Effects of 3D 2% Weight/Volume Chitosan Scaffold on New Bone Formation in Rabbit Calvarial Defect

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Abstract

Objective: The purpose of this study was to evaluate the effect of 3D 2% w/v chitosan scaffold on new bone formation in the rabbit's calvarial defect.

Methods: Two bicortical skull defects were prepared in 16 male New Zealand white rabbits, divided into two groups. Eight rabbits were used as control group(skull defects filled with autogenous bone chip), and the other eight rabbits were used as experimental group(skull defects filled with 3D 2% w/v chitosan scaffold). The animals were sacrificed at 2, 4, 8 and 12 weeks. New bone formation was evaluated by radiographic densitometry Micro-CT and histomorphometric analysis.

Results: All animals well tolerated with surgical procedure without any evidence of infection or wound dehiscence. The macroscopical showed the graft well incorporated in experimental group in all specimens. Histological observation, 3D 2% w/v chitosan scaffold showed viable lamellar bone with osteoblasts forming bone from the defect margin. Radiomorphometric analysis showed increased bone densitometry over time (0.018±0.0037, 0.022±0.076, 0.024±0.004 and 0.03±0.0044) respectively. Histomorphometirc analysis showed increased percentage of new bone formation over times (2.19±0.47, 4.49±0.96, 9.71±2.11, and 13.35±1.9) respectively and Micro-CT analysis also showed increased percentage of bone volume fraction over times (3.42±0.62, 4.57±0.82, 6.17±0.7, and8.74±0.98) respectively.

Conclusions: 3D 2% w/v chitosan scaffold presented good biocompatibility, osteoconductive properties and enhanced new bone formation in animal model. Therefore, it should be a scaffold for new bone formation.

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List of Abbreviations and Symbols

BVF	=	Bone volume fraction
BV/TV	=	Bone volume per total volume
et al	=	And others
H&E	=	Hematoxylin eosin
kg	=	Kilogram
kVp	=	Kilovoltage peak
mg	=	Miligram
min	=	Minute
micro-CT	=	Micro computed tomography
mm	=	Millimeter
MV/TV	=	Mone volume per total volume
NB	=	Newly formed bone
OB	=	Original bone
OD	=	Optical density
ROI	=	Region of interest
SD	=	Standard deviation
sec	=	Second
SC	=	3D 2% w/v chhitosan scaffold
µA	=	Microampere
μm^3	=	Cubic micrometer
µm	=	Micrometer
w/v	=	weight/volume
TCP	=	Tricalcium phosphate
W	=	Watt
µA	=	Microampere
µl	=	Microliter
µm	=	Micrometer
μm^3	=	Cubic micrometer

Chapter 1

Introduction

Bone is a dynamic tissue, in constant resorption and formation, permitting the maintenance of bone tissue, the repair of damaged tissue and the homeostasis of the phosphocalcic metabolism. Through these balanced phenomena, known as the remodeling process, about 5% of cortical bone and 20% of trabecular bone is renewed per year.[1] Bone contains three main bone-specific cell types: the osteocyte is a mature cell that sits in bone lacunae, communicates with other osteocytes through long cellular processes, senses mechanical stress in bone, and sends signals for bone remodeling as a result of mechanical stress. Secondly, the responding cells are osteoblasts, being cells specialized to secrete the unique collagen-rich extracellular matrix in bone that enables mineralization. Thirdly, osteoclasts are macrophage-like cells that degrade the bone structure through a combination of localized acidification (removes the minerals) and protease secretion (breaks down the matrix). Osteoclasts tunnel through bone and are usually followed close behind by osteoblasts. Bone is in a constant state of remodeling in healthy individuals.[2]

Bone reconstruction in the oral and maxillofacial region permits the recovery of esthetics and function of the craniofacial skeletal. The management of large bone defects due to trauma, degenerative disease, congenital deformities, and tumor resection remains a complex issue for orthopedic reconstructive surgeons. The requirement is for an ideal bone replacement which is osteoconductive, osteoinductive, and osteogenic. Autologous bone grafts are still considered the gold standard for reconstruction of bone defects, but donor site morbidity and size limitations are the major concerns. 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.[3] 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, apromising new approach for bone repair.[4] While these sources produce excellent results, autogenous bone grafts are subject to limitations of an available amount of bone, secondary donor site defects, length of hospital stay, and cost. These limitations increase

interesting bone substitute materials, including allogenic, xenogenic and alloplastic bone materials. A bone graft substitute should be osteoconductive, osteoinductive, biocompatible, bio-resorbable, structurally similar to bone, easy to use, and cost-effective.[5] A bone substitute material can be used instead of or in combination with an autograft to increase graft volume.

Osteogenesis, osteoinduction, and osteoconduction are the three essential elements of bone regeneration along with the final bonding between host bones and the grafting material, which is called osteointegration. Osteoprogenitor cells living within the donor graft may survive during transplantation but can potentially proliferate and differentiate to osteblasts and eventually to osteocytes. These cells represent the ‘‘osteogenic’’ potential of the graft. ‘‘Osteoinduction’’ on the other hand is the stimulation and activation of host mesenchymal stem cells from the surrounding tissue, which differentiate into bone forming osteoblasts. This process is mediated by a cascade of signals and the activations of several extra and intracellular receptors, the most important of which belong to the TGF-beta super family.[6]Osteoconduction describes the facilitation and orientation of blood-vessels and the creation of the new Haversian systems. Finally, ‘‘osteointegration’’ describes the surface bonding between the host bone and the grafting material.[7]

Scaffolds or extracellular matrices are templates that are utilized in the formation of tissues by acting as a support system or framework for tissues or organs of the body. In addition to acting as frameworks for proliferating cells, scaffolds can also act as delivery systems. Cells and other factors are placed in the scaffold and the scaffolds promote cellular growth, adhesion, migration and differentiation of the tissues.[8]

A biodegradable scaffold in bone tissue engineering serves as a temporary skeleton inserted into the sites of defective or lost bone to support and stimulate bone tissue regeneration while it gradually degrades and is replaced by new bone tissue. Both bioactive ceramics and polymers have been developed and analyzed for use as tissue engineering scaffolds. Bioactive ceramics have a chemical composition resembling that of natural bone, allow osteogenesis to occur, and can provide a bony contact or bonds with host bone chitosan alginate hybrid scaffolds for bone tissue engineering.[9]

Chitosan is a naturally derived polysaccharide. It has gained much attention as a biomaterial in diverse tissue engineering applications due to its low cost, large scale availability, anti-microbial activity, and biocompatibility.[10] Chitin is widely found in shells of crustaceans

such as crabs and shrimp and is the second most abundant polymer after cellulose. Chitin forms strong inter and intramolecular hydrogen bonds, which is not easily broken by common solvents. Therefore, its solubility in common solvents is rather constrained. For this reason, limited utilization of this natural resource has been reported. Until the present, the majority of usage of chitin has mainly been related to chitosan, which is a cationic polymer derived from chitin comprising copolymers of β (1$\longrightarrow$4)-glucosamine and N-acetyl-d glucosamine. The physicochemical and biological properties of chitosan make it an excellent material for the preparation of drug delivery systems and for the development of new biomedical applications in many fields from skin to bone or cartilage.[11]

Recently, a tissue engineering strategy has been suggested to create a bone substitute material which acts as a scaffold and induces osteogenesis. Chitosan is a biocompatible and biodegradable polymer which is currently receiving a great deal of interest for medical and clinical applications due to its interesting intrinsic properties. Regarding its high biocompatibility, it is employed in various implantable and injectable systems such as orthopedic/periodontal composites, drug delivery systems, wound healing management, and scaffolds for soft and hard tissue regeneration.

Bone Graft Material

Bone graft materials are classified into three groups: autogenous, allogenous and alloplastic bone grafts.

Autogenous bone grafts are considered to be the gold standard for graft materials because of the three essential available components of new bone formation which include 1) a high population of pluripotential cells, 2) structures of cancellous bone acting as scaffolds, and 3) growth factors for stimulating mesenchymal cell recruitment, proliferation and differentiation. Autogenous bone grafts are usually harvested from the iliac crest, rib, or calvaria bone,[12] mandibular symphysis,[13] ramus, maxilla tuberosity, and exostoses.[12] The advantages ofautogenous grafts include early revascularization, high population of pluripotential cells, resistance to infection and immune activation. However, autogenous bone grafts also have several disadvantages such as limitation of graft volume available, required donor site surgery, prolongation of the operation time, and donor site morbidity.[14] The amount of cancellous bone graft volume available from the iliac crest is about 30-80 cm^2 and about 10-15cm^2 from

asinglerib. The hematoma, pain, paresthesia, hernia and gait disturbance are principle complications of iliac bone harvesting. Many studies indicated that autogenous bone grafts have unpredictable resorption rates in maxillary sinus augmentation procedure.[15] The site of the harvesting bone affects the success rate of osseointegration for dental implant.[16] Significant adverse events after surgery grafts include edema, rash (erythema), pain, sensory loss, and gait disturbance.[17] Thus, many alternative bone substitutes have been used including allogenous and alloplastic bone materials. The ideal bone substitute should be biocompatible and accepted by the host tissue with high porosity, large inner surface area, and gradually replaced by new bone, and as well as having the osteoinductive or osteoconductive properties. The osteoinduction is a healing process in which local stimulating factor causes messenchymal cells to migrate, proliferate and differentiate into the chondroblast or osteoblast cells. In the osteoconductive process, the bone substitute material serves as a scaffold for ingrowth of new vessels and osteoprogenitor cell from the host bone.

Allogenic bone materials are composed of tissue taken from another individual of the same species. The allogenic materials that available are demineralized freezed-dried bone (DFDBA) and freezed dried bone allografts (FDBA). The allogenic bone materials have osteoinductive and osteoconductive properties.[12] In the osteoinductive properties, the porous structures of allogenic bone grafts are a collagen matrix that supports the mesenchymal cells to facilitate the attachment, migration and differentiation to osteoblast cells. For the osteoinductive properties, allogenic grafts contain many growth factors which are embedded in the bone matrix and can be liberated when the bone matrix are resorped by osteoclast cells. The growth factors include insulin-like growth factors, transforming growth factor-beta, platelet derived growth factors, fibroblast growth factors, and bone morphogenic proteins (BMPs). Allogenic bone graft materials have several advantages over autogenous bone grafts. First, the mobility and morbidity of harvesting autogenous bone is eliminated. Secondly, allogenic bone grafts provide an essentially unlimited volume of graft material. And finally, allogenic bone grafts can provide a wide variety of physical forms which can be customized to specific application. However, the allogenic bone grafts have some disadvantages. They include, the materials are expensive; and the materials have risk of disease transmission, especially HIV and hepatitis B. The risk still exists, although this risk has been minimized by extensive donor screening and testing, extensive washing to remove donor cells and cell debris, and sterilization using high dose radiation. The

study about maxillary sinus augmentation with allogenic bone grafts indicated that these materials had rapid resorption rates with complete replacement of bone within 10-12 months.[18] For this reason, researchers have developed a biosynthetic bone material for substituting autogenous bone.

Alloplastic materials are synthetic or natural materials used as a substitute for bone grafting. Alloplastic materials have the potential to eliminate or at least reduce second surgical site morbidity. Also they are easy to use and are frequently less expensive than the overall cost for bone harvesting. The most common alloplastic grafting materials are deproteinized bovine bone hydroxyapatite, coralline hydroxyapatite, bioactive glass, and beta-tricalciumphosphate (TCP). In recent years, polymers have taken the place as the new generation for bone substitution materials.

