Published in final edited form as:

Biomaterials. 2018 December ; 187: 39–46. doi:10.1016/j.biomaterials.2018.09.030.

The Effects of a Functionally-Graded Scaffold and Bone Marrow-derived Mononuclear Cells on Steroid-induced Femoral Head Osteonecrosis

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Abstract

Osteonecrosis of the femoral head (ONFH) is a debilitating disease that may progress to femoral head collapse and subsequently, degenerative arthritis. Although injection of bone marrow-derived mononuclear cells (BMMCs) is often performed with core decompression (CD) in the early stage of ONFH, these treatments are not always effective in prevention of disease progression and femoral head collapse. We previously described a novel 3D printed, customized functionally-graded scaffold (FGS) that improved bone growth in the femoral head after CD in a normal healthy rabbit, by providing structural and mechanical guidance. The present study demonstrates similar results of the FGS in a rabbit steroid-induced osteonecrosis model. Furthermore, the injection of BMMCs into the CD decreased the osteonecrotic area in the femoral head. Thus, the

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Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All of the data are included in the paper.

combination of FGS and BMMC provides a new therapy modality that may improve the outcome of CD for early stage of ONFH by providing both enhanced biological and biomechanical cues to promote bone regeneration in the osteonecrotic area.

Keywords

Osteonecrosis; Femoral Head; Corticosteroids; Scaffold; Bone Marrow-derived mononuclear cells

Introduction

Osteonecrosis of the femoral head (ONFH) is a potentially debilitating disease that results in progressive collapse of the femoral head and subsequent degenerative arthritis. Greater than 20,000 new cases of femoral head osteonecrosis are diagnosed annually in the United States [1]. From 1992 through 2008 in the United States, the total number of surgeries for osteonecrosis of the femoral head increased from 3570 to 6400 per year, nearly doubling over a 16-year time period [2]. In addition, the prevalence of joint-preserving procedures such as core decompression, bone grafting and femoral osteotomy has consequently decreased from 24% to 12%, whereas the prevalence of joint-replacing surgeries including total hip replacement (THA) have increased from 76% to 88% [2].

Core decompression is a surgical procedure for joint preservation, and involves drilling into the osteonecrotic lesion [3]. It is often performed in the early stage of ONFH, in which the articular surface of the joint has not collapsed [4]. This procedure is thought to reduce the intramedullary pressure in the femoral head and, by inducing local bone trauma, trigger revascularization, bone formation and remodeling [5]. However, it has been reported that 37% of cases with core decompression treated progressed to femoral head collapse and 30% needed additional surgery [4].

The ideal adjunct to core decompression for the treatment for early stage of ONFH is the promotion of biomechanical and biological support to increase mechanical strength, accelerate bone regeneration in the osteonecrotic area, thus further delaying disease progression and preventing the collapse of the femoral head.

Implants used for early ONFH without collapse of the articular surface include a non-vascularized fibular graft (NVFG), a vascularized fibular graft (VFG) and a tantalum rod. However, the survival rate at seven years for early stage of ONFH treated with a NVFG was 30%, which was worse compared to VFG (86%). NVFGs are associated with poor clinical and radiographic outcomes [6]. The VFG is limited by the extensive surgical time and rehabilitation, and the possibility of infection as well as donor site morbidity [7, 8]. A porous tantalum metal implant is associated with little histological evidence of bone ingrowth (1.9%) into the necrotic area [9] and insufficient mechanical support of the subchondral bone [9, 10]. In addition, subtrochanteric stress fractures at the surgical entry point have occurred after this procedure [11].

We have used a tissue engineering approach to reconstruct necrotic bone tissue in the femoral head and improve the outcome of early stage of ONFH. First, we developed a novel

customized, functionally-graded scaffold (FGS) [12] with spatially controlled porosity, degradation, and mechanical properties, that is made of FDA cleared polycaprolactone (PCL) and β -tricalcium phosphate (β -TCP), and is fabricated using 3D printing [13–15]. We previously reported that the FGS improved bone ingrowth in femoral heads after core decompression in a normal healthy rabbit [12]. However, the potential therapeutic effect of the FGS for ONFH is still unknown.

