pISSN 1598-298X / eISSN 2384-0749
J Vet Clin 32(5) : 417-421 (2015)
http://dx.doi.org/10.17555/jvc.2015.10.32.5.417

Journal of Veterinary Clinics
www.ksvc.or.kr

Evaluation of the Biocompatibility of Cuttlebone in Mouse

Sangcheol Won, Joo Myoung Lee[1], Jongtae Cheong, Hyunjung Park and Jongpil Seo

College of Veterinary Medicine, Jeju National University, Jeju 63243, Korea

(Accepted: October 23, 2015)

Abstract : Bone grafting is widely used to bridge major bone defects or to promote bone union. Natural calcium carbonate (CC) has been used as a bone substitute material and used to scaffold for bone morphogenetic protein (BMP). The aims of this study is to evaluate the biocompatibility of cuttlebone (CB) and hydroxyapatite from CB (CBHA). Each material was shaped into disks (5 mm in diameter and 2 mm in thickness). To test biocompatibility, the disks were implanted into the dorsal subcutaneous tissue in mice. Fibrous capsule thickness around each disk was evaluated histologically at 2 and 4 weeks after implantation. Concerning biocompatibility, fibrous capsule thickness of CBHA was significantly thinner than that of CB and CHA ($p < 0.05$) at 2 and 4 weeks after implantation. Based on the clinical and histological results, CBHA would be a safe material for use inside the body and has more effective osteoconduction than CB.

Key words **:** Bone graft, Cuttlebone (CB), Hydroxyapatite (HA), Scaffold.

Introduction

The development of new biomedical devices from various materials has received a great deal of attention recently. When a material is intended for safe use inside the body, its *in vivo* performance and biocompatibility must be scrupulously verified (17).

Measuring the thickness of an encapsuling membrane around the implant is a basic tool for estimating biocompatibility (17). Utilization of the thickness of the scar capsule around an implant alone is problematic because there are factors other than the material itself that can affect capsular thickness (17). The fibrous tissue includes inflammatory components such as macrophages, fibroblasts, neutrophils, collagen, and numerous blood vessels (5), and capsule formation depends on various factors, including implant size (1), shape (16), surface texture (3), surface chemistry (8), pore size (20), and implantation site (2). Subcutaneous implantation of biomaterials induces acute and chronic inflammatory reactions resulting in fibrous tissue formation around the device (5). Thickness appears to directly correspond with the other cellular components present in the fibrous tissue matrix (5). The tissue and cellular responses to implants are screened on the basis of morphologic observations on routine histologic evaluation (5).

Commonly, biocompatibility of bone graft substitutes and scaffolds are investigated by two types of *in vivo* tests: subcutaneous implant in mice (12) or rats (5), and calvarial defect models employing rabbits (11) or rats (4). Typically, before the implant, biomaterials are prepared to eliminate the immune response.

[1]Corresponding author.
E-mail : dol82@jejunu.ac.kr

The purpose of this study was to compare tissue responses after implantation of CB in a mouse model. Furthermore, the thickness of fibrous capsule surrounding specimens was measured by histologic analysis.

Materials and Methods

Fabrication of implants

Cuttlebone after defatting and freeze-drying (CB1), cuttlebone after removing organic components (CB2) and hydroxyapatite from CB2 (CBHA) were prepared from the same genus of cuttle bone (*Sepia esculenta*). The CHA implants used consisted of hydroxyapatite (HA) from coral (BoneMedik®, Metabiomed, Korea). The CB1 implants were processed in several steps that included defatting, freezing, drying, and sterilization (Fig 1) (6). The CB2 implants were processed by removing organic components, washing, drying, and sterilizing (Fig 2) (13).

CBHA implants were processed in hydrothermal synthesis: CB2 was put in $2M(NH_4)_2HPO_4$ in a Teflon® lined hydrothermal bomb (Hydrothermal Reactor System®, Hanwoul Engineering, Korea) and heated for 16 h at 180ºC. Then, the block was immersed in 2M $(NH_4)_2HPO_4$ and treated at 200ºC for 24 h hydrothermally. After thoroughly washing with distilled water, the block was dried at 90ºC. After X-ray diffraction (XRD) examination of the block, it was used as CBHA. These implants were shaped into cylindrical disks about 5 mm in diameter and 2 mm in thickness (Fig 3) and were sterilized by ethylene oxide gas.

Experimental animals and surgical procedure

Twenty 9-week-old, 22 ± 0.2 g male BALB/c mice were used in the experiments. They were housed under a standard light-dark schedule, were fed a stock diet and had access to tap water *ad libitum*.

