Effect of JJYMD-C, a novel synthetic derivative of gallic acid, on proliferation and phenotype maintenance in rabbit articular chondrocytes *in vitro*

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Abstract

Tissue engineering encapsulated cells such as chondrocytes in the carrier matrix have been widely used to repair cartilage defects. However, chondrocyte phenotype is easily lost when chondrocytes are expanded *in vitro* by a process defined as "dedifferentiation". To ensure successful therapy, an effective pro-chondrogenic agent is necessary to overcome the obstacle of limited cell numbers in the restoration process, and dedifferentiation is a prerequisite. Gallic acid (GA) has been used in the treatment of arthritis, but its biocompatibility is inferior to that of other compounds. In this study, we modified GA by incorporating sulfamonomethoxine sodium and synthesized a sulfonamido-based gallate, JJYMD-C, and evaluated its effect on chondrocyte metabolism. Our results showed that JJYMD-C could effectively increase the levels of the collagen II, Sox9, and aggrecan genes, promote chondrocyte growth, and enhance secretion and synthesis of cartilage extracellular matrix. On the other hand, expression of the collagen I gene was effectively down-regulated, demonstrating inhibition of chondrocyte dedifferentiation by JJYMD-C. Hypertrophy, as a characteristic of chondrocyte ossification, was undetectable in the JJYMD-C groups. We used JJYMD-C at doses of 0.125, 0.25, and 0.5 μg/mL, and the strongest response was observed with 0.25 μg/mL. This study provides a basis for further studies on a novel agent in the treatment of articular cartilage defects.

Key words: Sulfamonomethoxine sodium; Gallic acid; Pro-chondrogenic agent; Chondrocyte; Rabbit articular cartilage; Dedifferentiation

Introduction

Articular cartilage has poor regenerative capacity after injury (1-3). Catabolic factors such as pro-inflammatory cytokines, which can induce a gradual self-destruction of cartilage, are activated after injury, finally resulting in secondary osteoarthritis. The poor healing capability of cartilage and dense extracellular matrix (ECM), which prevent chondroprogenitors from migrating to the injury site, altogether contribute to irreversible cartilage loss (2,4).

Tissue engineering encapsulated cells in carrier matrix have been widely used to repair cartilage defects (5-8) and are considered to be a promising strategy for regeneration of cartilage defects. Shaped cartilage has been regenerated *in vitro* and in immunocompromised animals by using autologous, allogeneic, or xenogeneic transplants (9). However, translation to immunocompetent animals or clinical use has proven to be difficult because post-injury inflammation and sustained inflammatory reactions may inhibit transplanted chondrocytes to synthesize sufficient ECM (10). Another difficulty is dedifferentiation of chondrocytes during expansion *in vitro*, which is necessary for cell-based therapy. Since dedifferentiated chondrocytes produce a non-cartilage-specific ECM characterized by inferior mechanical properties, they are not suitable for cell-based therapy. Finding anti-inflammatory mediators that can also restore

cartilage function is a prerequisite to support neo-cartilage formation and inhibit post-traumatic cartilage inflammation.

Gallic acid (GA) and its derivatives are a group of polyphenolic compounds known to affect several pharmacological and biochemical pathways, in addition to their strong anti-oxidation (11-13) and anti-inflammatory properties (14,15). It was reported that GA could induce apoptosis in rheumatoid arthritis fibroblast-like synoviocytes through regulation of apoptosis-related protein expression and reduction in the expression of pro-inflammatory mediators, such as pro-inflammatory cytokines, chemokines, cyclooxygenase-2, and matrix metalloproteinase-9 (16). Another study reported that GA could attenuate the pro-inflammatory and pro-oxidant effects caused by tumor necrosis factor-α, interleukin-6, NADPH oxidase, and thioredoxin-interacting protein. It can also attenuate DNA damage and suppress hyperglycemia-induced activation of pro-inflammatory and pro-oxidant gene expression (17). The study by Kim et al. (18) showed that GA was a histone acetyltransferase inhibitor and could suppress β-amyloid neurotoxicity by inhibiting microglial-mediated neuroinflammation. However, GA was reported to suppress cell proliferation. The investigation by Ou et al. (19) indicated that GA was an effective anti-atherogenic agent in vascular smooth muscle cells by attenuating cell cycle progression via AMP-activated protein kinase-mediated activation of endothelial nitric-oxide synthase (19). Being much more hydrophilic than its esters, GA displayed much weaker pharmacological effects in cell systems (11). Thus, modification of GA to improve its cytocompatibility is necessary.

