Food & Function

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c9fo01089k

Troxerutin suppresses the inflammatory response in advanced glycation end-product-administered chondrocytes and attenuates mouse osteoarthritis development

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As a chronic degenerative joint disease, osteoarthritis (OA) is clinically characterized by a high incidence, long-term pain, and limited joint activity but without effective preventative therapy. Troxerutin (Tx) is a natural flavonoid, also called vitamin P4, which is widely present in plants consumed as part of our daily diet, such as cereals, various fruits and vegetables, tea, and coffee, and possesses various biological activities, especially an anti-inflammatory effect. Here, we aimed to investigate the potential chondroprotection of Tx in experimental OA development. In *in vitro* studies, human chondrocytes were isolated and exposed in advanced glycation end-products (AGEs) to simulate OA development. It was found that Tx pretreatment inhibited the AGE-induced production of pro-inflammatory factors in chondrocytes, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). Meanwhile, AGE-medicated extracellular matrix (ECM) degradation was decreased in Tx-pretreated chondrocytes. Furthermore, we found that Tx pretreatment suppressed the activation of the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in AGE-exposed chondrocytes. *In vivo*, Tx treatment prevented the narrowing of the joint space, the calcification of cartilage, and the loss of proteoglycans in the mouse OA model. In brief, Tx is considered as a potential therapeutic agent for OA.

Received 22nd May 2019,
Accepted 12th July 2019

DOI: 10.1039/c9fo01089k

rsc.li/food-function

1. Introduction

As the population ages, osteoarthritis (OA) gradually becomes the most prevalent senile joint disorder, which is the fourth leading cause of disability and affects the life quality of at least 10% of the population.[1,2] Unfortunately, the clinical treatment of OA is limited to relieving symptoms by pharmaceuticals and surgeries, while there is little control over OA development.[3,4] For non-pharmacological therapies, some researchers have reported that physical activity, nutrition, and stem cell transplantation promote cartilage restoration in an OA animal model.[5–8]

Currently, the aetiology and pathogenesis of OA is still not completely clear, but aging has been identified as one of the critical risk factors for OA development.[9,10] With the senescence process, the nonenzymatic glycation of macromolecules (called the Maillard reaction) causes the generation of advanced glycation end-products (AGEs), which are poorly degenerated.[11–13] These phenomena lead to the plentiful accumulation of AGEs in the joint cartilage; subsequently, accumulated AGEs affect the mechanical performance of articular cartilage and induce an imbalance between extracellular matrix molecule (ECM) catabolism and anabolism.[14,15] In addition, the receptor of AGE (RAGE) has been found in articular chondrocytes and synoviocytes.[16] The activation of RAGE by AGEs in chondrocytes causes the production of multiple pro-inflammatory factors and catabolic mediators, including TNF-α, interleukin, matrix metalloproteases (MMPs), and thrombospondin motifs (ADAMTS), contributing to chondrocytes dysfunction and ECM degeneration.[17] Thus, we utilized AGEs as a potent agent in our *in vitro* experiments of OA.

The inflammatory response and ECM degradation stimulated by AGEs in chondrocytes are related to nuclear factor

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kappa B (NF-κB) signaling.[18] It is reported that AGE treatment in human chondrocytes could activate the NF-κB pathway in a concentration- and time-dependent manner.[14,15] Also, it increases nuclear p65 expression and decreases cytoplasmic IκBα expression. Meanwhile, the pretreatment of chondrocytes with pyrrolidine dithiocarbamate (PDTC), a recognized NF-κB inhibitor, could suppress AGE-induced ECM degeneration.[14] Moreover, AGEs have induced the phosphorylation of c-Jun N-terminal kinase (JNK) and p38, two key members of the mitogen-activated protein kinase (MAPK) signal family, in human and rabbit chondrocytes, but the extracellular signal-regulated kinase (ERK) phosphorylation only showed a slight and transient increase.[19,20] Also, small molecule-specific inhibitors of JNK and p38, but not ERK, could potentially reverse AGE-induced collagen II reduction in chondrocytes.[14] Therefore, the NF-κB and MAPK pathways are considered as potential therapeutic targets of OA.

Troxerutin (Tx) is a natural flavonoid widely present in plants consumed as part of our daily diet, such as cereals, various fruit and vegetables, tea, and coffee.[21] Tx is also called vitamin P4 and exerts abundant biological activities, including anti-oxidant, anti-thrombosis, and anti-diabetic. It is worth mentioning that the anti-inflammatory effect of Tx was proved by performing extensive cellular and animal experiments.[22] Hu *et al.* reported that Tx alleviated the transcription of inflammatory genes in BDE-47(2,2′,4,4′-tetrabromodiphenyl ether)-induced mouse liver injury *via* inhibiting the nuclear transfer of p65.[23] In a gentamycin-induced acute kidney injury model, rats co-treated with Tx and gentamycin showed lower levels of TNF-α, IL-6, and IL-10 expression as well as the down-regulation of p38 phosphorylation relative to gentamycin-treated rats.[24] Due to these biological properties mentioned above, we plausibly hypothesized that Tx may protect chondrocytes against OA development. In the present study, we treated human chondrocytes with AGEs *in vitro* and established a mouse OA model by surgery to confirm the protective effects of Tx and to explore the underlining mechanisms.

