Theaflavins prevent cartilage degeneration via AKT/FOXO3 signaling *in vitro*

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Abstract. Theaflavins (TFs) are the main bioactive polyphenols in tea and contribute to protection against oxidative stress. Excessive reactive oxygen species (ROS) accumulation can lead to the disruption of cartilage homeostasis. The present study examined the potential effects of TFs on H_2O_2-induced cartilage degeneration *in vitro*. Cell Counting kit (CCK-8) was used to determine cell viability, and flow cytometric analysis was used to detect ROS, apoptosis and DNA damage. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to detect the expression levels of target factors. The present study revealed that TFs effectively reduced the expression of catabolic factors, including matrix metalloproteinase-13, interleukin-1 and cartilage glycoprotein 39. TFs inhibited ROS generation in cartilage degeneration, and suppressed apoptosis and DNA damage caused by oxidative stress. TFs also downregulated the expression levels of cleaved caspase-3 and B-cell lymphoma 2-associated X protein, and the DNA damage-related genes, ATR serine/threonine kinase and ATM serine/threonine kinase. Furthermore, TFs enhanced the activity of glutathione peroxidase 1 and catalase, but reduced the expression levels of phosphorylated (p)-AKT serine/threonine kinase (AKT) and p-Forkhead box O3 (FOXO3)a. Conversely, the effects of TFs on apoptosis and DNA damage were reversed by persistent activation of AKT. In conclusion, TFs prevented cartilage degeneration via AKT/FOXO3 signaling *in vitro*. The present study suggested that TFs may be a potential candidate drug for the prevention of cartilage degeneration.

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Introduction

Tea is one of the most widely consumed beverages worldwide, the health benefits of which have been recorded against numerous diseases in ancient China (1). In terms of worldwide distribution, black tea is mainly consumed in Western countries, whereas green tea is more common in Asian countries. It has been reported that tea polyphenols can inhibit osteoclast formation and differentiation in rats (2); however, the mechanism underlying the protective effects of tea polyphenols on cartilage cells have yet to be elucidated. Theaflavins (TFs) are the primary active polyphenols in black tea, which include theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3-3'-digallate (3). TFs have been reported to possess numerous properties including antioxidant, antiviral and anticancer activities, in various biological processes (4,5). Cartilage degeneration is associated with the progression of osteoarthritis, and is mainly induced by oxidative stress (6). The present study aimed to explore the potential effect of TFs, in particular theaflavin-3-3'-digallate, on an *in vitro* model of cartilage degeneration and the related mechanisms.

Progressive cartilage destruction can be attributed to several factors (7). Among these factors, reactive oxygen species (ROS) are responsible for the maintenance of cartilage homeostasis; ROS act as the critical signaling intermediate of intracellular signaling pathways, including the phosphoinositide 3-kinase/protein kinase B and c-Jun N-terminal kinase pathways (8,9). Over-accumulation of ROS may lead to the disruption of cartilage homeostasis (10,11). In addition, apoptosis is considered to be associated with cartilage degeneration (12,13). It has been reported that overproduction of ROS can trigger intracellular DNA damage, which serves as a cellular stress factor (8). Furthermore, it has been demonstrated that Forkhead box (FOX) transcription factors are implicated in cell cycle progression, immune regulation, tumor growth and the aging process (14). FOXO proteins are able to regulate oxidative stress resistance through controlling downstream antioxidant targets, including glutathione peroxidase 1 (Gpx1) and catalase (CAT) (15,16). In addition, AKT serine/threonine kinase (AKT) serves important roles in cell survival and its activation can phosphorylate several downstream proteins, including FOXO3a. The transcriptional inactivation of FOXO3a can be induced by phosphorylation via AKT (17). Based on these findings, it is likely that AKT/FOXO

signaling may be associated with the protective effect of TFs in cartilage cells.

The present study aimed to investigate the protective effect of TFs on cartilage cells and attempted to explore the underlying mechanisms.

Materials and methods

Cell culture and grouping. Human chondrocytes (cat. no. 4650; ScienCell Research Laboratories, Inc., San Diego, CA, USA) were cultured in a 6-well plate ($1x10^5$/well) at 37˚C in a 5% CO_2 incubator with Dulbecco's modified Eagle medium containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin and streptomycin (Sangon Biotech Co., Ltd., Shanghai, China). Cells were fixed with 95% ethanol for 15 sec at room temperature and then stained with 1% toluidine blue (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 5 min at room temperature and observed under a light microscope (magnification, x100). Theaflavin-3-3'-digallate (purity >90%) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). At ~80% confluence, the cells were serum-starved overnight and were then divided into three treatment groups for comparison: i) Control group; ii) model group, in which cells were treated with 0.3 mM H_2O_2 for 6 h; and iii) TF pretreatment groups, in which cells were pretreated with various doses of TFs (10 and 20 μg/ml) for 12 h, followed by 6 h H_2O_2 incubation. The TF concentrations employed were based on previous literature (18,19). For the activation of AKT, cells were pretreated with 50 ng/ml insulin-like growth factor I (IGF-I; R&D Systems, Inc., Minneapolis, MN, USA) for 10 min prior to H_2O_2 or TF treatment, according to previous studies (20,21).

Cell Counting kit (CCK)-8 assay. The cells were serum starved overnight in a 96-well plate ($1x10^5$ cells/well), and were then treated with H_2O_2 (0.1-0.5 μM) in serum-free medium for various durations (6, 12, 24 and 48 h). A CKK-8 kit was used to detect cell viability, according to the manufacturer's protocol (Beijing Kangwei Century Biotechnology Co., Ltd., Beijing, China). Briefly, the CCK-8 solution was added to each well and the cells were incubated for 4 h at 37˚C, after which, absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ELISA assay. The cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and were treated as aforementioned. Matrix metalloproteinase (MMP)-13 (cat. no. DY511) and interleukin (IL)-1β (cat. no. DLB50) activities were detected using ELISA kits (R&D Systems, Inc.), according to the manufacturer's protocols. The ELISA kit used to measure the expression of cartilage glycoprotein 39 (Cgp-39; cat. no. HC021) was purchased from Shanghai GeFan Biotechnology, Co., Ltd. (Shanghai, China).