Sulfamonomethoxine sodium has been used as a synthetic antibiotic for a long time because it is inexpensive and readily available. Most importantly, it easily penetrates through the cell membrane and into tissues and body fluids. Recently, new N-isopropoxy-arylsulfonamide-based hydroxamate inhibitors were shown to be effective in a collagen *in vitro* assay and cartilage degradation model (20). These compounds contain several sulfonamide groups that could benefit cell growth. This study indicates that modification of the GA with a sulfa drug may promote chondrogenesis.

In this study, we synthesized a novel sulfonamide-based gallate to further examine its effect on chondrocyte metabolism. This study provides evidence for its application in cartilage tissue engineering.

Material and Methods

Synthesis of JJYMD-C

3,4,5-Triacetoxy-N-{4-[(6-pyrimidin-4-yl)sulfamoyl]-phenyl}benzamide (JJYMD-C) was prepared from GA and sulfamonomethoxine sodium. A scheme of the synthetic route is shown in Figure 1. After reaction c, distilled water was added to the mixture. The raw product (powder) was then precipitated and separated by vacuum filtration. The powder was recrystallized from methanol/tetrahydrofuran.

Articular cartilage cell culture

Articular chondrocytes were dissociated from knee joint cartilage slices of 1-week-old New Zealand rabbits by enzymatic digestion. In brief, two New Zealand rabbits were used, and cartilage slices were dissociated with trypsin (0.25% aqueous solution; Solarbio, China; 30 min; 37°C) and then with type-II collagenase (2 mg/mL; Gibco, USA) in alpha-modified Eagle's medium (α-MEM; Gibco; 3 h; 37°C). After simple centrifugation (300 g, 5 min, 37°C), the chondrocytes were resuspended with α-MEM containing fetal bovine serum (20%, v/v; Gibco) and penicillin/streptomycin (1%, v/v; Solarbio) and cultured in a humidified incubator (5% CO_2; 37°C; 6 days). The culture medium was replaced every 3 days after plating. Rabbit chondrocytes (passage 1) were used for further studies.

JJYMD-C treatment

JJYMD-C dissolved in dimethylsulfoxide (DMSO; Sigma, USA) was prepared as stock solution and stored (-4°C). This stock solution was then added to the cell cultures to provide different concentrations of JJYMD-C. Culture media containing different concentrations of JJYMD-C were replaced every 3 days.

Cytotoxicity assay

The assay for JJYMD-C cytotoxicity to chondrocytes was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Gibco) method. Chondrocytes were digested using trypsin/EDTA (ethylenediaminetetraacetic acid) solution (0.25%; Solarbio) when cells of passage 1 reached 60-70% confluence. After centrifugation (300 g, 5 min, 37°C), the cells were resuspended in fresh medium, counted using a hemocytometer, and then seeded on 96-well plates. The final cell number in each well was 5×10^3. Concentrations of
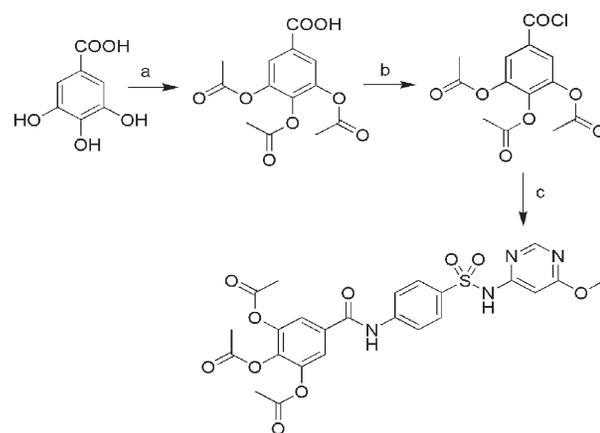

Figure 1. Schematic route and procedures for the synthesis of JJYMD-C. Reagents and conditions: (a) acetyl oxide, oil bath, 120°C; (b) $SOCl_2$, oil bath, 80°C; (c) sulfamonomethoxine sodium, tetrahydrofuran, pyridine, ice bath (0°C).