Injection of bone marrow-derived mononuclear cells (BMMCs) to the core decompression drill hole has recently been used in humans to augment the core decompression therapy [16–19]. BMMCs are thought to provide both angiogenic and osteogenic stimuli for bone healing [19–21]. We hypothesize that the combination treatment of the FGS and BMMC therapy after core decompression would improve the outcome for early stage of ONFH. The primary aim of this study was to investigate the effect of FGS for treatment of early stage ONFH using a rabbit steroid-induced osteonecrosis model. The secondary aim was to validate the FGS in combination with clinically available BMMC therapy for reconstitution of the osteonecrotic area.

Materials and Methods

Design and Fabrication of FGS

FGS for ONFH treatment was designed with spatially controlled porosity, degradation, and mechanical properties to reconstruct necrotic bone tissue in the femoral head, as previously described [12]. Briefly, polycaprolactone (PCL) and β -tricalcium phosphate (β -TCP) were used for the fabrication of FGS. The FGS was cylindrical shaped (2.4 mm diameter, 27 mm length) and consisted of three segments of spatially graded porosity, including 4 mm length proximal segment of 15% porosity, 17 mm length middle segment of 40% porosity, and 6 mm distal segment of 15% porosity (Fig. 1). The porosity of each segment was set to be similar to human bone porosity [22]. The graded scaffold was formed in one piece using our in-house-built layer-by-layer 3D fabrication system, Hybprinter [13, 14].

Animal Experiments

The experimental design was approved and performed following our institution's Animal Care and Use Committee guidelines. Twenty-four male New Zealand White rabbits (West Oregon Rabbit Company, Philomath, OR), ranging in weight from 3.5 to 4.0 kg, were used. A single intramuscular injection of 20 mg/kg methylprednisolone acetate (MPSL) was performed to establish the steroid-induced osteonecrosis model as previously described [23, 24]. All the treated animals lost weight and 2 rabbits died prematurely. Rabbits were divided into 4 groups: CD group, core decompression only ($N=8$); FGS group, core decompression + FGS ($N=8$); BMMC group, core decompression + BMMCs ($N=3$); FGS+BMMC group, core decompression + FGS+ BMMCs ($N=3$).

The surgical procedure was performed four weeks after steroid injection [24]. Animals were anesthetized by administering 40 mg/kg ketamine and 4 mg/kg xylazine. Additional inhalation anesthesia was performed with isoflurane. In the CD group, core decompression of the femoral head was performed on the left hip. Briefly, a 20 mm lateral skin incision was

created to expose the third trochanter. A small hole was created at the distal end of the third trochanter with a round burr. Under fluoroscopic guidance, a bone tunnel was created using a 2 mm diameter drill from the hole towards the femoral head through the mid axis of the femoral neck. The depth of bone tunnel was approximately 27 mm. The 2 mm tunnel was then widened with a 2.4 mm diameter drill bit. In the FGS group, the FGS was inserted and press fit into the bone tunnel manually. In the BMMC group, approximately 3 ml of bone marrow was harvested during surgery from the posterior iliac crest using a Monoject™ J-type Bone Marrow Biopsy/Aspiration Needle (Covidien, Mansfield, MA, USA) and collected into a heparinized syringe. BMMCs were isolated by using SepMate™ tube (STEMCELL™ Technologies Inc., Vancouver, BC, Canada) with Ficoll-Paque (GE Healthcare, 1.077 g/ml). A total of 1×10^7 cells was resuspended in 50 μ l of Phosphate-Buffered Saline (PBS). After the core decompression procedure, BMMCs were injected into the bone tunnel using a pipette. In the FGS+BMMC group, the FGS was soaked in the BMMCs/PBS solution for 3 minutes, then the remaining BMMCs were injected before implanting the FGS.

The wound was closed using a non-absorbable suture. Analgesia was administered by injection of 0.5 mg/kg buprenorphine twice a day for the first 2 days after surgery. After an 8-week postoperative period, rabbits were euthanized with a lethal dose of sodium pentobarbital. The whole femurs were resected, and the soft tissues were removed. The specimens were stored in the -80°C freezer.

