Original Article
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Use of Chitosan-Gelatin Sponge as a Bone Substitute Material in Rabbit Model

Saowaluck Limmonthol, Thongchai Nuntanaranont,† Kwunchit Oungbho,‡ Wilad Sattayasansakul§*

Abstract

This study aimed to investigate the bone healing capacity of using chitosan-gelatin sponge as bone substitute material in rabbit calvarial bone defects and to compare with autogenous bone grafting. Six New Zealand white rabbits were included in the study. Two identical 8 mm diameter cranial bone defects were prepared in each rabbit and grafting was done with autogenous bone from defect preparation and chitosan-gelatin sponge. After 12 weeks, bone defects of the two groups were evaluated qualitatively and quantitatively by means of radiography (step-wedge calibration, imaging densitometry) and histology (histomorphometry, digital imaging analysis). Results showed that chitosan-gelatin sponge promoted bone healing in part by its osteoconductive property. Up to 40-45% of areas of new bone formation were found in the specimens from chitosan-gelatin sponge group. There was statistical difference in both radiographic optical density and histomorphometric percentage bone area when comparing between the two groups ($p < .05$). To conclude, the use of only chitosan-gelatin sponge could not replace autogenous bone graft with regard to bone healing capacity. However, further studies of other modifications of chitosan-gelatin sponge grafting are indicated.

Key words : animal model; bone substitute material; chitosan-gelatin sponge

Introduction

Oncologic surgery, traumatic injury and congenital disorder are known to be contributing factors of large bony defects in the maxillofacial region of patients. In the reconstruction of large bony defects, autogenous bone grafting remains to be the most promising treatment modality. However, limitation in tissue supply and surgical morbidity of the different body donor sites have motivated the exploration in the use of bone substitute materials. These bone substitute materials, such as hydroxyapatite, ceramic and polymer, are noted to induce osteogenesis by acting as a biologic scaffold for ingrowth of various cells and tissues essential for bone formation.

Chitosan is a biocopolymer comprising of glucosamine and N-acetylglucosamine. It is derived from the partial deacetylation of chitin which is found in the exoskeleton of insects and marine invertebrates. The biochemical structure of chitosan is similar to that of glycosaminoglycan, an important constituent of the extracellular matrix of human hard tissue. During the past decade, a number of researchers have investigated the potential application of chitosan to promote bone regeneration.[1-5] However, as a bone graft substitute, chitosan itself is very viscous and slowly degradable.[1] Consequently, it is usually applied in combination with other materials to improve the degradation property.[6-8] One of these materials that are frequently employed is gelatin. Gelatin is a heterogenous mixture of water soluble proteins derived from

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hydrolysis of collagen. Being biodegradable and biocompatible, gelatin can form crosslinkage with chitosan to give a more optimal scaffold for tissue-engineered osteogenesis, especially in gelatin sponge form which has been found to be an effective carrier material for bone regeneration.[9-11] Recent investigations have documented the usefulness of chitosan-gelatin sponge as a biodegradable and biocompatible porous matrix and a biological scaffold for fibroblast proliferation based on *in vitro* studies.[1,12,13] However, there is still minimal data of *in vivo* application of chitosan-gelatin sponge in bone regeneration in the current literature.

The present study aims to investigate the bone healing capacity of using chitosan-gelatin sponge as a bone substitute material. Based on experimental calvarial bone defect in rabbits, the osseous regenerative potential of chitosan-gelatin sponge was examined and compared with autogenous bone grafting.

Materials and Methods

A. Chitosan-gelatin sponge preparation

One percent chitosan (Fluka® medium viscosity) in 1% acetic acid solution and 5% aqueous gelatin B solution (Sigma® medium viscosity, 225 bloom) were crosslinked by using 25% gluteraldehyde solution. Based on Oungbho's proportion,[1] 33.5 mg and 6.67 mg of gluteraldehyde were used for each gram of chitosan and gelatin B respectively.

