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Effect of guided bone regeneration with or without Pericardium® bioabsorbable membrane on bone formation

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지도교수 문 성 용

이 논문을 치의학 박사학위신청 논문으로 제출함.

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조선대학교 대학원

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안유석의 박사학위 논문을 인준함.

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조선대학교 대학원

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국문초록

Tutoplast 이식 후 Pericardium 막의 존재 유무에 따른 골형성 비교 평가

안유석

지도교수 : 문성용

조선대학교 치의학과 구강악안면외과학전공

이 연구의 목적은 동종골(allogenic bone)만을 사용하였을 때와, 동종골과 막(membrane)을 함께 사용하였을 때 골형성 정도를 평가하는 것이다. 60마리의 건강한 Sprague-Dawley rats을 6주와 12주로 반씩 나누고, 대조그룹과 특정 조건하의 그룹으로 나누었다. 이 실험에서 사용된 이식 재료는 동종골이식재인 Tutoplast Spongiosa Mikrochip와 MCBA(mineralized cancellous bone allograft)이다. 특히, MCBA는 특정한 가공 절차를 통해 항원성과 바이러스를 통한 감염을 감소시켰다. 콜라겐으로 구성되고 인체 심막으로 만들어진 Pericardium(Tutoplast Pericardium, Tutogen Medical GmbH)는 흡수성 막으로 사용된다. 실험그룹 2에서 가장 많은 양의 골형성이 이루어졌다. 대조그룹과 비교했을때, 실험그룹 1의 손상부위에서 상당량의 새로 형성된 골이 관찰되었으며 골형성은 6주차와 12주차 사이에서 진전되었다. 6주 후에 Pericardium으로 처치된 그룹은 rim area에 염증 세포가 침투하였고, 염증세포는 또한 12주 그룹에서도 관찰 되었다. 그러나, 12주 그룹에서도 막의 외형은 남아 있었으며, 막이 결합조직의 유입을 막음으로써 골의 재생을 가능케했다. 이 연구 결과에 의하면, 차단막사용시 골이식만 단독으로 하였을 때 보다 새로운 골형성을 더 자극하였으며, 차단막의 유지기간이 충분한 것으로 사료된다.

Abstract

The purpose of this study was to evaluate the bone formation after using the allogenic bone alone or with a membrane.

Sixty healthy Sprague–Dawley rats were divided equally into 6-week 12-week, and control groups and maintained under identical conditions. Graft materials used in this experiment were the allograft Tutoplast Spongiosa Mikrochip and a mineralized cancellous bone allograft (MCBA), which had reduced antigenicity and virus cross infection because it had been treated with a special process. Pericardium, which is made from human pericardium and composed of collagen, was used as an absorbable membrane. Grafting materials were not transplanted in the control group, the Tutoplast® was transplanted in experimental group 1, and the Tutoplast® was transplanted in experimental group 2.

The most abundant volume of new bone formation occurred in experimental group 2. Noticeable new bone was observed in the defect area of experiment group 1 when compared with the control group, and this progressed between 6 and 12 weeks. After 6 weeks, the group treated with Pericardium® showed infiltration of inflammatory cells into the rim area, and inflammatory cells were still observed in the 12-week group. However, the membrane outline remained even in the

12-week group, and the membrane facilitated bone regeneration by blocking the influx of connective tissue that impeded bone regeneration beneath the membrane.

Based on thesis results, the barrier membrane stimulated new bone formation more than did a bone graft alone. It was considered that the period for maintaining a barrier membrane was sufficient. The effect of guided bone regeneration was sufficient at 6 weeks.

I. Introduction

Many materials have been used for bone regeneration and research is ongoing in the area of bone generation in bone defect areas. In particular, with the wide use of implants, autogenous bone, allogenic bone, and diverse synthetic bones have been used to treat bone defect areas that develop in the temporomandibular area. The best bone regeneration results are obtained using fresh autogenous bone due to osteoinduction and osteoconduction. However, autogenous bone has disadvantages such as pain and edema caused by additional injury to the donor site, the limited volume that can be harvested, and high graft-fragment resorption[1,2]. Therefore, allogenic bone graft materials that do not have bone regenerative capacity but do not require an additional donor site for a bone graft have been widely used. Because tissues harvested from other people are grafted, they may act as an antigen in recipient tissues and induce an immune reaction. Therefore, allogenic graft materials are frozen, freeze-dried, demineralized freeze-dried, or irradiated in many cases[3,4].

