Original Paper

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Treatment of critical-sized calvarial non-union defect via collagen-polyglycolic acid scaffold loading with simvastatin in rabbits

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Keywords

collagen, polyglycolic acid, bone formation, simvastatin, scaffold

Abstract

The aim of the present study is to investigate the effects of a sustained-release simvastatin in collagen-polyglycolic acid scaffold on bone formation in the rabbit calvarial critical-sized defect. This study was carried out to examine if maximum bone regeneration with less inflammation would be attained by combining an optimal dose of simvastatin with Collagen-Polyglycolic acid scaffold, which is an osteoconductive biomaterial capable of releasing the drug slowly. To induce critical-sized calvarial defect in the 10 nominated adult New-Zealand rabbits we trephined four holes measured 5-mm-diameter into each head, and filled them with preparations of different doses of simvastatin (0.5 mg, and 1 mg) blended with Collagen-Polyglycolic acid, Scaffold alone or left empty. Five animals were sacrificed after 4 weeks and the rest of them after 8 weeks and examined histologically. Statistical analysis revealed that in the first time frame (the first four weeks), the difference between the control group and the simvastatin 0.5 mg group on one hand and the simvastatin 1 mg group and the control group on the other hand, there were statistically significant difference between ($p < 0.05$). In the second time frame (the next four weeks), there were statistically significant differences between the simvastatin 0.5 mg group and the control group, and between the scaffold group and the control group ($p < 0.05$). When combined with collagen-polyglycolic scaffold, 0.5 mg simvastatin is the optimal dose for the arousal of the maximum bone regeneration in rabbit calvarial defects without causing inflammation and it could be applied as an effective bone graft material.

Abbreviations

PGA: Polyglycolic Acid
BMP-2: Bone Morphogenetic Protein 2
SC: Scaffold
SIM: Simvastatin
BMSCs: Bone marrow-derived Mesenchymal Stem Cells
EPCs: Endothelial Progenitor Cells
VEGF = Vascular Endothelial Growth Factor
eNOS = Endothelial Nitric Oxide Synthase
α-TCP = Alpha Tri-Calcium Phosphate
CS = Calcium sulphate
CSD = critical size defect

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Introduction

The necessity to transplant bone to a deficient skeletal location has been identified for centuries. Bone occurs in two forms, trabecular and compact, and it carries out several functions. First, bone is the main reservoir of calcium in the body, and exchanges this mineral easily with the extracellular fluid environment. The concentration of calcium in the body fluids is strictly controlled, and the supply of calcium in bone is essential to this control. Second, the hematopoietic marrow that is situated in trabecular bone provides the body's cells, tissues and organs with their nutrient-carrying red blood cells and infection combating white blood cells (Yaszemski et al., 1996).

The alleviation of bone defects has continued to be a significant clinical orthopedic challenge. Clinical situations develop when bone is incapable of healing itself, similar to segmental bone loss, fracture non-union, and failed spinal fusion which is then result in serious morbidity and mortality. Recent attempts to improve bone healing have achieved limited success, giving power to the development of improved techniques. Although the autogenous bone graft is look up on as the gold standard, it is linked with many limitations such as increased operative time, limited availability and significant morbidity associated with blood loss, wound complications, local sensory loss and, above all, chronic pain. To circumvent these restrictions, various organic and inorganic biomaterials have been suggested such as ceramics, poly glycolic acid, poly lactic acid and collagen. However, no ideal material has yet been pinpointed. Bone tissue-engineering methods apply bone marrow-derived mesenchymal stem cells (BMSCs) or endothelial progenitor cells (EPCs) to facilitate restoration. Nevertheless, these methods are limited by the restricted availability of stem cell sources, the potential immune response, and the complication of the procedures (Yueyi et al., 2013; Hussain et al., 2014).

Bone grafts are commonly used to correct skeletal deficits. Moreover, they can add one or more forms of stimulation to a site where bone is required. Osteoinductive materials actively trigger the formation of new bone by prompting primitive stem cells to transform into osseous-forming cells and osteoconductive materials serving as scaffold. To develop an effective bone graft substitute, both osteoinductive and osteoconductive properties are required (Ozec et al., 2007).

Collagen has been extensively used as a material for cell scaffolds, and it has in vivo safety proven through long-term applications in clinical trials, cosmetics, and the food industries. Collagen is the most familiar protein in the body, which provides tissues in the body including skin, blood vessels, tendon, cartilage and bone with strength and structural stability. Along with hydroxyapatite, collagen is one of the two major components of bone. It forms eighty nine percent of the organic matrix and thirty two percent of the volumetric composition of bone. Therefore, its potential for culturing cells to produce bone appears to be significant. Some of the superiorities associated with collagen include the low immunogenic response across species, abundance in nature, hemostatic promotion, and ease of its manipulation into different forms. Collagens are weakly immunogenic in comparison with other proteins. The antigenicity of a collagen biomaterial can be declined by the process of cross-linking. Collagen sponges are highly permeable with interconnected pore structure, which is effective in infiltration of cells, supplying of nutrients and oxygen to cells and dispose of cell waste. Collagen, in terms of its protein nature, is a biodegradable molecule, which is decomposed in the tissues by catabolic processes, including degradation by specific collagenases and phagocytosis. The rate of degradation of an implant relies heavily on the location selected for implantation in the organism. Comparable to all natural polymers, one fundamental issue about using collagen as the major constituent of a scaffold for orthopedic tissue engineering is its relatively poor mechanical properties. However, it is illustrated in this paper that the compressive and tensile mechanical properties of collagen scaffolds can be improved using physical and chemical cross-linking methods (O'brien et al., 2011; Chevallay et al., 2000; Toosi et al., 2016). However, based on the previous studies, collagen sponge as a scaffold for cell growth and differentiation in hard tissue has weak mechanical strength and is not suitable to be used in bone tissue engineering. In order to overcome this problem with collagen sponge, mixing with other materials should be tried. The materials that are used for combination need to be bioabsorbable and it is preferable to choose material that has been clinically used. Some biodegradable synthetic polymers, such as poly glycolic acid (PGA) and its copolymers with L-lactic acid and D-lactic acid, have been blended into collagen sponges for tissue engineering. PGA has US FDA approval for clinical use in humans in a variety of medical applications. Studies have demonstrated that incorporation of PGA fibers assists collagen sponge in mounting resistance against compression in vitro. By reducing water absorption and providing a physical network between fibers and collagen, collagen sponge shrinks less. Shrinkage is among the most significant deficiencies of collagen sponges (Agrawal et al., 2001; Hiraoka et al., 2003; Toosi et al., 2016). In this study, we added PGA fibers to collagen sponge to use the advantages of each other.

