Macromolecular Research

Article

DOI 10.1007/s13233-017-5134-5

www.springer.com/13233 pISSN 1598-5032 eISSN 2092-7673

In vivo Bone Regeneration Evaluation of Duck's Feet Collagen/PLGA Scaffolds in Rat Calvarial Defect

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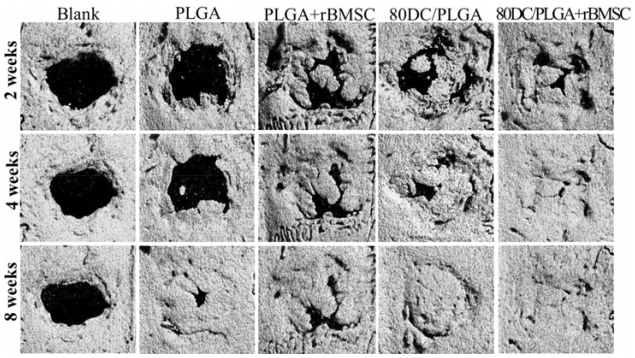
Received January 16, 2017 / Revised May 29, 2017 / Accepted June 3, 2017

Abstract: Tissue engineered bone substitutes should mimic natural bone characteristics to be highly-suitable for treating bone defects in addition to its biocompatibility and good mechanical stability. In this study, we performed a detailed *in vivo* bone regeneration evaluation of 80 wt% duck's feet collagen/poly(lactide-*co*-glycolide) scaffolds (DC/PLGA) fabricated by solvent casting/salt leaching strategy in a rat calvarial defect as model. We have already shown a strong influence of DC/PLGA scaffolds on bone regeneration in terms of biomaterial cohesion, architecture, mechanical features, and *in vitro* biological properties. The as-fabricated scaffold has shown significant increase in osteogenesis, initial bone formation and differentiation, ascribed to the high percentage of DC in the 80 wt% DC/PLGA scaffold. The *in vivo* implanted scaffold was found be well-attached to the bone defect region and eventually gets integrated with the surrounding tissues without any pronounced inflammatory reactions. Compared to bare PLGA, an increased recovery in bone volume was observed at 8th week post-surgery. Thus, the 80 wt% DC/PLGA scaffold can be envisioned as a potential alternative bone graft in bone tissue engineering.



Keywords: duck's feet collagen, poly(lactic-*co*-glycolide), scaffold, calvarial defect, bone regeneration.

1. Introduction

Bone fracture and defect caused by trauma such as accidents, disasters, and bone disease associated with decrease in bone density due to aging results in serious deformation and loss of function, thus effective and advanced treatments were highly desired.[1-3] Conventional bone defect treatment procedures include autografting and allografting, *etc.*[4-6] The autogenous bone graft is the best suitable protocol for osteogenesis, however it have few disadvantages including inflammation/infection, pain at harvest bone and lack of adequate supply of donor cells to meet the current demand. Other roadblocks of allogenous bone graft include disease transmission, hepatitis from donors and lower osteogenesis effect than autograft.[7,8] Hence, tissue engineering plays a pivotal role in treating bone related defects by culturing stem cells in bone-conductive scaffold followed by induction of bone cells differentiation.[9,10] Generally, biodegradable synthetic polymer materials such as polylactide, polyglycolide and poly(lactide-*co*-glycolide), *etc.* were most commonly used for fabricating tissue engineered scaffolds owing to their good processability, no requirement of secondary surgery and fast recovery of newly formed tissues with sufficient strength.[11-13] However, using single material also comes with a few downsides of causing low bone conduction and nonspecific inflammatory reaction. Hence, combination/composites of biomaterials (for example combination of biodegradable polymer with natural polymer biomaterial) have garnered wide interest in the tissue engineering field.

