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Intra-articular delivery of sinomenium encapsulated by chitosan microspheres and photo-crosslinked GelMA hydrogel ameliorates osteoarthritis by effectively regulating autophagy

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ABSTRACT

Reduced expression of autophagy regulators has been observed in pathological cartilage in humans and mice. The present study aimed to investigate the synergistic therapeutic effect of promotion of chondrocyte autophagy via exposure to sinomenium (SIN) encapsulated by chitosan microspheres (CM-SIN) and photo-crosslinked gelatin methacrylate (GelMA) hydrogel, with the goal of evaluating CM-SIN as a treatment for patients with osteoarthritis. First, we fabricated and characterized GelMA hydrogels and chitosan microspheres. Next, we measured the effect of SIN on cartilage matrix degradation induced by IL1-β in chondrocytes and an *ex vivo* model. SIN ameliorated the pathological changes induced by IL1-β at least partially through activation of autophagy. Moreover, we surgically induced osteoarthritis in mice, which were injected intra-articularly with CM-SIN and GelMA. Cartilage matrix degradation and chondrocyte autophagy were evaluated 4 and 8 weeks after surgery. Treatment with the combination of CM-SIN and GelMA retarded the progression of surgically induced OA. SIN ameliorated cartilage matrix degradation at least partially by inducing autophagy *in vivo*. Our results demonstrate that injection of the combination of GelMA hydrogel and CM-SIN could be a promising strategy for treating patients with osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a destructive joint disease that leads to degeneration of cartilage and changes in the subchondral bone and synovium, followed by bone damage and morphological changes [1]. Age, genetic predisposition, hereditary factors, obesity, mechanical injuries, and joint trauma are risk factors of OA [2,3]. Structure-modifying medications and nutraceuticals may be effective therapeutic agents for osteoarthritis and merit further investigation [4].

Autophagy is a lysosomal degradation pathway and a homeostatic cellular mechanism that is essential for survival, differentiation, development, and homeostasis [5]. Meanwhile, autophagy is a protective mechanism in normal cartilage [6]. Reduced expression of autophagy regulators was observed in pathological cartilage in humans and mice [7]. Activation of autophagy by rapamycin treatment reduced the severity of OA in experimental models [8]. Therefore, autophagy activation may be a novel therapeutic target for OA treatments. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein that is distributed ubiquitously in mammalian tissues and cultured cells and is used as a specific marker to monitor autophagy [9]. During autophagy, LC3 is recruited to the autophagosomal membrane. When autophagy is induced, LC3-I is conjugated to phosphatidylethanolamine to form LC3-II, which reflects autophagic activity [9,10].

Sinomenium (9α,13α,14α)-7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methyl-morphinane-6-one, SIN) is a natural alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum*. SIN has several pharmacological effects, including anti-rheumatism and immunomodulation [11]. The hydrochloride

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salt of SIN is widely used in the clinic as a treatment for patients with rheumatoid arthritis (RA) [12]. SIN has the protective capacity to antagonize cartilage degradation [13], and it reduces protein levels of the cartilage degradation marker matrix metallopeptidase 13 (MMP-13, also known as collagenase 3) in rats and blocks the pathogenesis of collagen-induced arthritis via NF-κB signaling and down-regulates MMP13 expression [14,15]. In addition, by regulating autophagy in animal models of sepsis, SIN improved survival, reduced organ damage, and attenuated the release of inflammatory cytokines [16]. These clues suggest that SIN protects chondrocytes by facilitating autophagy and preventing cartilage degradation. However, few investigations have been carried out to evaluate the therapeutic efficacy of SIN as a treatment for patients with OA.

Chitosan microspheres (CMs) are an efficient biopolymeric drug delivery system capable of delivering therapeutic agents in a controlled and/or sustained manner. CMs are characterized by biocompatibility, nontoxicity, a lack of allergenicity, and biodegradability, all of which have contributed to their successful utilization for site-specific drug delivery [17]. In this study, gelatin methacrylate (GelMA) hydrogel was used as a vehicle to facilitate intra-articular injection of chitosan. Compared with other hydrogels, GelMA crosslinks, when exposed to light irradiation to form hydrogel with tunable physical properties, have essential properties resembling native extracellular matrix, and show promise for cartilage tissue engineering applications [18,19]. However, thus far, no investigation has been carried out to evaluate its therapeutic efficacy in animal models of OA, making its suitability for this purpose uncertain. In this study, we report the results of experiments in which GelMA hydrogels containing chitosan microspheres encapsulating SIN were injected into mouse joints, with the goal of producing sustained SIN release and ameliorated OA changes.