Micro-CT Analysis

The proximal femurs were evaluated *in vivo* using a micro-CT scanner (eXplore CT120; TriFoil Imaging Inc., Northridge, LA, USA) with 50 μ m resolution. A cylindrical region of interest (2.4 mm diameter; proximal segment: 4 mm length, middle segment: 17 mm length, distal segment: 6 mm length) was co-centrally positioned over the core decompression site (Fig. S1). The volume of new bone and remaining FGS was determined by GEMS Micro View software (eXplore Micro View v.2.5, Analysis Plus, GE Healthcare, Toronto, Canada). Thresholds were applied to differentiate between new bone and residual scaffold material in the region of interest as reported previously [12, 25]. The degradation rate for each segment was measured using following formula.

$$\text{Degradation rate of FGS(\%)} = \frac{V_{\text{int}} - V_{\text{rem}}}{V_{\text{int}}} \times 100$$

V_{int} : initial scaffold volume, V_{rem} : remain scaffold volume at 8 weeks after surgery. The degradation rate of FGS in the both FGS and FGS+BMMC groups was compared among proximal, middle, and distal segments. In addition, the degradation rate between the FGS group and FGS+ BMMC groups was compared in each segment.

The Bone Ingrowth Ratio for each segment was measured using following formula.

$$\text{Bone ingrowth ratio (\%)} = \frac{V_{\text{bone}}}{V_{\text{total}} - V_{\text{rem}}} \times 100$$

V_{bone} : bone volume at 8 weeks after surgery, V_{total} : total volume of bone tunnel segment. The new bone volume and bone ingrowth volume were compared among all 4 groups.

Mechanical Testing

All femurs in the CD and FBS groups were used for structural evaluation. Specimens were stored in a -80°C freezer until the day of testing. Specimens were cut 60 mm distal to femoral head. The remaining diaphyseal bone was aligned vertically in an aluminum block and then potted with polymethylmethacrylate. Specimens were maintained moist with PBS throughout the preparation and testing procedures.

Mechanical testing was performed on an E10000 Materials Testing System fitted with a 10 KN load cell (Instron Corporation, Norwood, CA). The base of the experimental set-up incorporated a three-axis swivel vise mounted on an unconstrained xy stage. The xy stage was used to prevent off axis loading during tests. Specimens were secured in the swivel vise with the diaphyseal axis oriented 17° from vertical in the frontal plane, with the resulting load vector on the femoral head directed 17° lateral (Fig. S2). The loading direction approximates the frontal plane angle at peak load during a variety of daily activities in humans [26]. Although this loading direction may not be physiologic for rabbits, it was chosen to approximate loading that would cause femoral head collapse in cases of ONFH in humans. Forces were transmitted to the femoral head via an aluminum cylinder with a machined spherical recess. The diameter of the recess approximated that of the femoral head. The spherical recess was coated with lubricant to minimize friction during testing.

Following the application of a 10 N compressive preload, specimens were loaded at a displacement rate of 10 mm/min until catastrophic failure. Load and displacement data were recorded at 100 Hz. Outcome measures included stiffness, calculated from linear portion of load vs displacement curve, maximum load, and failure mode.

Histological Analysis

The specimens in the four groups was prepared for decalcified histology. All specimens except one in the CD and FGS group were used after the mechanical testing. In both the BMMC and FGS+BMMC groups, all femurs were cut at the site of the femoral neck after decalcification. The femoral heads and trochanters were fixed in 4% paraformaldehyde (pH 7.4), decalcified in 0.5M EDTA (pH 7.4), and embedded in paraffin. A 5- μm longitudinal section parallel to the direction of the drill hole/scaffold was obtained and stained with hematoxylin and eosin (H&E). To assess the changes in ONFH, a total of ten fields under $\times 100$ magnification were randomly selected and the number of the empty lacunae in the trabecular bone of the femoral head was counted [27]. To automatically and precisely quantify all the histologic results, an image analysis program was developed in MATLAB by our group (Fig. S3). Before importing the images to the MATLAB program, to avoid artifacts from the bone marrow regions, the bone marrow regions were removed by ImageJ. In the histologic images, the nucleus was dyed with blue color, while the cells without nuclei remained empty. The holes were filled with green color, which is same as background. The MATLAB program first separated the imported image into two categories, blue and green, based on the layers in the RGB image. The green regions can either be the background or the

cells without the nucleus, and if the selected part was noise or the background, it was removed. After separating the green and blue layers, the background and the noise were found by applying a gaussian filter. If the selected region was larger than the average cell size (in pixels), the region would remain after filtering, while the cells would not be shown in the background noise. Consequently, the background and the noise could be removed by subtracting them from blue and green images. Finally, the cells were found using the edge function in MATLAB, and the number of cells with and without a nucleus were retrieved from the blue and possible green groups, respectively.