Collagen is a natural polymer and a major protein found in the body, especially in skin, bone, cartilage, and muscle.[14] Collagen acts as a calcium stores, actively promotes the formation of bone composed of 30% calcium and 70% collagen.[15,16] In particular, the collagen extracted from duck's feet has a high content of type-I collagen which plays an important role in bone formation and depositing mineral, occupying most parts of bone extracellular matrix, and also significantly reduces inflammatory reaction.[17] However, scaffolds fabricated employing only collagen has shown insufficient mechanical strength for replacement of defect bone tissues.[18,19] On the other hand, poly(lactide-*co*-glycolide) (PLGA) is a well-known polymer, and widely popular in bone regeneration studied due to its biodegradability and capability in enhancing mechanical strength of the tissue scaffolds.[20]

Acknowledgments: This research was supported by Technology Commercialization Support Program (814005-03-3-HD020), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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In our previous reports, we have fabricated 0, 10, 20, 40, 60, 80 wt% DC/PLGA scaffolds by mixing poly(lactide-*co*-glycolide) (PLGA) and collagen extracted from the duck's flippers (DC) using solvent casting/salt leaching method to increase scaffold's mechanical properties, followed by *in vitro* bone regeneration potency evaluation.[21,22] An increased comprehensive strength was found for 80 wt% DC/PLGA scaffold *i.e.* 5.57 MPa. The 80 wt% DC/PLGA scaffold was shown as an efficient cell carrier, promoting cellular proliferation and osteogenic differentiation of bone marrow derived mesenchymal stem cells (rBMSCs). In particular, 80 wt% DC/PLGA (80 DC/PLGA) scaffolds showed a 1.8 time increase in rBMSCs proliferation rate at 28 days, and 2 times increase in alkaline phosphatase (ALP) activities at 7 days compared with the PLGA scaffolds. Furthermore, a pronounced upregulation of gene expression related to osteogenic differentiation and bone regeneration in 80 DC/PLGA scaffold was also observed. Inspired by all the above outcomes, herein we have performed a detailed *in vivo* bone regeneration examination on 80 DC/PLGA fabricated by solvent casting/salt leaching strategy in rat calvarial defect as model. Presenting a consistency with the *in vitro* results, an enhanced bone volume recovery was evident at 8th week post-surgery without any significant inflammatory reactions.

2. Experimental

2.1. Reagents and materials

Duck's feet were purchased from Korean local market. Poly(lactic-*co*-glycolic acid) (PLGA) (average molecular weight of 90,000 g/mol, 75:25 by mole ratio of lactide to glycolide, Resomer® RG756) was purchased from Boehringer Ingelheim Chem. Co. Ltd. (Germany). All reagents used in this experiment were of high-performance liquid chromatography (HPLC) grade.

2.2. Preparation of duck's feet collagen

DC was prepared according to our previous reported study.[21,22] Briefly, flippers were washed with distilled water, fat were removed using 0.5 M sodium hydroxide solution and stirred at 250 rpm for 24 h. Then, the flippers were washed using methanol and chloroform in 3:1 ratio, ethanol and acetone. Washed tissues were added to 5% citric acid at a ratio of 1:8 and stirred for 48 h at 250 rpm, 4 °C. Then the mixture was crushed in a blender. After removal of precipitate, the supernatant was filtered through a filter paper (pore size=3 μm) and 0.22 μm syringe filter. After filtering, the solution was adjusted to pH = 7 and centrifuged for 15 min at 12,000 rpm. The precipitated collagen was then washed with 100% ethanol, centrifuged for 5 min at 3,500 rpm, 4 °C and lyophilized. Lyophilized collagen was pulverized to fine powder (<180 μm) using Freezing Mill (6700 SPEX Inc., USA).

2.3. Fabrication of scaffolds

PLGA and 80 DC/PLGA scaffolds were prepared using solvent casting/salt leaching method (Figure 1). Briefly, 1 g PLGA was dissolved in 4 mL methylene chloride and 0.8 g DC powder was added. Then the DC/PLGA mixture solution was blended with 9 g sodium chloride (NaCl, Orient Chem. Co., Korea) for 3 h stirring at room temperature. The mixture was poured into a silicone mold of 4 mm diameter, 3 mm thickness and pressurized by Lab Press (MH-50Y, CAP 50 tons, Masada, Tokyo, Japan) with 60 kgf/cm^2 for 24 h at room temperature. Scaffolds were immersed into the distilled water to remove NaCl for 48 h. The distilled water was changed to fresh distilled water every 6 h. The scaffolds were freeze at -80 °C and lyophilized for 24 h.

