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A novel synthesized sulfonamido-based gallic acid – LDQN-C: Effects on chondrocytes growth and phenotype maintenance

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ABSTRACT

Chondrocyte based therapy is promising to treat symptomatic chondral and osteochondral lesions. Growth factors to accelerate the proliferation and retain the phenotype of chondrocytes *in vitro* are imperative. However, the high cost and rapid degradation of growth factors limited their further application. Therefore, it is significant to find substitutes that can preserve chondrocytes phenotype and ensure sufficient cells for cytotherapy. Antioxidant and anti-inflammatory agents or their derivatives that have effect on arthritis may be an alternative. In this study, we synthesized sulfonamido-based gallate – LDQN-C and investigated its effect on rat articular chondrocytes through examination of the cell proliferation, morphology, viability, glycosaminoglycans (GAGs) synthesis and cartilage specific gene expression. Results showed that LDQN-C could enhance secretion and synthesis of cartilage extracellular matrix (ECM) by up-regulating expression levels of aggrecan, collagen II and Sox9 genes compared to the GA treated group and control group. Expression of collagen type II was effectively up-regulated while collagen I was down-regulated, which demonstrated that the inhibition of chondrocytes dedifferentiation by LDQN-C. Range of 1.36×10^{-9} M to 1.36×10^{-7} M is recommended dose of LDQN-C, among which the most profound response was observed with 1.36×10^{-8} M. GA at concentration of 0.125 μg/mL was compared. This study might provide a basis for the development of a novel agent for the treatment of articular cartilage defect.

1. Introduction

Articular cartilage has regenerative capacity after injury, which may lead to morbidity and functional impairment [1,2]. Traditional cartilage repair including subchondral drilling, microfracture, osteotomy and etc. were not satisfactory. Chondrocyte based approaches like autologous chondrocyte implantation (ACI), allogenous chondrocyte implantation are useful to treat symptomatic chondral and osteochondral lesions [3]. But several issues are involved: (1) the number of chondrocytes is limited; (2) chondrocytes are subject to age-related changes; (3) loss of phenotype which is called dedifferentiation, is the main problem confronted chondrocytes during expansion *in vitro* [4,5]. To retain the phenotype and accelerate the proliferation of chondrocytes culture *in vitro*, lots of growth factors like transforming growth factor (TGF-β1) and insulin-like growth factor (IGF) are employed [6]. However, the high cost, lose of activity and rapid degradation of growth factors limited their application in clinic. Therefore, to exploit low-cost and stable agents that will substitute for growth factors in the maintenance of phenotype of chondrocytes is of significance.

Gallic acid (GA) and its derivatives are a group of polyphenol compounds which have strong anti-oxidant effect, and have been known to impact several pharmacological and biochemical pathways [7]. It was reported that a lyophilized extract of wine which contains large amounts of phenolic components have the protective effects in cartilage alteration [8]. However, bioactivity of GA was compromised because it is much more hydrophilic than its esters, resulting in much weaker antioxidant effects than its esters

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in cell systems [7]. It was reported that GA suppressed cell proliferation [9]. Therefore, to introduce some lipophilic compounds may improve the bioactivity of GA and broaden its application.

Several derivatives of GA showed excellent biological properties. A sulfonamide-based GA derivatives can promote articular chondrocytes metabolism [10]. Epigallocatechin-3-gallate (EGCG), a derivative of GA, enhances aggrecan and type II collagen synthesis and ameliorates IL-1β-mediated suppression of TGF-β synthesis in human chondrocytes [11]. Recently, a series of new N-isopropoxy-arylsulfonamide hydroxamate inhibitors which contain several phenyl group and sulfonamide group were proved to be effective in inhibiting ex vivo cartilage degradation [12]. This implied that incorporating sulfonamides to GA may enhance the bioactivity and therefore support the growth of chondrocytes.

In this study, we synthesized sulfonamido-based gallate – N-[4-(6-Chloro-pyridazin-3-ylsulfamoyl)-phenyl]-3,4,5-trihydroxy-benzmide (LDQN-C) and investigated its chondroprotective effect through examination of the cell proliferation and expression of cartilage specific genes and proteins of chondrocytes compared with GA at concentration of 0.125 μg/mL.

2. Materials and methods

2.1. Synthesis of LDQN-C

LDQN-C was prepared from GA and Sulfachloropyrazine sodium. The synthetic procedure is presented in Fig. 1.

Electrospray ionization mass spectrum (ESI-MS) was recorded on a Shimadzu LC-MS 2010A. ^{1}H and ^{13}C NMR spectra were obtained from a Bruker Advance III 300 at 400 and 125 MHz, respectively.

2.2. Articular cartilage cells culturec

Joint cartilage were dissected from new-born SD rats followed by enzymatic digestion with 0.25% trypsin (Solarbio, China) for 30 min and then with 2 mg/mL collagenase type II (Gibco, USA) in Minimum Essential Medium Alpha Medium (α-MEM, Gibco, USA) for 3 h. After centrifuge (1000r, 5 min), the cells were resuspended in a basal culture medium of α-MEM containing 20% Fetal Bovine Serum (FBS, Gibco, USA) and 1% antibiotics (penicillin 100U/mL, streptomycin 100 U/mL). Culture conditions in a incubator (Thermo Fisher Scientific, UK) at 5% CO_2 37 °C humidified atmosphere.

2.3. LDQN-C treatment

LDQN-C was dissolved in sodium hydroxide solution (NaOH, Sigma, USA) of 0.1 mg/mL as stock solution and stored at 4 °C. The stock solution of LDQN-C was then added to the cell cultures to provide various concentrations. Culture medium containing various concentrations of LDQN-C was replaced every 3 days. As control, stock solution of GA with the concentration of 0.125 μg/mL was prepared.

