Study on cartilage nano scaffolds with acellular matrix and collagen II

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Abstract: Objective: To explore the feasibility of preparing tissue-engineered nano scaffolds using acellular articular cartilage extracellular matrix (CAEM)and Type II collagen (COL II) via electrospinning technology. Methods: Rabbit costal cartilage was decellularized, defatted, and enzymatically digested, followed by drying to obtain CAEM.CAEM and COL II were mixed at a mass ratio of 1:2 and processed into tissue-engineered nano scaffolds using electrospinning technology. The physicochemical properties of the scaffolds were evaluated by measuring their water absorption rate and degradation rate. The cytotoxicity and cell adhesion properties of the scaffolds were assessed using the CCK-8 assay. Results: The fiber diameter of the CAEM-COL II nano scaffolds was (627±165.4) nm, with a water absorption rate of (623.0±27.4) %and a degradation rate of (45.6±5.8) %after 35 days. The CCK-8 assay results indicated that the CAEM-COL II composite scaffolds exhibited good adhesion properties for chondrocytes and favorable biological performance. Conclusion: The CAEM-COL II nano scaffolds provide an excellent microenvironment for the growth and proliferation of chondrocytes and have potential application value in tissue-engineered cartilage reconstruction.

Keywords: Tissue-engineered nano scaffolds, Type II collagen, Acellular articular cartilage extracellular matrix, Electrospinning

1. Introduction

Articular cartilage defects caused by trauma and degenerative joint diseases are common clinical conditions, often leading to joint pain, deformity, and functional impairment, which significantly reduce patients' quality of life and may even result in loss of work capacity. Current clinical treatments primarily rely on non-surgical therapies and joint replacement surgeries. Non-surgical treatments can significantly relieve pain and improve joint function to some extent in some patients. However, many patients who undergo non-surgical treatments achieve poor outcomes and are forced to opt for surgical interventions, which generally yield good short-term results. However, the risks and complications associated with surgery can leave a small number of patients in an even more painful situation. In the early 21st century, some researchers treated cartilage defects using methods such as chondrocyte injection or chondrocyte membrane transplantation, achieving certain therapeutic effects in some patients. However, unresolved issues, such as how to concentrate cells at the cartilage defect site and how to firmly fix the cell membrane, have prevented these methods from being widely accepted and applied by clinicians. Nevertheless, this has opened a new chapter in the treatment of cartilage defects using tissue-engineered cartilage. This study focuses on exploring the preparation of tissue-engineered cartilage nano scaffolds using electrospinning technology with cartilage acellular extracellular matrix and Type II collagen (Cartilage acellular extracellular matrix-Collagen II, CAEM-COL II). Through the evaluation of the scaffolds 'physicochemical properties and cytotoxicity, the results demonstrate that these scaffolds have potential application value in the construction of cartilage tissue engineering.

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2. Materials and methods

2.1. Experimental animals, reagents,and instruments

Ten adult Japanese large-ear rabbits and two 1-week-old Japanese large-ear rabbits were provided by the Experimental Animal Center of North Sichuan Medical College. All animal experimental protocols were approved by the Animal Experiment Management Committee of North Sichuan Medical College.

Reagents used included trifluoroethanol (Chengdu Best Reagent Co., Ltd., China), Triton X-100(Beijing Chemical Reagent Company, China), pepsin (Shanghai Lan ji Technology Development Co., Ltd., China), ethylenediaminetetraacetic acid (EDTA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), DNase, RNase, guanidine hydrochloride (Sigma Company, USA), Tris-HCl.

Instruments used included a high-voltage power supply (Tianjin Dong wen Company, China), a constant-flow injector (Baoding Lange Company, China), a scanning electron microscope (Hitachi Company, Japan), a CO_2 incubator (Thermo Company, USA), a vacuum drying oven (Shanghai Yiheng Technology Co., Ltd., China), an FD-1 freeze-dryer (Beijing Boyikang), and a Biofuge Primo R low-temperature centrifuge (Thermo Scientific, USA).