Polymers: there is a need for an alternative to autogenous bone as a grafting material for the reconstruction of oral and maxillofacial surgery. Bone tissue engineering promises such an alternative, and many groups have developed strategies based on seeding of cells onto 3 dimensional biodegradable polymeric scaffolds. The advantages of polymers are biocompatibility and biodegradability scaffolds with different features (forms, porosities and pore sizes, rates of degradation, and mechanical properties) to match tissue specific application.[19] There are three types of polymer-based materials: natural polymers, synthetic polymer and composites. Many natural polymers are found in living organisms of known biocompatibility. Such polymers can be used to replace or regenerate native tissue structures and allow positive cell interactions with surrounding tissues. Conversely, synthetic polymers are formed through controllable chemical processes to achieve desirable material and chemical properties for a wide range of biomedical applications. The synthetic polymers have a promising advantage over the natural polymers for scaffold developments because their mechanical and proliferation properties are comparatively more predictive and reproducible. Composite scaffolds are built by mixing two or more materialsto achieve desirable properties and characteristics by taking advantages from each of the materials.[20]

Natural polymers: many polymers from nature have the advantage of biocompatibility and biodegradability since they are composed from structural materials of tissue (i.e. collagen and glycosaminoglycans).[19] Natural polymers often possess highly organized structures and may contain an extracellular substance, which can be bound to cell receptors. Although they are of known biocompatibility, the lack of natural polymer in large quantities and

the difficulty in processing them into scaffolds limit them for clinical applications. Moreover, as natural polymers can guide cells to grow at various stages of development, they may stimulate an immune response at times. This leads to the concerns regarding antigenics and the delivery disease for allografts. Since the degradation of these polymers depends on the enzymatic processes, degradation rate may vary from patient to patient.[20] Nevertheless, the low mechanical strength and high rates of degradation of natural polymers often result in their use in composites or in chemical modification by cross-linking to improve properties and reduce degradation rates. However, these changes may cause cytotoxic effect and reduce biocompatibility.[21]

Synthetic polymers: Synthetic polymers are man-made polymers, which have advantages over the use of natural origin polymers as they are more flexible, more predictable and processable into different size and shapes. The physical and chemical properties of a polymer can be easily modified and the mechanical and degradation characteristics can be altered by their chemical composition of the macromolecule. The functional groups and side chains of these polymers can be incorporated, i.e., the synthetic polymers can be self cross-linked or cross linked with peptide or other bioactive molecules, which may be a desirable biomaterial for bone tissue engineering. Additionally, synthetic polymers are generally degraded by simple hydrolysis, which is desirable as the degradation rate does not have variations from host to host unless there are inflammations and implant degradation etc. to affect the local pH variation. The most extensively used synthetic polymers are poly-glycolic acid (PGA), poly lactic acid (PLA) and their co-polymers; polycaprolactone (PCL) and polyethylene glycol (PEG). The mechanical properties of PLA and PGA are relatively weak for high porosity scaffolds, which limit their usage on the hard tissue regeneration applications. PEG has a relatively high compressive modulus corresponding to a higher modular weight, but have worse degradation characteristics.[20]

Composite polymers: Scaffolds that are made of composites consist of two or more materials. These materials are used together to produce a better scaffold taking the advantage from each of the component materials independently.[20]

Biomedical Application of Chitosan

The design of artificial kidney systems has made possible repetitive hemodialysis and sustained life of chronic kidney failure in patients. Chitosan membranes have been proposed as an artificial kidney membrane because of their suitable permeability and high tensile

strength.[22] The most important part of an artificial kidney is the semipermeable membrane and are so far made from commercially regenerated cellulose and cuprophane. Since the primary action of the cellulose membrane is that of a sieve, there is little selectivity in the separation of two closely related molecules.[23] These novel membranes need to be developed for better control of transport, ease of formability and inherent blood compatibility.

Tissue Engineering

Tissue engineering is the development and manipulation of laboratory-grown cell, tissue or organs that would replace or support the function of defective or injured parts of the body. The many potential advantages of tissue engineering include the development or revolution of current technology in total hip, knee, cartilage, tendon, and vascular replacement. Many of these practices at present involve implantation of either an autologous or synthetic graft in place of the damaged area. Within the body the implant must satisfy requirements relative to biocompatibility as well as functional and mechanical stability. Many materials can react compatibly with the body. But unfortunately, they cannot meet the long-term mechanical, geometrical, and functional requirements of the body. Therefore, tissue engineering technology has been developed to construct artificial tissues that can mimic the natural ones by combining with modulated cells with different types of scaffolding materials, including natural and synthetic polymers. Among these materials, polylactide (PLA), polyglycolide (PGA) and their copolymer; polylactide-co-glycolide (PLGA) have received much attention because of their biodegradability and biocompatibility, which are suitable candidates for tissue engineering.[24] Chitosan and some of its derivatives have been studied for utilization in several biomedical applications including wound dressings, drug delivery systems, and space filling implants. However, in comparison to these, little has been done to explore use of chitosan within the tissue engineering paradigm. Chitosan has been found to have acceleratory effect on the tissue engineering processes owing to its polycationic nature. This enhances the cells attraction to this polymer. It has been found that the degree of cell attachment also depends on the percent of deacetylation of the chitosan.

In 2000, Prasitsilp et al. showed the effects of the degree of deacetylation in vitro cellular responses to chitosan from two different sources, shrimp and cuttlefish. They examined four chitosan substrates, two from each source, differing by about 10 percent deacetylation and ranging between 76 and 90 percent deacetylation. Results indicated that cells

more readily attach to more highly deacetylatedchitosans from both sources.[25] Recently, many efforts have been made on chitosan for using it as scaffolding material in tissue engineering. In 2001, Jarry et al[26] demonstrated that chitosan can be easily processed into porous scaffolds, films and beads.

Wound Healing/Wound Dressing[27, 28]

Chitosan has been found to have an acceleratory effect on the wound healing/wound dressing process. Regenerated chitin fibers, non-woven mats, sponges and films exhibit an increase in wound healing by over 30 percent. Chitin can also be used as a coating on normal biomedical materials. Standard silk and catgut sutures coated with regenerated chitin or chitosan show wound healing activities only slightly lower than the all-chitin fibers. Surgical gauze coated with regenerate chitin demonstrates a substantially greater amount of activity than an uncoated control group.

Burn Treatment[29]

Chitosan is a promising candidate for burn treatment. This is true since chitosan can form tough, water-absorbent, biocompatible films. These films can be formed directly on the burn by application of an aqueous solution of chitosan acetate. Another advantage of this type of chitosan treatment is that it allows excellent oxygen permeability. This is important to prevent oxygen-deprivation of injured tissues. Additionally, a chitosan films have the ability to absorb water and is naturally degraded by body enzymes. This fact means that the removal of chitosan is not necessary in most injuries (and specially burns). Removing the wound dressing can cause damage to the injury site.

Artificial Skin

The effect of treatment with chitosan and saline solution on healing and fibroplasia of wounds made by scalpel insertions in skin and subcutaneous tissue in the abdominal surface of dogs have been reported. The design for artificial skin applicable to long-term chronic use focuses on a non antigenic membrane which performs as a biodegradable template for the synthesis of neodermal tissue. It appears that chitosan polysaccharides having structural characteristics similar to glycosaminoglycans can be considered for developing such

substratum for skin replacement.[29-31] Nowadays the investigations on brain-scale damage and plastic skin surgery are being made by the use of chitosan.

Ophthalmology

Chitosan has replaced synthetic polymers in ophthalmological applications. Chitosan possesses all the characteristics required for an ideal contact lens; optical clarity, mechanical stability, sufficient optical correction, gas permeability, partially towards oxygen, wettability, and immunologically compatible. Contact lenses are made from partially depolymerized and purified squid pen chitosan by spin casting technology. These contact lenses are clear, tough, and possess other required physical properties such as modulus, tensile strength, oxygen permeability, antimicrobial content, and wound healing properties. These properties of chitosan along with excellent film forming capability, make chitosan suitable for development of an ocular bandage lens.[32]

Drug Delivery Systems

The applicability of natural polysaccharides such as agar, konjac, and pectin in the design of dosage forms for sustained release has been reported.[33, 34] Despite the medical applications of chitin/chitosan described above, they are still utilized in the pharmaceutical field. It is already known that compounds having a molecular weight lower than 2900 pass through membranes derived from chitosan. Since chitin and chitosan do not cause any biological hazard and are inexpensive, these polymers might be suitable for the preparation of dosage forms of commercial drugs.[35-37]

Antimicrobial Properties

Recent studies in antibacterial activity of chitosan have revealed that chitosan is effective in inhibiting growth of bacteria. The antimicrobial properties of chitosan depend on its molecular weight and the type of bacterium. Regarding gram-positive bacteria, chitosan with 470 KDa was the most effective, except for Lactobacillus sp., whereas for gram-negative bacteria, chitosan with 1,106 KDa was effective. Chitosan generally showed stronger bactericidal effects for gram-positive bacteria (Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, and L. bulgaris) than for gram-

negative bacteria (E.coli, Pseudomonas fluorescens, Salmonella typhymurium, and Vibrio parahaemolyticus) in the presence of 0.1% chitosan.[38]

Koide (1998) reported that chitin and chitosan in vitro show antibacterial and anti-yeast activities.[39] One of the chitosan derivatives, N-carboxybutyl chitosan, was tested against 298 cultures of different pathogenic microorganisms, where it showed bacteriostatic and bactericidal activities. It also showed marked morphological alterations in treated microorganisms when examined by electron microscopy.[40]

Biocompatibility and degradability of chitosan

Concerning the safety of chitosan, a range of toxicological studies has been performed for biocompatibility and implantation effects of chitosan in medical use. In general, results showed no toxicity in rats at up to 2000 mg/kg/day in gavages dosing and at up to 5% (3000 mg/kg/day) in the diet during a duration of up to 3 months.[41] In human beings, daily doses of 4.5 g chitosan have been taken orally in human volunteers with no adverse effects reported.[42] Even higher oral doses of up to 6.75 g were reported as safe.[43] It was reported that short-term human trials for cholesterol-lowering of up to 12 weeks showed no significant clinical symptoms, with no evidence of an allergic response.[44] In vitro degradation of chitosan with egg white and human lysozyme occurs mainly through depolymerization by the enzyme.[45] After primary ligament fibroblasts were seeded on compound photocrosslinked N-methacrylated glycol chitosan scaffolds, most of the cells (72% 64%) remained viable during a 4-week culture period, and immunohistochemistry staining revealed that extracellular matrix markers of ligament , including collagen type I, collagen type III, and decorin, were organized and accumulated within the composite scaffolds.[46]

Hou J et al (2012) evaluated segmental bone regeneration using rhBMP-2-loaded collagen/chitosan microspheres composite scaffolds in a rabbit model using micro-computer tomographic analysis. It showed that not only were the defects were bridged as early as 4 weeks, but that defect had also healed the defects and presented recanalization of the bone marrow cavity at 12 weeks.[47] Cho BC et al (2005) evaluated the effect of calcium sulfate-chitosan composite on the osteogenesis of defective tibia in rabbits. This study found that new bone formation began to be seen at 2 weeks, and at 6 weeks fibrous connective tissue still remained at the center. However, the fibrous connective tissue at the center was replaced with callus, the bony bridge was obvious,

and lamellation of the callus was also observed.[48] Besides that, chitosan membrane coated PLGA enhanced local bone formation at both 2 and 8 weeks.[49] Many of the scaffolds currently being designed have outstanding osteoconductive properties for rapidly stimulating the migration of fibrovascular and osteoprogenitor cells into a porous structure, which is subsequently incorporated into and replaced by bone tissue.[50] The most effective scaffolds should:

- Readily incorporate and retain mesenchymal cells in tissue culture.
- Rapidly induce fibrovascular invasion from the surrounding tissue.
- Have significant osteoconductive properties to improve incorporation with the host bone.
- Not induce significant acute immune responses or a chronic foreign body response.
- Have biomechanical properties similar to those of normal bone, which will limit stress shielding resulting in bone loss adjacent to the implant.
- Be biodegradable, with a controllable absorption rate that parallels the rate of new bone deposition.
- Have biodegradation products that are nontoxic and easily secreted by normal physiological pathways.
- Contain sites that can noncovalently bind osteogenic biomolecules to enhance osteoinduction.