Flow cytometric analysis of ROS levels. The cells ($1x10^4$ cells/well) were treated as aforementioned. Subsequently, the cells were stained with H2DCHF-DA (Invitrogen; Thermo Fisher Scientific, Inc.) for ROS measurement, as previously described (22). BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) running BD CellQuest™ software version 3.3 (BD Biosciences) was used to perform flow cytometric analysis. All data are representatives of at least three independent experiments.

Flow cytometric analysis of apoptosis. The cells ($1x10^5$/well) were cultured in 6-well plates. An Annexin V/propidium iodide (PI) apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to detect apoptosis. According to the manufacturer's protocol, Annexin-V and PI staining was analyzed using analyzed using a flow cytometer (BD FACSCanto II) with FACSDiva software version 6.1 (both BD Biosciences).

Flow cytometric analysis of DNA damage. According to a previous study (23), DNA damage was estimated using flow cytometry-based detection of γ-H2A histone family, member X (γ-H2AX). Briefly, the collected cells were suspended in BD Cytofix/Cytoperm fixation and permeabilization solution (BD Biosciences). After a 15-min incubation at 37˚C, the cells were washed using PBS and blocked with 10% normal goat serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 1 h. Cells were incubated with anti-γ-H2AX (pS139) antibody (cat. no. ab26350; 1:100; Abcam, Cambridge, UK) at 4˚C overnight and then incubated with fluorescein isothiocyanate-conjugated secondary antibodies (cat. no. ab7064; 1:1,000; Abcam) for 45 min at 37˚C. Subsequently, the fluorescent signals were measured using a BD flow cytometer (BD Biosciences).

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using RNAiso reagent (Takara Bio, Inc., Otsu, Japan). Subsequently, cDNA was reverse transcribed from total RNA using ReverTra Ace (Toyobo Life Science, Osaka, Japan) and oligo-dT (Takara Bio, Inc.), according to manufacturer's protocol. The mRNA expression levels were quantified using an ABI 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using AceQ qPCR SYBR Green Master Mix (Vazyme, Piscataway, NJ, USA). The thermocycling conditions were as follows: 95˚C for 5 min, followed by 30 cycles of 94˚C for 15 sec and 60˚C for 30 sec, and final extension at 72˚C for 5 min. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (24). Primer sequences for RT-qPCR were as follows: ATR serine/threonine kinase (ATR) forward, 5'-GGAATCACGACTCGCTGA; AC-3' reverse, 5'-AAATCGGCCCACTAGTAGCA-3'; ATM serine/threonine kinase (ATM) forward, 5'-CGAGGCGTACAATGGTGAAG-3' and reverse, 5'-CCTCCGGCTAAGCGAAATTC-3'; B-cell lymphoma 2-associated X protein (Bax) forward, 5'-GAGCGGCGGTGATGGA-3' and reverse, 5'-TGGATGAAACCCTGAAGCAAA-3'; and β-actin forward, 5'-CTCTTCCAGCCTTCCTTCC-3'; and reverse, 5'-AGCACTGTGTTGGCGTACAG-3'.

Western blot analysis. A Total Extraction Sample kit (Sigma-Aldrich; Merck KGaA) was used to extract total proteins. Protein concentration was determined using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). The proteins (20 μg/lane) were separated by 10% SDS-PAGE and were then