JJYMD-C (0.00-2.5 μg/mL) were then added to the cell cultures. After 3 days of culture, the cytotoxicity assay was carried out by MTT analysis. Briefly, a solution of MTT in phosphate-buffered saline, pH=7.0, was added to each well (final concentration: 5 mg/mL) and incubated (37°C; 4 h). After the medium was removed, DMSO (200 μL) was added to dissolve the MTT formazan formed by the metabolically viable cells. Absorbance (570 nm) of MTT was measured by an enzyme-labeled meter (Thermo Fisher Scientific, UK). All experiments were performed in triplicate.

Cell proliferation analysis and biochemical assay

After culture (2, 4, and 6 days), the cells removed from the old media were digested with a proteinase K solution (Sigma) for the following biochemical assays. Intracellular secretion of glycosaminoglycans (GAGs) was assayed by the 1,9-dimethylmethylene blue dye (Sigma) and DNA content was quantified using the Hoechst 33258 dye (Sigma) assay. In each sample, absorbance (460 nm) of total intracellular DNA content, as indicated by the Hoechst 33258 dye, was measured using a spectro-fluorometer. Absorbance (525 nm) of total intracellular GAG secretion was measured spectrophotometrically and converted into concentration using a chondroitin sulfate (Sigma) standard curve. Production of GAGs by each chondrocyte was normalized to the total DNA content of all chondrocytes, which displayed their biosynthetic activity in the various culture media.

Safranin-O staining

Histology of chondrocytes was performed to assess GAG synthesis using safranin-O/Fast Green staining. After the cells were fixed in 95% ethanol (30 min), they were successively stained with 0.02% aqueous Fast Green for 5 min (Sigma) and 0.1% safranin O for 10 min (Sigma), immediately washed with tap water, and dried naturally at room temperature. The cells were then sealed with a neutral gum, observed, and photographed under an inverted phase-contrast microscope (Zeiss Corporation, Germany).

Morphological examination

After 2, 4, and 6 days of incubation, the chondrocytes were fixed in 95% ethanol before hematoxylin-eosin staining. Cells were initially stained with the nuclear (3 min) and then with the cytoplasm (5 s) dye. Subsequently, the cells were rinsed in tap water, dried naturally at room temperature and sealed with neutral gum. They were then examined and photographed using an inverted phase-contrast microscope (Zeiss Corporation).

Cell viability assay

Cell viability of chondrocytes was determined by fluorescein diacetate (FDA; Genway Biotech Inc., USA)/ propidium iodide (PI; Sigma) staining at days 2, 4, and 6. Briefly, FDA and PI stock solutions were added to the cells (final concentration: 2 μM and 2 μg/L, respectively), and they were incubated in the dark (37°C; 5 min). Images were taken using a laser-scanning confocal microscope (Nikon A1, Japan).

Immunohistochemical staining

Secretion of type I and II collagens was characterized immunohistochemically using an immunohistochemical staining kit (Boster, China). To visualize collagen, the cells were first fixed in paraformaldehyde (4%, w/v) and treated with Triton X-100. To exclude endogenous peroxidase activity, the cells were then incubated at room temperature with H_2O_2 (3%; 10 min). In addition, the plates were blocked at room temperature with normal goat serum (10 min). Human anti-rabbit antibody (type I and II collagens) was added after dilution (1:200) and the cells were then incubated with the second antibody and biotin-labeled horseradish peroxidase. Subsequently, antibody binding was visualized with 3,3′-diaminobenzidine tetra-hydrochloride (DAB kit, Boster) before brief counterstaining with hematoxylin. Cells were gradually dehydrated, sealed with neutral gum, observed, and photographed using an inverted phase-contrast microscope (Zeiss Corporation).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The genetic information for type I, II, and X collagens, aggrecan, and Sox9 genes was detected by qRT-PCR (Table 1). Total intracellular RNA was extracted employing the RNA isolation kit (Tiangen Biotechnology, China) according to the manufacturer's instructions. About

Table 1. Genes and primer design for quantitative real-time polymerase chain reaction analysis.

| Genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
GAPDH	GTCATCATCTCAGCCCCCTC	GGATGCGTTGCTGACAATCT
Aggrecan	TTGCCTTTGTGGACACCAGT	GAGCCAAGGACGTAAACCCA
Sox9	GACGCACATCTCGCCCAAC	TCTCGCTTCAGGTCAGCCTT
Type I collagen	CCCAGCCACCTCAAGAGAAG	CGGGGCTCTTGATGTTCTCA
Type II collagen	TCCGGAAACCAGGACCAAAG	CTTTGTCACCACGGTCACCT
Type X collagen	CTACGCTGAGCGGTACCAAA	GGCTTCCCAGTGGCTGATAG
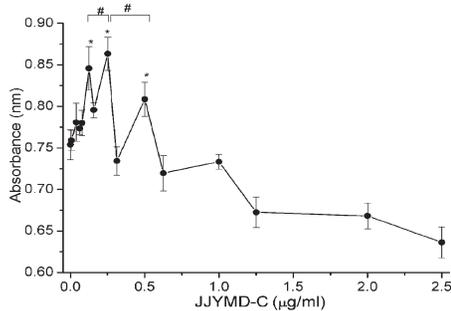
Figure 2. Cytotoxicity analysis of chondrocytes after 3 days of treatment with different concentrations of JJYMD-C. Data are reported as means ± SD for n = 20. *P<0.05 compared to control; #P<0.05 comparisons as indicated (one-way ANOVA followed by the Dunnett *post hoc* test).

300 ng total RNA was used as a template and reverse-transcribed into cDNA using a reverse transcription kit (Fermentas Company, USA). qRT-PCR was performed using the Quantitative PCR Detection System (Realplex 4, Eppendorf Corporation, USA) with FastStart Universal SYBR Green Master Mix (Roche Company, Germany) at 95°C (10 min) and 60°C (1 min). The melting curve data were collected to verify PCR specificity. Each gene was analyzed in triplicate to diminish operation error. Relative gene expression levels were calculated using the 2-ΔΔCt method relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

Statistical analysis

Results are reported as means ± SD. Statistical differences were determined using one-way ANOVA followed by the Dunnett *post hoc* test. The level of significance was set at P<0.05.

Results

Preparation of JJYMD-C

The procedure for synthesis of JJYMD-C from GA and sulfamonomethoxine sodium is shown in Figure 1. JJYMD-C has the following properties: white powder, yield 59%, m.p. >270°C, MS-ESI: m/z: 557.0 [M-H]-, ^{1}H-NMR (400 MHz, DMSO) δ 10.69 (s, 1H, -CO-NH), 8.41 (s, 1H, Py-H), 7.92 (m, 4H, 4 × Ar-H), 7.82 (s, 2H, Ar-H), 6.73 (s, 1H, Py-H), 3.83 (s, 3H, -OCH3), 2.33 (s, 3H, -CH3), 2.31 (s, 6H, 2 × -CH3). ^{13}C-NMR (125 MHz, DMSO) δ 169.99, 168.05, 166.99, 163.73, 158.56, 143.22, 142.85, 137.68, 132.45, 128.10, 120.79, 120.14, 90.94, 54.23, 20.34, 19.90.

Cytotoxicity assay

Cytotoxicity of JJYMD-C on articular chondrocytes was examined by the MTT assay. Articular chondrocytes were treated with three different increasing concentrations of JJYMD-C (range: 0.0-2.5 μg/mL). As shown in Figure 2, absorbance values for JJYMD-C concentrations were comparable to those of the control in the range of 0.125-0.5 μg/mL.

Cell proliferation

In this study, cell proliferation in the experimental and control groups was analyzed by measurements of DNA content. Comparatively, cells cultured with JJYMD-C (J1, J2, and J3: JJMYD-C of 0.125, 0.25, and 0.5 μg/mL, respectively) grew more than those in the control group
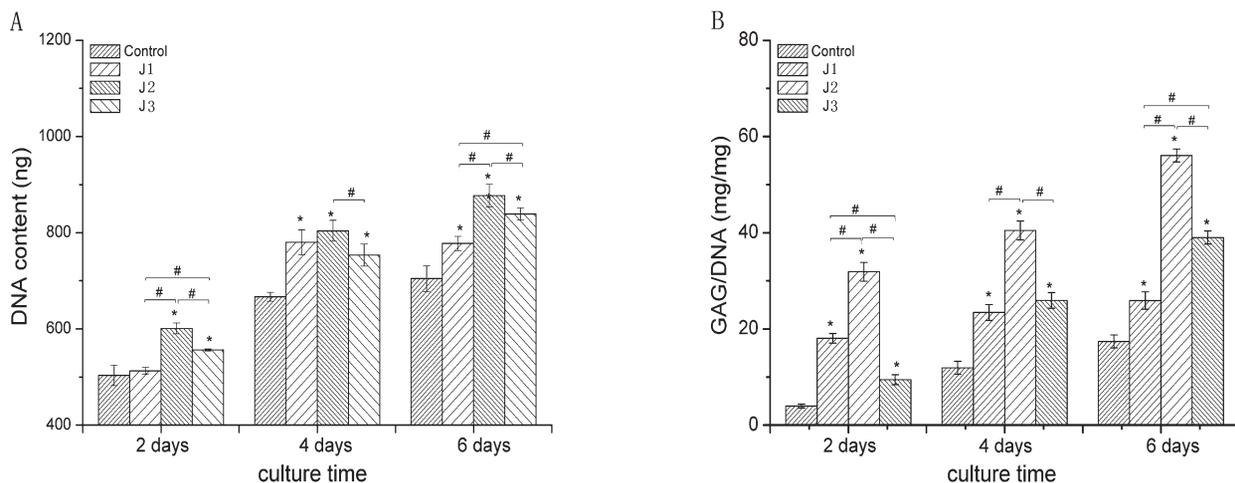