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis of the parametric data was performed using a one-way ANOVA with Tukey's multiple comparisons tests and Student's *t*-tests. Statistical analysis of the nonparametric data was performed using Kruskal-Wallis tests with Dunn's multiple comparison tests. All plotting of data and statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

All animals survived the operative procedure and follow-up period. One sample in FGS group was excluded because of bending of the FGS implant, confirmed on micro CT.

FGS increased bone ingrowth on micro CT analysis

Representative micro CT reconstructed images of the proximal femur are shown in Fig. 2A-H. In the CD group and the BMMC group, mineralized tissue was noted in the proximal part of the drill channel. In the both FGS group and FGS+BMMC group, mineralized tissue was observed inside the pores of FGS around the scaffold. Furthermore, the proximal segment of the FGS was observed to degrade.

Using micro CT image analysis, degradation rates of the scaffold in the FGS group were $19.3\% \pm 12.5\%$ at the proximal segment, $4.4\% \pm 3.0\%$ at the middle segment, and $10.4\% \pm 5.3\%$ at the distal segment of the scaffold, respectively. The degradation rate at the proximal segments was higher than those at the middle segments ($P < 0.05$) (Fig. 2I). In the FGS+BMMC group, the degradation rate of the scaffold was $42.5\% \pm 14.0\%$ at the proximal segment, $5.3\% \pm 1.9\%$ at the middle segment, and $5.5\% \pm 3.2\%$ at the distal segment, respectively. The degradation rate at the proximal segment was significantly higher than those at both the middle and distal segments ($P < 0.05$). When comparing the FGS group and the FGS+BMMC group, the degradation rate at the proximal segment in the FGS+BMMC group was significantly higher than in the FGS group ($P < 0.05$).

The results of the new bone volume and bone ingrowth ratio are shown in Fig. 2J-K. There were no significant differences among the 4 groups with respect to the total volume of new bone. However, bone ingrowth ratio in the proximal segment was $92.3\% \pm 6.2\%$ in the FGS group, which was significantly higher than $69.5\% \pm 18.3\%$ in the CD group ($p < 0.01$) and $54.0\% \pm 22.2\%$ in the BMMC group ($P < 0.005$). In addition, bone ingrowth ratio in the proximal segment was $96.3\% \pm 1.7\%$ in the FGS+BMMC group, which was significantly

higher than in the BMMC group ($P < 0.005$). In the distal segment, bone ingrowth ratio in the FGS group ($25.5\% \pm 6.6\%$) was significantly higher than in the BMMC group ($9.1\% \pm 7.6\%$, $P < 0.005$). Bone ingrowth ratio in the middle segment was not significantly different among the four groups.

No differences in structural properties between the FGS group and the CD group

Mechanical testing demonstrated that all samples in both the core and scaffold groups fractured at the femoral neck (Fig. 3A). There were no significant differences between the two groups in stiffness (CD group: 1608 ± 562 N/mm, FGS group: 1511 ± 562 N/mm, $P = 0.51$) or maximum load to failure (CD group: 1111 ± 159 N, FGS group: 1097 ± 173 N, $P = 0.87$) (Fig. 3B-C).

BMMCs histologically decreased the osteonecrotic area in the femoral head

The femoral head sections stained with H&E demonstrated trabecular bone in the drill channel in the CD and BMMC groups (Fig. 4), but little to no bone formation in the trochanteric area of the drilled tunnel (Fig. 5). In contrast, bone growth was noted around and within the porous FGS in both the femoral head and the trochanteric areas in the FGS and FGS+BMMC groups. It is worth noting that the empty spaces intertwined with new bone in the FGS and FGS+BMMC groups are artifacts left behind by scaffold struts dissolved during the sample preparation.

To quantify the amount of necrotic bone we determined the percentage of empty osteocyte lacunae within the trabecular bone of the femoral head in each group (Fig. 6A-D). The percentage of the empty lacunae was $53.4\% \pm 13.8\%$ in the CD group, $39.6\% \pm 7.5\%$ in the FGS group, $21.4\% \pm 1.4\%$ in the BMMC group and $19.9\% \pm 14.2\%$ in the FGS+BMMC group. The percentage of empty lacunae in the CD group was significantly higher than in the BMMC and the FGS+BMMC groups ($P < 0.05$) (Fig. 6E).

