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Thermosensitive hydrogel made of ferulic acid-gelatin and chitosan glycerophosphate

Yung-Hsin Cheng[a,b,1], Shu-Hua Yang[c,2], Chia-Ching Liu[a,1], Amit Gefen[d,3], Feng-Huei Lin[a,b,*]

[a] Institute of Biomedical Engineering, College of Engineering and College of Medicine, National Taiwan University, No. 1, Jen Ai Road, Section 1, Taipei, 10051, Taiwan
[b] Division of Medical Engineering, National Health Research Institute, Chun-Nan, Taiwan
[c] Department of Orthopedics, National Taiwan University College of Medicine and National Taiwan University Hospital, No. 1, Jen Ai Road, Section 1, Taipei, 10051, Taiwan
[d] Department of Biomedical Engineering, Tel Aviv University, Tel Aviv, 69978, Israel

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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

* Corresponding author at: Division of Medical Engineering, National Health Research Institute, No. 35, Keyan Road, Zhunan, Miaoli County 350, Taiwan. Tel.: +886 2 23641541; fax: +886 2 23641541.
E-mail addresses: d97548005@ntu.edu.tw (Y.-H. Cheng), shuhuayang@ntu.edu.tw (S.-H. Yang), gefen@eng.tau.ac.il (A. Gefen), double@ntu.edu.tw (F.-H. Lin).
[1] Tel.: +886 2 23641541; fax: +886 2 23641541.
[2] Tel.: +886 2 2123456x63981; fax: +886 2 23224112.
[3] Tel.: +972 3 6408093; fax: 972 3 6405845.

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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

H_2O_2 (RDH, USA) for 30 min. The 200 μl of FA–G/C/GP or C/G/GP solution were added to the transwell culture insert mounted on 24-well plates incubated with 100 μM H_2O_2-induced oxidative stress NP cells and then cultured at 37 °C. The 100 μM H_2O_2-induced oxidative stress NP cells with C/G/GP hydrogel and with FA–G/C/GP hydrogel were abbreviated as C/G/GP and FA–G/C/GP group respectively.

2.8. RNA extraction and gene expression of NP cells

Total RNA was extracted from NP cells using RNeasy Protect Mini kit (QIAGEN, Germany) after 2.5 h as described in Section 2.7. RNA was stored in −80 °C for reverse transcription polymerase chain reaction (RT-PCR). The first strand complementary DNA (cDNA) was synthesized from RNA and SuperScript™ III First-Strand Synthesis System (Invitrogen, USA) for RT-PCR (PTC-200, MJ Research, USA) in accordance with the manufacturer's instruction. 1 μl of primer, 9 μl of cDNA and 10 μl of 2× TaqMan Universal PCR Master Mix (ABI, USA) were mixed in a final volume of 20 μl for single reaction. Reaction was performed by ABI PRISM 7700 Sequence Detection System. The target genes of real-time PCR reaction were inducible nitric oxide synthase (iNOS, GenBank accession no. AF469048), interlukin-1β (IL-1β, GenBank accession no. M26295), collagen-II (Col II, GenBank accession no. AF027122), aggrecan (Agn, GenBank accession no. L38480), collagen-I (Col I, GenBank accession no. D49399), matrix metalloproteinase-3 (MMP-3, GenBank accession no. M25664), tissue inhibitor of metalloproteinase-1 (TIMP-1, GenBank accession no. J04712) and transforming growth factor-β (TGF-β, GenBank accession no. AF000133). Each target gene was calibrated for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession no. L23961). The relative quantitation value of gene expression was determined using ΔΔCt method.

2.9. Analysis of cell numbers

The cell number in each well was evaluated by crystal violet assay at day 1. Crystal violet was dissolved in the 10% ethanol. NP cells were washed with PBS and 50 μl of 0.2% crystal solution was then added for 10 min. The 24-well cell culture plate was washed with running water and the 200 μl of 33% acetic acid was then added. 100 μl of each sample was transferred to a 96-well microplate. The absorbance was measured at the wavelength of 570 nm using an ELISA reader. The cell number of each sample was calculated from the linear standard curve with known numbers of NP cells.