2. Materials and methods

2.1 Ethics statement

The collection of human articular cartilage tissues was carried out according to the terms of the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical University, following the guidelines of the Declaration of Helsinki. Also, all the experiments were performed in accordance with the guidelines of the Declaration of Helsinki, and approved by the ethics committee at Wenzhou Medical University (ethic code: 2018-078). Informed consents were obtained from all human participants of this study.

2.2 Reagents and antibodies

Troxerutin (purity ≥95%) was purchased from Selleck Chemicals (Houston, TX, USA). The primary antibodies against COX-2, IκBα, p65, p-p38, p38, p-JNK, and JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies against collagen II, ADAMTS5, iNOS, Lamin B, and GADPH were acquired from Abcam (Cambridge, UK). Goat anti-rabbit and anti-mouse IgG-HRP were from Bioworld (OH, USA). Alexa Fluor®488 labeled Goat Anti-Rabbit IgG (H+L) second antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Bovine serum albumin (BSA) and AGE-BSA (AGEs), safranin-O, fast green, and type II collagenases were purchased from Sigma-Aldrich (St Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). The 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Beyotime (Shanghai, China). Bicinchoninic acid (BCA) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The cell culture reagents were purchased from Gibco (Grand Island, NY, USA).

2.3 Primary human chondrocytes culture

Joint cartilage tissues were obtained from eight OA patients (aged 63–70 years, three men and five women) who undergoing total knee replacement surgery at the Second Affiliated Hospital of Wenzhou Medical University. Full ethical consent was obtained from all patients. The hyaline cartilage tissues were cut into smallish pieces and washed three times with PBS. After washing by PBS, tissue was dealed with 0.2% type II collagenase collagenase II, which could digest cartilage tissues, for 5 h at 37 °C. After washing by PBS and resuspension, chondrocytes were cultured into tissue culture flasks in DMEM/F12 supplemented with 10% FBS and 1% antibiotic in an atmosphere of 5% CO_2 at 37 °C. At about 80% confluence, chondrocytes were starved in serum-free medium overnight. Then, the cells were replanted into 10 cm culture plates at the appropriate density. The second-passage chondrocytes were used for the following experiments to avoid the phenotype loss.

2.4 Animal model

Forty-five eight-week-old C57BL/6 male wild-type (WT) mice were purchased from the Animal Center of the Chinese Academy of Sciences Shanghai, China. All of the experimental procedures involving animal care and use were according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the approval of the Animal Care and Use Committee of Wenzhou Medical University. The mouse osteoarthritis model was induced by surgical destabilization of the medial meniscus (DMM).[25] That is, the mice were tranquilized with an intraperitoneal injection of 2% (w/v) pentobarbital (40 mg kg^{-1},) and then the joint capsule of the right knee was incised just medial to the patellar tendon and the medial meniscotibial ligament was cut with microsurgical scissors. Surgery, including an arthrotomy, was also performed in the mice of a dummy group as a control, which lacked a snipped medial meniscotibial ligament. After surgery, the mice were randomly assigned to three groups: the dummy group, DMM group, and DMM + Tx treatment group.

2.5 Experimental design

For the *in vitro* study, to investigate the chondroprotective effect of Tx, the chondrocytes were treated with 50 μg mL^{-1} AGEs, alone or in combination with Tx pretreatment at different concentrations (5, 10, 20 μM). A control group was left untreated except for the change of the medium. To explore the potent mechanism of Tx-induced protection, the dose and duration of AGEs stimulation was 50 μg mL^{-1} and 2 h and the dose of Tx was 20 μM. However, to explore the functional changes, such as the inflammatory or ECM markers, the duration of AGEs was extended to 24 h.

For the *in vivo* experiments, mice suffered surgical destabilization of the medial meniscus (DMM) as described above. After surgery, the DMM + Tx group were administered Tx dissolved in water (30 mg kg^{-1}) by oral gavage once a day for eight consecutive weeks. At the same time, animals in the DMM group received an identical volume of water. All the experimental animals were sacrificed after eight weeks post-surgery, and mouse knee joint tissue samples were collected for histological and X-ray analysis.

2.6 Cell viability assay

The second-passage chondrocytes (8000 cells per well of a 96-well plate) were treated with Tx (2.5, 5, 10, 20, 40 μM) for 24 h or AGEs (12.5, 25, 50, 100, 200 μg ml^{-1}) in serum-free medium and the viability was determined using the cell counting kit-8 (CCK-8; Dojindo Co, Kumamoto, Japan) following the manufacturer's instructions. All the experiments were performed five times.

2.7 NO measurement and ELISA

The interaction of NO in culture medium was measured by Griess reagent. After the treatment of the OA chondrocytes, culture supernatants were collected to estimate the levels of PGE2, TNF-α and IL-6, collagen II, aggrecan, MMP13, and ADAMTS-5 in the supernatants using a commercially available ELISA kit (R&D Systems, Minneapoils, MN, USA) according to the manufacturer's instructions. All the assays were performed five times.

2.8 Western blotting

After the treatments, the chondrocytes were harvested, washed with cold PBS, and lysed in ice-cold RIPA lysis buffer with 1 mM PMSF (phenylmethanesulfonyl fluoride) and the lysate was prepared for 10 min followed by 15 min centrifugation at 12 000 rpm and 4 °C. Then, the BCA protein assay kit (Beyotime) was used to detect the concentrations of each protein. Equivalent amounts of lysate protein (40 ng) were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). After blocking with 5% nonfat milk for 2 h, the blots were incubated with primary antibodies against GADPH (1 : 5000), iNOS (1 : 1000), COX-2 (1 : 1000), p65 (1 : 1000), IκBα (1 : 1000), and Lamin B1 (1 : 1000), JNK (1 : 1000), p-JNK (1 : 1000), p38 (1 : 1000), p-p38 (1 : 1000), diluted in 2% TBST, overnight at 4 °C. Blots were then incubated with the respective secondary antibody for 2 h at room temperature, followed by washing with TBST three times. Finally, the blots were visualized by electrochemiluminescence plus the reagent (Invitrogen). The intensity of these blots was quantified with Image Lab 3.0 software (Bio-Rad).

