Journal of Bioscience and Bioengineering
VOL. 118 No. 4, 469–475, 2014

ELSEVIER

www.elsevier.com/locate/jbiosc

Effects of fibrinogen concentration on fibrin glue and bone powder scaffolds in bone regeneration

Beom-Su Kim,[1,2] Hark-Mo Sung,[2] Hyung-Keun You,[3] and Jun Lee[1,*]

Wonkwang Bone Regeneration Research Institute, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea,[1] Bonecell Biotech Inc., 77 Dunsan-dong, Seo-gu, Daejeon 302-830, Republic of Korea,[2] and Department of Periodontology, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea[3]

Received 23 December 2013; accepted 27 March 2014
Available online 24 April 2014

Fibrin polymers are widely used in the tissue engineering field as biomaterials. Although numerous researchers have studied the fabrication of scaffolds using fibrin glue (FG) and bone powder, the effects of varied fibrinogen content during the fabrication of scaffolds on human mesenchymal stem cells (hMSCs) and bone regeneration remain poorly understood. In this study, we formulated scaffolds using demineralized bone powder and various fibrinogen concentrations and analyzed the microstructure and mechanical properties. Cell proliferation, cell viability, and osteoblast differentiation assays were performed. The ability of the scaffold to enhance bone regeneration was evaluated using a rabbit calvarial defect model. Micro-computed tomography (micro-CT) showed that bone powders were uniformly distributed on the scaffolds, and scanning electron microscopy (SEM) showed that the fibrin networks and flattened fibrin layers connected adjacent bone powder particles. When an 80 mg/mL fibrinogen solution was used to formulate scaffolds, the porosity decreased 41.6 ± 3.6%, while the compressive strength increased 1.16 ± 0.02 Mpa, when compared with the values for the 10 mg/mL fibrinogen solution. Proliferation assays and SEM showed that the scaffolds prepared using higher fibrinogen concentrations supported and enhanced cell adhesion and proliferation. In addition, mRNA expression of alkaline phosphatase and osteocalcin in cells grown on the scaffolds increased with increasing fibrinogen concentration. Micro-CT and histological analysis revealed that newly formed bone was stimulated in the scaffold implantation group. Our results demonstrate that optimization of the fibrinogen content of fibrin glue/bone powder scaffolds will be beneficial for bone tissue engineering.

[**Key words:** Fibrin glue; Fibrinogen; Scaffold; Bone tissue engineering; Bone powder]

Bone defects are caused by a variety of conditions, such as tumors, trauma, disease, and bone fractures. Although small bone defects can heal themselves, large bone defects cannot and require bone graft replacements. In tissue engineering, reconstruction of bone often requires a biodegradable porous scaffold (1) because the porous scaffold provides necessary support for cell growth, adhesion, and differentiation (2). In addition, the selection of biomaterials with which to construct the scaffold is an important factor because their properties will determine scaffold properties like biocompatibility, osteoinductivity, and osteoconductivity (3,4).

To construct a biocompatible scaffold for bone tissue engineering, several biomaterials, including hydroxyapatite (5), tricalcium phosphate (6), and allogeneic bone powder (7) were used. Among these, allogeneic bone powders have been frequently used to reconstruct bone defects. Because the molecular structure of bone is identical across species, it is possible to use bone from animal sources for dental implant bone grafts with good results (8).

Furthermore, biodegradable natural polymers, such as a collagen (5), gelatin (9), chitosan (10), and fibrin (11), have been used to fabricate scaffolds. Fibrin polymers have been shown to have excellent adhesiveness and biocompatibility (12,13). Fibrin glues (FG) are mainly composed of fibrinogen and thrombin (14). Thrombin converts fibrin, a biopolymer, into fibrin monomers. Monomeric fibrin forms a fibrous clot that has biological adhesive properties, and because of these properties, these clots are widely used in various surgeries, including orthopedic surgery (15,16). In addition, fibrin structures function as a temporary matrix during the rebuilding and repair of tissues (17). Although some studies demonstrate that a composite biomaterial containing FG can exhibit increased biocompatibility and osteoconductivity when compared with the biomaterial alone (18,19), there is insufficient evidence for the utility of scaffolds composed of FG and bone powder in such applications. In an attempt to improve its utility for bone tissue engineering, we sought to optimize the composition of FG and bone powder scaffolds.

In this study, FG and bone powder scaffolds were fabricated using various concentrations of fibrinogen, and their microstructural and mechanical properties were characterized. The *in vitro* biocompatibility of the scaffold was evaluated using human mesenchymal stem cells (hMSCs) *in vitro*, and their tissue response and ability to induce bone formation *in vivo* were evaluated using a rabbit calvarial defect model.

