Original Articles

Construction of tissue-engineered cartilage with different biological scaffolds in vitro

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Abbreviations: bone matrix gelatin (BMG), haematoxylin and eosin (H&E), toluidine blue (TB), extracellular matrix (ECM)

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This study aims to find out which polymeric scaffolds were more appropriate for the construction of tissue engineering cartilage. Rat costal cartilage chondrocytes were cultured in vitro. The first generation of chondrocytes was used as seed cells and inoculated in chitosan/gelatin and bone matrix gelatin (BMG)/fibrin glue scaffolds. Haematoxylin and eosin staining, toluidine blue staining, safranin O, immunological detection, and scanning electron microscopy (SEM) observations were implemented. After 2 weeks, all stains of BMG/fibrin glue showed that chondrocytes were uniformly distributed on the surface and inside of the material, and collagen and type II collagen staining was positive. There were slightly more cells on chitosan/gelatin than on BMG/fibrin glue. However, the number of internal cells was small with non-uniform distribution and the staining result was not as apparent as that of BMG/fibrin glue. As the culture time lengthened, results displayed a large quantity of expression of specific collagen and typed II collagen of chondrocytes, with chitosan/gelatin showing obvious advantages over BMG/fibrin glue material. The chitosan/gelatin contributed to maintaining chondrocyte phenotype and secretion of the extracellular matrix. Tissue engineering cartilage was successfully constructed in vitro. Moreover, such tissue growth, proliferation and matrix secretion of chondrocytes on chitosan/gelatin were superior to those on BMG/fibrin glue scaffold.

Key Words: chondrocytes; tissue engineering; chitosan/gelatin; BMG/fibrin glue

INTRODUCTION

Cartilage regeneration is essential to repair cartilaginous defects in orthopaedic or plastic surgeries. The limited regenerative capacity of cartilage tissue makes it challenging to treat severe cartilaginous lesions. Current treatment options such as osteochondral transplantation and autologous chondrocyte implantation have potential risks and limitations, including inadequate availability of donor tissues, donor site morbidity and poor attachment of the graft to the surrounding chondral surface. Recently, tissue engineering has been established as a promising approach to repair damaged cartilage, which involves a combination of cells, scaffolds and bioactive factors to fabricate functional new tissues.¹⁻³

An important aspect of tissue engineering is the design of polymeric scaffolds that have good mechanical and biological

properties.⁴ Scaffolds are capable of facilitating cell attachment, proliferation and differentiation. A variety of scaffolds, either

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natural or synthetic, have been developed for cartilage tissue engineering.⁵⁻⁷

Cartilage tissue engineering mainly cultures and amplifies chondrocytes in vitro and plants them on scaffolds at high density with favourable biocompatibility and degradability. Tissue engineering cartilage is then formed under the effect of multiple regulating factors within a certain period; therefore, large quantities of chondrocytes in good growing states and proper scaffolds play critical roles.⁸⁻⁹

In the present study, we attempt to combine different materials to compensate for their respective defects and therefore construct improved tissue engineering cartilage. Bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin scaffolds were utilized and a comparison was made to determine scaffolds more appropriate for the construction of tissue engineering cartilage.

MATERIALS AND METHODS

Materials

Female Sprague-Dawley rats weighing approximately 120g were purchased from the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, China). Dulbecco's Modified Eagle Medium and fetal bovine serum were purchased from Invitrogen (St. Louis, MO, USA). Hyaluronidase, collagenase II, trypsin, and Alcian Blue 8GX (Sigma, St. Louis, MO, USA), mouse anti-rat type II collagen monoclonal antibody (Neomarker, California, USA), goat anti-rat aggrecan monoclonal antibody (Santa Cruz), DAB kit (Zhongshan, Beijing, China), RNA isolation kit (Feijie Company, Shanghai, China), First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania), chitosan/gelatin (Tianjin University of Traditional Chinese Medicine), BMG (Fourth Military Medical University) and fibrin glue (Bioseal Biotech Company, Guangzhou, China) were purchased from different sources.

Isolation and culture of chondrocytes

The isolation and culture of rat costal chondrocytes were performed as described previously.¹⁰ All procedures were approved by the Ethics Committee of Xi'an Jiaotong University (Xi'an, China). Briefly, rats were killed by cervical dislocation, ribs were removed

and the cartilage was collected. Tissue samples were digested with hyaluronidase and collagenase II and the isolated cells were cultured at 37°C under a humidified atmosphere of 5% CO₂. Cells were subcultured at a ratio of 1:1 after treatment with 0.25% trypsin/EDTA. In the present study, cells at passage 2 were utilized.