Osteogenic, osteoinductive and osteoconductive capacity of chitosan

Many previous studies have shown the osteogenic capacity of chitosan and the first report describing an attempt to regenerate bone in vivo using chitosan dates back to 1988[51] when Muzzarelli and colleagues implanted chitosan membranes and chitosan ascorbate gel into cranial defects in cats. Their findings suggested that chitosan seems to induce a stimulatory and/or attractive effect on stromal cells of surrounding tissues. Subsequent studies from the same authors describe the use of methylpirrolidone chitosan in defects created in the rabbit tibia[52]and in the femoral head of sheep.[53] These studies confirmed previous observations[51]of the possible stimulatory and/or attractive effect of chitosan on adjacent cells. Chitosan has also been used as a carrier for growth factors such as PDGF-BB, to promote bone formation in a critical-sized calvaria defect in rats.[54]Osteoconductive chitosan/tricalcium phosphate (TCP) sponges were

observed to promote osseous healing of rat calvarial defects versus controls (without scaffolds). The addition of PDGF-BB to a carrier further enhanced bone regeneration.[55] These authors observed that chitosan /TCP sponges without bioactive PDGF-BB resulted in more bone formation than chitosan-TCP without the bioactive agents.[54]PDGF growth factor is produced by platelets, osteoblasts, and monocytes/ macrophages and it is believed to have a role in the migration of MSCs to wound sites.[56] The combination of chitosan-PBS scaffolds with human BMSCs implanted into critical-sized cranial defects in nude mice resulted in enhanced integration with the surrounding tissue and significant bone formation. This was more evident for the scaffolds cultured with human cells.[57]Electrospun chitosan nanofiber membranes evidenced new bone formation at 4 weeks in rabbit cranial defects compared to the controls (empty bone defects) , where only soft tissue formation was observed.[58] Chitosan combined with nano HA, in the form of microspheres, implanted in rat calvaria defects for 12 weeks were observed to promote bone regeneration.[59] Moreover, chitosan-PLAGA microspheres conjugated in a scaffold by particle aggregation, with or without heparin and recombinant human bone morphogenetic protein 2 (rhBMP-2), promoted bone regeneration in vivo, with more pronounced results for the scaffolds with the incorporated growth factor.[60] A study by Ríos and coworkers used a model mimicking the clinical bone free flaps, by using a cranial flap that involves the design of the desired tissue at an ectopic site in the patient's own body. This study used chambers containing silk fibroin-chitosan scaffolds implanted on top of the grafted periosteum over the latissimusdorsi muscle of sheep.[61] Bone grafts were used as positive controls and empty defects as negative controls. The authors found that the same amount of bone was regenerated in the defects with the tested scaffolds as for the defects with bone graft.[62]

Klokkevold et al (1996) evaluated the effect of chitosan on osteoblast differentiation and bone formation in vitro by treating the mesenchymal stem cells harvested from fetal Swiss Webster mice calvaria prior to osteoblast differentiation and calcification with chitosan suggesting that chitosan potentiates the differentiation of osteoprogenitor cells and may facilitate the formation of bone.[62] Yang et al (2011) compared different proportion of chitosan/allogeneic morselizedbone composites. They were implanted at the ratio of 1:50 and 1:25, respectively. It showed that the bone formation was earlier in the ratio 1:50 than in 1:25 at 4 and 8 weeks after operation.[63]

In the past decade, chitosan has been used for biodegradable scaffolds as bone graft substitute. Chitosan was earlier used in combination with approved bone substitute material such as hydroxyapatite to improve the migration of an HA graft in a rabbit model, demonstrating that it has osteoconductive properties.[64] Biodegradable chitosan containing osteoblast cells have been used to enhance bone healing as demonstrated by Mattioli-Belmonte et al. They seeded the osteoblastic cells from newborn mouse calvaria into N, N-dicarboxymethyl chitosan and 6-oxychitin scaffolds in Prague-Dawley rat's femoral condyle bone defect. The result of this study showed that bone regeneration occurred in both scaffolds but enhanced scaffolds containing osteoblasts.[65]Porous three-dimensional scaffolds have been used extensively as biomaterials in the field of tissue engineering, it provides an extracellular matrix analog which functions as a necessary template for host infiltration and a physical support to guide the proliferation and differentiation of cells into the functional tissues or organs and, there is various percent compositions are utilized to produce a chitosan scaffold that has a desirable pore size and porosity properties. The pore formation is influenced by the chitosan solution concentration [66]. Hsieh et al. [66] showed that the mechanical properties of the scaffold were increased when the chitosan concentration was increased from 1 to 3% (w/v). This range allowed for proper pore formation. Above this range the viscosity was too high and below the range the viscosity was too low. In this study was used 2% chitosan concentration as scaffold for new bone formation is appropriately.

Chapter 2

Objective of the Study

Objectives

General objective:

To evaluate the effects of 3D 2% w/v chitosan scaffold on new bone formation in the rabbit's calvarial defect

Specific objectives

1. To evaluate the compatibility of 3D 2% w/v chitosan scaffold.
2. To assess the amount of new bone formation of 3D 2% w/v chitosan scaffold.
3. To compare new bone formation between a 3D 2% w/v chitosan scaffold and autogenous bone.

Benefit of the study

To provide the specific knowledge of a 3D 2% w/v chitosan scaffold related to biocompatibility and new bone formation.

Hypothesis

3D 2%w/v chitosan scaffold could enhance the new bone formation and biocompatibility in a rabbit's calvarial defect.

Chapter 3

Materials and methods

Experimental models

Sixteen male New Zealand white rabbits aged between 5 - 7 months and weighing 3 - 4.5 kg were used in this study. Housing and feeding of animals were performed according to standard animal care protocols of the Faculty of Science and performed in accordance with the regulations and approval of the animal experiment ethics committee of the Prince of Songkla University (Figure 1).

Figure 1. New Zealand white rabbit

Group of study

The animals were divided into 2 groups. Group 1 was the experimental group (n=8) using a 3D 2% w/v chitosan scaffold and group 2 was the control group (n=8) using autogenous bone chip; the defects were created on both sites of the parietal bone on the cranium of the rabbit. The details of the groups of the study are presented in Table 1.

Table 1. Groups of the study

Groups	Description	Animals	Number of defects
1	3D 2% w/v chitosan scaffold	8	16
2	Autogenous bone chip	8	16
	Total	16	32

Materials

❖ 3D 2% weight/volume chitosan scaffold preparation

Two percent of chitosan solution was prepared by dissolving 5.7 ml of 100% acetic acid and 494.3 ml of distilled water. Then the prepared solution was kept at room temperature for 24 hours and filtered. This chitosan solution was injected by using a 10 ml syringe (needle size 22 gauze, long $1^{5/8}$ inch) into 1 M of sodium hydroxide (NaOH), pressed by a steel ball (weight 6.25 kg) and blended together to make fibril-like chitosan. They were filtered and washed with 200 ml distilled water twice to remove excess sodium hydroxide. This fibril-like chitosan was put in Polypropylen Cylindrical Molds (14 mm in diameter, height 11cm), centrifuged at 3000rpn for 5 minutes. After excess water was rinsed out, the chitosan was refrigerated at 4°c for 24 hours then frozen at -20°c for 24 hours, after that they were placed into 96% ethanol for 1 hour. The chitosan scaffolds were scaffolds with thickness of 2 mm. Finally, the chitosan scaffolds were frozen in liquid nitrogen to maintain the poresize and dried at 37°c.

Surgical procedure

1. Anesthetic Phase

All rabbits were placed on the surgical table in a prone position. Anesthesia was induced by 25 mg/kg Ketamine Hydrochloride and 5 mg/kg diazepam intramuscularly into the gluteal region. Three minutes later, an intravenous catheter was placed into the marginal ear vein for intravenous anesthesia. (Figure2). 5 mg/kg Thiopental was administered intravenously and then titrated at the rate of 2 mg/kg every 15 minutes (maximum dose not more than 30 mg/kg) until achieving unconsciousness. At the beginning of the surgery, Penicillin G solution (50.000-100.000 U/Kg) was also given for prophylaxis.

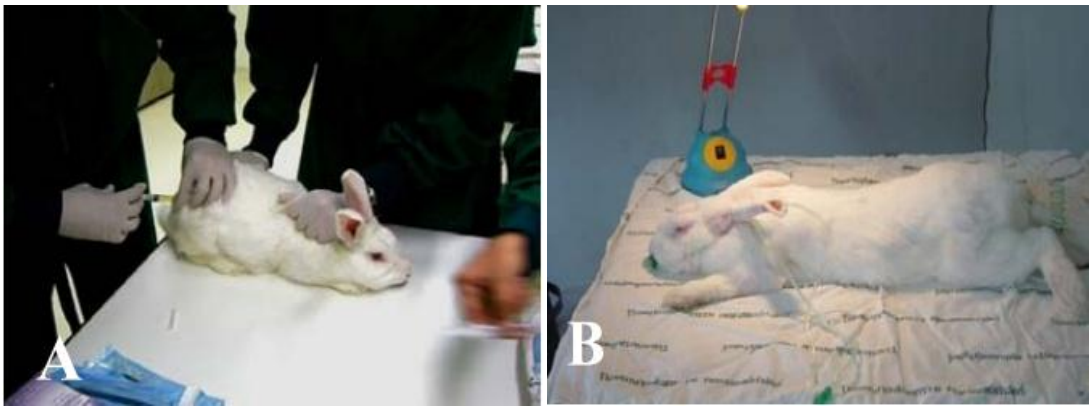

Figure 2. The animal was anesthetized by 25 mg/kg ketamine and 5 mg/kg diazepam intramuscularly into the gluteal region (A). Then intravenous anesthesia was administered with thiopental (B)

2. Operative phase

All surgical procedures were performed under aseptic conditions by the same surgical team. The surgical field was shaved and disinfected with 10% Providone-Iodine over the cranium area between both ears of the rabbit. A mid-sagittal incision of 2-3 cm was made after local infiltration of 2% lidocaine hydrochloride with 1:100.000 epinephrines of 1.8 ml at the skin area of the surgical field (Figure 3). Subperiosteal dissection was carried out and 2 bicortical bony defects 10 mm in diameter were created in the left and right parietal bone with a small round and fissure burs using a micromotor. During surgery the drilled holes were carefully rinsed with 0.9% NaCl solution and cleaned out in order to remove abraded particles, reduce drilling temperature and avoid bone necrosis. Both defects were made far from the sagittal suture, approximately 2 mm (Figure 4). A sterilized aluminum template was used as a guide to ensure the same sized defect in each animal. A 1 mm deep circular mark was made with a small round bur and filled with preheated gutta-percha for later identification of the defect edges on the histological sections (Figure 5). In the control group, autogenous bone from the removed calvarium was minced with a bone morselizer (SalvinDentalSpacialtiesInc, Charlotte, NC, USA). The amount of the autogenous bone was calibrated to have the equal volume with the chitosan scaffold using the acrylic mold and were further weighed(Figure 6) and inserted in one side of the defect. In the experimental groups, a 3D 2% w/v chitosan scaffold 10 mm in diameter was soaked with 0.9% normal saline for 10 minutes and inserted into the defects.

Then the periosteum, muscle and skin were sutured in two layers with Vicryl® 3-0. (Figure 7) The graft materials were randomly filled into each side of the cravalial defect as shown in Table 2

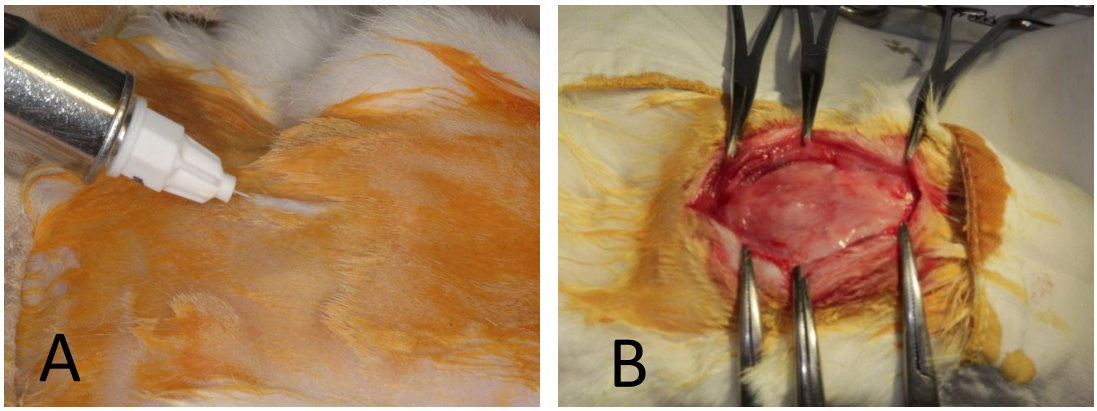

Figure 3. Local infiltration (A) Mid-sagittal incision to expose cavalial defect (B)

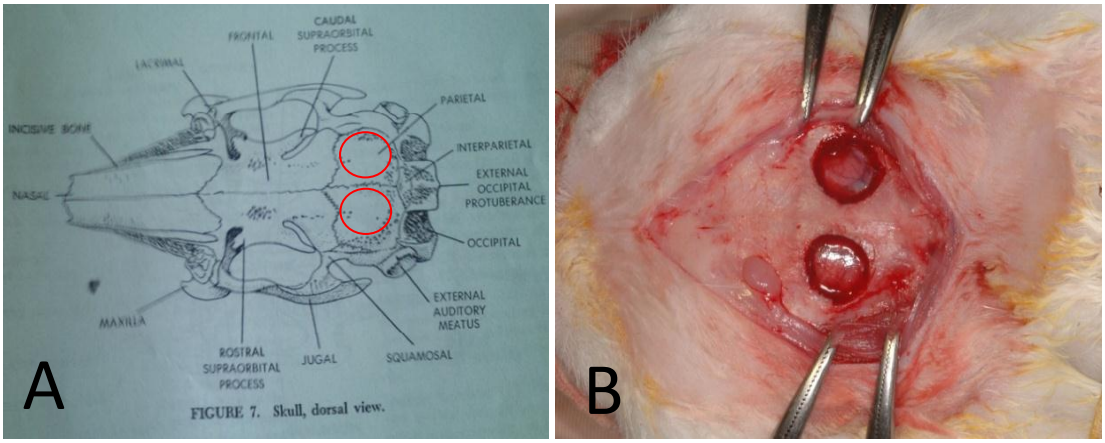

Figure 4. Schematic presentation of the critical size defects in rabbit cravaria (A). Two round defects 10 mm in diameter were created in the parietal bone on both sides of the cravalium (B)

