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In vitro effect of a synthesized sulfonamido-based gallate on articular chondrocyte metabolism

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ABSTRACT

Autologous chondrocyte implantation (ACI) is a promising strategy for cartilage repair and reconstitution. However, limited cell numbers and the dedifferentiation of chondrocytes present major difficulties to the success of ACI therapy. Therefore, it is important to find effective pro-chondrogenic agents that restore these defects to ensure a successful therapy. In this study, we synthesized a sulfonamido-based gallate, namely *N*-[4-(4,6-dimethyl-pyrimidin-2-ylsulfamoyl)-phenyl]-3,4,5-trihydroxy-benzamide (EJTC), and investigated its effects on rabbit articular chondrocytes through an examination of its specific effects on cell proliferation, morphology, viability, GAG synthesis, and cartilage-specific gene expression. The results show that EJTC can effectively promote chondrocyte growth and enhance the secretion and synthesis of cartilage ECM by upregulating the expression levels of the aggrecan, collagen II, and Sox9 genes. The expression of the collagen I gene was effectively downregulated, which indicates that EJTC inhibits chondrocytes dedifferentiation. Chondrocyte hypertrophy, which may lead to chondrocyte ossification, was also undetectable in the EJTC-treated groups. The recommended dose of EJTC ranges from 3.125 μg/mL to 7.8125 μg/mL, and the most profound response was observed with 7.8125 μg/mL. This study may provide a basis for the development of a novel agent for the treatment of articular cartilage defects.

Autologous chondrocyte implantation (ACI) is considered a promising strategy for cartilage repair and reconstitution,[1,2] but limited cell numbers and the dedifferentiation of chondrocytes are the major obstacles in this therapy process. For this reason, it is of significance to find effective pro-chondrogenic agents for the restoration of defects in order to ensure a successful ACI therapy.[3–5] Gallic acid (GA) is an endogenous plant polyphenol found abundantly in tea,[6] grapes,[7] berries,[8] and other plant species,[9,10] and it has been documented to have strong pharmacological activity and to affect several pharmacological and biochemical pathways.[11–14] GA has been reported to have pro-apoptotic and anti-inflammatory effects in the therapy of arthritis.[8,15] Some studies also indicate its chondroprotective and chondrogenic roles.[16,17] However, GA has weaker pharmacological effects compared with its esters, which may be due to its higher hydrophobicity.[18] This may result in its inferior bioactivity.[19] The modification of GA with substances containing large groups may improve its pharmacological properties and thus contribute to its development as a potential pro-chondrogenic agent.

The sulfonamide family consists of a broad spectrum of synthetic bacteriostatic antibiotics and was commonly used in human and veterinary medicine for therapeutic and prophylactic purposes in last century due to their easy penetration through the membrane and into body fluids and tissues.[20] However, some sulfonamide drugs were also documented to be suitable for the treatment of degenerative joint disorders and inflammatory processes, such as osteoarthritis and rheumatism.[21,22] Recently, a new series of arylsulfonamido-based hydroxamates were synthesized and evaluated, and the findings show that these can effectively block ex vivo cartilage degradation without cytotoxic side effects.[23] These compounds contain several phenyl groups and sulfonamide groups, which may shed light on the synthesis of GA derivatives by the introduction of sulfonamide groups. The modification of GA with sulfonamide, which has the property of being easily absorbed, may enhance the pharmacological activity of GA.

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In this study, we synthesized a sulfonamido-based gallate, namely *N*-[4-(4,6-dimethyl-pyrimidin-2-ylsulfamoyl)-phenyl]-3,4,5-trihydroxy-benzamide (EJTC, Fig. S1), and examined its effect on chondrocyte growth through an examination of its effect on cell proliferation, morphology, ECM synthesis, and cartilage-specific gene expression. This investigation was intended to test the hypothesis that the modification of GA with sulfonamide may improve the bioactivity of GA. The synthesized drug may be a potential pro-chondrogenic agent for ACI applications.

EJTC was prepared from GA and sulfadimidine. The detailed synthetic route is presented in Figure 1. After the reactions, an appropriate volume of distilled water was added to the mixture. The raw product was then precipitated and separated by vacuum filtration. The raw product was recrystallized in a THF-methanol solvent system. The molecular formula of EJTC was confirmed to be $C_{19}H_{18}N_4O_6S$ by the analyses of MS-ESI, ^{1}H NMR and ^{13}C NMR data.[24]

EJTC was dissolved in 0.1 M sodium hydroxide solution (NaOH, Sigma, USA) to obtain the EJTC stock solution, and the stock solution was stored at −4 °C. The EJTC stock solution was then added to the cell cultures to obtain various final concentrations. The culture medium containing various concentrations of EJTC was replaced every 3 days.