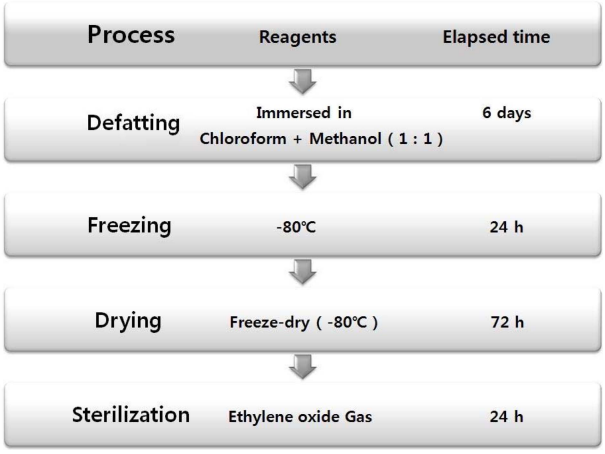

Fig 1. Preparation of CB1 for implantation. The CB1 implants were processed through defatting, freezing, drying, and sterilization.

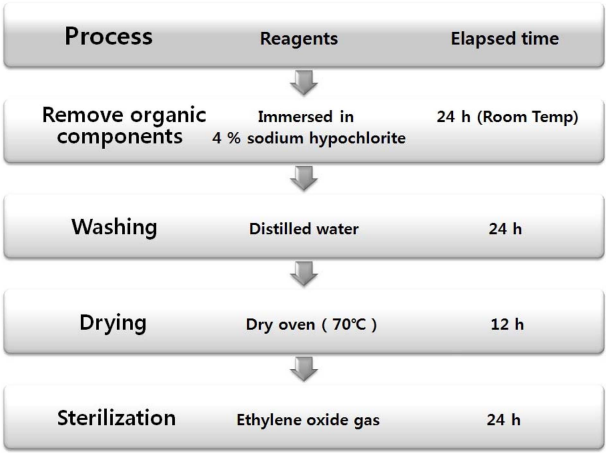

Fig 2. Preparation of CB2 for implantation. The CB2 implants were processed through removing organic components, washing, drying, and sterilization.

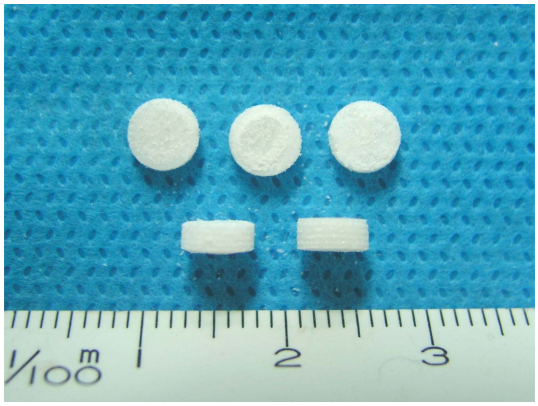

Fig 3. CB1, CB2, CBHA, and CHA implants were shaped cylindrical disks about 5 mm in diameter and 2 mm in thickness and were sterilized by ethylene oxide gas.

The experimental protocol was approved by the Animal Care and Use Committee, Jeju National University (approval number 2010-0042). The mice were divided into four experimental groups as shown in Table 1. Aseptic surgical technique was applied during the surgical procedure. Mice were anesthetized by the intramuscular injection of a dose of tiletamine/zolazepam (Zoletil50®, Virbac, France). After the anesthesia, the middle of the back of each mouse was shaved. The incision sites were washed with 70% ethanol and scrubbed with 10% povidone iodine, and a skin incision with 1.5 cm in length was made. Sterilized implants were inserted subcutaneously through the incision site (Fig 4) and the wound was closed with 4-0 nylon. Immediately following implantation, the mice were injected subcutaneously with a dose of gentamicin sulfate (Gentamicin 5% Inj.®, Daesung Microbiological Labs., Korea) for 3 days.

