as a material for the preparation of nanofiber membrane. Pluronic® F127 (Sigma-Aldrich, Missouri, USA) was used as surface hydrophilic modification additive. Dimethylformamide (Sigma-Aldrich, Missouri, USA) and Tetrahydrofuran (Sigma-Aldrich, Missouri, USA) were used as solvents. Bio-gide® (Geistlich Pharma AG, Wolhusen, Switzeland) was used as collagen membrane in positive control group and Osteon Ⅲ® (Dentium, Seoul, Korea) was used as bone graft material.

2. Fabrication of nanofiber membrane

PLGA was dissolved to be 18 wt.% in DMF/THF (80/20 vol.%) mixed solvent. The manufactured radiation solution was supplied to an electrospinning pack equipped with nozzle at 50 to 150 ㎕/hole per minute using a quantitative pump and was discharged after grounding the (-) electrode using a high voltage generator (AU-100R6, Matsusada Precision, Shiga, Japan). Temperature and humidity of the room which is installed quantitative pump were kept at 30℃ to 32℃ and 50% respectively and PLGA nanofiber with an average diameter of 200~400 ㎚ was obtained by electrospinnig while controlling the applied high voltage at 25 ㎸. To prepare the hydrophilic PLGA nanofiber, hydrophilic modification additive F127 was added at 0.3, 1.0, 5.0 wt.% and hydrophilic PLGA nanofiber with a thickness of 80 ㎛ were prepared in the same method. The manufactured PLGA

nanofiber and hydrophilic PLGA nanofiber were thermocompressed at 60 ℃ to 1MPa for 15 seconds to produce a membrane with a thickness of 0.25 ㎜. The fabricated membranes were cut to 10×10 ㎜ size and sterilized through Ethylene Oxide (EO) gas before surgical procedure. PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane samples and its surface analysis data were provided by AMOGreenTec co., Ltd (Kimpo, Korea).

3. Characterization of membrane

The morphology of PLGA nanofiber membrane and hydrophilic PLGA nanofiber membranes were observed using field emission scanning electron microscope (FE-SEM, S-4200, Hitachi, Japan). The water contact angle (WCA) was measured to evaluate the hydrophilicity of the manufactured nanofiber membranes. After dropping distilled water on the PLGA nanofiber membrane and the hydrophilic PLGA nanofiber membranes, the angle between water droplet and membrane was investigated using a contact angle meter (Phoenix-Smart, SEO, Suwon-si, Korea) and the average value was calculated. In order to evaluate the water absorption of the membranes, the membranes were cut to 10×30 ㎜ and then immersed in phosphate buffered solution (PBS, pH=7.4) under 37℃, 60 rpm condition. The membranes were dried for 24 hours before weight measurement and the water absorption rate was calculated using the following equation after measuring the weight of each membrane over time:

$$\text{Water absorption rate (\%)} = \frac{W_1 - W_0}{W_0} \times 100$$

Where W_1 represents the wet weight of membrane and W_0 is the initial weight of the membrane. And the surface of the water absorbed PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane were observed using FE-SEM.

4. Animals

The selection, surgical treatment and postoperative management of experimental animals were conducted with the approval of Institutional Animal Care Use Committee (IACUC) of Chosun University (approval number: CIACUC2017-A0051). A total of 32 New Zealand White male rabbits weighing 2.5~3.0 ㎏ were used in this study. The experimental animals were randomly selected and assigned 8 animals in each of the 2 weeks and 6 weeks group but 2 animals of 6 weeks group died during the experiment.

5. Experimental design

Four circular bone defects with a diameter of 8 ㎜ were formed on each of the calvaria of rabbits and each material was applied as shown in Table 1 and Figure 1.

Table 1. Experimental groups

	Experimental groups	
PLGA nanofiber membrane	Negative control	No membrane
	Positive control	Collagen membrane
	Test Ⅰ	Bone graft with PLGA nanofiber membrane
	Test Ⅱ	PLGA nanofiber membrane
	Experimental groups	
Hydrophilic PLGA Nanofiber membrane	Negative control	No membrane
	Positive control	Collagen membrane
	Test Ⅲ	Bone graft with hydrophilic PLGA nanofiber membrane
	Test Ⅳ	Hydrophilic PLGA nanofiber membrane

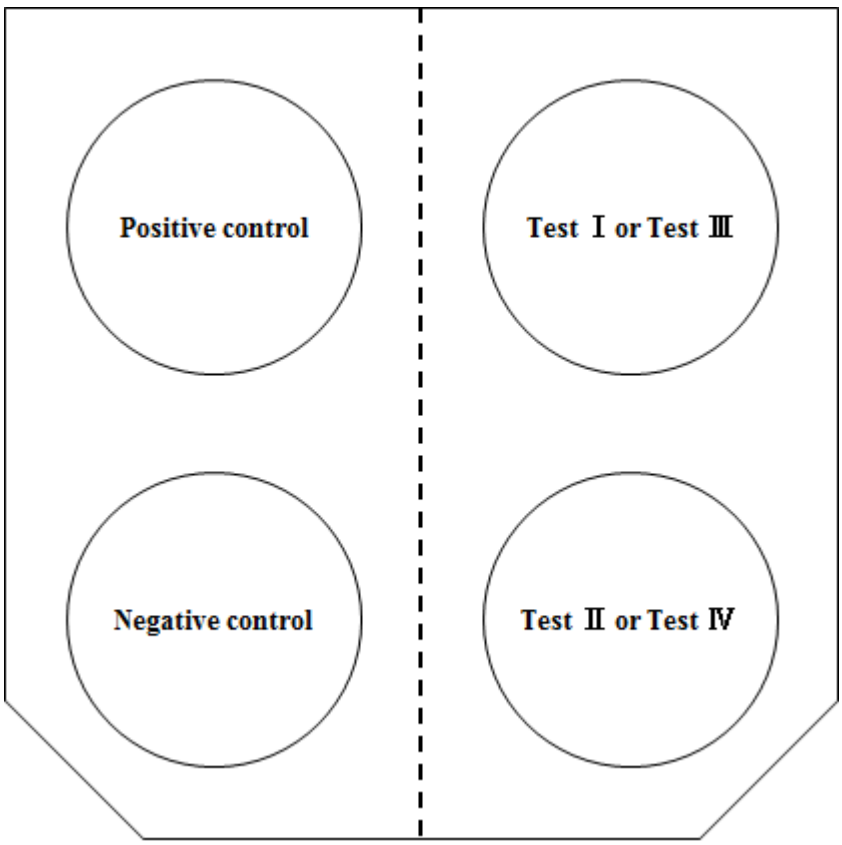

Figure 1. Schematic diagram of experimental groups.

6. Surgical procedure

A mixture of 5 ㎎/㎏ xylazine hydrochloride (Rompun®, Bayer Korea, Seoul, Korea) and 15 ㎎/㎏ ketamine hydrochloride (Ketalar®, Yuhan, Seoul, Korea) was injected into the femoral muscle to induce general anesthesia in rabbits. After anesthesia, depilation of the surgical site was performed and disinfected with povidone iodine. Infiltration anesthesia was carried out with 2% lidocaine HCl (Huons, Seoul, Korea) and the upper surface of calvaria was exposed after incision of the frontal bone. Four cicular bone defects were formed on the calvaria using a trephine bur with an external diameter of 8 ㎜. After then, four circular bone defects were divided and each experimental material was applied according to the experimental group. The periosteum was repositioned to fix the membrane with 5-0 Vicryl® (Ethicon, Somerville, USA) and the scalp was sutured with 4-0 Blue Nylon (AILEE, Busan, Korea) (Figure 2A-D). 1 ㎎/㎏ of Gentamycin (Dong-wha pharm, Seoul, Korea) was intramuscularly injected to prevent infection during 3 days after surgery and sacrificed 2 and 6 weeks after.

