



Evaluation of bone matrix gelatin/fibrin glue and chitosan/gelatin composite scaffolds for cartilage tissue engineering

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ABSTRACT. This study was designed to evaluate bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin composite scaffolds for cartilage tissue engineering. Chondrocytes were isolated from costal cartilage of Sprague-Dawley rats and seeded on BMG/fibrin glue or chitosan/gelatin composite scaffolds. After different *in vitro* culture durations, the scaffolds were subjected to hematoxylin and eosin, Masson's trichrome, and toluidine blue staining, anti-collagen II and anti-aggrecan immunohistochemistry, and scanning electronic microscopy (SEM) analysis. After 2 weeks of culture, chondrocytes were distributed evenly on the surfaces of both scaffolds. Cell numbers and the presence of extracellular matrix

components were markedly increased after 8 weeks of culture, and to a greater extent on the chitosan/gelatin scaffold. The BMG/fibrin glue scaffold showed signs of degradation after 8 weeks. Immunofluorescence analysis confirmed higher levels of collagen II and aggrecan using the chitosan/gelatin scaffold. SEM revealed that the majority of cells on the surface of the BMG/fibrin glue scaffold demonstrated a round morphology, while those in the chitosan/gelatin group had a spindle-like shape, with pseudopodia. Chitosan/gelatin scaffolds appear to be superior to BMG/fibrin glue constructs in supporting chondrocyte attachment, proliferation, and biosynthesis of cartilaginous matrix components.

Key words: Chondrocyte; Tissue engineering; Composite scaffold; Bone matrix; Cartilage; Chitosan

INTRODUCTION

Cartilage injury is a common health problem worldwide and is difficult to treat owing to the scarcity of therapeutic options. Tissue engineering is emerging as a promising approach for repairing cartilage defects (Makris et al., 2015). A key component of tissue engineering is the use of appropriate biocompatible scaffolds (He et al., 2014), which provide physical support for the adhesion, migration, and differentiation of seeded cells. Numerous scaffolds have been developed and tested in cartilage tissue engineering (Liu et al., 2013; He et al., 2014; Makris et al., 2015). However, no standard or ideal scaffold is currently available for tissue regeneration.

In the present study, we compared the effects of bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin composite scaffolds in supporting chondrocyte attachment, proliferation, and synthesis of cartilaginous matrix components.

MATERIAL AND METHODS

Chondrocyte isolation and culture

Chondrocytes were isolated from costal cartilage of 4-week-old Sprague-Dawley rats, according to the method described by Reinholt et al. (Wang et al., 2010b), which was approved by the Ethics Committee of Xi'an Jiaotong University and the experiments followed the appropriate animal care guidelines. Briefly, pieces of cartilage were minced and digested with collagenase and hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 4-6 h. The isolated cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C, and allowed to grow to confluence. Cells were detached with 0.25% trypsin/ethylenediaminetetraacetic acid (Invitrogen) and reseeded on 25-cm² dishes. At 90% confluence, cells were trypsinized, centrifuged, and resuspended in DMEM as passage 1 cells.

Scaffold preparation and cell seeding

For preparation of the BMG/fibrin glue composite scaffold, BMG (obtained from

the Fourth Military Medical University, Xi'an, China) was immersed in DMEM for 5 min, then placed in thrombin solution. After drying, the BMG scaffold was incubated at 37°C for 10 min with a mixture of fibrinogen (Guangzhou Bioseal Biotech. Co., Ltd., Guangzhou, China) and passage 1 chondrocytes in DMEM. The chitosan/gelatin composite scaffold was purchased from Tianjin University of Traditional Chinese Medicine (Tianjin, China) and also seeded with passage 1 chondrocytes. The composite scaffolds loaded with chondrocytes were transferred to 24-well plates and cultured in DMEM containing 20% FBS. Half of the culture medium was replaced daily for 8 weeks.

Scanning electronic microscopy (SEM)

The cell-scaffold samples were collected after 2 and 4 weeks of culture and fixed in 2.5% glutaraldehyde for 5 min at 37°C, before being dehydrated through an ethanol gradient. Scaffolds were cut, glued on steel stubs, and coated with gold-palladium. The attachment to and morphology of cells on the surface of scaffolds were assessed by SEM.

Histology and immunohistochemistry

The cell-scaffold samples were collected at different time points and fixed in 4% paraformaldehyde. The samples were dehydrated, embedded in paraffin, and cut into 4- μ m sections. These sections were then stained with hematoxylin and eosin, Masson's trichrome, or toluidine blue. For immunohistochemistry, sections were deparaffinized, rehydrated, and immunostained with mouse monoclonal anti-collagen II (Neomarker, Fremont, CA, USA; 1:200 dilution) or goat monoclonal anti-aggrecan (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilution) antibodies. Fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology) was used to bind the primary antibody. Sections were examined under a fluorescence microscope (Nikon, Tokyo, Japan).