Table 1. Experimental design for the assessment of biocompatibility of the implants in mice

Mice	Group	Application	
		Site	Type of implants
n = 10	CB1	L. Dorsal back SQ	Cuttlebone 1
	CHA	R. Dorsal back SQ	HA from coral
n = 10	CB2	L. Dorsal back SQ	Cuttlebone 2
	CBHA	R. Dorsal back SQ	HA from cuttlebone

L.: Left, R.: Right, n: number of experimental animal, CB: Cuttlebone, HA: Hydroxyapatite, SQ: Subcutis

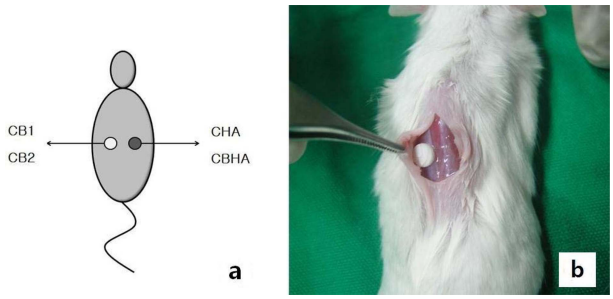

Fig 4. Schematic drawing for applied implants in subcutaneous tissue in mice (a). Photograph of applied implants (b). Sterilized implants were inserted subcutaneously through the incision site.

Histologic investigations and analysis of fibrous capsular membrane thickness

Ten mice were euthanized at 2 and 4 weeks after surgery. Implanted disks and the surrounding tissue were removed as a single mass and immediately immersed in 10% phosphate-buffered formalin for 3 days. The mass was decalcified for at least 7 days using 5% formic acid. Implants were dehydrated in an ethanol series, embedded in paraffin, and cut into 6 mm-thick sections. The sections were stained with hematoxylin and eosin (H&E) stain and Masson's trichrome stain. The fibrous capsular membrane thickness around the implants was determined with a CCD camera-based digital image analysis system. The system consisted of a microscope (Olympus BX41; Japan) and an Olympus DP20 video camera. The fibrous capsular membrane thickness was determined at each point of the horizontal and vertical lines. The fibrous capsular membrane thickness was expressed as a mean value of

eight hits.

Statistical analysis

The Statistical Package for the Social Science version 17.0 software (SPSS, USA) was used for data analysis. Mann-Whitney's *u*-test was used to evaluate differences between each group. A p value ≤ 0.05 was considered statistically significant.

Results

Characterization CBHA

The typical X-ray diffraction (XRD) patterns of product prepared by hydrothermal reaction of CB2 at 200°C for 24 h are shown in Fig 5. The XRD patterns of the CB were confirmed as HA on the basic of JCPDS card #09-0432. They were completely transformed into HA by hydrothermal reaction.

Analysis of fibrous capsular membrane thickness

The analytical results of fibrous capsular membrane thickness are shown in Fig 6. At 2 weeks, the thickness of fibrous capsule in group CBHA was significantly thinner than that of other groups. Groups CB1, CB2, and CHA displayed no significant differences. At 4 weeks, the thickness of the fibrous capsule in group CB1 was significantly thicker than that of other groups. In group CBHA, the thickness of the fibrous capsule was significantly thinner than that of other groups. There was no significant difference between group CB2 and CHA. In all groups, the thickness of fibrous capsules at 4 weeks was thinner than at 2 weeks.

Histologic evaluation

At 2 weeks after implantation, all graft materials in the subcutis were surrounded by mild to moderate dense fibrous stroma composed of abundant collagenous fiber than stained blue upon Masson's trichrome staining (Fig 7). Variable numbers of inflammatory cells such as neutrophils, macrophages, and foreign body giant cells had infiltrated around the graft materials. Some blood vessels in the subcutis showed marked congestion. At 4 weeks after implantation, fibrous stromal reaction and inflammatory reaction around graft

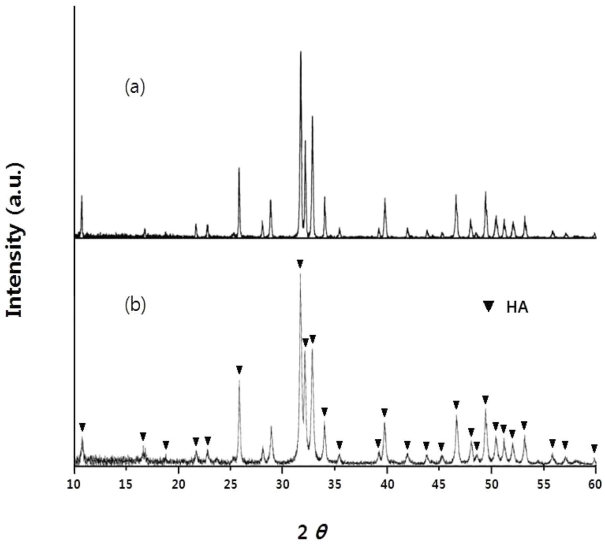

Fig 5. X-ray diffraction patterns of HA (JCPDS # 09-0432) (a) and products prepared by hydrothermal reaction of CB2 (b). Arrows indicate the picks of HA.