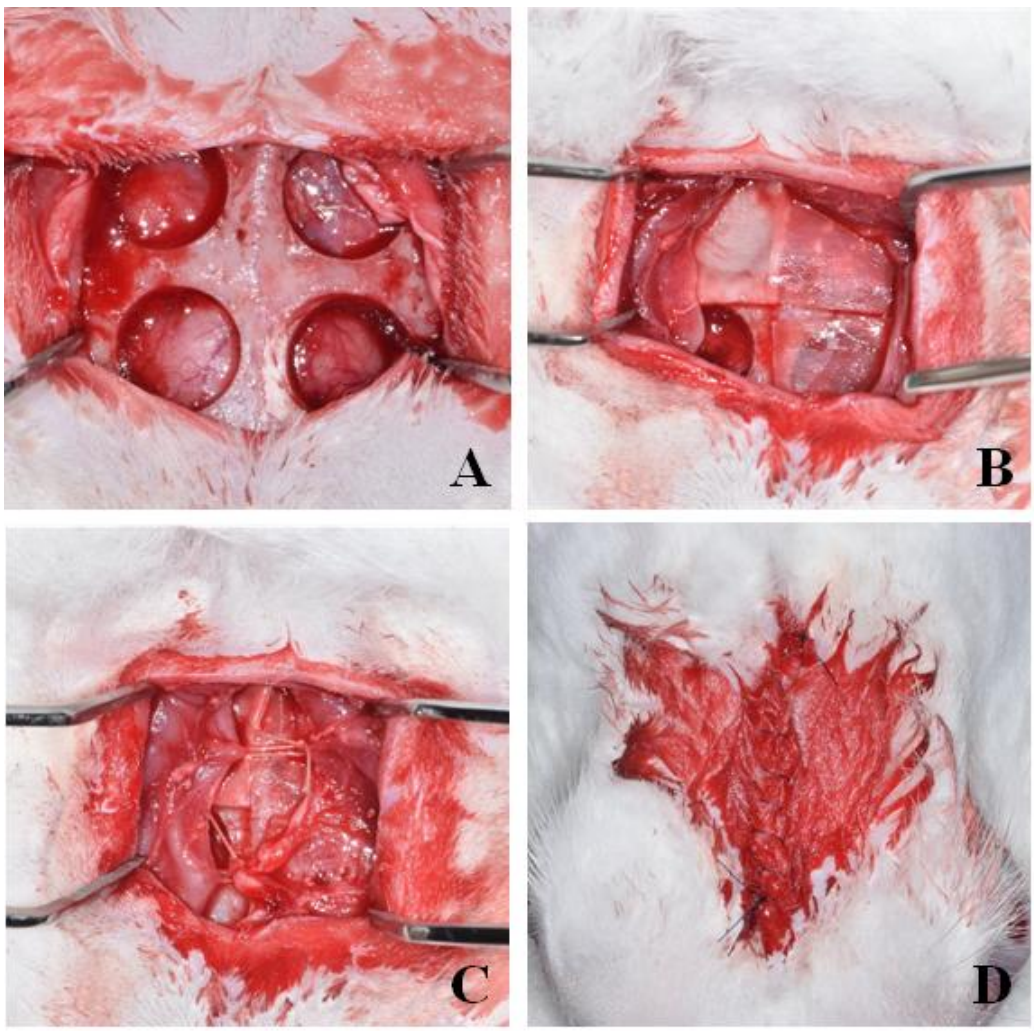

Figure 2. Surgical procedure. (A) The 8 ㎜ diameter bone defects were formed on the rabbit calvaria. (B) Each experimental material was applied to the calvarial defect. (C) The periosteum was repositioned and the membranes were fixed. (D) The scalp was sutured.

7. Radiographic evaluation

After 2 and 6 weeks, the rabbits were sacrificed and bone tissue block including bone defects was collected from the calvaria of rabbits. The bone specimen was fixed in 10% formaldehyde and analyzed by Micro-Computed Tomography (Micro-CT) scanning. Micro-CT scanning was performed using the Quantum GX μCT imaging system (PerkinElmer, Hopkinton, USA) of Korea Basic Science Institute (KBSI, Gwanju, Korea) under conditions of tube voltage 90 ㎸, tube current 88 ㎂ and voxel size 90 ㎛. The scanned image was reconstructed three-dimensionally using Analyze software 12.0 (AnalyzeDirect, Overland Park, USA) and evaluated the volume of mineralized new bone tissue within the defects in all directions and in all widths.

8. Histological evaluation

The bone specimens were fixed in 10% formaldehyde and decalcified for 14 days using 15% formic acid. Dehydration was performed using ethanol and then the samples were embedded in paraffin. The bone paraffin sections, 5 ㎛ thickness, were stained with hematoxylin and eosin (H&E) stain and Masson's trichrome (MT) stain. The sections were observed histologically using a light microscope (Leica DM750, Leica Microsystems, Wetzlar, Germany) and digital images were acquired using a digital microscope camera (Leica ICC50®, Leica Microsystems, Wetzlar, Germany).

9. Statistical analysis

The experimental values of each group were presented as mean and standard deviation (SD). The data was analyzed by SPSS 18.0 Statistical analysis system (SPSS, Chicago, USA). The Kruskal-Williams test was

carried out to evaluate the statistical significance of differences among the experimental groups and statistical significant difference between each pair of groups was confirmed through the Mann-Whitney test. The $p < 0.05$ was considered statistically significant and confidence level was verified at 95%.

Ⅲ. Results

1. Characterization evaluation

The surface morphology and water contact angle of PLGA nanofiber membrane and hydrophilic PLGA nanofiber membranes according to F127 content prepared by electrospinning were evaluated. The PLGA nanofiber membrane and PLGA containing 0.3 wt.% of F127 nanofiber membrane were showed low hydrophilicity. When the content of F127 was 5.0 wt.%, the water contact angle was almost 0° that was indicated complete hydrophilization. However, a large amount of bead was formed and crumbled property of nanofiber was confirmed through FE-SEM image in PLGA nanofiber membrane with 5.0 wt.% of F127 (Figure 3).

As a result of evaluating water absorption, PLGA nanofiber membrane and PLGA nanofiber membrane containing 0.3 wt.% of F127 was showed insignificant water absorption rate until 8 weeks. The PLGA nanofiber membrane containing 1.0 wt.% or 5.0 wt.% of F127 exhibited the significant water absorption after 4 weeks. In addition, when the content of F127 was 5.0 wt.%, the observation of surface of membrane at 4 weeks was confirmed that the nanofiber was constricted by being hydrolyzed (Figure 4).

Based on these results, in this study, it was decided to use the hydrophilic PLGA nanofiber membrane containing 1.0 wt.% of F127 with appropriate hyderophilicity and water absorption rate.

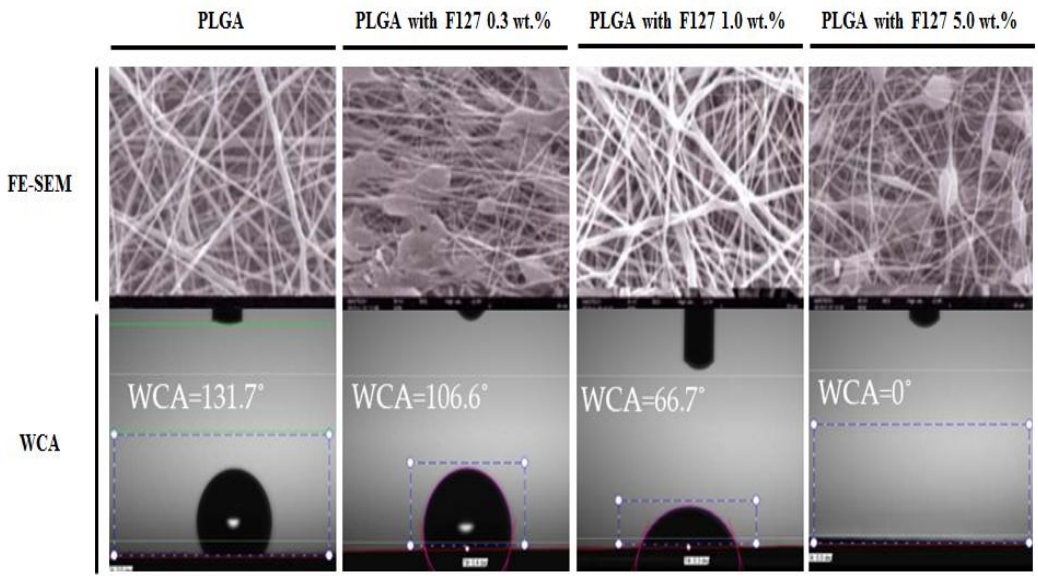

Figure 3. Field emission scanning electron microscope (FE-SEM) and water contact angle (WCA) evaluation of PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane according to F127 content. The surface morphology of PLGA and hydrophilic PLGA nanofiber membranes were observed using FE-SEM (×2000). And The hydrophilicity of each membrane was determined by water contact angle measurement.

