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*Original Article*

# Effect of chitosan scaffold on bone healing in rabbit calvarial defect

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**Abstract**

The aim of this study was to evaluate the effect of 3D 2% w/v chitosan scaffolds on new bone formation in a rabbit calvarial defect. Two bi-cortical skull defects were prepared in 16 male rabbits and divided into two groups: autogenous (positive controls) and chitosan scaffolds (experimental groups). The animals were sacrificed at 2, 4, 8, and 12 weeks. New bone formation was evaluated by micro-CT and histomorphometric analysis. The positive control group demonstrated significantly greater bone formation. The experimental groups showed viable lamellar bone with osteoblasts forming bone from the defect margin. Histomorphometric analysis showed an increased percentage of new bone formation over time, and a micro-CT analysis also showed an increased percentage of bone volume over time. Although the new bone formation was not as good as the positive control groups, our in-house fabricated chitosan scaffolds presented good biocompatibility and osteoconductive properties in a rabbit calvarial defect.

**Keywords:** boneformation, chitosan, scaffold

## 1. Introduction

Bone reconstruction in the oral and maxillofacial region permits esthetic and functional recovery of the craniofacial skeleton. Management of large bone defects due to trauma, degenerative disease, congenital deformities, and tumor resections remain a complex issue for orthopedic reconstructive surgeons**.** Autologous bone grafts are still considered the gold standard for reconstruction of bone defects, but donor site morbidity and size limitations are a major concern. The use of bioartificial bone tissues may help to overcome these problems. The reconstruction of large volume defects remains a challenge despite the success of reconstruction of small-to-moderate sized bone defects using engineered bone tissues (Marcacci *et al.*, 2007). However, the major disadvantage of autografts is the limited tissue supply, which makes the repair of large defects problematic. An alternative to traditional graft sources is bone tissue engineering. It is a promising new approach for bone repair (Miranda *et al.*, 2011).

Biomedical polymers, such as surgical sutures, wound dressing, and braces/fixation materials, are widely used as medical service materials. Furthermore, due to their biocompatibility and bioabsorbability, they do not induce antibodies from the immune system. They are also hypo-allergenic and do not cause inflammation to human tissue (Niklason, 2000; Saito *et al.*, 2001). Therefore, biomedical polymers have become an extremely popular material in this new research domain of tissue engineering. In order to effectively and massively cultivate the tissue cells, these

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biomedical polymers are usually designed as three-dimensional (3D) scaffold structures of interconnected pores with high porosities, making it easy to implant them into the human body. Hence this kind of scaffold must have good biocompatibility, biodegradability, high porosity, and possess the appropriate mechanical properties (Austin *et al.,* 1981; Chen *et al.,* 2000; Hutmacher, 2000; Petite *et al.,* 2000; Price *et al.,* 2003; Yang *et al.,* 2001).

Chitosan is a well-known natural polymer that is biodegradable, biocompatible, and non-toxic (VandeVord *et al.,* 2002). It can be made into gelatin, fiber, and membrane shapes for various uses. Moreover, since chitosan can be obtained easily and conforms to tissue engineering application requirements, it can be implanted into the human body causing no harm (Hutmacher *et al.,* 2001; Suh & Matthew, 2000). It is a well-known suitable material for use in tissue engineering (Chenite *et al.,* 2000).

Previously, we developed a novel chitosan scaffold that demonstrated good physical and biological properties *in vitro* (Guan *et al.,* 2015). Therefore, the purpose of this study was to further investigate the *in vivo* effects of our in-house fabricated 3D 2% w/v chitosan scaffolds on new bone formation in a rabbit calvarias defect.

## 2. Methods

### 2.1 Fabrication of chitosan scaffolds

Chitosan (Sea Fresh Chitosan Lab Co, Thailand) with 85% deacetylation and a molecular weight of 57,000 daltons was utilized. To construct the scaffolds, chitosan was dissolved in 0.2 M acetic acid in final concentrations of 2% (w/v) and injected into 1M NaOH. Fibril-like chitosan particles were formed. They were then filtered through a sheet cloth and placed in 15 ml centrifuge tubes, then centrifuged at 3,000 rpm for 5 min, after which they were kept at 4 °C for 24 h and subsequently frozen at –20 °C. After 24 h, the cylindrical scaffolds were immersed in 96% alcohol for 1 h, 1 M NaOH for 5 min, and 70% alcohol for 12 h. The formed scaffolds with a diameter of 10 mm were sectioned into slices of 2 mm thickness. They were submerged into liquid nitrogen for a few seconds and then placed into 24-well plates and dried at 37 °C for 2 days.