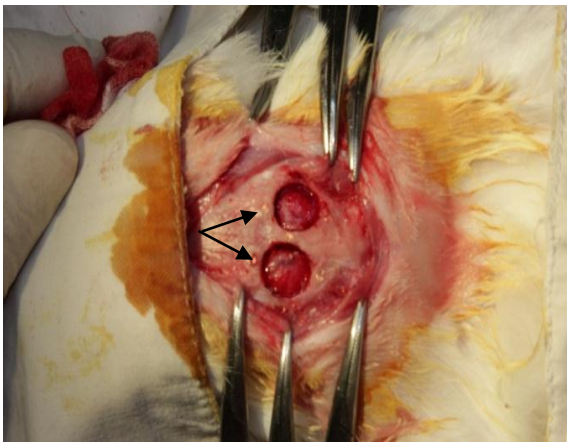

Figure 5. Gutta-percha markers were used to locate bone defect (arrow)

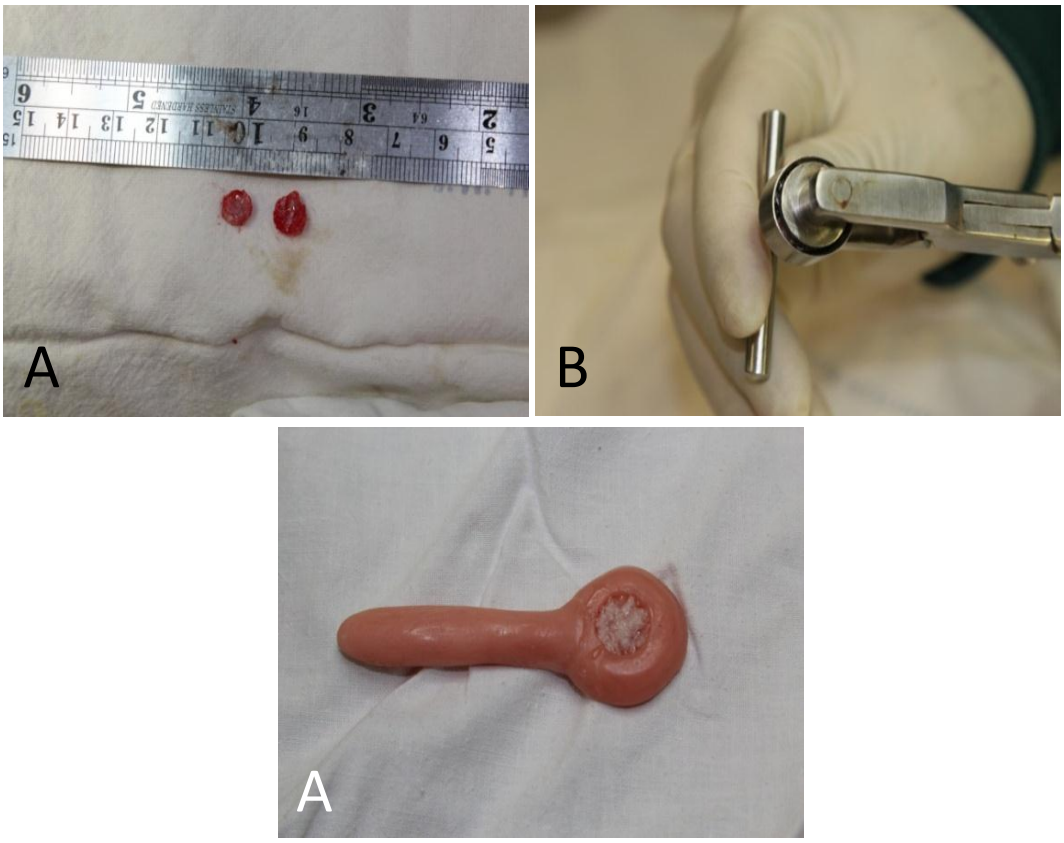

Figure 6.The 2 bicortical bone defects size is 10 mm in diameter (A). Harvested bone was minced with a bone morselizer(Salvin Dental SpacialtiesInc, Charlotte, NC, USA) (B).The autogenous bone was measured by an acrylic mold (C)

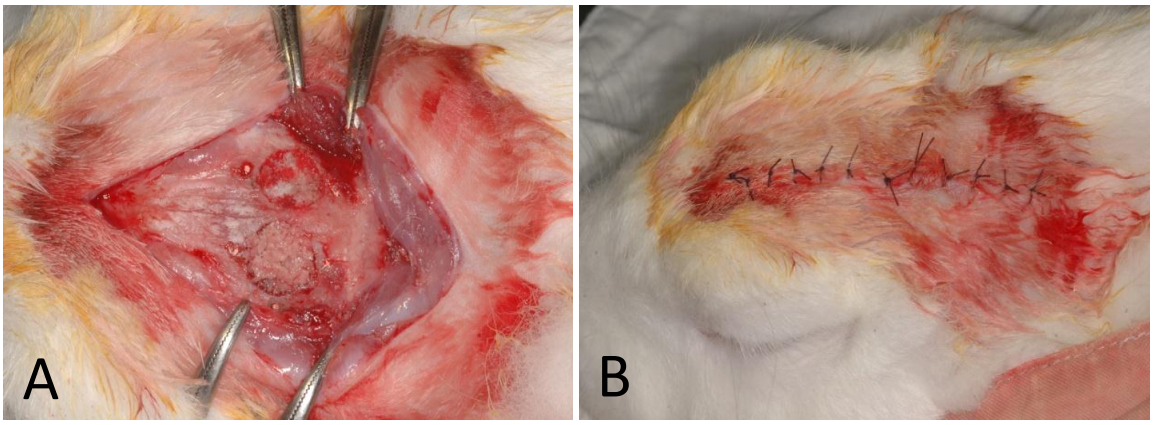

Figure 7. 3D 2% w/v chitosan scaffold and autogenous bone were implanted into both bone defects (A). Suture by Vicryl®3-0 (B)

Table 2. Graft materials randomly selected for each side of the test animals

Nnumber of rabbits	**Test site**	**Graft material**	**Specimen label**
1	1L	3D 2% w/v chitosan scaffold	1a
	1R	Autograft	1b
2	2R	3D 2% w/v chitosan scaffold	1a
	2L	Autograft	1b
3	3R	3D 2% w/v chitosan scaffold	1a
	3L	Autograft	1b
4	4L	3D 2% w/v chitosan scaffold	1a
	4R	Autograft	1b
5	5L	3D 2% w/v chitosan scaffold	1a
	5R	Autograft	1b
6	6R	3D 2% w/v chitosan scaffold	1a
	6L	Autograft	1b
7	7L	3D 2% chitosan scaffold	1a
	7R	Autograft	1b
8	8R	3D 2% w/v chitosan scaffold	1a
	8L	Autograft	1b

Table 2. Graft materials randomly selected for each side of the test animals (Continued)

Number of rabbits	Test site	Graft material	Specimen label
9	9R	3D 2% w/v chitosan scaffold	1a
	9L	Autograft	1b
10	10R	3D 2% w/v chitosan scaffold	1a
	10L	Autograft	1b
11	11L	3D 2% w/v chitosan scaffold	1a
	11R	Autograft	1b
12	12L	3D 2% w/v chitosan scaffold	1a
	12R	Autograft	1b
13	13R	3D 2% w/v chitosan scaffold	1a
	13L	Autograft	1b
14	14L	3D 2% w/v chitosan scaffold	1a
	14R	Autograft	1b
15	15L	3D 2% w/v chitosan scaffold	1a
	15R	Autograft	1b
16	16L	3D 2% w/v chitosan scaffold	1a
	16L	Autograft	1b

R=Right side L=left side

a =3D 2% w/v chitosan scaffold and b=Autogenous bone chip

3. Postoperative phase

The rabbits were carefully monitored for good recovery and then the animals were stabled in single cages, a standard pellet and water ad libitum, under standard environment conditions. The single dose of pethidine (10mg/kg) was administered intramuscularly for post-operative analgesic. As antibiotic therapy, PGS (50.000 - 100.000 U/kg) was injected intramuscularly once daily for three days and the wound was dressed once a day during the three days post-operative period. Thereafter, clinical changes of the subject including swelling, color, inflammation, tissue necrosis were closely followed up and recorded accordingly.

4. Sacrifice period

Two, four, eight, and twelve weeks after surgery, the animals were sacrificed with an overdose (1.2-1.3 ml) of pentobarbital sodium 200 mg/ml administered intravenously. Then the calvarium was removed in one piece with a fissure bur in a low-speed micro-motor under copious saline irrigation and then immediately immersed to 10% formalin at least 24 hours for tissue fixation before submitting for radiographic examination, micro-computerized tomography and microscopic analysis for histomorphometric analysis. The groups of sacrifice are shown in Table3

Table 3. Time of sacrifice of the study groups

Groups	**Number of defect site**				
	2 weeks	**4 weeks**	**8 weeks**	**12 weeks**	**Total**
1 (3D 2% w/v chitosan scaffold)	4	4	4	4	16
2 (Autogenous bone)	4	4	4	4	16

Radiographic examination

Radiographs of the specimens were taken by the same dental radiographic machine before the histological section with 60 kvp, 10 mA, at 1.25 sec., using the occlusal films (Kodak, Ultra-speed DF-49). The distance between the film and the x-ray tube was kept at 40 cm in every specimen and the aluminium 5 steps wedge was used for film calibration (Figure 8). The film was taken by automatic processing using Dent-X9000 processor (Figure 9). Then resulting radiographs were scanned using a Bio-Red ® Model GS-700 imaging Densitometer (Figure 10) and analyzed under software (Image Pro Plus version 7.0). The radiographic optical density (Mean OD) or count of the pixels within the object per area of each defect was measured three times to minimize the measuring error and calculated for comparing the amount of mineralized tissue produced in response to each type of graft material.

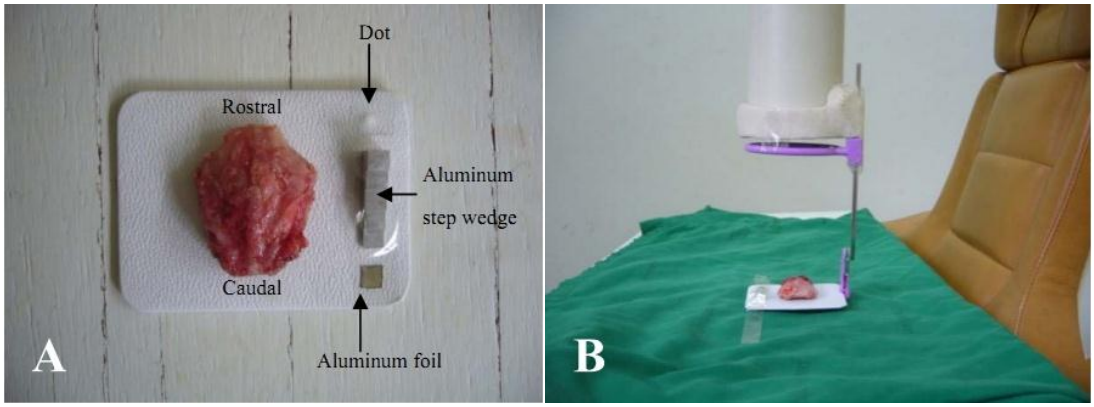

Figure 8. The bone specimen was placed on an x-ray film (A). The parallel film holder device was used for parallel technique radiograph (B)

Figure 9. An automatic film processor (Dent X 9000, Dent X/Logetronics GmbH Kornberg, Germany

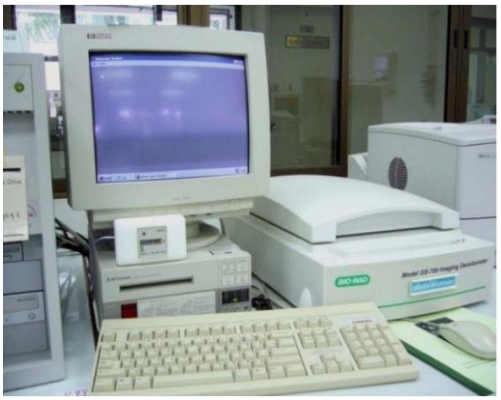

Figure 10. Bio-RAD GS-700 Image Densitometer; USA

Histological examination

Following radiographic examination, the calvarial specimens were decalcified in 10% formic acid which was subsequently changed every day for three weeks and cut into two pieces, each piece containing either an autogenous graft defect or a 3D 2% w/v chitosan scaffold defect**.** Both pieces were trimmed until they encroached on the graft area, then they were divided into 2 pieces at the center before dehydrating in graded series of alcohol with an automatic tissue processor(Leica TP 1020) (Figure 11) and embedded in paraffin blocks (Figure 12). The specimen was sectioned along a sagittal plane to the bone surface by using a diamond saw microtome in 5 μm thickness of three serial sections of each specimen (Figure13). Each histologic section was stained with Haematoxylin and Eosin (H&E) in one mounted glass slide as shown in figure14 . In summary, 6 slides from the center of each autogenous graft defect or 3D 2% w/v chitosan scaffold defect was represented for each defect and examined under a light microscope to evaluate newly formed bony trabeculae (woven bone), fibrous tissue (collagen boundless) and mesenchymal cells with osteoblasticmophology.