2.10. Analysis of sulfated-glycosaminoglycans

The sulfated-glycosaminoglycan (GAG) content was analyzed by 1,9-dimethylmethylene blue (DMMB) assay. The culture medium of each well was collected at day 1. 40 μl of each sample was mixed with 250 μl of DMMB solution in a 96-well microplate. The sulfated-GAG-DMMB complex was detected by ELISA reader at the wavelength of 595 nm (Chou, Kuo, Lin, Tsai, & Lin, 2008).

2.11. Caspase-3 activity

NP cells were collected at the end of 1-day culture. Total protein content was determined using BCA protein assay kit (Pierce, USA) according to the manufacturer's instructions. Caspase-3 activity was evaluated by CaspACE assay system (Promega, USA). 30 μg of total protein of each sample was mixed with a reaction buffer containing 2 μl of DMSO, 10 μl of 100 mM DTT and 32 μl of caspase assay buffer in a 96-well microplate. 2 μl of DEVD-pNA was then added and incubated at 37 °C for 4 h. The absorbance was measured at the wavelength of 405 nm using an ELISA reader.

Table 1
The percentage of residual amino groups in the gelatin and FA–gelatin group.

Group	Residual NH_2 group (%)
Gelatin	100 ± 5.3
FA–gelatin	39.1 ± 3.2

Table 2
The gelation temperature and gelation time of FA–G/C/GP solution.

Gelation temperature	32.17 ± 0.58 °C
Gelation time (at 37 °C)	57.65 ± 2.74 s
Gelation time (at 25 °C)	>900 s

2.12. TUNEL staining

At the end of 1-day culture, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed by using ApopTag® plus fluorescein in situ apoptosis detection kit (Chemicon, USA) according to the instructions provided by the manufacturer.

2.13. Statistical analysis

Statistical analysis of residual amino group content, cytotoxicity, mRNA gene expression, sulfated-GAG production and caspase-3 activity was performed using one-way analysis of variance (ANOVA). Results were considered significant if $p < 0.05$ and expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. TNBS assay

Residual amino group of FA–gelatin were evaluated by TNBS assay. TNBS reacts with amino groups of gelatin to form the TNBS–amino acid complex products. The residual amino group of gelatin (without FA) was designed as 100% than compared with FA–gelatin group. The residual amino group of FA–gelatin was significantly decreased 61.9% compared with gelatin group as shown in Table 1.

3.2. Reheological characterization

Gelation temperature and gelation time of the FA–G/C/GP solution were determined at which G' is equal to G''. As shown in Table 2, the gelation temperature of FA–G/C/GP solution was 32.17 ± 0.58 °C. The gelation time of FA–G/C/GP solution was 57.65 ± 2.74 s at 37 °C. The FA–G/C/GP solution was kept in liquid form even the time was extended to 15 min at 25 °C.

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

As described in Section 2.5, the mitochondrial activity was evaluated by WST-1 assay. As shown in Fig. 1(a), the OD value of the control group at day 1 and day 3 were 1.256 ± 0.069 and 1.467 ± 0.059, respectively. The OD value of the FA–G/C/GP group at day 1 and day 3 were 1.223 ± 0.033 and 1.493 ± 0.112, respectively. The results of WST-1 showed that there was no significant difference in OD value between the control group and FA–G/C/GP group at day 1 and day 3.

