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Three-dimensional dynamic fabrication of engineered cartilage based on chitosan/gelatin hybrid hydrogel scaffold in a spinner flask with a special designed steel frame

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ABSTRACT

Cartilage transplantation using in vitro tissue engineered cartilage is considered a promising treatment for articular cartilage defects. In this study, we assessed the advantages of adipose derived stem cells (ADSCs) combined with chitosan/gelatin hybrid hydrogel scaffolds, which acted as a cartilage biomimetic scaffold, to fabricate a tissue engineered cartilage dynamically in vitro and compared this with traditional static culture. Physical properties of the hydrogel scaffolds were evaluated and ADSCs were inoculated into the hydrogel at a density of 1×10^7 cells/mL and cultured in a spinner flask with a special designed steel framework and feed with chondrogenic inductive media for two weeks. The results showed that the average pore size, porosity, swelling rate and elasticity modulus of hybrid scaffolds with good biocompatibility were 118.25 ± 19.51 μm, 82.60 ± 2.34%, 361.28 ± 0.47% and 61.2 ± 0.16 kPa, respectively. ADSCs grew well in chitosan/gelatin hybrid scaffold and successfully differentiated into chondrocytes, showing that the scaffolds were suitable for tissue engineering applications in cartilage regeneration. Induced cells cultivated in a dynamic spinner flask with a special designed steel frame expressed more proteoglycans and the cell distribution was much more uniform with the scaffold being filled mostly with extracellular matrix produced by cells. A spinner flask with framework promoted proliferation and chondrogenic differentiation of ADSCs within chitosan/gelatin hybrid scaffolds and accelerated dynamic fabrication of cell–hydrogel constructs, which could be a selective and good method to construct tissue engineered cartilage in vitro.

1. Introduction

Osteochondral defects caused by degenerative joint disease, wounds and others bring about great pains for many patients, and are becoming more of an increasing problem. The ability to self-repair in cartilage tissue is very limited due to its simple structure and lack of nervus vascularis and lymphoid tissue. Besides, while existing clinical practices to repair osteochondral defects and wounds are diverse, they are not ideally effective to recover the cartilage tissue completely [1–3]. With the rapid development in cartilage tissue engineering, this approach appears to be rather promising in repairing osteochondral defect in clinical practice.

Fabricating constructs through a combination of cartilage cells and suitable scaffold is vital to cartilage tissue engineering, thus the selection of a suitable cell source and supporting scaffold would determine the success rate of manufactured artificial tissues [4,5]. Recently, given its multi-potential differentiation ability, abundant proliferation in vitro, easy access, smaller pain and minimum immunological rejection [3], adipose-derived stem cells (ADSCs) have become a much more attractive cell source for tissue engineering than bone mesenchymal stem cells (BMSCs), and have now been used to fabricate tissue engineering cartilage by many researchers [6,7].

The selection and design of cartilage scaffold as a basic framework for tissue engineered cartilage are vital to repair injured cartilage tissue successfully. There are numerous scaffolds involved in the current research of our field, each with their advantages in their application,

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and some weaknesses that are simultaneously revealed [8]. Improved methods to reduce the weaknesses of existing scaffolds are mainly by material recombination or surface modification. Chitosan, whose structure is similar to the cartilage matrix-glycosaminoglycans (GAG) and has a degradation product that is a glucosamine monomer [9], is a natural high molecular weight polymer with many appealing characteristics including no adverse reactions to human body, good biocompatibility and degradability, nontoxicity, immunogenicity, inhibition of inflammation, antibiosis and so on. Gelatin, which is a degradation product of collagen, possesses a strong biological activity and facilitates adhesion, proliferation, differentiation and extracellular matrix secretion of cartilage cells on the scaffold [10]. At present, these two natural biopolymers have been combined by several researchers as a cartilage scaffold [11,12]. However, their physical properties and cell growth profiles on the scaffold can vary significantly due to differences in preparation conditions. Herein, the merits of the hybrid scaffold will be retained while shortcomings of each will be overcome. We achieve this by adjusting the ratio of the two components in order to regulate and control the performance of the scaffold. Finally, the formation of a chitosan/gelatin hydrogel with an optimum content using the method of freeze drying pore-forming will be determined as the scaffold for constructing an engineered cartilage.

In constructing a tissue engineered cartilage, the culture environment in vitro is also a crucial aspect in ensuring the success of an artificial tissue given the appropriate seeding cells and scaffold material. With growing interest and in-depth research in cell culture technology, researchers are beginning to realize the advantage of a dynamic environment in bioreactors [13–15], as compared to traditional static culture for creating tissue engineered cartilage. Noticeably, the spinner flask which has a simple design, is able to provide a dynamic microenvironment owing to fluid flow created by stirring to enhance mass transfer and facilitate proliferation and differentiation of cells. Its simplicity and functionality has proven for its wide use in the field of tissue engineering [16,17]. In 2001, cartilage cells were seeded onto PLLA scaffold by Gooch et al. [18], and cultured in the spinner flask. Consequently, the secretion of collagen and glycosaminoglycan increases on account of the mixing during stirring. In 2010, Zhu et al. [19] demonstrated that culturing in the spinner flask improves the survival rate, proliferation, and expression of specific genes in ADSCs.