2.4. Cytotoxicity test

The cytotoxicity of LDQN-C on chondrocytes was performed by the 3-(4, 5)-dimethylthiahiazo-(-z-y1)-3,5-di-phenytetrazoliumromide (MTT, Gibco, USA) method. Chondrocytes were seeded on 96-plates. The final cell number in each well was 5×10^3. Concentrations of LDQN-C ranged from 1.36×10^{-10} M to 54.5×10^{-3} M and GA ranged from 6.25×10^{-8} ~25 μg/mL were added to cell cultures.

After 3 days of culture, cytotoxicity test was carried out by MTT analysis. Briefly, a solution of MTT in PBS was added into each well with the final concentration of 5 mg/mL and incubated at 37 °C for 4 h. After the media was removed, 200 μL dimethylsulfoxide (DMSO; Sigma) was added, which was used to dissolve MTT formazan formed by metabolically viable cells. The absorbance was detected by an enzyme-labeled meter (Thermo Fisher Scientific, UK) at 570 nm. All experiments were performed in triplicate.

As determined by MTT analysis, the concentrations of LDQN-C at 1.36×10^{-9}, 1.36×10^{-8} and 1.36×10^{-7} M and of GA at 0.125 μg/mL, among which a peak was presented, were chosen for further investigation. Three groups were divided: (1) control group: chondrocytes without any treatment; (2) LDQN-C treatment groups: chondrocytes treated with various concentrations of LDQN-C (1.36×10^{-9}, 1.36×10^{-8} and 1.36×10^{-7} M); (3) GA treatment groups: chondrocytes treated with GA at concentration of 0.125 μg/mL.

2.5. Cell viability assay

The cell viability of chondrocytes was determined by Fluorescein diacetate (FDA, Genway Biotech, Inc, USA)/propidium iodide (PI, Sigma, USA) staining at day 2, 4 and 6 respectively. Briefly, FDA and PI stock solutions were added to the cells at a final concentration of 2 μmol/L and 2 μg/L respectively and incubated in

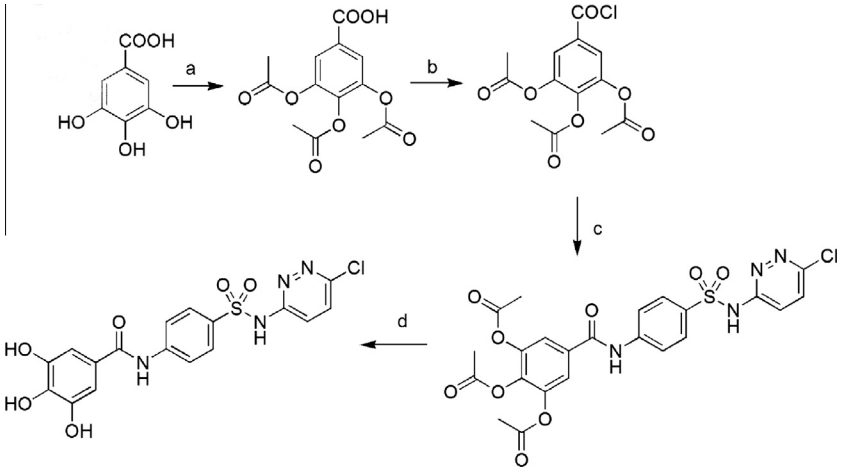

Fig. 1. Synthesis route of LDQN-C. Reagents and conditions: (a) acetyl oxide, oil bath, 120 °C (b) $SOCl_2$, oil bath, 80 °C (c) Sulfachloropyrazine sodium, THF, Pyridine, ice bath.

the dark for 5 min at 37 °C. Images were captured via a laser scanning confocal microscope (Nikon, Japan).

2.6. Cell morphological analysis

After being culture with different concentrations of LDQN-C for 2, 4 and 6 days, the cells were washed by PBS for three times and fixed with 95% alcohol for 30 min. Then the cells were washed by PBS again and stained with hematoxylin and eosin kit (HE, JianCheng Biotech, China) to observe the cell morphology. Images were photographed by an inverted phase contrast microscope (Zeiss Corporation, German).

Cells were stained for 30 min at room temperature with rhodamine phalloidin (Invitrogen, USA), followed by Hoechst 33258 (Beyotime, China) for 5 min to visualize nuclei. Imaging was performed using scanning confocal microscope (Nikon, Japan).

2.7. RNA extraction and qRT-PCR analysis

The real-time quantitative polymerase chain reaction (qRT-PCR) was to analysis the gene expression of aggrecan, collagen II, Sox9, collagen I and collagenX. The primer sequences and genbank accession numbers used for qRT-PCR are summarized in Table 1. Chondrocytes were seeded in 6-well plates and cultured alone or with LDQN-C (concentrations of 1.36×10^{-9}, 1.36×10^{-8} and 1.36×10^{-7} M) and GA at concentration of 0.125 μg/mL. After 2, 4 and 6 days, total RNA was successively extracted with a Total isolation RNA kit (Invitrogen, USA) according to the instructions of manufactures. Reverse transcription of RNA was carried out at 25 °C for 5 min, 42 °C 60 min and then 72 °C for 5 min using a reverse transcription kit (Fermentas Company, USA). The qRT-PCR reactions were performed using a Quantitative PCR Detection System (realplex 4, Eppendorf Corporation, USA) with a FastStart Universal SYBR Green Master (Mix, Roche Company, Germany) under the condition of 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C. The dissociation curve of each primer pair was analyzed to confirm the primer specificity. Marker gene expression of chondrocytes was analyzed by the $2^{-\Delta\Delta CT}$ method using β-actin. Each sample was repeated three times for each gene.