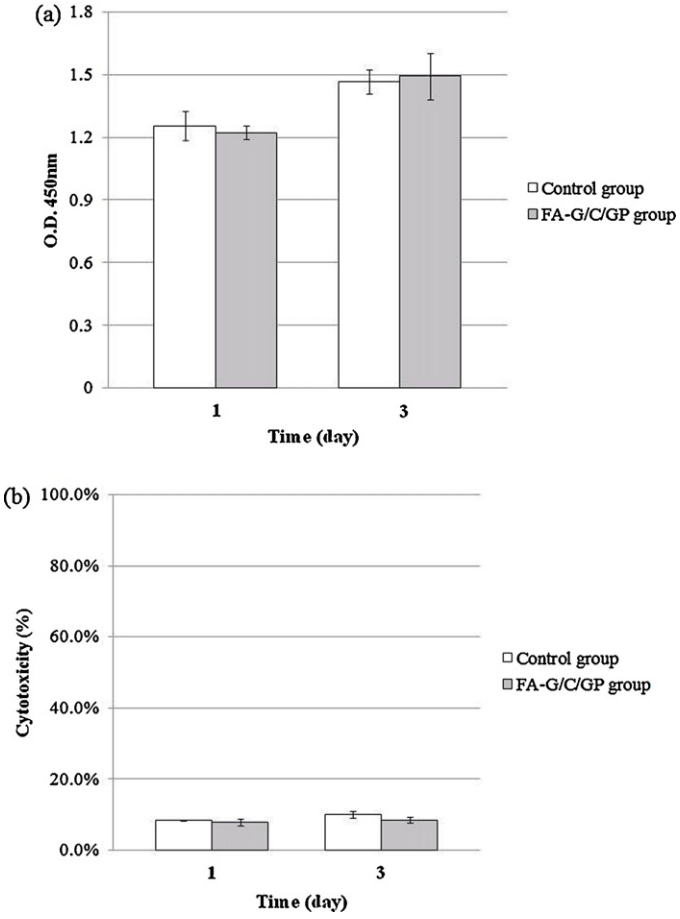

Fig. 1. Cytotoxicity of FA–G/C/GP to NP cells: (a) WST-1 assay and (b) LDH assay ($n = 3$, $p > 0.05$).

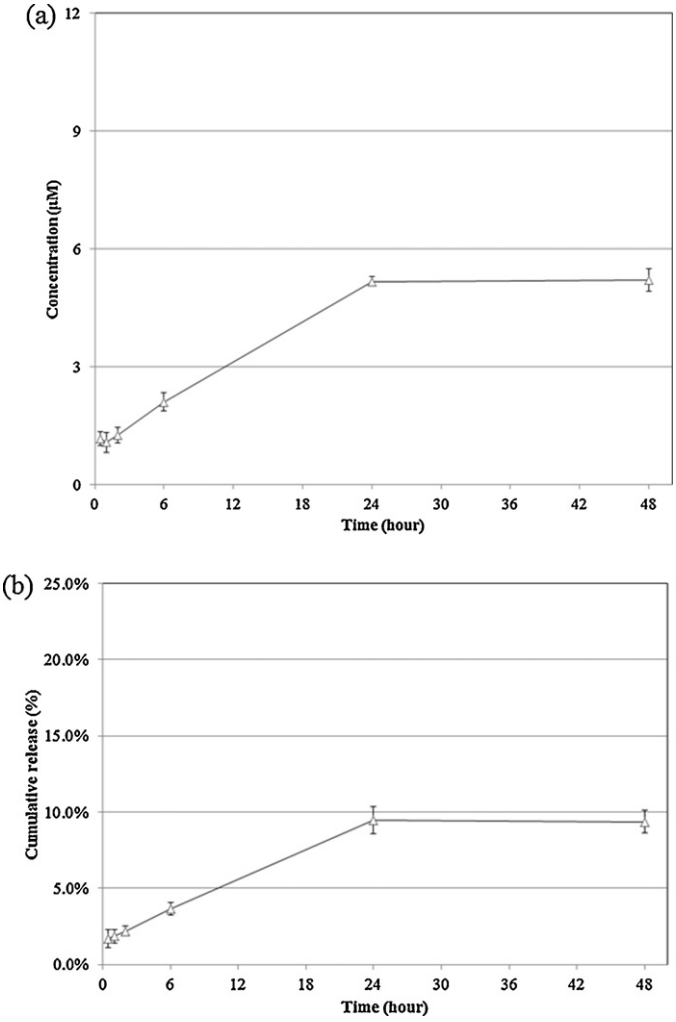

Fig. 2. The release profile of FA from FA–G/C/GP hydrogel: (a) cumulative percentage and (b) the cumulative concentration of FA in the PBS at 37 °C.

The results of LDH assay was expressed in percentage of cytotoxicity which was calculated by the following equation:

$$\text{cytotoxicity}\,(\%) = \frac{\text{OD}_{\text{exp}} - \text{OD}_{\text{medium}}}{\text{OD}_{\text{total lysis}} - \text{OD}_{\text{medium}}} \times 100$$

As shown in Fig. 1(b), the cytotoxicity of control group at day 1 and day 3 were 8.5 ± 0.1 and $10.2 \pm 0.9\%$, respectively. The cytotoxicity of FA–G/C/GP group at day 1 and day 3 were 7.8 ± 0.9 and $8.6 \pm 0.8\%$, respectively. There was no significant difference in cytotoxicity between control group and FA–G/C/GP group.