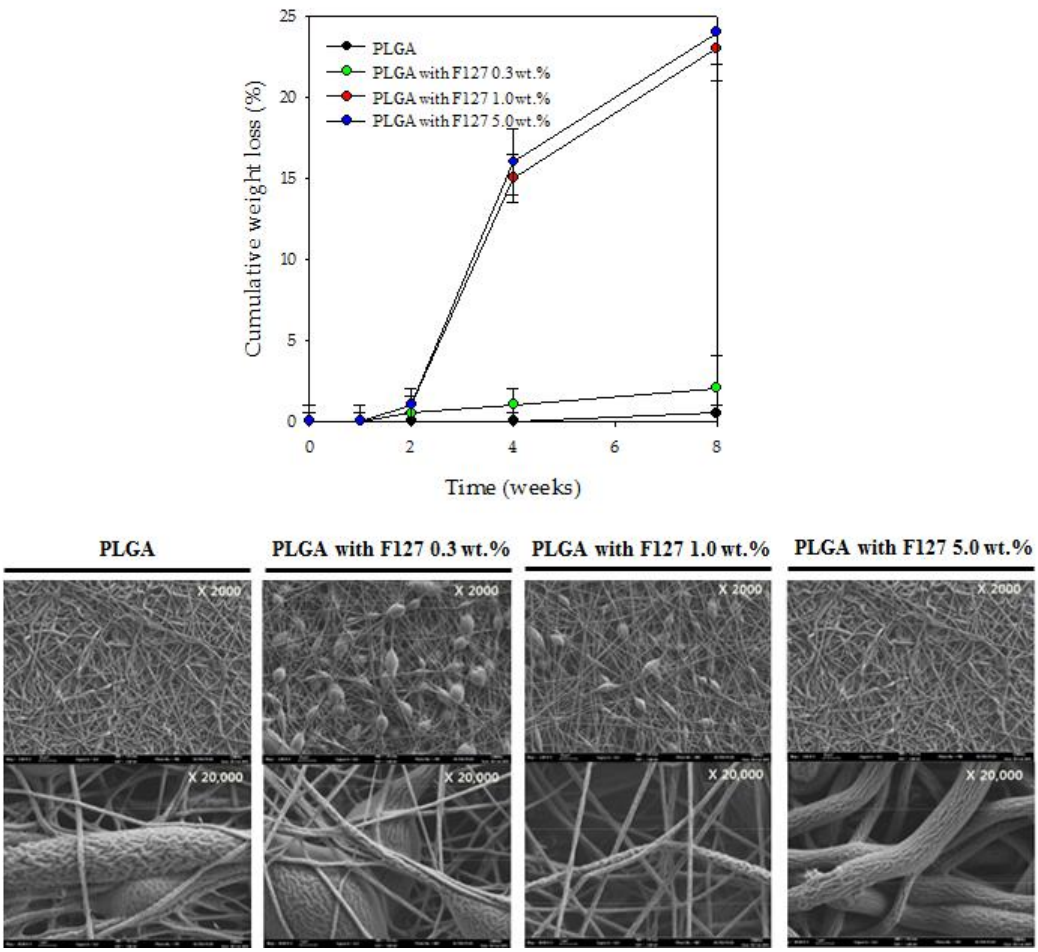

Figure 4. Water absorption evaluation of PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane according to F127 content. The PLGA nanofiber membrane and hydrophilic PLGA nanofiber membranes were immersed in PBS for 8 weeks. Thereafter, the water absorption rate was confirmed by measuring the weight of each membrane over time. Also, the surface of the water absorbed PLGA nanofiber membrane and hydrophilic PLGA membranes were confirmed by FE-SEM images at 4 weeks.

2. Radiographic evaluation

1) PLGA nanofiber membrane

In the 2 weeks and 6 weeks groups, the least amount of new bone was formed in the negative control group that were not used bone graft material or membrane in bone defect and the largest amount of new bone was formed in the experimental group that used PLGA nanofiber membrane with bone grafting material (Figure 5).

In 2 weeks experimental group, it was found that the average amount of new bone formation in the positive control group using collagen membrane was 5.24 ㎣ and 3.76 ㎣ in experimental group using PLGA nanofiber membrane. Also, when used with bone graft material, the average amount of new bone formation was 23.56 ㎣, which was 10 times more than the negative control group. In the case of 6 weeks experimental group, The average of new bone formation was 9.02 ㎣ in the negative control group and 15.66 ㎣ in the PLGA nanofiber membrane group, which was 1.5 times more than the negative control groups (Table 2).

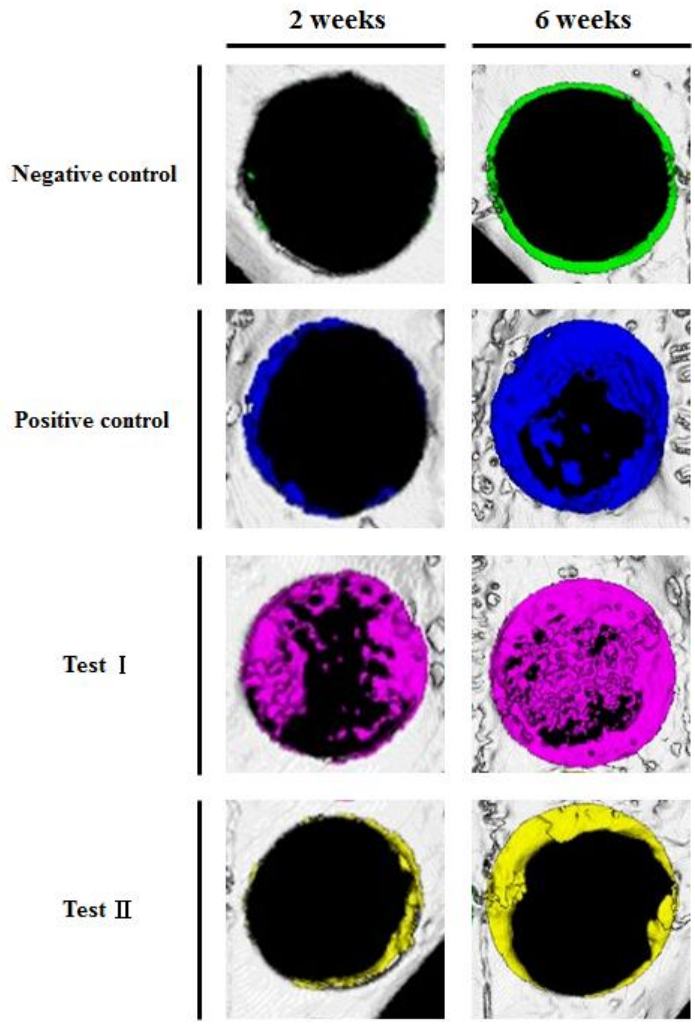

Figure 5. Micro-CT images of the PLGA nanofiber membrane in 2 weeks and 6 weeks experimental groups. After 2 weeks and 6 weeks, the bone tissues were harvested and immediately fixed using 10% formaldehyde. After fixation, the bone tissues were subjected to Micro-CT analysis to evaluate bone regeneration at the clavarial defects.

Table 2. Micro-CT analysis of PLGA nanofiber membrane

	Groups		bone volume (mm^3)	*p* value				
				Kruskal-Wallis test	Mann-Whitney U test			
					A	B	C	D
PLGA nanofiber membrane	2 weeks							
	A	Negative control	2.58 ± 1.65	0.000[a]	-	-	-	-
	B	Positive control	5.24 ± 2.80		0.021[b]	-	-	-
	C	Test Ⅰ	23.56 ± 8.65		0.000[b]	0.000[b]	-	-
	D	Test Ⅱ	3.76 ± 1.90		0.574	0.161	0.000[b]	-
	Groups		bone volume (mm^3)	*p* value				
				Kruskal-Wallis test	Mann-Whitney U test			
					A	B	C	D
	6 weeks							
	A	Negative control	9.02 ± 3.63	0.001[a]	-	-	-	-
	B	Positive control	19.90 ± 11.23		0.011[b]	-	-	-
	C	Test Ⅰ	33.80 ± 8.46		0.001[b]	0.017[b]	-	-
	D	Test Ⅱ	15.66 ± 8.12		0.053	0.902	0.001[b]	-

Values are presented as mean±standard deviation.

A, negative control; B, positive control; C, test Ⅰ; D, test Ⅱ.

[a] Statistically significant difference ($p<0.05$) (Kruskal-Wallis test)

[b] Statistically significant difference ($p<0.05$) (Mann-Whitney U test)

2) Hydrophilic PLGA nanofiber membrane

As with PLGA nanofiber membrane, the 2 weeks and 6 weeks groups showed the least amount of new bone in the negative control group and the largest amount of new bone was formed in the experimental group using the hydrophilic PLGA nanofiber membrane with bone graft material (Figure 6).

In the 2 weeks experimental group, the average amount of new bone formation was 3.31 ㎣ in the negative control group and 30.33 ㎣ in experimental group used with bone graft material, which was 9 times more new bone formation. The new bone formation amount of 9.28 ㎣ and 5.01 ㎣ was observed in positive control group using collagen membrane and hydrophilic PLGA nanofiber membrane. In the 6 weeks experimental group, The new bone formation amount of 13.47 ㎣ new bones, similar to collagen membrane, in experimental group using only hydrophilic PLGA nanofiber membrane. And experimental group with bone graft material showed the new bone formation amount of 26.52 ㎣ (Table 3).