Figure 3. Quantification of cell proliferation by detection of DNA content and matrix production by glycosaminoglycan (GAG) analysis. *A*, Proliferation of chondrocytes cultured *in vitro* with 0 (control), 0.125 (J1), 0.25 (J2), and 0.5 μg/mL (J3) JJYMD-C for 2, 4, and 6 days. *B*, GAG level (mg) normalized to DNA content (mg). Data are reported as means ± SD for 4 independent experiments *P<0.05 compared to control; #P<0.05 comparisons as indicated (one-way ANOVA followed by the Dunnett *post hoc* test).
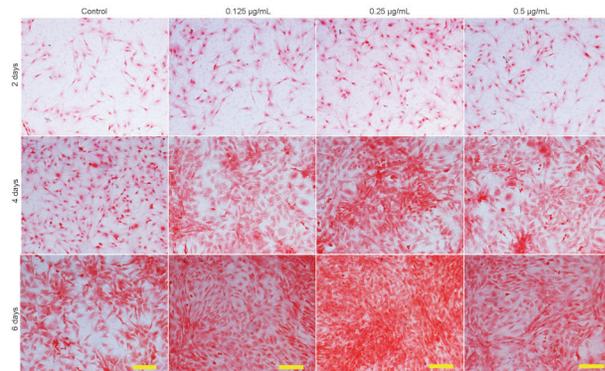
Figure 4. Safranin-O-staining showing the synthesis of extracellular matrix by chondrocytes cultured *in vitro* with 0 (control), 0.125, 0.25, and 0.5 µg/mL JJYMD-C for 2, 4, and 6 days. Scale bar: 100 µm.

Figure 6. Laser-scanning confocal microscopy images showing the viability of chondrocytes cultured *in vitro* with 0 (control), 0.125, 0.25, and 0.5 µg/mL JJYMD-C for 2, 4, and 6 days. Scale bar: 100 µm.

(P<0.05), as indicated by higher DNA values (Figure 3A), at the same period. Furthermore, among different concentrations of JJYMD-C, the highest cell proliferation was achieved at 0.25 µg/mL.

Secretion of GAGs

Extracellular production of GAGs by rabbit articular chondrocytes was measured by biochemical assay after 2, 4, and 6 days of culture. Regarding intracellular production of GAGs, given as a ratio of GAGs to DNA at different concentrations of JJYMD-C (Figure 3B), production of GAGs was time-dependent in all groups. Quantitatively, GAG production in culture media treated with JJYMD-C was increased significantly more than that in the control at the same times. JJYMD-C at 0.25 µg/mL promoted the highest GAG synthesis among the three concentrations.

The safranin-O-positive stain in the JJYMD-C group indicated that GAGs were abundant and homogeneously
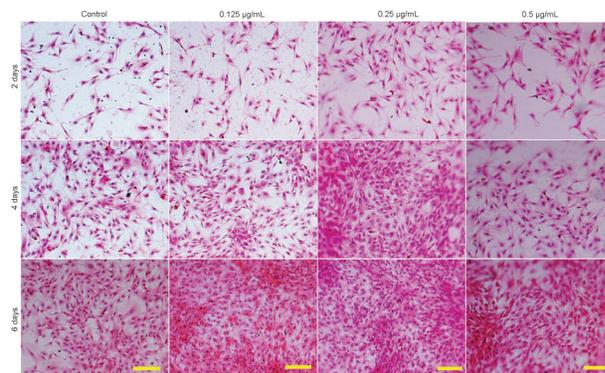
distributed around the chondrocytes (Figure 4).