Simvastatin is a chemical alteration of lovastatin, a rate-limiting enzyme of the cholesterol synthesis pathway. Moreover, Statin is a certain inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, rate-limiting enzyme of the cholesterol synthesis pathway. Simvastatin is claimed to enhance osteoblastic activity and impede osteoclastic activity. Additionally, It is appears to have an anti-inflammatory effect that works by reducing the production of interleukin-6 and interleu-

kin-8. The success in use of simvastatin to promote bone formation in vivo is dependent on the local concentration. In fact, there have been continuous attempts to find an appropriate delivery system. The effects produced depend on the doses, which means doses should be prescribed with caution considering the benefits and risks. Several studies have been carried out showing the bone-promoting effect of local application with different carriers in various animal models. Simvastatin is reported to increase cancellous bone volume, bone formation rate, and cancellous bone compressive strength (Park et al., 2009). The purpose of the present study is to inspect the effects of loading simvastatin in the collagen-polyglycolic acid scaffold on rabbit calvarial bone defects and investigate the amount of new bone formation histopathologically.

Materials and Methods

Preparation of collagen-polyglycolic acid and simvastatin combination

Type I collagen in the form of an aqueous solution, made from porcine tendon by pepsin treatment (6.33 mg/mL, pH 3.0) in HCl, and was bought from Nitta Gelatin Inc. The non-woven fabric of PGA fiber (Figure 1), 20 mm in diameter (0.5 mm in thickness, 200–210 g/m^2) was a gift from Dr. Hossein Hosseinkhani (Taipei, Taiwan) (Toosi et al., 2016).

Collagen sponges with various amounts of PGA fiber were made using the dehydrothermal method. To eliminate oils and fats, PGA non-woven fabric was submerged in acetone for 1 h and washed three times with double distilled water for 10 min at 25°C (Toosi et al., 2016). PGA fiber was loosened by using tweezers to gain the PGA component. Then, collagen/fiber ratio (w/w) 0.52 was selected. 1.8 mg PGA and 0.94 mg collagen (150 µl) were placed into the 96-well plate homogenously. The resulting collagen solution was frozen at -20°C for 24 h to obtain collagen sponge incorporating PGA fiber. The sponge which underwent freeze-drying was dehydrothermally cross-linked at 140°C for 12 h under 0.1 torr vacuum situations. Since cross-linking method is toxicologically more appropriate than the chemical cross-linking alternative, the former method was selected (Toosi et al., 2016).

In vitro assay of simvastatin release from simvastatin-loaded collagen-polyglycolic acid scaffolds

The simvastatin-loaded Collagen-Polyglycolic Acid scaffolds with different dose of simvastatin (0.5 mg , 1 mg) were laid down in 5 ml PBS at 37˚C and the PBS was altered in 1, 3, 4, 6, 8, 11, 14 or 21 days, respectively. At each point, the solution absorbance was investigated at a wavelength of 238 nm using an ultraviolet-visible spectrophotometer, while the simvastatin concentration was assessed through a standard curve made of different amounts of simvastatin

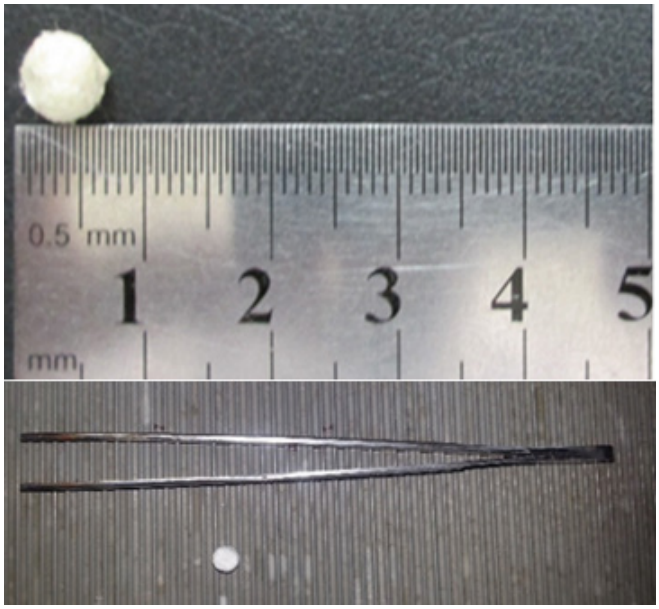

Figure 1
Frame structure of a collagen sponge with PGA fibers

(Figure 2A, 2B) (Huang et al., 2014).