A mixture of the crosslinked chitosan solution and the crosslinked gelatin B solution was then prepared in the ratio of 1:10 by weight to get the stable chitosan-gelatin foam.[1] This was finally put under freeze-drying to obtain the chitosan-gelatin sponge (CGS). Disks of 8 mm diameter and 3 mm thickness were cut from the CGS (Fig.1 a). Before using in the animal experiment, the CGS disks were treated by ethylene dioxide gas for sterilization.

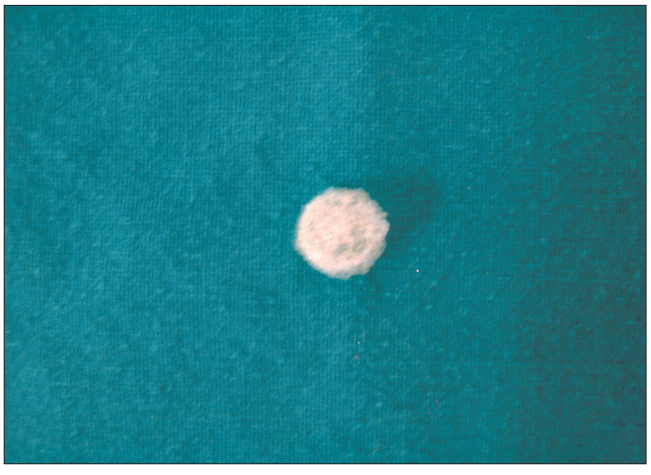

Fig. 1 ***(a)*** *Chitosan-gelatin sponge disk*

B. Macroscopic and SEM features of chitosan-gelatin sponge

The CGS appeared brittle when dehydrated but became soft, malleable and slightly expanded after soaking with 0.9% NaCl solution prior to application into bone defects (Fig. 1 b). Under SEM, CGS exhibited a three-dimensional porous structure carrying pores of 200 µm that created an anastomosing network throughout the chitosan-gelatin matrix.

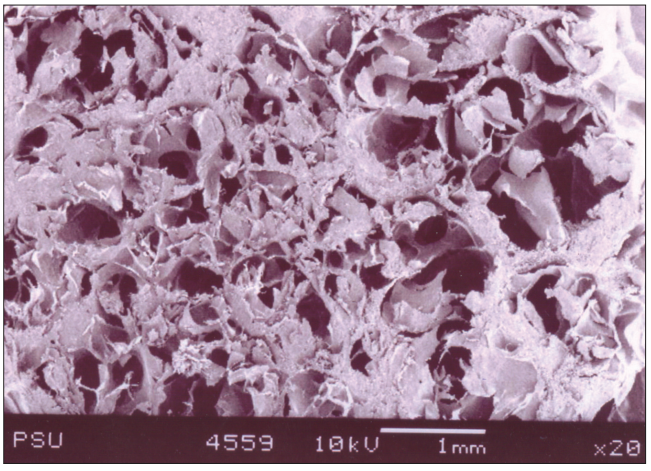

Fig. 1 ***(b)*** *SEM of chitosan-gelatin sponge.*

C. Animal preparation and specimen evaluation

Six New Zealand white rabbits were used in the study. Two identical circular bone defects, each of 8 mm diameter, were created on the cranium of each rabbit. The bone removed during defect preparation was crushed and stored for following use. Each defect was then immediately grafted with either autogenous bone or CGS disk. Landmark-holes of 4 points per defect were made and filled with amalgam for easy identification after the sacrifice period (Fig. 2). The animals were euthanized at 12 weeks post-surgery. All animal procedures were approved by the Prince of Songkla University Animal Research Ethical Committee.

All bone defects were evaluated both radiographically with step-wedge calibration and histologically with qualitative and quantitative assays. The radiographs were scanned by Imaging Densitometer (Bio-Rad® Model GS-700) and analysed by using computer software (Molecular Analyst®, Bio-Rad Laboratories Incorporation.) to obtain *the average radiographic optical density (Mean OD)* of each defect. The Mean OD or