2.8. Cell proliferation analysis and biochemical assay

After cultured for 2, 4 and 6 days respectively, chondrocytes were washed by PBS, and then digested with 0.25% trypsin/ETDA. Cells were centrifuged and then responded in PBS containing 60 μg/mL proteinase K (Sigma, USA) for 16 h at 60 °C. The DNA content was determined by spectrofluorometer using Hoechst 33258 dye at 460 nm with calf thymus DNA as standard and the absorption of Hoechst 33258 dye as the baseline (13). The total secretion of glycosaminoglycan (GAG) was measured using 1, 9-Dimethylmethylene Blue (DMMB, SIGMA-ALORICH, USA) with Chondroitin Sulfate as standard. The GAG content was qualified on standard curve and accordingly normalized to the total DNA content. All the experiments were carried out in triplicate.

2.9. Safranin O staining

Safranin O-stained cells were scored for glycosaminoglycans (GAGs) and Rhodamine phalloidin (Cytoskeleton, USA)/Hoechst 33258. The cells were fixed with 95% alcohol for 30 min and then stained with 0.1% Safranin O (Sigma, USA) for 10 min. Subsequently, cells were rinsed with water and scaled with neutral gum. Finally, the cells were observed and photographed under an inverted phase contrast microscope equipped with a computer (Zeiss Corporation, German).

2.10. Immunohistochemical examination

For immunohistochemical examination, monoclonal antibody to type II collagen (Boster, China) and type I collagen (Boster, China) were used according to the instructions. Cells were incubated with a primary antibody at a dilution of 1:200 for 2 h. Whereafter, second antibody and biotin labeled horseradish peroxidase were added. Subsequently, 3′-diaminobenzidine tetrahydrochloride (DAB) kit (Boster, China) was used according to the instructions with cells counterstained with haematoxylin. Finally, cells were gradually dehydrated and sealed with neutral gum. An inverted phase contrast microscope (Zeiss Corporation, German) was used to evaluate and photograph the cells.

2.11. Statistical Analysis

Results were demonstrated as means ± SD. Statistical significance was determined using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The level of significance was set to $P < 0.05$.

3. Results

3.1. Preparation of LDQN-C

The procedure of synthesis of GA and Sulfachloropyrazine sodium was shown in Fig. 1 LDQN-C has the following properties: light yellow powder, yield 55%, MS-ESI: m/z: 457.1[M-H]$^-$, ^{1}H NMR (400 MHz, DMSO) δ 10.27 (s, 1H, –CO–NH), 7.94–7.85 (dd, J = 8.9 Hz, 4H, Ar–H), 7.77 (d, J = 9.5 Hz, 1H, Py–H), 7.55 (d, J = 9.5 Hz, 1H, Py–H), 6.94 (s, 2H, Ar-H).^{13}C NMR (125 MHz, DMSO) δ 166.06, 154.33, 145.58, 143.83, 137.34, 127.96, 124.35, 119.66, 107.45.

3.2. Cell cytotoxicity

The cytotoxicity of different concentrations of LDQN-C on chondrocytes was detected by MTT assay. Articular cartilage cells of SD rats were treated with LDQN-C of various concentrations (1.36×10^{-10}–54.5×10^{-3} M) and GA (6.25×10^{-8} ~25 μg/mL). As shown in Fig. 2A, LDQN-C which ranged from 1.36×10^{-10} to 1.36×10^{-5} M exhibited nearly no cytotoxicity to chondrocytes. Especially, LDQN-C from 1.36×10^{-9} to 1.36×10^{-7} M promoted cell growth evidently ($p < 0.05$). In contrast, LDQN-C at the

Table 1
Primers for RT-PCR performance.

Gene name	Forward primer	Reverse primer
B-actin	5′-CCCATCTATGAGGGTTACGC-3′	5′-TTTAATGTCACGCACGATTTC-3′
Aggrecan	5′- CCGCTGGTCTGATGGACACT-3′	5′- AGGTGTTGGGGTCTGTGCAA-3′
Collagen II	5′- CTGGTCCTTCCGGCCCTAGA-3′	5′- GGATCGGGGCCCTTCTCTCT-3′
Sox9	5′-TCCAGCAAGAACAAGCCACA-3′	5′- CGAAGGGTCTCTTCTCGCTC-3′
Collagen I	5′- CATGAGCCGAAGCTAACCC-3′	5′- CTCCTATGACTTCTGCGTCTGG-3′
Collagen X	5′- TCTGCTGCTAGTGTCCTTGACG-3′	5′- GGAATGCCTTGTTCTCCTCTTACT-3′

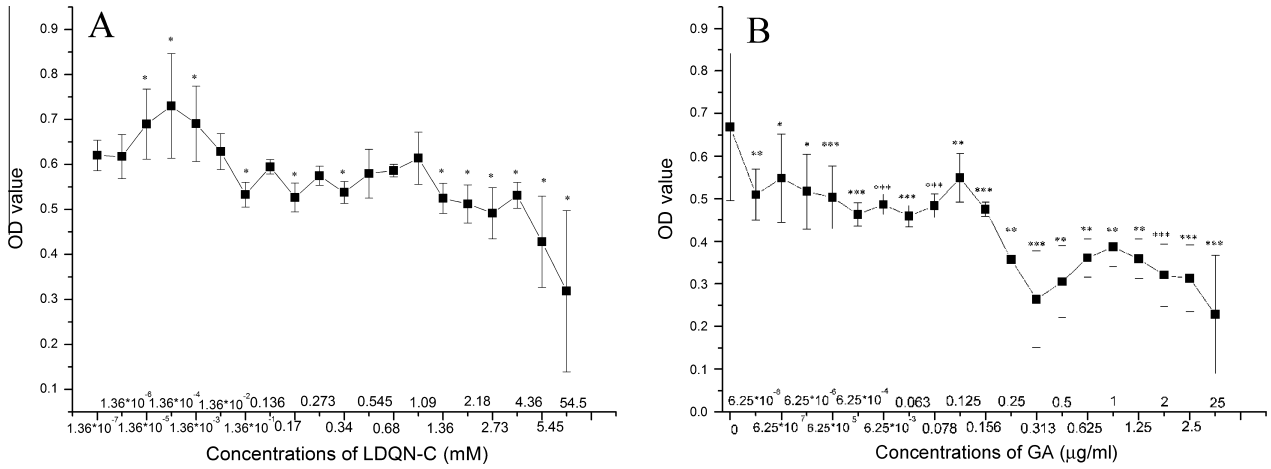

Fig. 2. Cytotoxicity analysis of chondrocytes treated by different concentrations of LDQN-C (A) and GA (B) after 3 days (mean ± SD, $n = 20$).

