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Comparison of three-dimensional printing and vacuum freeze-dried techniques for fabricating composite scaffolds

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ABSTRACT

In this study, the performances of different preparation methods of the scaffolds were analyzed for chondrocyte tissue engineering. Silk fibroin/collagen (SF/C) was fabricated using a vacuum freeze-dried technique and by 3D printing. The porosity, water absorption expansion rates, mechanical properties, and pore sizes of the resulting materials were evaluated. The proliferation and metabolism of the cells was detected at different time points using an MTT assay. Cell morphologies and distributions were observed by histological analysis and scanning electron microscopy (SEM). The porosity, water absorption expansion rate, and Young's modulus of the material obtained via 3D printing were significantly higher than those obtained by the freeze-dried method, while the pore size did not differ significantly between the two methods. MTT assay results showed that the metabolism of cells seeded on the 3D printed scaffolds was more viable than the metabolism on the freeze-dried material. H&E staining of the scaffolds revealed that the number of cells in the 3D printed scaffold was higher in comparison to a similar measurement on the freeze-dried material. Consequently, stem cells grew well inside the 3D printed scaffolds, as measured by SEM, while the internal structure of the freeze-dried scaffold was disordered. Compared with the freeze-dried technique, the 3D printed scaffold exhibited better overall performance and was more suitable for cartilage tissue engineering.

1. Introduction

Cartilage cells and their extracellular matrix (ECM) are the main component of cartilage. Cartilage contains no blood vessels, nerves, or lymphatic drainage and gains access to main nutrients from the synovial fluid [1]. The structure of the cartilage determines its capacity for regeneration and self-repair. Due to trauma, inflammation, tumors, and other causes, cartilage injury is extremely common in clinical settings. Treatment of cartilage defects using a source of autologous chondrocyte transplantation (ACT) can cause secondary damage [2]. Further, the use of allograft cartilage transplantation can not only reject native cartilage–tissue contact within the affected area, but such treatment of cartilage-like tissues are far different from normal cartilage tissue with respect to their biological properties and functions and can be susceptible to easy degeneration [3,4]. Other treatment methods have obvious deficiencies. Current treatments also cannot restore native cartilage; thus, clinical needs have motivated cartilage tissue engineering. The ideal scaffold material should have the ability to guide and promote the formation of new tissue, have good biocompatibility, cause minimal injury and inflammation to the surrounding tissue, and provide mechanical support. Natural materials such as collagen(COL) and silk fibroin(SF) have been the most extensively explored for these applications [5].

With the development of molecular biotechnology, SF has become more and more widely used in the field of biological materials and medical treatments [6]. The main advantages of SF are that it is non-toxic, low in price, comes from abundant sources, andexhibits good biocompatibility and strength, as well as surface and viscoelastic stability [7]. COL is the main component of a cartilage matrix; the scaffold of collagen protein is conducive to cell adhesion, cell support and protection [8]. The disadvantage of collagen scaffolds is that the speed of degradation is fast, and the mechanical strength is insufficient [9]. COL is inadequate as a scaffold material unless it is blended with other materials [10]. In a

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previous study [11], silk fibroin blended with collagen was found to improve the mechanical properties and to facilitate the formation of thicker pore walls and a rougher surface.

Among the many methods available to make composite cell scaffolds, the freeze dried method is a simple and convenient method for making porous structures with controllable microstructures and properties. Therefore, many studies have focused on freeze dried and have gotten good results [12]. With the development of 3D printing, several researchers have attempted to use this method for fabricating cell scaffold type structures, some of them leading to exciting results [13]. However, studies examining and comparing the two aforementioned methods are scarce. Thus, in the present study, we tested our hypothesis that the 3D printing method can be used to fabricate scaffold structureswith superior physical characteristics, cell adhesion, microstructures and overall mechanical properties compared to those prepared via freeze-dried. Moreover, this study can provide direction to further the progress of cartilage tissue engineering scaffolds.