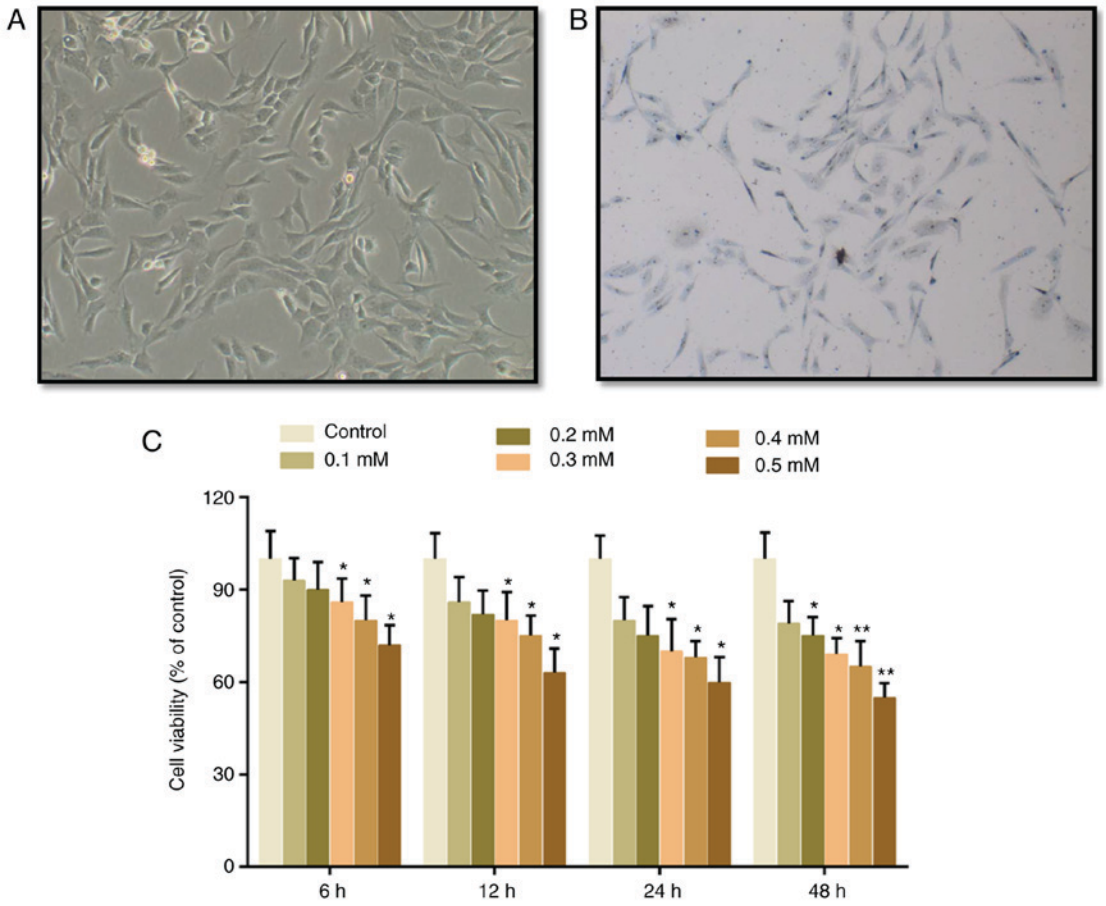

Figure 1. (A) Unstained and (B) toluidine blue-stained cartilage cells were observed under light microscope; magnification, x100. (C) Effects of H_2O_2 on cartilage cells. Cells were treated with various doses of H_2O_2 (0.1, 0.2, 0.3, 0.4 and 0.5 mM) for 6, 12, 24 and 48 h. *P<0.05 and **P<0.01 vs. control.

transferred onto a polyvinylidene fluoride membrane. To block non-specific proteins, non-fat milk (3%) was used to incubate the membrane for 2 h at room temperature. Following incubation with primary antibodies overnight at 4˚C, the membrane was incubated with secondary antibodies for 2 h at room temperature, and the bands were developed using an enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Little Chalfont, UK). Blot density was determined using Quantity One software version 4.6.9 (Bio-Rad Laboratories, Inc.). The primary antibodies used were as follows: Anti-ATR (cat. no. ab2905; 1:1,000), anti-Bax (cat. no. ab53154; 1:1,000; both Abcam, Cambridge, UK), anti-cleaved caspase-3 (cat. no. 9664; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ATM (cat. no. ab78; 1:2,000), anti-γH2AX (cat. no. ab11175; 1:8,000; both Abcam), anti-phosphorylated (p)-AKT (cat. no. 13038; 1:1,000; Cell Signaling Technology, Inc.), anti-AKT1/2 (cat. no. ab182729; 1:5,000), anti-p-FOXO3a (cat. no. ab53287; 1:1,000; both Abcam), anti-FOXO3a (cat. no. 2497; 1:1,000; CST), anti-Gpx1 (cat. no. ab22604; 1:1,000), anti-CAT (cat. no. ab16731; 1:2,000; both Abcam) and anti-β-actin (cat. no. 4970; 1:1,000; Cell Signaling Technology, Inc.). Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit; cat. no. ab205718; 1:2,000 and goat anti-mouse; cat. no. ab205719; 1:5,000) were obtained from Abcam.

Statistical analysis. All experiments were independently performed ≥3 times. Data are presented as the means ± standard deviation. GraphPad software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to compare differences between groups by one-way analysis of variance followed by Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

TFs inhibit ROS generation in cartilage degeneration. The cartilage cells presented with spindle morphology, and toluidine blue staining was conducted to identify the cells. It was identified that the cytoplasm was stained light blue and the nucleus was stained dark blue, indicating that these cells were chondrocytes (Fig. 1A and B). Subsequently, CCK-8 assay was conducted to evaluate the effects of H_2O_2 on the viability of cartilage cells. It was demonstrated that cell viability was suppressed by H_2O_2 in a dose-dependent manner. A significant difference emerged in the group that was treated with 0.3 mM H_2O_2 for 6 h, in which cell viability was deceased by 14% (Fig. 1B). Therefore, 0.3 mM H_2O_2 was subsequently used to treat cartilage cells for 6 h, in order to mimic the progression of cartilage degeneration. To measure cartilage degeneration following H_2O_2 treatment, the expression levels of catabolic factors, MMP-13, IL-1β and Cgp-39, were detected. It was demonstrated that the expression levels of MMP-13, IL-1β and Cgp-39 were increased by H_2O_2, but were decreased by TF pretreatment (Fig. 2A). These findings suggested that TFs