concentrations of 1.36×10^{-4}–54.5×10^{-3} M showed an inhibitive effect on chondrocytes. Therefore, the concentrations of LDQN-C ranged from 1.36×10^{-9} M to 1.36×10^{-7} M were chosen for further investigation. The cytotoxicity of GA was showed in Fig. 2B. Among the chosen concentrations, 0.125 μg/mL of GA promoted chondrocytes growth the most significantly.

3.3. Cell viability assay

Cell viability was determined by FDA/PI staining (Fig. 3). The results demonstrated that LDQN-C exerted potent effect on chondrocytes survival compared to control and GA groups. The FDA/PI staining images indicated that live cells in LDQN-C groups were more than the ones in the control and GA groups, which was consistent with the result of cell proliferation. The results implied that LDQN-C has a positive effect on cell growth. Among the experimental groups, concentration of 1.36×10^{-8} M was superior to others.

3.4. Cell morphology

Fig. 4 showed the morphology of articular chondrocytes cultured for 2, 4 and 6 days. The chondrocytes treated by LDQN-C grew better in the same period compared with the control and GA groups. More round cells that represent typical morphology of chondrocytes were found in LDQN-C groups. Especially at the concentration of 1.36×10^{-8} M, LDQN-C facilitated cell proliferation more than others. Fig. 5 showed the actin filaments of chondrocytes by the rhodamine phalloidin/Hoechst 33258 staining, which was in agreement with the HE analysis. It was shown that cells in LDQN-C treated groups grew in clumps with densely distributed ECM. While in control and GA groups, less cells and ECM were present.

3.5. Cartilage specific gene expression

The effect of LDQN-C on chondrocytes relative gene expression of collagen I, collagen II, collagen X, Sox9, aggrecan (a proteoglycan

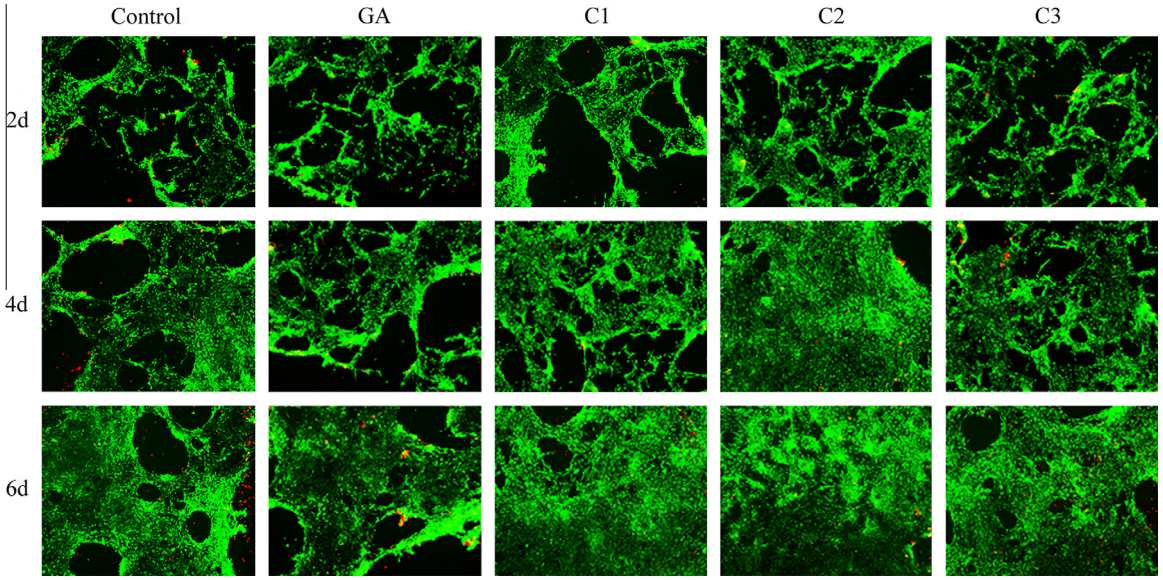

Fig. 3. Confocal laser scanning microscopy images showing the viability of chondrocytes cultured *in vitro* alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) for 2, 4 and 6 days. Cell seeding density: 2×10^4/mL (original magnification 100×, scale bar is 100 μm).

