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Cytocompatibility and osteogenesis evaluation of HA/GCPU composite as scaffolds for bone tissue engineering

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ABSTRACT

Porous scaffolds for bone repair were prepared from newly designed segmented aliphatic polyurethane based on glyceride of castor oil and isophorone diisocyanate. To promote the scaffolds' biological and mechanical properties, hydroxyapatite powder was incorporated into the polymer matrix. The scaffold (named as HA/GCPU) with 40 wt% HA had an average pore size of 500 μm and a compressive strength of 4.6 MPa. The *in vitro* cell culture studies demonstrated that the HA/GCPU scaffold owned good cytocompatibility. The scaffold and cell-seeded scaffold were implanted in defects (Φ3 mm × 3 mm) of femoral condyle of Sprague–Dawley rats, respectively. New bone could extensively form in both the scaffold and cell-seeded scaffold. It indicates that the HA/GCPU composite scaffold has good prospect for bone repair and regeneration.

1. Introduction

There are a large number of patients suffering from bone defects caused by trauma, tumor or bone related diseases [1]. Current strategies for repair of bone defects include autograft, allograft and synthetic materials [2]. Although autograft remain to be the gold standard due to its osteoconductive, osteoinductive and non-immunogenicity [3], it is subjected to donor shortage and donor site morbidity [4]. In addition, allograft has the risk of immunological and disease transmission [5]. Synthetic materials are often used to substitute autograft and allograft, however, they are sometimes subjected to fatigue failure and wear over time [6]. To overcome aforesaid disadvantage, bone tissue engineering (BTE) has emerged as an alternative for bone repair and regeneration. The researches in BTE focus on a three-dimensional porous scaffold which can be loaded with specific living cells or growth factors to launch a bone tissue regeneration in natural way [7]. In previous study, several porous scaffolds composed of hydroxyapatite (HA) and castor oil-based polyurethane (PU) were prepared and their potential for bone repair were evaluated in our group. In order to enhance the mechanical strength of HA/PU scaffolds, a new attempt is made in this study to use the glyceride of castor oil as the soft segment combined with isophorone diisocyanate (IPDI) as the hard segment for preparation of HA/PU scaffold. This paper aims at a twofold objective, on one hand, to study the cytocompatibility of this novel scaffold, on the other, to evaluate the effect of the scaffold on bone regeneration *in vivo*.

2. Materials and methods

2.1. Materials

Hydroxyapatite precipitate was synthesized in our lab [8]. The obtained HA slurry was then spray-dried, and screened through a mesh sieve (~75 μm). Castor oil, glycerol and isophorone diisocyanate were supplied by Aladdin Co. Ltd., China. F-12 nutrient mixture and newborn calf serum (cell-culture grade) were from Invitrogen Corporation, USA. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (purity: >98%) was from Amresco, USA. Live/dead viability/cytotoxicity kit was from Molecular Probes, USA. 1,4-butanediol (BDO) and stannous octoate were from Chengdu Kelong Co. Ltd., China and of analytical grade.

2.2. Preparation and characterization of scaffold

The HA/GCPU porous scaffold was synthesized by glyceride of castor oil and isophorone diisocyanate (IPDI) with addition of

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hydroxyapatite (HA) powder. Firstly, glyceride of castor oil (GCO) was prepared by transesterification of castor oil with glycerol according to the literature [9]. The reaction was carried out at 200 °C for 1 h while stirring, the mole ratio of glycerol to castor oil was 2.5:1, CaO was used as catalyst. The porous scaffold with 40 wt% HA was prepared in a three-necked flask at 70 °C under nitrogen atmosphere. GCO and IPDI were mixed thoroughly by a ratio of −OH to −NCO at 1:1.1, then a certain HA powder was added. Stannous octoate, BDO and distilled water were added successively as catalyst, chain extender and foaming agent. The final mixture turns into HA/GCPU scaffold after dumping into a mold and foamed in an oven at 110 °C for 4 h. The scaffold morphology was observed by scanning electron microscopy (SEM) (JSM-6500LV, JEOL, Japan) at 30 kV after gold coated. The compressive strength of the scaffold was tested according to the procedure set by the ASTM D695-96. The scaffold porosity was measured by the liquid displacement method. The scaffold with a known volume (V) and weight (M) was immersed in distilled water. A series of evacuation–repressurization cycles was conducted to force the distilled water to penetrate and fill the pores of the scaffolds. After that the weight of the scaffold was recorded as M_1. The porosity was calculated according to the following formula: porosity $= (M_1 - M)/V \times 100\%$. Five samples were tested in each group.

