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Conjugated icariin promotes tissue-engineered cartilage formation in hyaluronic acid/collagen hydrogel

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ABSTRACT

Bioactive factors were added into hydrogel scaffolds to overcome the shortcomings of their low cell affinity and poor bioactivity, as well as limited cell spreading ability for anchorage-dependent cells such as chondrocytes, MSCs, etc. But the direct addition of these bioactive factors may lead to high cost, short effective time and possible side effects etc. In this study, a flavonol glycosides named icariin was chemically conjugated to hyaluronic acid/collagen hydrogel (HA–Ica/Col). It was observed that the presence of HA–Ica macromolecules had no significant effect on mechanical and degradation property of the hydrogel. However, the fixed icariin in HA–Ica/Col hydrogel gradually released, effectively maintained the chondrocytes morphology and promoted the biosynthesis of cartilage matrix. The gene expressions of sox9, aggrecan, type II collagen (Col II) of the seeded chondrocytes were obviously increased. The productions of glycosaminoglycan (GAG) and Col II in HA–Ica/Col hydrogel were much more. The formation of new cartilage tissue in HA–Ica/Col hydrogel was obviously better than which in hydrogel without fixed icariin. These results suggested that integrating icariin into scaffold by chemical conjugation will increase the quality of formed cartilage, and may decrease the risks of excessive release, therefore is valuable in cartilage tissue engineering.

1. Introduction

Articular cartilage damage is the most common disease in clinical and its repair has been a challenge in orthopedic medicine because of the limited ability of self-repair of cartilage tissue [1,2]. Many methods were proposed for the repair of cartilage damage. Among them, cartilage tissue engineering is believed a promising technique because it eliminates the barriers of the limited sources and the donor site morbidity. In cartilage tissue engineering, active seed cells proliferate and differentiate in proper designed scaffold to form cartilage tissue to restore the damage. With or without the cooperation of growth factors, the scaffold support the seeded cells, offer suitable microenvironment and even promote and induce the formation of new cartilage tissue. Comparing with the seed cells, the scaffold can be easily designed and optimized on many aspects, such as component, structure and mechanical property etc. Therefore, the scaffold is an adjustable critical factor [3,4]. Ideal scaffold may initialize good biological effects, and give better restoration of articular cartilage.

Hydrogels are widely used as cartilage tissue engineering scaffolds. Their high equilibrium swelling properties allow certain mass transport of the signaling molecules, nutrients, and metabolic wastes in and out [5–7]. The partially stress transformation and the hypoxia condition in hydrogels offer similar environment as natural cartilage to the seed cells. Some components of cartilage have been proved can form hydrogel scaffolds, such as hyaluronic acid (HA) and collagen. Many studies have demonstrated that HA hydrogels can support the chondrogenesis of MSCs and chondrocytes in vitro and promote neocartilage formation and the integration of the neocartilage with the surrounding native cartilage in the articular cartilage defect [5]. The self-assembly of collagen can help it to form hydrogel, and some reports have indicated that collagen hydrogel has endogenous ability to induce chondrogenesis differentiation of the seeded cells [8]. Even, collagen hydrogel can help

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to reduce the immune rejection for the allogeniec seed cells [9]. Meanwhile, HA and collagen can be conveniently composited to form hydrogel and combined the advantage of them both. In previous study [10], we have proved that when HA and collagen (HA/Col) composited hydrogel formed, the interaction of HA and collagen gave better cartilage inducing ability than single component hydrogel. However, present HA/Col composited hydrogel has far lower mechanic property than natural cartilage. The molecular morphology and the combination way of HA and collagen in the composited hydrogel are different from natural cartilage, makes the cartilage inducing ability, the integration of the neocartilage with the surrounding native cartilage and restoration effects of the composited hydrogel still limited.

In order to endow hydrogel scaffolds preferable chondrogenesis, some exogenous growth factors and chondro-promotive drugs are incorporated into scaffolds [4,11]. In them, a flavonol glycoside named icariin (Ica) has proved its promoting effect on cartilage formation. It can improve the proliferation of chondrocytes [12], reduce the degradation of extracellular matrix (ECM) [13], promote the expressions of sox9, collagen II and aggrecan genes of chondrocytes and the synthesis of cartilage-specific matrices [14,15]. In previous study, when Ica was loaded in collagen hydrogel, Ica could promote the growth of neocartilage and the integration of the neocartilage with the native cartilage and subchondral bone [14]. Meanwhile, Ica has advantages of low cost, steady activity and security compared to exogenous growth factor. However, Ica is a small molecular weight (only 676.65) component. When it was added into the hydrogel directly, Ica will release fast into the surrounding tissue fluid. The fast release leads to high carrying dose requirement and short duration of validity, and even more risk of side effects because of the high initial released concentration.