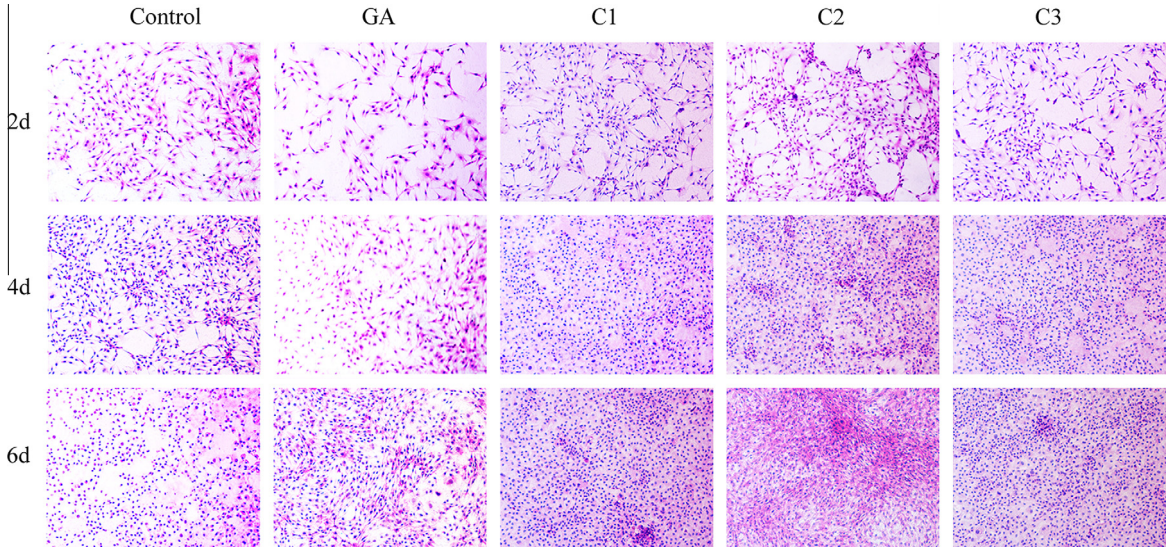

Fig. 4. Hematoxylin-eosin staining images showing the morphology of chondrocytes cultured *in vitro* alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) for 2, 4 and 6 days. Cell seeding density: 2×10^4/mL (original magnification 100×, scale bar is 100 μm).

composed of GAGs) after 2, 4 and 6 days culture was detected by Real time-PCR. As shown in Fig. 6, aggrecan, collagen II and Sox9 were notably promoted by LDQN-C compared to the control and GA groups. Particularly, LDQN-C at concentration of 1.36×10^{-8} M showed the highest collagen II, aggrecan and Sox9 expression. The results indicated that LDQN-C could upregulate collagen II, aggrecan and Sox9 expressions relative to control and GA groups. In contrast, collagen type I was down-regulated by LDQN-C but not in GA and control groups. The results suggested that LDQN-C may either delay or prevent the chondrocytes from dedifferentiation. In addition, collagen X expression was scarcely detectable in all groups, which suggested that cell hypertrophy could not be detected. Among all the groups, concentration of 1.36×10^{-8} M showed the best performance, as demonstrated by the highest expression of aggrecan and collagen II expression.

3.6. Cell proliferation

In this study, the cell proliferation in LDQN-C- treaded groups, GA group and control group were analyzed by DNA content measurements in 2, 4 and 6 days respectively. As shown in Fig. 7A, the chondrocytes cultured with LDQN-C grew faster than control

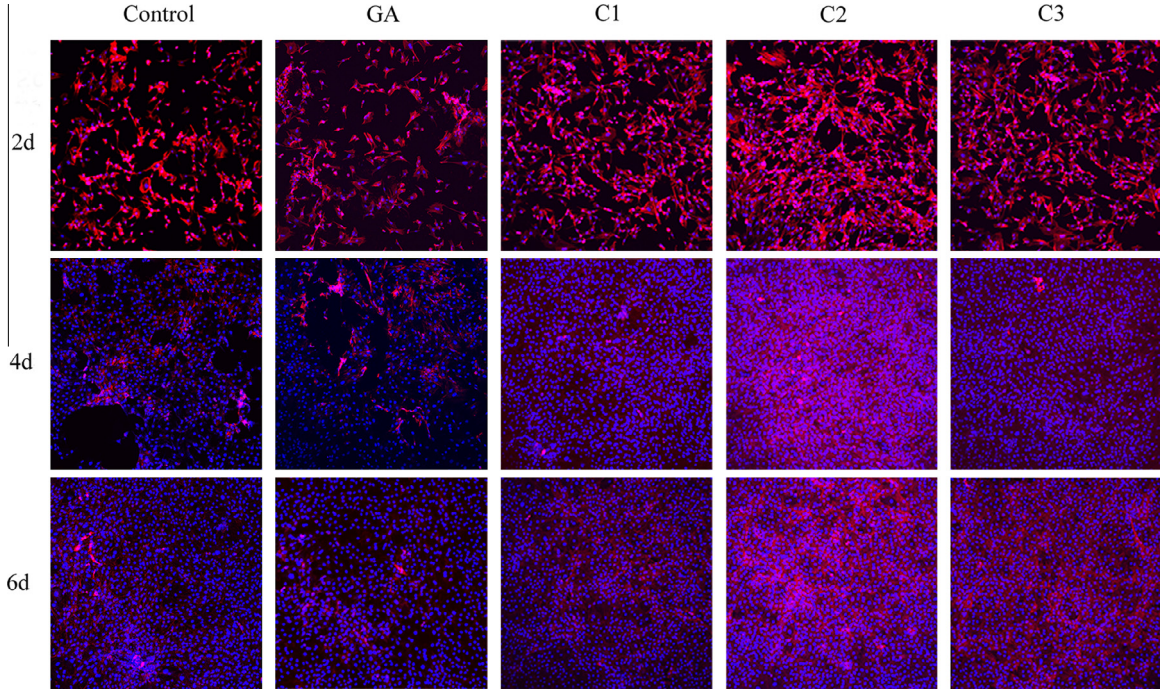

Fig. 5. Phalloidin/Hoechst 33258 staining images showing the morphology of chondrocytes and actin filaments after treated alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) for 2, 4 and 6 days. Cell seeding density: 2×10^4/mL (original magnification 100×, scale bar is 100 μm).