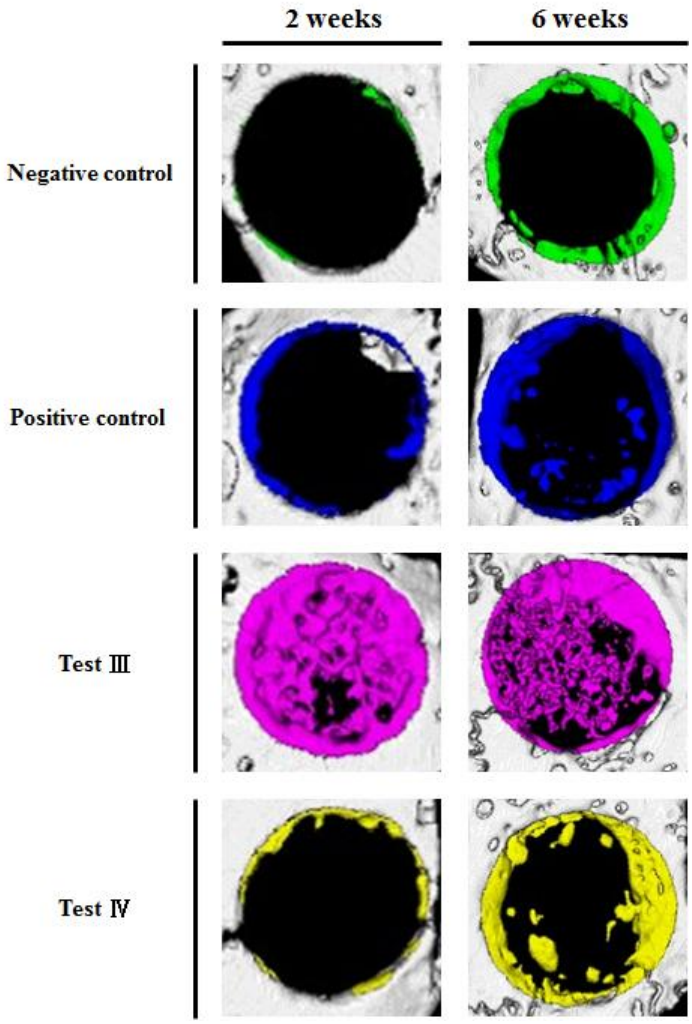

Figure 6. Micro-CT images of the PLGA nanofiber membrane in 2 weeks and 6 weeks experimental groups. After 2 weeks and 6 weeks, the bone tissues were harvested and immediately fixed using 10% formaldehyde. After fixation, the bone tissues were subjected to Micro-CT analysis to evaluate bone regeneration at the clavarial defects.

Table 3. Micro-CT analysis of hydrophilic PLGA nanofiber membrane

	Groups		bone volume (mm^3)	*p* value				
				Kruskal-Wallis test	Mann-Whitney U test			
					A	B	C	D
Hydrophilic PLGA Nanofiber membrane	2 weeks							
	A	Negative control	3.31 ± 2.13	0.000[a)]	-	-	-	-
	B	Positive control	9.28 ± 4.31		0.005[b)]	-	-	-
	C	Test Ⅲ	30.38 ± 10.83		0.000[b)]	0.000[b)]	-	-
	D	Test Ⅳ	5.01 ± 4.27		0.574	0.050[b)]	0.000[b)]	-
	Groups		bone volume (mm^3)	*p* value				
				Kruskal-Wallis test	Mann-Whitney U test			
					A	B	C	D
	6 weeks							
	A	Negative control	10.47 ± 4.41	0.026[a)]	-	-	-	-
	B	Positive control	13.81 ± 6.07		0.318	-	-	-
	C	Test Ⅲ	26.52 ± 12.56		0.007[b)]	0.038[b)]	-	-
	D	Test Ⅳ	13.47 ± 7.19		0.318	1.000	0.038[b)]	-

Values are presented as mean±standard deviation.

A, negative control; B, positive control; C, test Ⅲ; D, test Ⅳ.

[a)] Statistically significant difference ($p<0.05$) (Kruskal-Wallis test)

[b)] Statistically significant difference ($p<0.05$) (Mann-Whitney U test)

4. Histological evaluation

1) PLGA nanofiber membrane

In the 2 weeks experimental group, irregular connective tissue that significantly reduced vertical thickness was formed in bone defect of negative control group. The new bone was formed at the margin of the bone defect and consistent granulation tissue was observed under the membrane in positive control group. The PLGA nanofiber membrane group showed new bone formation with continuity than positive control group at margin of defect. When used with bone graft material, it was confirmed that thickness of new bone was formed at the margin of the defect and it is similar to normal bone, and the new bone was formed at the margin of defect along with granulation tissue to the center with uniform thickness (Figure 7 and 8).

in the case of 6 weeks group, the negative control group of PLGA membrane showed inflammatory pattern with incomplete filling of the bone defect. In PLGA nanofiber membrane group, continuous bone formation was observed under the membrane, which was similar to positive control group. Also, When used with bone graft material, it was confirmed that new bone with a sufficient thickness was formed vertically compared to 2 weeks group (Figure 9 and 10).

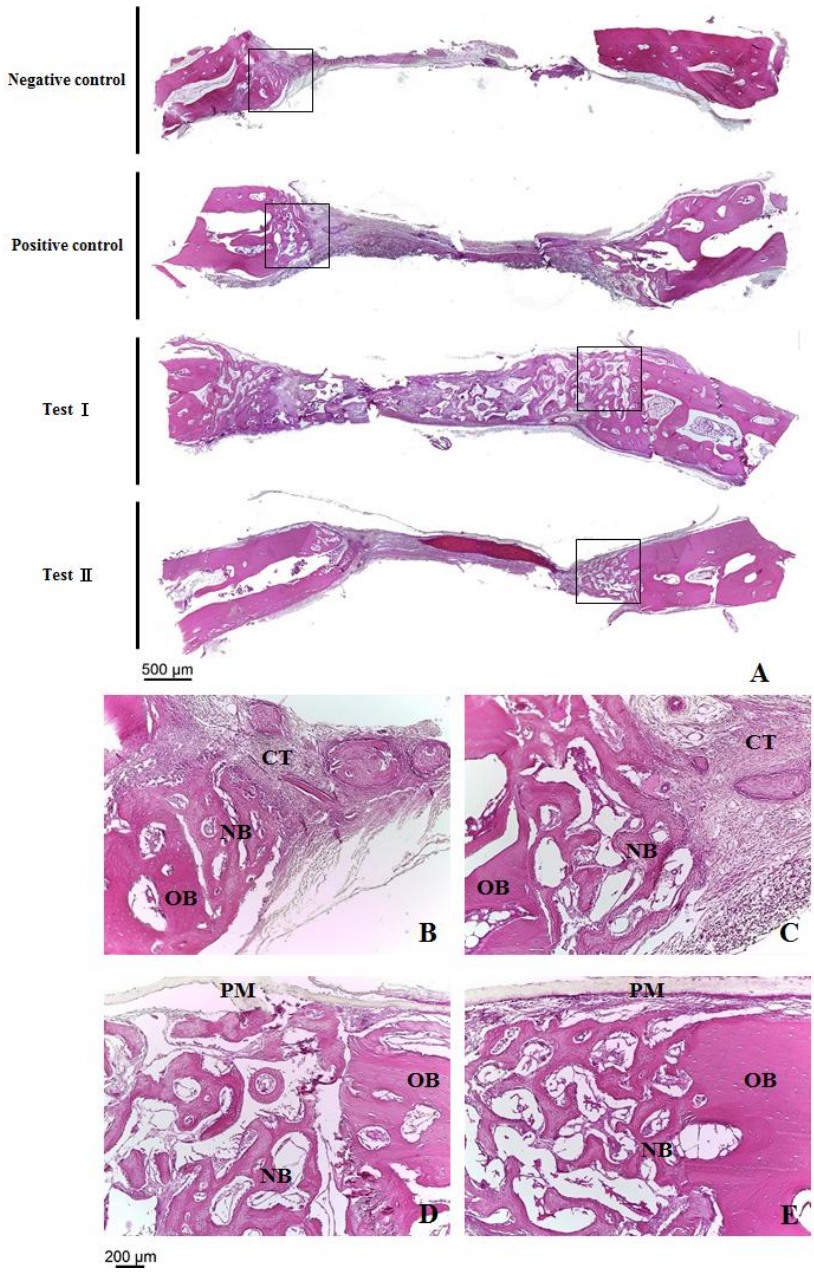

Figure 7. Histologic observation of PLGA nanofiber membrane in 2 weeks experimental groups using H&E staining. (A) The bone paraffin sections were observed by histological evaluation using the H&E staining (40×). (B) In negative control group, a small amount of new bone and irregular connective tissue was observed at the margin of bone defect

(100×). (C) In the positive control group, a large amount of new bone was formed at the margin of bone defect compared to the negative control group (100×). (D) The test Ⅰ group confirmed that the thickness of new bone was formed similar to normal bone thickness in under the membrane (100×). (E) In the test Ⅱ group, the new bone with continuity was formed at the margin of bone defect compared to the positive control group (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, plga nanofiber membrane.

