Anti-inflammatory and anti-osteoarthritis effects of tectorigenin

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ABSTRACT

Osteoarthritis (OA) is a common and dynamic joint disease, including the articular cartilage, underlying bones, and synovium. In particular, OA is considered as the degeneration of the cartilage. Tectorigenin (Tec) can affect many biological processes. However, its effect on articular chondrocytes remains unclear. This study aimed to assess the role of Tec in articular cartilage. In vitro, Tec inhibited the expression levels of type X collagen, cyclooxygenase-2, matrix metalloproteinase (MMP)-3, and MMP-13 gene but enhanced those of Runx1, type II collagen, and aggrecan in the presence of IL-1β. Meanwhile, Tec inhibited apoptosis through the Bax/Bcl-2/caspase-3 pathway, upregulating p-Bad, downregulating Bax/Bcl-2 ratio, and activating caspase-3 compared with IL-1β treatment only. Moreover, this process was partially regulated by NF-κB P65. In vivo, the chondroprotective effect of Tec was assessed by establishing a model of surgically induced OA. Tec-treated joints exhibited fewer osteoarthritic changes than saline-treated joints. Meanwhile, 1.5 μg/kg Tec treatment produced better protective effect than 0.75 μg/kg. Osteoarthritis Research Society International scoring system were employed to assess histopathological grading of the models, as well as the outcomes of immunohistochemistry for Aggrecan Neoepitope and MMP-3, further confirmed the results. In conclusion, this study showed that Tec played a chondroprotective role on the OA process by preventing articular cartilage degeneration and chondrocyte apoptosis via the NF-κB P65 pathway.

KEYWORDS: Tectorigenin; Runx1; Apoptosis; NF-κB p65; Osteoarthritis
INTRODUCTION

Osteoarthritis (OA) is a common joint disease; it is regarded as a local inflammatory response caused by joint instability and accompanied by the progressive degeneration of articular cartilage[1], particularly in sites where stress exceeds the value that can be sustained by the joint. Over 12% of the aging Western population has been reported to suffer from OA, particularly those who are 45 years and older [2, 3]. Given the aging population in the developed world, the OA prevalence is projected to increase in the coming decades[4]. However, the exact OA pathogenesis remains a subject of debate and research.

During the OA process, a number of inflammatory mediators have been reported[5]. Interleukin (IL)1β is considered as a main inflammatory mediator resulting the occurrence of OA by damaging articular cartilage via NF-κB pathway activation[5-7]. The destructive effect is also affected by cyclooxygenase-2 (Cox-2), which produces prostaglandin E2(PGE2), resulting inflammation and pain in OA [8, 9]. Cox-2 does harm to superficial layers of articular cartilage, furthermore NF-κB pathway play an important role during the process.

At present, disease-modifying OA drug (DMOAD) that can prevent and rescue OA damage is yet to be available [10, 11]. Common treatments for OA include nonsteroidal anti-inflammatory drugs, analgesics, locally administered corticosteroids, and viscosupplementation. These medications only provide patients with symptomatic relief, and require surgical intervention in the end. Nowadays, many attentions have been brought to improve the the degeneration of articular cartilage, with minimal success [12].

Tectorigenin (Tec), which is an effective component of traditional Chinese medicine derived from Belamcanda chinensis, has attracted considerable interest because of its antiproliferative, anti-inflammatory, and antioxidant activities [13-16]. Tec inhibits inflammatory responses caused by interferon-γ/lipopolysaccharide in the murine macrophage RAW264.7 cells[17]. Moreover, its anti-inflammatory effect inhibits NO synthase [18] and Cox-2 expression, NO and PGE2 production, IL-1β secretion, and NF-κB signaling blockage [17, 19, 20].

Although Tec is involved in many biological activities, information regarding its effects on ameliorating OA remains minimal, particularly on the prevention of the cartilage degeneration. Thus, our study aims to clarify the role of Tec OA, as well as to unveil the mechanisms by which Tec affects articular cartilage degeneration. We report that Tec has anti-inflammatory and anti-osteoarthritis effects during the process of OA.
MATERIALS AND METHODS

Reagents

Tec (purity, >98%; CAS number, 548-77-6; molecular weight, 300.26) was supplied by Cdmust Bio, Ltd. (Chengdu, China).