Cell morphology

We assessed the morphology of articular chondrocytes by inverted microscopy after treatment with JJYMD-C at concentrations of 0.125, 0.25, and 0.5 µg/mL (Figure 5). No significant differences were observed in cartilaginous morphology between the experimental groups after 2, 4, and 6 days of culture in the presence of increasing concentrations of JJYMD-C. Compared with the control, the chondrocytes grew better in the presence of JJYMD-C and showed a distinctive proliferation tendency with gradually increasing culture time. In addition, JJYMD-C could enhance proliferation of chondrocytes at the concentration of 0.25 µg/mL better than in other groups.

Cell viability assay

Viable cells and dead cells were visualized using the calcein-acetoxymethyl ester (AM)/PI double stain (Figure

Figure 5. Hematoxylin-eosin staining showing the morphology of chondrocytes cultured *in vitro* with 0 (control), 0.125, 0.25, and 0.5 µg/mL JJYMD-C for 2, 4, and 6 days. Scale bar: 100 µm.
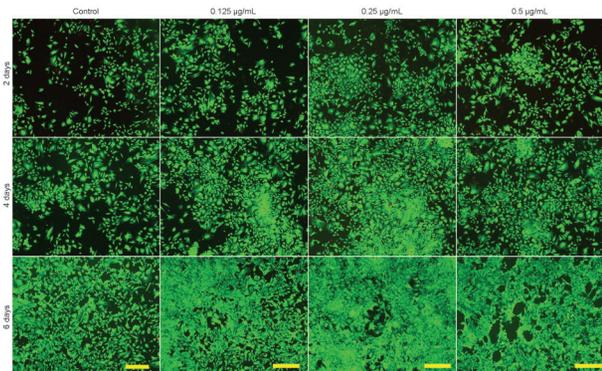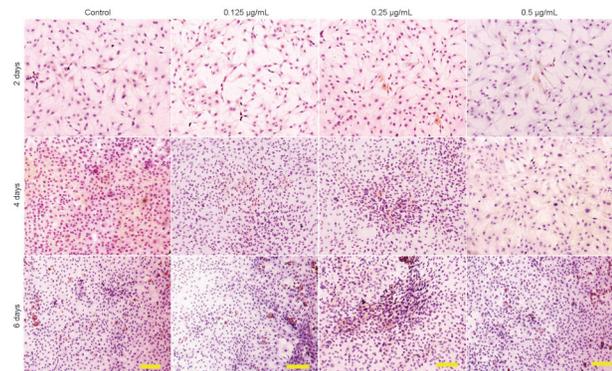

Figure 7. Immunohistochemical staining revealed the presence of type I collagen. Chondrocytes cultured *in vitro* with 0 (control), 0.125, 0.25, and 0.5 µg/mL JJYMD-C for 2, 4, and 6 days. Scale bar: 100 µm.
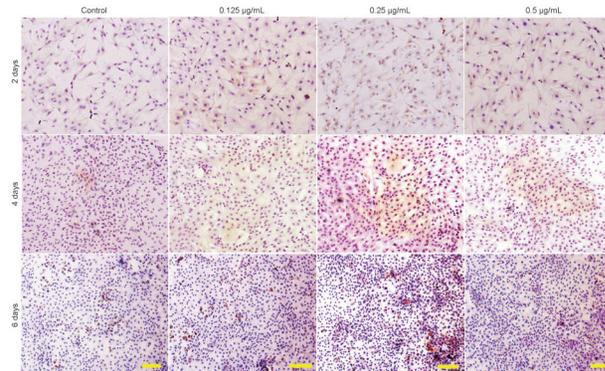
Figure 8. Immunohistochemical staining revealed the presence of type II collagen. Chondrocytes cultured *in vitro* with 0 (control), 0.125, 0.25, and 0.5 μg/mL JJYMD-C for 2, 4, and 6 days. Scale bar: 100 μm.

6). Calcein-AM/PI staining showed that survival in the JJYMD-C groups was more frequent than in the control group. Consistent with the results of cell proliferation (Figure 3), viable cells were far more frequent than dead cells in different concentrations of JJYMD-C. As evidenced by the higher number of live cells, 0.25 μg/mL JJYMD-C was superior to others.