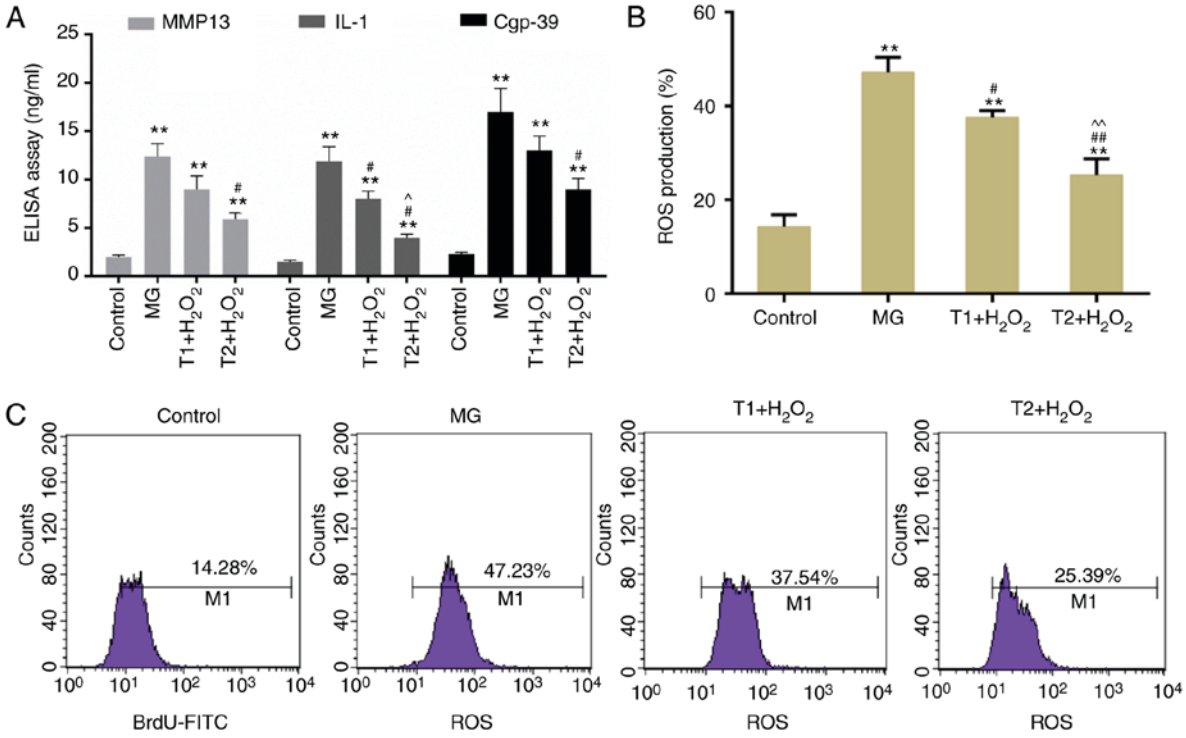

Figure 2. (A) Detection of catabolic factors, MMP-13, IL-1β and Cgp-39. (B) ROS production rate was determined following treatment with TFs and H_2O_2. (C) ROS levels were measured by flow cytometry. **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MG; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 vs. T1 + H_2O_2. Cgp, cartilage glycoprotein; IL, interleukin; MG, model group; MMP, matrix metalloproteinase; ROS, reactive oxygen species; T1, pretreatment with 10 μg/ml TFs; T2, pretreatment with 20 μg/ml TFs; TFs, theaflavins.

may inhibit cartilage degeneration. Furthermore, according to flow cytometric analysis, it was demonstrated that ROS levels were markedly decreased in the TF pretreatment groups (Fig. 2B and C).

TFs suppress apoptosis and DNA damage following oxidative stress. The results of flow cytometric analysis revealed that H_2O_2-induced apoptosis was suppressed by pretreatment with TFs (Fig. 3A and B). DNA double-strand breaks (DSBs) are a type of detrimental DNA damage, for which γH2AX is considered a surrogate marker. In response to DSBs, H2AX is phosphorylated at Ser139 (γH2AX) (25). The present results demonstrated that TFs could decrease γH2AX expression compared with in the model group (Fig. 4A and B). In addition, western blotting confirmed that TFs inhibited the expression levels of γH2AX (Fig. 4C). The expression levels of apoptosis-associated factors, including cleaved caspase-3 and Bax, were decreased in the TF pretreatment groups compared with in the model group. The expression levels of DNA damage-response genes, ATM and ATR, were also decreased following TF pretreatment (Fig. 5A-C). However, the protein expression levels of ATR were slightly, but not significantly, increased in the T1 + H_2O_2 group.

TFs decrease the activity of AKT, FOXO3a, Gpx1 and CAT. Emerging evidences have demonstrated that FOXO proteins are important mediators of oxidative stress (15,16). Compared with in the model group, the protein expression levels of p-AKT and p-FOXO3a were mitigated by TF pretreatment, whereas the expression levels of Gpx1 and CAT were enhanced (Fig. 6A and B).

AKT activity is necessary for the protective effects of TFs. To further confirm the role of AKT in the present study, apoptosis and DNA damage were detected following treatment with the AKT activator, IGF-I. The results demonstrated that TF pretreatment did not significantly reverse H_2O_2-induced apoptosis following persistent activation of AKT (Fig. 7A and B). In addition, TF-induced inhibition of DNA damage was reversed following persistent activation of AKT (Fig. 8A-C).

Discussion

Tea is one of the most widely consumed beverages worldwide (26). The potential health benefits of tea have been widely reported, particularly with regards to the prevention of cardiovascular disorders and cancer. Phenols and polyphenols are the primary bioactive substances in tea that exert health effects (27); therefore, attention has been paid to the antioxidative effects of tea polyphenols (28). Cartilage degeneration is a serious complication of osteoarthritis, which is mainly caused by oxidative stress (29). A previous study demonstrated the positive function of tea polyphenols in maintaining bone homeostasis (2). TFs are the primary active content of tea phenols (30); however, little is currently known about the effects of TFs on cartilage degeneration.

Studies have revealed the connection between structural degeneration and biochemical markers (31-34). Several biochemical markers, including MMP-13 (32), IL-1β (33) and Cgp-39 (34), are used to diagnose patients with a high risk of joint degeneration. The present study demonstrated that TFs inhibited H_2O_2-mediated cartilage degeneration by decreasing the levels of MMP-13, IL-1β and Cgp-39. This study also aimed