Experimental animals

Animal experiments were conducted according to protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Shanghai Jiaotong University (Shanghai, China).

Primary cell culture

The primary chondrocytes were isolated from the femoral condyles and tibial plateau of male Sprague Dawley (SD) rats (160–180 g). Rat articular cartilage was cut into small fragments and digested first with 0.25% trypsin (Gibco Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C and then with 0.2% collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 5 h at 37 °C. After dissociation, the cell suspension was filtered through a 40 μm cell strainer (BD Falcon, Bedford, MA, USA), and the cells were collected via centrifugation at 800×g for 10 min. Chondrocytes were resuspended in DMEM/F-12 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA, USA). The primary chondrocytes were cultured following a previously presented method[21]. The chondrocytes were seeded in six-well plates (2×10^5/well), and the sub-confluent cells were pre-incubated with five Tec concentrations (25, 50, 100, 200, and 400 μM, as determined in the preliminary tests) for 1 h; stimulation with IL-1β (10 ng/mL) for 24 h followed. The cells were harvested, and the related gene mRNA expression and protein levels were determined to assess Tec concentrations, which were used in subsequent experiments.

MTT assay

To determine the cytotoxicity of Tec on chondrocytes, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (#M5655; Sigma-Aldrich, St. Louis, MO, USA) assay was applied based on the previously described protocol [25].

Apoptosis assay

Annexin V and propidium iodide (PI) double stainings were conducted to determine the apoptosis level caused by the Tec. Chondrocytes were seeded at 2×10^5 cells/well in six-well
plates and incubated for 24 h. The images were recorded using a fluorescent inverted microscope. The remainder of the cells were collected and resuspended in 1 × binding buffer to a proper concentration of approximately 1 × 10^6 cells/mL. The cell suspension added with 5 μL annexin V and 1 μL PI (#V23200; Life Technologies) was incubated for 15 min in the dark at room temperature. Thereafter, 400 μL 1 × binding buffer was added to the suspension, and the samples were examined through flow cytometry (FACSA, BD Biosciences) at a wavelength of 488 nm.

**Surgical induction of OA**

Animal handling and experimental procedures were performed following approval from the Institute of Health Sciences Institutional Animal Care and Use Committee. Eight-week-old male SD rats (200 g) were randomly divided into four groups: (1) 1.5 μg/kg Tec-treated animals, (2) 0.75 μg/kg Tec-treated animals, (3) saline-treated animals, and (4) sham group (n = 10 mice in each group). OA was induced via medial collateral ligament transection and medial meniscal tear on the knee joints, as previously described[22]. The animals were anesthetized, and surgery was performed to transect the medial collateral ligament and cut through the full thickness of the medial meniscus to induce joint destabilization of the right knee. Sham animals underwent the same surgical procedure without any ligament transection or meniscal tear. After surgery, each rat was given penicillin once a day for the first 3 days. For intra-articular injection, Tec (Cdmust Bio, Ltd. Chengdu, China) was dissolved in 100 μM in DMSO (#94563; Sigma-Aldrich, St. Louis, MO, USA) before being used in sterile saline (0.9% NaCl; JinTong Pharmaceutical Factory, Shanghai, China). The mice were re-anesthetized and administered with 10 μL intra-articular injection of Tec or saline immediately after surgery every 5 days for 8 weeks. The animals were sacrificed at 8 weeks post-surgery, and samples of the knee joints were collected for further molecular and histological analyses.

**Real-time polymerase chain reaction (PCR) and Western blot analysis**

PCR and Western blot analysis based on the previously described protocol[23]. The primers were produced by Sangon Biotech (Shanghai, China). The primer sequences are listed in Supplementary Table 1.
**Statistical analysis**

All numerical data are expressed as mean±SD. Statistical differences among groups were analyzed by one-way ANOVA. All statistical analyses were performed with SPSS software, version 16.0. Statistical differences between two groups were determined by the Student’s t-test; p<0.05 was considered statistically significant.