Secretion of type I and type II collagens

Figures 7 and 8 show expression of type I and II collagens in the cytoplasm of chondrocytes at different treatment times with and without drug-treated culture media. Strongly positive staining is evident in large areas for cartilage-specific type II collagen and very sparse with light staining for type I collagen in the presence of JJYMD-C. In contrast, less positive staining of collagen II and less negative staining of collagen I were present in the control. At 0.25 μg/mL, JJYMD-C could maintain the phenotype of chondrocytes better than at other concentrations.

Gene expression

The effect of JJYMD-C on synthesis of ECM by chondrocytes was further investigated through gene expression of collagens I, II, and X, Sox9, and aggrecan (a proteoglycan composed of GAGs) after 2, 4, and 6 days of culture. As shown in Figure 9, expression of cartilage-specific genes, such as aggrecan, collagen II, and Sox9, was significantly boosted by JJYMD-C at the three concentrations compared with the control. Moreover, the highest expression of collagen II, aggrecan, and Sox9 in the presence of different concentrations of JJYMD-C was observed at 0.25 μg/mL. JJYMD-C could up-regulate collagen II, aggrecan, and Sox9 expression. At the same time, collagen X expression was scarcely detectable in the presence of JJYMD-C, indicating that cell dedifferentiation and hypertrophy were not prominent. JJYMD-C at different concentrations induced lower
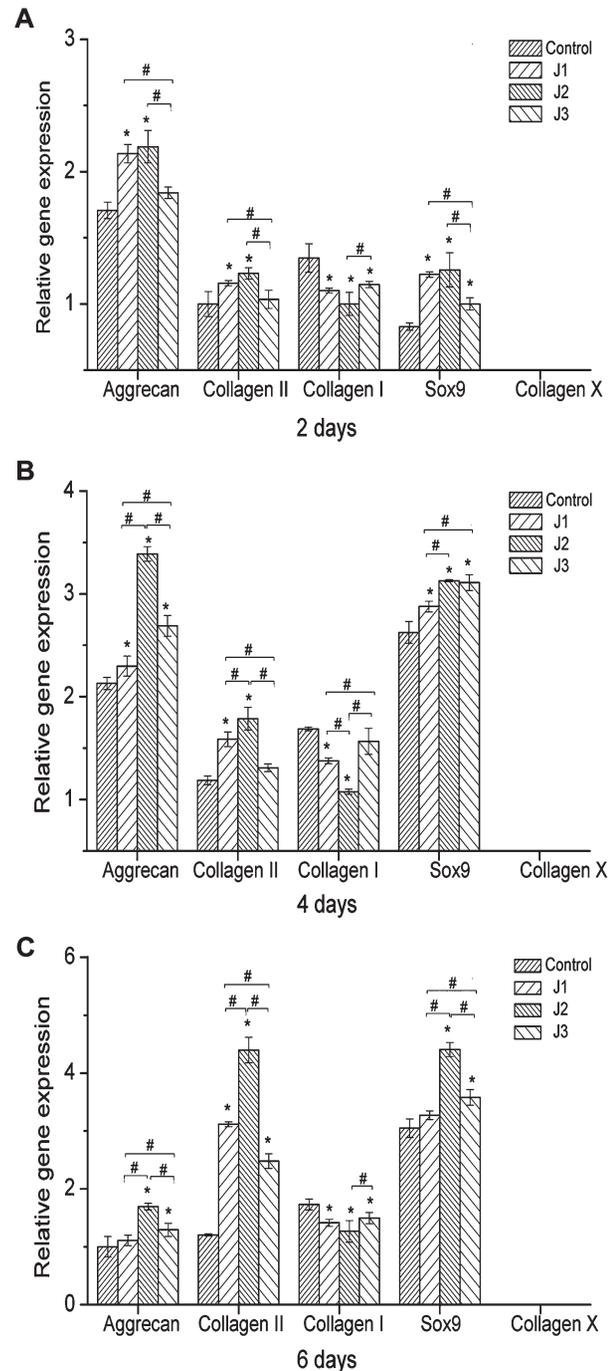

Figure 9. Quantitative comparison of extracellular matrix-related gene expression by quantitative real-time polymerase chain reaction. The chondrocytes were cultured with 0 (control), 0.125 (J1), 0.25 (J2), and 0.5 μg/mL (J3) JJYMD-C for 2 days (*A*), 4 days (*B*), and 6 days (*C*) (n = 3 for each experiment). The levels of gene expression in media containing JJYMD-C were analyzed by the 2-$\Delta\Delta$CT method, and glyceraldehyde-3-phosphate dehydrogenase was used as internal control. Data are reported as means ± SD of three independent culture experiments. *P < 0.05 compared to control; #P < 0.05 comparisons as indicated (one-way ANOVA followed by the Dunnett *post hoc* test).

expression of collagen I after culture for 2, 4, and 6 days compared with the control group. Moreover, the levels of collagen I with 0.25 µg/mL JJYMD-C were lower than with the other two concentrations.