Freeze-dried allogenic bones are supplied in a both non-demineralized and demineralized form. Demineralization may enhance osteoconductive capacity by removing minerals within the graft and exposing collagen, growth factors, and particularly, bone morphogenic proteins.[5–7]

Freeze-dried bone allograft (FDBA) is mineralized, so it hardens faster than demineralized FDBA (DFDBA). The Tutoplast Spongiosa Mikrochip (Puros, Zimmer Dental, Carlsbad, CA, USA) is a mineralized cancellous bone allograft (MCBA) in which the possibility of antigenicity and viral cross infection is reduced by special treatments.[8-10]

Barrier membranes mediate very important functions in guided bone regeneration (GBR), and various membranes have been used. Among these is e-polytetrafluoroethylene (Gore-tex), but it has shortcomings in that it is non-absorbable, so a second surgery is required to remove the membrane. It is also expensive, and the possibility for inflammation is high.[11,13] Therefore, absorbable membranes that maximally exert effects similar to nonabsorbable membranes have been used recently. We selected an absorbable membrane derived from human pericardial membrane (Pericardium). Pericardium (Tutoplast Pericardium®, Tutogen Medical GmbH) has been used widely for regenerating the bone defect areas near implants or as a barrier membrane to perforate the maxillary sinus, but too few clinical studies have been conducted on these procedures.

The purpose of this study was to evaluate the bone formation after using the allogenic bone alone or with a membrane.

II. Experimental materials and methods

A. Experimental materials

1. Animals

Sixty healthy Sprague–Dawley rats (200–300 g) were divided equally into 6-week, 12-week, and control groups and maintained under identical conditions.

2. Materials

Graft materials used in this experiment were the allograft Tutoplast Spongiosa Mikrochip (Tutoplast®, particle size 1000–2000μm, Tutogen Medical GmbH, Neunkirchen, Germany) and a mineralized cancellous bone allograft (MCBA). Pericardium (Tutoplast Pericardium®, Tutogen Medical GmbH), which is made from human pericardium and composed of collagen, was used as an absorbable membrane.

B. Experimental methods

1. Anesthesia of experimental animals

Anesthesia was induced with an intramuscular injection of 10 mg/kg ketamine HCl (Ketara®, Yoohan Yanghang) and 0.3 mg/kg xylazine (Rompun®, Bayer Korea). Inhaled ethyl ether anesthesia was applied additionally if needed.

2. Surgical methods

Hair was removed from the skin of the rat cranium, and the area was sterilized and locally anesthetized (2% lidocaine, 1:100,000 epinephrine, Gwang Myung Drugs). An approximately 2-cm incision was made from the occipital bone to the frontal bone, subcutaneous tissues were resected and elevated together with the periosteum bilaterally, and the cranial bone was exposed. A full thickness defect area was made using a low speed dental drill, being careful not to injure the dura and blood vessels in the cranial parietal area. The area was approximately 8 mm in diameter, which was the critical bone defect size. To prevent excess heat, the areas were washed with saline while forming the defect area. The incision for the control group was sutured by closing the upper periosteum of the defect area without either graft material membranes. Two treatment modalities were applied for the defected area. In experimental group 1, the Tutoplast® was transplanted to the defect area in a relatively even thickness and sutured in place by closing the periosteum. In experimental group 2, the Tutoplast® was transplanted to the defect area in a relatively even thickness, covered with Pericardium® attached tightly to graft materials, and sutured by closing the periosteum. After surgery, 5 mg/kg gentamicin (Daesung Microbiological Labs. Co., Ltd, Uiwangsi, Korea) was injected intramuscularly for 5 days to prevent infection.

3. Preparation of tissue samples and examination

Ten animals were anesthetized as described above and sacrificed in each experimental group. Then, the defect area including the upper periosteum, the scalp, and adjacent healthy bone was resected, fixed in 10% neutral formalin, and decalcified by acid immersion (Calci-Clear Rapid®, National Diagnostics, Atlanta, USA) for approximately 4 hours. In the middle of the bone defect area, three sections of 3-mm thickness were harvested consecutively, washed, and embedded in paraffin using an automatic tissue preparation instrument (Hypercenter XP, Shandon, UK). Paraffin blocks 4–5 μm in thickness were prepared, attached to glass slides, maintained at 68°C on a heat plate for longer than 1 hour, and stained with hematoxylin-eosin or Masson's trichrome stains. The prepared specimens were observed under microscopy and images were captured with the MagnaFire digital camera system (Optronics, Goleta, CA, USA). The region of interest was measured and analyzed for new bone formation using the Visus Image Analysis System (Image and Microscope Technology, Daejeon, Korea).