**RESULTS**

**Effects of Tec on viability**

The chemical structure is shown in Fig. 1A. The different concentrations (0, 25, 50, 100, 200, and 400 μM) exerted on the primary cultured chondrocytes were assessed via MTT assay. Tec concentrations of ≥200 μM exhibited mildly toxic effects on chondrocytes, particularly at 48 h and 72 h of culture. The effect heightened at 400 μM after 48 h of culture (Fig. 1B, P < 0.05). Thus, 25, 50, and 100 μM Tec concentrations were used for the in vitro tests.

**Chondroprotective effects of Tec on primary cultured articular chondrocytes**

To determine the role of Tec on primary cultured articular chondrocytes, the chondrocytes were pre-incubated with Tec for 1 h, then stimulated with IL-1β for 24 h. IL-1β-stimulated chondrocytes exhibited upregulation of Col10a1, Cox-2, MMP-3, and MMP-13 levels but downregulation of Runx1, Col2a1, and Acan expression levels (##P < 0.01, ###P < 0.001). Tec inhibited the IL-1β-mediated induction of Col10a1, Cox-2, MMP-3, and MMP-13 levels and enhanced levels of Runx1, Col2a1, and Acan with IL-1β (Fig. 1C, *P < 0.05, **P < 0.01). Additionally, these changes were more significant at 50 μM and 100 μM concentrations in contrast to 25 μM. Moreover, an immunofluorescence staining was conducted to further demonstrate the effects of Tec in Col2a1 and MMP-13 expression (Fig. 1D). IL-1β treatment dramatically decreased the Col2a1 expression and increased MMP-13 expression. Nevertheless, Tec reversed the trend at 100 μM. Furthermore, 100 μM Tec further enhanced the cartilage matrix synthesis (Alcian blue and toluidine blue staining) compared with IL-1β treatment only (Fig. 1E).
Tec downregulated Cox-2 and upregulated Runx1 via NF-κB P65

To evaluate which pathway was involved in the chondroprotective effects of Tec, we conducted an experiment on Tec (50 μM and 100 μM) at the protein level. Consequently, Western blot and densitometric analyses showed that Tec downregulated the expression levels of P-p65, MMP-3, Col10a1, and Cox-2, but upregulated that of Runx1, particularly in Tec 100 μM when compared with IL-1β treatment only. The results indicated that NF-κB P65 was involved in the mechanism behind the chondroprotective effects of Tec (Figs. 2A and 2B). By using a specific NF-κB inhibitor, BAY-11-7082, more obvious trend of Col10a1, MMP-3, Runx1 and Cox-2 was detected compared with those of the IL-1β and Tec treatment group. These results further demonstrated that Tec exerted chondroprotective effects partially through NF-κB P65 (Figs. 2C).

Inhibitory effect of Tec exerted on IL-1β-induced apoptosis in chondrocytes through NF-κB pathway

To elucidate the role of Tec upon apoptosis in chondrocytes, we performed an experiment on chondrocytes that were pre-incubated with Tec for 1 h, then stimulated with IL-1β for 24 h; following which, flow cytometry as well as the proteins were examined. Based on the quantitative results, we observed less apoptosis in chondrocytes (Figs. 3A and 3B) and decreased protein level of p-p65, Bax, and caspase-3 and increased Bcl-2 and p-Bad in Tec group compared with the IL-1β treatment only group (P < 0.05) (Figs. 3C and 3D). This result showed that Tec played a protective role in IL-1β-induced apoptosis in chondrocytes through NF-κB pathway.

Protective effects of Tec on surgically induced OA

We also assessed the role of Tec on OA mouse model to test if Tec has a protective effect in vivo. Periodic Tec or saline were injected into the knee joints (after OA model establishment) for 6 weeks. The representative histological sections showed that the Tec-treated joints had less osteoarthritic damage than the saline-treated group. Furthermore, the protective effects were more evident in 1.5 μg/kg mice group than in 0.75 μg/kg mice group. The histopathological grading using the OARSI scoring system [24] further confirmed the result (Figs. 4A and 4B). Immunohistochemistry for Aggrecan Neoepitope and MMP-3 indicated decreased number of Aggrecan Neoepitope and MMP-3 in Tec group (0.75 μg/kg) compared
with saline-treated group. Moreover, the trend of decreased number of Aggrecan Neoepitope and MMP-3 was more significant in Tec group (1.5 μg/kg) (Figs. 4C).