Anesthesia and surgical procedures

The institutional committee for animal experiments consented to this study. Ten New Zealand rabbits were used in this study. The rabbits were at adult stage and weighed approximately 2kg. The animals were kept in cages and fed with a solid diet and water ad libitum. Four bone circular defects (5mm in diameter) per rabbit were created in the calvarial bone while protecting the dura mater. The first experimental group of holes were filled with 1 mg simvastatin loaded in collagen-polyglycolic acid, whereas in the second group 0.5 mg simvastatin replaced 1 mg of it. The third group of holes had only the collagen-polyglycolic acid without simvastatin. No material was implanted in the control group. Five animals were killed postoperatively after 4 weeks and the rest were sacrificed after 8 weeks (Mukozawa et al., 2011).

The animals were administered anesthetic with a mixture of ketamine–xylazine (40 and 4 mg/kg), and Isoflurane in 100% oxygen were used as a maintenance of the anesthesia. The dorsal part of the cranium was shaved and aseptically prepared for surgery. A 40-mm-long cut was made along the calvarial bone, so that the skin, subcutaneous tissue and periosteum were reflected, uncovering the calvarial bone. To produce the four full-thickness bone defects of 5mm diameter the dorsal part of the calvarium was trephined (Figure 3A). A 5mm trephine bur was used to induce the defects under constant irrigation with sterile physiologic solution to avoid overheating of the bone edges. The surgical procedure was conducted with proper care to minimize damage to dura matter (Nyan et al., 2009). The holes in each rabbit were rotated clock-wise, they filled with 0.5 mg simvastatin loaded in collagen-polyglycolic

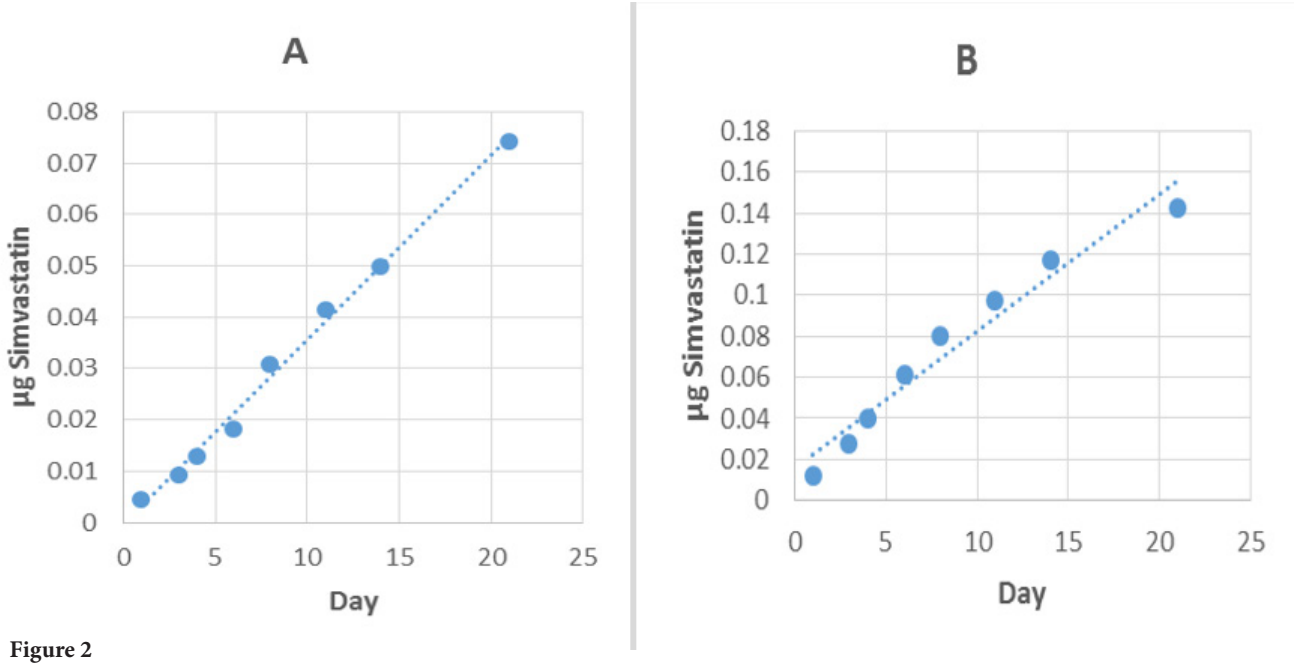

Figure 2
Cumulative release of simvastatin in vitro. Simvastatin-loaded Collagen-Polyglycolic Acid scaffold (0.5 mg Simvastatin; A) and Simvastatin loaded Collagen-Polyglycolic Acid scaffold (1 mg Simvastatin; B).

acid scaffold, 1 mg simvastatin loaded in collagen-polyglycolic acid scaffold, collagen-polyglycolic acid alone, and finally one hole left empty (Figure 3B).