2.4. Cell culture

Rabbit bone marrow mesenchymal stem cells (rBMSCs) were isolated from New Zealand White rabbits (4 weeks-old, female, Hanil laboratory animal center, Wanju, Korea) as described previously. Briefly, the extracted femurs were washed in phosphate buffer saline (PBS), pH 7.4, containing 2% penicillin (Invitrogen), to remove blood or other contaminants. Bone marrow was collected using 18-gauge syringe after cutting femurs. The cell suspension was cultured in alpha-minimum essential medium (α-MEM) (Lonza, Walkersville, MD, USA) supplemented with 20% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), and 1% antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA)). BMSCs incubated in 5% CO_2 incubator at 37 °C. The medium was changed every other day.

2.5. Cell attachment on scaffolds

First, the scaffolds were cut in 4 mm diameter and 0.5 mm thickness, sterilized in 70% of alcohol for 30 min followed by 3 times washing in PBS solution. Then, rBMSCs were seeded into the scaffolds at a density of 1×10^5/scaffold and cultured for 7 days *in vitro*.

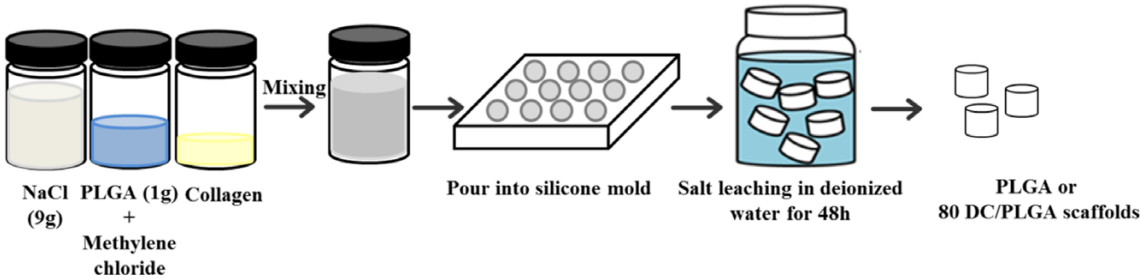

Figure 1. Schematic fabrication process of PLGA and 80 DC/PLGA scaffolds.

2.6. Rat calvarial defect model

The Sprague-Dawley rats (SD rat, 6 weeks, female) were used for *in vivo* test. Rats were anesthetized with intramuscular injection with 60 μL of Zoletil and Domitor (2:1 ratio). The surgical field hairs on rat's head were shaved using an electric shaver. The surgical site was washed with povidone-iodone. Midline coronal surgical incision was made and dissected to expose the calvarias. Two 4 mm diameter sized circular full-thickness bone defects were created using a surgical drill. Scaffolds (0.5 cm×4 mm) were inserted into bone defects. Empty defects were used as control. Experiment group of four were PLGA, 80 DC/PLGA scaffolds without/with 1×10^5 rBMSC. After the implantation, subcutaneous tissue was closed and the skin incisions were sutured (Figure 2). All the animal experiments were performed in accordance with the guidelines and approval of Chonbuk National University Animal Care Committee, Jeonju, Republic of Korea (CBNU 2016-50).

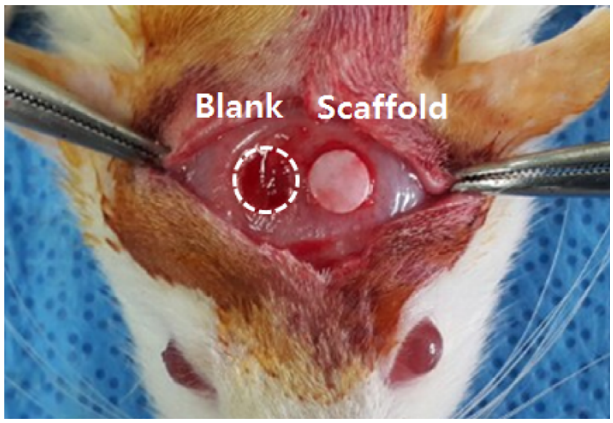

Figure 2. Implant surgery process in the rat calvarial defect model.