DISCUSSION

Tec, which inhibits the inflammation of acute lung injury in mice, is derived from the Chinese herb B [25]. Another study shows that antibacterial activity against methicillin-resistant Staphylococcus aureus is also confirmed [26]. Therefore, Tec may represent a potentially effective option to treat inflammation. However, the mechanisms of Tec in OA are yet to be determined. The current research aims to investigate whether or not Tec has an anti-inflammatory activity against OA. Our data showed that Tec suppressed the OA process. In particular, Tec prevented chondrocytes degeneration and chondrocytes apoptosis without inducing cartilaginous hypertrophy.

OA is a common disease and its incidence mushrooms with age. At present, OA is managed through various treatment modalities, including pharmacological and nonpharmacological therapies [27, 28]. Till now, however, no effective treatment has been found. Most therapeutic methods, particularly the DMOAD, attempt to preventing or delaying the articular cartilage degeneration and achieved minimal success [12]. Thus, repairing damaged articular cartilage may provide another way for OA patients. In the present study, we observed that Tec a new potential candidate for OA treatment.

Previous reports showed that OA development may be resulted from hypertrophic differentiation of articular chondrocytes [3]. This result is further supported by our findings that Tec may improve OA without inducing hypertrophy, as determined by the assessments of the increased expression of Runx1, which induces chondrogenic differentiation and suppresses subsequent hypertrophy [18, 29], as well as the decreased hypertrophic marker expression (Collagen type X) (Figs. 3A and 3B). In OA, however, articular cartilage abnormally suffers from hypertrophy and followed by endochondral ossification, which result in arthrosis degeneration [30]. Multiple signaling pathways, including WNT and NF-κB, are involved in the hypertrophic differentiation of articular chondrocytes, [31, 32]. The outcomes were consistent with our finding that Tec partially inhibited NF-κB p65, accompanied by increasing Runx1 level and declining Collagen X level. Moreover, NF-κB inhibitor BAY-11-7082 intensified this trend (Fig. 2C).
Chondrocytes, which are the only cell in articular cartilage, maintaining the dynamic balance between synthesis and degradation of the extracellular matrix (ECM). Apoptotic cell death has been observed in OA cartilage, which was related to matrix degradation and calcification, suggesting a role in OA pathogenesis. Previous study has shown that Caspase-3 is regarded as an main factor in the process of occurrence of apoptosis [33]. Furthermore, Bcl family, which can be divided into anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) and pro-apoptotic proteins (Bax, Bad, Bak, Bik, and Bid), have been reported to play an important part during apoptosis. [34]. In our study, we observed that Tec inhibited apoptosis via the Bax/Bcl-2/caspase-3 pathway, upregulating p-Bad, downregulating Bax/Bcl-2 ratio, and activating caspase-3 when compared to IL-1β treatment only. To our interest, Tec decreased protein level of p-p65, compared to IL-1β treatment only (Fig. 3C). The result showed that Tec played a protective role in IL-1β-induced apoptosis in chondrocytes partially through NF-κB pathway. However, the inhibitors of apoptosis may have potential side effects, such as carcinogenesis [35]. Thus, modifying apoptosis safely to inhibit or suppress apoptosis in OA remains obscure.

In conclusion, this study demonstrated the positive role of Tec in OA restriction. In vitro, we identified Tec as an apoptosis inhibitor in chondrocytes partially via the NF-κB P65 pathway. In vivo investigation showed that Tec could ameliorate manifestation of knee joint cartilage in OA model. The outcomes indicate that Tec may be a potential therapeutic candidate for OA treatment.

**Conflict of interest**

All authors have no conflict of interest to state.

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References

Figure 1: Chondroprotective effects of Tec on chondrocytes.