Discussion

PCL- β TCP scaffolds are osteoconductive and thereby facilitate bone ingrowth *in vivo* [28–32]. However, to our knowledge, there have been no *in vivo* studies to document the efficacy of PCL-TCP scaffolds in inducing bone ingrowth in an osteonecrosis model. In the present study, we used novel 3D printed PCL- β TCP scaffolds with functionally graded porosity in an established steroid-induced osteonecrosis rabbit model.

The scaffold design is intended to support the mechanical loads that are present within the subchondral bone in the femoral head (the proximal segment) and the cortical bone at the distal part of the drilled hole (the distal segment), and progress from each end toward the trochanteric region (the middle segment). The porosities of cortical bone and trabecular bone in humans are 5–10% and 50–90%, respectively [22]. Thus, the porosity of each segment was set to be close to the human bone porosity, and therefore set as 15% in the proximal and distal segments and 40% in the middle segment. The porosity was also determined based on the compressive strength for human trabecular bone in the proximal femur, which was in the range of 0.6–10.2 MPa [33], and the compressive strength of the scaffold with 15% and 40% porosities was in respective range [12]. The ultimate load bearing capability is assumed,

from the result of bone remodeling, to regenerate the subchondral and cortical bone, not from the FGS itself.

In both the FGS and FGS+BMMC groups, the degradation rate of the FGS in the proximal segment was higher than in the middle and distal segments, which was consistent with our previous observation using a normal healthy rabbit [12]. It can be postulated that the bone at the proximal segment is more metabolically active than at the middle or distal segments thus resulting in more rapid degradation of the scaffold in this region. In addition, degradation rate of the FGS in the FGS+BMMC group was higher than in the FGS group. It has been reported that BMMCs accelerate a neovascularization and modulate inflammation [20]. Thus, the accelerated degradation of the FGS is probably associated with the BMMCs capabilities to regulate angiogenesis and inflammation. Our previous study demonstrated that the porosity of the scaffold affected the degradation rate of the scaffold in vitro; porosity may also affect the cellular activities on the PCL- β TCP scaffold [12]. The optimal porosity of the FGS is still debatable; however, the porosity of the scaffold is tunable when translating this device to human patients.

Micro CT analysis demonstrated that the bone ingrowth ratio in the FGS group was higher than that in the CD group at both the proximal and distal segments. The results of the micro CT analysis were similar to our previous study using normal healthy rabbits. Moreover, decalcified histological analysis confirmed bone formation bridging the FGS and bone tunnel, and new bone formation within the pores of FGS in the femoral head and trochanteric region. These results clearly indicated that the bone grew into the FGS inserted in the bone tunnel created by core decompression in the femoral head and in the surgical entry point. Our results differ from those reported using a tantalum rod; the latter device demonstrated little bone ingrowth (1.9%) into the necrotic area histologically [9], and insufficient mechanical support of the subchondral bone [9, 10]. In addition, subtrochanteric stress fractures at the surgical entry point have occurred after core decompression with placement of a tantalum implant [11]. Previous studies have reported that use of a tantalum rod for ONFH stimulated a dense circumferential cortical rim that would inhibit the ingress of bone and vascular progenitor cells, as well as other important cellular elements [9, 34]. Although the FGS is not mechanically as robust as a tantalum rod, the FGS has mechanical properties between that of cancellous and cortical bone and has been shown to facilitate guided tissue regeneration. Therefore, our novel FGS facilitates bridging between the bone tunnel and the FGS, and accelerates bone ingrowth, consequently reducing the potential for subtrochanteric fractures and preventing femoral head collapse.

Because there is no existing mechanical testing protocol to evaluate the osteonecrotic femoral head, and there are different ways for subtrochanteric fractures and femoral head collapse to occur, we designed a mechanical testing protocol to mimic physiological loading of the proximal femur in humans [26]. A limitation of our protocol is the fact that the loading direction selected may not be physiologic for rabbits. However, if differences in structural properties were present between groups, the protocol in the current study would still have been able to identify those differences. During mechanical testing, all specimens fractured in the femoral neck, with femoral heads maintaining their structural integrity. No differences in structural properties were observed between the FGS and CD groups. As such,

further mechanical testing protocols need to be developed to evaluate the potential benefits of FGSs.