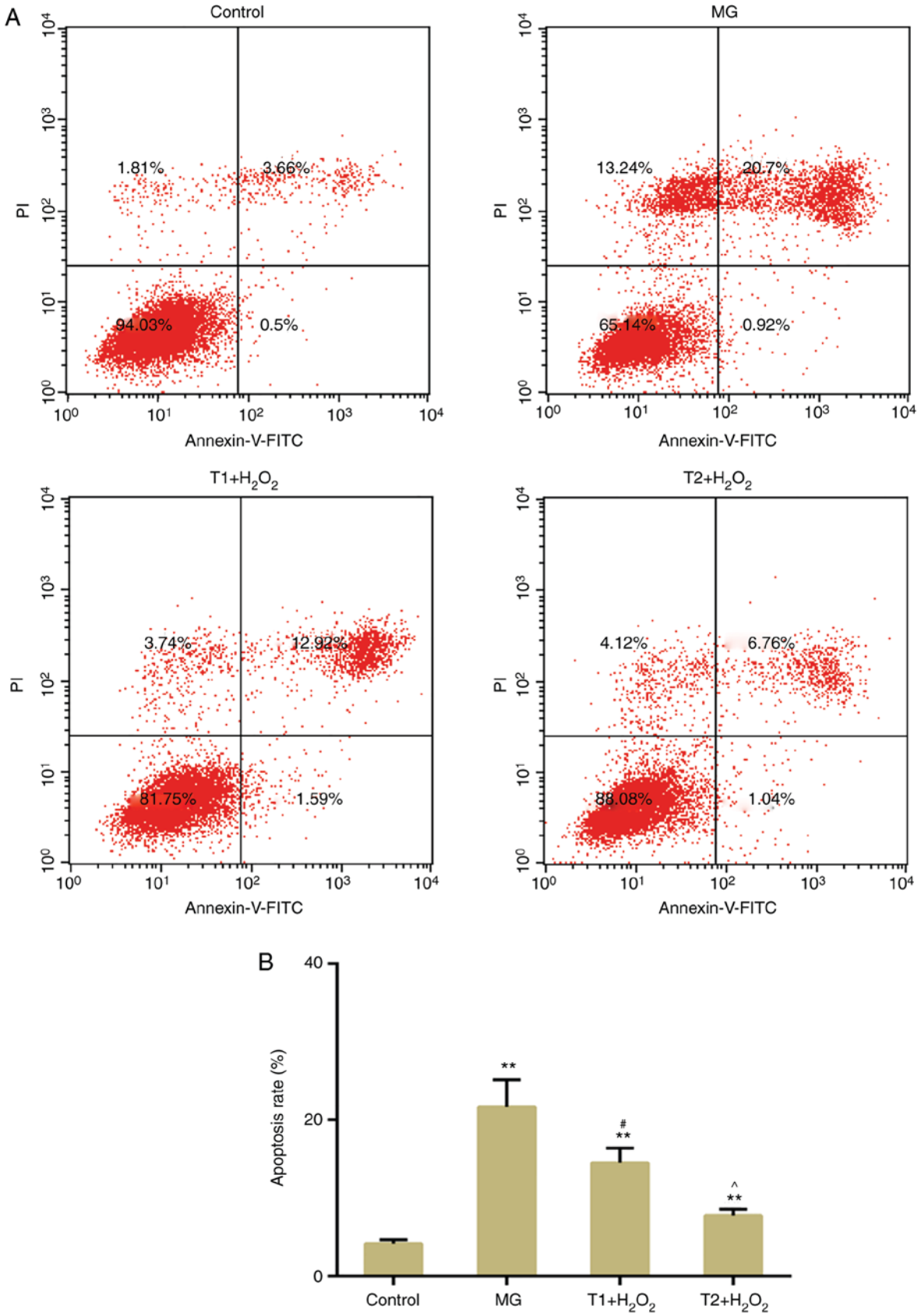

Figure 3. (A) Flow cytometric analysis of cartilage cell apoptosis. (B) Determination of cell apoptosis rate. **P<0.01 vs. control; $^{\#}$P<0.05 vs. MG; $^{\wedge}$P<0.05 vs. T1 + H_2O_2. FITC, fluorescein isothiocyanate; MG, model group; PI, propidium iodide; T1, pretreatment with 10 μg/ml TFs; T2, pre-treatment with 20 μg/ml TFs; TFs, theaflavins.

to illustrate the molecular mechanisms underlying the effects of TFs on the cartilage cells. The results demonstrated that ROS production was markedly increased in the model group, whereas pretreatment with TFs significantly decreased ROS levels.