2.9 Immunofluorescence

For collagen II and ADAMTS-5 staining, the chondrocytes were planted in glass plates in a six-well plate together with AGEs or co-treated with 50 μg ml^{-1} AGEs and 20 μm Tx for 24 h. For p65 staining, the duration of AGEs was reduced to 2 h. After treatment, the cells were washed with 500 μL of 1× PBS three times and fixed using 400 μL of 4% paraformaldehyde (pH 7.4) for 10 min at 37 °C, followed by permeation by adding 400 μL of 0.1% Triton X-100 in 1× PBS for 15 min. Next, 5% bovine serum albumin was added at 37 °C for 60 min to block the cells. The cells were rinsed with PBS and incubated with primary antibodies diluted in PBS : collagen II (1 : 100), ADAMTS5 (1 : 100), and p65 (1 : 100) in a humid chamber overnight at 4 °C. After treatment, the samples were rinsed three times in PBS and incubated with Alexa Fluor®488 labeled conjugated second antibodies (1 : 200) for 1 h at room temperature and then labeled with DAPI (Invitrogen) for 5 min. Images could easily be seen through a fluorescence microscope (Olympus Inc., Tokyo, Japan).

2.10 Molecular modeling

The molecular structure of ISO was drawn with ChemBioDraw, and ChemBio3D was used to minimize its energy. p65 (PDB ID: 1LE9), JNK (PDB ID: 4IZY), and p38 (PDB ID: 1OUK) were chosen for the docking studies and prepared for docking after being downloaded from PDB (https://www.rcsb.org/).[26–28] After being minimized using PyMoL (version 1.7.6), the lowest energy conformations for docking were determined *via* the default parameters. The protein–ligand docking analysis was conducted using AutoDockTools (version 1.5.6), which can provide ligand binding flexibility with the binding pocket residues. The images were finally generated using UCSF PyMoL.

2.11 X-ray imaging method

After 8 weeks of surgery within or with no treatment, the mice were given the X-ray examination. X-ray imaging was performed on all the mice to evaluate the joint space, osteophyte formation, and calcification changes of the cartilage surface using a digital X-ray machine (Kubtec Model XPERT.8; KUB Technologies Inc.). Proper images were obtained in the following settings: 50 kV and 160 μA.

2.12 Histopathologic analysis

Slides of each mouse knee joints were prepared for histological analysis. Briefly, the knee joints were prepared by removing skin and muscle and then immediately fixed in 4% paraformaldehyde. The knee joints were further decalcified in 20% formic acid and embedded in paraffin. Slides of each joint

were stained with safranin O-fast green (S–O). The cellularity and morphology of the cartilage and subchondral bone were examined by another group of experienced histology researchers in a blind manner using a microscope, and evaluated by using an Osteoarthritis Research Society International (OARSI) scoring system for medial femoral condyle and medial tibial plateau as described previously.[29] Fifteen mice in each group were used for histomorphometric scoring.

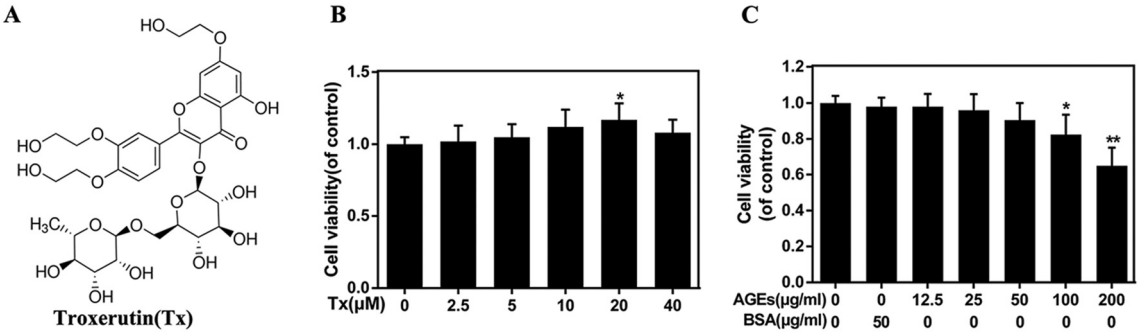

Fig. 1 Effect of Tx and AGEs on human chondrocytes viability. (A) Chemical structure of troxerutin. (B and C) Cytotoxicity of Tx and AGEs on chondrocytes determined at various concentrations for 24 h by CCK8 analysis. The data in the figures represent the averages ± S.D. Significant differences among different groups are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control group, $n = 5$.