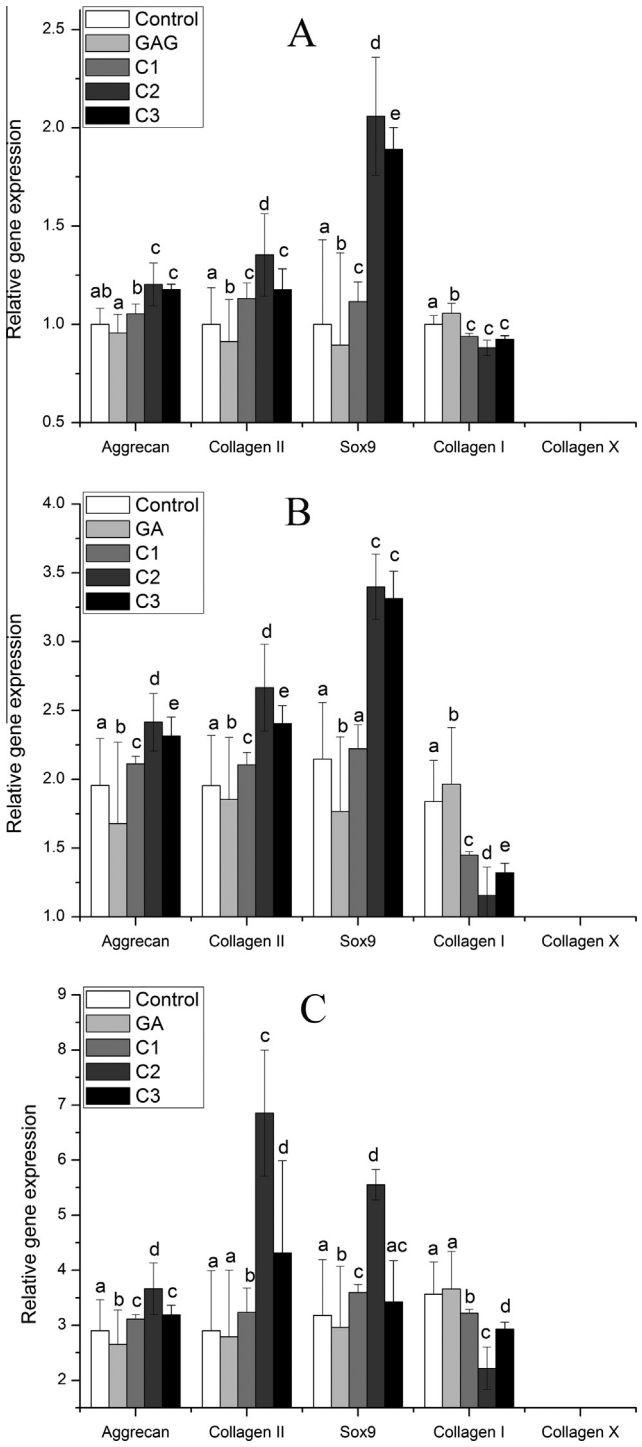

Fig. 6. Quantitative compare of ECM-related gene expression by qRT-PCR. The chondrocytes were cultured alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) for 2 days (A), 4 days (B) and 6 days (C) ($n = 3$ for each experiment). The gene expression levels in LDQN-C media relative to the control group were analyzed by the $2^{-\Delta\Delta CT}$ method using GAPDH as the internal control. The data represent the mean ± SD of three independent culture experiments.

and GA groups, which was evidenced by the significantly higher DNA contents ($P < 0.05$) in the same culture period. The results also indicated that LDQN-C at the concentration of 1.36×10^{-8} M promoted cell growth the most in all the LDQN-C groups.

3.7. GAGs synthesis

Biochemical assays were used for the quantitative investigation of the production of GAG after 2, 4 and 6 days of culture. The histogram showed the GAG production given as a ratio of GAG to DNA in Fig. 7B. Quantitatively, GAG production in LDQN-C-treated groups was significantly enhanced than that in control and GA groups at the same time point. The effect of LDQN-C is dose-dependent.

3.8. ECM secession

Qualitative assessment by using Safranin-O staining also showed a deeper staining in LDQN-C groups compared with control and GA groups (Fig. 8). At concentration of 1.36×10^{-8} M, LDQN-C exhibited the most GAG synthesis among the three concentrations.

3.9. The production of collagen type I and type II

Expression of type I and type II collagen by immunohistochemical staining with untreated, GA and LDQN-C-treated culture media was shown in Fig. 9. Large areas of positive staining for cartilage-specific type II collagen was shown in Fig. 9B, while only very sparse and light staining for type I collagen was observed in Fig. 9A in LDQN-C-treated groups. It is converse in control and GA groups, especially in GA group. The results confirmed the maintenance of chondrocytic phenotype after treated by LDQN-C (especially at 1.36×10^{-8} M), which indicated that LDQN-C may effectively inhibit de-differentiation of chondrocytes cultured *in vitro*.

4. Discussion

It has been reported that GA derivatives appeared to play an important role in protection of chondrocytes. In this study, LDQN-C was synthesized by coupling sulfonamide groups with GA and its effects on the chondrocytes growth and phenotype maintenance were investigated, Comparison with GA was also conducted. The results indicated that LDQN-C could enhance chondrocytes growth, as evidenced by more rapid cell proliferation than control and GA groups (Fig. 7A). LDQN-C could obviously promote GAGs deposition in cultured chondrocytes which was shown by biochemical assay, but not in control and GA groups (Fig. 7B). As the main component of cartilage matrix, GAG is crucial in maintaining cartilage load-bearing capacity [13]. Consistent with the increase of GAG production, LDQN-C could upregulate the expression of aggrecan, collagen II and Sox9 (Fig. 6). Sox9 was considered as the primary chondrogenic marker that enhanced the production of collagen and aggrecan [14] and acted a key role in chondrogenesis [15]. The results suggested that LDQN-C could facilitate chondrocytes growth and stimulate exuberant cartilage matrix secretion through regulating the key activator of the chondrocyte-specific enhancer.