### 2.2 Animal preparation

Sixteen male New Zealand white rabbits aged 5-7 months and weighing 3-4.5 kg were used in this study. All authors hereby declare that the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments were examined and approved by the Animal Research Ethics Committee; Prince of Songkla University (#0521.2.03/1189).

### 2.3 Surgical procedure

Anesthesia was induced using 25 mg/kg of ketamine and 5 mg/kg of diazepam intramuscularly 30 min before surgery. Thiopental (5 mg/kg) was administered intravenously and then titrated at the rate of 2 mg/kg every 15 min (with a maximum dose of 30 mg/kg) until unconsciousness was achieved.

The surgical field was disinfected with 10% povidone-iodine. A mid-sagittal incision was made after local infiltration of 1.8 ml of 2% lidocaine hydrochloride and 1:100,000 epinephrine. A subperiosteal dissection was carried out and two identical bicortical bone defects 10 mm in diameter were carefully created using small round and fissure burs with normal saline irrigation.

Sixteen rabbits were randomly divided into 2 groups. Two bicortical bony defects with a 10 mm diameter were created in the left and the right parietal bone with a small fissure bur using a micromotor. In the control group, defects were filled with autogenous bone chips that were minced with a bone morselizer (Salvin Dental Specialties Inc, Charlotte, NC, USA) and calibrated to have an equal volume with the chitosan scaffold using an acrylic mold. In the experimental group the defects were filled with a 3D 2% chitosan scaffold of 10 mm in diameter which was soaked with 0.9% normal saline for 10 min (Figure 1). The periosteum, muscle, and skin were sutured using vicryl® 4/0. Then 4 rabbits were sacrificed at the end of 2, 4, 8, and 12 weeks (Table 1) and fixed in 10% formalin before submitting them to micro-computerized tomography (micro-CT) and microscopic analysis for histomorphometric analysis.

### 2.4 Histomorphometric analysis

The specimens were trimmed until the graft area was encroached. They were then divided into 2 pieces with a stainless steel fissure bur at the center before decalcification and embedding in paraffin blocks. The specimens were sectioned along a sagittal plane to the bone surface using a

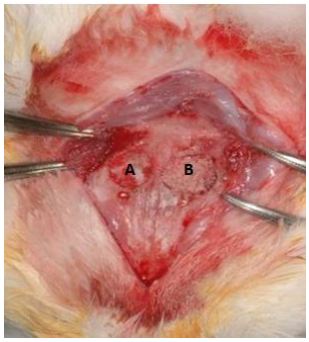


Figure 1. Bilateral calvarial defects filled with different materials: (A) 3D 2% w/v chitosan scaffold and (B) autogenous bone chip).

Table 1. Time of sacrifice in the study groups.

| Groups | Number of rabbits | | | | |
|---|---|---|---|---|---|
| | 2 weeks | 4 weeks | 8 weeks | 12 weeks | Total |
| 3D 2% w/v chitosan scaffold<br>Autogenous bone | 4 | 4 | 4 | 4 | 16 |

microtome cutting into 5 µm thickness serial sections of each specimen. Each histologic section was stained with haematoxylin and eosin (H&E). Six slices from the center of each autoginous graft defect or 3D 2% w/v chitosan scaffold defect were randomly selected to represent each defect. The sections were examined under light microscope to evaluate newly formed fibrous tissue (collagen boundless) and bony trabeculae (woven bone) with osteoblastic morphology.

### 2.5 Micro computed tomography (micro-CT)

A high resolution micro-CT system (Micro-CT80, Scanco, Medica AG, Basseesdorf, Switzerland) was used. After calibration the specimens were scanned perpendicularly to the cranium vault at 55 kVp, 72µA and 4w in high-resolution mode (18.5µm$^3$/voxel). Scanned data were reconstructed by built-in software. The region of interest (ROI) was analyzed using the following parameter for bone volume fraction (BVF): percentage of bone volume divided by total defect volume (BV/TV) (Figure 2).

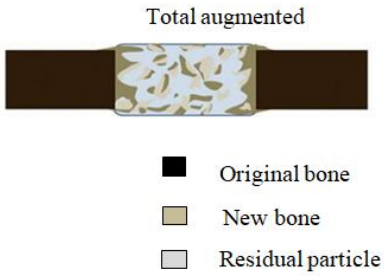


Figure 2. Schematic drawing shows the ROI (region of interest) for micro-CT analysis.