2.2. Methods

2.2.1. Preparation of acellular matrix

After removing the surface soft tissues from the costal cartilage of adult rabbits, the cartilage was cut into pieces approximately 0.5 cm in size. Subsequently, five times the volume of 0.25%trypsin was added to the cartilage pieces, which were then digested at 37°C for 35 minutes. The mixture was centrifuged at 600g for 5 minutes, and the supernatant was discarded. The cartilage pieces were washed three times with PBS. Next, a Tris-HCl buffer solution (10 mmol/L, pH 7.5) containing 1%Triton X-100 and 0.35 mg/L PMSF was added at twice the volume of the cartilage. The mixture was continuously agitated at 4°C for 24 hours to remove cells. After centrifugation at 600g for 5 minutes, the cartilage pieces were washed three times with PBS. Then, a solution containing 50 U/mL DNase and 1 U/mL RNase at one times the volume of the cartilage was added, and the mixture was digested at 37°C overnight. After centrifugation at 400g for 5 minutes, the cartilage pieces were washed three times with dd H_2O. The resulting acellular cartilage matrix fragments were collected. The cartilage fragments were placed into a 50-mL centrifuge tube, and an adequate amount of liquid nitrogen was added to thoroughly freeze the fragments. The frozen cartilage fragments were immediately ground into a powder using a grinder. An appropriate amount of methanol-chloroform solution (volume ratio of 2:1) was added to the powdered cartilage for defatting over 24 hours. After centrifugation at 400g for 5 minutes, the cartilage was washed three times with PBS to obtain the insoluble cartilage extracellular matrix(ECM)precipitate. At least five times the volume of 1%pepsin(w/v) was added to the insoluble ECM precipitate, and the mixture was digested at 20°C for 36 hours. After centrifugation at 400g for 5 minutes to remove the sediment, the resulting supernatant was lyophilized(freeze-dried) at low temperature for 84 hours to obtain the soluble acellular cartilage matrix. Extraction of COL II was performed according to the previously reported literature [1].

2.2.2. Preparation of nano scaffolds

The acellular cartilage matrix and COL II were mixed at a ratio of 1:2(w: w) and dissolved in a solvent mixture of trifluoroethanol and dd H_2O (v: v=1:1). After magnetic stirring at room temperature for 72 hours, a 10%(w/v)electrospinning solution was prepared. Based on preliminary experiments, the electrospinning conditions were set as follows: the room temperature was controlled at 25°C–30°C with a humidity of 40%, the spinning voltage was set at 15 kV, the feed rate was 6 ml/h, and the collection distance was 10 cm. Electrospinning was performed using a copper plate as the collector. The copper plate with the collected nanofibers was placed in a vacuum drying chamber at room temperature with a vacuum degree of-0.1 MPa and dried for 3 days. The fiber membranes were then collected for further characterization and parameter analysis.

2.2.3. Scanning electron microscopy(SEM)observation of scaffolds and fiber diameter measurement

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Following the method described in the literature [2]: The surface of the material was coated with gold using an ion sputtering instrument and then observed under a scanning electron microscope (SEM)to examine its surface morphology, fiber diameter, and pore structure. For each material, SEM images at 10,000×magnification were selected. Using the Image-Pro Plus 6.0 professional image analysis software, the diameters of 20 randomly selected fibers in each image were measured. The average fiber diameter and standard deviation were then calculated using SPSS 18 software.

2.2.4. Measurement of scaffold water absorption rate

Several samples were weighed to obtain W_1. The samples were then immersed in double-distilled water. At predetermined time points, the samples were removed and weighed again to obtain W_2. The water absorption rate of the material was calculated using the formula: Water Absorption Rate=$(W_2-W_1)/W_1$.

2.2.5. CCK8 assay for cytotoxicity and cell adhesion evaluation

The scaffold materials were cut to appropriate sizes, sterilized with ethylene oxide, and placed into a 96-well culture plate. Chondrocytes were seeded at a density of 5×10^4 cells/m L. At various time points(3h,6h,9h,12h,and 24h),the old culture medium was aspirated, and the wells were washed three times with PBS.Subsequently,200μL of serum-free and antibiotic-free culture medium and 20μL of CCK8 solution were added to each well and incubated for 1–4 hours. The absorbance at 450 nm was measured, with three replicates for each time point. As a control, chondrocytes of the same density were seeded into a cell culture plate and measured at corresponding time points for comparison.

2.2.6. Statistical analysis

All data are presented as mean± standard deviation (x± s). Parametric data were analyzed using the t-test. Statistical analysis was performed using SPSS 18.0 software, with $P<0.05$ considered statistically significant.