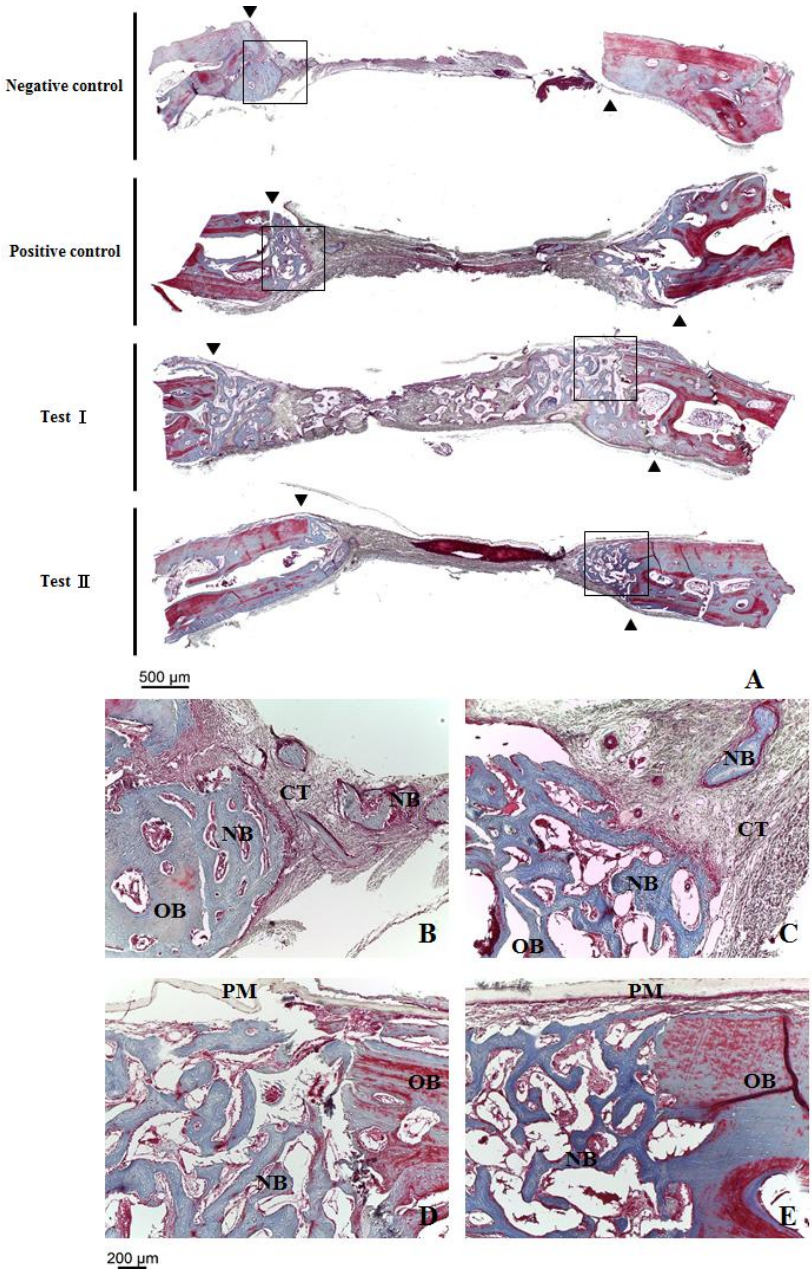

Figure 8. Histologic observation of PLGA nanofiber membrane in 2 weeks experimental groups using MT staining. (A) The bone paraffin sections were observed by histological evaluation using the MT staining (40×). (B) In negative control group, a small amount of new bone was observed at the margin of bone defect (100×). (C) In the positive control

group, new bone and connective tissue was formed at the margin of bone defect (100×). (D) In the test Ⅰ group, it was confirmed that a large amount of new bone was formed under the PLGA nanofiber membrane (100×). (E) In the test Ⅱ group, it was confirmed that the new bone of sufficient thickness was formed at margin of bone defect (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, plga nanofiber membrane.

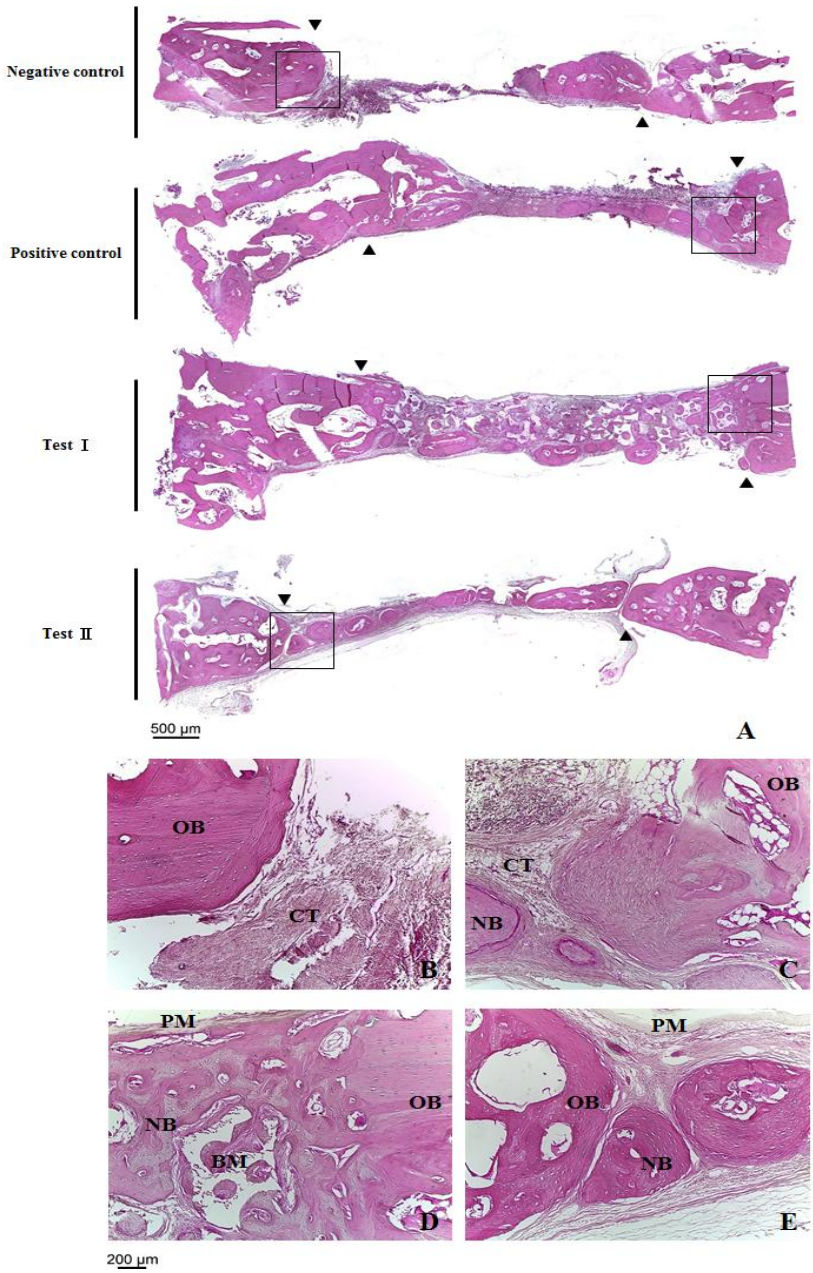

Figure 9. Histologic observation of PLGA nanofiber membrane in 6 weeks experimental groups using H&E staining. (A) The bone paraffin sections were observed by histological evaluation using the H&E staining (40×). (B) In negative control group, it was confirmed that the connective tissue was formed at the margin of bone defect. (100×). (C) In the positive

control group, the new bone was formed at the margin with more ossified bone than the 2 weeks group. (100×). more (D) The test Ⅰ group confirmed that the new bone with a sufficient thickness was formed vertically (100×). (E) Inflammation was not observed under the membrane, and ossified new bone was found in the test Ⅱ group.(100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, plga nanofiber membrane; BM, bone graft material.

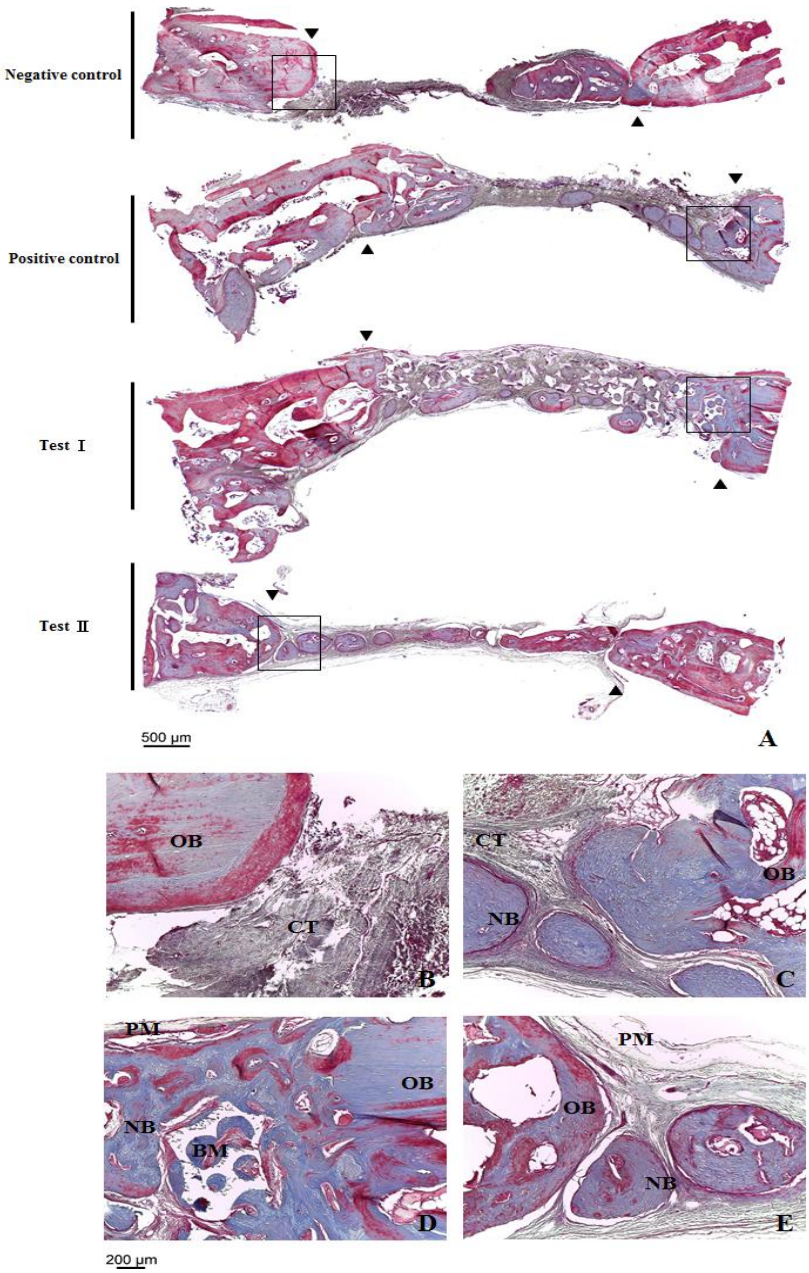

Figure 10. Histologic observation of PLGA nanofiber membrane in 6 weeks experimental groups using MT staining. (A) The bone paraffin sections were observed by histological evaluation using the MT staining (40×). (B) In negative control group, the formation of new bone was limited to the margin of bone defect and mostly filled with connective tissue (100×).