### 2.6 Statistical analysis

Statistical analysis was performed using statistical analysis software (SPSS ver16.0). Significant differences among groups were identified using the Mann Whitney U test. Data are presented as mean±SD, and where the P-value was less than 0.05 the result was considered statistically significant.

## 3. Results

### 3.1 Animals

All animals tolerated the surgical procedure and the anesthesia well. They recovered rapidly after surgery and the entire wound healed gradually without evidence of wound infection or wound dehiscence being found during the study period.

### 3.2 Histomorphometric analysis

The new bone formation in the autogenous bone chip showed significantly greater difference than the 3D 2% w/v chitosan scaffold ($P<0.05$) during the time period of the study (Figure 3).

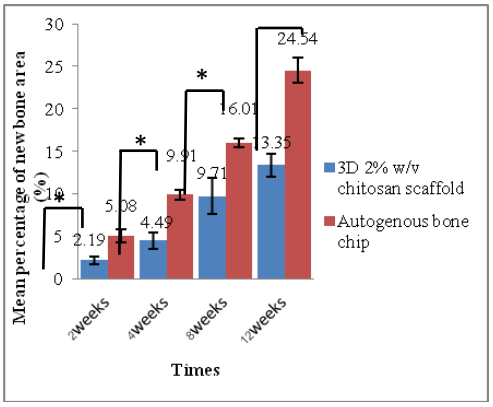


Figure 3. Histomorphometric analysis of the mean percentage of new bone area.

At 2 weeks, defects in the autogenous group demonstrated that the connective tissue infiltrated from the periphery of the defects. At 4 weeks, some autogenous bone chips had disappeared and were replaced by new bone. The defect was completely bridged and gained good continuity at 8 and 12 weeks and newly formed bone incorporated well with the graft and host bone. Clinical evaluation of 3D 2% w/v chitosan scaffolds at 2, 4, 8, and 12 weeks demonstrated difference, and histological evaluation of these specimens revealed a thin fibrous scar across the defect with some amounts of new bone being occasionally seen at the bony margin of the defect. This finding was seen more frequently in the 12-week specimens. No inflammatory cell infiltration could be detected in either group throughout the experiment periods (Figures 4 and 5).

### 3.3 Micro computed tomography analysis

At 2 weeks, the percentages of bone volume fraction for autogenous and 3D 2% w/v chitosan were 17.71±2.62 and 3.42±0.62, respectively. At 4 weeks, the percentages of bone volume fraction for autogenous and 3D 2% w/v chitosan were 21.54±1.66 and 4.57±0.82, respectively. At 8 weeks, the percentages of bone volume fraction for autogenous and 3D 2% w/v chitosan were 25.83±0.64 and 6.17±0.7, respectively. At 12 weeks, the percentages of bone volume fraction for

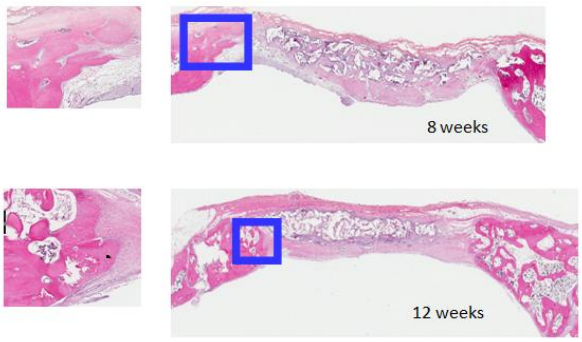


Figure 4. Histology images (H&E stained) of 3D 2% w/v chitosan scaffold.

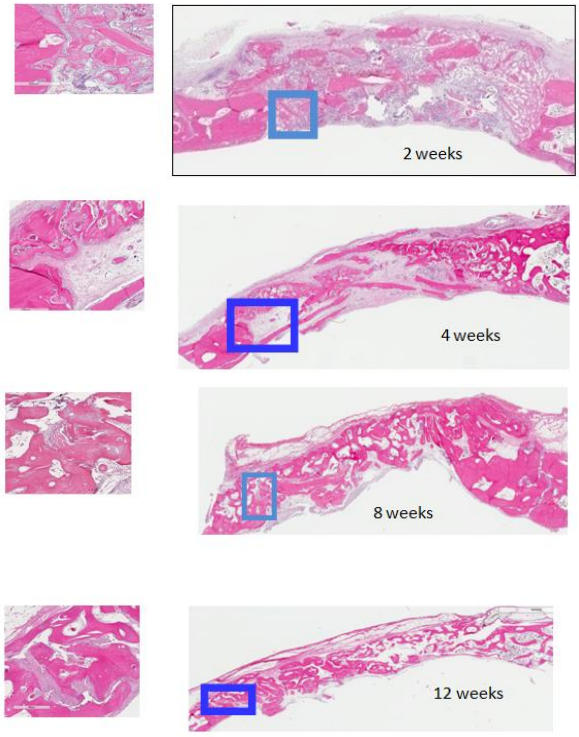


Figure 5. Histology images (H&E stained) of autogenous bone chip.