Tissue processing

All animals' lives were humanly taken after 4 and 8 weeks postoperative, respectively. The area of the original surgical defect and the tissues surrounding it were separated en bloc. The blocks were placed in 10% neutral formalin, washed with water, and then decalcified in 10% nitric acid solution. After the first decalcification, each specimen was split longitudinally into two blocks precisely along the center line of the original surgical defect (Marianoet al., 2010). After further decalcification, they were processed and placed in paraffin. Serial sections 6-µm thick were lacerated in a longitudinal direction starting at the middle of the original surgical defect. The sections were spattered with hematoxylin and eosin (H & E) to be analyzed by light microscopy (LABO AMERICA INC USA (LABO MED)) (Mariano et al., 2010).

Statistical methods

All measurements and histological inspections were conducted blindly without knowing if a treated or untreated specimen was assessed. Statistical analyses were performed by the statistical program SIGMA STAT version 3.5 for Windows. Because the data were non-parametric; between two different time frame (4 weeks and 8 weeks)

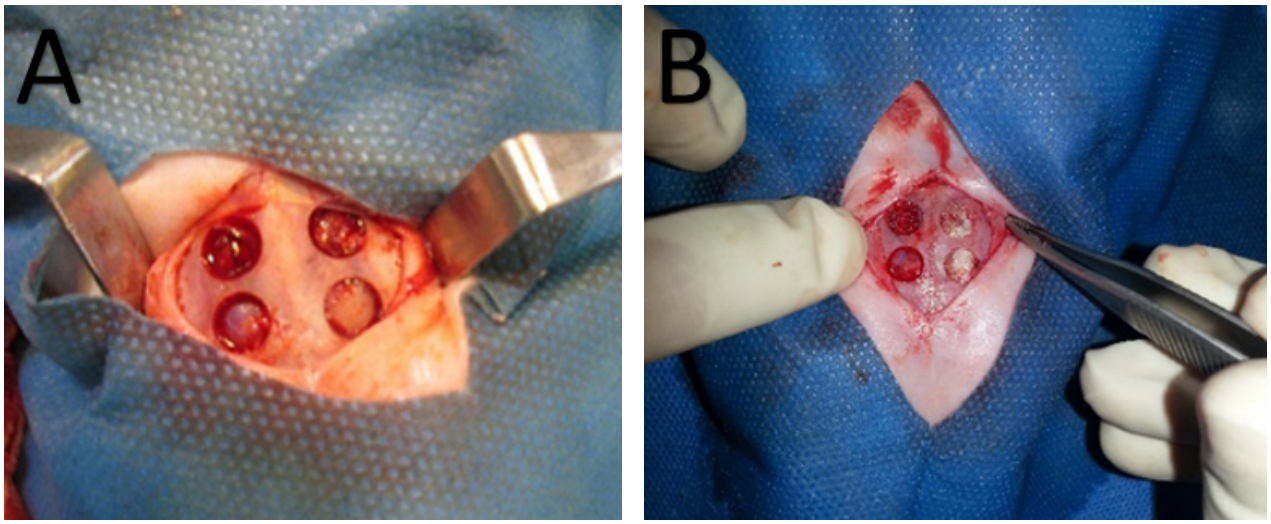

Figure 3
Intraoperative finding. (A) Bone defects were made at the calvarial region. (B) Showing the sites of four surgically – created bone defects filled with different types of treatment (1) 0.5 mg simvastatin with collagen-PGA, (2) 1 mg simvastatin with collagen-PGA (3) collagen-PGA alone (4) Control (no material) that were implanted within the defects.

Table 1
Histological Scoring System. A scoring system was used to assess histopathological survey (Khadra et al., 2004; Karimi et al., 2013; Yuehuei H. An et al., 2003).

Parameter	Criteria	Score	Parameter	Criteria	Score	Parameter	Criteria	Score
Quantity of new bone formation	No new bone formation	0	Numbers of fibroblasts	None to very minimal	0	Union	No evidence of union	0
	Mild bone formation	1		Few fibroblasts	1		Fibrous union	1
	Moderate bone formation	2		Predominantly fibroblasts	2		Osteochondral union	2
	Full bone formation	3		Fewer number of fibroblasts	3		Bone union	3

Kruskal-Wallis One Way Analysis of Variance on Ranks was used and between the four groups (Control, Scaffold, Scaffold with Simvastatin 0.5 mg and Scaffold with Simvastatin 1 mg) Mann-Whitney Rank Sum Test was used. A P value below 0.05 was considered to indicate a statistically significant difference between the groups.

Results

Macroscopic observation

All animals recovered uneventfully after the surgery. No macroscopic infection of the wounds was noted. Side effects such as paralysis, paroxysm, respiratory problems or signs of pain were not seen. In all animals of negative control, Collagen-PGA without simvastatin, Collagen-PGA with 0.5 mg simvastatin and Collagen-PGA with 1 mg simvastatin groups, the soft tissue wounds healed uneventfully without showing clinical signs of inflammation.

Histological observation

A scoring system, illustrated in Table 1, was used to assess histopathological survey (Khadra et al., 2004; Karimi et al., 2013; Yuehuei H. An et al., 2003).

In the 4 weeks' time frame group only the difference between the control group and scaffold with simvastatin 0.5 mg, and control group and scaffold with simvastatin 1 mg were statistically significant; however, in the 8 weeks' time frame group the difference between control group and scaffold with 0.5 mg simvastatin, and control group and scaffold without simvastatin were statistically significant (p <0.05) (Table 2).