3. Results

3.1. Macroscopic observation of materials

The extracted cartilage extracellular matrix (Figure 1) and the macroscopic morphology of the nano scaffold (Figure 2) were observed. Light microscopy (Figure 3) and scanning electron microscopy (SEM)images (Figure 4) of the scaffold showed that the nanofibers had a relatively uniform diameter without significant bead formation. The pores between the nanofibers were interconnected, and the surface morphology was well-defined.

3.2. Physical property testing of the scaffold

The water absorption capacity of the material reached saturation at 4 hours, with an average water absorption rate of (623.0±27.4) % (Figure 5). Degradation of the material gradually stabilized after 14 days, with a degradation rate of (45.6±5.8) %at 35 days (Figure 6). The diameter of the nanofibers was measured to be(627±165.4)nm.

Figure 1: Cartilage Extracellular Matrix

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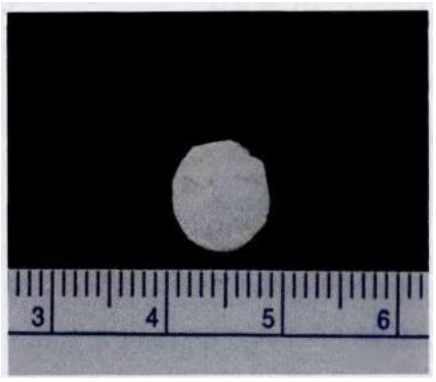

Figure 2: Macroscopic Image of the Material

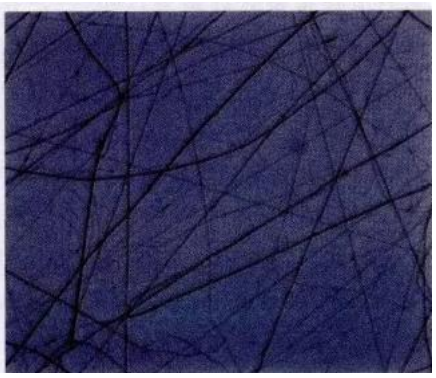

Figure 3: Light Microscopy Observation of Nanofibers (×400)

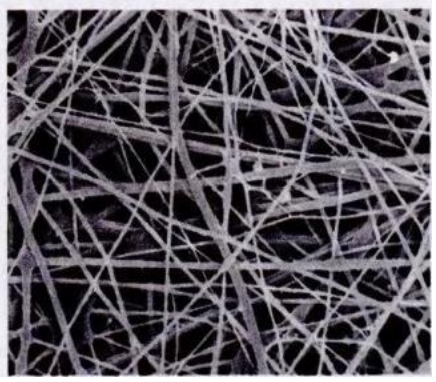

Figure 4: Scanning Electron Microscopy Observation (×5000)

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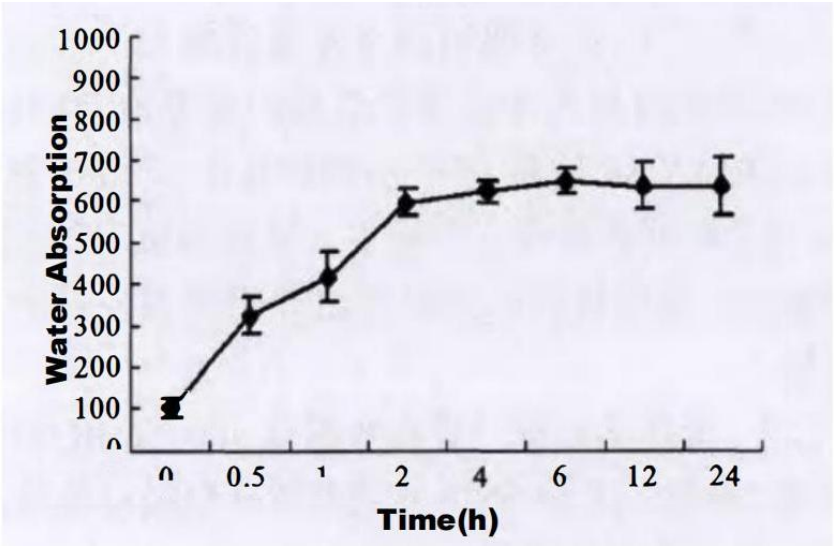