The articular cartilage was harvested from one-week-old New Zealand rabbits by enzymatic digestion. Cartilage cells in the logarithmic phase of growth and with 80–90% confluence were used for the following experiments.

The effect of EJTC on chondrocyte cytotoxicity was assessed by the MTT method. After treatment with various concentrations of EJTC (3.125–62.5 μg/mL) for 3 days, the cells were harvested and subjected to a cytotoxicity test. As shown in Figure 2, EJTC at concentrations ranging from 3.125 to 25 μg/mL exhibited low cytotoxicity. Within this range, EJCT at a concentration of 7.8125 μg/mL promoted evident cell growth. In contrast, EJTC at concentrations ranging from 31.25 to 62.5 μg/mL exhibited an inhibitory effect on chondrocytes. Hence, EJTC concentrations ranging from 3.125 to 25 μg/mL were used in the subsequent assays.

After treatment with EJTC (3.90625, 7.8125, and 15.625 μg/mL) for 2, 4, and 6 days, respectively, the cells were digested using proteinase K (Sigma, USA) and then subjected to cell proliferation analysis and biomechanical assay. The cell proliferation was measured by assessing the DNA content through the fluorescence of Hoechst 33258 (Sigma, USA) at 460 nm using the absorbance value of the Hoechst 33258 dye alone as the baseline. The total glycosaminoglycans (GAGs) in each treatment group was measured by

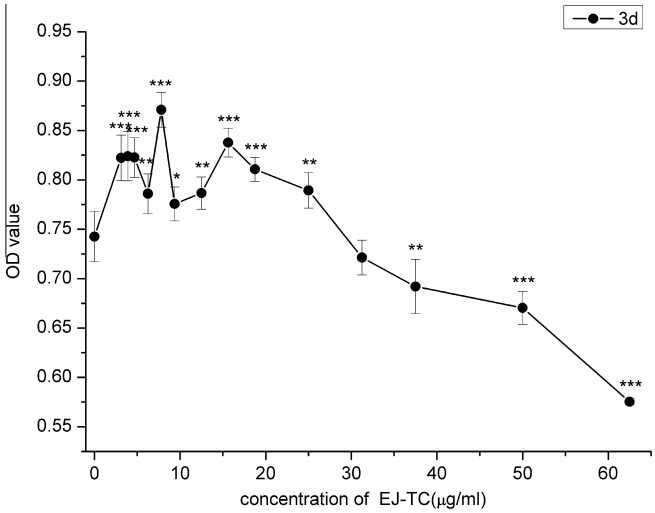

Figure 2. Cytotoxicity analysis of chondrocytes treated with different concentrations of EJTC for 3 days (means ± SD, $n = 4$). ($^*P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001).

the 1,9-dimethylmethylene blue (DMMB; Sigma, USA) spectrophotometric assay at 525 nm. Chondroitin sulfate (Sigma, USA) was used as the control to plot the standard curve. The amount of GAGs in each group was normalized to the total DNA content of the cells. As shown in Figure 3A, the chondrocytes cultured with EJTC grew faster than the control cells, as was evidenced by the significantly higher DNA contents (P <0.05) observed in these cells compared with the controls at the same time points. The results also indicate that the EJTC concentration of 7.8125 μg/mL promotes a higher rate of cell growth compared with the other EJTC concentrations, and this finding is in accordance with the MTT results. In Figure 3B, GAG production was gradually increased over time. Comparatively, the amount of GAGs produced by chondrocytes treated with EJTC was significantly higher than that observed in the control group at the same time point. In line with the cell proliferation results, the EJTC concentration of 7.8125 μg/mL exhibited the strongest promoting effect on GAG synthesis among the three tested concentrations.

Safranin-O/fast green-stained cells were scored to assess their glycosaminoglycan (GAG) contents. The cells were fixed with 95% alcohol for 30 min and then stained with 0.1% Safranin O (Sigma, USA) for 10 min. Subsequently, the cells were rinsed with water and sealed with neutral gum. The cells were then observed and

