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

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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(lactic-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 to 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 allogeneous 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(lactic-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(lactic-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

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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. An increased comprehensive strength was found for 80 wt% DC/PLGA scaffold 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 observed. Inspired by all the above outcomes, herein we have performed a detailed in vitro 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. 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/cm² 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% CO₂ 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 1×10⁵/scaffold and cultured for 7 days in vitro.

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