3.4. FA release from FA–G/C/GP hydrogel

The FA concentration of each sample was calculated from the linear standard curve of FA. Fig. 2 shows the release profile of FA from the FA–G/C/GP hydrogel. The cumulative concentration of FA in the PBS at 0.5, 1, 2, 6, 24 and 48 h were 1.167 ± 0.179, 1.064 ± 0.262, 1.251 ± 0.189, 2.097 ± 0.230, 5.166 ± 0.124 and 5.206 ± 0.291 μM, respectively (Fig. 2(a)). The percentage of cumulative release at 0.5, 1, 2, 6, 24 and 48 h were 1.7 ± 0.6, 1.8 ± 0.5, 2.2 ± 0.3, 3.6 ± 0.4, 9.5 ± 0.9 and $9.3 \pm 0.7\%$, respectively (Fig. 2(b)).

3.5. Gene expression

NP cells stimulated with 100 μM H_2O_2 for 30 min and then incubated with FA–G/C/GP hydrogel or C/G/GP hydrogel for 2 h were designed and abbreviated as C/G/GP and FA–G/C/GP group, respectively. The results of mRNA gene expression were reported as fold increase compared with fresh NP cells (control group).

Inducible nitric oxide synthase (iNOS) and interleukin-1β (IL-1β) are pro-inflammatory genes. As shown in Fig. 3(a), the expression of iNOS and IL-1β were significantly up-regulated in C/G/GP group with 2.6237 and 1.8636-folds to the control group, respectively. There was no significant difference in the expression of iNOS and IL-1β between the control group and FA–G/C/GP group. The expression of iNOS was significantly higher in C/G/GP group (2.6237 ± 0.3886) compared with FA–G/C/GP group (1.0987 ± 0.0989). The expression of IL-1β was significantly up-regulated in C/G/GP group (1.836 ± 0.1295) compared with FA–G/C/GP group (1.2175 ± 0.1664). Collagen-II, aggrecan and collagen-I are the ECM component genes. As shown in Fig. 3(b), the expression of collagen-II and aggrecan were significantly down-regulated in C/G/GP group with 0.7856 and 0.6737-folds to the control group, respectively. The expression of collagen-II was significantly higher in FA–G/C/GP group (1.1600 ± 0.0477) compared with other groups. There was no significant difference in the expression of aggrecan between the control group and FA–G/C/GP group. The expression of collagen-I was significantly lower in FA–G/C/GP group (0.7214 ± 0.1977) compared with C/G/GP group (1.2029 ± 0.1301). There was no significant difference in the expression of Col I between the control group and experimental group (C/G/GP group and FA–G/C/GP