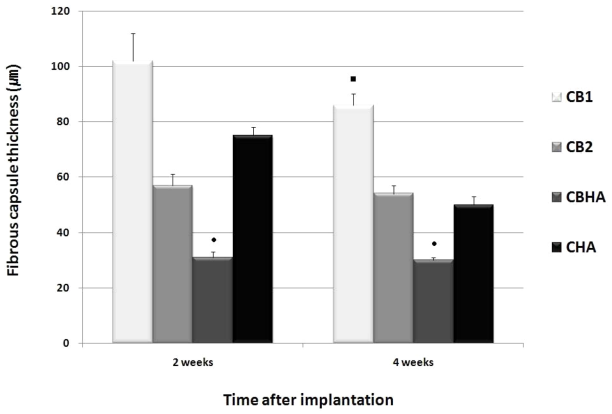

Fig 6. Thickness of fibrous capsule surrounding CB1, CB2, CBHA, and CHA implants measured at 2 and 4 weeks after implantation in mice. Values are expressed as mean ± SE.
● Significantly lower than other groups at 2 and 4 weeks after implantation ($p < 0.05$).
■ Significantly higher than other groups at 4 weeks after implantation ($p < 0.05$).

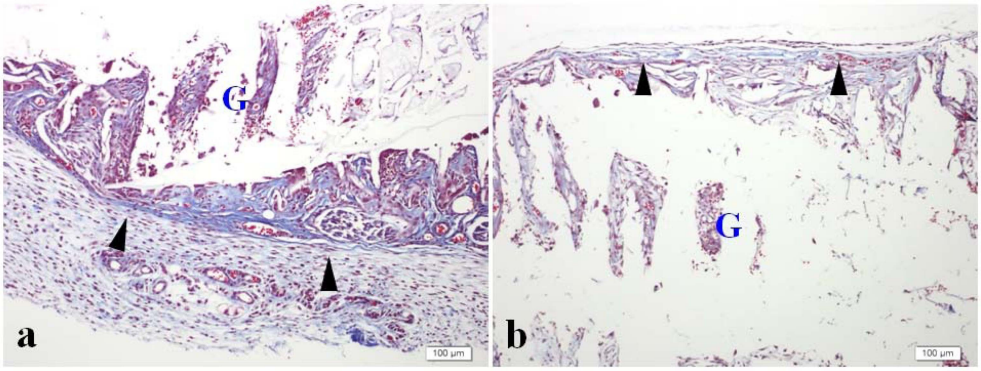

Fig 7. Granulation tissue formation (arrowheads) around graft materials (G) in group CB2 (a) and CBHA (b) at 2 weeks after implantation. Group CB2 showed more thick fibrous tissues and inflammatory reactions than group CBHA. The sections were stained using Masson's trichrome.

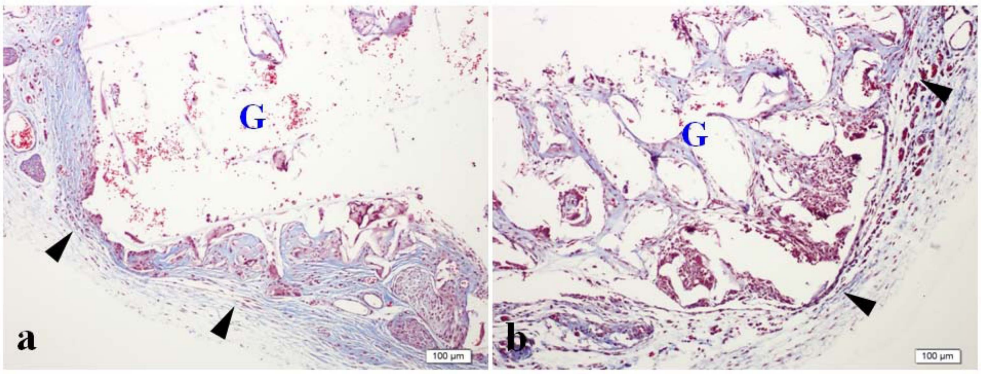

Fig 8. Granulation tissue formation (arrowheads) around graft materials (G) in group CB2 (a) and CBHA (b) at 4 weeks after implantation. Group CB2 still showed thicker fibrous tissues than group CBHA. The sections were stained using Masson's trichrome.

materials had gradually decreased compared to the mice observed 2 weeks (Fig 8). Occasionally, new formed capillaries were observed in the proliferated fibrous stroma.