Among three different concentrations, JJYMD-C at a concentration of 0.25 µg/mL demonstrated the highest expression of aggrecan and collagen II.

Discussion

GA, an endogenous plant polyphenol, which exists abundantly in tea, grapes, berries, and other plants, has important biological and pharmacological properties, such as anti-cancer (21-24), anti-oxidant (17,25), and anti-inflammatory (26) activities. It has been suggested that a more hydrophilic GA derivative would be more efficient in diffusing through the membrane polar phase (11,27). In this study, we synthesized a novel GA derivative, JJYMD-C, and examined its effect on chondrocyte metabolism. Our results indicated that JJYMD-C exhibited an effect of promoting chondrocyte growth compared with the control group, especially at the concentration of 0.25 µg/mL (Figures 3, 5, 6, and 7). As demonstrated by biochemical assay and safranin-O staining (Figures 3 and 4), JJYMD-C could obviously promote GAG deposition in cultured chondrocytes, which play a pivotal role in maintaining cartilage load-bearing capacity (28). In addition, our results (Figures 8 and 9) indicated that JJYMD-C in the range of 0.125-0.5 µg/mL enhanced the expression of collagen II, Sox9, and aggrecan genes, which are cartilage-specific markers (29,30). In JJMYD-C groups, 0.25 µg/mL showed the highest expression of aggrecan and collagen II.

Expression of type I collagen, which indicates dedifferentiation of chondrocytes (31-33), was significantly inhibited by JJYMD-C (Figures 8 and 9). In most cases, dedifferentiation occurred whereas the differentiated phenotype of chondrocytes consists primarily of type II collagen. Cartilage-specific proteoglycan is lost and replaced by a complex collagen phenotype consisting predominately of type I collagen with a low level of proteoglycan synthesis (34,35). Results from PCR, biochemical, and immunohistochemical analyses have shown that type I collagen expression was significantly down-regulated by JJYMD-C. Moreover, secretion of type X collagen, considered as characteristic of hypertrophic chondrocytes and endochondral ossification, was nearly undetectable in the presence of JJYMD-C (Figure 9) (36), implying that hypertrophy of chondrocytes would be

hardly induced by JJYMD-C. Therefore, dedifferentiation and hypertrophy may be prevented by JJYMD-C as evidenced by the reduced collagen I and the barely expressed collagen X.

As for the recommended dose of JJYMD-C, our results showed that chondrocyte proliferation is enhanced in the concentration range of 0.125-0.5 µg/mL (Figure 2). This figure shows that among the three different concentrations, that of 0.25 µg/mL contributed to the highest cell proliferation and matrix secretion stimulation.

Due to the poor pharmacological effects and biological properties of GA (17,21,22,26), the changes achieved with JJYMD-C are significant. Epigallocatechin-3-gallate (EGCG), a GA derivative, was found to inhibit degradation of human cartilage proteoglycan and type II collagen, and selectively inhibits ADAMTS-1, ADAMTS-4, and ADAMTS-5 (disintegrins and metalloproteinases with thrombospondin motifs) (37,38). It was also reported that EGCG improves IL-1β-mediated suppression of TGF-β synthesis and enhances type II collagen and aggrecan core-protein synthesis in human articular chondrocytes (39,40). In this study, JJYMD-C, a novel GA derivative, could also support chondrocyte growth maintaining the chondrocytic phenotype. This implies that suitable modifications in GA may lead to improvements in its pharmacological effects.

In conclusion, we showed that JJYMD-C could effectively promote proliferation of chondrocytes, and enhance secretion and synthesis of cartilage ECM. Meanwhile, it could prevent chondrocyte dedifferentiation by up-regulating the expression levels of the aggrecan, collagen II, and Sox9 genes while down-regulating the expression of collagen I gene. Hypertrophy leading to chondrocyte ossification was not detected in the media containing JJYMD-C. The best performance was obtained with the concentration of 0.25 µg/mL. Thus, JJYMD-C may be useful as a pro-chondrogenic agent for chondrocyte-based therapy.

Acknowledgments

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