2.7. Micro computerized tomography

At 2nd, 4th, and 8th week post-surgery, micro computerized tomography (μCT) analysis was performed using μCT system (Skyscan 1076, Optoscan, Belgium). Before photographing, experimental animals were anesthetized, placed on stand, and then taken under external surveillance. The scanner was equipped with the following settings: voltage 101 kV, current 98 μA, filteration 0.5 mm aluminium. The obtained data is reconstructed using the Skyscan program (DataViewer, CTAn, CTvol), numerical analysis of bone mineral density (BMD), bone volume (BV), percent bone volume (BV/TV).

2.8. Histological evaluation

Histological evaluation of the calvarial samples was performed after 2nd, 4th, and 8th week post-surgery. Rats were sacrificed

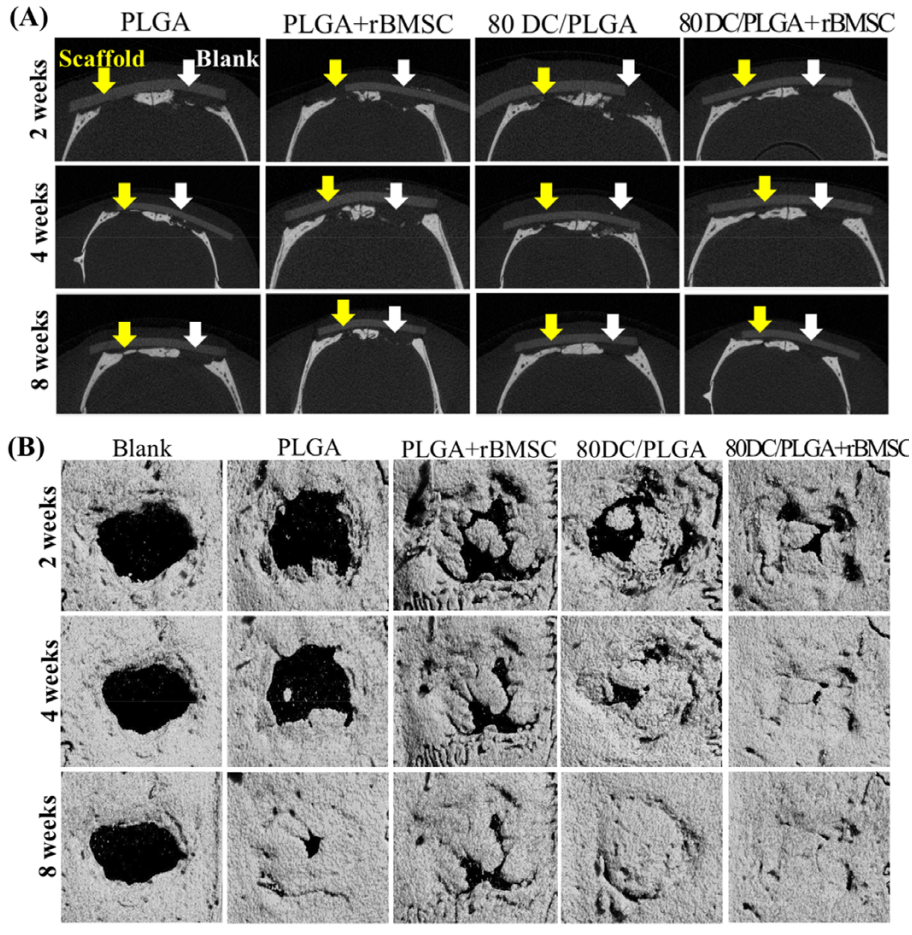

Figure 3. Micro computerized tomography images. (A) Cross-sectional. (B) 3D reconstruction of implanted scaffolds in calvarial defect after 2nd, 4th, and 8th week of surgery.

and the extracted bones with scaffolds were washed in PBS, and fixed in 10% formaldehyde (Sigma-Aldrich). The constructs were decalcified in decalcifying solution (Sigma-Aldrich), dehydrated with a series of graded ethanol, and embedded in paraffin. Paraffin sections of 7 μm thickness were cut using microtome (Thermo Scientific), and fixed on poly-L-lysine (PLL) coating slide. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome staining (MTS) after de-paraffin process for histological examination. All the samples were analyzed under an optical microscope (Nikon TE-2000, Japan).

