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Pore size effect of collagen scaffolds on cartilage regeneration

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ABSTRACT

Scaffold pore size is an important factor affecting tissue regeneration efficiency. The effect of pore size on cartilage tissue regeneration was compared by using four types of collagen porous scaffolds with different pore sizes. The collagen porous scaffolds were prepared by using pre-prepared ice particulates that had diameters of 150–250, 250–355, 355–425 and 425–500 μm. All the scaffolds had spherical large pores with good interconnectivity and high porosity that facilitated cell seeding and spatial cell distribution. Chondrocytes adhered to the walls of the spherical pores and showed a homogeneous distribution throughout the scaffolds. The in vivo implantation results indicated that the pore size did not exhibit any obvious effect on cell proliferation but exhibited different effects on cartilage regeneration. The collagen porous scaffolds prepared with ice particulates 150–250 μm in size best promoted the expression and production of type II collagen and aggrecan, increasing the formation and the mechanical properties of the cartilage.

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1. Introduction

Porous materials and scaffolds are important for many biomedical and biological applications [1–4]. In tissue engineering, porous materials can provide a temporary microenvironment to promote cell adhesion, proliferation and differentiation to guide the formation of new tissues and organs [5,6]. Biodegradable polymers, ceramics and their composites have been formed into porous structures for use in tissue engineering [7–9]. Controlling the pore structure, including pore size and interconnectivity, is key to creating ideal porous biomaterials and scaffolds [10–13]. Cell functions as well as new tissue regeneration rely heavily on the size of the pores [14–25]. The pore size should be in a range that facilitates cell penetration and migration during cell seeding, nutrient diffusion and removal of metabolic substances, and provides a three-dimensional microenvironment inducing cell assembly and differentiation [23]. Many reports show the effects of the pore size of a porous scaffold on tissue regeneration [14–25]. However, some of the reported results do not agree with each other. Some results indicate that chondrocyte phenotype and biosynthetic activity is improved in collagen matrices containing smaller pores [16,19], while some conclude that the larger pores promote the production of cartilaginous matrix proteins and cartilage regeneration [17,21]. The contradictory results may be due to the complexity of factors in three-dimensional culture, which may affect cell penetration, distribution and nutrient diffusion [12,14,16–25]. As well as pore size, pore interconnectivity can also affect the smooth delivery of cells into the pores, nutrient diffusion, exchange and removal of metabolic molecules during cell culture [11,22,26,27]. Therefore, to compare the effects of the pore size, interconnectivity should also be considered to ensure homogeneity of cell distribution throughout the scaffolds [27,28].

Many methods have so far been developed to control the pore structure characteristics of scaffolds, such as porogen leaching, freeze-drying, gas foaming and rapid prototyping [29–33]. Among these methods, porogen leaching offers many advantages for the easy manipulation and control of pore size and porosity. Although the porogen materials used in this method can leave replica pores after leaching, they cannot initiate the formation of interconnected pores [34]. As a result, cells cannot enter the isolated pores and the void space cannot be filled with new tissue, so those pores remain in the newly regenerated tissues as defects [35]. To improve pore interconnectivity, porogen materials are bonded before mixing them with polymer matrix. However, the bonded porogen materials require organic solvents for leaching and the residual solvents are toxic to cells. Such problems should be avoided when engineering tissues. To solve these problems, we have developed a method that uses ice particulates as a porogen material to prepare collagen scaffolds with precisely controlled pore structure [36]. Ice particulates were pre-prepared and mixed with collagen aqueous solution. The prepared ice particulates were embedded in the col-

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lagen aqueous solution. During the freezing process, the ice particulates functioned as nuclei to initiate the formation of new ice crystals in the surrounding aqueous solution. The newly formed ice crystals bonded the pre-prepared ice particulates and became interconnecting pores after freeze-drying. Therefore, this method did not use organic solvent and the porogen materials could be simply removed by freeze-drying. Using this method it was possible to create large pores with good interconnectivity in collagen porous scaffolds that could be used for comparison of pore size on tissue regeneration.

Collagen was chosen as a base material because it is a natural polymer and highly abundant in various tissues including skin, blood vessels, tendon, cartilage and bone. Collagen-based porous scaffolds have shown favorable results with regard to chondrocyte adherence and their ability to maintain a differentiated chondrocyte phenotype [37]. In this study, collagen porous scaffolds with four different ranges of pore sizes were prepared by using pre-prepared ice particulates. The four types of collagen porous scaffolds were used for the in vitro culture and in vivo implantation of articular chondrocytes to compare the effect of the pore size on cartilaginous matrix production and cartilage regeneration.