Discussion

Transformation of CB into CBHA through a hydrothermal reaction process has been widely-reported (10,19), and CBHA has been confirmed by X-ray diffractometry analysis in this experiment. CB was completely transformed into the same CBHA, although the conditions of time and temperature were different in this study.

Tissue reactions that are important from the standpoint of biocompatibility mainly relate to an inflammatory reaction (17). In this study, inflammation process were observed for 4-week period. Within 24 hours, macrophages were found in close contact with the implant surface (18). Then, fibroblasts and connective tissue proliferated and, finally, the implant was encapsulated. The thickness of fibrous tissue was widely variable and the fibrous tissue surrounding subcutaneous implants was thinner than that surrounding intraperitoneal ceramic (5). In this experiment, implantation was inserted subcutaneously. Previous tests of biocompatibility established that more biocompatible implants had thinner surrounding connective tissue (9). The same result from mouse abdominal connective tissue was reported in other study (12). In this experiment, significantly thinner connective tissue was observed in the CBHA group ($p < 0.05$). CBHA was confirmed to have higher biocompatibility than CB1, CB2, and CHA. The rough-textured surfaces generate thicker capsules, and smooth surfaces usually generate thinner capsules (9). Superior tissue compatibility should be associated with smooth, well-contoured implants with no acute angles (18). The long-term biological response clearly implicates macrophages as the dominant cell type at implant surfaces and that implant stability depends largely on the dynamic behavior of macrophages. These observations suggest that the CHA surface is rougher than that of CBHA. This was grossly evident in the present study. Furthermore, group CB2 displayed thinner connective tissue than that of group CB1 in 2 and 4 weeks. These results indicate that CB2 preparation is more efficient than that of CB1, but no significant difference between them. The thickness of connective tissues tended to be thinner in all groups at 4 weeks. Many researchers have been trying to find out the ideal conditions in the preparation of allograft or xenograft materials. The most generally-used methods are demineralization, freezing, freeze-drying, defat-freezing, and freeze-drying after defatting. Freezing after defatting confers a higher bone neogenesis effect than freeze-drying after defatting (7). Specimens processed with freezing after defatting, however, are more limited with respect to transport and storage than freeze-drying after defatting (14). Also, as described above, the preparation methods associated with allograft and xenograft uniquely involve materials that contain BMP. Bone allograft or xenograft materials containing BMP are reported as being stable when they are processed with freeze-drying after defatting (6). But, CB is a natural calcium carbonate (CC) ceramic that is different from the implant materials applied in the aforementioned preparation methods. CB1 was processed with freeze-drying after defatting (Fig 1) and CB2 was processed as described previously (Fig 2) (13).

The ratio of cell components of fibrous capsules changes with the time after implantation (15). In their study, the population of fibroblasts in fibrous capsules decreased gradually and the population of fibrocytes increased gradually after implantation. Presently, the population changes of fibroblasts and fibrocytes showed a similar trend at 4 weeks after implantation.

Presently, CBHA was the most biocompatible materials among the experimental implants. However, further experiments are necessary to find out more details about the suitable preparation of CB.

References

1. Aalto M, Heppleston AG. Fibrogenesis by mineral fibres: an in-vitro study of the roles of the macrophage and fibre length. Br J Exp Pathol 1984; 65: 91-99.
2. Bakker D, van Blitterswijk CA, Hesseling SC, Grote JJ. Effect of implantation site on phagocyte/polymer interaction and fibrous capsule formation. Biomaterials 1988; 9: 14-23.
3. Behling CA, Spector M. Quantitative characterization of cells at the interface of long-term implants of selected polymers. J Biomed Mater Res 1986; 20: 653-666.
4. Bosch C, Melsen B, Karin V. Guided bone regeneration in calvarial bone defects using polytetrafluoroethylene mem-