4. Statistical analysis

The area of bone formed from the edge of defect area to the center was measured (n = 3/sample), and an ANOVA was performed. Scheffe's test was performed to assess significance among the experimental groups.

III. Experiment results

A. Histological findings

In the 6-week control group, new bone was formed, which was thin toward the center. New bone was not detected in the rim and center of the defect area, and most areas were filled with loose connective tissue (Fig. 1).

Compared with the 6-week group, the 12-week control group showed an increase in thickness at the edge of new bone that formed near the defect margins. An increase in the loose connective tissue was also observed compared to the 6-week group (Fig. 2).

2. Experimental group 1 (allogenic bone)

In the 6-week group, limited new bony spurs proliferated substantially in the defect margins, and infiltration and activation of osteoblasts was observed in the grafted bone. Edges between grafted bone fragments and old bone were indistinguishable due to ingrowth of osteoblasts, a type of organization (Fig. 3). New bone showed noticeable fusion with adjacent bone fragments, and bone marrow formation was detected in some new bony spurs.

In the 12-week group, the pattern of new bone formation had progressed substantially, and bone from new bony spurs and host bone

were observed on the edge of the defect area (Fig. 4). Proliferation of blood vessels was abundant, and active bone formation features were evident. Resorption and bone formation were ongoing simultaneously at the edge of the grafted bone due to osteoclast and osteoblast activity, and a change was noted in the original shape of the grafted bone. Compared to the 6-week group, denser bone fusion was present due to the remodeling. However, in all animals, the center of the defect area remained in an immature ossified condition showed delayed bone fusion compared with the rim area.

3. Experimental group 2 (allogenic bone and absorbable barrier membrane graft)

In the 6-week group, the membrane was partially absorbed in the graft area, numerous inflammatory cells had formed colonies and infiltrated, and phagocytes and multinucleated cells colonies were observed (Fig. 5). Substantial new bone formation was observed on the grafted bones in the center of the graft. Similarly, an increase in new bone formation was noted at the rim area and, compared with experimental group 1, a noticeably greater increase in new bone formation was found in the center area.

In the 12-week group, the membrane boundary had disappeared in several areas, indicating that membrane absorption had progressed

substantially (Fig. 6). The center defect area was connected with the rim area and was proliferating continuously when compared with the 6-week group. In all animals, a bone-filling pattern was observed at the center of the defect area, and connective tissues were hardly detected.

B. Histomorphometric analysis

In the 6-week groups, the amount of new bone formation in the control group, experimental group 1, and experimental group 2 were 0.22 ± 0.03㎟, 0.43 ± 0.04㎟, and 0.71 ± 0.64㎟, respectively (control vs. groups 1 and 2, p = 0.00; group 1 vs. group 2, p = 0.34).

In the 12-week groups, the amount of new bone formation in the control group, experimental group 1, and experimental group 2 were 0.29 ± 0.02㎟, 0.55 ± 0.06㎟, and 0.70 ± 0.03㎟, respectively (control vs. groups 1 and 2, p = 0.00; group 1 vs. group 2, p = 0.00).

Overall, the new bone formation in the 12-week groups was significantly higher than that in the 6-week groups (p = 0.00) (Table 1)

Table 1. Mean amounts of new bone formation in control, experimental group 1 and 2 at 6 and 12 weeks after placement (㎟)

	Mean ± SD		
Time	Control	Experimental group 1	Experimental group 2
6 weeks	0.20 ± 0.13	0.41 ± 0.14*	0.69 ± 0.64*,†
12 weeks	0.27 ± 0.11	0.54 ± 0.16*	0.70 ± 0.03*,†

* Statistically significant difference relative to control ($p<0.05$).

† Statistically significant difference relative to experimental group 1 ($p<0.05$).

SD = standard deviation.