Figure 5: Water Absorption Rate Curve of the Material

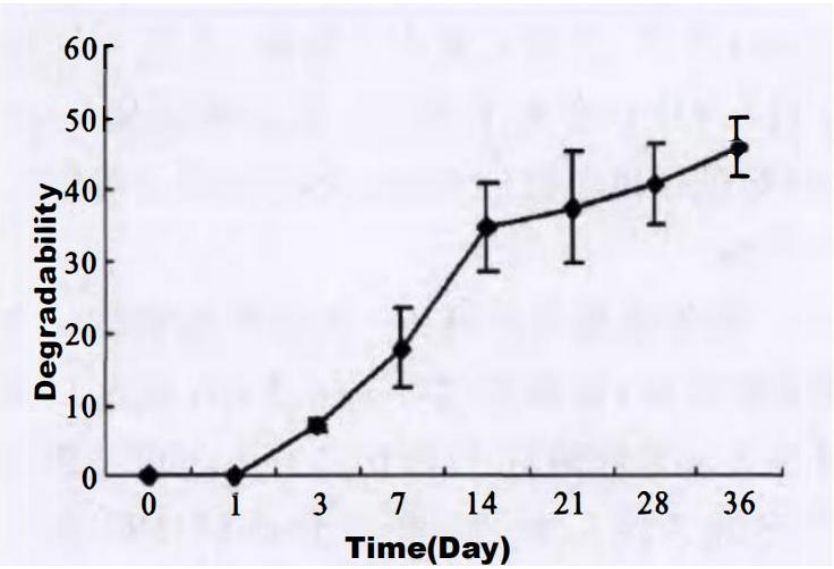

Figure 6: Degradation Curve of the Material

3.3. Cell adhesion test

No significant differences were observed between the experimental and control groups at 3h,6h,9h,12h, and 24h($P>0.05$).

The CCK8 assay results indicated that the acellular cartilage matrix-COL II nano scaffold exhibited good cell adhesion properties and favorable biological performance, as shown in Figure 7.

4. Discussion

4.1. ntroduction to tissue-engineered cartilage

Tissue-engineered cartilage is one of the most extensively studied and rapidly developing fields in current scientific research. The exploration of seed cells has been extensive; however, maintaining the phenotype of these cells effectively has long been a challenging issue for researchers, with no breakthrough progress achieved to date. Currently, cytokines are commonly used to preserve the phenotype of chondrocytes or induced chondrocyte-like cells. Nevertheless, as the duration of in vitro culture extends, the

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phenotypic characteristics of seed cells gradually diminish, and they tend to differentiate into "fibroblast-like" cells. This transformation poses significant difficulties in the successful construction of tissue-engineered cartilage. Consequently, improving the biological performance of tissue engineering scaffolds has increasingly garnered the attention of scientific researchers.

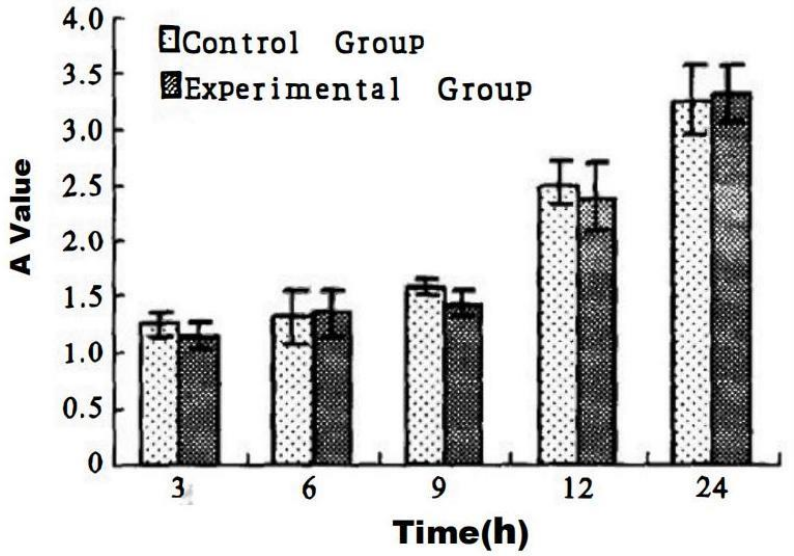

Figure 7: Evaluation of Cell Adhesion

4.2. Importance of scaffolds in tissue engineering

The physicochemical properties and biological functions of a scaffold are one of the keys to the success of tissue-engineered cartilage construction. Scaffolds not only provide a space for seed cells to grow, differentiate, and metabolize but also determine the basic shape and function of the reconstructed tissue or organ [1].