As is known to all that dedifferentiation occurred while the differentiated phenotype of chondrocytes consists primarily of type II collagen and cartilage-specific proteoglycan is lost and replaced by a complex collagen phenotype consisting predominately of type I collagen and a low level of proteoglycan synthesis [16,17]. Results of PCR, biochemical and immunohistochemical assay, collagen type I expression all showed downregulation of collagen type I, which suggested that LDQN-C could effectively delay or prevent dedifferentiation of chondrocytes. On the other hand, collagen type X that is specifically associated with hypertrophic chondrocytes and precedes the onset of endochondral ossification could not be detected in all the LDQN-C-treated groups. This implied that

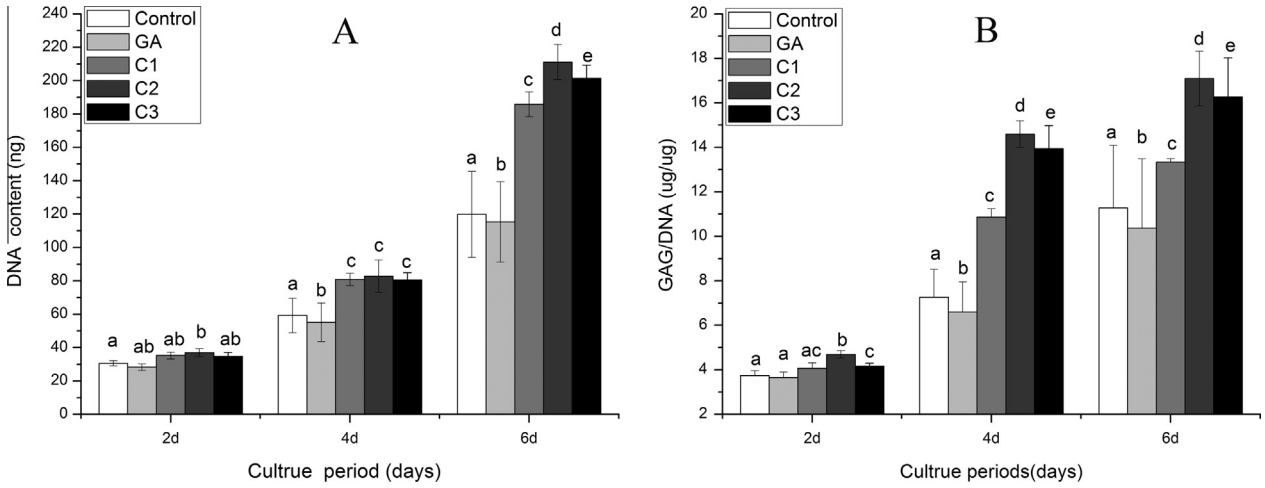

Fig. 7. Quantification of cell proliferation by detected of DNA content (A) and matrix production by glycosaminoglycan (GAG) analysis (B). A. the proliferation of chondrocytes cultured *in vitro* alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) B. GAG (mg) normalized to DNA (mg). Data from four independent experiments were evaluated, and the mean ± SD is shown.

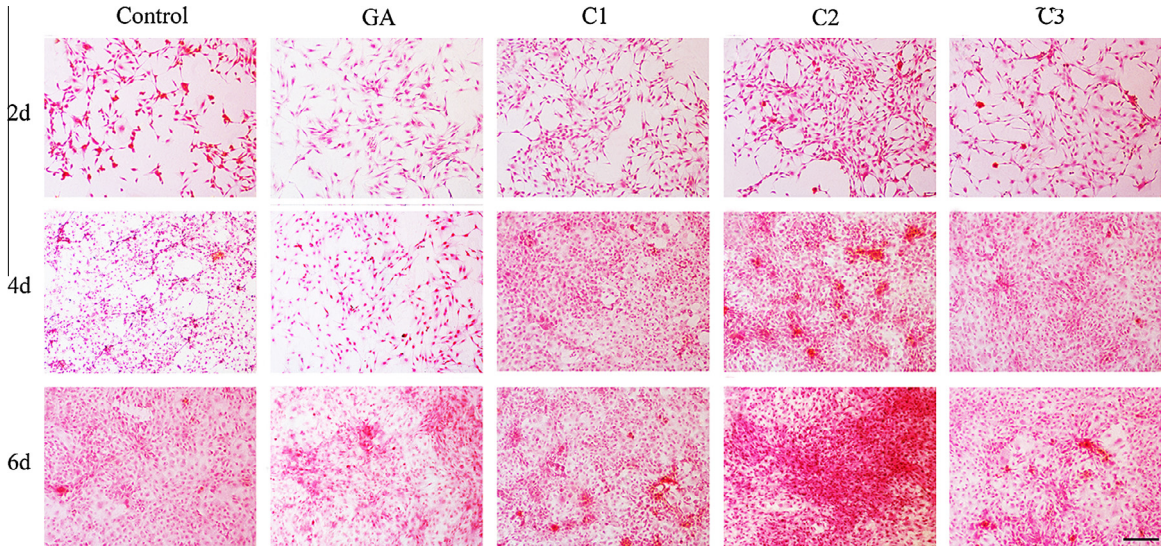

Fig. 8. Safranin O staining showing the ECM synthesis of chondrocytes culture *in vitro* alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) for 2, 4 and 6 days. Cell seeding density: 2×10^4/mL (original magnification 100×, scale bar is 100 μm).

hypertrophy of chondrocytes would not be induced by LDQN-C. Therefore, LDQN-C has the ability to maintain the phenotype of chondrocytes, as proved by the reduced collagen I messages and the barely evident messages of collagen X indicating the inhibition of the dedifferentiation and hypertrophy.