(C) In the positive control group, the more new bone was formed than the 2 weeks group (100×). (D) In the test Ⅰ group, it was observed that new bone was formed around bone graft material (100×). (E) In the test Ⅱ group, the new bone formation was confirmed to be similar to the positive control group (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, plga nanofiber membrane; BM, bone graft material.

2) Hydrophilic PLGA nanofiber membrane

In the 2 weeks group of the hydrophilic PLGA nanofiber membrane, some new bone was formed at the margin of the bone defect in the negative control group, but it was limited to the bone margin and only irregular continuous connective tissue was formed below the defect. On the other hand, in positive control group, new bone formation was observed not only at the margin of the bone defect but also at the central part, and uniform granulation tissue was formed. In the experimental group using the hydrophilic PLGA nanofiber membrane, continuous and thin new bone formation was observed, but no granulation tissue formation was observed when compared with positive control group using collagen membrane. When TCP-containing bone graft material and hydrophilic PLGA nanofiber membrane were used together, new bone formation pattern was observed to be similar to normal bone at the under part of the membrane and bone graft material was uniformly filled with bone defect (Figure 11 and 12).

After 6 weeks, in the negative control of the hydrophilic PLGA nanofiber membrane, thin new bone was formed in the bone defect, but it showed irregular shape and the formation of the new bone was limited to the margin of bone defect. In the experimental group of hydrophilic PLGA nanofiber membrane, the new bone was formed not only at the margin of defect but also the center, and continuous new bone was formed under the membrane. In addition, when the bone graft material used together, the bone graft material was maintained more uniformly than the PLGA membrane, which showed that the new bone with a similar thickness to the normal bone was formed and the continuity was completely restored (Figure 13 and 14).

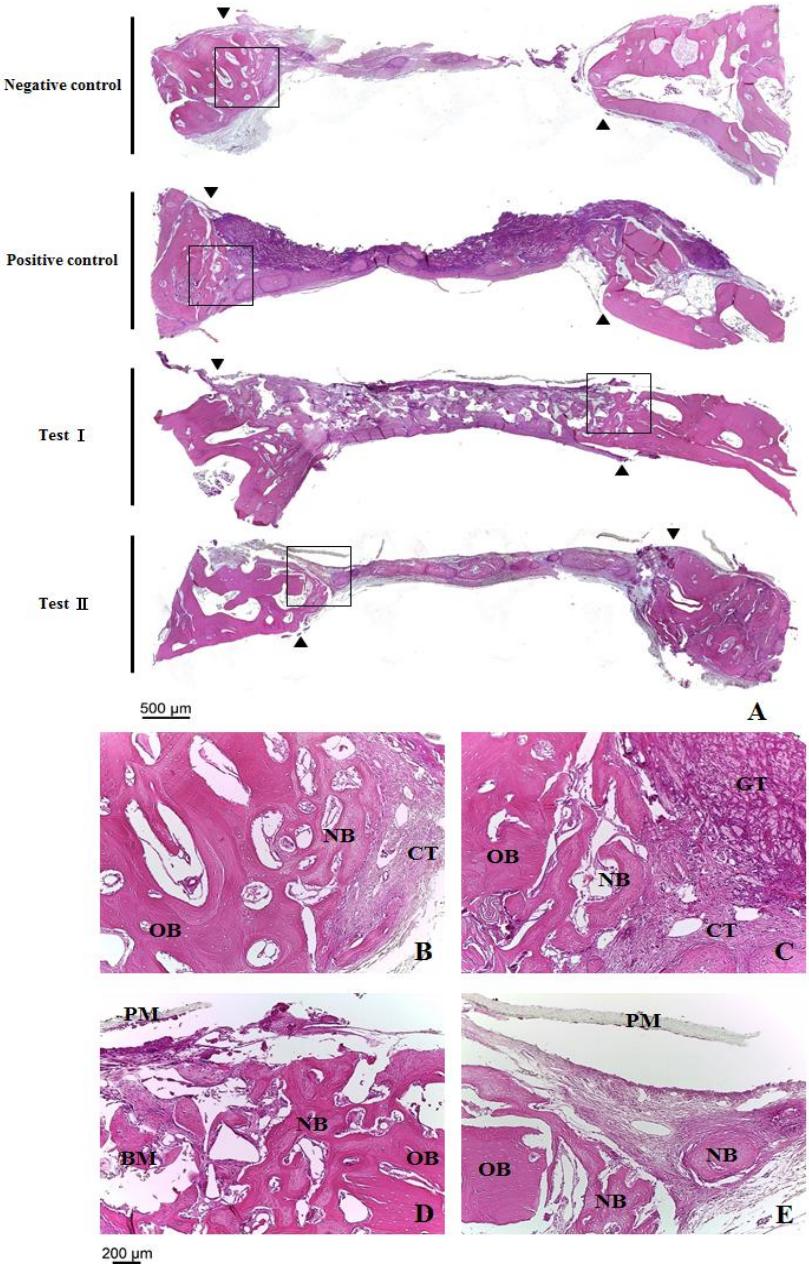

Figure 11. Histologic observation of hydrophilic PLGA nanofiber membrane in 2 weeks experimental groups using H&E staining. (A) The bone paraffin sections were observed by histological evaluation using the H&E staining (40×). (B) In negative control group, a small amount of new bone formed on the margin of bone defect was observed (100×). (C) In

the positive control group, the new bone formed on the margin of defect and granulation tissue formation was observed under the membrane (100×). (D) In the test Ⅲ group, it was confirmed that the new bone was formed in the margin of defect with a thickness similar to original bone (100×). (E) In the test Ⅳ group, Inflammation was not observed under the PLGA nanofiber membrane and new bone was formed in the margin of defect (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, hydrophilic plga nanofiber membrane; BM, bone graft material; GT, granulation tissue.

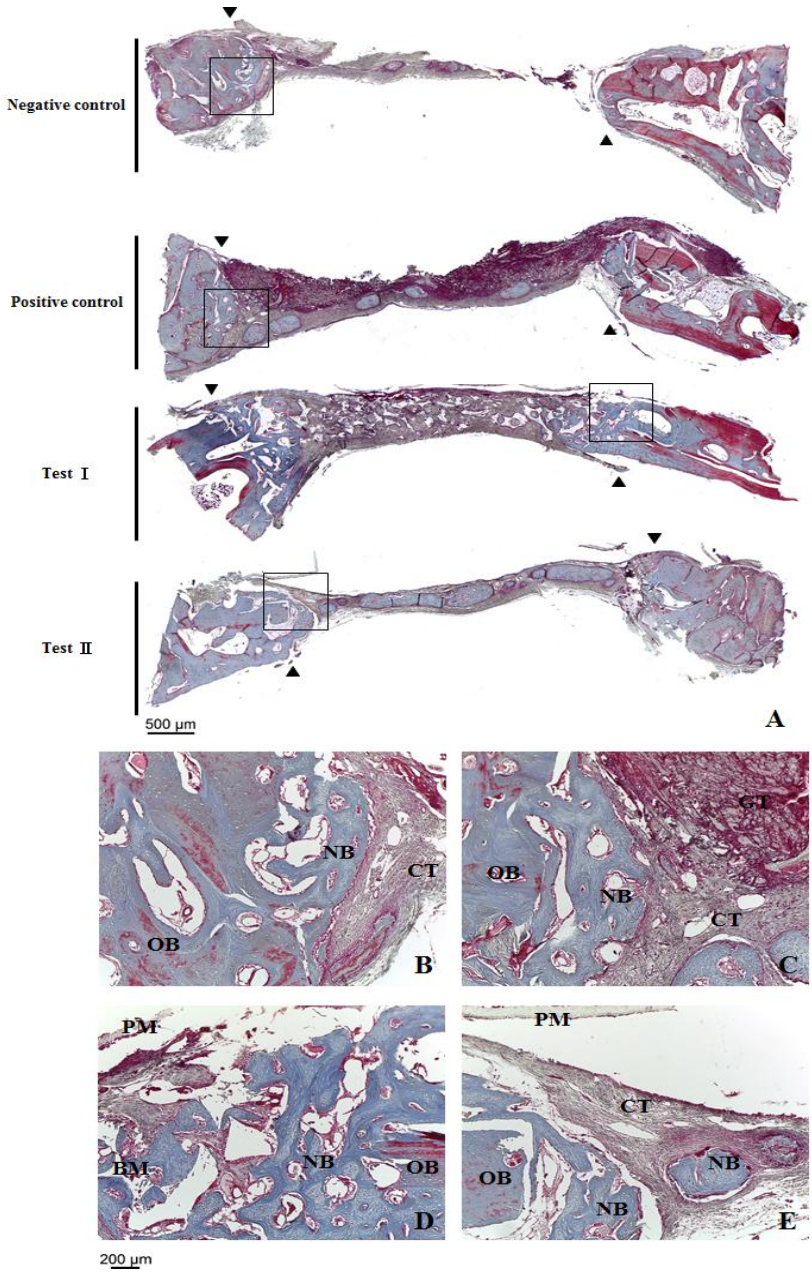

Figure 12. Histologic observation of hydrophilic PLGA nanofiber membrane in 2 weeks experimental groups using MT staining. (A) The bone paraffin sections were observed by histological evaluation using the MT staining (40×). (B) In negative control group, a small amount of new bone and irregular connective tissue were formed in the bone defect (100×).