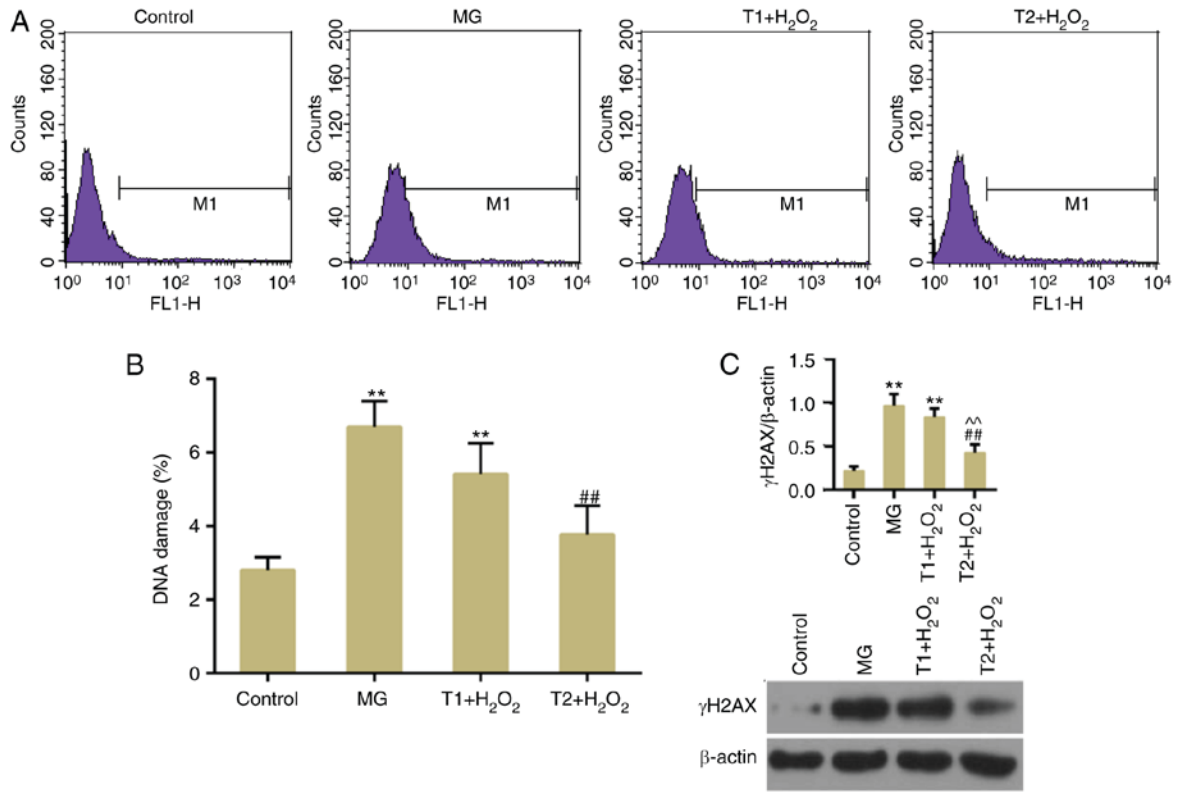

Figure 4. (A) Flow cytometric analysis of DNA damage in cartilage cells. (B) Determination of DNA damage rate. (C) Western blot analysis of the expression of γH2AX. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. MG; $^{\wedge\wedge}$P<0.01 vs. T1 + H_2O_2. H2AX, H2A histone family, member X; MG, model group; T1, pretreatment with 10 μg/ml TFs; T2, pretreatment with 20 μg/ml TFs; TFs, theaflavins.

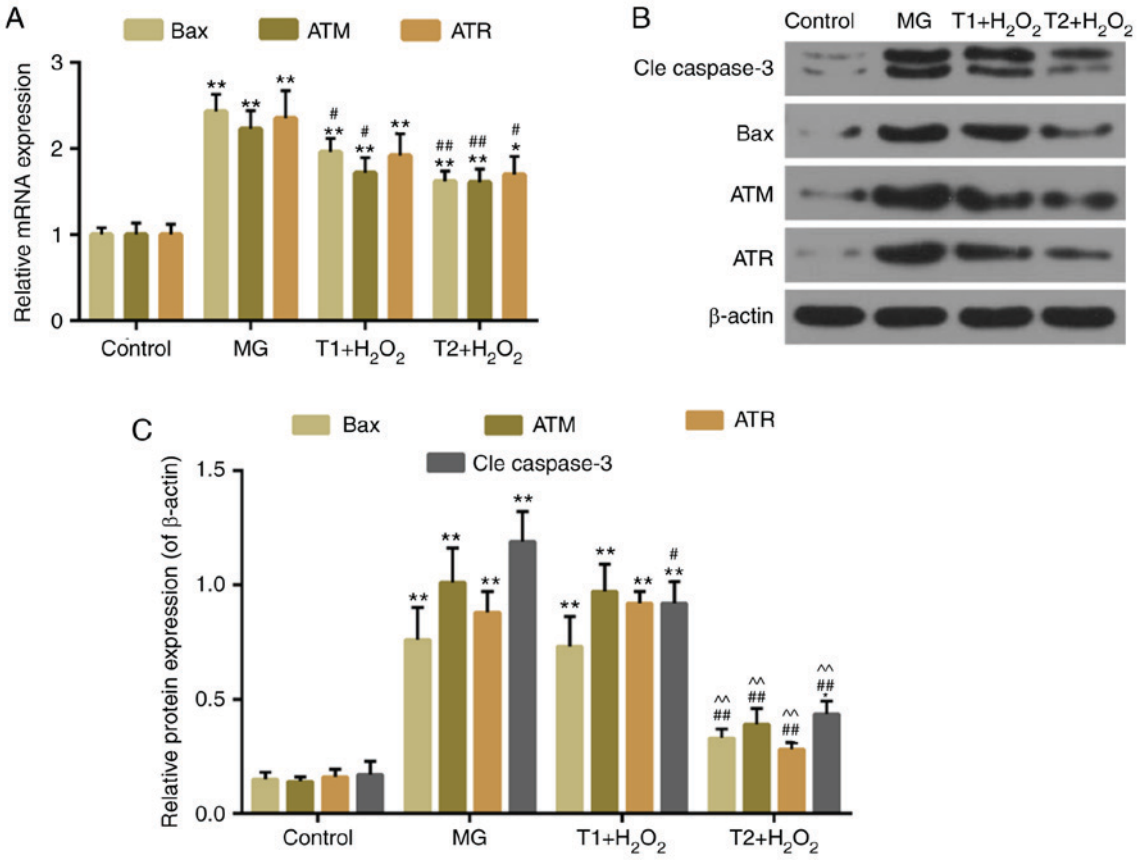

Figure 5. (A) Quantitative analysis of Bax, ATR and ATM mRNA expression. (B and C) Western blot analysis of cleaved caspase-3, Bax, ATR and ATM. *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MG; $^{\wedge\wedge}$P<0.01 vs. T1 + H_2O_2. ATM, ATM serine/threonine kinase; ATR, ATR serine/threonine kinase; Bax, B-cell lymphoma 2-associated X protein; MG, model group; T1, pretreatment with 10 μg/ml TFs; T2, pre-treatment with 20 μg/ml TFs; TFs, theaflavins.