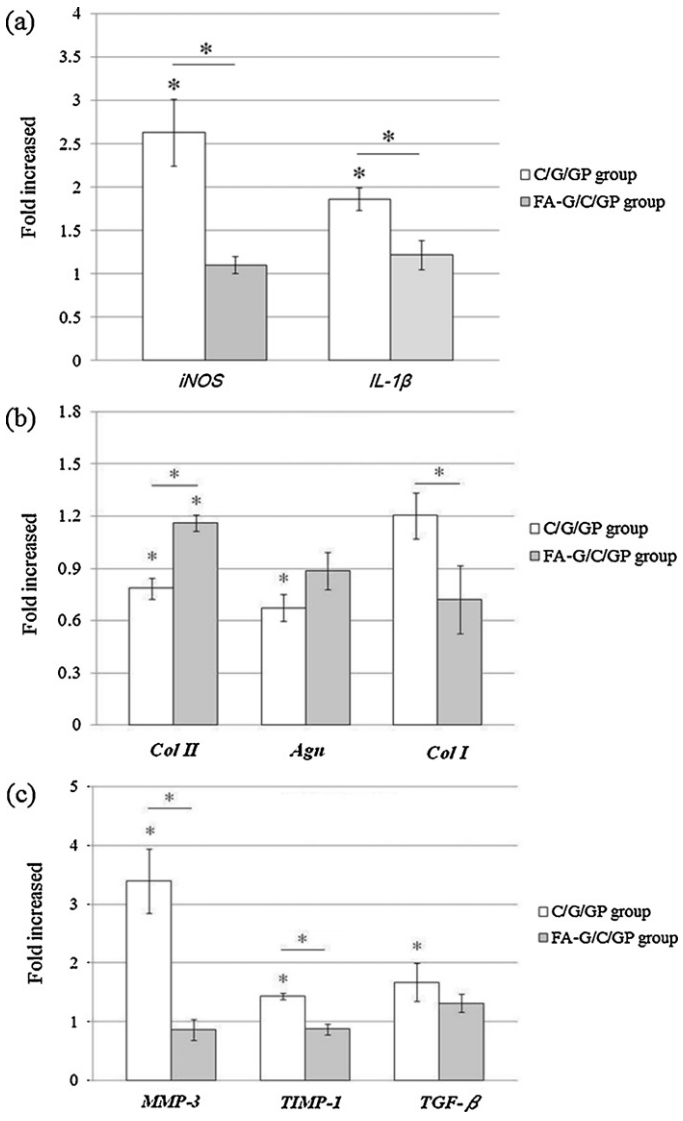

Fig. 3. The expression of (a) inducible nitric oxide synthase (iNOS) and interlukin-1β (IL-1β), (b) collagen-II (Col II), aggrecan (Agn) and collagen-I (Col I) and (c) matrix metalloproteinase-3 (MMP-3), tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-β (TGF-β) in the normal NP cells (without treatment, control group), post-treatment of C/G/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (C/G/GP group) and post-treatment of FA–G/C/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (FA–G/C/GP group). The result was expressed as fold increase compared with normal NP cells. Each target gene was normalized to GAPDH ($n = 3$, $^*p < 0.05$).

group). Matrix metalloproteinase-3 (MMP-3) and tissue inhibitor of metalloproteinase-1 (TIMP-1) are catabolic gene and anti-catabolic gene respectively. As shown in Fig. 3(c), the expression of MMP-3 and TIMP-1 were significantly up-regulated in C/G/GP group with 3.3934 and 1.4314-folds to the control group, respectively. There was no significant difference in the expression of MMP-3 and TIMP-1 between the control group and FA–G/C/GP group. The expression of MMP-3 and TIMP-1 were significantly down-regulated in FA–G/C/GP group compared with C/G/GP group. As shown in Fig. 3(c), the expression of TGF-β was significantly higher in C/G/GP group (1.6735 ± 0.3179) compared with control group. There was no significant difference in the expression of TGF-β between the control group and FA–G/C/GP group.

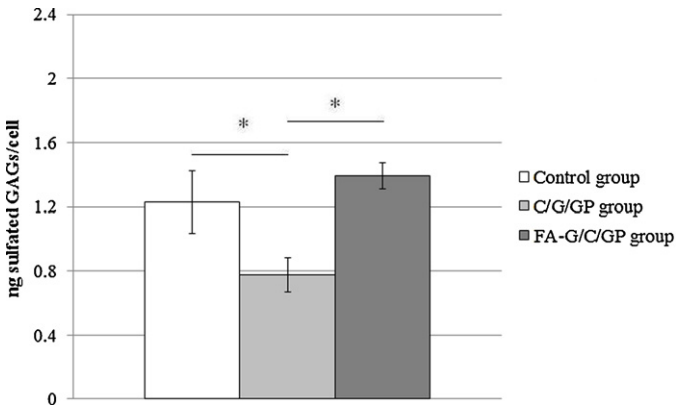

Fig. 4. The sulfated-GAGs production per cell in normal NP cells (without treatment, control group), post-treatment of C/G/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (C/G/GP group) and post-treatment of FA–G/C/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (FA–G/C/GP group) ($n = 3$, $^*p < 0.05$).

3.6. Sulfated-GAG production

The sulfated-GAG production was calculated form the standard curve which was constructed by condroitin-6-sulfate. The results of sulfated-GAG production were normalized to cell number. As shown in Fig. 4, the sulfated-GAG production per cell was significantly decreased in C/G/GP group (0.775 ± 0.105) compared with other groups. There was no significant difference in the sulfated-GAG production between the control group (1.228 ± 0.196) and the FA–G/C/GP group (1.389 ± 0.082).

3.7. Caspase-3 activity

Activation of caspase-3 was highly associated with apoptosis. The caspase-3 concentration of the sample was calculated by the linear standard curve of pNA. The results of caspase-3 activity were expressed in percentage. As shown in Fig. 5, the caspase-3 activity was significantly increased in C/G/GP group ($126.5 \pm 5.5\%$) compared with control group ($97.7 \pm 1.0\%$). There was no significant difference in caspase-3 activity between FA–G/C/GP group ($96.1 \pm 4.8\%$) and control group.