In our survey of the control groups in the four-week category and the eight-week category, no significant difference (P=1.000) was observed. However, a significant difference (P=0.008) between the scaffold groups of the two categories was noticed. Moreover, Between simvastatin 0.5 mg groups in the four-week category and that of the eight-week category no significant difference was detected (p = 0.095), which was also the case for simvastatin 1 mg with p = 0.690 (Table 3).

Descriptive Histological Examination

Control Groups

In both four and eight-week categories, only a thin layer of new bone was seen in the defect margins. The central portion of defect was filled with tissue debris or left empty. There was no difference between control groups neither in

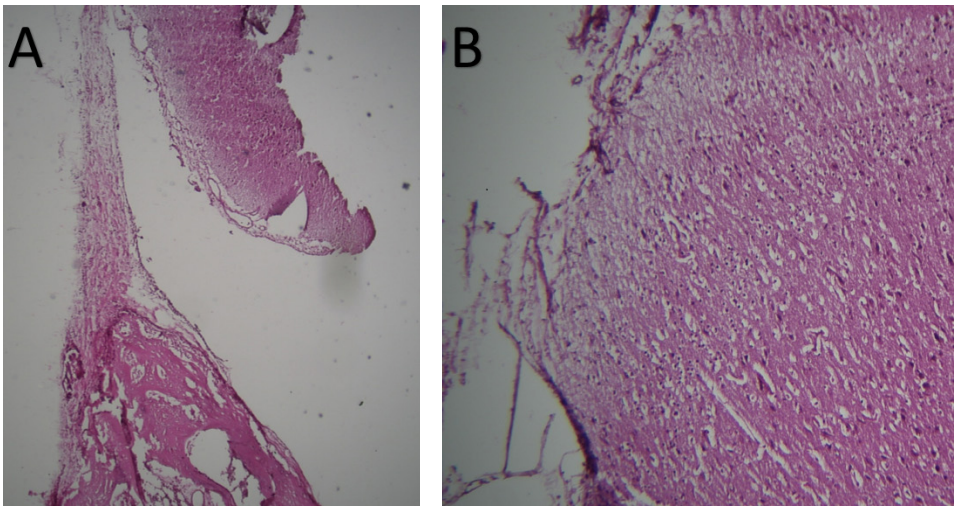

Figure 4
Photomicrographs of the calvarial defects of the control group. (A) Avulsion of the necrosis tissue (Hematoxylin and eosin stain, original magnification × 4) (B) Tissue necrosis without any sign of bone repair (Hematoxylin and eosin stain, original magnification ×10)

Table 2
Data of Histological Analysis Between Two Time Frame

Group	Number		Median		25%		75%	
Time	4w	8w	4w	8w	4w	8w	4w	8w
Control	5	5	0.00	0.00	0.00	0.00	0.00	0.00
Scaffold	5	5	4.00	7.00	3.00	5.75	4.00	7.50
Simvastatin 0.5 mg	5	5	6.00	9.00	4.75	6.75	8.00	9.00
Simvastatin 1 mg	5	5	5.00	5.00	4.00	4.75	5.00	5.00

In the 4 weeks' time frame group only the difference between the control group and scaffold with simvastatin 0.5 mg, and control group and scaffold with simvastatin 1 mg were statistically significant; however, in the 8 weeks' time frame group the difference between control group and scaffold with 0.5 mg simvastatin, and control group and scaffold without simvastatin were statistically significant ($p < 0.05$)

four nor in eight-week categories (Figure 4).

Collagen-Polyglycolic Acid Group

In the four-week category, a scant amount of bone formation was observed at the periphery of the defect. The middle of the defect was filled with collagen fiber and connective tissue (fibroplasia); however, in some places evidence of new bone formation were detected (Figure 5A). The remnant of the polyglycolic acid fibers which were light yellow in color were evident between the collagen fibers (Figure 5B). In the eight-week category, the amount of bone formation was much higher than the four-week category; although connective tissue which was filling the defect was still evident and fibroplasia was less than that of the four-week category (Figure 5C).

Collagen-Polyglycolic Acid with simvastatin 1 mg Group

In the four-week category, bone formation in the margin of the defect and around PGA fibers was mild (Figure 6A). Center of the defect was mostly filled with connective tissue and bone matrix was little. Moreover PGA fibers were surrounded with newly formed bone (Figure 6B).

In the eight-week category, PGA fiber became less in their number than that of the four-week category (Figure 6D). Nonetheless, Osteochondral bone formation was more than that of the four-week category. Besides thickness of the newly formed bone was more than that of 4 weeks (Figure 6C).

Collagen-Polyglycolic Acid with simvastatin 0.5 mg Group

In the four-week category, new bone formation was more than the rest of the other group. Furthermore, newly formed vessels were completely evident. Bone formation in the margin of the defect was more than the other groups in the terms of quality (Figure 7A). Periphery of the newly formed bone was surrounded with active osteoblasts (Figure 7B).