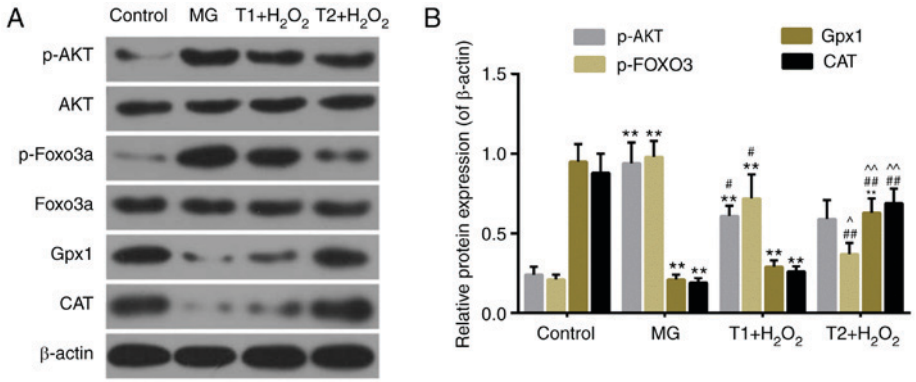

Figure 6. (A and B) Western blot analysis of AKT, p-AKT, FOXO3a, p-FOXO3a, Gpx1 and CAT. **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MG; $^{\wedge\wedge}$P<0.01 vs. T1 + H_2O_2. CAT, catalase; FOXO3a, Forkhead box O3a; MG, model group; Gpx, glutathione peroxidase 1; p-, phosphorylated; T1, pretreatment with 10 μg/ml TFs; T2, pre-treatment with 20 μg/ml TFs; TFs, theaflavins.

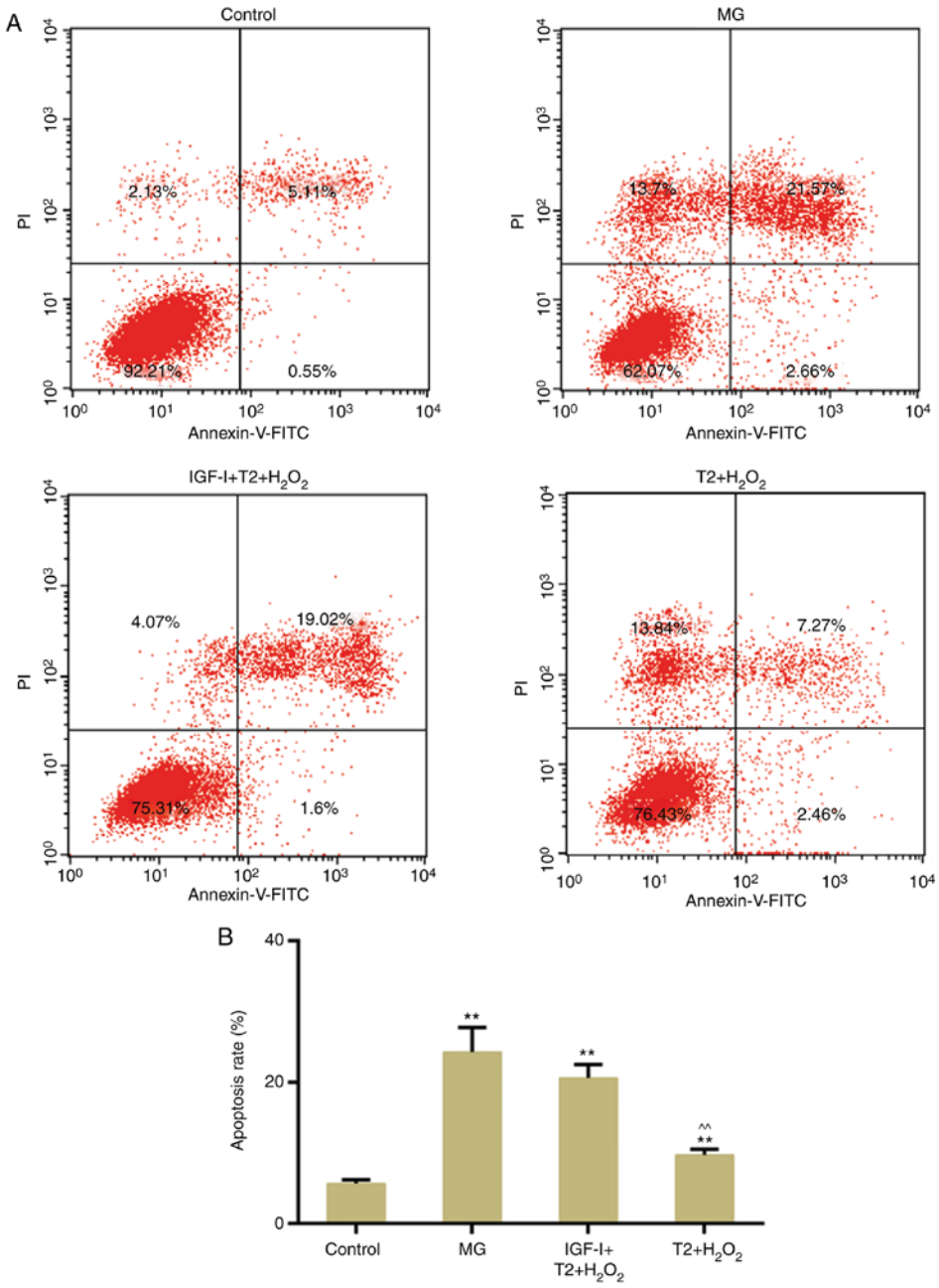

Figure 7. (A and B) Flow cytometric analysis of apoptosis. **P<0.01 vs. control; $^{\wedge\wedge}$P<0.01 vs. IGF-I + T2 + H_2O_2. FITC, fluorescein isothiocyanate; IGF, insulin-like growth factor; MG, model group; PI, propidium iodide; T1, pretreatment with 10 μg/ml TFs; T2, pre-treatment with 20 μg/ml TFs; TFs, theaflavins.