To assess the therapeutic potential of microsphere-encapsulated SIN as a treatment for patients with OA, we developed and evaluated the characteristics of a photo-crosslinked GelMA hydrogel, after which chitosan microspheres were utilized to produce controlled SIN release. Finally, we investigated the effects of SIN on OA cartilage degeneration. We hypothesized that the combination of chitosan microsphere-encapsulated SIN (CM-SIN) with our newly developed photo-crosslinked GelMA hydrogel would synergistically prevent the progression of degenerative changes in surgically induced OA.

2. Materials and methods

2.1. Materials

Gelatin (Type A, 300 bloom from porcine skin) and methacrylic anhydride (MA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless specifically mentioned.

2.2. Methacrylated gelatin synthesis

Type A porcine skin gelatin was mixed into phosphate-buffered saline (PBS) at 60 °C and stirred until fully dissolved at a concentration of 10% (w/v). MA was added to the gelatin solution at a rate of 0.5 mL/min until the target volume was reached, stirred at 50 °C, and allowed to react for 1 h. Following a 5 × dilution with additional warm (40 °C) PBS to stop the reaction, the mixture was dialyzed against distilled water using 12–14 kDa cutoff dialysis tubing for 1 week at 40 °C to remove salts and methacrylic acid. The solution was lyophilized for 1 week to generate a white porous foam and stored at −80 °C.

2.3. Hydrogel preparation

The prepolymer hydrogel solution was prepared by mixing 6 wt % GelMA solution into PBS containing 1% (w/v) 2-hydroxy-1- (4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) as a photoinitiator at 80 °C. The GelMA prepolymer solution was vigorously stirred at room temperature for 10 min to generate a homogeneous solution, which was pipetted into a 24-well culture dish (200 μL/well) and exposed to UV light (320–500 nm, 7.0 mW/cm^2) for 2 min to allow for photo-crosslinking. The samples were incubated in a free-floating manner at 37 °C in PBS for 24 h, followed by storage at −20 °C.

2.4. Characterization of GelMA-SF hydrogel

The hydrogel swell ratio was analyzed as follows. The hydrogels were lyophilized until dry and dry weight (Wd) was measured. Dried hydrogel samples (n = 3) were immersed in 50 mL of PBS at 37 °C and allowed to swell. Swollen hydrogel samples were weighed to determine swollen weight (Ws) at different time points. The swelling ratio (Q) was calculated by the following equation: Q = Ws/Wd.

To characterize the enzymatic degradation properties of GelMA hydrogel, we placed the hydrogel samples in 1.5-mL centrifuge tubes with 1 mL PBS containing 1–2 U/mL collagenase type II at 37 °C. At a pre-defined time, the hydrogels for each condition were removed, frozen, and lyophilized. Mass loss was determined as the ratio of the final weight to the original dry weight. All experiments were repeated 3 times.

2.5. Preparation of chitosan microspheres

Chitosan microspheres were prepared using the water-in-oil (W/O) emulsion solvent diffusion method. Chitosan solution (2% w/v) was prepared by dissolving chitosan (Shanghai Bio Science and Technology) in 2.5% (v/v) acetic acid aqueous solution (Sinopharm Chemical Reagent) at room temperature. The chitosan solution was mixed with SIN by stirring overnight with a magnetic stirrer to produce a homogeneous mixture. Next, 5 mL of the resulting mixture was aspirated into a syringe pump and added drop-wise into the oil phase (24.72 mL), which consisted of 14 mL liquid paraffin (Sinopharm Chemical Reagent), 10 mL petroleum ether, and 0.72 mL Span 80 (Sangon Biotech), at a flow rate of 4 mL/h with continuous stirring at 1500 rpm. A syringe needle with an internal diameter of 0.2 mm was used for this process. After the solvent diffusion procedure, the suspension was cross-linked using 25% (v/v) glutaraldehyde solution as a cross-linking agent. The addition of the cross-linker was carried out three times, at time intervals of 15 min, with the following volumes of glutaraldehyde: 0.64, 0.64, and 0.32 mL. Subsequently, the suspension was stirred at room temperature to produce cross-linking and centrifuged at 3000 rpm for 5 min, after which the supernatant fluid was discarded. Next, the microspheres were washed with petroleum ether (three times) (Sinopharm Chemical Reagent), methanol (two times) (Sinopharm Chemical Reagent), acetone (one time) (Sinopharm Chemical Reagent), isopropyl alcohol (one time) (Sinopharm Chemical Reagent), ethanol (one time), and distilled water (three times). After washing, the microspheres were collected after lyophilization with a freeze-dryer to remove residual water. For the control group, pure chitosan microspheres were prepared by directly dropping the chitosan solution into the oil phase under the same conditions.