In the eight-week category, the defects were completely filled with the osteoid matrix (Figure 7C). PGA fibers were still evident. Fibroplasia was diminished significantly and

Table 3
Data of Histological Analysis Between Four Groups

Group	Number	Missing	Median	25%	75%
Control 4w	5	0	0.000	0.000	0.000
Control 8w	5	0	0.000	0.000	0.000
Scaffold 4w	5	0	4.000	3.000	4.000
Scaffold 8w	5	0	7.000	5.750	7.500
Simvastatin 0.5 mg 4w	5	0	6.000	4.750	8.000
Simvastatin 0.5 mg 8w	5	0	9.000	6.750	9.000
Simvastatin 1 mg 4w	5	0	5.000	4.000	5.000
Simvastatin 1 mg 8w	5	0	5.000	4.750	5.000

In our survey of the control groups in the four-week category and the eight-week category, no significant difference ($p = 1.000$) was observed. However, a significant difference ($p = 0.008$) between the scaffold groups of the two categories was noticed. Moreover, Between simvastatin 0.5 mg groups in the four-week category and that of the eight-week category no significant difference was detected ($p = 0.095$), which was also the case for simvastatin 1 mg with $p = 0.690$

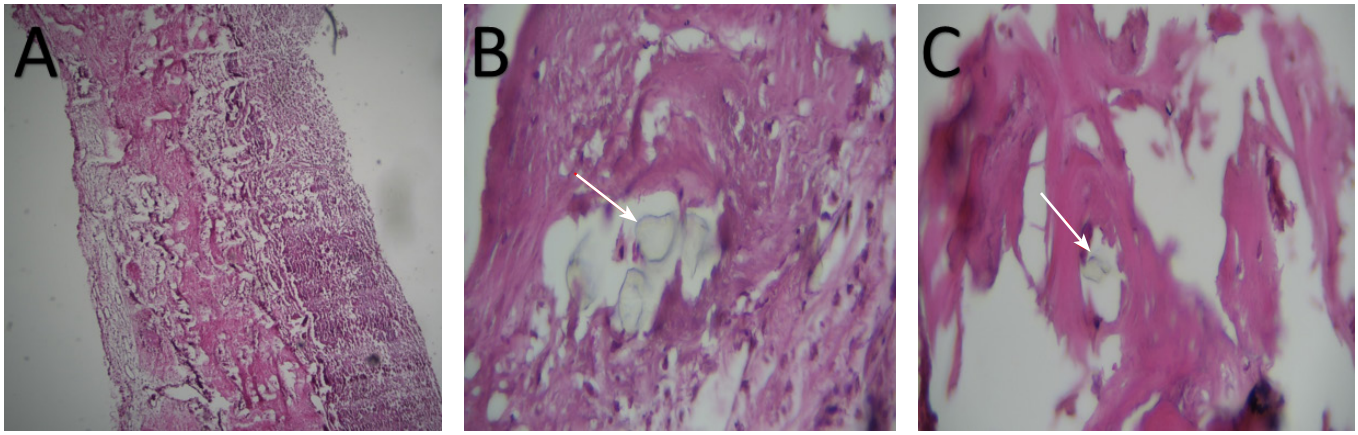

Figure 5
Photomicrographs of the calvarial defects of the Collagen-Polyglycolic acid group. (A) Fibroplasia and mild bone formation at 4 weeks (Hematoxylin and eosin stain, original magnification ×4). (B) Polyglycolic acid remnant between collagen fiber (Hematoxylin and eosin stain, original magnification ×40). White arrow shows PGA remnant.(C) Moderate bone formation at 8 weeks (Hematoxylin and eosin stain, original magnification ×40). White arrow shows PGA remnant.

newly bone formation was occurred at the center of the defect (Figure 7D).

Discussion

In spite of treatment for bone fractures, be it surgical or non-surgical, some of them do not succeed to heal, and become non-unions. Non-union can be explained as the failure of a fracture to recover after 6 months, without further advancement towards healing. However, the most advanced available treatments are not sufficiently effective and often lead to some complications. As a result, an alternative strategy is essential to treat a severe non-union fracture. The field of tissue engineering seeks to incorporate engineering technology into the principles of biological science to devise plans for the regeneration and repair of lost or harmed tissue (Toosi et al., 2016; Tseng et al., 2008).

Simvastatin is one of the cholesterol lowering drugs, which is commonly prescribed and has been proved to up-regulate BMP-2 and VEGF gene expression in osteoblasts; moreover, simvastatin triggers endothelial nitric oxide synthase (eNOS), and alkaline phosphatase. It is claimed that administering statins either systemically or locally boosts bone growth and/or regeneration (Nyan et al., 2009; Thylin et al., 2002).

Despite studies reporting the anti-inflammatory effects of statins (Weitz-Schmidt et al., 2002), high-dose simvastatin generates inflammation around the site of local application. It has been proved that local simvastatin application at 2.2mg induces inflammation and scabbing of the skin lying on top of the murine calvaria (Thylin et al., 2002). Stein et al. used simvastatin in methyl cellulose gel in a polylactic acid membrane locally on rat mandible at different doses and discovered that by cutting back on the simvastatin dose, the signs of inflammation can be decreased. They deduced that 0.5 mg simvastatin is the optimum dose for the sole local application (Stein et al., 2005). Therefore, the local effects of simvastatin depend on dose and carrier. We conjecture that a carrier which degrades rapidly during the early phase of bone healing would not be advisable for bone