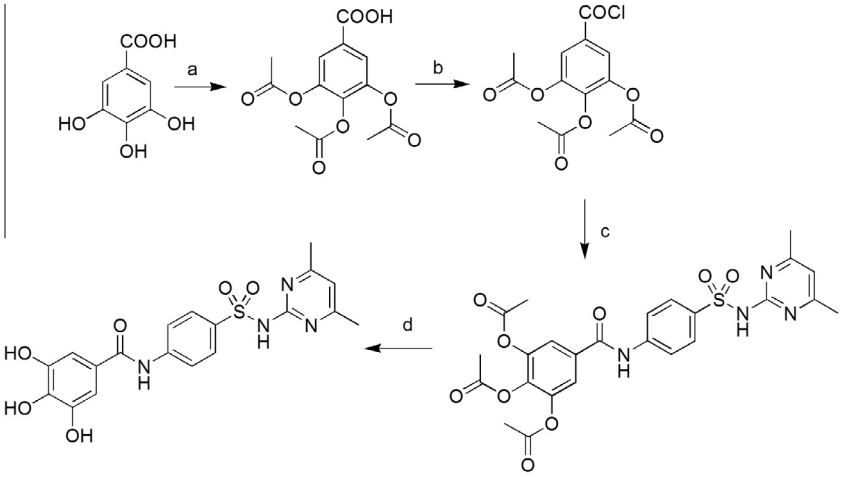

Figure 1. Schematic description of the synthesis route and synthetic procedures for EJTC. Reagents and conditions: (a) acetyl oxide, oil bath, 120 °C; (b) $SOCl_2$, oil bath, 80 °C; (c) sulfadimidine, THF, pyridine, ice bath; (d) HCl, THF, 60 °C.

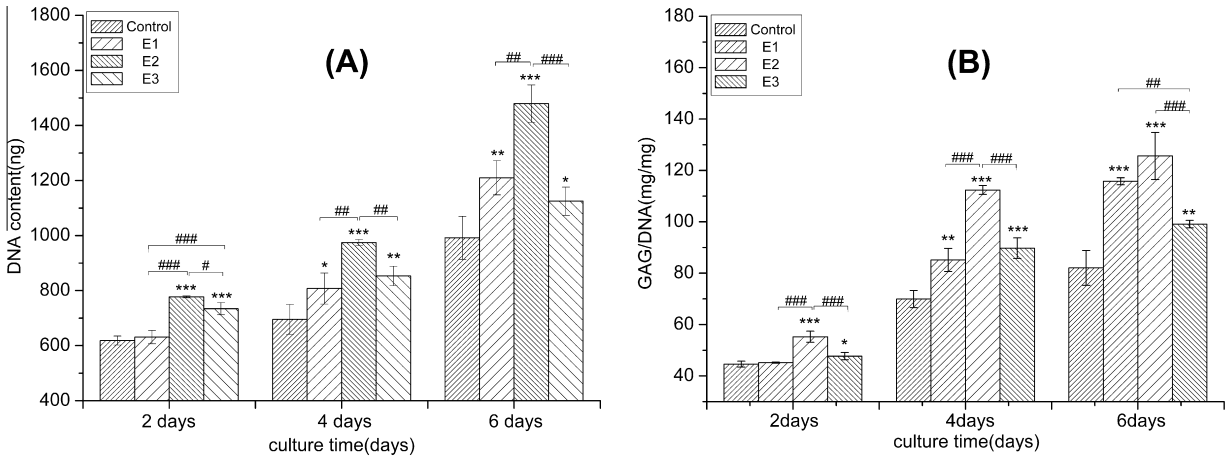

Figure 3. Quantification of cell proliferation by the detection of the DNA content and matrix production through glycosaminoglycan (GAG)) analysis. (A) Proliferation of chondrocytes cultured in vitro with 0 μg/mL (Control), 3.90625 μg/mL (C1), 7.8125 μg/mL (C2), and 15.625 μg/mL (C3) EJTC for 2, 4, and 6 days. (B) The level of GAG (mg) was normalized to the DNA content (mg). The data from four independent experiments were evaluated, and the means ± SD are shown. ($^{*,\#}P$ <0.05; $^{**,\#\#}P$ <0.01, $^{***,\#\#\#}P$ <0.001).

photographed under an inverted phase contrast microscope equipped with a computer (Zeiss Corporation, Germany). The safranin O staining results also indicate that more GAGs were secreted by the chondrocytes treated with EJTC compared with the control cells (Fig. 4), as shown by the deeper staining. In particular, the cells treated with 7.8125 μg/mL EJTC exhibited the highest proteoglycan and GAG deposition.

The cell viabilities of the cultures on days 2, 4, and 6 were determined through the use of a live-dead viability assay kit (Invitrogen, USA). Briefly, calcein-AM and propidium iodide stock solutions were added to the cells at final concentrations of 2 μmol/L and 2 μg/L, respectively, and the cells were incubated in the dark for 5 min at 37 °C. The images were captured using a laser scanning confocal microscope (Nikon A1, Japan). The results demonstrate that EJTC exerts a potent effect on chondrocyte survival (Fig. 5). The calcein-AM/PI staining images indicate that the numbers of live cells in the EJTC groups are higher than those observed in the control group, which is consistent with the cell proliferation results. These findings indicate that EJTC has a positive effect on cell growth. Among the experimental groups, the EJTC concentration of 7.8125 μg/mL was superior to the others.