4.3. Types of scaffold materials

Currently, the materials commonly used for the preparation of tissue engineering scaffolds are mainly divided into three categories: natural materials, synthetic materials, and their composites. Each has its own advantages and disadvantages, and they are difficult to replace each other. Scaffolds constructed from synthetic materials have the advantage of good mechanical properties, providing a better surface morphology for tissue construction. However, synthetic materials generally have relatively poor biocompatibility, lack cell adhesion factors, and their degradation rate in vivo is not easy to control. Moreover, the degradation products may have certain cytotoxicity[3-5].However, this does not mean that synthetic materials cannot have a better application prospect. Pan et al. used a poly (lactic acid) (PLA)bilayer scaffold with different porosities combined with bone marrow stromal stem cells (BMSCs)to repair rabbit articular cartilage defects. After 12 weeks of observation, it was found that the PLA scaffolds with different porosities had better effects on cartilage defect repair than single-porosity scaffolds [6]. Hung et al. prepared a three-dimensional scaffold using polyurethane as the raw material and carrying growth factors, using low-temperature 3D printing technology. This scaffold combined with BMSCs showed good repair functions for rabbit knee joint defects[7].

Natural materials and their derivatives have good biocompatibility and degradability, support the adhesion and colonization of chondrocytes, and can better maintain the phenotype of chondrocytes, thus being favored by many researchers. Common methods for preparing scaffolds from natural cartilage extracellular matrix materials include decellularized cartilage blocks and scaffolds prepared from decellularized cartilage ECM by lyophilization. Sun et al. prepared a scaffold by decellularizing cartilage

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and then seeded BMSCs onto the scaffold for continuous in vitro culture. The results showed that BMSCs could grow on the surface of the scaffold[8].This indicates that the scaffold prepared by decellularization has sparse pores, making it difficult for cells to penetrate into the interior of the scaffold and concentrate a large number of seed cells within the scaffold. Therefore, it cannot serve as an ideal scaffold for cartilage tissue engineering. The lyophilization method generally involves grinding decellularized cartilage and then lyophilizing it at low temperatures to prepare a three-dimensional porous scaffold of a specific shape. In vitro experiments have shown that ECM scaffolds prepared by this method have good biological properties[9-11].

4.4. Role of type ii collagen in cartilage scaffolds

Type II collagen(COL II)is the primary solid component of the extracellular matrix of articular cartilage, accounting for 50%to 80%of the dry weight of articular cartilage(AC).It mainly constitutes the fibrous framework of AC and, together with the high-water-content proteoglycan(PG)chains, forms a network-like matrix[1].Numerous studies have confirmed that COL II promotes the adhesion, proliferation, and maintenance of the chondrocyte phenotype[12-15].In previous studies, scaffolds constructed using cartilage ECM particles as basic units were prone to brittleness, and the molecular signals of collagen, hyaluronic acid, and other components within the ECM particles could not be fully exposed.

4.5. Development of a new scaffold using electrospinning technology

In this study, the cartilage acellular matrix was subjected to defatting, decellularization, and antigen removal, followed by enzymatic digestion to convert it into a soluble matrix at the molecular level. Type II collagen was then added and thoroughly mixed to adjust the viscosity of the solution. Using electrospinning technology, a nanoscale tissue-engineered cartilage scaffold was prepared, achieving preliminary satisfactory results. After multiple comparative experiments, the optimal electrospinning conditions were found to be: a collagen-to-ECM mass ratio greater than 2:1 in the spinning solution, a spinning solution concentration of 10%, a temperature of 25°C, a humidity of 40%, a collector distance of 10 cm, and an electrospinning rate of 1 mL/h.

5. Conclusion

The tissue-engineered cartilage scaffold constructed from acellular cartilage matrix and COL II has a relatively uniform nanoscale diameter, homogeneous pore size, high water absorption rate, and good cell adhesion properties. This scaffold can effectively simulate the microenvironment for chondrocyte growth in terms of structure and composition, and it holds potential for application in cartilage tissue engineering.

6. References

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