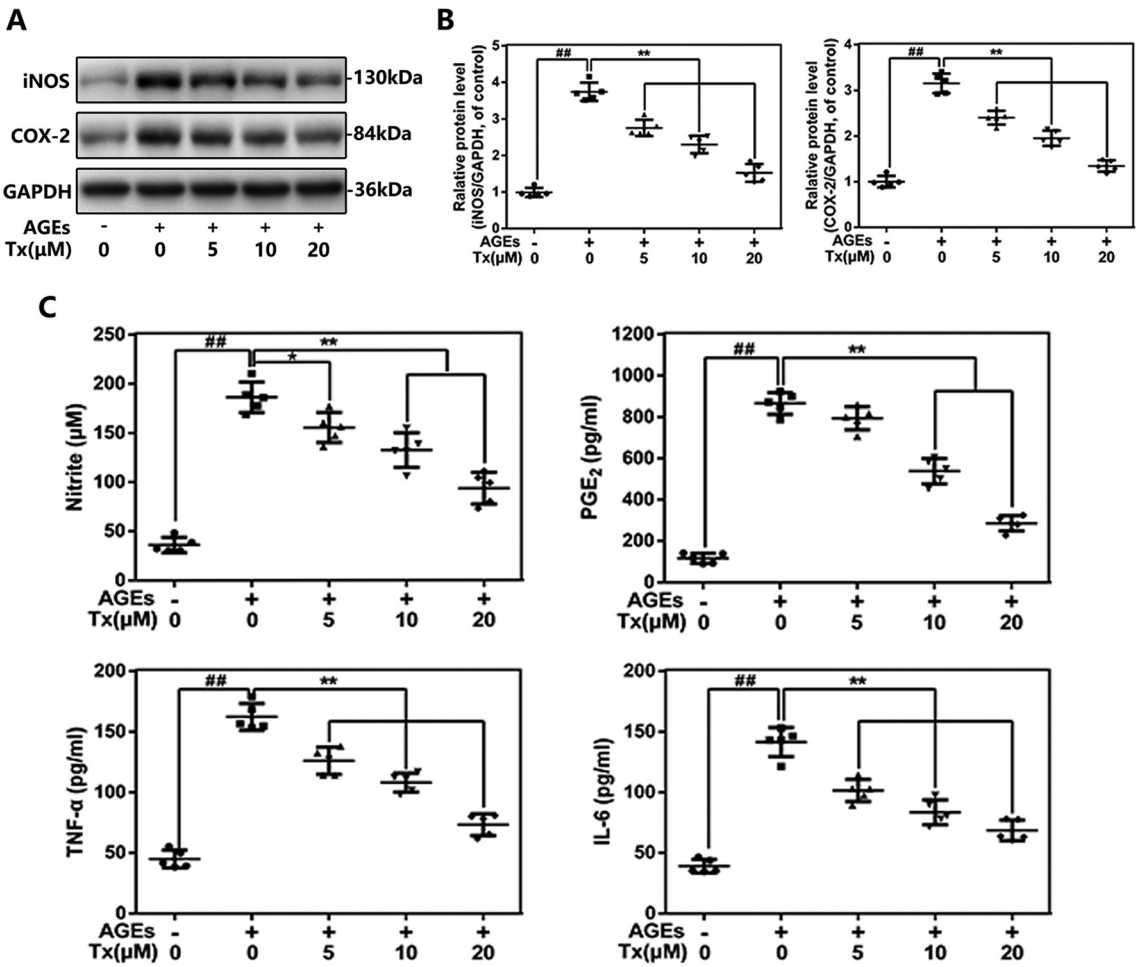

Fig. 2 Effect of Tx in AGE-induced inflammatory response in human chondrocytes. (A and B) The protein expressions of iNOS and COX-2 in chondrocytes treated as above detected by western blot. (C) Effect of Tx on AGE-exposed PGE_2, NO, TNF-α, and IL-6 production in human chondrocytes. The data in the figures represent the averages ± S.D. Significant differences among different groups are indicated as $^{\#\#}P < 0.01$, *vs.* control group; $^{**}P < 0.01$ *vs.* AGEs alone treatment group, $n = 5$.

2.13 Statistical analysis

The experiments were carried out at least five times. The results are presented as the mean ± S.D. Statistical analyses were performed using SPSS statistical software program 20.0. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test for comparison between the control and treatment groups. Nonparametric data (like the OARSI score) were analyzed by the Kruskal–Wallis H test. *P* values less than 0.05 were considered significant.

3. Result

3.1 Effect of Tx and AGEs on human chondrocytes' viability

The chemical structure of Tx is shown in Fig. 1A. Chondrocytes' viability in Tx and AGEs treatment were analyzed by a cell counting kit (CCK8) kit. As shown in Fig. 1B, Tx did not decrease chondrocytes viability after 24 h at 2.5, 5, 10, 20, and 40 μM, indicating that Tx displayed no cytotoxicity for human chondrocytes at the above dosages and times. Also, we used Tx with the concentrations of 5, 10, and 20 μM and a duration of 24 h in our subsequent experiments. However, the CCK8 result in Fig. 1C shows that AGEs inhibited human chondrocyte viability at the doses of 100 and 200 μg ml^{-1} for 24 h.

3.2 Tx alleviates inflammatory response in AGE-exposed human chondrocytes

Next, to assess the anti-inflammatory effect of Tx in AGE-treated human chondrocytes, several inflammatory productions were detected by western blot and ELISA. As shown in Fig. 2A and B, the expression of iNOS and COX-2 were upregulated after AGEs administration, but Tx pretreatment reversed these changes in a dose-dependent manner. Furthermore, it was found by ELISA analysis that Tx suppressed the AGE-induced increase of endogenous NO, PGE2, TNF-α, and IL-6 production in a concentration-dependent manner. These results suggest that Tx could effectively prevent AGE-caused inflammatory reactions.