2.9. Statistical analysis

All the results were plotted as means±Standard Deviation (SD) of experiments performed in triplicate. Statistical significance was analyzed using Student's t-test and P value as zero is accepted to indicate statistical significant differences.

3. Results and discussion

3.1. Micro computerized tomography

After 2nd, 4th, and 8th week of bone transplantation, all the experimental animals were photographed using micro-CT, centered on the graft site in skull. Figure 3 shows the (A) cross-sectional and (B) 3D reconstruction micro-CT images of implanted scaffolds in calvarial defect after 2nd, 4th, and 8th week of surgery. Overall, a reduction in bone defects was evident in all groups at all time points. As compared to PLGA, 80 DC/PLGA group displays more new bone formation and reduced bone defect over a period of 8th week (A). Furthermore, the 3D reconstruction micro-CT image shows a distinct the bone defect in a circular shape with no bone regeneration symptoms in blank (B). In case of PLGA, weak new bone formation was evident in the central part of the bone defect at 2nd and 4th week and bone formation was confirmed on 8th week except in the middle portion. Notably, the 80 DC/PLGA demonstrated a signification new bone formation at all time points along complete bone regeneration in the bone defect area at 8th week.

Figure 4 shows the numerically analyzed bone defects portion as values of BMD, BV and BV/TV using an analysis program. First, the region of interest (ROI) measured, was specified and the BMD value was measured. As shown in Figure 4(A), the lowest BMD values *i.e.* 0.037±0.0038 g/cm^3 was found for the control groups at 2nd week. On the other hand, the BMD value for 80 DC/PLGA+rBMSC group was calculated as 0.371± 0.0014 g/cm^3, which is 10 times higher than control group and 2 times more than PLGA, 80 DC/PLGA and PLGA+rBMSC groups. At 4th week, an increased BMD values were observed in all the groups (*i.e.* control, 80 DC/PLGA, PLGA+rBMSC and 80 DC/PLGA+rBMSC) except PLGA. However at 8th week, BMD values of 80 DC/PLGA group were found as 0.546±0.0215 g/cm^3 which is higher than PLGA+rBMSC group (with 0.417± 0.01 g/cm^3 BMD values), and other groups were able to confirm that the bone was regenerated with the same trend as 4th week.

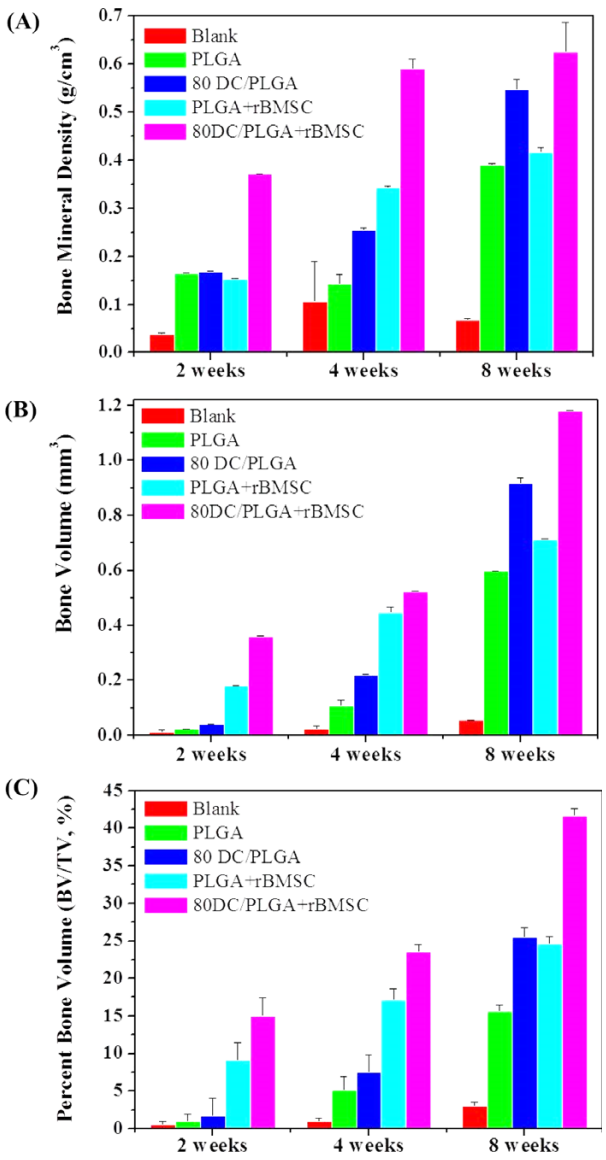

Figure 4. Micro computerized tomography analysis of calvarial defect after implantation, (A) bone mineral density, and (B) bone volume and (C) percent bone volume (Since the blank group has the lowest bone volume density, hence no statistically significant difference between other groups were found compared to blank).