autogenous and 3D 2% w/v chitosan were 36.07±3.34 and 8.74±0.98, respectively(Figures 6, 7, and 8). Throughout the period of study, the autogenous bone chips as the gold standard positive control had significantly greater percentage of bone volume fraction than the 3D 2% w/v chitosan scaffolds ($P<0.05$).

## 4. Discussion

Chitosan is a naturally derived polysaccharide. It is a desirable material for the development of drug delivery systems and new biomedical applications in various tissues from skin to bone or cartilage due to its physicochemical and biological properties (Duarte *et al.*, 2010). The main advantages of our in-house fabricated scaffolds were related to the use of chitosan. It is not expensive and it is locally available. Even though fabrication is simple, it provides good biocompatibility *in vitro* (Guan *et al.*, 2015) and it is a promising scaffold for tissue engineering. Before clinical studies can be carried out, an *in vivo* study is required.

The results of our in-house-fabricated 3D 2% w/v chitosan scaffolds demonstrated many ideal properties for use as a bone substitute. It is a natural polymer material which can be manipulated into any shape and size including 3D scaffolds. In addition, the scaffolds in 10 mm circular shapes were easy to handle in this intraoperative experiment. Moreover, the defects repaired with these scaffolds showed very good tissue response without postoperative infection and no inflammatory cells could be detected during any time period of the experiment.

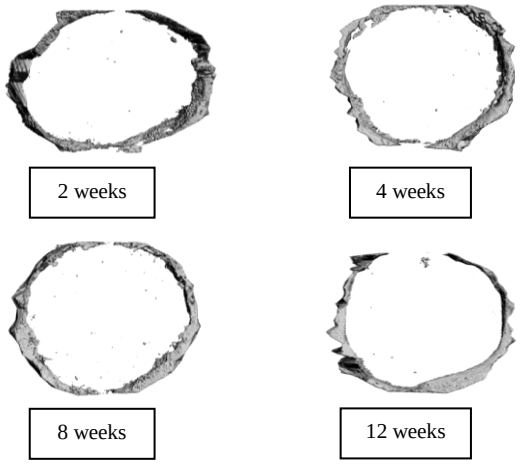


Figure 6. Micro-CT images of bone volume fraction of the 3D 2% w/v chitosan scaffold.

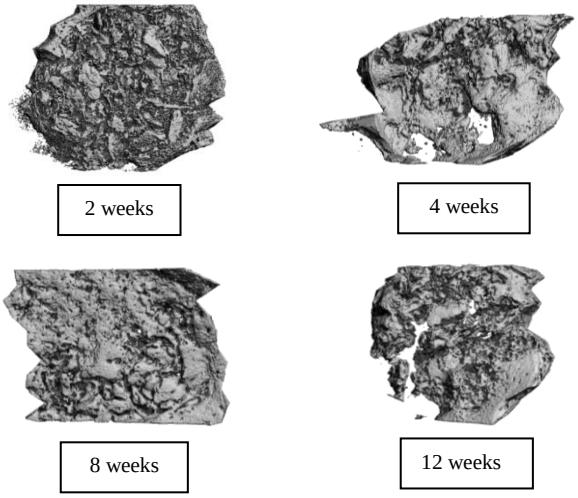


Figure 7. Micro-CT images of bone volume fraction of autogenous bone chip.

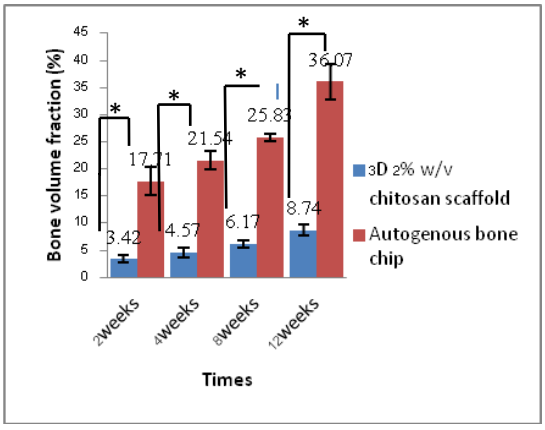


Figure 8. Micro-CT analysis of bone volume fraction.