2. Materials and methods

2.1. Scaffold preparation

Collagen porous scaffolds were made by using pre-prepared ice particulates as a porogen material. Initially, an aqueous collagen solution as well as ice particulates were made. The 2% (w/v) aqueous collagen solution was prepared by dissolving freeze-dried porcine type I collagen (Nitta Gelatin, Osaka, Japan) in a mixture of ethanol and acetic acid (10:90 v/v, pH 3.0) at 4 °C. The ethanol/acetic acid mixture was used to reduce the freezing temperature of the aqueous collagen solution. The prepared collagen solution did not freeze at −4 °C, guaranteeing homogeneous mixing with the ice particulates. Three batches of collagen aqueous solution were prepared and stored in a refrigerator (at 4 °C) until use. The ice particulates were prepared by spraying Milli-Q water into liquid nitrogen using a sprayer. The ice particulates were sieved by sieves with mesh pores of 150, 250, 355, 425 and 500 μm to obtain ice particulates having diameters of 150–250, 250–355, 355–425 and 425–500 μm. The sieving process occurred at −15 °C in a low-temperature chamber (Espec, Osaka, Japan). The sieved ice particulates were stored in closed glass bottles in a −80 °C freezer until use. Then, each batch of the 2% (w/v) aqueous collagen solution was mixed with the sieved ice particulates. Before mixing, the collagen solution and the ice particulates were moved to a −4 °C low-temperature chamber for 6 h to balance their temperatures. The four sets of ice particulates, each with different diameters, were separately added to four batches of pre-cooled collagen aqueous solution in a 50:50 (v/w) ratio. The components were mixed thoroughly with a steel spoon. Each of the four types of mixtures of collagen solution and ice particulates was poured into a silicone frame that was then placed on a PFA film-wrapped copper plate, and the mixture surface was flattened with a steel spatula. The constructs were then frozen at −80 °C for 6 h, and freeze-dried for 3 days in a Wizard 2.0 freeze-dryer (VirTis, Gardiner, NY). The freeze-dried constructs were cross-linked with glutaraldehyde vapor by placing the constructs in a closed box with 20 ml of a 25% aqueous glutaraldehyde solution at 37 °C. After cross-linking, the constructs were washed with Milli-Q water three times and immersed in 0.1 M aqueous glycine solution for 24 h to block the unreacted aldehyde groups. After the glycine treatment, the constructs were washed with Milli-Q water six more times. The collagen porous scaffolds were freeze-dried again after washing for the subsequent experiments. Four collagen porous scaffolds of each pore size were prepared. A control collagen porous scaffold was prepared by the same procedure with a 2% (w/v) concentration of collagen solution without the use of ice particulates. The control collagen porous scaffold was only used for scanning electron microscopy observation to compare the different pore structures of scaffolds prepared with or without ice particulates.

2.2. Scaffold characterization

The microstructures of the collagen porous scaffolds were observed using a JSM-5610 scanning electron microscope (JEOL, Tokyo, Japan). The cross-sections of the collagen porous scaffolds were coated with platinum by an ECS-101 sputter coater (Elionix, Tokyo, Japan) before observation. The mean pore size and pore density of the collagen porous scaffolds was measured from their scanning electron microscopy (SEM) images by a MetaVue Image System (Universal Imaging Corp., Buckinghamshire, UK). Six images were taken of each scaffold and used for the mean pore size calculation.

The mechanical properties of the collagen porous scaffolds were measured by a static compression mechanical test machine (TMI UTM-10T; Toyo Baldwin Co., Ltd., Tokyo, Japan). Before testing, the samples were punched into cylindrical samples (8 mm diameter × 4 mm height) with a biopsy punch, and wetted in PBS for 1 h. Each test sample was compressed at a rate of 2.0 mm min^{-1} at room temperature. Load–deformation curves were obtained from a chart record. The compressive modulus was calculated from the curves and the sample dimensions. The average values were calculated from four samples ($n = 4$).

The porosity of collagen porous scaffolds was measured according to Archimedes' principle ($n = 3$) [38]. The porosity was calculated according to the following formula: porosity = ((W2 – W1)/(W2 – W3)) × 100%, where W1 is the dry weight of the scaffold, W2 is the wet weight of scaffold (including PBS solution), and W3 is the weight of the scaffold in PBS solution (subtracting buoyancy from W1).

2.3. In vitro cell culture

For cell culture use, the collagen porous scaffolds were punched into cylindrical samples (8 mm diameter × 4 mm height). The samples were sterilized with 70% ethanol, washed three times with Milli-Q water and conditioned with Dulbecco's Modified Eagle Medium (DMEM) at 37 °C for 30 min. Bovine articular chondrocytes were cultured in the scaffolds. The chondrocytes were isolated from articular cartilage from the knees of a 9 week old female calf, which were obtained from a local slaughterhouse and digested with an aqueous solution of 0.2% w/v collagenase type II (Worthington Biochemical, Lakewood, NJ). The isolated primary chondrocytes were cultured in 75 cm^2 tissue culture flasks in DMEM with an atmosphere of 5% CO_2 at 37 °C. The DMEM was supplemented with 10% fetal bovine serum, 4500 mg l^{-1} glucose, 4 mM glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 μg ml^{-1} ascorbic acid. The cell culture medium was refreshed every 3 days. The chondrocytes were harvested by treatment with a trypsin/EDTA solution when the cells reached a confluence of 80%. The harvested chondrocytes (P1 chondrocytes) were resuspended in DMEM to prepare a cell suspension solution of 2.5×10^7 cells ml^{-1} for cell seeding. The P1 chondrocytes were seeded into the scaffolds twice by adding 200 μl of the cell suspension solution (1.0×10^7 cells per scaffold) to each of the cylindrical sides of the scaffolds. The second seeding was performed 3 h after the first to allow for cell adhesion. Cell seeding efficiency was analyzed at 6 h after cell seeding on both sides (before adding the medium). The cells on the surface of culture plates were

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