Hematoxylin and eosin (HE) staining images (Fig. 6) shows the morphology of articular chondrocytes cultured for 2, 4, and 6 days. The chondrocytes treated with EJTC grew better and appeared to have an obvious proliferation tendency compared with the control cells. A higher number of round cells, which represents the typical morphology of chondrocytes, were found in the EJTC-treated groups. In particularly, the EJTC concentration of 7.8125 μg/mL facilitated a higher degree of cell proliferation compared with the other tested concentrations.

Cartilaginous intracellular matrix deposition was evaluated by immunohistochemical assay, monoclonal antibody to type II

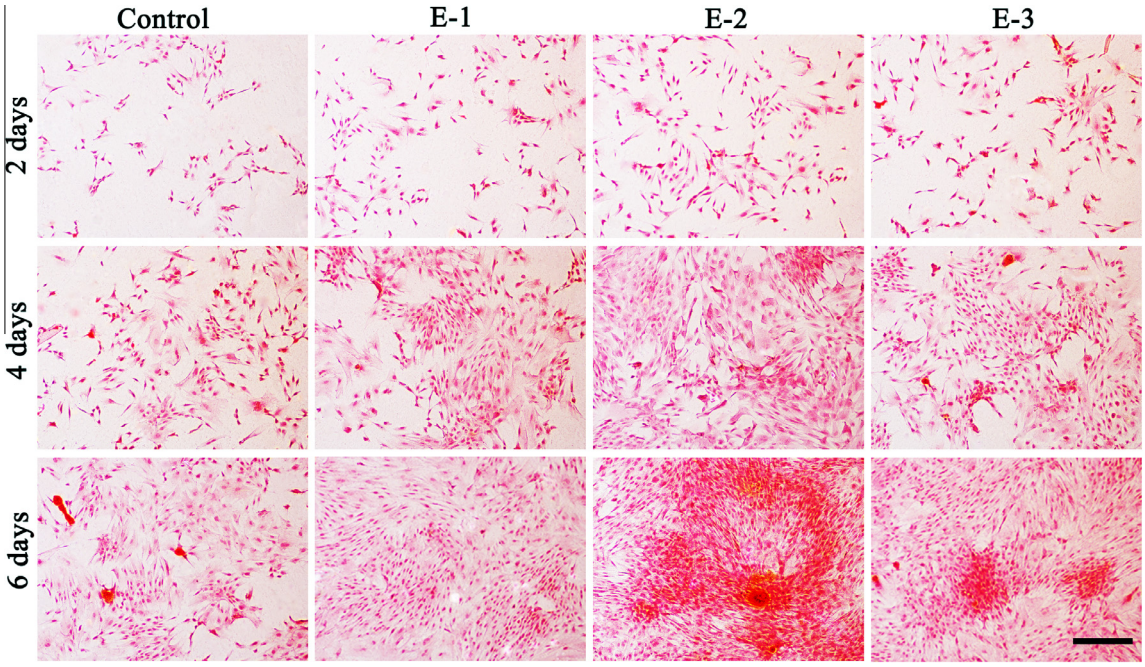

Figure 4. Safranin O staining showing the ECM synthesis by chondrocytes cultured in vitro with 0 μg/mL (Control), 3.90625 μg/mL (E-1), 7.8125 μg/mL (E-2), and 15.625 μg/mL (E-3) EJ-TC for 2, 4, and 6 days. Scale bar = 200 μm.