Figure 4(B) shows the bone volume (BV) measured to determine the amount of new bone formed in the bone defect. The trend of BV was found to be similar to the previous BMD result, and the amount of new bone was increased with time in 2nd, 4th, and 8th week. At 2nd and 4th week, the differences in BV of PLGA scaffold without and with rBMSC were 8 and 4 times and the differences in BV of DC/PLGA without and with rBMSC were 9 and 2.4 times, respectively. At 8th week, the amount of new bone of scaffold groups with rBMSC was also higher than the scaffold groups without rBMSC. Interestingly, a higher new bone formation was observed for the DC/PLGA group with a value of 0.916±0.0204 mm^3 compared to the PLGA+rBMSC group with 0.7116±0.004 mm^3.

Furthermore to confirm the substantial amount of new bone formation, the bone volume (BV) was divided by the tissue volume (TV) and the value of percent bone volume (BV/TV) was

shown in Figure 4(C). We found that the BV/TV shows similar tendency as the previously reported BMD and BV.[20] The substantial amount of new bone is formed in an increasing order of control, PLGA, 80 DC/PLGA, PLGA+rBMSC and 80 DC/PLGA+rBMSC scaffolds in 2nd and 4th week, and increased in order of control, PLGA, PLGA+rBMSC, 80 DC/PLGA, and 80 DC/PLGA+rBMSC scaffolds at 8th week. These results suggest that rBMSCs were differentiated into osteocytes and impacted bone regeneration in the early stage, and collagen, a bone protein was affected after 8th week. In particular, it was reported that DC occupies a large proportion of type-I collagen, which is the main component of bone and promotes differentiation of osteoblasts *via* facilitating calcium and phosphorus deposition on the network structure of collagen fibers to accelerate bone formation.[23,24] Therefore, the 80 DC/PLGA group exhibits faster bone regeneration than the rBMSC groups.

3.2. Histological evaluation

Full-thickness defect were formed in both parietal bones of rats and sacrificed at 2nd, 4th, and 8th week. The defect site was visually observed, and a tissue section through the defect center was prepared and histologically observed. The results of hematoxylin and eosin (H&E) staining are shown in Figure 5(A). At 2nd week, no osteoblast, inflammatory cell, and neovascularization in the defect area, and new bone formation in the center were observed. In all groups, connective tissues were observed throughout the defect site, and new bone formation was slightly observed in the 80 DC/PLGA+rBMSC group. The 4th week post-surgery results showed that the scaffold was distributed over the entire defect in all groups and weak regeneration of new bone was observed at the periphery of the defect, but bone formation was not found in the other part. Compared to 80 DC/PLGA, the PLGA+rBMSC groups showed more number of new blood vessels and bone formation. In the 80 DC/PLGA+rBMSC group, bone regeneration sites were widely observed at a significantly faster rate than the other groups. At 8th week in the PLGA group, new bone formation was observed between the tissues. In the PLGA+rBMSC group, graft material was observed in the central region of the bone defect. Similar to 80 DC/PLGA, 80 DC/PLGA+rBMSC group showed dense new bone on the central part excluding the surgical site boundary, and new bone was regenerated sporadically on the border area.

