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Thermosensitive hydrogel made of ferulic acid-gelatin and chitosan glycerophosphate

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ABSTRACT

Reactive oxygen species-induced oxidative stress is involved in apoptosis of nucleus pulposus (NP) cells that can alter cellular phenotype and accelerate disc degeneration. Ferulic acid (FA) possesses an excellent antioxidant and anti-inflammatory properties. In the study, we developed the thermosensitive FA–gelatin/chitosan/glycerol phosphate (FA–G/C/GP) hydrogel which was applied as a sustained release system of FA to treat NP cells from the damage caused by oxidative stress. The gelation temperature of the FA–G/C/GP hydrogel was 32.17 °C. NP cells submitted to oxidative stress promoted by H_2O_2, and post-treated with FA–G/C/GP exhibited down-regulation of MMP-3 and up-regulation aggrecan and type II collagen in mRNA level. The sulfated-glycosaminoglycan production was increased and the apoptosis was inhibited in the post-treatment group. The results suggest that the thermosensitive FA–G/C/GP hydrogel can treat NP cells from the damage caused by oxidative stress and may apply in minimally invasive surgery for NP regeneration.

1. Introduction

Intervertebral disc lies between vertebral bodies and consists of central nucleus pulposus (NP) and outer annulus fibrosus. The main components of disc are water, proteoglycans and collagen, which provide mechanical support to resist the external stress (Humzah & Soames, 1988). Disc degeneration is generally believed that originate in the NP with decrease of cell number, decrease of collagen-II and loss of proteoglycans. In the degenerative NP, cells may lose their phenotype and change extracellular matrix (ECM) composition by decreasing anabolism or increasing catabolism (Anderson & Tannoury, 2005; Urban & Roberts, 2003). It has been suggested that degenerative process is accelerated by catabolic factors such as pro-inflammatory mediators, matrix metalloproteinases (MMPs) and apoptotic factors (Walker & Anderson, 2004). Current clinical treatments for disc degeneration includes medication, physical therapy, fusion, artificial disc replacement and discectomy, however, these treatments attempt to relieve pain rather than repair the degenerative disc (An et al., 2003). Novel biological treatments are under investigation to treat degenerative disc in the early stages of degenerative process by promoting synthesis or inhibiting degradation of ECM, which have gained more attention in recent years (Paesold, Nerlich, & Boos, 2007; Yoon, 2005).

Overproduction of reactive oxygen species (ROS) including superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) can cause oxidative stress which is associated with age-related diseases (Finkel & Holbrook, 2000). ROS-induced oxidative stress has been reported to involve in senescence and apoptosis of NP cells which may alter cellular phenotype and associate with the disc degeneration (Gruber, Ingram, Norton, & Hanley, 2007; Kim, Chung, Ha, Lee, & Kim, 2009; Kim et al., 2007). In the previous studies, we found that ferulic acid (4-hydroxy-3-methoxy cinnamic acid) (FA) from Chinese herb medicine may have ability to treat ROS-induced diseases (Chen et al., 2010; Cheng, Yang, & Lin, 2011; Cheng, Yang, Yang, et al., 2011). FA is a member of polyphenol family, which possesses an excellent antioxidant property due to the resonance structure. Therapeutic potential of FA in various diseases such as cardiovascular, cancer and diabetes have already been proved (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002; Srinivasan, Sudheer, & Menon, 2007).

In recent years, thermosensitive hydrogel formation by simple sol–gel transition and without chemical reaction has been

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increasing interest in a wide range of biomedical and pharmaceutical applications (Muzzarelli, 2009; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012; Ruel-Gariépy & Leroux, 2004) Chitosan-based thermosensitive hydrogel is currently a great deal of interest for drug and protein delivery (Bhattarai, Gunn, & Zhang, 2010; Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005). Drug delivery system via covalent bond between drug and polymer can prolong the period of release compared with the direct incorporation (Bhattarai et al., 2010). In previous studies (Cheng, Yang, & Lin, 2011; Cheng et al., 2010; Cheng, Yang, Yang, et al., 2011), we developed thermosensitive chitosan/gelatin/glycerol phosphate (C/G/GP) hydrogel as a cell carrier for NP regeneration and the therapeutic effects of FA on H_2O_2-induced oxidative stress NP cells have also been demonstrated. In the study, we are going to develop the thermosensitive FA covalently linked gelatin/chitosan/glycerol phosphate (FA–G/C/GP) hydrogel as a sustained release system of FA for NP regeneration.

2. Materials and methods

2.1. Isolation of NP cells

All experimental procedures were approved by the Animal Experimentation Ethics Committee of National Taiwan University Hospital and maintained in accordance with the guidelines for the care and use of laboratory animals. Four-month-old New Zealand white rabbits weighing approximately 2 kg were used. All chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The NP were harvested from the IVD and treated with the 10% penicillin of PBS at 37 °C for 10 min and then immersed in Dulbecco's modified eagle's medium-nutrient mixture F-12 ham medium (DMEM-F12) containing 10% fetal bovine serum (Gemini Bio-products, USA), 1% penicillin and 0.05% L-ascorbic acid with 0.2% collagenase at 37 °C for 18 h. NP cells were collected and cultured in DMEM-F12 at 37 °C, 5% carbon dioxide and 95% relative humidity (Cheng et al., 2010).