MATERIALS AND METHODS

Preparation of scaffolds FG from a Greenplast kit (Green Cross Corp., Seoul, Korea) and calcium-phosphate-coated bovine bone powder (Biocera; Oscotec, Chunan, Korea) were used in scaffold preparation. To construct the scaffolds, 450 mg of bone powder was placed in each hexahedron-shaped mold (10 mm × 10 mm × 5 mm). Next,

* Corresponding author. Tel.: +82 42 341 2800; fax: +82 42 341 2809.
E-mail addresses: omslee@wonkwang.ac.kr, omslee@wku.ac.kr (J. Lee).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.03.014

0.2 mL of fibrinogen solution (10, 20, 40, or 80 mg/mL in PBS) was added to the bone powder and mixed well. Then, 0.1 mL of thrombin solution (Greenplast Kit; 5 U/mL) was added, and the composites were rapidly blended. Polymerization of the resultant mixture was achieved at room temperature after 1 h of incubation. The blocks were then freeze-dried for 3 days to obtain FG/bone powder scaffolds.

Micro-computerized tomography analysis To evaluate the entire scaffold structure, samples were scanned with an aluminum filter using micro-computerized tomography (Micro-CT; Sky-Scan 1172TM; Skyscan, Kontich, Belgium). Three-dimensional and trans-sectional images were obtained, and the data were reconstructed using the CT-analyzer software (Sky-scan).

Microstructural analysis To observe the structure, the scaffolds were examined using a scanning electron microscope (SEM; EM-30; Coxem, Daejeon, Korea). Before observation, the samples were sputter-coated with gold for 120 s under vacuum.

Porosity measurement Porosity was measured using a mercury intrusion porosimeter (AutoPore IV9500, Oak Ridge, TN, USA). Briefly, scaffolds were sealed in a penetrometer, weighed, and subjected to analysis (20). The porosity of 5 samples per scaffold was measured and reported as average percent porosity.

Compressive strength analysis To evaluate the mechanical properties of the scaffolds, compressive strength was measured. The fabricated scaffold (10 mm × 10 mm × 5 mm) was subjected to a compression test using an Instron model 4505 universal test machine (Instron, Canton, MA, USA) by applying load via a 1 N load cell at a crosshead speed of 0.5 mm/min under ambient conditions.

Cell culture Human bone marrow-derived mesenchymal stem cells (hMSCs) were obtained from Prof. HK You (Wonkwang University, Iksan, Korea). The cells were cultured in α-Minimal Essential Medium (α-MEM) (Gibco-BRL, Gaithersburg, MD, USA) with 1% penicillin/streptomycin and 10% fetal bovine serum (Gibco-BRL) at 37°C, 5% CO_2, and 100% humidity. Passages 4–6 were used in each experiment. The differentiation of hMSCs into osteoblasts was induced by treatment with osteoblast stimulant solution (OS; 10 mM β-glycerophosphate, 0.05 mg/mL ascorbic acid, and 0.1 mM dexamethasone [Sigma–Aldrich, St. Louis, MO, USA]). The culture medium and OS were replaced every 2 days during the experiment.

Cell proliferation assay and evaluation of cytotoxicity CellTiter96 Aqueous One Solution (Invitrogen, Carlsbad, CA, USA) was used to measure cell proliferation. The hMSCs were seeded and cultured on the FG/bone powder scaffolds. After culture (1, 5, 10, or 15 days), 100 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent was mixed with 500 μL of culture media and added to each well. After incubation for 2 h, 200 μL of the supernatant was removed and its absorbance was measured at 490 nm using a microplate reader (SpectraMAX M3; Molecular Devices, Sunnyvale, CA, USA). In addition, a Live/Dead Viability/Cytotoxicity staining kit (Invitrogen) was used to evaluate the cytotoxicity of the produced scaffolds. According to the manufacturer's protocol, cells were seeded and cultured for 3 days. Then, the sample was rinsed with PBS to remove the phenol red and serum, and reagent solution was added. After incubation for 30 min in a CO_2 incubator, the samples were observed using an inverted fluorescence microscope (DM IL LED Fluo; Leica Microsystems, Wetzlar, Germany).

Cell adhesion observation SEM was used to observe cell adhesion to the scaffold. After 5 days of culture, scaffolds were briefly washed with PBS. Then the samples were fixed with 2.5% glutaraldehyde, and postfixation was performed with 0.1% osmium tetroxide (OsO_4, Sigma). The samples were dehydrated with a graded ethanol series (50%, 75%, 95%, 100%, and 100%), sputter-coated with gold, and visualized by SEM (EM-30).

Real-time polymerase chain reaction To quantify osteoblast differentiation, cells were cultured on the FG/bone powder scaffolds for 10 days, and the mRNA expression of alkaline phosphatase (ALP) and osteocalcin (OC) marker genes was assessed using a quantitative real-time polymerase chain reaction (qRT-PCR) assay. To obtain total mRNA, the scaffolds were rinsed with PBS and chopped, and the cells were dissociated from the scaffold by sonication. Total mRNA was extracted using an RNA isolation kit (Ribospin; GeneAll, Seoul, Korea), according the manufacturer's protocol. PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), TaqMan Universal PCR Master Mix, TaqMan primers, and probe sets specifically targeting ALP (Hs01029144_m1), OC (Hs01587814_g1), and 18S rRNA (Hs99999901_s1; Applied Biosystems). The 18S rRNA gene was used as an internal standard.