In a previous report [35], prolonged -80°C cryopreservation for over 5 years was shown to preserve the biomechanical and histological properties of bone allografts. In our study, the femurs were processed for histology after -80°C cryopreservation and biomechanical testing. However, the specimens were immediately fixed after biomechanical testing. Thus, this delayed processing was assumed to have little effect on the histological evaluations.

A previous study reported that the BMMC implantation with core decompression increased vascularization and bone regeneration in the early stage of ONFH using the rabbit model [36]. However, our micro CT analysis results did not show significant differences in newly formed bone volume between the BMMC group and the CD group. In order to evaluate the necrotic extent of the femoral head, we developed a protocol to assess the percentage of empty osteocyte lacunae in the femoral head as an index of the extent of necrotic segment [27]. The present study demonstrated that the BMMC group histologically decreased the percentage of empty lacunae in the femoral head. The decrease in percentage of the empty lacunae in the femoral head was also observed in the FGS+BMMC group, in addition to accelerated bone ingrowth in the proximal and distal segments. The FGS as a scaffold facilitates bone formation during implantation, and more importantly, facilitates the BMMCs' loading and preservation during the injection into the bone tunnel. Thus, the accelerated new bone formation could be the result of enhanced BMMC preservation and bone ingrowth aided by the FGS and decreased osteonecrosis aided by BMMCs. The effects could potentially result in improvement of the clinical outcome for early stage of ONFH.

There are several limitations in this study. First, a steroid-induced osteonecrosis model in rabbits was used to simulate the early stage of ONFH. However, this rabbit model differs to some degree from the clinical entity seen in humans. In a recent review [37], although multiple animal models have attempted to study early stage ONFH, the ideal animal model has not yet been established. Qin et al. [38] reported a protocol for ONFH in rabbits with a single low-dose $10\text{ }\mu\text{g/kg}$ lipopolysaccharide (LPS), followed by three injections of 20 mg/kg MPSSL, and the incidence of osteonecrotic lesions was as high as 93% [39]. However, the mortality rate in this protocol was as high as 20% and many animals lost significant weight [36, 39]. Our first experience with this protocol, i.e. low dose LPS and high dose MPSSL was less successful. The majority of rabbits died or had to be euthanized because of serious loss of body weight. As a result, our institution's Animal Care and Use Committee prohibited the use of this protocol on ethical grounds. Alternatively, Motomura et al. [24] reported that the incidence of ONFH was 96% (24/25) with one rabbit death using a single injection of 40 mg/kg MPSSL; the incidence of ONFH was 70% with no rabbit deaths at 4 weeks when using a single injection of 20 mg/kg MPSSL. Thus, we selected the protocol using a single injection of 20 mg/kg MPSSL. Despite using this lower dose of MPSSL, 2 of 24 rabbits in our study were euthanized. We believe that this protocol optimizes safety and efficacy and is the best model currently available. Second, radiological examination to evaluate ONFH was not performed one month after administration of the MPSSL, immediately prior to the surgery. In the early stage of osteonecrosis, radiographic examination is of little value to detect the osteonecrotic area; even magnetic resonance

imaging (MRI) requires more than 6–12 weeks after the onset of the disease [40, 41]. Thus, no specific imaging was performed prior to the surgical intervention. Third, although the sample size in the BMMC and FGS+BMMC groups was small, the power analysis using $\alpha = 0.05$ and effect size calculated from the data in this study showed that more than 80% statistical power ($1-\beta = 1.00$) to detect significant differences of percentage of the empty lacunae among the groups using analysis of variance (ANOVA). Thus, the sample size in both the BMMC and FGS+BMMC groups was not increased. Fourth, FGS was degraded by xylene in the paraffin-embedded and H&E staining processes. Thus, in the histological analysis, the FGS could not be directly evaluated. Furthermore, some degree of new bone formation around and inside the FGS might have been washed out during processing. Finally, new mechanical testing protocols need to be developed to evaluate the potential benefits of different therapies for femoral head preservation in ONFH.