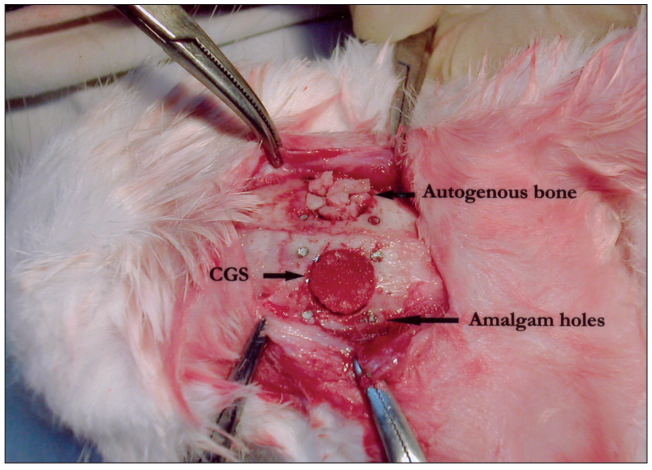

Fig. 2 *Autogenous graft and chitosan-gelatin sponge (CGS) in rabbit bony defects.*

pixel count per unit area of each defect was repeatedly measured for three times in order to minimize the measuring error.

The decalcified H&E preparation of the specimens from both types of defects were sectioned in 5 μm thickness. Two slides from the center of each defect were randomly chosen to evaluate the amount of new bone formation by measuring the area containing osteoblastic cells in five microscopic fields (four peripheral, one central). The areas of new bone formation were summated and the value was expressed as a percentage of the total field of vision or mean percentage of bone area by using Leica Qwin Imaging Analysis. Each slide was repeatedly evaluated for three times to test the reproducibility of measuring equipment.

D. Statistical analysis

All data were processed on the computer and analysed using the statistical package for social sciences software (SPSS 10.0, SPSS Incorporation). Pair t-test was used to compare the values of mean OD and mean percentage of bone area obtained from the two types of grafted bone defects. The level of statistical significance was set at $p < .05$.

Results

Following the grafting procedure, all wound healing and recovery of the rabbits were uneventful without any postoperative complications. No osteolysis, hyperplasia or other negative tissue responses were found in the bone defects throughout the 12 weeks healing period. On gross examination of the specimens, all autogenous graft samples revealed greater area of smooth and hard bony surface than the CGS graft samples, when comparing site by site in the same animal.

The specimen radiographs demonstrated that both grafting materials could integrate with the surrounding bone but for the same specimen, the autogenous graft sites revealed higher radiopacity than the CGS graft sites (Fig. 3). Moreover, some autograft-filled defects showed a speckled pattern of radiographic density, suggesting the presence of residual mineralized graft material that had not been resorbed or remodeled.

In H&E microscopic sections, the presence of new bone growth, vascular and osteoid tissues at various stages of maturity was detected over the periphery of all defects.

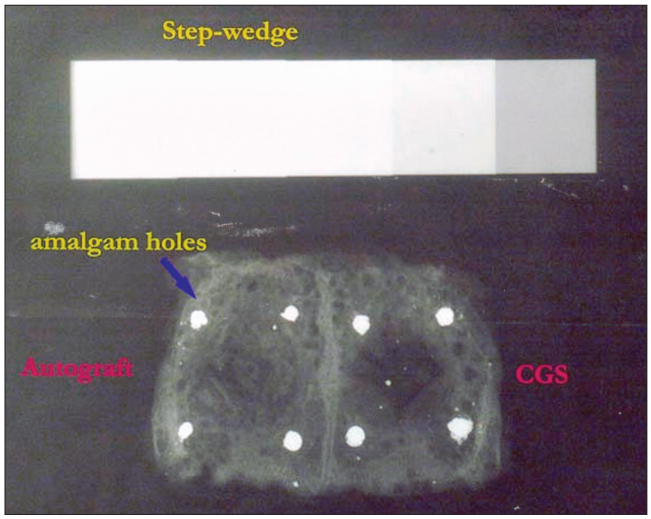

Fig. 3 *Radiograph of specimen.*

However, regions of active bone healing, as reflected by the turnover of graft material and dense areas of bone formation, were more readily seen in the autograft defects than in the CGS graft defects (Fig. 4 a, b). In all CGS graft samples, fibrous connective tissue stroma over the residual network of CGS graft was observed at the center of the defect without infiltrate of inflammatory cells (Fig. 5 a, b). On the other hand, different sizes of dead bone spicules containing empty lacunae were revealed in some regions of the autograft defects, suggesting the presence of significant amount of residual autograft bone remaining in the defects.