Figure 11. Pathcentre™ enclosed tissue processor; Shandon; USA

Figure 12. Parafin Embedding Station; EG 1160; Leica; Germany

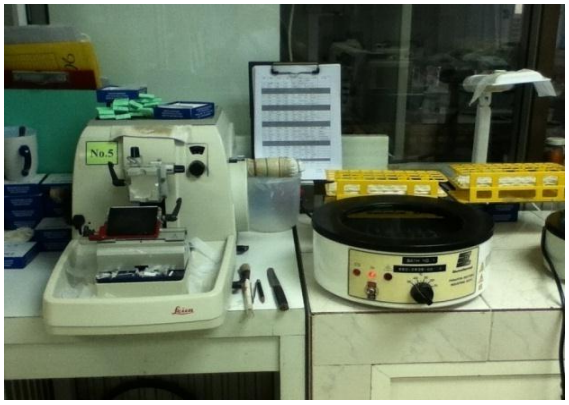

Figure 13. Rotary microtome; RM2235; Leica; Germany

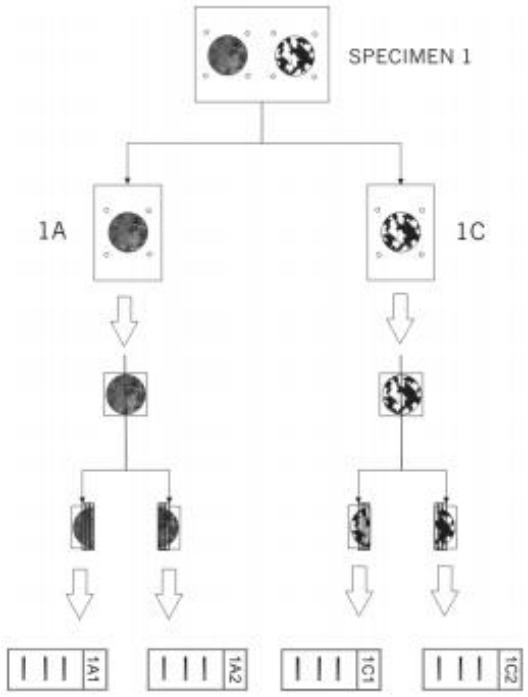

Figure 14. Slide-Labeled diagram

Histomorphometric analysis

One piece of sectioned specimen from each glass slide was randomly chosen to be quantifiably examined by using Leica Qwin Image analysis (Figure15). The amount of new bone formation was determined by measuring the area containing osteoblast cells in five microscopic fields (four peripheral, one central) on the representative section from each defect. The amount of new bone formation was summed and expressed as a relative percentage

area of the total field of view or mean bone area %. One representative section was measured three times to minimize the measuring error and calculated to compare the amount of new bone formation produced in response to each type of graft material. Images of newly formed bone were identified by a given color in each image. These were digitalized and transferred to the computer for image processing and analysis of the quantity fraction of the total area of the defect using the formula:

$$\% \text{ new bone} = \frac{\text{Area of new bone in the defect}}{\text{Total area of the defect}} \text{ x } 100$$

Figure 15. LeicaQwin Imaging Analysis Unit; Germany

Micro-computerized tomography

A high resolution micro-CT system (Micro-CT80, Scanco, Medica AG, Basseersdorf, Switzerland) was used (Figure16). After calibration, the specimens were scanned perpendicularly to the cranium vault at 55 kVp, 72 µA and 4W in high-resolution mode (18.5µm^3/voxel). Scanned data were reconstructed by built-in software. This allowed the creation of a 3D reconstruction of the defect, which was referred to as the region of interest (ROI). The region of interest was analyzed using the following parameters, bone volume fraction (BVF): percentage of bone volume by total defect volume (BV/TV)

Figure 16. Micro-CT (µCT20, Scano Medical AG, BAssersdorf, Switzerland)

Statistical analysis

A descriptive study was used to evaluate the clinical, radiographic examination and histological aspects. The total bony defect area, area of new bone formation and percentage of bone area was measured three times separately to minimize bias. The numerical data were presented in mean values ± SD. The Mann Whitney U test was used to find out the difference of radiographic bone density and percentage of bone area between the experimental and control group, and the difference of each study period (2, 4, 8, 12 weeks) was analyzed by the Kruskal – Wallis test with multiple comparison. A P-value less than 0.05 were considered statistically significant. All the data were subjected to SPSS window version 16.

Chapter 4

Results

Clinical observation

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 in all of the autogenous and 3D 2% w/v chitosan scaffolds containing samples through the study period of 2, 4, 8 and 12 weeks. There were no accidental deaths of rabbits throughout the study period. After full recovery, they were able to eat the pellet food and drink water ad libutum (Figure 17). After euthanasia and epiperiosteal exposure of the calvarias no infection of the hard and soft tissues was detectable. The underlying brain and dura were kept intact.

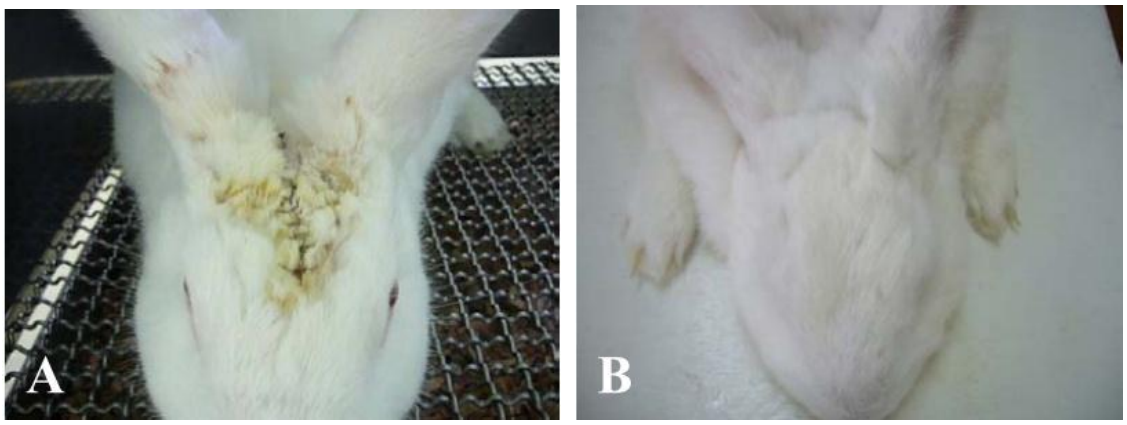

Figure 17. Clinical examination at 1 week postoperatively. There was no wound dehiscence, exposure of the bone grafting materials or infection (A). All of the experimental animals had good wound healing before sacrifice at 2, 4, 8 and 12 weeks after the surgery (B)

Gross morphological evaluation

The macroscopic examination showed that the graft blended more into the surrounding host bone and was denser in control groups than test groups. In control group, the autogenous bone chip side was filled with dense bone-like tissue in all specimens. The defect area

had a flat surface. In the experimental group, the 3D 2% w/v chitosan scaffold side was occupied with soft fibrous tissue in all specimens at 2 weeks and 4 weeks. The surface of the defect area was flat and soft tissue in consistency. However, the bone defect edge projected into the defect with bone-like tissue projection at 8 weeks and 12 weeks, which was dense and with bone –like hardness in consistency. The filled surface of the defect was under the margin of the surrounding host bone (Figure18).

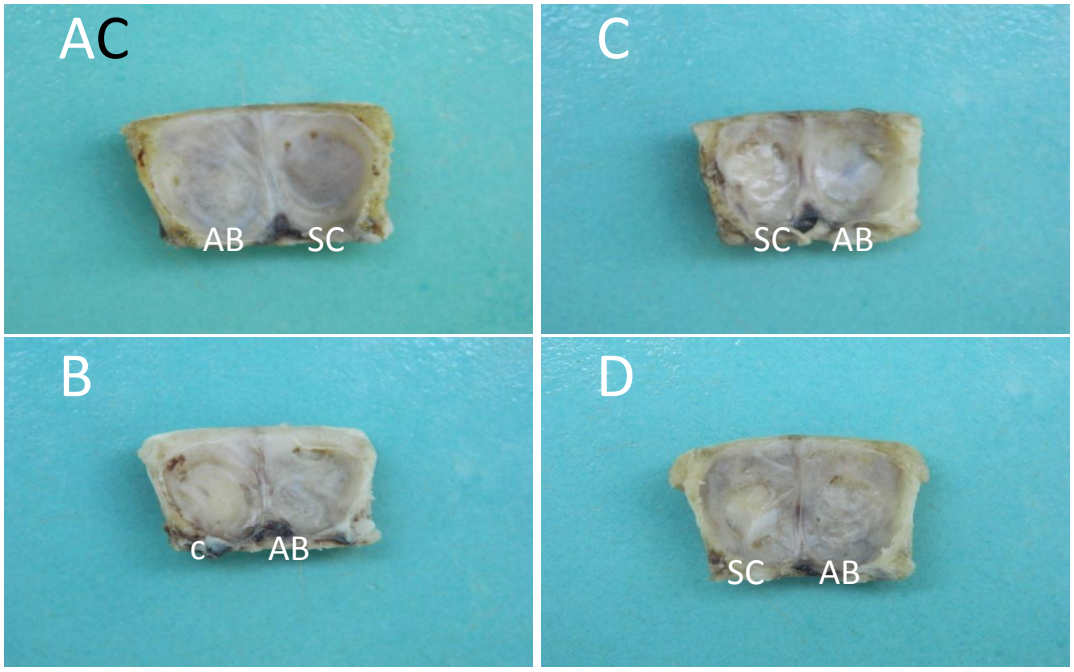

Figure 18. The specimen of rabbit calvarium at 2 weeks (A), 4 weeks (B), 8 weeks (C) and 12 weeks (D). AB= Autogenous bone chip and SC= 3D 2% w/v chitosan scaffold

Radiographic evaluation

The control group at 2 weeks and 4 weeks on the autogenous bone chip side showed a radiopaque mass of bone chip with varying sizes and density. At 8 weeks and 12 weeks, showed radiopaque mass of none chip varying sizes and densities. Homogenous radiopaque areas were apparent near the margins of the defects with similar density to that normal bone. The test group at 2 weeks and 4 weeks on the 3D 2% w/v chitosan scaffold side showed a homogenous radiolucent area over nearly the entire bony defect. At 8 weeks and 12 weeks, radiographs showed the margin of the defects being replaced by a radiopaque mass, and its density was similar to that of normal bone, but at the center of the defects there was still a radiolucent area (Figure 18).