Formation of in vitro constructs

Chitosan/gelatin chondrocytes engineered constructs: The chitosan-gelatin scaffolds were pre-wet overnight with 100-μL culture medium in an incubator for gas exchange and then a 100-μL cell suspension was added to each scaffold. After the attachment of cells to the scaffolds, 900-μL fresh medium was added into each well, and the cell-scaffold constructs were maintained at 37°C in 5% CO₂ humidified atmosphere. Culture medium was changed every two days.

BMG/fibrin chondrocytes engineered constructs: Cells were seeded onto the BMG/fibrin scaffolds using the commercially available fibrin glue kit. Fibrinogen was diluted in phosphate buffer saline, while thrombin was diluted in calcium chloride to obtain working solutions according to the manufacturer's instructions. Chondrocytes were incorporated into the fibrinogen solution at a concentration of 1×10⁷ cells/mL, which generated final fibrin/BMG/cell constructs with approximately one million cells per scaffold. The BMG scaffolds were soaked in the chondrocyte-fibrin solution and polymerised by subsequently dropping the constructs into the thrombin-calcium chloride solution. Then, the constructs were cultured in Dulbecco's Modified Eagle Medium containing 20% fetal bovine serum.

Histological evaluation

Sections of engineered cartilage were de-paraffinised, rehydrated and stained with haematoxylin and eosin (H&E), toluidine blue (TB) or Safranin O.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using TRIzol reagent and cDNA was reverse transcribed from total RNA using the First Strand cDNA Synthesis Kit. PCR amplification was performed using the specific primers summarized in Table 1. PCR products were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide. The bands were quantified by densitometry.¹¹

Table 1. PCR primers.

Gene	Primer sequences	Product size (bp)
Aggrecan	Sense 5'-GCTACACAGGTGAAGACTTTGTAGACATCC-3'	478
	Antisense 5'-GCTGTGCCTCCTCAAATGTCAGAGAGTATCT-3'	
Collagen II	Sense 5'-TGGTGCTGCTGACGCTGCTCATCGCCACGGTCCTA-3'	339
	Antisense 5'-GCCTTCTGATCAAATCCTCCAGCCATCTGGGCCCGC-3'	
β-actin	Sense 5'-GAGGGAAATCGTGCGTGAC-3'	353
	Antisense 5'-TAGGAGCCAGGGCAGTAATCT-3'	

Scanning electron microscopy (SEM)

SEM was used to observe the structure of constructs without or with chondrocytes. The constructs were fixed for 24 h in 2.5% glutaraldehyde in phosphate buffer saline, washed in 0.164 M potassium (pH 7.4) and dehydrated in a graded series of ethanol (30-100%). Samples were critical-point dried (Balzers Union Critical Point Dryer, BU-11120 Balzers, Liechtenstein) and sputter coated with gold (10 nm thickness) using a Balzers Union

sputtering device (BU-07120, Balzers, Liechtenstein). Each sample was then visualised with a Philips XL 30-FEG scanning electron microscope (Philips, Eindhoven, The Netherlands).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). All experiments were performed in duplicate and samples in all experimental groups were analyzed in triplicate.

Data are presented as mean±standard deviation (SD). Results were analyzed using Student's t-test and the differences were considered significant when $P<0.05$.

RESULTS

Histological observation

After two-week culture, chondrocytes were uniformly distributed on the surface and inside of the BMG/fibrin glue. The number of cells extended on the surface of the chitosan/gelatin was slightly greater than that on the BMG/fibrin glue; however, the number of cells inside the material was less than that on the surface of the material. As the culture time lengthened, quantities of cells on the surface and inside the chitosan/gelatin gradually increased (Figure 1). At 8 week, a large number of laminated chondrocytes existed on the surface of the chitosan/gelatin and all staining results showed a large number of secretions of collagen and type II collagen. At the same time, the BMG/fibrin glue was in the degrading phase, during which interruption of material continuity occurred and chondrocyte and secretion quantities of the extracellular matrix (ECM) were obviously smaller than those in the chitosan/gelatin group (Figure 2).

Figure 1. Staining results of material cell compounds during the 2nd week (H&E staining, toluidine blue staining and safranine O staining).

Figure 2. Staining results of material-cell compounds during the 8th week (TB staining and Safranine O staining).

SEM observation

SEM showed the structure of two materials and both were suitable for tissue engineering. In the 2nd weeks, chondrocytes adhered on the surface of the BMG/fibrin glue scaffold and cells existed independently. A small number of chondrocytes adhered on the chitosan/gelatin surface, cells had a typical spindle-shaped morphology, contact between cells was compact and there was an existing form of ECM between cells (Figure 3).

Figure 3. SEM graph of two materials and with cells at 2nd week

RT-PCR

RT-PCR showed that after 8 weeks, aggrecan and type II collagen expression quantities by cells on chitosan/gelatin were higher than those on BMG/fibrin glue, indicating that chitosan/gelatin maintained the chondrocyte phenotype, contributed to the secretion of ECM of chondrocytes and formed tissue engineering cartilage which was better than BMG/fibrin glue (Figure 4).