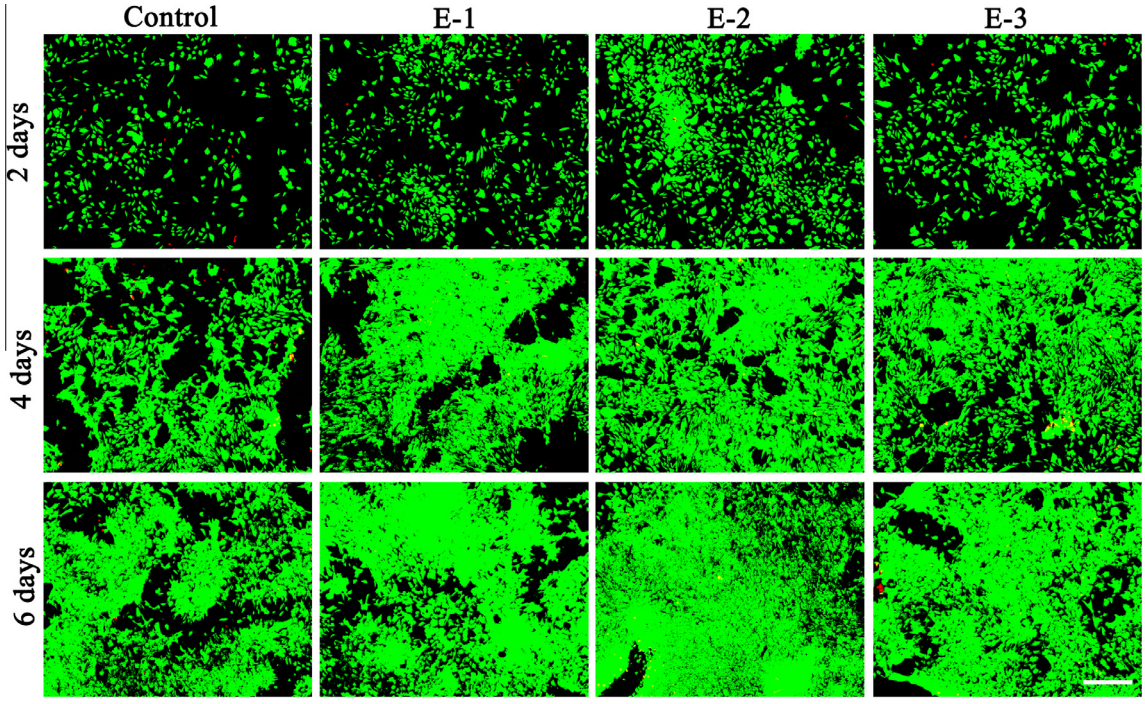

Figure 5. Confocal laser scanning microscopy images showing the viability of chondrocytes cultured in vitro with 0 μg/mL (Control), 3.90625 μg/mL (E-1), 7.8125 μg/mL (E-2), and 15.625 μg/mL (E-3) EJTC for 2, 4, and 6 days. Scale bar = 200 μm.

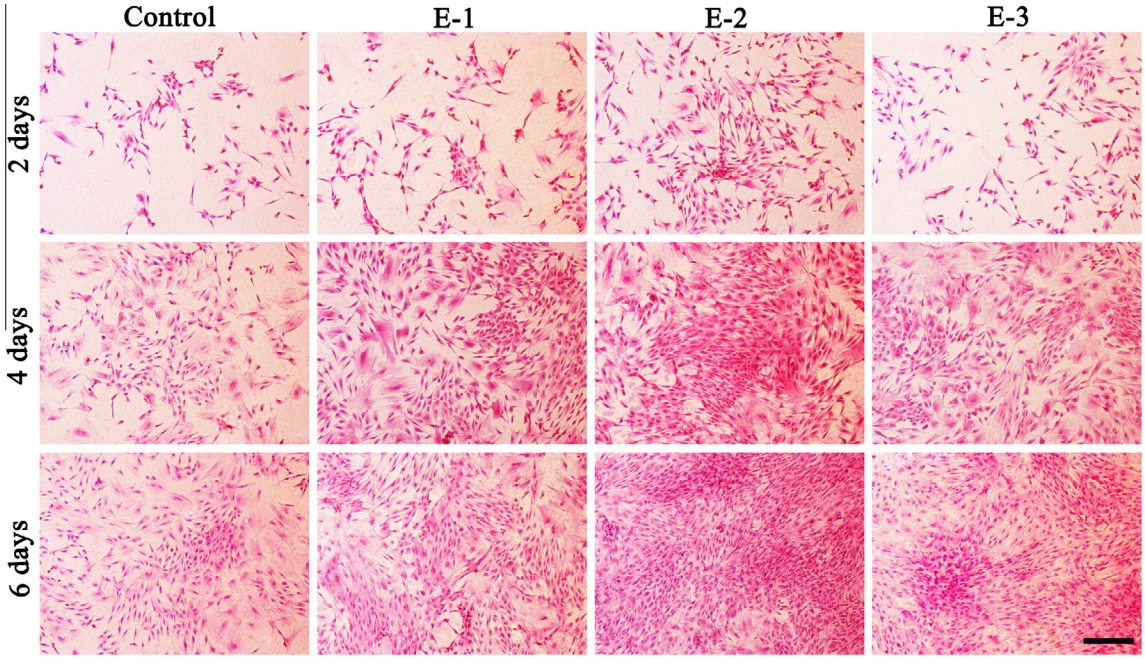

Figure 6. Hematoxylin-eosin staining images showing the morphology of chondrocytes cultured in vitro with 0 μg/mL (Control), 3.90625 μg/mL (E-1), 7.8125 μg/mL (E-2), and 15.625 μg/mL (E-3) EJ-TC for 2, 4, and 6 days. Scale bar = 200 μm.

collagen (Boster, China) and type I collagen (Boster, China) were used according to the manufacturer's instructions, an inverted phase contrast microscope (Zeiss Corporation, Germany) was used to evaluate and photograph the cells. As shown in Figures 7 and 8, Obvious positive staining for cartilage-specific type II collagen was observed in large areas in the EJTC groups after treatment for 2, 4, and 6 days. In contrast, only very sparse and light staining was observed for type I collagen, which is an indicator of the dedifferentiation of chondrocytes. The control group exhibited less positive staining of type II collagen and more positive staining of type I collagen. These results confirm the maintenance of the chondrocytic phenotype after treatment with EJTC.