Biochemical composition

Levels of amino sugars and hydroxyproline (Hypro), indicators of glycosaminoglycan (GAG) and collagen, respectively, were measured in 10 tissue-engineered chondral constructs after 4 weeks of culture. DNA content was analyzed using the Hoechst 33258 dye assay (Sigma-Aldrich) with a calf thymus DNA standard. The concentration of amino sugars, as a measure of GAG, was determined using a dimethylmethylene blue colorimetric assay (Sigma-Aldrich) with chondroitin sulfate C (Sigma-Aldrich) as a standard. For the Hypro assay, an aliquot of papain digest was hydrolyzed in 6 N HCl for 16 h at 110°C, and a dimethylaminobenzaldehyde/chloramine T colorimetric assay was used with hydroxy-L-proline (Sigma-Aldrich) as a standard. Standards were processed in duplicate and samples in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and $P < 0.05$ was considered statistically significant.

RESULTS

Histological findings

After 2 weeks of culture, chondrocytes were distributed evenly on the surfaces of the BMG/fibrin glue and chitosan/gelatin scaffolds (Figure 1). However, few cells were detected inside the latter (Figure 1). Importantly, total cell number in the chitosan/gelatin scaffold was markedly increased at 8 weeks, coupled with abundant generation of proteoglycan and collagen (Figure 2). Cell number and the concentration of extracellular matrix components were remarkably lower in the BMG/fibrin glue scaffold than in the chitosan/gelatin scaffold (Figure 2). In addition, the former showed signs of degradation after 8 weeks of culture.

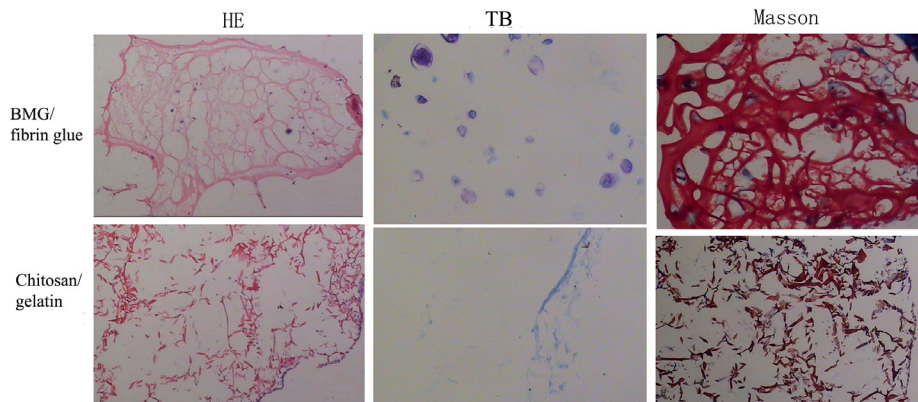


Figure 1. Hematoxylin and eosin (HE), Masson's trichrome, and toluidine blue staining (TB) analysis of the bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin scaffolds 2 weeks after being seeded with chondrocytes.

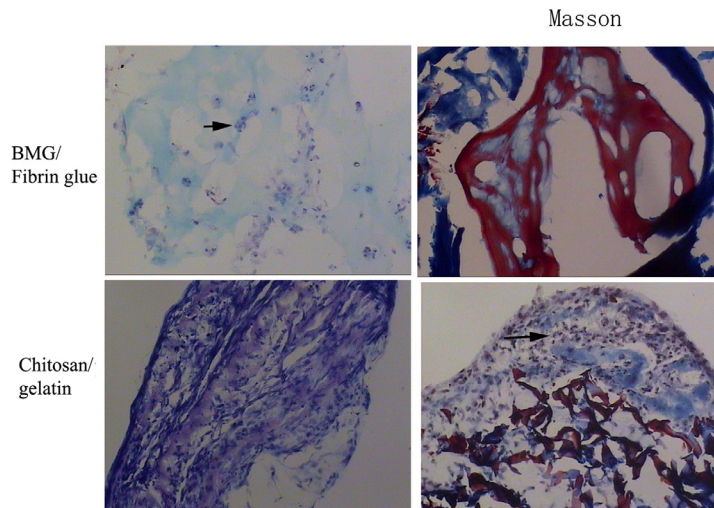


Figure 2. Masson's trichrome and toluidine blue staining analysis of the bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin scaffolds 8 weeks after being seeded with chondrocytes (arrows mean cells).

Immunohistological findings

Immunofluorescence analysis confirmed that levels of collagen II and aggrecan produced by chondrocytes were greater in the chitosan/gelatin scaffold relative to the BMG/fibrin glue scaffold after 8 weeks of culture (Figure 3).