3.8. TUNEL staining

Detection of apoptosis was performed by TUNEL assay which can label the nuclei of apoptotic cells. There were lots of TUNEL-positive cells in C/G/GP group (Fig. 6(b)). In contrast, there

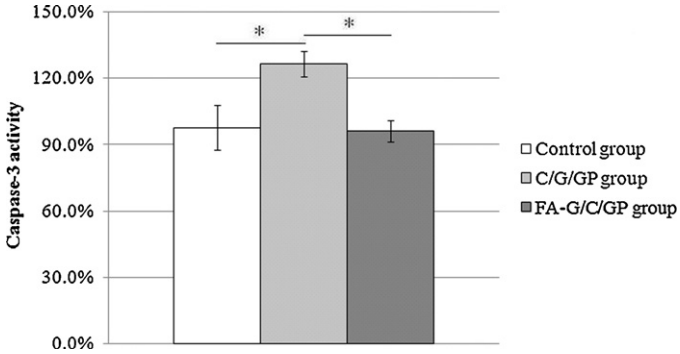

Fig. 5. The caspase-3 activity in normal NP cells (without treatment, control group), post-treatment of C/G/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (C/G/GP group) and post-treatment of FA–G/C/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (FA–G/C/GP group) ($n = 3$, $^*p < 0.05$).

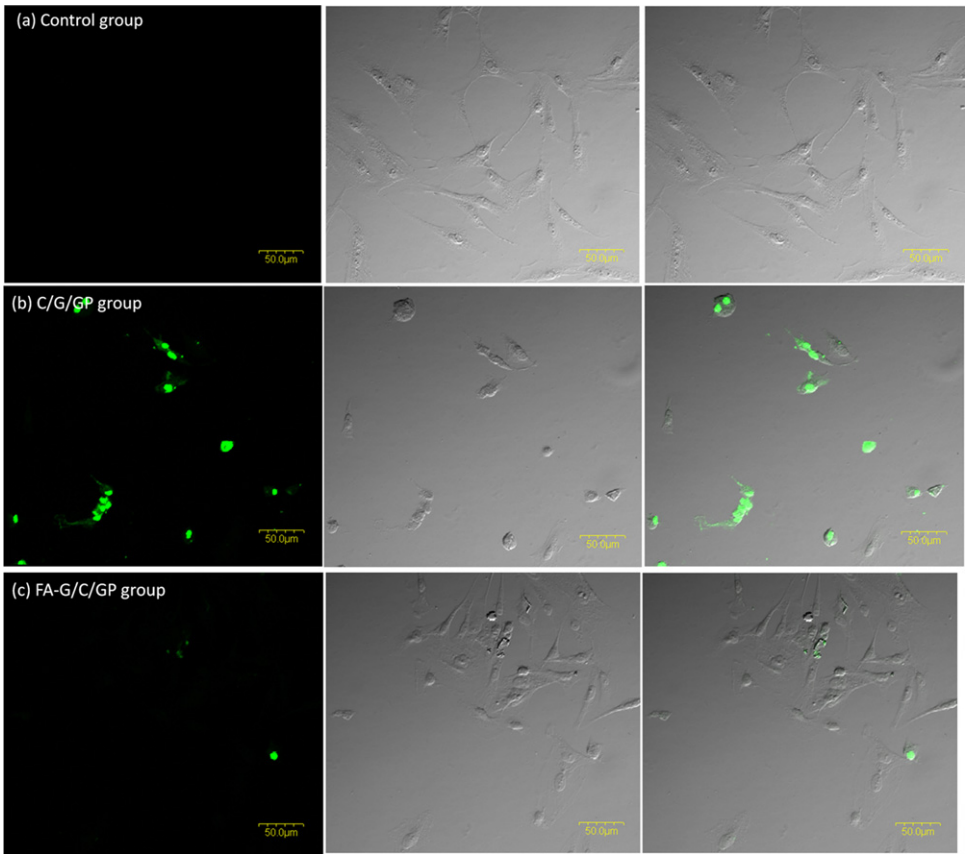

Fig. 6. TUNEL staining of (a) normal NP cells (without treatment, control group), (b) post-treatment of C/G/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (C/G/GP group) and (c) post-treatment of FA–G/C/GP hydrogel on 100 μM H_2O_2-induced oxidative stress NP cells (FA–G/C/GP group).

were significantly less TUNEL-positive cells in both control group (Fig. 6(a)) and FA–G/C/GP group (Fig. 6(c)).