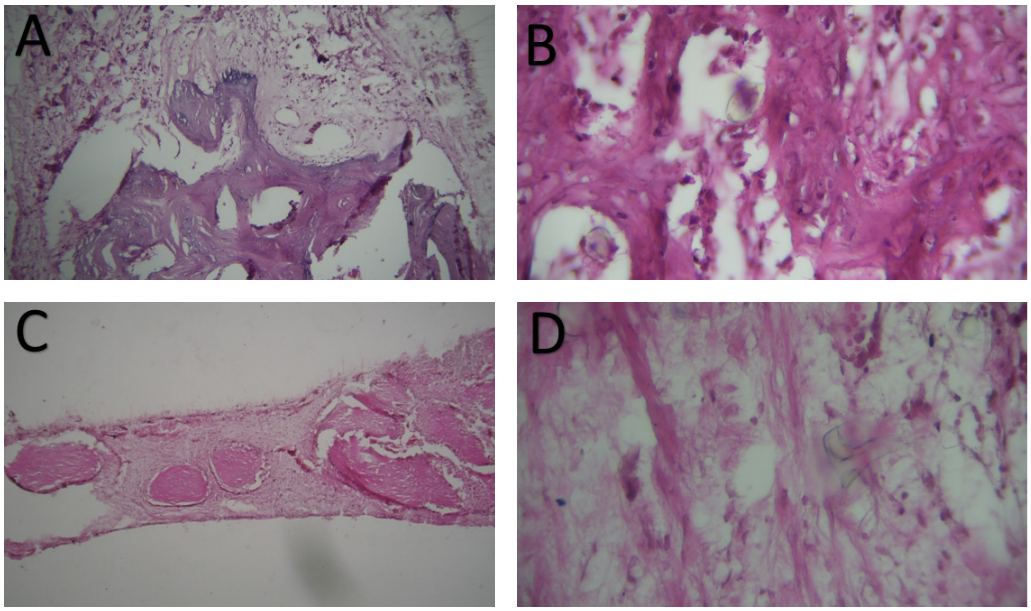

Figure 6
Photomicrographs of the calvarial defects of the Collagen-Polyglycolic acid with simvastatin 1 mg group. (A) Osteochondral bone formation at the margin of the defect (Hematoxylin and eosin stain, original magnification × 10). (B) Newly formed bone around the PGA fibers (Hematoxylin and eosin stain, original magnification ×40). (C) Osteochondral bone formation at the center of the defect (Hematoxylin and eosin stain, original magnification ×4). (D) PGA fibers at the center of the newly formed bone (Hematoxylin and eosin stain, original magnification ×40).

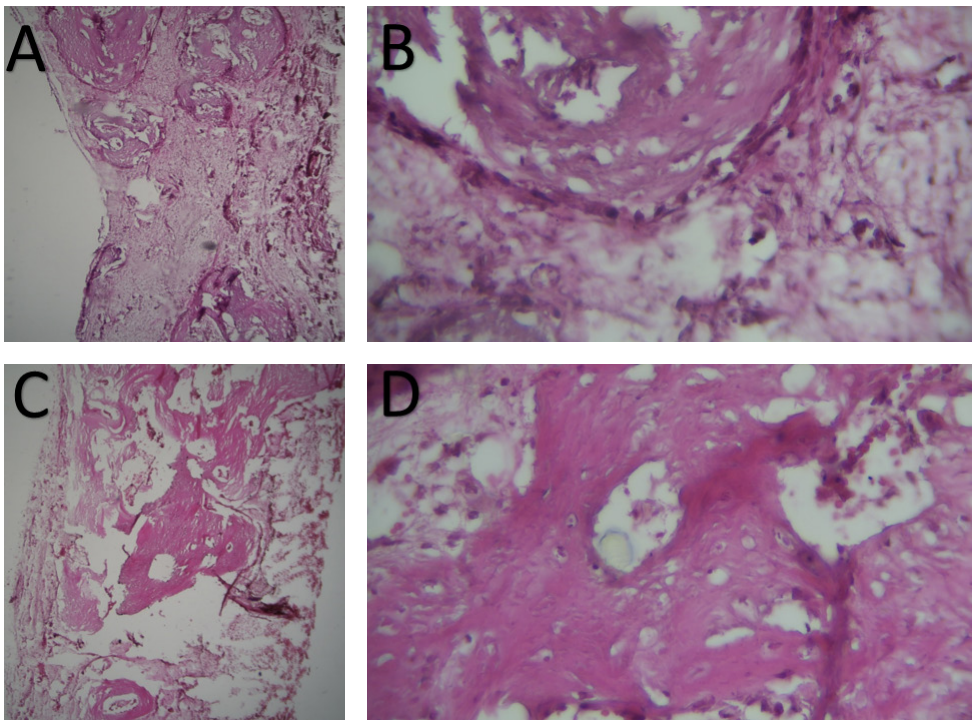

Figure 7
Photomicrographs of the calvarial defects of the Collagen-Polyglycolic acid with simvastatin 0.5 mg group. (A) Osteochondral bone formation at the center of the defect at 4 weeks (Hematoxylin and eosin stain, original magnification ×4). (B) Active osteoblast at the periphery of the newly formed bone at 4 weeks (Hematoxylin and eosin stain, original magnification ×40). (C) Full bone formation at the center of the defect at 8 weeks (Hematoxylin and eosin stain, original magnification ×4). (D) Newly formed bone around the PGA fiber and osteocyte formation (Hematoxylin and eosin stain, original magnification ×40).

regeneration with simvastatin.