(C) In the positive control group, the new bone was formed at the margin of bone defect. The connective tissue and granulation tissue were also confirmed. (100×). (D) In the test Ⅲ group, the bone graft material was well maintained and the new bone was formed under the hydrophilic PLGA membrane (100×). (E) In the test Ⅳ group, the new bone was formed under the hydrophilic PLGA membrane, but no granulation tissue was observed (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, hydrophilic plga nanofiber membrane; BM, bone graft material; GT, granulation tissue.

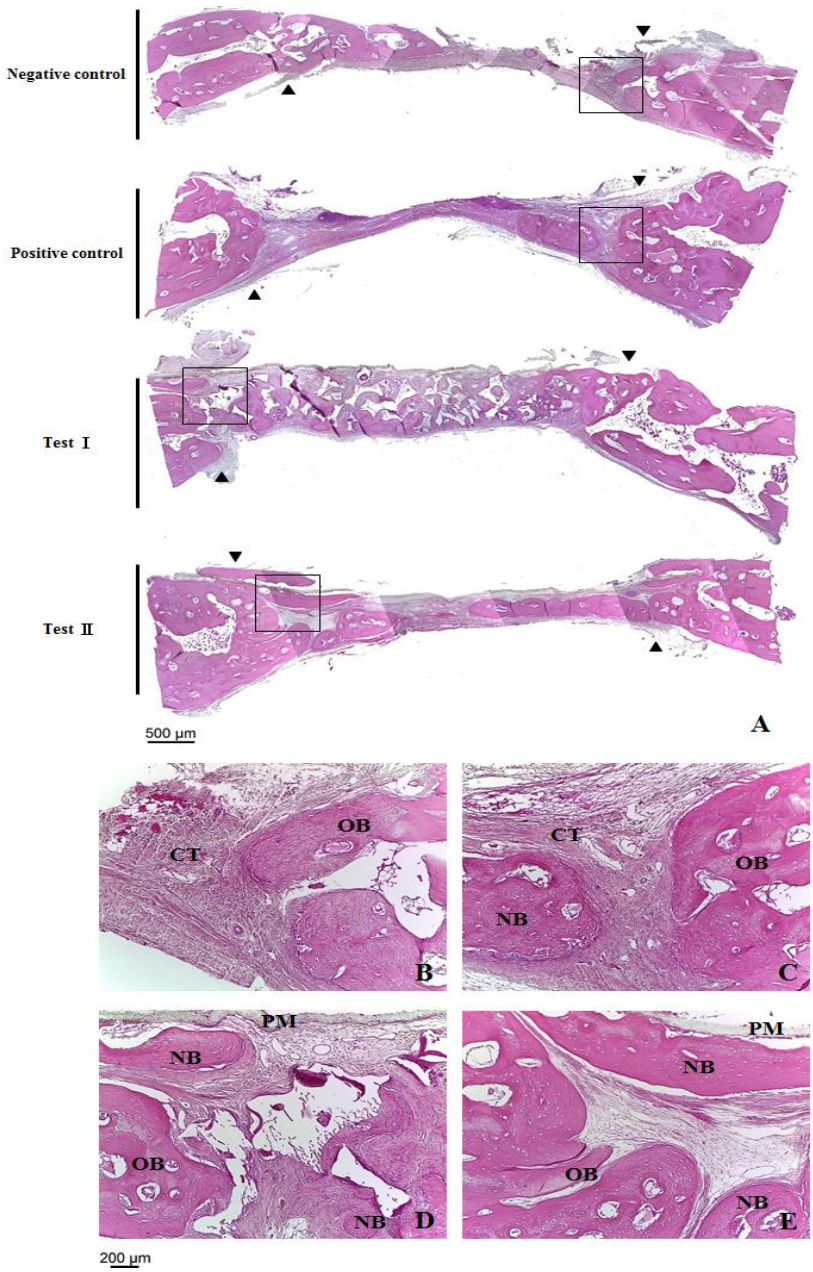

Figure 13. Histologic observation of hydrophilic PLGA nanofiber membrane in 6 weeks experimental groups using H&E staining. (A) The bone paraffin sections were observed by histological evaluation using the H&E staining (40×). (B) In negative control group, connective tissue was formed in bone defect (100×). (C) In the positive control group, the new

bone and connective tissue were confirmed. (100×). (D)In the test Ⅲ group, the new bone with continuity was observed under the hydrophilic PLGA membrane (100×). (E) In the test Ⅳ group, the ossified new bone was formed under the hydrophilic PLGA membrane compared to the 2 weeks group (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, hydrophilic plga nanofiber membrane.

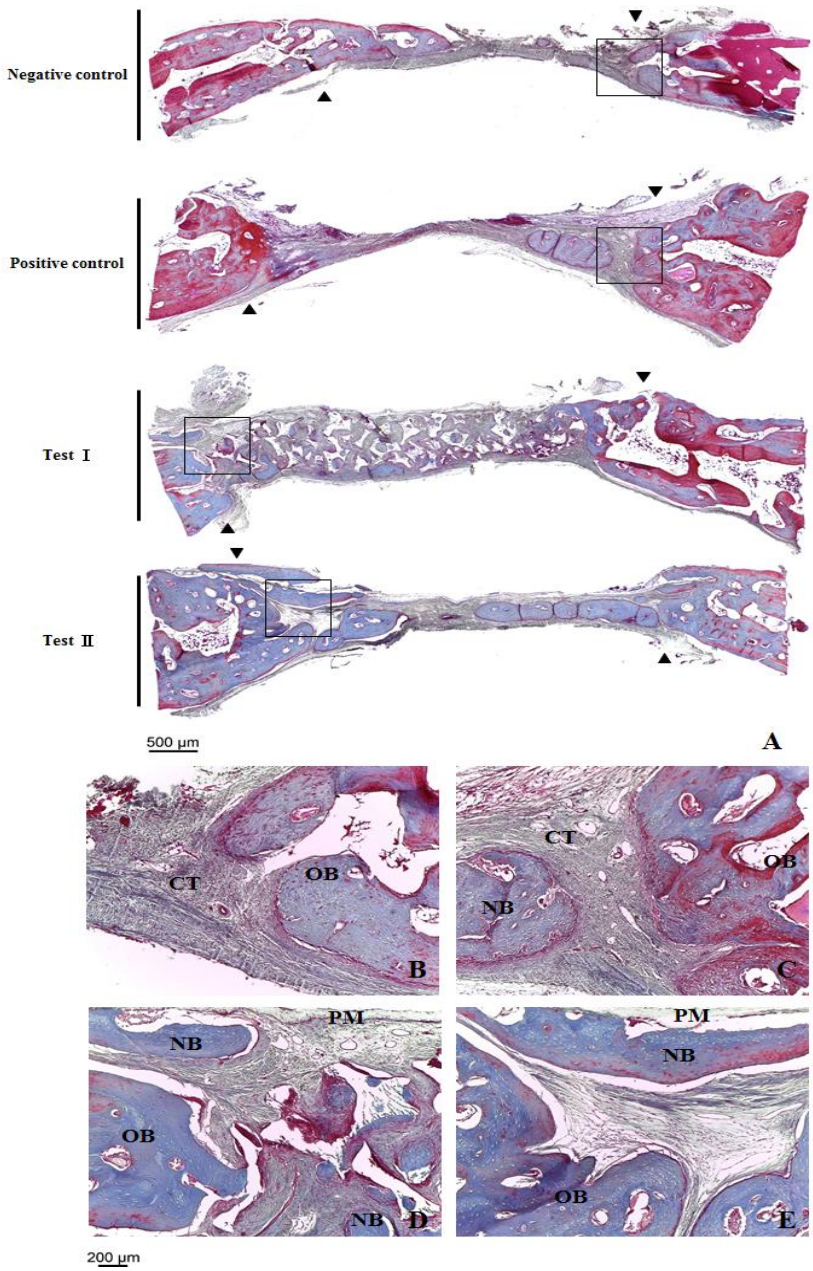

Figure 14. Histologic observation of hydrophilic PLGA nanofiber membrane in 6 weeks experimental groups using MT staining. (A) The bone paraffin sections were observed by histological evaluation using the MT staining (40×). (B) In negative control group, the new bone formation

was not observed at the margin of bone defect (100×). (C) In the positive control group, it was confirmed that the connective tissue and new bone formation was formed at the margin of defect (100×). (D) In the test Ⅲ group, a large amount of new bone was formed compared to the positive control group (100×). (E) In the test Ⅳ group, (100×). Black arrow heads, margin of bone defect; OB, original bone; NB, new bone; CT, connective tissue; PM, hydrophilic plga nanofiber membrane.