4. Discussion

The residual amino group of FA–gelatin was significantly decreased compared with gelatin group as shown in Table 1. The mechanism of antioxidant action of FA is through the hydroxyl group of FA which has the ability to donate a proton, and then to form the phenoxy radical. Phenoxy radical is a stable structure, which has five different resonance structures due to electron delocalization (Srinivasan et al., 2007). In this study, FA was linked to gelatin through amide bond formation and this chemical reaction would not affect the antioxidant property of FA.

In the previous study (Cheng et al., 2010), we suggested that the gelation mechanism of the thermosensitive C/G/GP hydrogel included hydrophobic interaction, hydrogen bonding, electrostatic interaction and molecular chain movement. The hydrophobic interactions have been assumed to be the main driving force to form the hydrogel consisting of chitosan and gelatin at 37 °C. FA is a hydrophobic compound. In the study, FA was covalently cross-linked to gelatin that may influence the hydrophilic properties of gelatin. As shown in Table 2, the gelation temperature of FA–G/C/GP was 32.17 °C that was lower than C/G/GP hydrogel 33.88 °C (Cheng et al., 2010). The results suggested that FA–gelatin may accelerate the gelation process through increasing hydrophobic interaction of the hydrogel. This type of gelation mechanism has also been reported in other cases (Bhattarai et al., 2005; Roughley et al., 2006). The results of rheological analysis indicated that FA–G/C/GP solution could turn into a gel within 1 min at 37 °C and remain liquid for 15 min at 25 °C (Table 2). The results suggested that developed hydrogel also had good gelation and handling property for clinical application.

The results of cytotoxicity test showed that FA–G/C/GP hydrogel was biocompatible (Fig. 1). The concentration of FA in the FA–G/C/GP hydrogel was 500 μM. In the previous study (Cheng, Yang, & Lin, 2011; Cheng, Yang, Yang, et al., 2011), we demonstrated that there was no significant cytotoxicity to NP cells if the concentration of FA was lower than 500 μM. Recent studies indicated that the apoptosis of NP cells could be induced by 100 μM H_2O_2 and the content of H_2O_2 was 0.13 ± 0.09 μmol in the human degenerative NP (Finkel & Holbrook, 2000; Wei, Brisby, Chung, & Diwan, 2008). In previous study (Cheng, Yang, & Lin, 2011; Cheng, Yang, Yang, et al., 2011), we found that the oxidative stress induced by 100 μM H_2O_2 can be significantly decreased to normal level by 0.5 μM of FA. As shown in Fig. 2(a), the cumulative concentration of FA released from developed hydrogel was 1.167 ± 0.179 μM within 30 min that was enough to stop the free radical reaction induced by 100 μM H_2O_2. Thermosensitive chitosan-based hydrogels have been reported that almost 80% of hydrophilic compounds were released in the first 2 days (Ruel-Gariépy, Chenite, Chaput, Guirguis, & Leroux, 2000). In contrast, hydrophobic compounds were released from hydrogel for at least 1 month (Ruel-Gariépy et al., 2004). As shown in previous study (Cheng, Yang, & Lin, 2011; Cheng, Yang, Yang, et al., 2011), the percentage of cumulative release of FA from FA-incorporated C/G/GP hydrogel was 16.6 ± 2.1% in the first 2 h, and it could be significantly decreased to 9.3 ± 0.7% in the FA–G/C/GP (Fig. 2(b)). The results demonstrated that FA–G/C/GP hydrogel could sustain the delivery and prolong the release period of FA compared with the direct incorporation of FA.

Oxidative stress can induce inflammation through activation of nuclear factor-kappa B (NF-κB) and then up-regulate the expression of pro-inflammatory gene such as IL-1β and iNOS (Martindale

& Holbrook, 2002; Rahman, Biswas, & Kirkham, 2006). As shown in Fig. 3 (a), the mRNA gene expression of iNOS and IL-1β were significantly up-regulated in C/G/GP group compared with control group. FA is a phenolic acid which can inhibit the activation of NF-κB by inhibition of IκB kinase activity and down-regulate the expression of pro-inflammatory genes (Rahman et al., 2006). The results indicated that FA release from the developed hydrogel could reduce the level of inflammation caused by oxidative stress.