In term of quantitative measurements, comparison of the values of mean OD and mean percentage of bone area between the two grafting materials was illustrated in Figure 6a and b. When comparing the data between the CGS group and autogenous graft group, the difference in the radiographic optical density test ($p = .004$) and the histomorphometric new bone area analysis ($p = .001$) was both statistically significant.

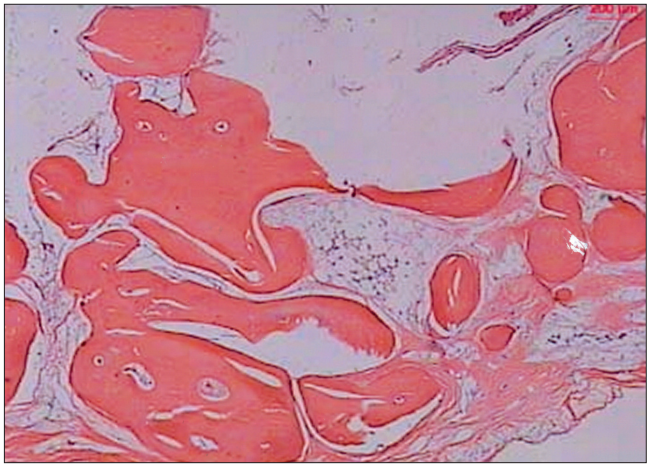

Fig. 4 *H&E section of autogenous graft specimen at x5 magnification*
(a) *new bone formation area at central region of specimen*

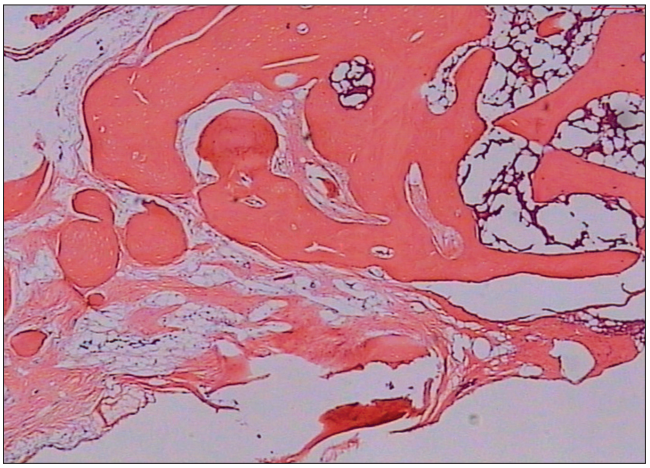

Fig. 4 ***(b)*** *new bone formation area at peripheral region of specimen*

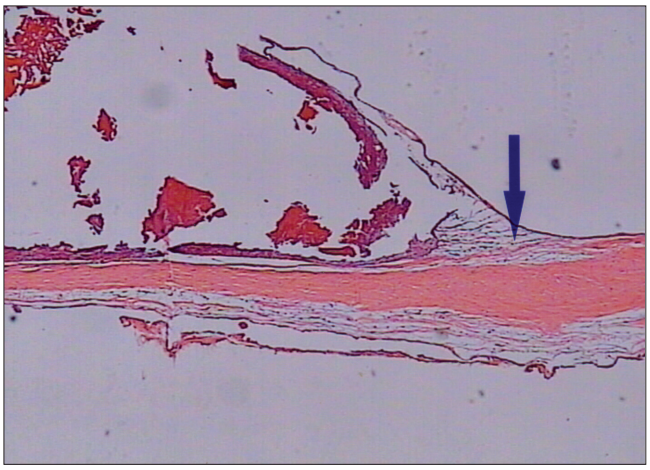

Fig. 5 *H&E section of CGS specimen at x5 magnification*
(a) *fibrous connective tissue (blue arrow) at central region of specimen*