As for the recommended dose of LDQN-C, our results showed that the concentration of LDQN-C concerning enhancing chondrocytes proliferation ranged from 1.36×10^{-10} M to 1.36×10^{-5} M (Fig. 2A). DNA synthesis of rat chondrocytes was increased in a dose-dependent manner when chondrocytes were cultured in the medium containing LDQN-C at the concentration of 1.36×10^{-9} M, 1.36×10^{-8} M and 1.36×10^{-7} M groups could support the greatest cell proliferation and stimulate the most matrix secretion.

Due to inferior pharmacological effects and biological property of GA [18,19], the modification is of significance. As one of the derivatives of GA, Epigallocatechin-3-gallate (EGCG) was found to inhibit the degradation of human cartilage proteoglycan and type II collagen, and selectively inhibit ADAMTS-1, ADAMTS-4, and ADAMTS-5 [20,21]. It was also found that EGCG ameliorates IL-1β-mediated suppression of TGF-β synthesis, and enhances type II collagen and aggrecan core protein synthesis in human articular chondrocytes [11]. Furthermore, recent studies indicated that sulfonamide-based gallates effectively inhibited IL-1β induced osteoarthritis [22] and exerted effects on cartilage growth [23–25]. In agreement with these studies, LDQN-C which is a novel derivative of GA, can also support the chondrocytes growth and maintain the phenotype. As sulfonamide-based gallate, LDQN-C may be potential candidate for treatment of cartilage degenerative diseases. This implied that suitable modification of GA may lead to the improvement of the pharmacological effects in cartilage defect and may be osteoarthritis.

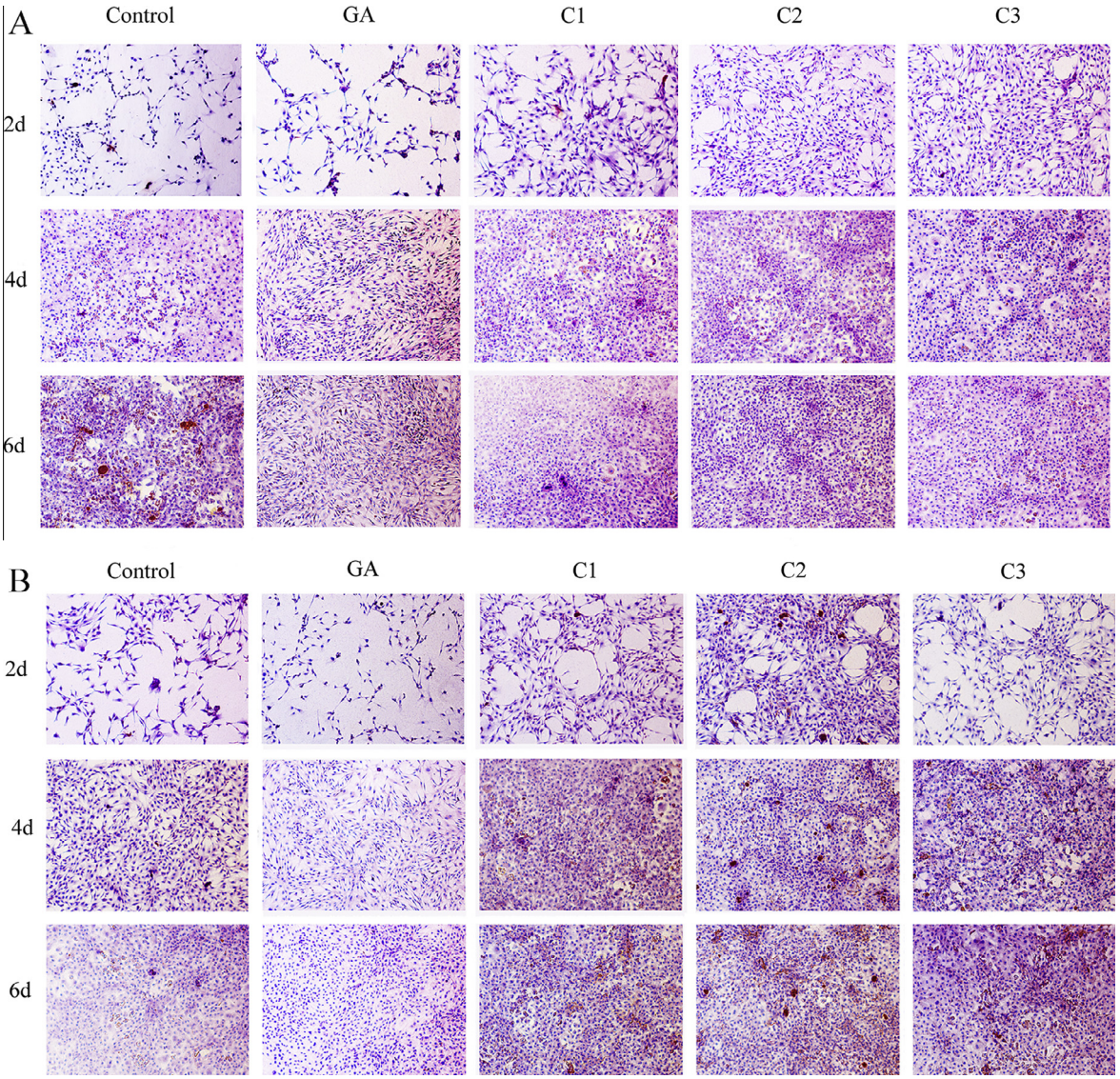

Fig. 9. Immunohistochemical staining images revealed the presence of type I (A) and type II (B) collagen. Chondrocytes cultured *in vitro* alone (control) or with GA (GA = 0.125 μg/mL) and LDQN-C (C1 = 1.36×10^{-9} M, C2 = 1.36×10^{-8} M, C3 = 1.36×10^{-7} M) for 2, 4 and 6 days. Cell seeding density: 2×10^4/mL (original magnification 100×, scale bar is 100 μm).

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