2.3. In vitro cell culture

ROS 17/2.8 cells were provided by the West China School of Stomatology, Sichuan University. The cells were derived from rat osteosarcoma and expressed the characteristic features of osteoblasts. Osteoblastic ROS 17/2.8 cells were seeded on the surface of the scaffold samples ($1 \times 1 \times 0.1$ cm^3, 1×10^4 cells/sample). The scaffold/cells constructs were incubated in a 5% CO_2 incubator at 37 °C. After 7 days, cell morphology on the HA/GCPU scaffold was observed by SEM. Cell viability was evaluated using the Live/Dead viability/cytotoxicity assay (Molecular Probes, USA), and the cells were imaged using fluorescence microscope (TE2000-U, Nikon, Japan). Cell proliferation was assessed by MTT assay. The absorbance was measured at 490 nm on a microplate reader (1420, Perkin Elmer Co. Ltd., USA) after 1, 4, 7 day (s). Osteocalcin levels in the culture media were evaluated by an assay reagent kit (Biomedical Technologies Inc., MA) after 1, 4, 7 day (s).

2.4. In vivo animal studies

To investigate the osteogenesis of the composite porous scaffold, female Sprague–Dawley rats weighing between 160 and 200 g were used in the experiment. The HA/GCPU scaffold with 40 wt% HA was sterilized by ethylene oxide before use. The ROS 17/2.8 cells (10^5 cells/mL) were injected on the HA/GCPU scaffolds. The cell-scaffold constructs were kept in an incubator at 37 °C for 7 days. A total of sixteen SD rats were divided into two groups. A bone defect with a diameter of 3 mm was created in the region of femoral condyle. In group 1, the defects were implanted with cylindrical HA/GCPU scaffolds (Φ3 mm × 3 mm), and in group 2, HA/GCPU scaffolds seeded with ROS 17/2.8 cells were implanted into the defects. Then musculature and skin were closed with nylon sutures. Gentamicin (10 mg/kg) was administered for 3 days postoperatively to prevent infection. The rats were allowed to move freely in their cages and sacrificed at 4 and 8 weeks respectively. The femurs were dissected and fixed in 4% phosphate-buffered paraformaldehyde. After that scaffold samples with surrounding bone were decalcified, dehydrated in a series of gradient ethanol solutions, embedded in paraffin, and subsequently sectioned to slices of 5 mm in thickness. The sections were processed for Masson staining, and examined under light microscope (TE2000-U, Nikon, Japan).

2.5. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). Statistical significance was determined using SPSS 10.0. Statistical comparison of two experimental groups was performed using the Student's t-test. $p < 0.05$ was considered to be significant.

3. Results and discussion

The digital and SEM images in Fig. 1 clearly show the macrography and micrograph of 40 wt% HA/GCPU scaffold. The scaffold has an average pore size of 500 μm and maintains an interconnected porous structure. The scaffold porosity and compressive strength are measured for about 60% and 4.6 MPa, respectively. The pore size and strength are suitable for the use of bone tissue engineering.

The synthesis of scaffolds for bone tissue engineering must consider the nature of raw materials [10]. Polyols, such as polyesters and polyethers, are usually used to provide flexible segment and cross-linking site for the resulting polymers [11]. Castor oil as a renewable source is also a polyol and recently adopted in the synthesis of PU polymer and scaffolds. However, the shortage of using castor oil is the low number of hydroxyl groups in its molecular structure. Compared to castor oil, the glyceride of castor oil can provide more hydroxyl groups (from 155 to 288 of the hydroxyl value) for reaction with isocyanate, therefore, the efficiency of the polymerization can be greatly improved, resulting in an increase of the scaffold compressive strength (from hundreds of kPa to 4.6 MPa). Moreover, the HA filler in the PU matrix is also helpful for the increase of mechanical strength [12]. The results show that the novel synthetic route of HA/GCPU composite scaffolds is promising for bone tissue engineering.