3.3 Tx regulates ECM catabolism in AGE-treated human chondrocytes

As the crucial products of chondrocytes, the ECM synthesis and degradation level is considered as a pivotal index to

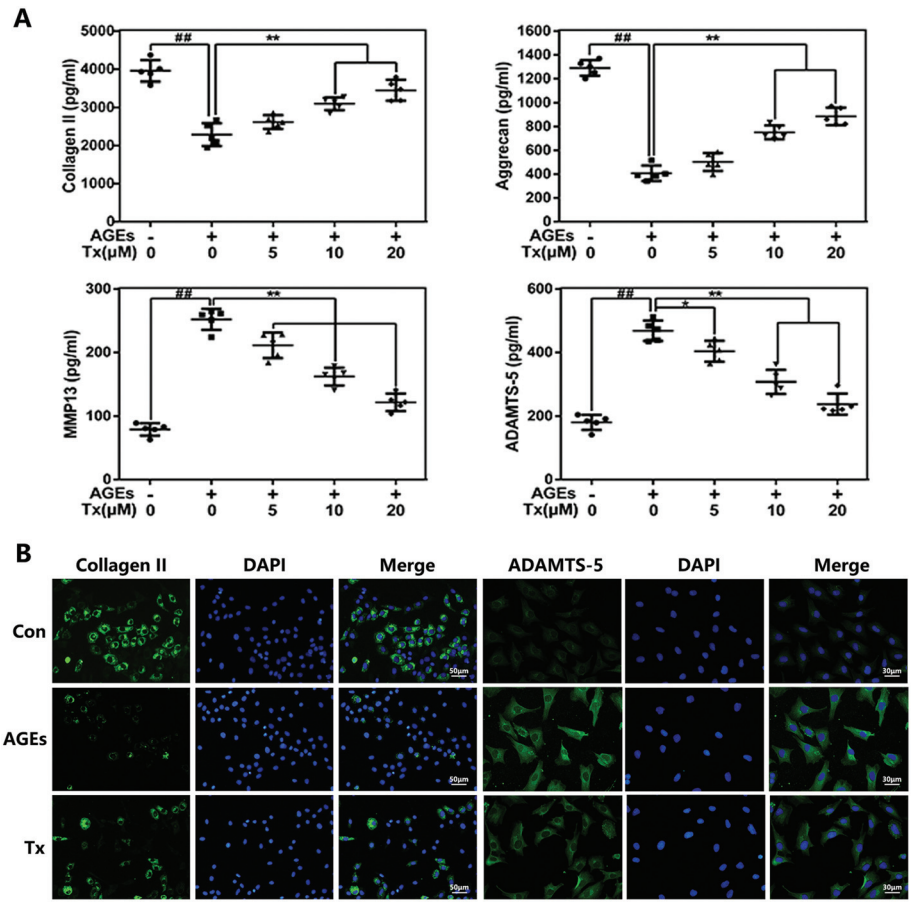

Fig. 3 Effect of Tx in AGE-medicated ECM degeneration in human chondrocytes. (A) The level of collagen II, aggrecan, MMP13, and ADAMTS-5 in chondrocytes treated as above were visualized by ELISA. (B) The representative collagen II and ADAMTS-5 was detected by the immunofluorescence combined with DAPI staining for nuclei (scale bar: 50 μm or 30 μm). The data in the figures represent the averages ± S.D. Significant differences among different groups are indicated as $^{\#\#}P < 0.01$, *vs.* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* AGEs alone treatment group, $n = 5$.

evaluate chondrocyte function.[30] The ELISA results found that AGEs treatment inhibited collagen II and aggrecan expression but promoted ADAMTS-5 and MMP13. Treatment with Tx reversed these trends in a concentration-dependent manner (Fig. 3A). Moreover, our immunofluorescence staining indicated the same result as that with the ELISA assay (Fig. 3B). In a word, these data demonstrate that Tx could alleviate ECM degradation at the protein level.

3.4 Tx inhibits the AGE-medicated activation of the NF-κB pathway in human chondrocytes

In order to explicit the potential mechanism about the Tx-induced chondroprotective effect, we measured the expression of the NF-κB signaling associated gene by western blot. We found that AGEs accelerated the cytoplasmic IκBα degradation and nuclear p65 translocation, whereas Tx pretreatment inhibited these phenomena (Fig. 4A and B). Meanwhile, Tx treatment alone did not affect the NF-κB signaling activity. Additionally, immunofluorescent staining intuitively manifested that Tx reduced the AGE-induced increase of the p65 fluorescence intensity in the nucleus, consistent with the western blot analysis results.

3.5 Tx suppresses AGE-increased MAPK signaling in human chondrocytes

The MAPK pathway has been demonstrated to be involved in AGE-medicated inflammation, ECM degeneration, and oxidative stress in human chondrocyte. After AGEs stimulation for 2 h, the JNK and p38 phosphorylation levels were increased, whereas pretreatment with Tx suppressed this change. However, the administration of Tx alone did not show a clear difference in p-JNK and p-p38 expressions relative to the control group (Fig. 5A and B).