Figure 4. Aggrecan and type II collagen expression quantities of chondrocytes from different materials during the 8th week

DISCUSSION

After treatments including decalcification, degreasing, deproteinisation 95% of non-collagen materials except for the basic metabolic panel and lipid components with retardative effects are removed from the BMG and antigenicity is reduced.¹²⁻¹⁴ Meanwhile, a small quantity of basic metabolic panel and other internal growth factors facilitating chondrogenesis are still reserved in the BMG.¹⁵ However, pure BMG does not have strong adsorbability to chondrocytes; therefore, chondrocytes are poorly adhered on BMG easily but will be lost, and then cartilage tissues will not be easily formed. Fibrin glue also called fibrin sealant, provides tissue engineering new chondrogenesis with an available polymer scaffold by virtue of its strong plasticity and favourable biocompatibility.¹⁶ Hommiga et al. reported that rabbit articular chondrocytes could proliferate, synthesise matrix and maintain its phenotype in human fibrin glue. However, fibrin glue is revealed to degrade quickly.¹⁷⁻¹⁸ This deficiency prompts us to combine BMG and fibrin glue in the current study. Benefited from this combination, the two materials could compensate for their respective defects and conform better to the requirements for tissue engineering materials.

As a mammal-source polysaccharide, chitosan is similar to the glycosaminoglycan molecular structure of ECM with favourable

biocompatibility.¹⁹⁻²⁰ With the positive charge, chitosan results in nonspecific adsorption between the material surface and cytomembrane therefore contribute to cell adhesion on the material surface.¹² The degradation products showed no toxic or side effects on the human body.²¹⁻²² However, due to poor hydrophilia and low degradation velocity of pure chitosan material, it is challenging to meet the requirements of tissue engineering. Many scholars attempted to combine chitosan material with various materials and obtained favorable results.²³⁻²⁵ As chitosan has poor hydrophilia while gelatin can promote cell adhesion and growth, combining chitosan and gelatin was considered in the present study to form an ECM structure contributing to cell growth, which can facilitate cell adhesion and proliferation on the surface of the material.²⁶⁻²⁷

After tissue culture for eight weeks, it could be seen that collagen and type II collagen were generated on the surfaces and inside in both materials, which indicates successful construction of tissue engineering cartilage in vitro. With the culture time lengthened, the contents of collagen and type II collagen gradually increased, with these increases more obvious in chitosan/gelatin material than in BMG/fibrin glue material. All the histomorphological results showed that the positive staining in the chitosan/gelatin material was stronger than in the BMG/fibrin glue material group. After in vitro culture for two weeks, a small number of chondrocytes adhered on the surfaces of both materials and cells were seen to be uniformly distributed after conventional staining. Chondrocytes were hardly seen inside the chitosan/gelatin material; however, a small number of chondrocytes with uniform circular or oval distribution was seen inside the BMG/fibrin glue material. The chondrocytes were first evenly mixed in the bulk glue solution and then reacted with thrombin adsorbed on the BMG/fibrin glue scaffold to form fibrin glue, and chondrocytes were mainly embedded in fibrin glue and uniformly distributed on the BMG/fibrin glue scaffold surface and inside the grid mesh. Then, the chondrocytes were effectively adsorbed and fixed inside the scaffold so that they would not be easily lost. The drop-adding method was applied to add dropwise chondrocytes onto the chitosan/gelatin material. The permeation capacity into the material was weak and chondrocytes were mainly distributed on the surface of the material. Meanwhile, as oxygen and nutrition supply is adequate on the material surface, cells would grow easily on the chitosan/gelatin material surface, which was consistent with previous research.²⁸

As the culture time lengthened, the numbers of cells on both scaffold materials significantly increased and chondrocytes gradually grew inside materials with more uniform distribution. Revealed by SEM, more cells were observed on the BMG/fibrin glue surface than that on the chitosan/gelatin material. In our opinion, chondrocytes adhered on the surface of the chitosan/gelatin material meaning they would fall off more easily during the manufacturing process of SEM samples. However, the cell stretching status in the BMG/fibrin glue material was not as good as the chitosan/gelatin material, possibly because the degradation velocity of fibrin glue material is too high, which resulted in loss of a large number of chondrocytes, reduction of cell quantity, reduction of ECM secretion and significant reduction of connection and mutual influence between cells.

Chondrocytes could grow and proliferate well on both BMG/fibrin glue and chitosan/gelatin materials, which indicated that both these materials are appropriate scaffold materials for tissue engineering cartilage. However, in vitro research demon-

strated that chitosan/gelatin material was superior to BMG/fibrin glue material, and its concrete mechanism requires further in vivo experiments.

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