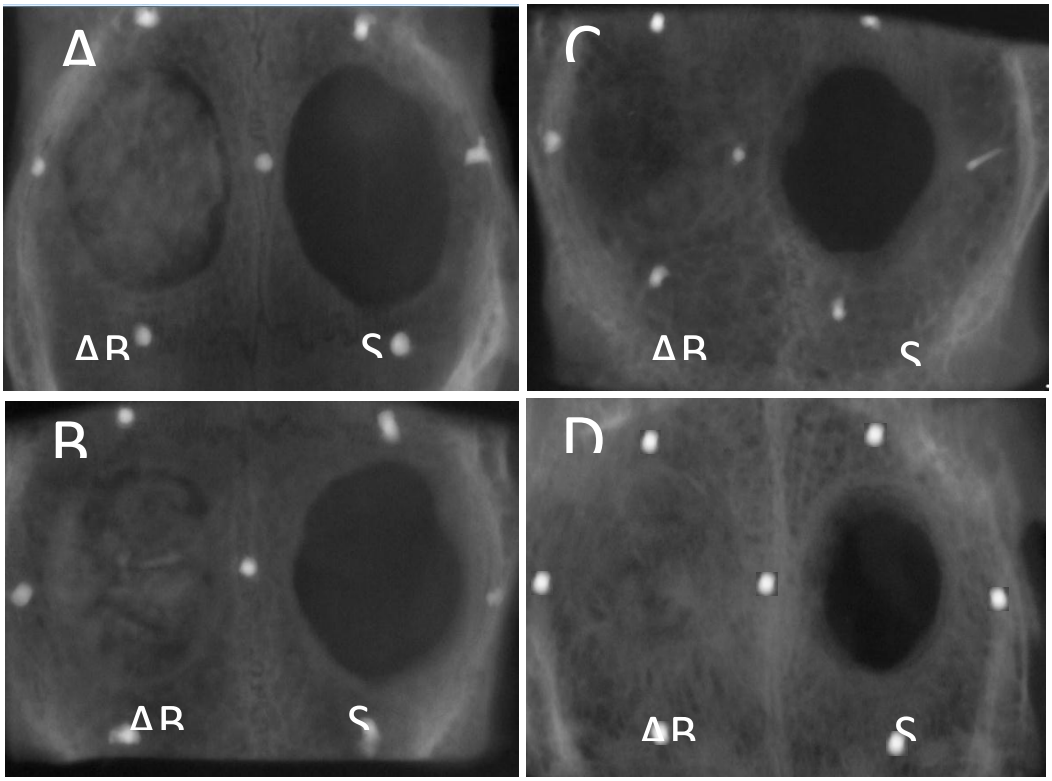

Figure 19. Radiograph of the rabbits' calvarium at 2 weeks (A); 4 weeks (B); 8 weeks (C) and 12 weeks (D) of healing. AB = Autogenous bone chip and SC = 3D 2% w/v chitosan scaffold

Radiomorphometric Analysis

The results of the radiomorphometric analysis are shown in Table 4. At 2 weeks postsurgery, mean radiodensity (±SD) for 3D 2% w/v chitosan scaffolds (group 1) and autogenous bone chips (group 2) were 0.0183±0.00377 and 0.0723±0.01345 respectively, with significant statistical difference ($p<0.05$). At 4 weeks postsurgery, mean radiodensity (±SD) for 3D 2% w/v chitosan scaffolds and autogenous bone chips were 0.0223±0.0767 and 0.0523±0.00293 respectively, with significant statistical difference ($p<0.05$). At 8 weeks postsurgery, mean radiodensity (±SD) for 3D 2% w/v chitosan scaffolds and autogenous bone chips were 0.0224±0.00402 and 0.0533±0.00253 respectively, with significant statistical difference ($p<0.05$. At 12 weeks postsurgery, mean radiodensity (±SD) for 3D 2% w/v chitosan scaffolds and autogenous bone chips were 0.0304±0.00446 and 0.0557±0.00438 respectively, with significant statistical difference ($p<0.05$) (Figure 20) and the experimental group found that

bone density mean had increased over the times of study, at 2 weeks had significant statistical difference with 12 weeks ($p<0.05$).

Table 4. Show data of radimorphometric (Bone density) in group1: 3D 2% chitosan scaffold and group 2: autogenous bone chip

Groups	Bone densitometry (mean± SD) OD			
	2 weeks	4 weeks	8 weeks	12 weeks
3D 2% w/v chitosan scaffold	0.018±0.0037	0.022±0.076	0.024±0.004	0.03±0.0044
Autogenous bone chip	0.072±0.013	0.052±0.0029	0.053±0.0025	0.055±0.0043

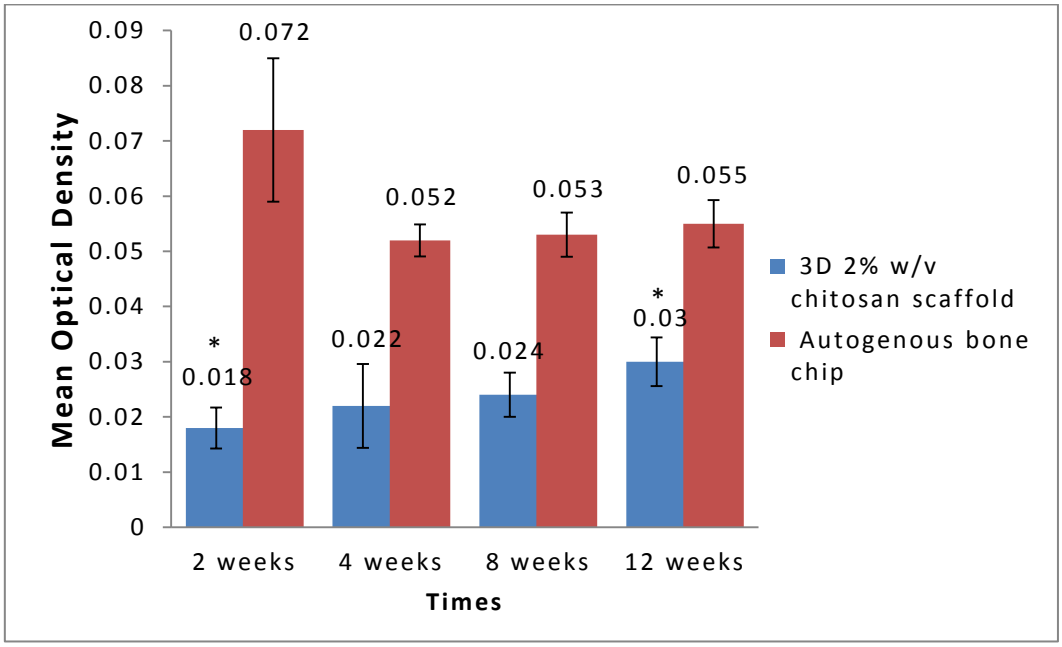

Figure 20. Mean optical density in each group after 2, 4, 8 and 12 weeks of healing

*= statistical significant difference ($p<0.05$)

Micro-CT Analysis

The total volumes of newly formed bone within the ROI (bone volume fraction or BV/TV) were summarized in Table5.At 2 weeks; the percentages of bone volume fraction for autogenous bone chips and 3D 2% w/v chitosan scaffolds were 17.71±2.62 and 3.42±0.62 respectively. At 4 weeks, percentages of bone volume fraction for autogenous bone chips and 3D 2% w/v chitosan scaffolds were 21.54±1.66 and 4.57±0.82 respectively. At 8 weeks, percentages of bone volume fraction for autogenous bone chips and 3D 2% w/v chitosan scaffolds were

25.83±0.64 and 6.17±0.7 respectively.At 12 weeks, percentages of bone volume fraction for autogenous bone chips and 3D 2% w/v chitosan scaffolds were 36.07±3.34 and 8.74±0.98 respectively**.** Throughout the period of study, the autogenous bone chips had a significantly greater percentage of bone volume fraction than the 3D 2% w/v chitosan scaffolds ($p<0.05$) (Figure 21), and 3D-reconstruction of bone volume fraction and material volume fraction of both groups were shown in Figure 22 and Figure 23. The experimental group found that bone volume fraction had increased over the times of study, at 2 weeks had significant statistical difference with 8 weeks and 12 weeks, at 4 weeks had significant statistical difference with 12 weeks ($p<0.05$).

Table 5. Bone volume fraction of micro-CT results at 2, 4, 8 and 12 weeks

Groups	BV/TV%			
	2 weeks	4 weeks	8 weeks	12 weeks
3D 2% w/v chitosan scaffold	3.42±0.62	4.57±0.82	6.17±0.7	8.74±0.98
Autogenous bone chip	17.71±2.62	21.54±1.66	25.83±0.64	36.07±3.34

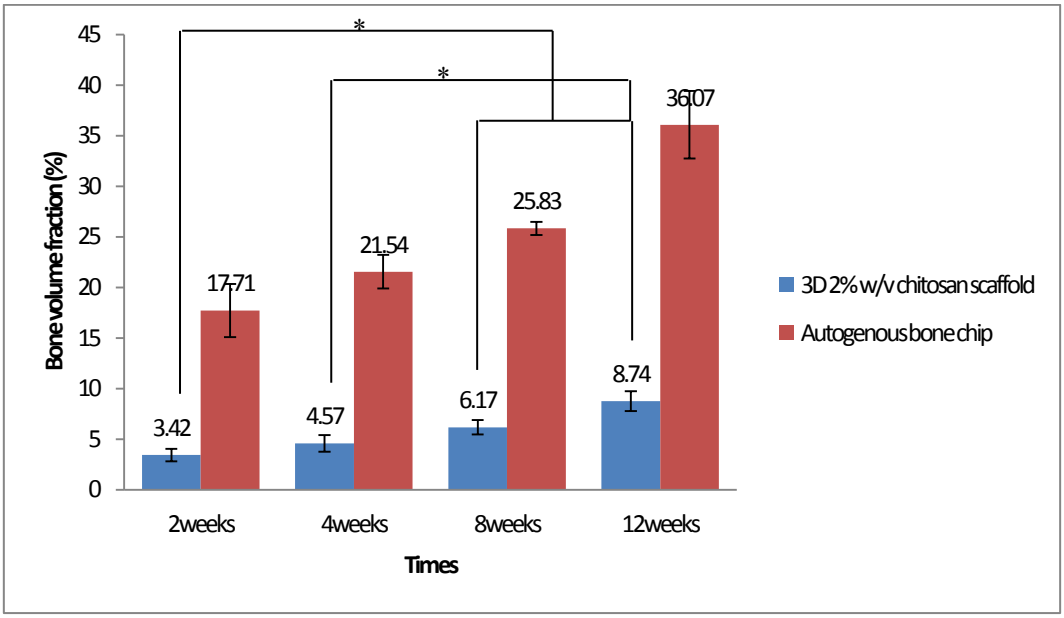

Figure 21. Micro-CT analysis; bone volume fractionof 3D 2% w/v chitosan scaffold and autogenous bone chips at 2, 4, 8, and 12 weeks

*= statistical significant difference ($p<0.05$)

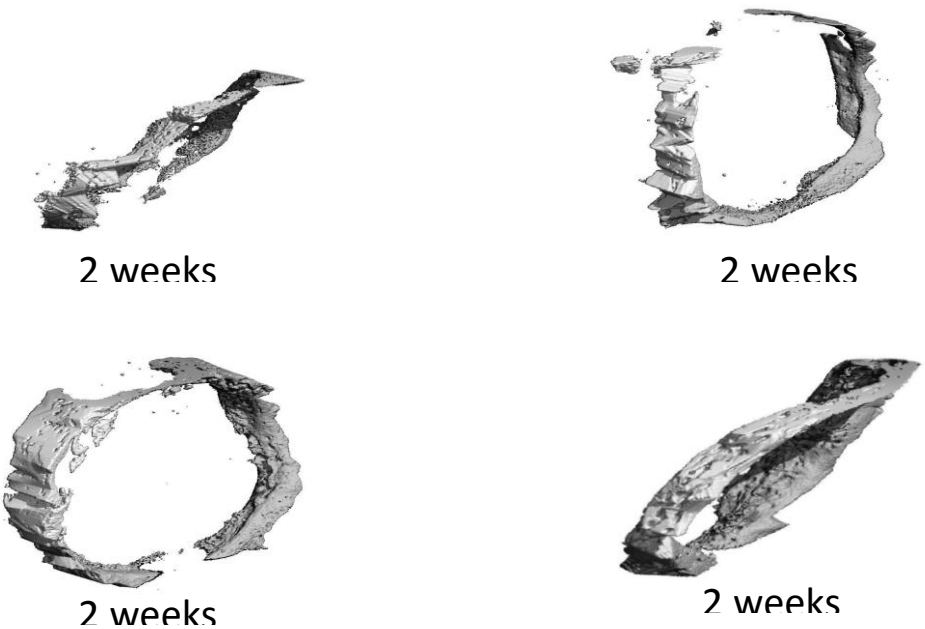

Figure 22. 3D-reconstruction of the bone volume fraction of 3D 2% w/v chitosan scaffolds at 2, 4, 8, and 12 weeks over the healing period

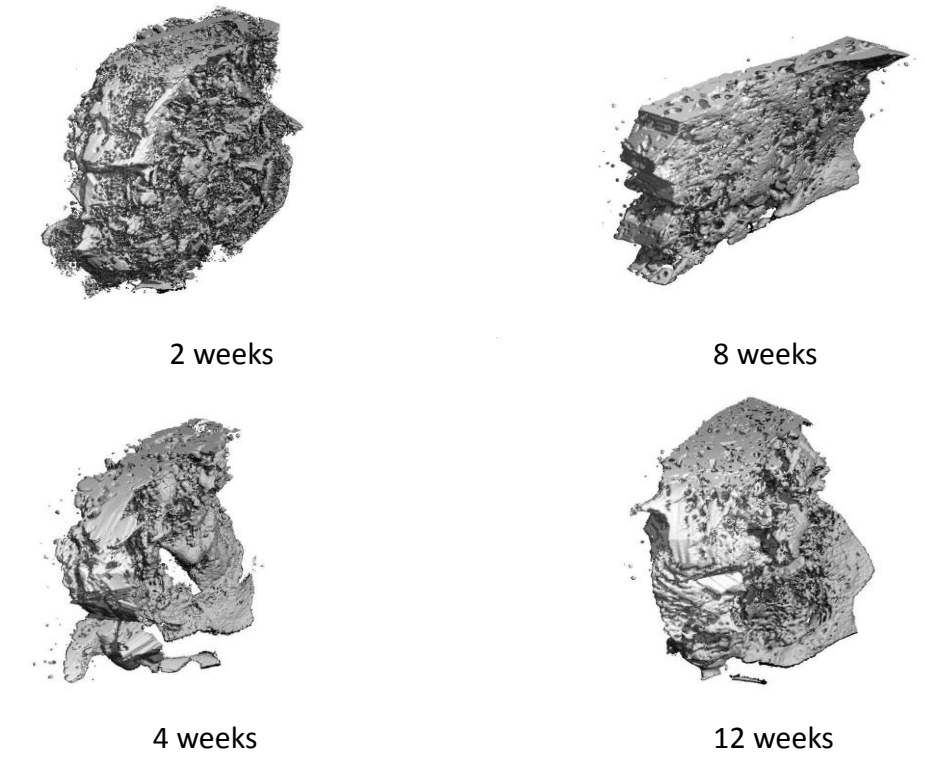

Figure 23. 3D-reconstruction of the bone volume fraction of autogenous bone chips at 2, 4, 8, and 12 weeks over the healing period

Histological evaluation

In H&E section slides, the presence of new bone growth, blood vessels and osteoid in various states of maturity were observed from the periphery of all defects, concluding that the cranial site in the rabbit is capable of generating physiologic healing in response to both of the graft materials. However, regions of active bone healing, evidenced by the turnover of graft material and dense area of bone formation, were seen greatly in the autograft-filled defects than presented in the 3D 2% w/v chitosan scaffold-filled defects (Figure 24 and Figure 25). The fibrous connective tissue stroma revealed over the residual 3D 2% w/v chitosan scaffold graft network at the center of the defect were observed mainly in all 3D 2% w/v chitosan scaffold graft samples, but no area of inflammatory cells were found. In addition, some regions of the autograft-filled defects presented with varying size of dead bone spicules containing some empty lacunae, suggesting that significant amounts of residual autograft bone remained in the defect.