IV. Discussion

Various bone graft materials have been used to restore bone defect areas. The use of autogenous bone is the most effective for bone regeneration, but its use is restricted due to the required second surgery on the donor area and the consequent discomfort of patients. Therefore, the use of commercialized bone graft materials has increased substantially. The limitations of autogenous bone are unstable fixation, rapid resorption, poor local tissue reaction, and graft materials reacting only passively. Furthermore, autogenous bone does not undergo osseointegration with host bone[15,16].

Lindhe[2] and Dahlin et al.[12] recommended the use of allogenic bone as a substitute for autogenous bone. Allogenic graft materials are obtained from cadavers or living donors and are treated with sterilization procedures at tissue banks approved by the American Association of Tissue Banks, where they are stored and dispensed. Allogenic graft materials have advantages in that they are available at any time, and a donor area for the bone graft is not required, which may shorten anesthesia and surgery time, resulting in fewer complications.

Osteoinductive regeneration is a method to augment bone healing

and regeneration, and the use of a barrier membrane is essential for successful osteoinductive regeneration. Nyman et al.[17] and Karring et al.[18] introduced GBR by placing a physical barrier membrane that blocked invagination of connective tissue in the alveolar tissue defect area to facilitate migration of alveolar ligament cells or osteoblasts required for alveolar bone regeneration. Dahlin et al.[14] reported a study in which 5-mm penetration windows were formed on both right horn areas of rats, and e-PTFE membranes were placed on the medial and lateral side, with the other side serving as the control with no membrane. Nyman et al.[17] reported that complete new bone formation was obtained in 6 months in a defect area using Teflon membranes in the vicinity of implants. Numerous studies have reported that the biocompatibility of such barrier membranes is excellent, and the procedure can be performed readily during surgery; thus, clinical outcomes were excellent. Nevertheless, because the material is not degradable, it has shortcomings in that a second surgery is required to remove the barrier membranes from alveolar tissues. The second surgery itself exerts harmful effects on growing granular tissue and causes financial and physical burdens on patients. To resolve such problems, the use of an absorbable membrane is increasing.

The results from experimental group 2 at 6 weeks showed that the membrane was partially absorbed in the bone graft area, and by 12

weeks, absorption of the membrane had progressed substantially. The membrane inhibited connective tissue invasion, which impedes bone formation, and thereby facilitated new bone formation.

Brunel et al.[18] reported that severe inflammation around an absorbable collagen barrier membrane in the rat cranium stimulates membrane degradation. In this study, animals in experimental group 2 at 6 weeks revealed numerous phagocytes and multinucleated giant cells forming infiltrating colonies, and the inflammatory cell infiltration was observed even in the 12-week group. Such inflammatory cell infiltration may be associated with membrane absorption; however, these findings were not observed in the bone graft area below the membrane and were restricted to the membrane rim. Thus, the infiltration appeared not to have a significant effect on bone regeneration. In cases in which absorbable barrier membranes are used, a period during which the membrane is not absorbed is required to prevent interference with new bone regeneration. Hurzeler et al.[19] referred to this as the critical maintenance period, and approximately 2–8 weeks are required for alveolar tissue regeneration. Becker et al.[20] reported that sufficient bone regeneration could be induced in 4–6 weeks in experiments using e-PTFE membranes. Similarly, we found that new bone formed in 12 weeks. Thus, the membrane should be maintained for a minimum of 4 weeks. However, the experiment was

performed on rat cranium, and other areas in humans may be different.

In a study reported by Dahlin et al.,[14] using graft materials to secure the maintenance of space was recommended for cases using absorbable membranes alone, because a depression in the membrane within the defect area frequently occurs. In cases in which absorbable barrier membranes are used and bone graft materials are used together with GBR, the interval for bone formation should be maintained because the membrane also has osteoinductive or osteoconductive abilities, so better bone formation could be obtained. In a study reported by Anderegg et al.,[21] better bone formation was obtained in cases using barrier membranes together with demineralized freeze-dried bone. Similarly, we found that the new bone formation amount of experimental group 2 was significantly higher than that in experimental group 1. The histological findings revealed firmer bone union in the 12-week group of experimental group 2 than in experiment group 1, which was in good agreement with the results reported by Brugnami et al.,[22] who reported that a barrier membrane prevents the coverage of graft bones with fibrous connective tissues, resulting in improved formation of new bony spurs by osteoblasts.