2. Materials and methods

2.1. Fabrication of scaffolds

Silkworm silk was boiled in 60 °C, 0.5% Na2CO3 solution for 30 min (to remove sericin), then kept at 60 °C, purified fibers was dissolved in CaCl2·CH3CH2OH·H2O(Mole ratio 1:2:8) at 60°Cabout 2 h and centrifuged 10 min at 8000 rpm/min to obtain up clear liquid, dialyzed for 72 h, concentrated 7 h in 40% PEG and got 8 wt% silk fibroin solution [1,3]. Fresh bovine tendon(Solebo Biological Technology Co., Ltd. Beijing, China)was stripped the outer membrane, removed the fat, crushed and soaked in 0.05 Tris mol/L buffer for 24 h, then collected precipitation, added the stomach Protease of the acetic acid solution and 3.5 mol/L NaCl solution, salting out sediment was collected, dialysed in deionized water for 5 days and obtained 2.5 wt% slurry of type II collagen [1,3]. Briefly, an 8 wt% silk fibroin solution (molecular weight 5,000, Sigma Chemical Co., St. Louis, MO, USA) was mixed with a 2.5 wt% slurry of type II collagen. Thus, the SF/C scaffolds were fabricated using a vacuum freeze-dried technique (the mixed solution was placed in a vacuum freeze dryer at −50 °C for 48 h) and 3D printing method. The printing conditions were as follows: 10 mm/s deposition rate, 0.3 mm slice thickness, −20 °C forming platform temperature, 0.09 mm/min extrusion rate, and 0.36 mm nozzle tip size. The fabricated SF/C scaffolds were sterilized using 3kGY Co60 for 30 min for subsequent in vitro experiments, and no physical changes were observed due to the irradiation in the next study about scaffolds.

2.2. Physical characterization

The porosity of the scaffolds was determined using the principle of liquid displacement [1]. The inner structure and pore size were evaluated by randomly measuring at least 30 pores using an image J program of SEM (Scanning Electron Microscope)image analysis program. Biomechanical properties of the silk fibroin/collagen scaffolds were measured using a universal testing machine (Instron5865) equipped with 0.1 N load at 0.5 mm/min under environmental conditions, compression of 10%, dynamic displacement of 5% amplitude at frequencies of f = 0.5 Hz, cycled three times. Young's modulus was calculated to obtain a stress-stain curve. After 14 days of culture, the thickness and Young's modulus of the scaffolds were tested again to compare the biomechanical properties with the original material.

2.3. Cell viability and SEM examination of the microstructure

BMSCs of Wistar rats were separated by density gradient centrifugation [1], and maintained in a humidified incubator (5% CO_2, 95% air) at 37 °C; after the third passage, the cells were used for further experiments. The third passage (P3) BMSCs were seeded on the two different kinds of scaffolds. Briefly, the scaffolds were each seeded with 100 μl of BMSC suspension containing 2×10^7 cells. Then, fresh induced medium was added into each well after 4 h and incubated continuously for 14 days; the media was changed every other day. The thiazolyl blue tetrazolium bromide (MTT) assay was used to assess cell viability. The principle of the assay is based on the metabolic activity of live cells. The time intervals were 1, 3, 5, 7, 9, 11, and 13 days. Briefly, at the determined times, the samples were removed and transferred to new 24-well plates. Solution of 2 mg/ml thiazole blue (MTT) was added to each well, and the samples were incubated for another 4 h. The medium was discarded, and 2 ml dimethyl sulfoxide (DMSO) was added to each well and dissolved fully, resulting in the formation of purple crystals. Then, 150 μl of each sample was transferred to a 96-well plate and measured at 520 nm by spectrophotometry.

After 14 days in culture, the scaffolds with attached cells were washed with PBS (pH 7.4), fixed with 4% glutaraldehyde solution and then with 1% osmic acid at 4 °C for 4 h. The scaffolds were subsequently dehydrated in graded ethanol solutions and air-dried. Finally, the scaffolds on aluminium stubs were coated with gold-palladium, and microstructures were observed using a scanning electron microscope.

2.4. Biochemical analysis

The glycosaminoglycan (GAG) content of the two different scaffolds was measured using the alcian blue method (GAG assay kit, Biocolor, Newtonabbey, UK), used for the determination of acidic glycosaminoglycan in the supernatant of the culture medium. Briefly, after continual induced for 14 days, time intervals were same with MTT, supernatant solution was collected and centrifuged at 1500 r/min for 10 min and 1.5% wt% alcian blue solution was added, and the absorbance was measured using enzyme standard instrument (Thermo Co., USA) at 520 nm.

2.5. Histological and immunohistochemical

After 14 days in culture, the scaffolds were washed with PBS (pH 7.4) and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin (H&E) to observe cell growth and distribution under a light microscope. To detect the expression level of type II collagen immunohistochemically after 2 weeks of induced culture, the scaffolds were fixed with 4% polyformaldehyde for 30 min, 3% H2O2 and 0.5% hyaluronic acid were added, and incubated for 30 min at 37 °C. Rabbit anti-rat type II collagen monoclonal antibody was added and left overnight at 4 °C. This mixture was washed twice with PBS, dropping 1:300 dilution of Sheep anti-rabbit biological biotinylated second antibody, and incubated at 37 °C. 1:1000 streptomycin was added, incubated for 30 min, 5 min color in DAB, and the results were observed under a microscope.

2.6. Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). All data were reported as the mean ± standard deviation (SD). One-way ANOVA was employed for all statistical analyses, followed by Student-Newman-Keul's test. Values were compared using multiple comparisons, where p-values of 0.05 or less were

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