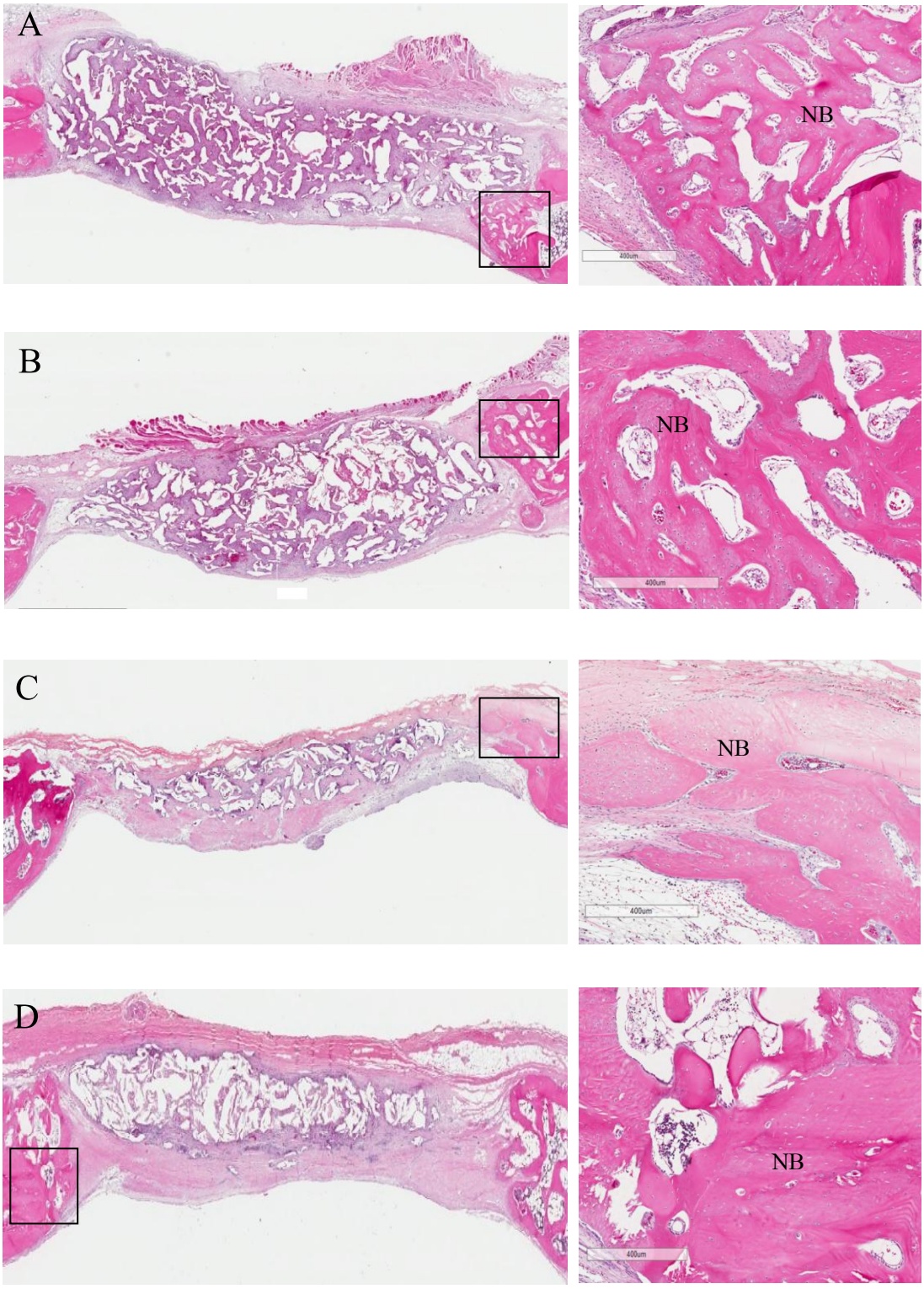

Figure 24. Histology examination of the test group at 2 weeks (A), 4 weeks (B), 8 weeks (C) and 12 weeks (D). SC(3D 2% w/v chitosan scaffold), NB(New bone) and OB(original bone)

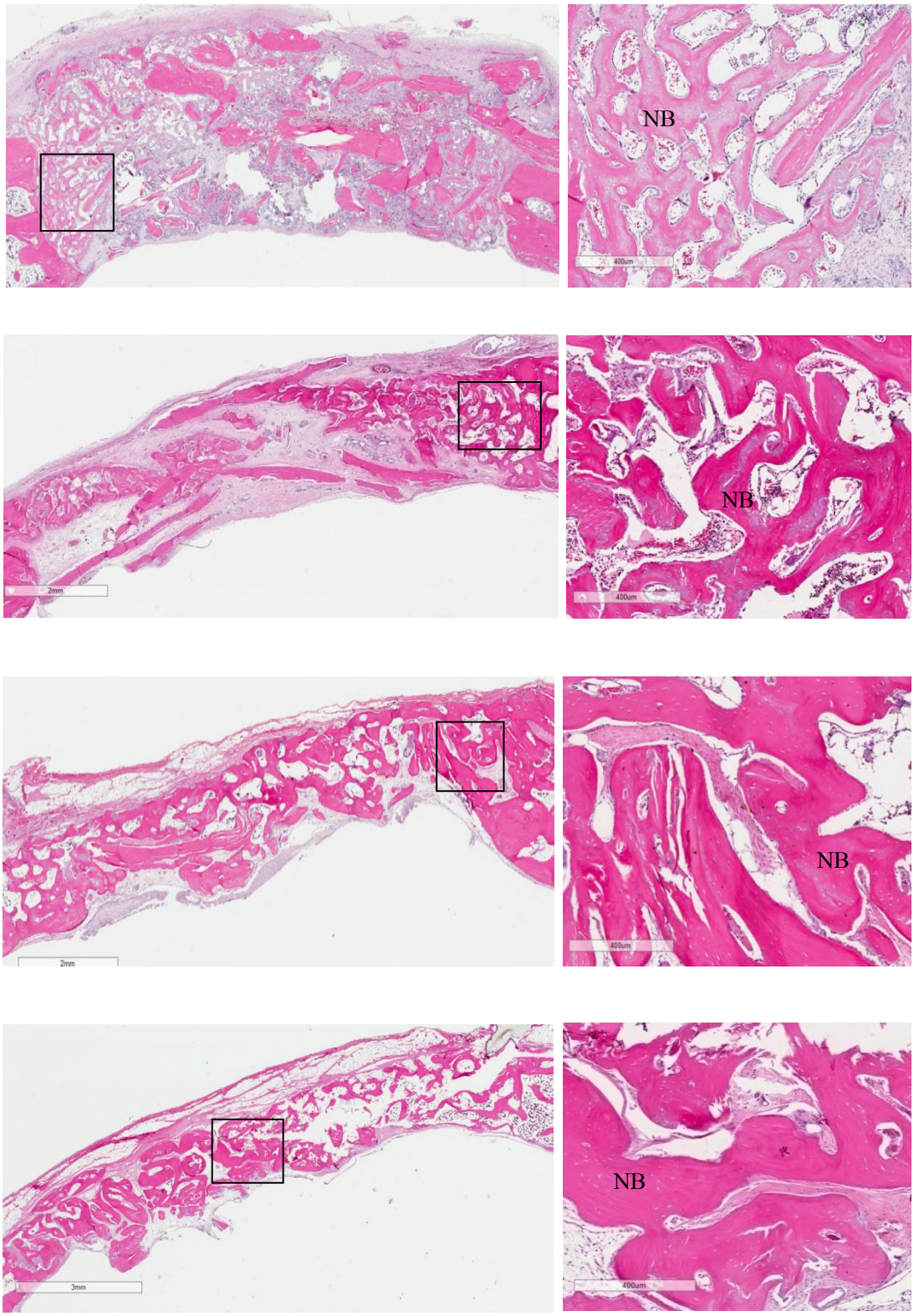

Figure 25. Histology examination of the control group at 2 weeks (A), 4 weeks (B), 8 weeks (C) and 12 weeks (D). NB(New bone) and OB(Original bone)

Histomorphometric Analysis

The values established to characterize the amount of new bone area produced in response to each type of graft material from each defect were represented as the average percentage of mean bone area from two representative slides that were measured three times for the mean bone area percentage value expressed in micron^2 units (Figure.23). The results of the histomorphometric analysis are shown in Table 6. At 2 weeks postsurgery, the mean new bone formation that revealed in the bone percentage area (±SD) for the control group and experimental group were 2.19±0.47and5.08±0.76 respectively, with significant statistical difference ($p<0.05$). At 4 weeks postsurgery, the mean new bone formation for the control group and experimental group were 4.49±0.96 and 9.91±0.57 respectively, with significant statistical difference ($p<0.05$). At 8 weeks postsurgery, the mean new bone formation for the control group and experimental group were 9.71±2.11 and 16.01±0.49 respectively**,** with significant statistical difference ($p<0.05$). At 12 weeks postsurgery, the mean new bone formation for the control group and experimental group were 13.35±1.39 and 24.54±1.47 respectively, with significant statistical difference ($p<0.05$). The experimental group found that percent of new bone formation had increased over the times of study, at 2 weeks had significant statistical difference with 8 weeks and 12 weeks, at 4 weeks also had significant statistical difference with 8 weeks 12 weeks ($p<0.05$).