In the 6-week experimental groups, a difference was observed in the volume of new bone formed compared with the control group. When compared with results at 12 weeks, more new bone was formed in

experimental group 1 than the other groups. It was considered that during the early phase of bone formation, the barrier membrane blocked the invasion of connective tissue, facilitating new bone formation. Additionally, the amount of bone formation was higher in the experimental group in which only a bone graft was used compared with the control group, suggesting that the bone graft materials exerted an osteoconductive effect that induced the growth of blood vessels and fibrous tissues and contributed to the proliferation and stability of the bone[23,24].

The results of this study showed when the absorbable membrane (Pericardium®) and allogenic bone (Tutoplast®) were used in combination during GBR, better bone formation resulted than cases in which bone graft materials alone were used.

V. Conclusion

1. The experimental group 2 showed the greatest amount of new bone at both 6- and 12- week compared with control and experimental group 1.
2. The experimental group 1 showed the greater amount of new bone at both 6- and 12- week compared with control group.
3. After 6 weeks, the group treated with Pericardium® showed infiltration of inflammatory cells into the rim area, and inflammatory cells were still observed in the 12-week group. However, the membrane outline remained even in the 12-week group, and the membrane facilitated bone regeneration by blocking the influx of connective tissue that impeded bone regeneration beneath the membrane.

Based on theses results, the barrier membrane enhanced the new bone formation and believed to be potentially useful in combination with allogenic bone in GBR. In addition, it was considered that the 6 weeks for maintaining a barrier membrane was sufficient.

References

1. Alberius P, Dahlin C, Linde A. Role of osteopromotion in experimental bone grafting to the skull: a study in adult rats using a membrane technique. J Oral Maxillofac Surg 1992;50:829-834.
2. Linde A, Alberius P, Dahlin C, Bjurstam K, Sundin Y. Osteopromotion: a soft-tissue exclusion principle using a membrane for bone healing and bone neogenesis. J Periodontol 1993;64:1116-1128.
3. Buck BE, Malinin TI, Brown MD. Bone transplantation and human immunodeficiency virus. An estimate of risk of acquired immunodeficiency syndrome (AIDS). Clin Orthop Relat Res 1989;240:129-136.
4. Mulliken JB, Glowacki J, Kaban LB, Folkman J, Murray JE: Use of demineralized allogeneic bone implants for the correction of maxillocraniofacial deformities. Ann Surg 1981;194:366-372.
5. Lane JM. Bone graft substitutes. West J Med 1995;163:565-566.
6. Açil Y, Springer IN, Broek V, Terheyden H, Jepsen S. Effects of bone morphogenetic protein-7 stimulation on osteoblasts cultured on different biomaterials. J Cell Biochem. 2002;86:90-98.
7. Wikesjö UM, Sorensen RG, Kinoshita A, Wozney JM.

RhBMP-2/alphaBSM induces significant vertical alveolar ridge augmentation and dental implant osseointegration. Clin Implant Dent Relat Res 2002;4:174-182.

8. Collins TA. Onlay bone grafting in combination with Branemark implants. Oral Maxillofac Surg Clin North Am. 1991;3:893-9029.
9. Feuille F, Knapp CI, Brunsvold MA, Mellonig JT. Clinical and histologic evaluation of bone-replacement grafts in the treatment of localized alveolar ridge defects. Part 1: Mineralized freeze-dried bone allograft. Int J Periodontics Restorative Dent 2003;23:29-35.
10. Gunther KP, Scharf HP, Pesch HJ, Puhl W. Osteointegration of solvent-preserved bone transplants in an animal model. Osteologie 1995;5:4-12.
11. Caffesse RG, Nasjleti CE, Morrison EC, Sanchez R. Guided tissue regeneration: comparison of bioabsorbable and non-bioabsorbable membranes. Histologic and histometric study in dogs. J Periodontol 1994;65:583-591.
12. Dahlin C, Linde A, Gottlow J, Nyman S. Healing of bone defects by guided tissue regeneration. Plast Reconstr Surg 1988;81:672-676.
13. Dahlin C, Gottlow J, Linde A, Nyman S. Healing of maxillary and mandibular bone defects using a membrane technique. An experimental study in monkeys. Scand J Plast Reconstr Surg Hand Surg 1990;24:13-19.