(A) Chemical structure of Tec. (B) Primary cultured chondrocytes were initially plated in each well of a 96-well plate and treated with different Tec concentrations (0, 25, 50, 100, 200, and 400 μM) for 24, 48, and 72 h. The values are expressed as mean ± SD of three independent experiments. Cells incubated in the culture medium without Tec were used as controls and considered 100% viable. (C) Primary cultured articular chondrocytes were pretreated with different Tec concentrations (25, 50, and 100 μM) for 1 h and subsequently stimulated with IL-1β (10 ng/mL) for 24 h. qRT-PCR was performed to determine the expression levels of Col2a1, Col10a1, MMP-3, MMP-13, Runx1, Cox-2, and Acan in chondrocytes. Data are expressed as mean ± SD. * Compared with cells stimulated with IL-1β alone (*P < 0.05, **P < 0.01). # vs. cells cultured without Tec and IL-1β (##P < 0.01, ###P < 0.001). (D) Primary cultured articular chondrocytes were treated with or without 10 ng/mL IL-1β and 100 μM Tec for 24 h. Expression levels of Col2a1 and MMP-13 were detected via immunofluorescence staining. Meanwhile, quantified positive cells of Col2a1 and MMP-13 (right). (E) Alcian blue and toluidine blue staining in chondrocytes at 14 day cultured with or without IL-1β (10 ng/mL) and Tec (100 μM) for 72 h. Employing Aggrecan content to quantify Alcian blue and toluidine blue staining.
Figure 2: Tec downregulated Cox-2 and upregulated Runx1 via NF-κB P65.

(A) The primary cultured articular chondrocytes were treated with or without IL-1β (10 ng/mL) and Tec (50 μM and 100 μM) for 72 h. The expression levels of Col10a1, MMP3, Cox-2, Runx1, and p-P65 were analyzed via Western blot. GAPDH was used as the loading control. (B) Densitometric analysis of the immunoblot band intensities for Col10a1, MMP-3, Cox-2, Runx1, and p-P65 was normalized using GAPDH. (C) qRT-PCR was performed to determine the expression levels of Col10a1, MMP-3, Runx1 and Cox-2 by using a specific NF-κB inhibitor, BAY-11-7082. Data are expressed as mean ± SD of three independent experiments. [n = 3; * compared with cells stimulated with IL-1β only (*P < 0.05, **P < 0.01, *** P < 0.001). # vs. cells cultured without Tec and IL-1β (#P < 0.05, ##P < 0.01, ###P < 0.001). & compared with cells stimulated with Tec and IL-1β (&P < 0.05, &&P < 0.01) ]
Figure 3: Effects of Tec on IL-1β-induced apoptosis in chondrocytes.

(A) Apoptosis assayed via flow cytometry using annexin V/PI double staining. (B) Quantitative analysis of apoptotic chondrocytes in each group. (C) Apoptosis-related proteins were analyzed through Western blot after Tec treatment (50 μM) and without Tec treatment. (D) Quantitative analysis of expression of p-Bad, Bax, Bcl-2 and caspase-3. Data are expressed as mean ± SD of three independent experiments. Data of the treatment group was expressed as fold change vs. that of control group (labeled as “1.00”) after normalized to Gapdh. [* compared with cells stimulated without Tec and IL-1β(* P < 0.05, ** P < 0.01, *** P < 0.001). # vs. cells cultured with IL-1β only (#P < 0.05, ##P < 0.01, ###P < 0.001)].
Figure 4: Effects of Tec treatment on surgically induced OA.

(A) Knee joints were harvested at 6 weeks postsurgery and analyzed histologically via Safranin O–fast green staining. Representative images are shown. (B) The quantification of OA on the femoral and tibial cartilage was scored histologically using the OARSI scoring system at 8 weeks postsurgery. (C) Immunohistochemistry for Aggrecan Neoepitope and MMP-3 was conducted. Red arrows indicate positive immunohistochemical staining of Aggrecan Neoepitope and MMP-3. Data are expressed as mean ± SEM (n = 10). *P < 0.05, **P < 0.01 vs. saline. Scale bar, 50 μm.