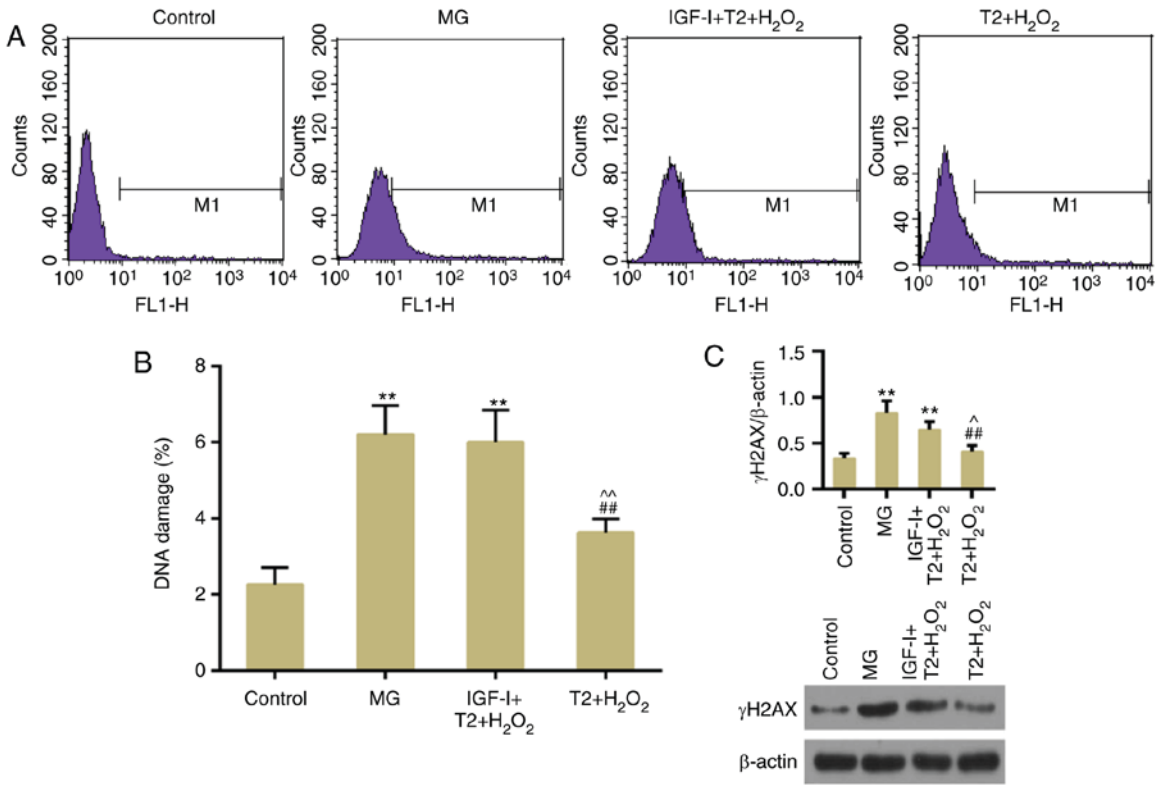

Figure 8. (A and B) Flow cytometric analysis of DNA damage. (C) Western blot analysis was conducted to detect the expression of γH2AX. **P<0.01 vs. control; ##P<0.01 vs. MG; ^P<0.05 and ^^P<0.01 vs. IGF-I + T2 + H_2O_2. H2AX, H2A histone family, member X; IGF, insulin-like growth factor; MG, model group; T1, pretreatment with 10 μg/ml TFs; T2, pre-treatment with 20 μg/ml TFs; TFs, theaflavins.

Furthermore, pretreatment with TFs reduced cell apoptosis and DNA damage caused by H_2O_2, and decreased the expression levels of cleaved caspase-3 and Bax, which are closely associated with cell apoptosis (35,36). The expression levels of ATR and ATM, which is the master kinase that controls the DNA damage check point (37,38), were decreased in the TF pretreatment groups compared with in the model group. These findings indicated that TFs may prevent cartilage matrix degeneration by inhibiting DNA damage and apoptosis. The inhibitory effects of TFs on apoptosis were supported by a recent study in PC12 neural cells (39). In addition, DNA damage can be modified by TFs in human lymphocytes (40). However, numerous studies have demonstrated that TFs inhibit proliferation and induce apoptosis in cancer cells (41-43). These contradictory results may be due to the distinct cell types used in each study model.

To explore the possible underlying mechanisms, the effects of TFs on the activity of AKT/FOXO3 signaling were investigated. It was noted that TFs mitigated the expression of p-AKT and p-FOXO3a, and enhanced Gpx1 and CAT activities compared with in the model group. It has previously been demonstrated that the reduced activity of AKT/FOXOs mitigates cell dysfunction in diabetic kidney disease (44). Notably, the present study revealed that TF-induced inhibition of apoptosis and DNA damage was reversed following persistent activation of AKT. Therefore, it may be hypothesized that the effects of TFs on cartilage cells may be tightly linked to AKT/FOXO signaling. Since phosphorylation of FOXO3 results in its inactivation, TFs may reduce inactivation of FOXO3 by suppressing AKT. However, this speculation was not validated in the present study. The protective effect of FOXO3 inactivation on cartilage still requires further investigation. In addition, the activity of FOXOs can be regulated by other signals (45); however, the regulation is rather complex, and parts of it are contradictory. For example, FOXO3 can be activated by the phosphorylation of 5'AMP-activated protein kinase, c-Jun N-terminal kinase and macrophage-stimulating 1 (46). Therefore, it would be useful to investigate how the upstream signals co-regulate FOXO3 signaling in future studies.

In conclusion, the present study demonstrated that TFs inhibited the ROS burst in cartilage destruction. TFs suppressed apoptosis and DNA damage by reducing the expression levels of cleaved caspase-3, Bax, ATR and ATM. Furthermore, TFs enhanced the activity of Gpx1 and CAT, and decreased the expression levels of p-AKT and p-FOXO3a. Notably, AKT signaling was necessary for the effects of TFs on apoptosis and DNA damage. The results of the present study demonstrated that TFs may be a potential candidate drug for the prevention of cartilage degeneration.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL and JZ designed the study, performed the experiments and performed the data analysis. JL wrote the manuscript. JL and JZ revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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