branes. Cleft Palate-Craniofacial Journal 1995; 32: 311-317.
5. Butler K, Benghuzzi H, Puckett A. Cytological evaluation of the tissue-implant reaction associated with S/C and I/P implantation of ALCAP and HA bioceramics in vivo. Pathol Res Pract 2001; 197: 29-39.
6. Choi IH, Lee CI. Effectiveness of transplantation by freeze-dried bone of goat to dogs. Korean J Vet Clin Med 1998; 15: 442-449.
7. Choung PH. An experimental study of undemineralized freeze-dried human bone. J Korean Assoc Maxillofac Plast Reconstr Surg 1996; 18: 164-174.
8. Clark AE, Hench LL, Paschall HA. The influence of surface chemistry on implant interface histology: A theoretical basis for implant materials selection. J Biomed Mater Res 1976; 10: 161-174.
9. Dupoirieux L, Pourquier D, Neves M, Téot L. Resorption kinetics of eggshell: An in vivo study. J Craniofac Surg 2001a; 12: 53-58.
10. Dupoirieux L, Pourquier D, Picot MC, Neves M. Comparative study of three different membranes for guided bone regeneration of rat cranial defects. Int J Oral Maxillofac Surg 2001b; 30: 58-62.
11. Durmuş E, Çelik I, Aydin MF, Yildirim G, Sur E. Evaluation of the biocompatibility and osteoproductive activity of ostrich eggshell powder in experimentally induced calvarial defects in rabbits. J Biomed Mater Res Part B: Appl Biomater 2007; 86B: 82-89.
12. Kim CB, Lee MH, Kim BI, Min BW, Kim MH, Choe ES, Cho HW. Comparative biocompatibility of metal implants in connective tissue of abdominal wall of the mouse. J Toxicol Pub Health 2004; 20: 13-20.
13. Kim, JJ, Kim HJ, Lee KS. Evaluation of biocompatibility of porous hydroxyapatite developed from edible cuttlefish bone. Key Engineering Materials 2008b; 361: 155-158.
14. Lee JI, Song HN, Kim NS, Choi IH. Conparison of osteoinductive effect of freezing, freeze-drying and defat-freezing implant preparation for allograft in rabbit. Korean J Vet Res 2007a; 47: 219-228.
15. Li DJ, Ohsaki K, Li K, Cui PC, Ye Q, Baba K, Wang QC, Tenshin A, Takano-Yamamoto T. Thickness of fibrous capsule after implantation of hydroxyapatite in subcutaneous tissue in rats. J Biomed Mater Res 1999; 45: 322-326.
16. Matlaga BF, Yasenchak LP, Salthouse TN. Tissue response to implanted polymers: The significance of sample shape. J Biomed Mater Res 1976; 10: 391-397.
17. Ryhänen J, Kallioinen M, Tuukkanen J, Junila J, Niemelä E, Sandvik P, Serlo W. In vivo biocompatibility evaluation of nickel-titanium shape memory metal alloy: Muscle and perineural tissue responses and encapsule membrane thickness. J Biomed Mater Res 1998; 41: 481-488.
18. Salthouse TN. Some aspects of macrophage behavior at the implant interface. J Biomed Mater Res 1984; 18: 395-401.
19. Sivakumar M, Kumart TSS, Shantha KL, Rao KP. Development of hydroxyapatite derived from Indian coral. Biomaterials 1996; 17: 1709-1714.
20. White RA, Hirose FM, Sproat RW, Lawrence RS, Nelson RJ. Histopathologic observations after short-term implantation of two porous elastomers in dogs. Biomaterials 1981; 2: 171-176.

쥐에서 오적골 생체적합성 평가

원상철 · 이주명[1] · 정종태 · 박현정 · 서종필

제주대학교 수의과대학

요 약 : 골대체재는 지연유합이나 유합부전 그리고 골절술과 관절고정술 시 골편의 연속성 확립이 필요한 경우 골절의 주요 결손부위를 채우는데 주로 활용되고 있다. 자가골을 대체할 수 있는 천연 골이식재의 대표적인 것이 calcium carbonate (CC)이며, 갑오징어의 오적골(Cuttlebone, CB) 또한 천연 CC로 이루어져 있다. 본 연구에서는 오적골의 다양한 전처리 후 직경 5 mm 두께 2 mm의 형태로 가공하여 생체적합성을 평가하고자 하였다. 조직검사에서 결합조직 두께는 2, 4주차 모두 CBHA군에서 가장 유의성 있게 얇았다 ($p < 0.05$). 이상의 결과들은 CBHA가 생체 내에 적용하는 골대체재로서 생체적합성이 매우 높은 것으로 나타났다. 따라서 CBHA는 편평골에 있어 생체적합성이 뛰어난 골대체재로 그 가치가 있는 것으로 생각되며, 수의 임상에 있어서 활용성이 매우 높을 것으로 사료된다.

주요어 : 골이식, 오적골, 수산화인회석, 담체