SEM images in Fig. 2A, B show the morphology of ROS 17/2.8 cells adhered on the surface of scaffold after 7 days of culture. The cells with typical osteoblastic morphology have formed cell sheet on the scaffold and spread in the pores of scaffold. This means the scaffold does not inhibit cell adhesion and proliferation. It is believed that the HA filler may provide plenty of cellular binding sites and the porous scaffold can offer ample space for cell ingrowth, which are the major approach to realize enhanced cellular activity and cytocompatibility of scaffolds [13]. The fluorescent image in Fig. 2C demonstrates an efficient cell attachment on the scaffold and a high affinity of the scaffold to the osteoblastic cells. Fig. 2D summarizes the metabolic activity of ROS 17/2.8 cells cultured on the scaffolds for 1, 4 and 7 day (s). During the 7 days of culturing, the cell metabolic activity and osteocalcin production increase significantly with the incubation time ($p < 0.05$). The results indicate that the HA/GCPU scaffold has good cytocompatibility and can provide favorable 3D microenvironment for cells.

The histological images are presented in Fig. 3. No inflammatory response is observed at 4 weeks. The newly formed bone tissue has appeared around the scaffold and in the pores of scaffold. At 8 weeks, there is more new bone formation around and within the porous scaffold, and no adverse inflammatory response can be noticed. In contrast, the amount of new bone is more in cell seeded scaffold than unseeded scaffold. Although the cell seeded group seems to perform slightly better than unseeded group, both scaffolds show good *in vivo* osteogenesis.

4. Conclusion

Porous HA/GCPU composite scaffold has been synthesized using glyceride of castor oil, aliphatic IPDI and bioactive HA powder. Such

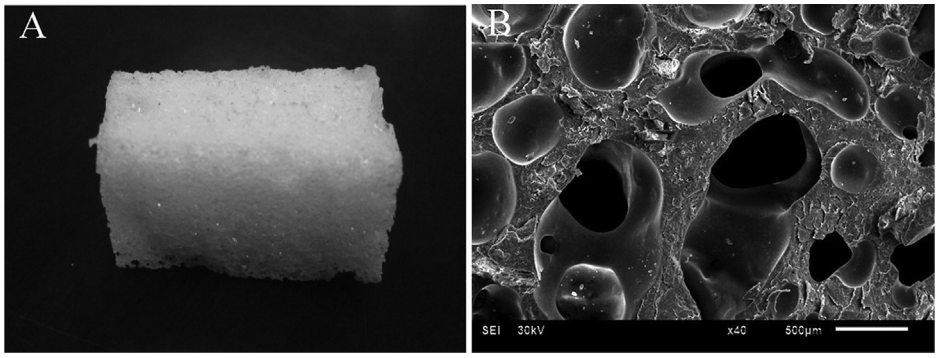

Fig. 1. Digital (A) and SEM (B) images of 40 wt% HA/GCPU scaffold.