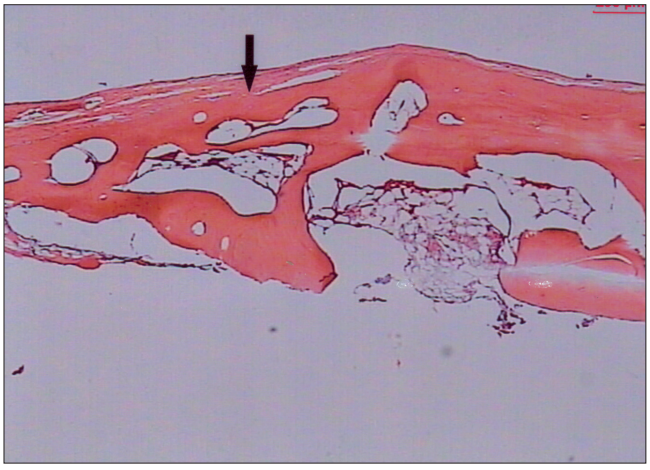

Fig. 5 ***(b)*** *new bone formation area (black arrow) at peripheral region of specimen*

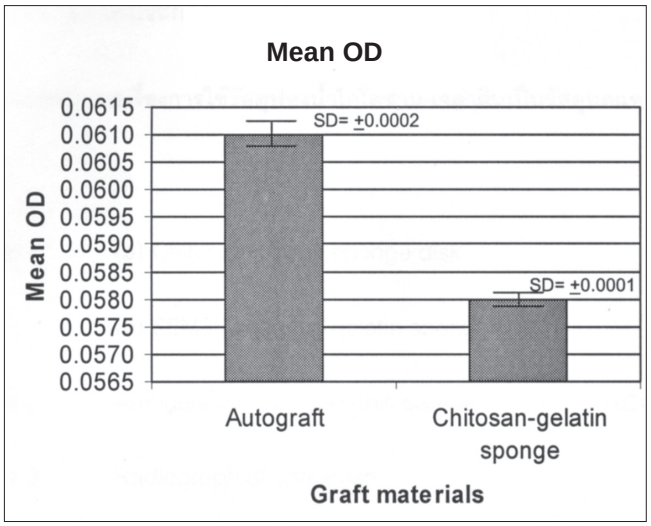

Fig. 6 ***(a)*** *the average radiographic optical density or Mean OD bar chart*

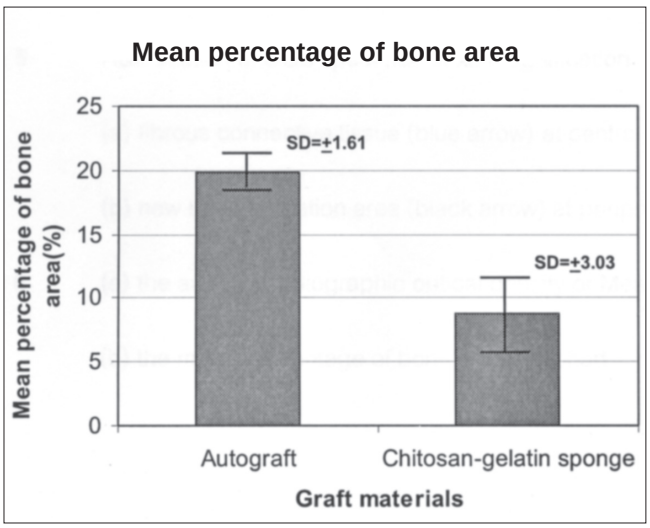

Fig. 6 ***(b)*** *the mean percentage of bone area bar chart*

Discussion

An ideal bone graft substitute should possess the following biological and physical properties: biocompatible, osteoconductive, osteoinductive, resorbable, strong, malleable inexpensive and user-friendly. It should also facilitate cellular adhesion, proliferation and differentiation.[14-16] In this study, CGS has revealed many properties of an ideal bone substitute. Being malleable, it can be manufactured to appropriate shape and size for surgical use. Its high biocompatibility is reflected by the favourable tissue response of the rabbits without any incidence of postoperative infection. Its ability to promote new bone formation was further substantiated in the study qualitatively and quantitatively. Hence, there is positive potential for the clinical use of CGS as bone graft substitute.