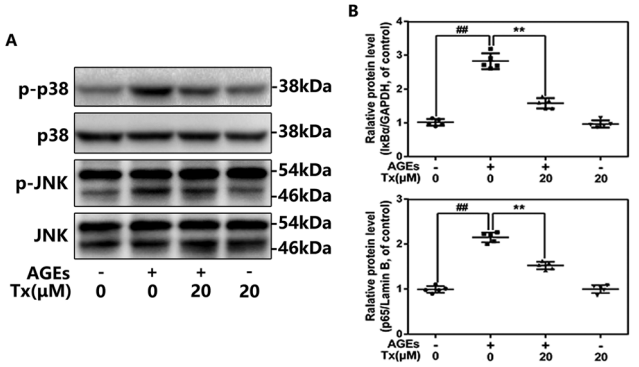

Fig. 5 Effect of Tx in AGE-increased MAPK signaling in human chondrocytes. (A and B) The protein expression of IκBα in cytoplasm and p65 in the nuclei in chondrocytes treated as above were detected by western blot. The data in the figures represent the averages ± S.D. Significant differences among different groups are indicated as $^{\#\#}P < 0.01$, *vs.* control group; $^{**}P < 0.01$ *vs.* AGEs alone treatment group, $n = 5$.

3.6 Molecular docking

To further confirm the underlying mechanism of Tx, we used molecular docking analysis to evaluate the direct affinity of Tx with pivotal proteins in the NF-κB and MAPK pathways. According to the molecular docking analysis, Tx exerted a high

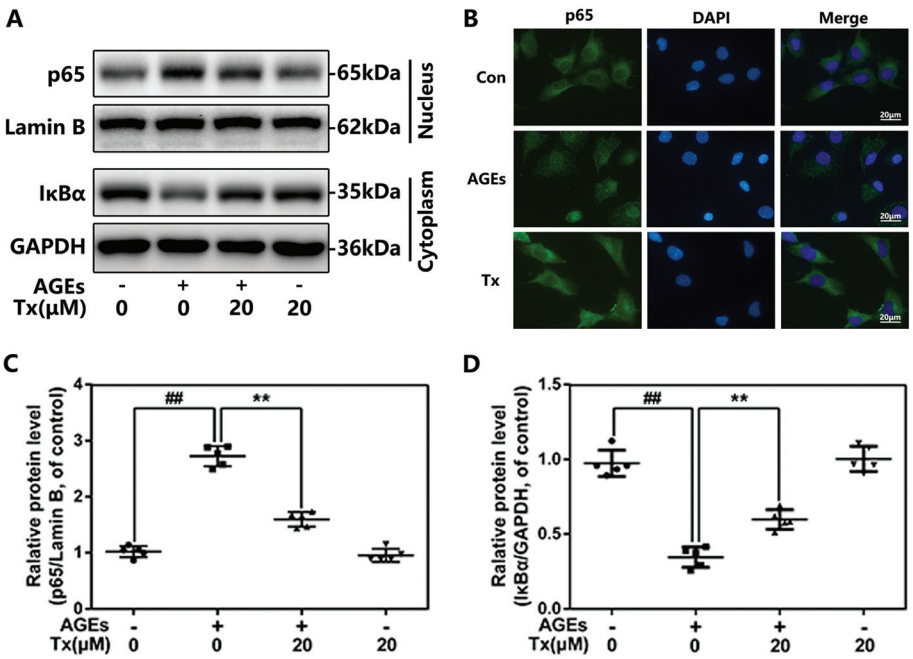

Fig. 4 Effect of Tx in AGE-medicated activation of the NF-κB pathway in human chondrocytes. (A, C and D) The protein expressions of IκBα in cytoplasm and p65 in the nuclei in chondrocytes treated as above were detected by western blot. (B) The nuclei translocation of p65 was detected by the immunofluorescence combined with DAPI staining for nuclei (scale bar: 20 μm). The data in the figures represent the averages ± S.D. Significant differences among different groups are indicated as $^{\#\#}P < 0.01$, *vs.* control group; $^{**}P < 0.01$ *vs.* AGEs alone treatment group, $n = 5$.