Figure 5(B) shows the Masson's trichrome (MTS) staining of implanted scaffolds in calvarial defect after 2nd, 4th, and 8th week

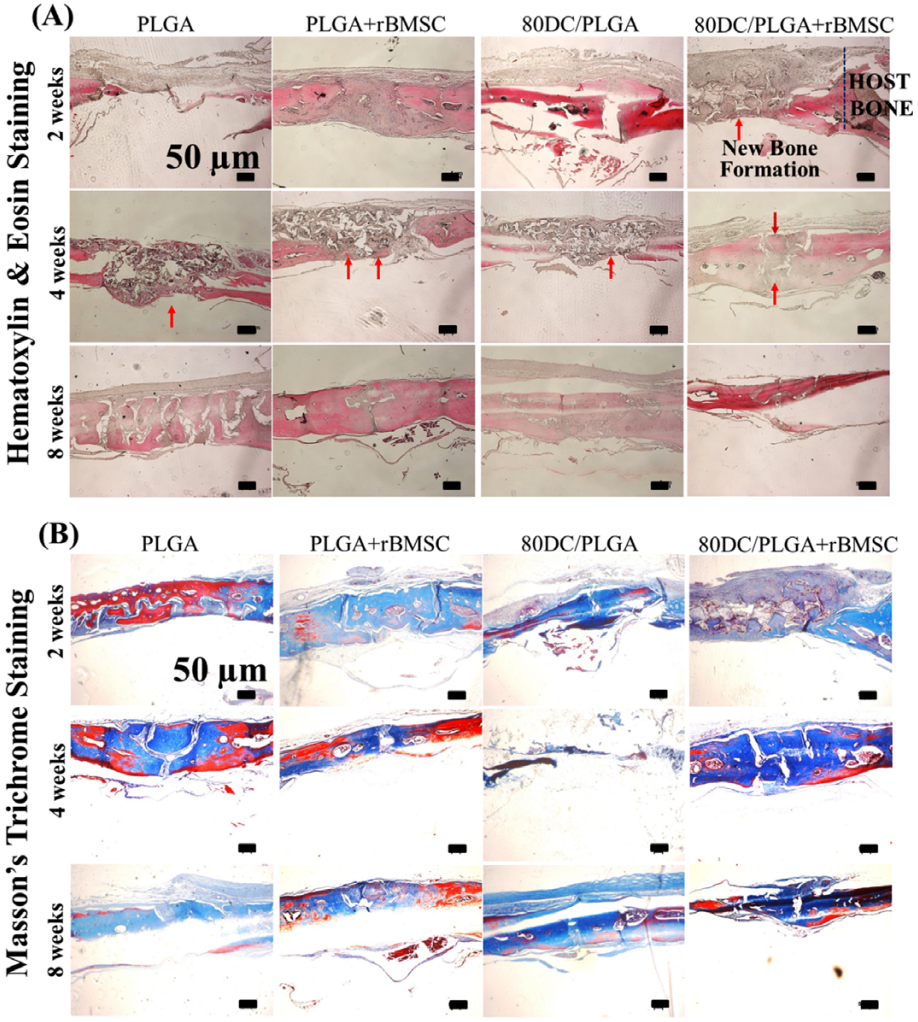

Figure 5. (A) Hematoxylin and eosin (H&E), and (B) Masson's trichrome (MTS) staining of implanted scaffolds in calvarial defect after 2nd, 4th, and 8th week of surgery. The red arrows mark the newly generated bone formation.

of surgery. Initially at 2nd week, the area of scaffolds and tissue regeneration was observed in blue at the implantation site and further it changes to red color which signifies new bone regeneration at 4th and 8th week with an increase in time. Thus, it can be confirmed that the formation of new bone increased in 80 DC/PLGA and 80 DC/PLGA+rBMSC group compared with other group. This increase in bone formation can be ascribed to the presence of DC which promotes new bone formation, increases bone conduction according to the cell affinity of collagen, the biocompatible environment.

4. Conclusions

In conclusion, we have successfully designed DC/PLGA scaffolds with enhanced bone regeneration and further confirmed the potency of the scaffolds with/without rBMSC in *in vivo* bone defect model. Micro-CT and histological evaluation images showed bone regeneration in increasing order of negative control, PLGA, PLGA+rBMSC, 80 DC/PLGA, and 80 DC/PLGA+rBMSC. Overall, 80 DC/PLGA scaffolds were found to be superior to the PLGA scaffold owing to the presence of type-I collagen, which is a major constituent of collagen extracted from the duck feet and promotes differentiation of osteoblasts and accelerates bone formation. Therefore, it can be suggested that the 80 DC/PLGA scaffolds provides a friendly favorable environment for bone regeneration scaffolds and further can be applied as bone graft materials.

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