Myat Nyan and coworkers in 2008 scrutinized the effects of the mixture with α-tricalcium phosphate and simvastatin on bone regeneration, and they arrived at the conclusion that when combined with α-TCP particles, 0.1 mg simvastatin is the optimum dose for stimulation of the maximum bone regeneration in rat calvarial defects without causing inflammation and it could be employed as an effective bone graft material; however, our survey shows that 0.5 mg simvastatin is an optimal local dose (Nyan et al., 2009). Ozec and coworkers used simvastatin gelatin sponge graft on mandibular critical-sized defect in 2007. They demonstrated that the use of simvastatin gelatin sponge promoted bone defect healing in the mandible of rats (Ozec et al., 2007). Both collagen and gelatin are suitable carriers for gradual release of simvastatin. Mukozava and coworkers in 2010 implanted a statin with two different carriers (hydrogel and atelocollagen sponge) in rabbit nasal bone using histological and immunohistochemical methods and assessed bone healing. Their study discovered that both the simvastatin with hydrogel and simvastatin with ACS implants demonstrated similar BMP-2 expression and newly formed bone, and there were no significant differences between the two carriers (Mukozawa et al., 2011). Stein and coworkers in 2005 employed different dose of 0.1, 0.5, 1.0, 1.5, or 2.2 mg simvastatin in methylcellulose gel in a polylactic acid membrane on the lateral aspect of the mandible. They reported that decreasing simvastatin dose from 2.2 mg to 0.5 mg declined inflammation to a more clinically-receivable level without sacrificing bone-growth potential (Stein et al., 2005). Their investigation was in the evident of our survey which shows 0.5 mg is an optimal dose of simvastatin locally. Huang et al. in 2014 enquired into the release of simvastatin from simvastatin loaded calcium sulphate (CS) scaffolds for the treatment of the segmental critical-sized defect of the rabbit ulna and they came to the conclusion that simvastatin loaded CS scaffolds is likely to have great potential in bone tissue engineering (Huang et al., 2014). Thylin and coworkers in 2002 applied methylcellulose gel as a carrier of simvastatin for bone regeneration on murine calvarial bone. They summarized that high dose of simvastatin methylcellulose gel can trigger murine calvarial bone apposition (Thylin et al., 2002).

Aybar and coworkers tried to observe the two different critical size defect (CSD) of rat calvaria that is unable to experience spontaneous bone regeneration. Circular surgical defects, 3 mm (Group A) and 5 mm (Group B) in diameter, were made in the parietal bones. The animals were left without medical care and sacrificed 1, 2, 3 and 6 weeks after surgery. Group A demonstrated bone formation at the experimental site, increasing from 1 week (4.5%) to 6 weeks (46%). However, Group B exhibited scant bone formation (less than 10%) during the experimental period (Aybar et al., 2004). It can be concluded that a defect 5 mm in diameter is a critical size defect (CSD) as it is the minimum bone defect size that needs treatment to cure. Based on their experience, the region of calvarial bone of rabbit was relatively flat and wide, so that bone defects with equal size could be produced with ease. Thus, it was possible to compare the four bone defects with similar condition in a rabbit. We relied on rabbit four calvarial defect model to examine our hypothesis, as it is a convenient model for the study of bone regenerative materials for the absence of fixation demands. Experimental models with very young animals are viewed undesirable to assess osteopromotive materials. The animals used in this study were at adult stage (3 to 4 months); therefore, increased bone regeneration associat-

ed with growth is not expected.

Fabrication of collagen sponge combined with biodegradable PGA fibers as bone tissue-engineered scaffold have been displayed to be osteoconductive and notably biocompatible and these materials have been successfully selected as a bioresorbable composite scaffold for bone-tissue engineering (Toosi et al., 2016).

In our survey we used simvastatin with two different dose of 0.5 mg and 1 mg as an osteoinductive material and a mixture of collagen-polyglycolic acid as a scaffold to work as an osteoconductive material. Myat Nyan (Nyan et al., 2009) derived the conclusion that when combined with α-TCP particles, 0.1 mg simvastatin is the optimum dose for triggering the ultimate bone healing in rat calvarial defects without inflammation; however, we concluded that the optimal dose of simvastatin is 0.5 mg which cause maximum bone regeneration in the rabbit calvarial defect when blended with collagen-polyglycolic acid carrier, in which the study of Stein and coworkers (Stein et al., 2005) support the result of our survey. Thus, the local effects of simvastatin would depend on dose carrier.

In our study bone regeneration in the collagen-polyglycolic acid without simvastatin group was achieved acceptably in 8 weeks' time period and the difference between this two time frames (4 weeks and 8 weeks) were statistically significant in this group ($p < 0.05$). The amount of bone formation in 4 weeks' time period was much less than that of the 8 weeks period. In the treatment groups with simvastatin, bone regeneration was achieved almost in 4 weeks, which suggest that simvastatin promote bone healing at an earlier stage than scaffold without simvastatin. The difference between the 0.5 mg groups in the four-week and the eight-week categories and in the case of 1 mg simvastatin groups was not statistically significant($p = 0.095$ and $p = 0.690$ respectively). Interestingly in the 4 weeks' time frame category only the difference between the control and scaffold groups with simvastatin 0.5 mg and control groups, as well as, scaffold with simvastatin 1 mg groups were statistically significant; however, in the 8 weeks' time frame category the difference between the control and the scaffold group with 0.5 mg simvastatin and control groups as well as scaffold without simvastatin were statistically significant ($p < 0.05$) (Table 2).

In conclusion, our study confirmed the bone-enhancing effect of local simvastatin and ascertained that 0.5 mg is the optimum dose for the maximum bone regeneration of 5-mm-diameter bone defects in rabbit calvaria when employed in combination with collagen-polyglycolic acid scaffold. Further studies are required to validate the effect of this optimal dose of simvastatin with collagen-polyglycolic acid combination in different clinical situations.

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