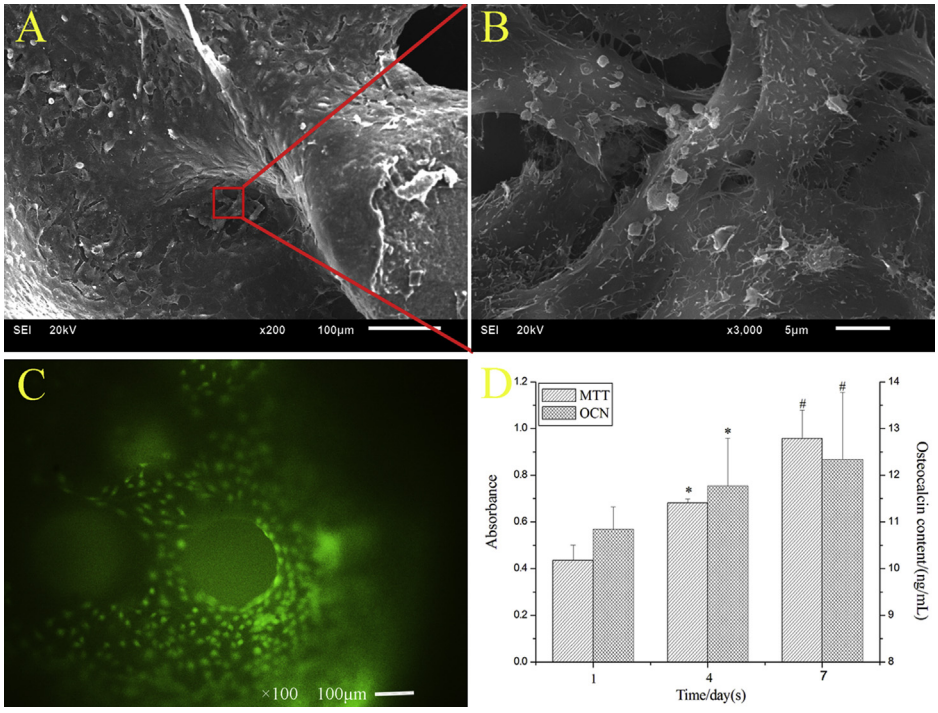

Fig. 2. SEM image of ROS 17/2.8 cells cultured on 40 wt% HA/GCPU scaffold for 7 days (A) and higher magnification image (B), fluorescent image (C), and MTT assay for cell proliferation and osteocalcin content in media (D). (Note: same symbols represent no significant difference ($p > 0.05$); different symbols represent significant difference ($p < 0.05$).)

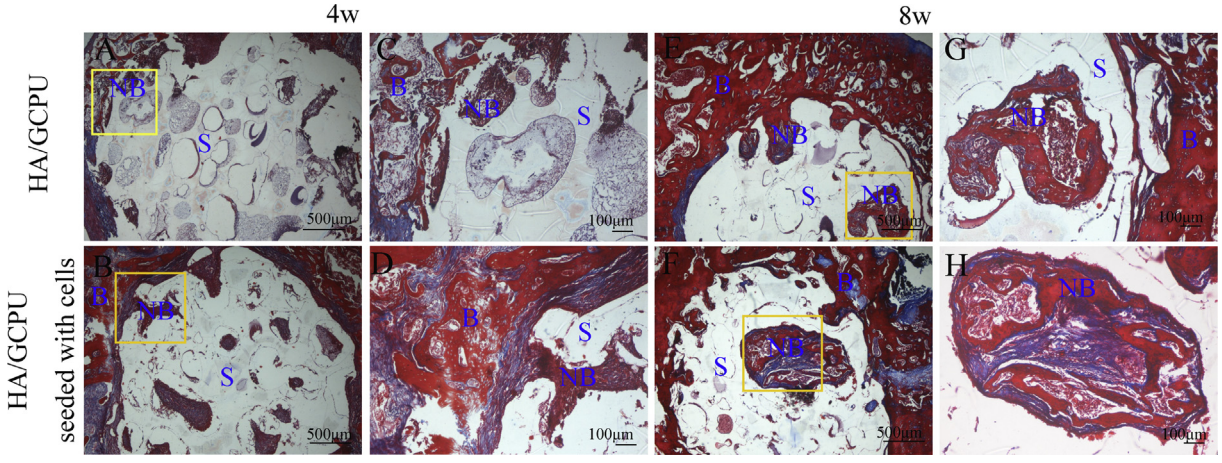

Fig. 3. Histological sections (Masson staining) of HA/GCPU and cell seeded HA/GCPU scaffolds at 4 (A, B) and 8 (E, F) weeks. (C, D) and (G, H) corresponding to each left box. S-scaffold, NB-new bone, B-bone.

scaffold with optimized mechanical and biological properties, i.e., good compressive strength, interconnected porous structure, high affinity to osteoblastic cells and bone-bonding ability via HA component, could be good candidate for bone repair and regeneration. Further animal experiments should be carried out on bone regeneration and scaffold degradability.

Conflict of interest

No conflicts of interest.

Ethical approval

The protocol was approved by medical center of Sichuan university.

Author contribution

Jingjing Du: data collections and writing.
Qin Zou: data collections and study design.
Yi Zuo: data analysis.
Yubao Li: study design.

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