The effect of EJTC on chondrocyte ECM synthesis was further investigated through the detection of the gene expression of collagen I, collagen II, collagen X, Sox9, and aggrecan (a proteoglycan composed of GAGs) after 2, 4, and 6 days of culture. The sequences

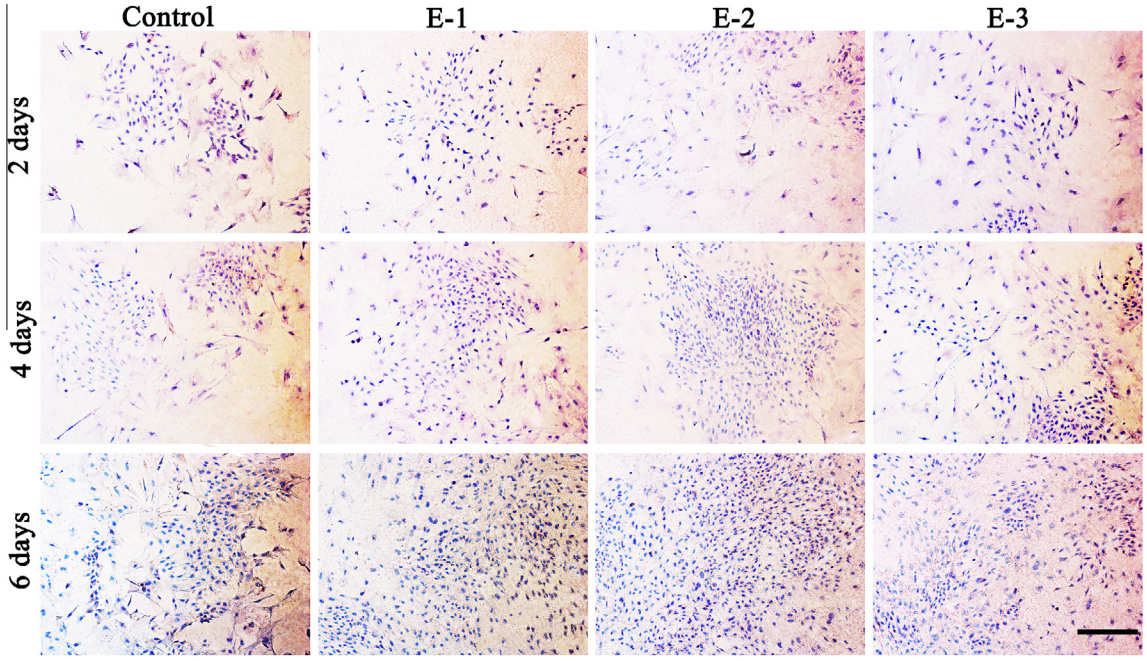

Figure 7. Immunohistochemical staining images revealing the presence of type I collagen in chondrocytes cultured in vitro wit h0 μg/mL (Control), 3.90625 μg/mL (E-1), 7.8125 μg/mL (E-2), and 15.625 μg/mL (E-3) EJTC for 2, 4, and 6 days. Scale bar = 200 μm.