It was known that proper conjugation of the bioactive factor to a scaffold could suppress the initial burst of the release [16,17]. With the break of chemical bonds and the degradation of the scaffolds, the bioactive factors can be release gradually, and maintain effective concentration for long time. As described above, both HA and collagen are believed high potential drug loading scaffolds [18,19]. If Ica can be fixed on HA/Col composited hydrogel, the risk of side effects may be effectively reduce and the cartilage restoration effects may be effectively enhanced. Therefore, in this study an Ica-modified covalently HA/Col hydrogel (HA–Ica/Col) was developed for cartilage tissue engineering. The hydrogel, the release kinetics of icariin, the seeded cell viability, the cartilage-related genes expression and the histology of the constructs were characterized. We hope the chemical conjugation of Ica may offer more efficient release and better cartilage formation in the hydrogel.

2. Materials and methods

2.1. Materials

Collagen type I (Col) was extracted from the skin of new-born calf with pepsin in acetic acid, and then purified using sodium chloride fractionation and fibril assembly [8]. Hyaluronic acid (HA, MW: 300–400 kDa) was purchased from Furuida Bioengineering Co (Shangdong, China). Icariin (Ica, purity ≥98%) was purchased from Nanjing TCM institute of Chinese materia medica. Methacrylic anhydride (MA), Fluorescein diacetate (FDA) and propidium iodide (PI) were obtained from Sigma–Aldrich Co. (St. Louis, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) was bought from Amreso Co. (USA). Collagenase type I was purchased from Gibco Co. (USA). All other stated chemicals are from Kelong Chemical Co. (Chengdu, China) without further purified.

2.2. Synthesis of Ica–MA and HA–MA

2.2.1. Synthesis of methacrylate icariin (Ica–MA)

Ica-MA was synthesized as previously reported method [20]. In brief, 500 mg Ica was dissolved in 5 mL pyridine. Then 600 uL methacrylic anhydride (MA) was added into the pyridine solution with stirring at room temperature. After 4 h, the reaction mixture was dialyzed against alcohol for 1 day and water for 3 days with repeated changes of water. The product Ica–MA was obtained by lyophilization and characterized by liquid chromatography-mass spectrometry (LC–MS) and proton nuclear magnetic resonance spectra (^{1}H NMR).

2.2.2. Synthesis of methacrylate HA (HA–MA)

HA-MA was prepared by the previous method [10]. In brief, HA powder was dissolved in distilled water to obtain 1 wt% HA solution. Then 500 mL above solution was cooled to 4 °C and 24 mL MA was added dropwise with stirring. The pH value of the reaction system was controlled at 8.0 by adding 5 mol/L NaOH solution. After stirring for 24 h at 4 °C, alcohol was added into the mixture solution to precipitate the reaction product, HA–MA. Then the precipitate was filtered, washed with alcohol for four times, then re-dissolved in distilled water and dialyzed against water for 4 days with repeated changes of water. The HA–MA sponge was obtained by lyophilization and stored at −20 °C.

2.3. Preparation of hydrogels

Ica-MA was dissolved in DMSO to obtain the stock solution with Ica–MA concentration of 0.02 mol/L. Then 500 μL stock solution was added into 10 mL phosphate buffered saline (PBS) containing 0.2 wt% of the photoinitiator 2-methyl-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959) with stirring to form suspension solution. Subsequently, 200 mg HA–MA was dissolved in the above suspension to obtain the mixed solution of Ica–MA and HA–MA. 2 mL acidic collagen solution with concentration of 12 mg/mL was adjusted to neutralization by NaOH solution with stirring at 4 °C. Subsequently, 2 mL mixed solution of Ica–MA and HA–MA was blended rapidly with the above neutral collagen solution with stirring at 4 °C to obtain the hydrogel precursor solution. Then each 100 μL hydrogel precursor solution was injected a cylinderical mold (about 8 mm in diameter and 2 mm in height) and exposed to the UV light (360–480 nm) for 30 s and then incubated at 37 °C for 15 min to form HA–Ica/Col hydrogel. The final concentration of HA–MA, Col and Ica–MA were 10 mg/ml, 6 mg/ml and 5×10^{-4} mol/L, respectively. HA/Ica/Col hydrogel (Ica-encapsulated hydrogel, with the Ica simply mixed into, but not fixed) and HA/Col hydrogel (without Ica, as control) were prepared by similar procedure.

2.4. Characterization of hydrogels

2.4.1. Mechanical test

Compression tests were carried out using dynamic mechanical analysis (DMA, Q800 TA, USA) to characterize the viscoelastic properties of cylindrical hydrogel samples. Experiments were carried out in compression mode with a fixed frequency of 1 Hz at room temperature and constant strain amplitude of 30 μm. The associated software was used to record the storage modulus (G') and loss modulus (G") of hydrogel samples. The resulting data were used to draw plots between the storage modulus and loss modulus with time.

2.4.2. Degradation and swelling measurement

For degradation analysis, the hydrogel samples ($n = 4$) were rinsed with deionized water for four times, then pre-swollen in