14. Dahlin C, Alberius P, Linde A. Osteopromotion for cranioplasty. An experimental study in rats using a membrane technique. J Neurosurg 1991;74:487-491.
15. Burchardt H. The biology of bone graft repair. Clin Orthop 1983;174:28-42.
16. Mellonig JT, Bowers GM, Cotton WR. Comparison of bone graft materials. Part II. New bone formation with autografts and allografts: a histological evaluation. J Periodontol 1981;52:297-302.
17. Nyman S, Lang NP, Buser D, Bragger U. Bone regeneration adjacent to titanium dental implants using guided tissue regeneration: a report of two cases. Int J Oral Maxillofac Implants 1990;5:9-14.
18. Brunel G, Piantoni P, Elharar F, Benqué E, Marin P, Zahedi S. Regeneration of rat calvarial defects using a bioabsorbable membrane technique: influence of collagen cross-linking. J Periodontol 1996;67:1342-1348.
19. Hürzeler MB, Quiñones CR, Caffesse RG, Schüpbach P, Morrison EC. Guided periodontal tissue regeneration in interproximal intrabony defects following treatment with a synthetic bioabsorbable barrier. J Periodontol 1997;68:489-497.
20. Becker W, Becker BE, Prichard JF, Caffesse R, Rosenberg E, Gian-Grasso J. Root isolation for new attachment procedures. A

surgical and suturing method: three case reports. J Periodontol 1987;58:819-826.

21. Anderegg CR, Martin SJ, Gray JL, Mellonig JT, Gher ME. Clinical evaluation of the use of decalcified freeze-dried bone allograft with guided tissue regeneration in the treatment of molar furcation invasions. J Periodontol 1991;62:264-268.
22. Brugnami F, Then PR, Moroi H, Leone CW. Histologic evaluation of human extraction sockets treated with demineralized freeze-dried bone allograft (DFDBA) and cell occlusive membrane. J Periodontol 1996;67:821-825.
23. Burchardt H. The biology of bone graft repair. Clin Orthop 1983;174:28-42.
24. Urist MR. A bone morphogenetic system in residues of bone matrix in the mouse. Clin Orthop 1973;91:210-220.

Figure Legends

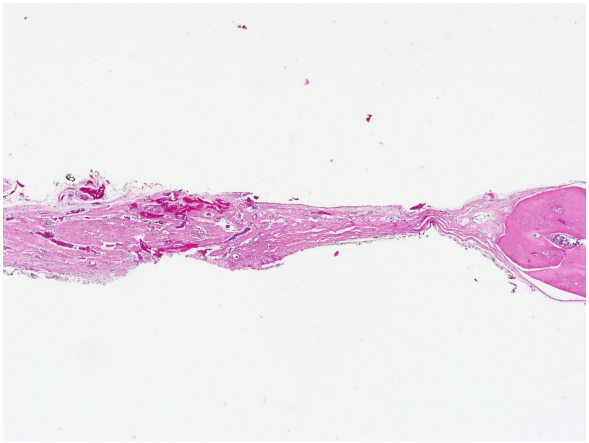

Fig. 1. Histopathological findings of the 6-week control group. A small amount of newly bone formed (asterisk) around the defect margin (arrows) was observed. Hematoxylin-eosin stain; original magnification x40 .

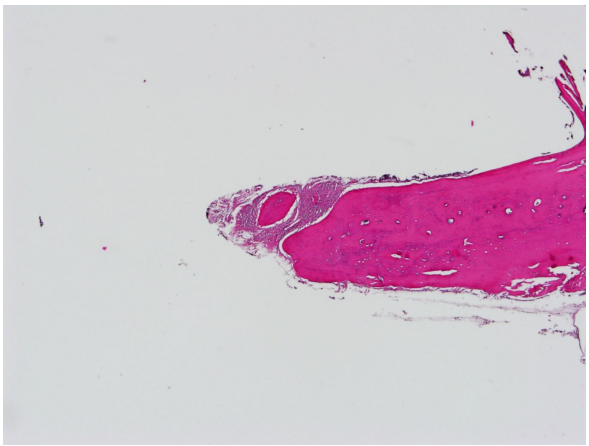

Fig. 2. Histopathological findings of the 12-week control group. A small amount of newly bone formed (asterisks) around the defect margin (arrows) was observed. Hematoxylin-eosin stain; original magnification x40 .