Various methods were employed to assess the formation of new bone in the rabbit cranial defects. Qualitative assessment was made on radiographs and histologic sections, and the quantitative measurements were based on imaging densitometer and histomorphometry. Moreover, the use of step wedge method for calibrating radiographic films and that of digital imaging analysis system for histomorphometry have enhanced the reliability and reproducibility of the experimental results.

Radiographic assay demonstrated a lesser degree of radio-opacity in all CGS graft defects when compared with the autograft defects which was understandable. In addition, some of autogenous graft specimens revealed a speckled radiographic pattern. This could be attributed to the presence of residual grafted bone that had not been resorbed or remodeled. Apparently, having a membranous bone origin from the rabbit cranium, the autogenous bone graft used in the study was characterized by lower content of osteogenic cells leading to relative difficulty or prolonged duration in resorption and remodeling.[17,18] This explanation appeared compatible with the radiographic quantitative finding of a significant difference in Mean OD between autograft defects and CGS grafted defects ($p < .05$). Presumably, the residual cortical bone was considered to cause the significantly higher Mean OD in all autogenous graft defects when compared with CGS defects.

Histologically, bone defects grafted with CGS showed viable lamellar bone with osteoblastic activities and vascular ingrowth at the periphery only, and autograft defects revealed new bone formation throughout the area. While it is currently understood that autogenous bone grafts exhibit osteoinduction and osteogenesis properties, one may surmise that CGS can promote new bone formation at least in part by means of osteoconduction. Such speculation is in fact compatible with the findings from previous studies.[19,20] Similar pattern of peripheral predominance of bone formation through osteoconduction with other bone graft substitutes has also been described in the literature using canine calvarial implantation site.[21,22] In addition, among the CGS defects, there was significant ingrowth of fibrous tissue with small amount of residual CGS at the center, confirming that CGS is biodegradable but having incomplete degradation within a period of 12 weeks. Accordingly, degradation is known to progress by lysozymic hydrolysis via the macrophage from the vascular tissues.[4] The relatively low vascularity of the cortical membranous bone in rabbit cranium might be contributing to the resultant degree of CGS degradation and extent of new bone formation. Furthermore, according to the study of Alberius and Johnell,[23] the observation of viable lamellar bone with osteoblastic activity in both CGS and autografted bone defects might suggest that CGS promoted new bone formation via intramembranous ossification in the same pattern as autogenous bone.

In the current study, CGS grafted defects demonstrated up to 40-45% of new bone formation based on histomorphometric analysis. Previous researchers working on other chitosan-containing, porous bone graft substitutes (such as porous chitosan matrix grafted in rat calvarium) also reported positive results but unfortunately, no quantitative measurement had been undertaken.[24] When considering together with other bone substitute materials, the present results from using CGS are comparable to the bioactive glass ceramic (40% of new bone formation)[25] and polylactic acid with alpha tricalcium phosphate (14% of new bone formation in a loaded implant model in sheep).[26]

Despite this study was based on only 12 cranial bone defects in 6 rabbits, the results demonstrated statistically significant difference between the two types of graft material with regard to histomorphometric and radiologic assessment. While the autogenous graft samples have provided a positive control in the experiment, it has to be admitted that the addition of non-grafting bony defects to serve as negative control would contribute to more direct evaluation of the bone healing capacity of CGS. Although, the size of the cranium bone defect used in this study did not reach the critical dimension commonly accepted in the rabbit model (15 mm),[27] limitation had come from the relatively small statue of the rabbits used in the study. Nevertheless, the current 8 mm bony defects had allowed proper preparation and evaluation in the comparison of the bone healing capacity between the two types of graft material.