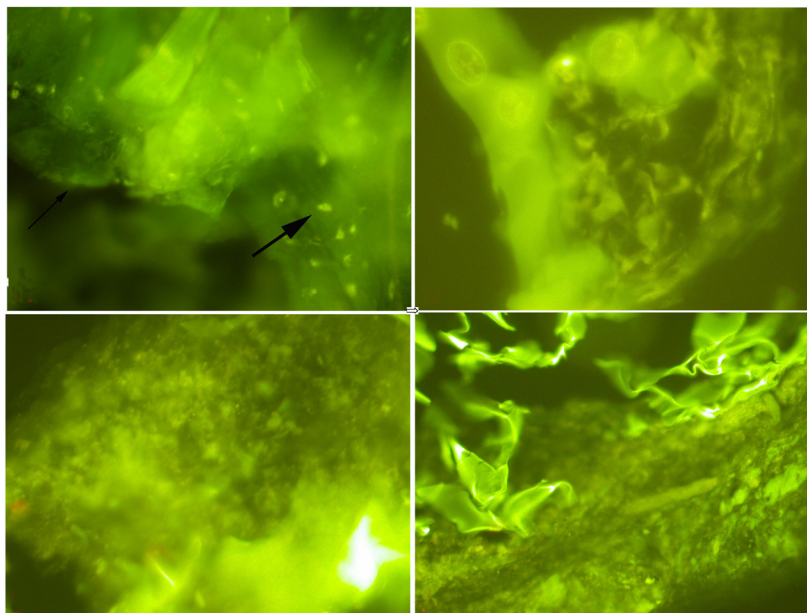


Figure 3. Immunohistochemistry analysis of the chondrocyte-containing bone matrix gelatin/fibrin glue and chitosan/gelatin scaffolds stained with anti-aggrecan and anti-collagen II antibodies (100X magnification). The thin arrow indicates the scaffold, while the thick arrow signifies a chondrocyte.

SEM analysis

At 2 weeks, the chondrocytes adhering to the BMG/fibrin glue scaffold showed pseudopodial extensions, while those attached to the chitosan/gelatin scaffold maintained a round morphology (Figure 4). At 4 weeks, greater numbers of cells were found on the surfaces of both composite scaffolds (Figure 4). Most of the cells on the surface of the BMG/fibrin glue scaffold showed a round morphology, while those of the chitosan/gelatin group exhibited a long spindle-like shape, with pseudopodia.

Biochemical composition analysis

After 4 weeks of culture, a significant increase in DNA-normalized GAG level was observed in the chitosan/gelatin group, compared to the BMG/fibrin glue constructs ($P < 0.05$). In addition, DNA-normalized Hypro measurements of the latter were only approximately 36.5% of those recorded in the chitosan/gelatin group, as shown in Figure 5 ($P < 0.05$).

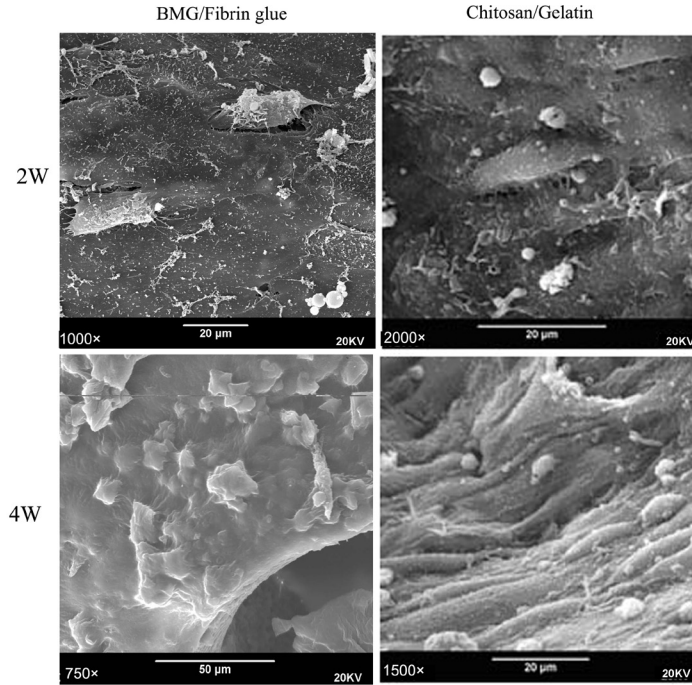


Figure 4. Scanning electron microscopy analysis of the chondrocyte-containing bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin scaffolds after 2 (2W) and 4 weeks (4W) of culture.