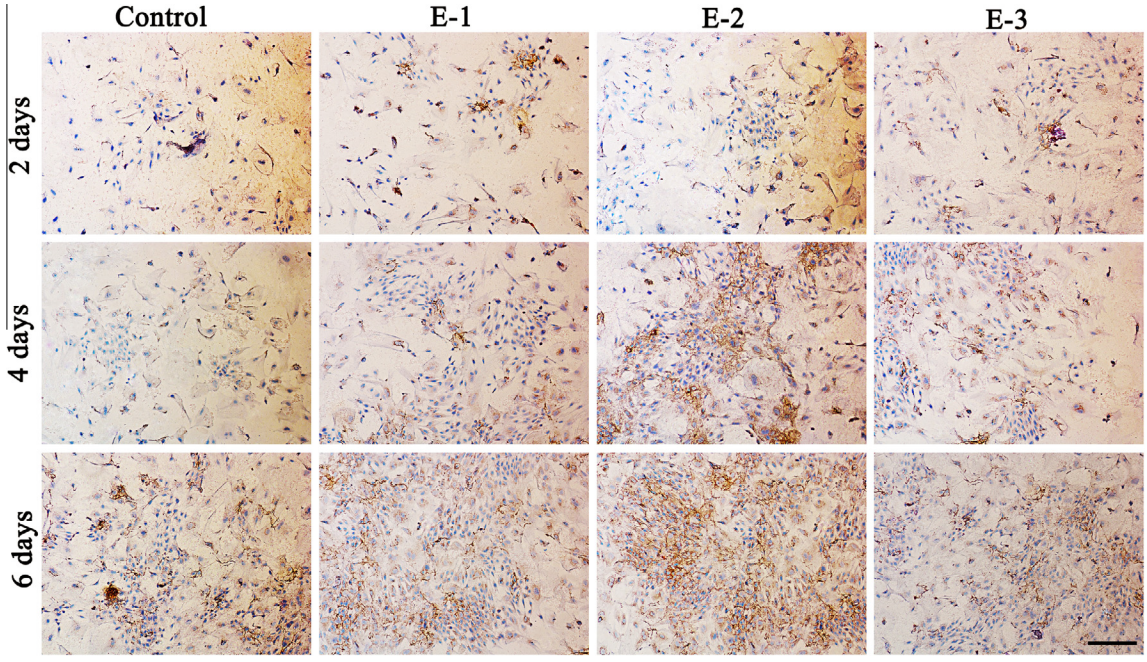

Figure 8. Immunohistochemical staining images revealing the presence of type II collagen in chondrocytes cultured in vitro with 0 μg/mL (Control), 3.90625 μg/mL (E-1), 7.8125 μg/mL (E-2), and 15.625 μg/mL (E-3) EJTC for 2, 4, and 6 days. Scale bar = 200 μm.

Table 1
Primer design for qRT-PCR analysis

mRNA	Forward primer	Reverse primer
GAPDH	5′-GTCATCATCTCAGCCCCCTC-3′	5′-GGATGCGTTGCTGACAATCT-3′
Aggrecan	5′-TTGCCTTTGTGGACACCAGT-3′	5′-GAGCCAAGGACGTAAACCCA-3′
Type I collagen	5′-CCCAGCCACCTCAAGAGAAG-3′	5′-CGGGGCTCTTGATGTTCTCA-3′
Type II collagen	5′-TCCGGAAACCAGGACCAAAG-3′	5′-CTTTGTCACCACGGTCACCT-3′
Type X collagen	5′-CTACGCTGAGCGGTACCAAA-3′	5′-GGCTTCCCAGTGGCTGATAG-3′
Sox9	5′-GACGCACATCTCGCCCAAC-3′	5′-TCTCGCTTCAGGTCAGCCTT-3′