Table 6. Shows data of histomorphometric analysis (% bone area) in both groups

Study group	% Bone area (Mean±SD)			
	2 weeks	4 weeks	8 weeks	12 weeks
3D 2% w/v chitosan scaffold	2.19±0.47	4.49±0.96	9.71±2.11	13.35±1.39
Autogenous bone chip	5.08±0.76	9.91±0.57	16.01±0.49	24.54±1.47

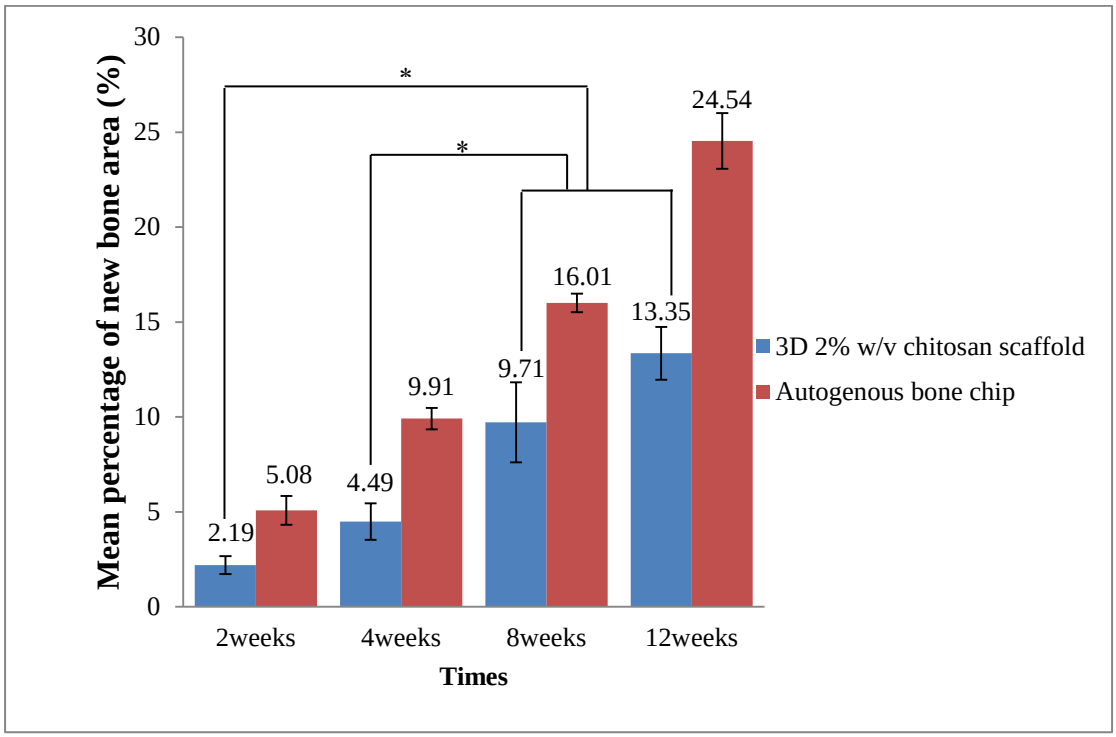

Figure 26. Histological finding of (H & E stained) the control group: Autogenous bone chips (A) and the experimental group: 3D 2% w/v chitosan scaffolds (B)

*= statistical significant difference ($p<0.05$)

Chapter 5

Discussion

The experimental model

The objective of this present study was to evaluate new bone formation using a 3D 2% w/v chitosan scaffold as the grafting material in a rabbit calvarial defect. The experimental model used in the present study was selected for the following reasons: 1) rabbits are widely used as experimental animals because they are cheaper and easy to keep; 2) the surgical procedure on the rabbit's calvarial bone is relatively simple to perform; 3) physiological bone healing is similar to humans;[67] 4) the calvarial defect model has many similarities to the maxillofacial region, as anatomically the calvarium consists of two cortical plates with a region of intervening cancellous bone similar to the mandible and physiologically, the cortical bone in the calvarium resembles an atrophic mandible;[68] and 5) a rabbit is easily manipulated and better ethically accepted for experiment than other animals such as goats, sheep, dogs, monkeys.

In rabbits, the bone metabolism is approximately three times faster than in humans.[67] Therefore, a healing period of 2 weeks and 4 weeks were chosen for evaluating the early phase of the healing response, such as the stability of the materials or the host reactions. Further healing periods of 8 weeks and 12 weeks were selected since such a time period is appropriate for evaluating the late phase of the healing response such as bone incorporation, resorption of materials, bone remodeling, or the amount of bone regeneration.[69]

Gross Specimen Observation

The 3D 2% w/v chitosan scaffold in this study, has many properties of an ideal bone substitute. It is a natural polymer material which can be manufactured to any shape, size and includes three dimensional (3D) scaffolds. A 3D 2% w/v chitosan scaffold in a 10 mm disk-shape was easy to use in this intraoperative experiment because the defects were also created in 10 mm-circular shapes. Moreover, whole defects repaired with 3D 2% w/v chitosan scaffolds showed very good tissue response without postoperative infection. At 2 weeks and 4 weeks in experimental group showed fibrous-like color and rubbery in consistency on palpation. It can said that phase of bone matrix production in a smaller amount of mineralized substance similarly to

fracture callus is structurally sound not as strong as mature bone. At 8 weeks and 12 weeks in experimental group showed color of regenerated tissue surface similarly to native bone hard in consistency. It can be said that mare mineralized substance deposition is similar to transitional stage phase I to phase II bone. In 3D 2% w/v chitosan scaffold showed fibrous-like color and rubbery in consistency. This may indicate that phase of bone matrix production in a smaller amount of mineralized substance similarly to fracture callus, which is structurally sound but not as strong as mature bone. In control group showed normal contour, normal color, hard in consistency, surface and texture of regenerated tissue similarly to native bone. Representative characters of ideal bone graft materials consist of osteoconduction (ideal scaffold for space maintenance), osteoinduction (growth factors, signaling molecule), and osteogenesis (osteoblastic cell). 3D 2% w/v chitosan scaffolds can promote new bone formation in qualitative and quantitative measurements by results obtained with a radiograph, a micro-CT and a histologic section.

Radiographic analysis

Radiographic results demonstrated the radiopacity in 3D 2% w/v chitosan scaffold graft defects but lesser amounts than observed in autogenous graft defects, which suggests that there were small amounts of mineralized bone tissue presented in 3D 2% w/v chitosan scaffold graft defects. In addition, some of the autogenous graft specimens revealed a speckled pattern in X-ray films which can be referred to the residual bone. Especially at 2 weeks, it was found that the mean OD increased higher than at other periods of the study because the autografting material in this study was cortical membranous bone from the rabbit cranium, which contains less osteogenic cells and is difficult for resorption and remodeling.[70] Moreover, in the preparation of autografting materials it was difficult to control the size of cortical bone particles so there was some different sizes of bone grafts in the defects that could not be completely resorbed and remodelled in every time period during the experimental study. Radiographic quantitative results obtained from this study revealed significant difference in mean OD between autograft-filled defects and 3D 2% w/v chitosan scaffold graft defects ($p<0.05$). The mean OD of the autografting group was significantly higher when compared with the 3D 2% w/v chitosan scaffold graft group. The Imaging Densitometer used in this current study was light and /or radiation detectors that are capable of converting biological signals into digital data. The digital

data are then displayed on the computer in a two-dimensional format using Molecular Analyst Softwae®. The intensity of the signals is directly proportional to the intensity of the gray levels displayed on the computer monitor. Therefore, the term "Optical Density" (OD) from this imaging Densitometer means the signal intensity and the term "Mean OD" is the average OD within the identified object or the measured frame. In this study, the measured frame was a circular shape that fitted to the created defects and was kept to the same size to observe the Mean OD from all tested defects including a step wedge bar in all X-ray films for calibrating the measurement.

Again, residual cortical bone should be suggested to cause a significant higher mean OD in all autogenous graft defects when compared with 3D 2% w/v chitosan scaffold graft defects. In addition, the method to identifying the defects after the healing period in this study was the created four gutta-percha holes encroaching each defect that could easily detect the defect margin during the specimen obtaining procedure and the radiographic evaluation. However, the disadvantage of this method was the contaminated gutta-percha flakes may have unintentionally got into some defects and may have been included in the Mean OD measured frame.

Micro-CT analysis

Conventional histologic or histomorphometric evaluation provides clear evidence of the bone healing process. However, the sample preparation process is tedious and destructive, and the three-dimensional (3D) anisotropic information of the bone trabeculae is compromised. Micro-computed tomography has been introduced as an alternative to these traditional evaluation methods. It has several advantages. As the specimens do not require special preparation in staining, embedding and cutting, non-invasive and provides a faster approach to evaluate and quantify cancellous bone. Most previous studies that used Micro-CT have focused on studying trabecular structures of cancellous bone. In this study, we used Micro-CT to analyze the amount of new bone formation using a rabbit cranial defect model,[(71)]but Micro-CT imaging data still has limitations in the discrimination of materials as it shows similar density such as bone chips and new bone. Results from Micro-CT revealed that the autogenous bone chip group had a significantly higher bone volume ($p<0.05$), but this data did not reflect the actual bone regeneration because the bone volume included both the grafted bone chips and new mineralized bone. The bone volume in the experimental groups represented actual new bone formed voxels.

However, Hou J et al (2012) evaluated segmental bone regeneration using rhBMP-2-loaded collagen/chitosan microspheres composite scaffolds in a rabbit model using micro-computer tomographic analysis. They showed that not only defects were bridged as early as 4 weeks, but also the defects were healed and presented recanalization of the bone marrow cavity at 12 weeks.[47]

Histological analysis

Histologic examination results revealed that defects repaired with 3D 2% w/v chitosan scaffold showed viable lamellar bone with osteoblast forming bone and blood vessels 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 grafts also have osteoconduction and osteogenesis properties.[72, 73] In addition, the predominance of bone formed through conduction from the periphery of the defect has also been described by others using the rabbit calvarial implantation site.[74, 75] 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 suggesting that the 3D 2% w/v chitosan scaffold is a biodegradable material, but it could not be completely degraded at any of the time periods in this study. The degradation of modified chitosan is known to be performed by lysozymic hydrolysis that require the macrophage cells from vascular tissue.[76] The lack of abundant vascular bundles from the cortical membranous bone in a rabbit cranium model would suggest causing slow degradation of 3D 2% w/v chitosan scaffolds and small amounts of new bone tissue response to this type of graft material. However, the periosteum which other studies[77] suggested to promote the bone healing was preserved and should refer to be the source of osteoprogenitor cells and fibrovascular tissue in this study. Moreover, result obtained with histomorphometric analysis also show significant difference in histologic bone area between autograft-filled defects and 3D 2% w/v chitosan scaffold graft defects at the $p<0.05$ level. The mean bone area percentage of the autograft group was significantly higher when compared with the 3D 2% w/v chitosan scaffold graft group. Again, the higher discrepancy of mean bone area percentage between both types of graft material 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 and the fact that some residual autograft bone was measured as new bone formation without intention.

Furthermore, histologic examination results revealed that the defects repaired with 3D 2% w/v chitosan scaffolds showed only a few areas of inflammatory cells, while autogenous-filled defects showed no area of inflammatory cells. These findings suggest that the 3D 2% w/v chitosan scaffold is a biocompatible material when used as a bone substitute. According to the study of Alberius and Johnell (1991)[78], the observation of viable lamellar bone with osteoblasts forming bone in both 3D 2% w/v chitosan scaffolds and autogenous-filled defects could suggest that 3D 2% w/v chitosan scaffolds promoted new bone formation by intramembranous ossification in the same pattern as autogenous grafts. Therefore, 3D 2% w/v chitosan scaffolds could promote new bone formation in histomorphometric analysis when compared with autogenousgrafts, it could promote new bone formation more than 50% at 8 weeks and 12 weeks

In previous study, use chitosan sponge[79]and chitosan/TCP sponge[54] as a carrier for platelet-riched plasma in rat calvarial bone has enhanced effectively bone formation. Due to the osteoinductive role of platelet-riched plasma, the bone healing capacity of these two types of chitosan-contained scaffold could not be evaluated. Although previous studies of other chitosan-contained porous scaffold such as porous chitosan matrice grafted in rat calvarial bone could promote new bone formation, unfortunately, no quantitative measurement had been undertaken[80]. When compared new bone formation capacity of 3D 2% w/v chitosan scaffold from this study to other types of bone substitute material, there were more than 50% new bon formation of 3D 2% w/v chitosan scaffold at 8 weeks and 12 weeks is comparable to bioactive glass ceramic promoting 40% of new bone formation[81], while poly-lactic acid with alpha tricalcium phosphate[82] shown only 14% of new bone formation in a load implant model in sheep and collagen sponge with rhBMP, an extracellular matric scaffold, promoting new bone formation in the same level of autogenous graft in sinus augmentation of rabbit[83]. However, high level of new bone formation of the collagen sponge with rhBMP resulted from the osteoinductive properties of rhBMP carried in the material.

Although this study was investigated in only 16 rabbits, the results showed statistical significant difference between two types of graft materials because the autografting material played as a positive control in this experiment. Therefore, the addition of a negative

control defect, which was an empty bony defect, would contribute to better evaluate the bone healing capacity of 3D 2% w/v chitosan scaffold. Moreover, the size of the defect used in this study was not a critical-sized cranial defect in the rabbit model which is 15 mm, due to the limitation in small size of rabbit cranium.[84] However, the 10 mm sized bony defect used in this current study was proper for comparing the bone healing capacity in both types of graft materials.

In this present study, the 3D 2% w/v chitosan scaffold was an inspirative bone substitute material because of the ability to produce it in our own laboratory and no previous studies have evaluated its healing capacity. Moreover, 3D 2% w/v chitosan scaffolds have many advantages for being a bone substitute material because of its bulk scaffold which can be grafted in bone defects, the controllable shape and size, and its porous structure which osteoprogenitor cells can easily migrate into 3D 2% w/v chitosan scaffolds.

Chapter 6

Conclusion

In conclusion, the 3D 2% w/v chitosan scaffold presented good biocompatibility, osteoconductive properties, and enhanced new bone formation in an animal model. Therefore, it should be a scaffold for new bone formation.

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Appendix

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This is to certify that the research project entitled "Effect of 3D 2% w/v chitosan scaffold on new bone formation in rabbit calvarial defect" which was conducted by Assoc. Prof. Thongchai Nuntanaranont, Faculty of Dentistry, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

Kitja Sawangjaroen

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