Conclusions

The present study demonstrates that a novel 3D printed, customized FGS improved bone growth in femoral head after CD in a rabbit steroid-induced osteonecrosis model. Furthermore, the injection of BMMCs into the CD decreased the osteonecrotic area in the femoral head. Therefore, the combination of a novel 3D printed customized FGS and the injection of BMMCs could open a new therapy modality that has the potential to improve the clinical outcome for early stage of ONFH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank Ms. Eiko Saito for technical assistance with tissue processing. We thank the staff of the Veterinary department.

Funding: This work was supported in part by the National Institutes of Health [R01AR072613 (NIAMS), NIH R01AR057837 (NIAMS), 1U01AR069395 (NIAMS/NIBIB)]; Stanford Wallace H. Coulter Seed Grants, and Boswell Foundation.

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Fig. 1. A functionally-graded scaffold (FGS).

(A) A schematic image illustrating the three segments of FGS in femoral head. (B) FGS was made of 80% polycaprolactone (PCL) and 20% β -tricalcium phosphate (β -TCP), created by our in-house-built layer-by-layer 3D fabrication system. The FGS was cylindrically shaped (2.4 mm diameter, 27 mm length), consisting of three segments of spatially graded porosity, including 4 mm length proximal segment of 15% porosity, 17 mm length middle segment of 40% porosity, and 6 mm distal segment of 15% porosity. (C) A micro CT scan image of FGS.

Fig. 2. Representative micro CT reconstructed image of the proximal femur.

(A-H) In the CD and the BMMC groups, mineralized tissue was noted in the proximal bone tunnel. In the FGS and FGS+BMMC groups, the proximal segment of the FGS was degraded, and mineralized tissue was confirmed inside and around the FGS. A, E: CD group, B, F: FGS group, C, G: BMMC group, D, H: FGS+BMMC group. (I) In the FGS group, the degradation rate at proximal segment was higher than those at middle segment ($P < 0.005$). In the FGS+BMMC group, degradation rate at proximal segment was higher than those at middle and distal segments ($P < 0.005$). In addition, at the proximal segment, degradation rate in the FGS+BMMC group was higher than those in the FGS group ($p < 0.05$, Student's t -test). *** $P < 0.005$ by Turkey post-hoc test. (J) New bone volumes in all three segments were not significantly different among the four groups. (K) In the proximal segment, bone ingrowth ratio in the FGS group was significantly higher than in the CD group ($P < 0.05$) and the BMMC group ($P < 0.01$). In addition, bone ingrowth ratio in the FGS+BMMC group was significantly higher than in the BMMC group ($P < 0.05$). In the

distal segment, bone ingrowth ratio in the FGS group was significantly higher than in the CD group and the BMMC group ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$ by Turkey post-hoc test.

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Fig. 3. Mechanical testing results.

(A) All samples in both CD group and FGS group fractured at the femoral neck area. (B and C) No significant difference in stiffness (B) or maximum load to failure (C) were found between the two groups (Student's *t*-test).

Fig. 4. H&E staining of the femoral head (magnification, x20).

(A-D) The CD group (A) and the BMMC group (C) showed bone ingrowth in the area of the drilled tunnel. The FGS group (B) and the BMMC+FGS group (D) showed bone ingrowth around the space of the FGS and some trabecular bone in the space of the FGS. A: CD group, B: FGS group, C: BMMC group, D: FGS+BMMC group. The area of the drilled tunnel: yellow dashed line, the space of the FGS: blue dashed line, Bar: 1 mm

Fig. 5. H&E staining of the femoral trochanter (magnification, x10).

(A-D) The CD group (A) and the BMMC group (C) showed little bone formation in the area of the drilled tunnel. The FGS group (B) and the BMMC+FGS group (D) showed bone ingrowth around the space of the FGS and trabecular bone in the space of the FGS. A:CD group, B: FGS group, C: BMMC group, D: FGS+BMMC group. The area of the drilled tunnel: yellow dashed line, Bar: 5 mm

Fig. 6. BMMCs histologically decreased the osteonecrotic area in the femoral head. (A-D) H&E staining in each group (magnification, x100). A: CD group, B: FGS group, C: BMMC group, D: FGS+BMMC group. Red arrow: empty lacunae, Black arrow: normal osteocyte, Bar: 200 μ m. (E) The percentage of the empty lacunae in the CD group was significantly higher than in the BMMC and the FGS+BMMC groups. * $P < 0.05$ by Dunn post-hoc test.