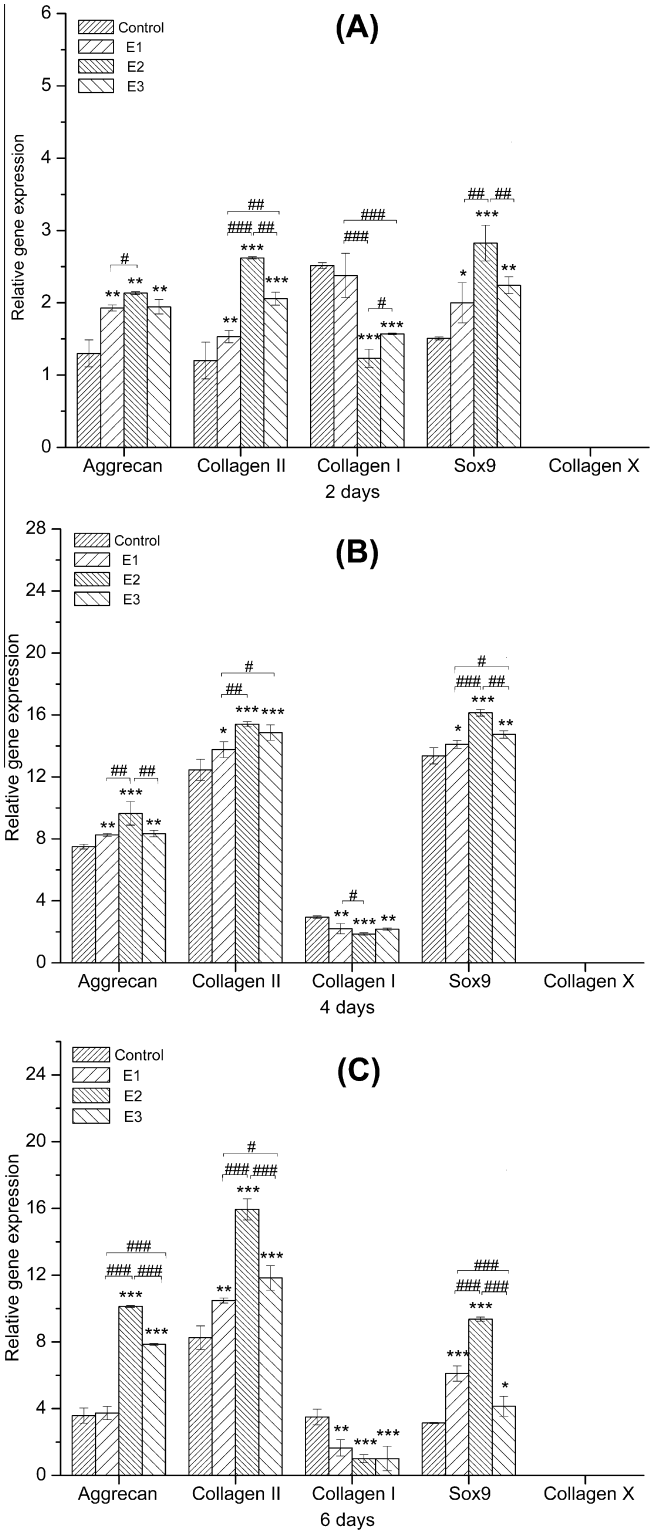

Figure 9. Quantitative comparison of ECM-related gene expression by qRT-PCR. Chondrocytes were cultured with 0 μg/mL (Control), 3.90625 μg/mL (E-1), 7.8125 μg/mL (E-2), and 15.625 μg/mL (E-3) EJ-TC for (A) 2 days, (B) 4 days, and (C) 6 days ($n = 3$ for each experiment). The gene expression levels in the cells treated with EJTC relative to those in the control group were analyzed by the 2-ΔΔCT method using GAPDH as the internal control. The data represent the means ± SD of three independent culture experiments. ($^{*,\#}P$ <0.05, $^{**,\#\#}P$ <0.01, $^{***,\#\#\#}P$ <0.001).

of the specific primers used are shown in Table 1. As shown in Figure 9, the transcription of aggrecan, collagen II, and Sox9 was notably promoted by EJTC. In particular, the cells treated with 7.8125 μg/mL EJTC showed the highest collagen II, aggrecan, and Sox9 expression. The results indicate that EJTC can upregulate collagen II, aggrecan, and Sox9 expression. In contrast, collagen type I was downregulated by EJTC. These results suggest that EJTC may either delay or prevent the dedifferentiation of chondrocytes. In addition, collagen X expression was scarcely detectable in all of the groups, which suggests the absence of cell hypertrophy. Among all of the groups, the EJTC concentration of 7.8125 μg/mL showed the best performance, as demonstrated by the highest expression levels of aggrecan and collagen II.

Based on the hypothesis that synthetic compounds of gallates and sulfonamides may improve the chondroprotective effect, we synthesized EJTC and examined its effects on chondrocyte growth and phenotype maintenance. The results suggest that EJTC can facilitate chondrocyte growth and stimulate exuberant cartilage matrix secretion. We hypothesize that its unique molecular structure plays a key role in its effects. As reported by Nuti,[23] the phenyl ring adjacent to the sulfonamide group allow its perfect docking into ADAMTS-5 and inhibits ADAMTS-5 activity on the native aggrecan substrate. This finding implies that the suitable modification of GA with a sulfonamide group may result in the improvement of its pharmacological effects. However, not all sulfonamido-based gallates had potent effect in our investigation, which implied that only suitable modification of sulfonamide group with GA could result in the improvement of the pharmacological effects. As for the recommended dose of EJTC, the MTT assay showed that the concentrations of EJTC that enhance chondrocyte proliferation range from 3.125 μg/mL to 25 μg/mL (Fig. 2). The rate of DNA synthesis in human articular chondrocytes was increased in a dose-dependent manner during culture in medium containing EJTC at concentrations ranging from 3.125 to 15.625 μg/mL, and the cells treated with 7.8125 μg/mL EJTC exhibited the highest cell proliferation and showed the greatest matrix secretion. In conclusion, we demonstrated that EJTC can promote cell growth and maintain the phenotype of human articular chondrocytes. Thus, EJTC may be a potential pro-chondrogenic agent for ACI in the treatment of cartilage repair.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.015. These data include MOL files and InChiKeys of the most important compounds described in this article.

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24. *EJTC:* a pale yellow powder with a yield of 59%. MS-ESI: m/z: 429.2 $[M-H]^-$, ^1H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H, –CO–NH), 7.91 (m, 4H, 4× Ar-H), 6.94 (s, 2H, 2× Ar-H), 6.73 (s, 1H, Py-H), 2.23 (s, 6H, 2× $-CH_3$). ^{13}C NMR (125 MHz, DMSO-d_6) δ 167.59, 165.99, 156.30, 145.57, 143.39, 137.29, 134.28, 129.05, 124.42, 118.99, 113.61, 107.84, 22.95.