Ⅳ. Discussion

In this study, the bone regeneration effect of PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane fabricated by electrospinning using rabbit calvarial defect model was investigated by radiological and histological evaluation.

Nanofiber produced by electrospinning is known to provide an optimal environment for cell attachment and proliferation because that resembles the physical shape of the extracellular matrix (ECM) structure of an organism [28-31]. In addition, nanofiber can be manufactured by using both synthetic polymers and natural polymers, and can be applied to various fields.

In the present study, electrospinning nanofiber was fabricated using the FDA approved material, lactic acid:glycolic acid ratio of 85:15, and hydrophilic PLGA nanofiber were fabricated by adding the FDA approved product Pluronic F127. The morphology observation, hyerophilicity evaluation and degradation evaluation were performed to investigate the properties of the fabricated membranes. As a result of evaluating water contact angle for measuring the hydrophilicity of PLGA nanofiber membrane and hydrophilic PLGA nanofiber membranes, it was confirmed that the water contact angle decreases as the content of F127 increases. This result is consistent to the result of study that the water contact angle increases with increasing roughness of hydrophobic surface and decreases with increasing roughness of hydrophilic surface [32]. Low water contact angle indicates high hydrophilicity, which may help to absorb blood or secretion in vivo. Water absorption was measured for 8 weeks to evaluate the degradation rate of the fabricated membrane. As a result, PLGA nanofiber membrane and hydrophilic PLGA containing 0.3 wt.% of F127 nanofiber membrane showed little decomposition until 8 weeks. However, it was observed that the degradation of the hydrophilic PLGA nanofiber membranes with F127 content of 1.0 wt.% and 5.0 wt.% started from 4 weeks. In general, PLGA is known to show

lower biodegradation rate of membranes as the glycolide content is lower [33]. But, in this experiment, rapid degradation was observed in the hydrophilic PLGA nanofiber membrane containing 1.0 and 5.0 wt.% of F127, which indicates that the degradation can be modulated by addition of a hydrophilizing content. This phenomenon can be interpreted as a result of the hydrolysis is promoted by hydrophilization, which can be seen that it represents the important material properties in the application of membrane for dental tissue regeneration.

Animal experiment was conducted to evaluate the efficacy of the electrospun PLGA nanofiber membrane and the hydrophilic PLGA nanofiber membrane. Rabbit calvaria is composed of an appropriate amount of bone marrow, and it has been widely used for the study of bone regeneration. The appropriate critical size of the calvarial defect for study is typically 10 to 15 ㎜ of external diameter [34-36]. The clavarial defect with an external diameter of 8 ㎜ is smaller than the commonly used critical size, but it is known to be suitable for comparative evaluation of the initial healing and bone regeneration response of the bone defect [37, 38]. Therefore, in this study, the calvarial defect model with a diameter of 8 mm was used. Dahlin et al. recommended the use of bone graft material with resorbable membrane to obtain sufficient space maintenance because the membrane is frequently dented inside bone defect when the resorbable membrane is used independently [39]. As such, the use of bone graft material and a membrane is important in GBR. Therefore, based on the results of this study, bone regeneration effect was evaluated using the fabricated membranes with bone graft material in this experiment. The experimental animals were sacrificed at 2 weeks and 6 weeks after surgical treatment and performed radiological and histological evaluations.

Micro-CT imaging and analysis were performed for radiological evaluation and bone volume values were calculated to compare the bone regeneration between the experimental groups. As a result, both the PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane of 2 and 6 weeks

experimental group showed more average new bone formation in the PLGA nanofiber membrane group compared to the negative control group, but there was no statistically significant difference. In the 6 weeks experimental group of the hydrophilic PLGA nanofiber membrane, the experimental group using the PLGA nanofiber membrane showed that the average new bone mass was formed similar to the positive control group. The result of using PLGA nanofiber membrane with bone graft material showed that the PLGA nanofiber membrane in the experimental group of 2 weeks were ten times more new bone formation than negative control group and the hydrophilic PLGA nanofiber membrane formed nine times more new bone formation than negative control group. In addition, the PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane in the 6 weeks experimental group showed an average bone mass three times higher than the negative control group. Both 2 weeks and 6 weeks experimental groups confirmed a statistically significant difference from the negative control group. However, it was confirmed that the average amount of new bone mass in the 2 weeks and 6 weeks experimental groups using PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane with bone graft material does not differ significantly in numerical values.

The study on bone regeneration is based on the evaluation of bone structure. Microcomputed tomography (Micro-CT) provides information about three-dimensional (3D) structure, facilitating the analysis of the actual structure of new bone [40]. However, there is a disadvantage that information such as presence or absence of inflammation and actual bone shape and thickness cannot be obtained. Therefore, histological evaluation in bone regeneration study complements these disadvantages of Micro-CT.

Kim et al. confirmed that when PLGA was subcutaneously implanted, PLGA produced an acidic environment, which resulted in inflammatory cells and giant cells at the implantation site [41]. In addition, Thomas et al. reported that invasion of inflammatory cells and fibrous capsule formation were observed around the PLGA resorbable membrane at 2 weeks after

surgery [42]. As a result of the histological evaluation of this study, no inflammatory cells were observed below the membrane in the experimental group using the PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane at 2 and 6 weeks after surgery. The new bone formation pattern was also formed similar to collagen membrane used in clinic, and the new bone formation was not limited to the margin but formed to the center of the bone defect. When the bone graft material used in combination with PLGA nanofiber membrane or hydrophilic PLGA nanofiber membrane, the absorption of bone graft material and the inflammatory response were not observed. In particular, after 6 weeks, when hydrophilic PLGA nanofiber membrane and bone graft material were used together, the bone graft material was maintained better than the PLGA nanofiber membrane and new bone was formed completely recovering continuity. These results suggest that the three-dimensional interconnected pore structure of electrospun nanofiber may promote cell respiration and hemostasis, thereby mitigating the inflammation caused by PLGA as well as bone regeneration and helping the healing process of the defect [43, 44].

Ⅴ. Conclusion

This study confirmed that the PLGA nanofiber membrane and hydrophilic PLGA nanofiber membrane fabricated by electrospinning have a similar ability of new bone formation to collagen membrane widely used in clinic and can complement the disadvantage of PLGA, which exhibits inflammatory response due to acidic environment during decomposition. Also, when used with bone graft material, sufficient interval for bone formation was provided and bone mass and bone quality was recovered similar to normal bone. Based on this, the electrospun nanofiber membrane using PLGA is expected to be useful as the membrane for GBR.

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감사의 글

5년간의 결실인 본 논문을 완성하면서 주위의 모든 분들에게 무한한 감사를 드립니다. 늘 따듯한 조언과 응원으로 아낌없이 지원해주신 지도 교수님 유상준 교수님께 진심으로 감사를 드립니다. 항상 많은 배려와 따뜻한 말로 아껴주신 김 병옥 교수님 감사합니다. 또한 바쁘신 와중에도 부족한 논문의 문제점과 미비점을 바로잡아주신 전남대학교 김 옥수 교수님과 김 희중 교수님, 김 병훈 교수님께도 깊은 감사를 드립니다. 언제나 가까이에서 애정으로 지도해주시고 끊임없이 격려와 조언을 해주신 국 중기 교수님 감사합니다. 좋은 논문을 작성할 수 있도록 많은 지원과 도움을 주신 김 찬 박사님 감사합니다.

학부생시절부터 진로에 대해 고민하고 있을 때 항상 올바른 방향을 제시해주시고 지도해주신 광주보건대학교 박 지일 교수님, 심 형순 교수님, 남 정란 교수님께 감사드립니다.

저를 친동생처럼 아껴주고 언제나 늘 곁에서 아낌없는 응원과 격려로 도움을 주는 나의 단짝 이 세림, 10년지기 친구이자 언제나 의지해 왔던 이 미리 부족한 제 옆에서 가족 이상으로 힘이 되어주어 감사합니다.

마지막으로 언제나 든든한 지원군이 되어준 우리 언니 이 지민과 친오빠 같은 형부 한 용희, 세상에서 가장 존경하며 사랑하는 우리 부모님 감사합니다. 끊임없는 사랑과 희생으로 지금까지 돌봐주시고 믿음으로 지켜봐주신 부모님께 이 논문을 바치며 앞으로 더욱 정진하며 한층 성장하는 존재가 되도록 노력하겠습니다.

2019년 12월 13일

이 경 현