A rabbit model has been used successfully in our previous studies concerning grafting materials (Pripatnanont *et al.*, 2007, 2009). The rabbit model has several advantages, such as standardization of experimental conditions for experiment repeatability, ease of handling (in terms of size), inexpensive costs, and rapid bone turnover rates (Muschler *et al.*, 2010). Although the critical-size defect in adult rabbits has been defined as at least 15 mm in circular diameter (Dodde *et al.*, 2000; Szpalski *et al.*, 2010), some studies reported that an 8 mm diameter defect is critical as it could not heal if bone graft was not used (Borie *et al.*, 2011). This present study used a 10 mm diameter circular defect because it was possible to create two defects in one animal. Sohn *et al.* (2010) showed no differences in bone healing between a circular defect created by a trephine sized 11 mm and 15 mm in diameters at each study period of 2, 6, 8, and 12 weeks, and there was also no differences of bone formation between 2 and 4 or between 8 and 12 weeks.

In rabbits, bone metabolism is approximately three times faster than in humans (Roberts *et al.*, 1987). Therefore, healing at 2 weeks and 4 weeks were chosen for evaluation of the early phase of the healing response in regard to the stability of the materials and the host reactions. Healing periods of 8 and 12 weeks were also selected since it is appropriate for evaluation of the late phase of the healing response in regard to bone incorporation, resorption of materials, bone remodeling, and the amount of bone regeneration (Sohn *et al.*, 2010). Although micro-CT imaging data observed throughout the entire study period revealed that the autogenous bone chip group had significantly higher bone regeneration than the experimental group ($P<0.05$), autogenous bone chip voxels and new mineralized bone voxels could not be distinguished while the experimental group represented only new bone formed voxels.

Histologic examination results revealed that defects repaired with 3D 2% w/v chitosan scaffolds showed viable lamella bone with osteoblast forming bone and blood vessel ingrowth only from the defect margin, while autograft-filled defects showed new bone formation areas throughout the defects. These findings suggest that 3D 2% w/v chitosan scaffolds promoted new bone formation by its osteoconduction property, while autogenous bone chip also has osteoconductive and osteogenesis properties (Aghaloo *et al.*, 2002; Bidic*et al.*, 2003). In addition, the predominance of bone formed through conduction from the periphery of the defect was also described by others using a rabbit calvarial implantation site (Oklund *et al.*, 1986; Sato *et al.*, 1985). Examination of 3D 2% w/v chitosan scaffold graft-filled defects also showed significant regions of fibrous tissue with small amounts of residual 3D 2% w/v chitosan scaffolds at the center. This finding suggests that 3D 2% w/v chitosan scaffolds are a biodegradable material although they did not completely degrade in this study during the time periods. The degradation of modified chitosan is known to be performed by lysozymic hydrolysis that requires macrophage cells from vascular tissue (Muzzarelli *et al.*, 1994). The lack of an abundant vascular bundle from the cortical membranous bone in a rabbit calvarium model would suggest the causes were slow degradation of 3D 2% w/v chitosan scaffolds and only small amounts of new bone tissue forming in response to this type of graft material. Moreover, results obtained with histomorhpometric analysis also showed that the mean percentage of new bone area of the autogenous group was significantly higher compared with the 3D 2% w/v chitosan scaffold group at the $P<0.05$ level. Again, the higher discrepancy of the mean percentage of new bone area between both types of graft materials was due to the osteogenesis and osteoconduction properties of the autogenous graft in promoting new bone formation, while the 3D 2% w/v chitosan scaffold has only an osteoconduction property. Additionally, the discrepancy could be due to the fact that some residual autograft bone was measured as new bone formation without intention. Therefore, the autologous bone implant material could be confounding with the new bone formation in this positive control group in bone volume fraction analysis by histomorphometry and micro-CT.

The advantages of our in-house fabricated 3D 2% w/v chitosan scaffolds include i) simple to fabricate, ii) locally available materials, and iii) the material costs are low. Our next step to improve the quality of this novel scaffold to be as good as the autogenous positive control is by incorporating collagen and silk fibronectin into the scaffold (Sangkert *et al.*, 2015, 2016). This could result in a promising scaffold for bone tissue engineering.

## 5. Conclusions

Although the new bone formation was not as good as the gold standard positive control groups, our in-house fabricated 3D 2% w/v chitosan scaffold presented good biocompatibility and osteoconductive properties in this animal model.

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