2.2. Preparation of thermosensitive FA–gelatin/chitosan/glycerol phosphate (FA–G/C/GP) solution

FA was dissolved in dimethyl sulfoxide (DMSO) with concentration of 0.1 M. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were dissolved in water with concentration of 1 M and 0.25 M respectively. 8% type A gelatin and 2.5% chitosan (degree of deacetylation > 95%, molecular weights = 340,000, Kiotek, Taiwan) were dissolved in water and 0.1 M acetic acid respectively. Both gelatin and chitosan solution were sterilized by autoclaving at 121 °C for 30 min. The viscous modulus (G'') was 0.634 ± 0.023 Pa and elastic modulus (G') was 0.271 ± 0.141 Pa of autoclaved chitosan solution. The G'' was 0.635 ± 0.010 Pa and G' was 0.328 ± 0.008 Pa of autoclaved gelatin solution. The rheological characterization were measured by Haake RheoStress 600 rheometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with parallel plate geometry sensor (PP35 Ti, 35 mm diameter and 0.105 mm gap) in oscillatory mode. The G' and G'' versus temperature were measured at a fixed frequency of 1 Hz and constant stress of 3 Pa. FA–gelatin solution was prepared by mixing 100 μl of 1 M EDC and 400 μl of 0.25 M NHS with 1 ml of 0.01 M FA and vortexed for 1 h. The mixture was filtered by 0.22 μm filter (Millex-GV, Millipore, USA) for sterilization and then mixed with 1 ml of 8% gelatin solution and vortexed for 1 h. FA–gelatin solution was then mixed with 2.5% chitosan solution to form the FA–gelatin/chitosan solution. 44.4% β-glycerophosphate disodium salt hydrate (β-GP) solution was sterilized by passing through a 0.22 μm filter and added drop by drop to the FA–gelatin/chitosan solution under stirring and adjusted the pH value to 7.4. The FA–G/C/GP solution was stored at 4 °C until further use.

2.3. Characterization of FA–gelatin

2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) assay was used for the detection of primary amino groups. As described in Section 2.2, the residual amino group of both gelatin and FA–gelatin were analyzed by TNBS assay. 10 μl of sample was mixed with 90 μl of 0.1 M sodium hydroxide. The mixture was transferred into a 96-well microplate and reacted with 50 μl of 0.1% TNBS for 2 h at 37 °C. After 2 h, 75 μl of stop solution containing 50 μl of 10% SDS and 25 μl of 1N HCl was added to terminate the reaction. The optical density (OD) value was measured at 420 nm using an enzyme-linked immunosorbent assay reader (ELISA, Sunrise remote, TECAN, USA). The residual amino group content of sample was determined by using a linear standard curve which was constructed by glycine.

2.4. Reheological characterization

Gelation temperature and gelation time of the FA–G/C/GP hydrogel were measured by Haake RheoStress 600 rheometer equipped with parallel plate geometry sensor (PP35 Ti, 35 mm diameter and 0.105 mm gap) in oscillatory mode. The G' and G'' versus temperature were measured at a fixed frequency of 1 Hz and constant stress of 3 Pa. In the study of gelation temperature, the temperature was raised from 20 to 50 °C. The gelation temperature and gelation time were defined at which the G' becomes larger than the G''.

2.5. Cytotoxicity of thermosensitive FA–G/C/GP hydrogel on NP cells

Cytotoxicity of FA–G/C/GP hydrogel on NP cells was performed by extraction method. The 0.1 g FA–G/C/GP hydrogel was immersed in 1 ml DMEM-F12 in a 48-well culture plate at 37 °C. The supernatant from each well was collected at day 3 for cytotoxicity test. NP cells were seeded in 96-well cell culture plates at a density of 5000 cells per well and cultured in DMEM-F12 for 18 h. Cells were then cultured in the extraction medium obtained from the developed hydrogel. WST-1 (Cell Proliferation Reagent WST-1, Roche, Germany) and lactate dehydrogenase (LDH, CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega, USA) were used to evaluate the cell viability and cytotoxicity of the developed hydrogel on NP cells at day 1 and day 3. The OD value of WST-1 and LDH assay were measured at 450 and 490 nm with an ELISA reader respectively (Chang, Tang, Hsu, Soung, & Wang, 2012).

2.6. In vitro FA release study

200 μl of FA–G/C/GP solution or C/G/GP (without FA) was added to the transwell mounted on 24-well plates (Corning, USA) and 1.5 ml of PBS was then added in each well and incubated at 37 °C. The 1.5 ml of PBS was collected and 1.5 ml of fresh PBS was then added at each time (0.5, 1, 2, 6, 24 and 48 h). The content of FA was evaluated by ultra violet–visible–near infrared (UV–vis–NIR) spectrophotometer (DU 7500, Beckman, USA) at the wavelength of 343 nm according to the absorption spectrum of FA.

2.7. Induction of oxidative stress and FA–G/C/GP hydrogel treatment

The NP cells were seeded in the 24-well cell culture plates with the density of 5×10^4 cells per well and cultured in DMEM-F12. After 18 h, cells were washed with PBS and 1.5 ml of DMEM-F12 was then added. Oxidative stress on NP cells was induced by 100 μM