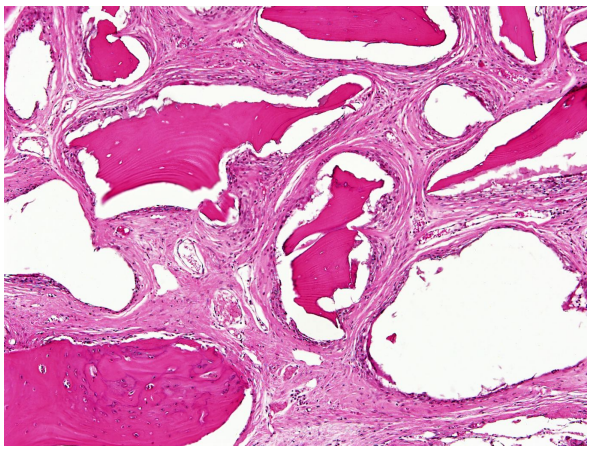

Fig. 3. Histopathological findings of the 6-week experimental group 1. A small amount of newly bone formed (asterisk) around the implant chips (arrows) was observed. Hematoxylin-eosin stain; original magnification x100 .

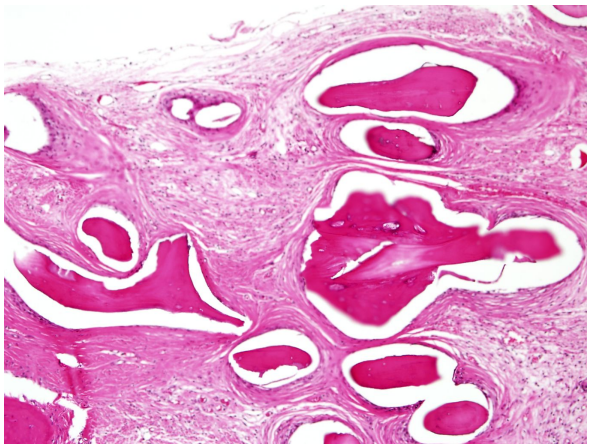

Fig. 4. Histopathological findings of the 12-week experimental group 1. A small amount of newly bone formed (asterisks) around the implant chips (arrows) was observed. Hematoxylin-eosin stain; original magnification x100 .

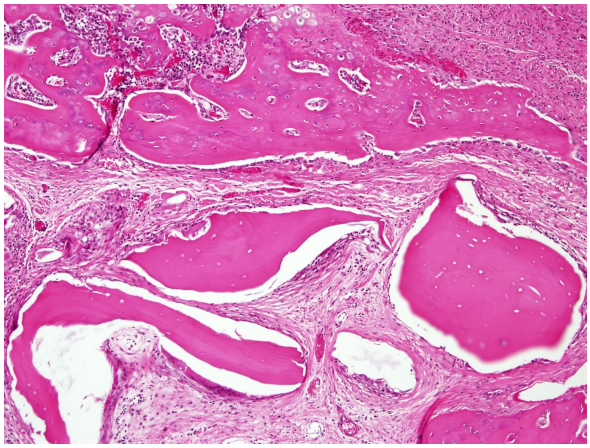

Fig. 5. Histopathological findings of the 6-week experimental group 2. Newly bone formed (asterisks) around the implant chips (arrows) and membrane was observed. Hematoxylin-eosin stain; original magnification x100 .

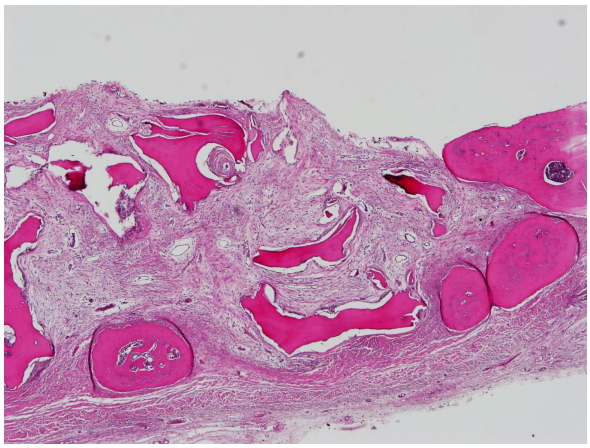

Fig. 6. Histopathological findings of the 12-week experimental group 2. Newly bone formed (asterisks) around the defect margin (arrows), implant chips and membrane was observed. Hematoxylin-eosin stain; original magnification x100 .