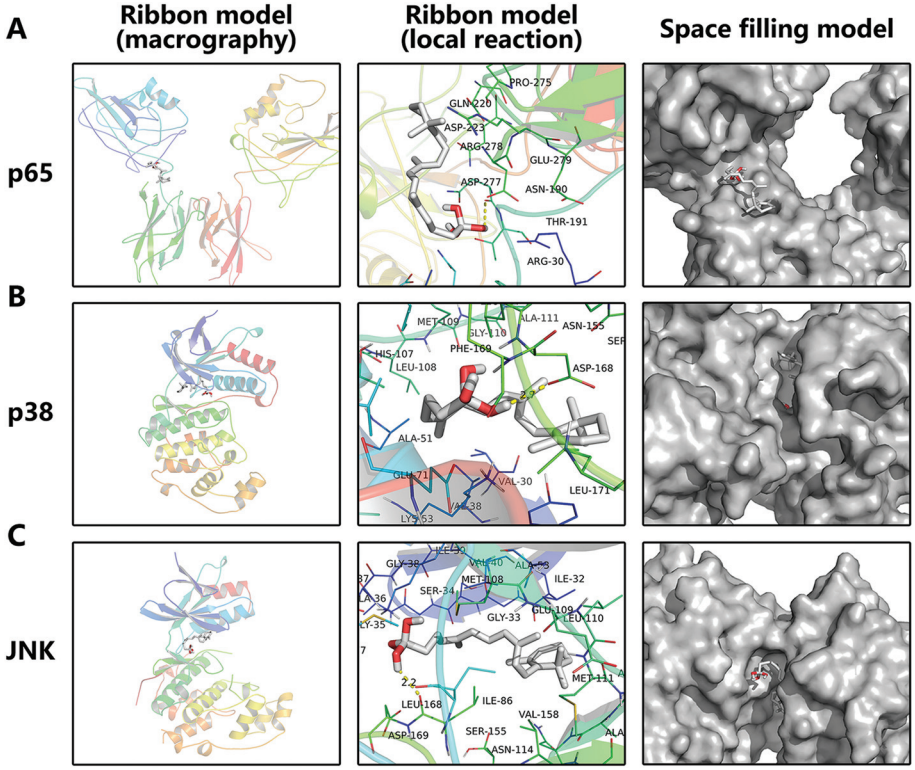

Fig. 6 Tx was docked with p65 (A), p38 (B), JNK (C) structures. Docking studies were performed as described in the Materials and methods. The protein residues are shown in a ribbon model. The proposed binding pose of Tx shows interactions with ASP277, ASP168, LEU168. The space filling models show the binding of Tx in the inhibitory binding pockets.

affinity for p65, p38, and JNK, *i.e.*, −4.6, −8.1, and −6.8 kcal mol^{-1}, respectively. The macrograhpy and local reaction between Tx and protein residues were shown in the form of a ribbon model. A space filling model showed the molecular structure of Tx was completely encapsulated in the inhibitory region of the corresponding proteins. As shown in Fig. 6A, a hydrogen bond was formed between Tx and the ASP-277 residue of p65. By the ribbon model, we observed a hydrogen bond linking the Tx molecule and the ASP-168 of p38 (Fig. 6B). Similarly, Tx could form a hydrogen bond with the LEU-168 of JNK, as shown in Fig. 6C. These data indicated that NF-κB and MAPK signaling might be involved in the protective effect of Tx in AGE-exposed chondrocytes.

3.7 Tx attenuates OA development in the DMM mouse model

Next, we established a mice DMM model by operation to evaluate the protective effect of Tx against OA development, followed by an oral gavage administration of Tx once daily for 8 weeks. Also, we examined histological and imaging changes in the knee joint of the mice by safranin O staining and radiographs. According to X-ray analysis, the mice suffering DMM surgery showed a narrowing of the joint space and calcification of the cartilage surface relative to the dummy group. However, Tx treatment obviously attenuated these phenomena (Fig. 7A). Furthermore, compared to the dummy group, the OA group showed a rough cartilage surface, obvious chondrocyte hypocellularity, and a large loss of proteoglycans, accompanied with higher OARSI scores. However, these pathological changes and OARSI scores appeared to be reduced in the Tx group (Fig. 7B and C).

4. Discussion

As a chronic degenerative joint disease, OA is clinically characterized by a high incidence, long-term pain, and limited joint activity, but without effective preventative therapy.[31] Nonsteroidal anti-inflammatory drugs (NSAIDs), as currently the main clinical drug for OA, only aim to relieve OA symptoms but fail to prevent the exacerbation of OA and—more profoundly—NSAIDs have side effects, which raise the risk of having a heart attack or stroke, especially in higher doses.[32] Therefore, finding a milder agent with fewer side effects has always been a hot topic in the research of OA treatment.

Tx is a trihydroxyethylated derivative of bioflavonoid rutin and is widely distributed in cereals, various fruit and vegetables, tea, and coffee. Due to the high water solubility of Tx, the gastrointestinal system can absorb it well without toxicity.[33] Tx is also called vitamin P4 and possesses multifarious biological properties, especially anti-inflammatory effects, which have been demonstrated in animal obesity, diabetes, and hepatitis models.[34–36] However its protection and specific

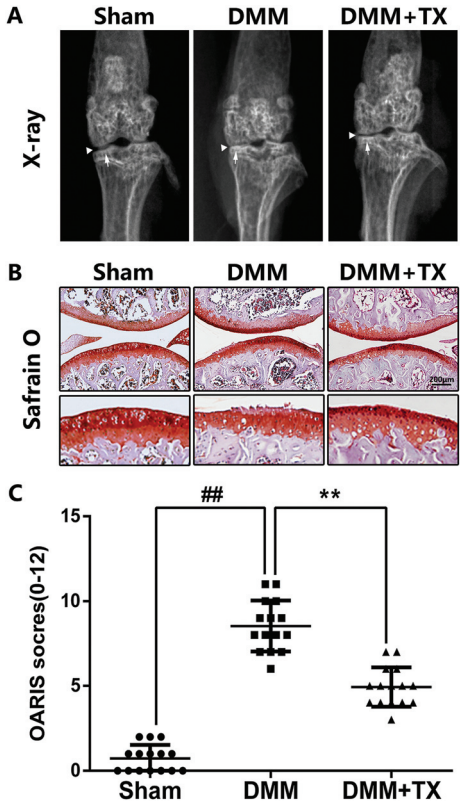

Fig. 7 Tx attenuates OA development in the DMM mouse model. (A). Digital X-ray image of mouse knee joints from different experimental groups. Narrowing of the joint space was found in both the OA and treatment groups (white triangles), the calcification of cartilage surface was obviously shown in the OA group (white arrows). (B). Representative S–O staining of cartilage from different experimental groups at 8 weeks post-surgery (scale bar: 200 μm). (C). Diagrams showed the OARIS scores of cartilage. The data in the figures represent the averages ± S.D. Significant differences among different groups are indicated as $^{\#\#}P < 0.01$, $^{**}P < 0.01$, $n = 15$.

mechanism in joint degeneration disease are still unknown. Thus, we performed this research to confirm Tx-induced anti-inflammation in OA development. In this study, we confirmed that Tx inhibited AGE-mediated iNOS-NO and COX-2-PGE2 expression and inflammatory factors production by suppressing the NF-κB and MAPK pathways. Besides, the chondroprotection of Tx is reflected by the suppression of collagen II and aggrecan degeneration and the alleviation of MMP and ADAMTS expression induced by AGEs. Our *in vivo* study found that Tx attenuated OA development.