In the degenerative NP, the imbalance between anabolisms and catabolism of the ECM results in loss of proteoglycans, decrease in collagen-II and increase in collagen-I (Anderson & Tannoury, 2005; Urban & Roberts, 2003). As shown in Fig. 3(b), the expression of collagen-II and aggrecan were significantly down-regulated in C/G/GP group compared with control group. The expression of collagen-II was significantly up-regulated in FA–G/C/GP group compared with other groups. Recent studies showed that polyphenol has the ability to prevent proteoglycan degradation (Li et al., 2008). In the study, we believe that FA might not only prevent proteoglycan degradation but also promote collagen-II synthesis.

In the normal NP, there is a balance between MMPs and TIMPs. MMPs have ability to degrade ECM components including proteoglycans and collagen-II (Goupille, Jayson, Valat, & Freemont, 1998). Recent studies indicated that overproduction of ROS can promote MMPs production through an increase of pro-inflammatory mediators (Hadjipavlou, Tzermiadianos, Bogduk, & Zindrick, 2008; Rahman et al., 2006). Activation of MMPs can induce the expression of TGF-β which can inhibit the MMPs activity by up-regulation of TIMPs (Liu & Gaston Pravia, 2010). The results suggested that the up-regulation of TGF-β might reflect that H_2O_2-induced oxidative stress NP cells attempt to balance the rate of synthesis and degradation of matrix by up-regulation of TIMP-1. High expression of TGF-β has been observed in the degenerative NP that is associated with inflammation (Peng et al., 2006; Sowa et al., 2008). The results showed that FA could reduce cellular inflammation caused by oxidative stress.

Aggrecan is a major proteoglycan in the NP and consists of core protein with attached glycosaminoglycan (GAG) including chondroitin sulfate and keratin sulfate. The decrease in the GAG content has been observed in the degenerative NP (Anderson & Tannoury, 2005). Fig. 4 showed that the sulfated-GAG production per cell was significantly decreased in C/G/GP group compared with other groups. The sulfated-GAG content was reached to the normal level in the FA–G/C/GP group.

Oxidative stress mediated apoptosis occurs via intrinsic pathway which promotes the release of cytochrome c from mitochondria and activates the caspase-9 activity. Activation of caspase-9 can lead to activation of caspase-3 which can cleave cellular substrate and result in apoptosis (Earnshaw, Martins, & Kaufmann, 1999; Hengartner, 2000). As shown in Fig. 5, the caspase-3 activity was significantly increased in C/G/GP group compared with control group. The caspase-3 activity was decreased to normal level in FA–G/C/GP group. The results of TUNEL staining showed that there were lots of TUNEL-positive cells in C/G/GP group (Fig. 6(b)), but only a few TUNEL-positive cells in both control group (Fig. 6(a)) and FA–G/C/GP group (Fig. 6(c)). Recent studies indicated that high level of ROS can directly damage both cells and ECM (Stern, Kogan, Jedrzejas, & Soltés, 2007; Walker & Anderson, 2004). Polyphenol family has been demonstrated to have ability to inhibit apoptosis by decreasing ROS levels (Li et al., 2008; Rahman et al., 2006). FA processes an excellent antioxidant property that can inhibit oxidant reaction caused by ROS. In the study, the results showed that FA could inhibit the apoptosis of H_2O_2-induced oxidative stress NP cells and prevent further cellular damage.

5. Conclusion

In the study, we developed FA–G/C/GP hydrogel which is liquid form at room temperature and turn into a gel at body temperature. FA–G/C/GP hydrogel is biocompatible. The FA–G/C/GP hydrogel can prolong the release period of FA. From the results of mRNA gene expression, FA–G/C/GP may treat H_2O_2-induced oxidative stress NP cells through down-regulation of MMP-3 and up-regulation aggrecan and collagen-II. The sulfated-GAG production of 100 μM H_2O_2-induced oxidative stress NP cells can be increased to the normal level in the post-treatment of FA–G/C/GP hydrogel group. The results of caspase-3 activity and TUNEL staining show that the apoptosis of NP cells caused by oxidative stress can be inhibited by post-treatment of FA–G/C/GP hydrogel. These results suggest that thermosensitive FA–G/C/GP hydrogel may have potential application for NP regeneration in the near future.

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