Animal experiments In this study, 3 months old New Zealand white rabbits, weighing 2.5–3.0 kg, were used. After the animal was anesthetized, the calvarium was exposed by making a skin incision. Circular calvarial defects were made using a trephine bur (8 mm in diameter), and FG/bone powder scaffolds were implanted in the induced calvarial defects.

The animals were sacrificed at 8 weeks of age, and the bone tissue defects were dissected out from the host bone. The extracted bone tissue was fixed with 4% paraformaldehyde buffered with 0.1 M phosphate solution (pH 7.2) for 3–5 days before further experiments. All animal experiments were performed according to the guidelines of the Wonkwang University Institutional Animal Care and Use Committee.

Micro-computed tomography The bone specimens were scanned using a micro-CT (Sky-Scan 1172TM). Then the image data were reconstructed using a CT-analyzer (Sky-Scan). We additionally scanned scaffolds prior to implantation using a micro-CT, and bone volume (BV) was obtained from the data sets. Then, the percentage of bone volume was calculated using the following equation:

$$(\%)\ \text{New bone volume} = (BV_{post} - BV_{sc})/TV \times 100 \quad (1)$$

where BV_{post} is the bone volume 8 weeks after implantation, BV_{sc} is the volume of scaffold prior to implantation, and TV is the total volume of the region of interest.

Histology After micro-CT scanning, the samples were dehydrated in a graded alcohol series (80–100%), decalcified in 8% formic acid/8% HCl, and embedded in paraffin. Sections of 5-μm thickness were prepared from the samples and mounted on slides. Following this, the samples were stained with hematoxylin/eosin (H&E) and Goldner's Masson trichrome (MT).

Statistical analysis All experiments were performed in triplicate, and statistical analyses were performed using statistical analysis software (Origin 8.0; OriginLab, Northampton, MA, USA). Significant differences among groups were identified by ANOVA followed by Dunnett's test. Values in the text are expressed as the means ± standard deviation (SD) and p values less than 0.05 were considered statistically significant.

RESULTS

Structural characterization of FG/bone powder scaffolds In the present study, the thrombin used had a concentration of 5 U/mL. Because this high concentration of thrombin accelerates cross-linking, making it difficult to handle, we used various concentrations (10–80 mg/mL) of fibrinogen to fabricate the scaffolds. In this study, we successfully fabricated porous scaffolds using bovine bone powder and FG (Fig. 1A). Micro-CT images of the scaffolds are shown in Fig. 1B. The micro-CT images showed that the bovine bone powders were uniformly distributed in all scaffolds. In addition, the trans-sectional layer micro-CT images indicated that several irregular and large pores were observed in the scaffolds fabricated with 10 and 20 mg/mL fibrinogen. SEM images of the fabricated scaffolds are shown in Fig. 1C. All of the scaffolds fabricated using fibrin glue contained fibrin networks and fibrin layers that branched among the bone powder particles. Fibril networks and fibrin layers were rarely observed in the scaffolds fabricated with 10 mg/mL fibrinogen, and more compact fibrin layers were observed in the scaffolds fabricated with 80 mg/mL fibrinogen when compared with those fabricated with 10 mg/mL fibrinogen. Furthermore, a fibrin layer closed the pores between adjacent bone powders in the scaffolds fabricated with 80 mg/mL fibrinogen.

Measurement of porosity and compressive strength SEM showed that fibrin layer formation closed many pores (Fig. 1C). However, in bone tissue engineering, the porosity of the scaffold is important for cell migration, angiogenesis, and nutrient supplementation (21,22). Therefore, we assessed porosity as a function of fibrinogen concentration (Fig. 2A). The porosities of 10 mg/mL and 20 mg/mL fibrinogen-fabricated scaffolds were not statistically different, with 76.1% ± 2.9% and 69.2% ± 3.6% porosity, respectively. However, the porosity of the scaffolds fabricated with 80 mg/mL fibrinogen (41.6% ± 3.6%) was significantly higher those fabricated with 40 mg/mL (64.2% ± 2.9%; $p < 0.05$). These results demonstrate that the porosity of the scaffold decreased as the concentration of fibrinogen increased due to the formation of fibrin layers. In addition, we measured the compressive strength of the scaffolds because mechanical properties are also crucial for bone reconstruction. The compressive strength of the scaffolds fabricated with 20 mg/mL fibrinogen was 0.46 ± 0.06 MPa and not significantly different when compared with those fabricated with 10 mg/mL fibrinogen (0.31 ± 0.02 MPa). However, the compressive strengths of both the 40 mg/mL and 80 mg/mL fibrinogen-fabricated scaffolds were significantly higher, at 0.93 ± 0.06 MPa and 1.16 ± 0.02 MPa, respectively, than the 10 mg/mL fibrinogen-fabricated scaffold (Fig. 2B).

Biocompatibility To evaluate the effects of fibrinogen concentration on cell proliferation, cytotoxicity, and cell adhesion, hMSCs were seeded and cultured on the scaffolds. As shown in