In this study, the porous chitosan/gelatin complex is first prepared through mixing gelatin with chitosan cross-linked via anionic interactions. Then, the ADSC induced cartilage seeded into the complex scaffold is dynamically fabricated in a spinner flask with a special steel frame, designed to boost the mass transfer rate of nutrients and timely discharge of metabolites inside the cell–scaffold complex. Meanwhile, the proliferation and matrix secretion of ADSCs are accelerated under the stress stimulus generated by convective flow. Accordingly, the three-dimensional fabrication for tissue engineering cartilage is preliminarily completed.

2. Materials and methods

2.1. Materials

The adipose tissue, provided by the Affiliated Hospital of Dalian Medical University, is taken from liposuction operation of 49 year old middle-aged women, strictly complied with the Helsinki declaration requirements and be agreed to sign by the patient. ADSCs were cultured at 37 °C and 5.0% CO_2 in a humidified incubator, with DMEM medium containing 10% FBS. The procurement of materials used in the experiment: The viscosity of chitosan is 300 cp, which was purchased from Haidebei Marine Bioengineering Co. Ltd, the molecular weight of which is less than 5000 and the degree of deacetylation is around 85.6%. Gelatin, the analytical agent, was purchased from Sinopharm Co. Ltd, the purity of which is more than 99.5%. Gelatin is from the skin of pig and only special for drug test (the Wo Kai brand of Sinopharm Co. Ltd). The Chitosan, $(C_6H_{11}NO_4)_n$ (GB29941-2013), original white, is from crab shells of Alaska Snow Crab. The absolute alcohol was purchased from Ante Food Co., Ltd. in Anhui. The acetic acid is from Shenyang chemical reagent plant. The carbodiimide and N-hydroxy-succinamide (NHS) were purchased from the Shanghai Medpep Co., Ltd. The 2-morpholino ethane sulfonate (MES) was purchased from Beijing Bio-lab Materials Institute. The sodium phosphate dibasic was purchased from Tianjin Damao chemical reagent plant.

2.2. Preparation of chitosan/gelatin hydrogel scaffolds

The gelatin solution and chitosan acetate solution were mixed slowly after being placed in an oven to dissolve at high temperature and centrifuged to remove bubbles. The mass ratio of gelatin and chitosan is 3:1. In order to study the relationship between swelling ratio, porosity and the total concentration of scaffolds, three groups of total concentrations of 3%, 4% and 5% hydrogel scaffolds were prepared separately, from which the group with optimal combination of physical properties would be prepared for the subsequent experiments.

Respectively, 50 mmol/L of 2-morpholino ethane sulfonate (MES), carbodiimide (EDC) and N-hydroxy-succinamide (NHS) were added to 40% ethanol solution to prepare for 300 mL cross-linking agent solution. The mixture of chitosan/gelatin was instilled into wells of culture plates and then put to a refrigerator for pre-freezing. After being dried in freeze drier, the scaffolds were cross-linked in carbodiimide/N-hydroxy-succinamide/morpholine ethane sulfonate (EDC/NHS/MES) for 6 h. Add 0.1 mol/L Na_2HPO4 for 2 h to neutralize acetic acid, cleaned with 40% ethanol 4 times per 30 min, after being pre-freezed in −20 °C refrigerator, they were transferred to an ultra-low temperature refrigerator to freeze and prepared for use after freeze drying once again.

2.3. Detection physical properties of hydrogel scaffolds

2.3.1. SEM observation surface morphology

The prepared hydrogel scaffolds were dried, sprayed in gold, and then observed under a scanning electron microscope (SEM). Images were captured to evaluate the surface morphology. According to the measurement results, multiple regions were selected randomly and then 20 pores were chosen from each region to calculate the average pore size.

2.4. Mechanical properties testing of hydrogel scaffolds

The elastic modulus of hydrogel scaffolds was measured using DDW-100 universal testing machine, the sample size was 5 mm × 5 mm × 5 mm, displacement control loading rate was 1 mm/min.

2.4.1. Detection of swelling rate and porosity of hydrogel

The swelling means that the volume of scaffolds would expand with the interaction force between the biological macromolecules being weakened as the solvent molecules diffused into the biological macromolecules. The porosity and swelling ratio of biological scaffolds must meet certain requirements before they can be used for cell culture. Appropriate porosity can promote the input of nutrients and output of metabolic waste and provide some room. Appropriate swelling rate, also called water absorption, is conducive to the cell adhesion and ensure that the water-soluble nutrients can penetrate into the inside of the scaffolds. Both of them are beneficial to the growth and metabolism of cells.

The porosity of hydrogel scaffolds was measured as following: the concentration of 3%, 4% and 5% hydrogel scaffolds was numbered as A, B and C, respectively. Each concentration has three samples. The results were expressed as mean ± SD. ① Nine test tubes were labeled as A_1, A_2, A_3; B_1, B_2, B_3; and C_1, C_2, C_3. A certain volume of water was added into