Although, chitosan itself has been proven to promote bone growth in previous reports,[2,3] it is difficult to assess such

osteogenic behaviour in the CGS used in this study. This is because the proportion of chitosan in the CGS used in the study was relatively small (chitosan:gelatin B ratio = 1:10 by weight). A higher content of chitosan used in the formula could adversely affect the stability of CGS because of its high viscosity which might prevent sponge formation.[1] Moreover, since it is generally accepted that autogenous bone graft is the only tissue transplant that possesses direct osteogenic effect due to its vital cellular contents, the CGS used in the study were not expected to be directly bone regenerative but rather, its porous structure could provide an optimal passive scaffold for the in-growth of bone-forming cells and tissues, possibly via *in vitro* cell-seeding before the experimental grafting procedures. Thus, it appears that the current findings may provide some solid background information for further studies in other modifications of CGS grafting.

Conclusion

Chitosan-gelatin sponge promotes bone healing in part by its osteoconductive property. Therefore, the use of only chitosan-gelatin sponge as a bone substitute material could not possibly replace autogenous bone grafting. However, the porous nature of chitosan-gelatin sponge may represent a biochemical scaffold that carries similar physical and biochemical properties of the cancellous bone matrix. Further studies should be performed by using combinations of chitosan-gelatin sponge with different platforms of osteogenic cells, having the target of approaching an ideal bone substitute material.

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บทวิทยาการ

การใช้วัสดุฟองน้ำไคโตซาน-เจลาตินเป็นวัสดุทดแทนกระดูกในสัตว์ทดลองกระต่าย

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบความสามารถในการหายของกระดูกเมื่อใช้วัสดุฟองน้ำไคโตซาน-เจลาตินเป็นวัสดุทดแทนกระดูกในแบบจำลองกระดูกกะโหลกศีรษะของสัตว์ทดลองกระต่ายและเปรียบเทียบกับการหายของกระดูกเมื่อใช้กระดูกปลูกถ่ายเนื้อเยื่อตนเอง โดยทำการศึกษาในกระต่ายพันธุ์นิวซีแลนด์ไวต์ จำนวน 6 ตัว แต่ละตัวจะทำรอยวิการบนกระดูกกะโหลกศีรษะเป็นรูปวงกลมขนาด 8 มิลลิเมตร 2 ข้างแล้วใส่วัสดุฟองน้ำไคโตซาน-เจลาตินและกระดูกปลูกถ่ายเนื้อเยื่อตนเองแต่ละข้างตามลำดับ ใช้ระยะเวลาการทดลองนาน 12 สัปดาห์ แล้วจึงนำตัวอย่างมาวิเคราะห์โดยภาพรังสีร่วมกับการเทียบมาตรฐานด้วยสเตปเวดส์ และลักษณะทางจุลพยาธิวิทยาเปรียบเทียบทั้งในเชิงคุณภาพและปริมาณ ผลการศึกษาพบว่า วัสดุฟองน้ำไคโตซาน-เจลาตินสามารถทำให้เกิดการหายของกระดูกได้โดยทำหน้าที่เป็นโครงร่างของเซลล์สร้างกระดูก รวมทั้งสามารถพบบริเวณที่มีการสร้างกระดูกใหม่ประมาณร้อยละ 40-45 เมื่อเปรียบเทียบกับกระดูกปลูกถ่ายเนื้อเยื่อตนเอง แต่อย่างไรก็ตามค่าความเข้มของภาพรังสีและปริมาณของกระดูกทางจุลพยาธิวิทยาที่วัดได้เป็นร้อยละระหว่างวัสดุปลูกกระดูกทั้ง 2 ชนิดนี้มีความแตกต่างกันทางสถิติอย่างมีนัยสำคัญ ($p < .05$) จากการทดลองนี้สามารถสรุปได้ว่า การใช้วัสดุฟองน้ำไคโตซาน-เจลาตินอย่างเดียวเป็นวัสดุทดแทนกระดูกสามารถทำให้เกิดการหายของกระดูกได้แต่มีคุณภาพไม่ดีนักเมื่อเปรียบเทียบกับกระดูกปลูกถ่ายเนื้อเยื่อตนเอง อย่างไรก็ดีควรจะมีการศึกษาเพิ่มเติมเกี่ยวกับการใช้วัสดุฟองน้ำไคโตซาน-เจลาตินในรูปแบบอื่นเพื่อเป็นวัสดุทดแทนกระดูก

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