During the OA process, senescence and local inflammation are the primary risk factors, but the specific mechanism and association between them remains to be further studied.[18] There is a significant linear correlation between the content of AGEs in human articular cartilage and human age.[17,37] Compared to young human articular cartilage (<20 years old), AGEs concentration is increased by at least 6 times in mature human articular cartilage (>60 years).[17] It is also reported that the AGEs level in elderly OA patients is 5 times higher than in younger patients.[12,38] Moreover, the AGEs level is increased in diabetic OA patients and is also considered to be an independent risk factor for diabetic OA development.[39] Therefore, in addition to IL-1β, TNF-α, and H_2O_2, AGEs are often used as an agent for *in vitro* experiments to mimic OA development, with the dose in previous studies ranging from 25 to 200 μg ml^{-1}.[40–43] In our study, we found that using AGEs in a concentration of 50 μg ml^{-1} did not affect the viability of chondrocytes by CCK8 analysis. In other research, the application of a high concentration of AGEs in chondrocytes has usually been used to investigate the apoptosis of chondrocytes, whereas lower concentrations of AGEs (50 μg ml^{-1}) for a total duration of 24 h might be more suitable to simulate inflammation in the OA cartilage. The accumulation of AGEs could stimulate the inflammatory responses and induce ECM degeneration by RAGE, which is widely expressed in chondrocytes and synoviocytes. Several cellular experiments have shown that AGEs treatment in chondrocytes activated the NF-κB and MAPK signaling and subsequently increased the production and secretion of MMPs, ADAMTS, TNF-a, IL-6, IL-8, and COX-2, contributing to ECM degeneration.[14,15]

Activation of NF-κB signaling is reputed to be important in the development of OA. In response to AGE-stimulated inflammatory property, IκBα is induced to phosphorylation, allowing the release and translocation of p65.[44] Previous studies demonstrated that p65 could speed up the gene transcription of pro-inflammatory mediators, such as PGE2, iNOS, COX-2, TNF-α, and IL-6. NO, tracing its root to the guanidine nitrogen of L-arginine with the catalyst of iNOS, induced the secretion of MMPs, which has an inhibitory effect on the synthesis of the ECM.[45] PGE2, generated from COX-2, induced cartilage degradation by MMPs and ADAMTS-5.[46] MMP-13 is a collagenase with a substrate preference for collagen II degradation, while ADAMTS-5 participates in cytokine-induced aggrecan degradation.[47,48] Our results displayed that Tx could significantly inhibit the overproduction of PGE2 and NO as well as COX-2 and iNOS at the mRNA and protein level in chondrocytes treated with AGEs. Similar results were also found for TNF-α and IL-6. For the NF-κB pathway, we found that Tx attenuated the AGE-mediated nuclear transfer of p65, which is consistent with Zi-Feng Zhang *et al.*[21] Their results showed that Tx repressed p65 acetylation at K310 *via* upregulating sirt1 activity in BDE-47-treated mouse livers.[21] However, our results from the docking analysis showed that Tx occupied the inhibitory binding pockets of p65 by interacting with the ASP277 of p65 and subsequently suppressed the phosphorylation process of p65.

The MAPK pathway was identified as the central node of various signaling transductions, which transduced extracellular stimuli into the nucleus and regulated the corresponding genes expression.[49] As a group of serine-threonine protein kinases, the MAPK pathway consists of three signaling pathways: ERK1/2, p38, and JNK, and these participate in multiple pathophysiological processes, such as cancer metastasis, cell differentiation, and especially inflammatory-associated diseases.[50] In the mouse OA model, the phosphorylation level of ERK1/2, p38 and JNK in OA cartilage tissue was higher relative

to normal.[51] Interestingly, in AGE-treated human chondrocytes, the phosphorylation of JNK and p38 was significantly increased but the ERK1/2 phosphorylation level only showed a slight and transient increase.[14,15] Also, only applying the specific inhibitors of JNK and p38, but not ERK1/2, could effectively inhibit AGE-induced inflammation in chondrocytes.[15] Our docking analysis seen in Fig. 6B showed that Tx occupied the inhibitory binding pockets of p38 and JNK by interacting with the ASP-168 of p38 and LEU-168 of JNK and subsequently suppressed the phosphorylation process of p38 and JNK.

Furthermore, we established a mouse DMM model to evaluate the protection of Tx for OA *in vivo*. The mice in the DMM group showed a rough cartilage surface, obvious chondrocyte hypocellularity, and a large loss of proteoglycans, accompanied with higher a OARSI score. However, treatment with Tx ameliorated these phenomena and reduced the OARSI score in DMM mice.

In conclusion, our *in vitro* study demonstrated that Tx inhibited the AGE-stimulated inflammatory response and ECM degeneration by repressing the NF-κB and MAPK signaling pathways in human chondrocytes. Moreover, the oral administration of Tx alleviated OA development in a surgery-induced mouse OA model. These data suggest Tx could be considered as a promising therapeutic agent for OA.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was funded by Wenzhou Municipal Science and Technology Bureau Foundation (2018Y0039).

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