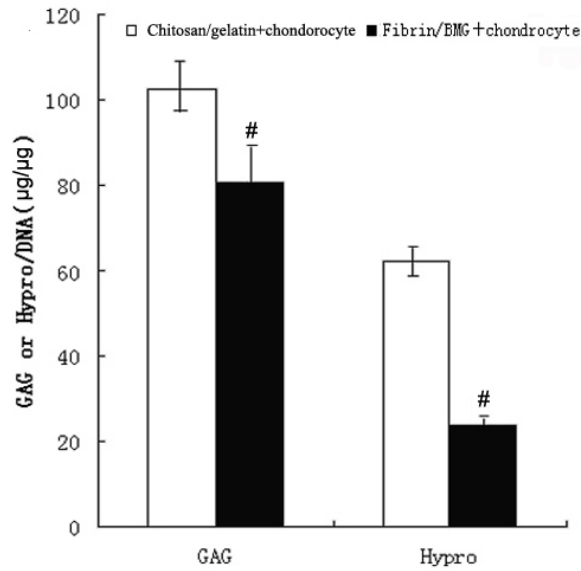


Figure 5. Chondrocyte-containing bone matrix gelatin (BMG)/fibrin glue and chitosan/gelatin constructs tested for proteoglycan and collagen composition after 4 weeks of culture, according to glycosaminoglycan (GAG) and hydroxyproline (Hypro) levels, respectively (# indicates $P < 0.05$).

DISCUSSION

BMG is processed to remove the majority of non-collagen proteins and lipids, but retains some amount of bone morphogenetic proteins and other chondrogenic factors (Hu et al., 1993). However, the attachment of chondrocytes to BMG scaffolds is challenging. Therefore, BMG is often used together with other biomaterials to form a composite scaffold (Zhuang et al., 2011). Fibrin glue demonstrates good biocompatibility and plasticity. Deponti et al. (2014) reported that collagenic-fibrin glue scaffolds are able to support chondrocyte survival and synthetic activity in static cultures. Moreover, Homminga et al. (1993) revealed that chondrocytes isolated from articular cartilage of young rabbits can proliferate well, maintain their morphology, and produce matrix in fibrin glue. However, fibrin glue is very readily biodegraded, thus limiting its application as a tissue engineering scaffold. In this study, to improve the biocompatibility of BMG, we used it to fabricate a composite scaffold with fibrin glue.

Chitosan is a natural polymer widely used in regenerative medicine, and has a GAG-like structure (Layek et al., 2015). Chitosan has a positively charged surface, which facilitates the attachment of cells with negatively charged groups (Wang et al., 2016). The main drawbacks of chitosan are its poor biodegradability and hydrophilicity. Many chitosan-based composite scaffolds have been developed and shown to have beneficial qualities for tissue engineering (Bini et al., 2005; Chen et al., 2007). Since gelatin is capable of promoting cell adhesion and growth, here, we produced a chitosan/gelatin composite scaffold, with a view to enhancing cell attachment and proliferation.

In this study, we found that after 8 weeks of culture, abundant collagen II and aggrecan were produced in the chitosan/gelatin and BMG/fibrin glue scaffolds, suggesting that both may be effective in cartilage tissue engineering. Compared to the BMG/fibrin glue scaffold, collagen II and aggrecan levels were more greatly elevated in the chitosan/gelatin group. In addition, histological analysis revealed that cell numbers and proteoglycan and collagen measurements were higher using chitosan/gelatin scaffolds than BMG/fibrin glue constructs. At 2 weeks, many cells were detected inside the latter, whereas few were observed within the former. This finding may be explained by the fact that the fibrinogen mixed with the chondrocytes prior to the experiment was converted to fibrin by thrombin, thus embedding chondrocytes in the composite scaffold. In contrast, in the chitosan/gelatin group, chondrocytes were dropped onto the scaffold, into which few cells subsequently migrated. The presence of sufficient oxygen and nutritional factors accounts for the predominant distribution of seeded cells on the surface of the scaffolds, consistent with a previous study (Wang et al., 2010a). After culturing, cells on the scaffold surfaces might proliferate and migrate, leading to their increased presence in the constructs' interiors. SEM imaging showed that surface cell number was higher for the BMG/fibrin glue scaffold than the chitosan/gelatin scaffold. However, most cells on the former's surface were round in shape. This may be the result of rapid degradation of the scaffold protein components, decreasing the generation of extracellular matrix and impairing cell-cell contact (Plaas et al., 1997).

In conclusion, the chitosan/gelatin scaffold appears to be superior to the BMG/fibrin glue construct in supporting chondrocyte attachment, proliferation, and biosynthesis of cartilaginous matrix components. The underlying mechanisms remain to be further clarified.

Conflicts of interest

The authors declare no conflict of interest.

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