Biochemical and Biophysical Research Communications 452 (2014) 682–688

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

5,7,3′,4′-Tetramethoxyflavone exhibits chondroprotective activity by targeting β-catenin signaling *in vivo* and *in vitro*

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ARTICLE INFO

Article history:
Received 5 August 2014
Available online 1 September 2014

Keywords:
5,7,3′,4′-Tetramethoxyflavone
Osteoarthritis
Chondrocytes
β-Catenin
PGE_2

ABSTRACT

Osteoarthritis (OA) is a progressive joint disorder, which remains the leading cause of chronic disability in aged people. This study is the first report which demonstrates the cartilage protective effect of 5,7,3′,4′-tetramethoxyflavone (TMF) by decreasing the concentration of IL-1β, TNF-α and PGE_2 in the knee synovial fluid in OA rat models *in vivo*. *In vitro*, after induced by PGE_2, the apoptosis rate of chondrocytes was significantly increased. In addition, PGE_2 increased the expression of cAMP/PKA signaling pathway in chondrocytes, stabilized and accumulated β-catenin, and activated the expression of β-catenin signaling pathway. These activities were counteracted by TMF dose-dependently. Collectively, TMF is a potential compound with chondroprotective activity by inhibiting both EP/cAMP/PKA signaling pathway and β-catenin signaling pathway.

1. Introduction

Osteoarthritis (OA) is the most common age-associated degenerative disease, characterized by articular cartilage breakdown, synovial membrane inflammation, osteophyte formation, and cartilage vascularization [1,2]. Cartilage homeostasis is essential for joint functionality [3], while in OA it is tilting towards disruption by combinations of biophysical and biochemical factors. It has been elucidated that the signaling pathways are the key molecular players [4]. While key chondrocyte signaling pathways include, but are not limited to, the p38, JNK and ERK MAP kinases, the PI3K-Akt pathway, Wnt/β-catenin and NF-κB/cytokines pathway [5,6]. Modulation of the activity in any of these pathways has been associated with various pathological states in cartilage.

The Wnt/β-catenin signaling pathway has been associated with postnatal cartilage matrix catabolism and articular chondrocyte dedifferentiation [3,7]. In the canonical Wnt/β-catenin signaling pathway, Wnt protein binds to cell-surface frizzled and the coreceptor low density lipoprotein receptor related protein 5 and 6 (LRP-5/6), leading to inhibition of β-catenin phosphorylation by glycogen synthase kinase 3 beta (GSK-3β) and proteasome-mediated degradation; β-catenin translocates to the nucleus, where it interacts with resident lymphoid enhancer factor/T-cell (LEF/TCF) transcription factors to activate target genes [7]. Cumulative studies mainly based on experimental animal models for OA have suggested an important pro-catabolic role for Wnt/β-catenin signaling in the pathogenesis of OA [8]. In *Col2a1-CreER^{T2} β-catenin$^{fx(Ex3)/wt}$* mice, overexpression of β-catenin protein was detected by immunostaining in the 3th month, reduction of Safranin O and Alcian blue staining could be found in the 5th month, cell cloning, surface fibrillation, vertical clefting, and osteophyte formation were observed in the 8th month. In addition, expression of chondrocyte marker genes, such as *aggrecan*, *MMP-9*, *MMP-13*, *Alp*, *Oc*, *colX*, and *Bmp2* were also significantly increased [9]. Thus, Wnt signaling can trigger cartilage damage by increasing degradation and decreasing the synthesis of cartilage matrix.

According to our previous studies, *Murraya exotica* shows antinociceptive and anti-inflammatory activities in rat knee osteoarthritis models [10]. It also exhibits anti-apoptotic chondroprotective activity probably through inhibiting β-catenin signaling [7]. 5,7,3′,4′-Tetramethoxyflavone (TMF), one of the major polymethoxyflavones (PMFs) isolated from *M. exotica*, has been reported to be associated with various bioactivities, including anti-fungal, anti-malarial, anti-mycobacterial, and anti-inflammatory activities [11]. We therefore undertook the study on the effects of TMF on β-catenin to determine whether it's a molecular target of TMF in chondrocytes and furthermore, a potential osteoarthritis chemoprevention molecular target.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.129
0006-291X/

2. Materials and methods

2.1. General

The study was approved by the Institutional Animal Care and Use Committee of Gannan Medical University. Rats in all treating groups were intragastrically administered with TMF at different doses. The control group animals received the same experimental handling as those of the treating groups except that the drug treatment was substituted for appropriate volumes of the dosing vehicle.

2.2. Rat knee OA models

Rat OA model was established by using Hulth's (1999) method [12]: the rat was anesthetized. After routine disinfection, 1 cm longitudinal incision was made by separating the medial parapatellar and cutting off the tibial collateral ligament, the articular cavity was opened and the cruciate ligament of knee was cut off, the medial meniscus was excised and the articular cavity rinsed and sutured layer by layer, then the rats underwent penicillin treatment for one week for prevention against infection. Six weeks after establishing the model, rats were sacrificed and the knee synovial fluid (SF) lavages were collected and stored at −20 °C for ELISA determination of IL-1β, TNF-α, and PGE_2. Other segments of the cartilage were collected for histological and immuno-fluorescence examination.

2.3. Primary cell cultures

Four-week-old rats were sacrificed. Immediately, cartilage was harvested from the knee joint under sterile conditions and digested with 0.25% pancreatic enzymes for 30 min to remove other tissues and cells, following with digestion of 0.2% collagenase II at 37 °C for 4 h. Cells were cultured to confluence in DMEM (low glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C with 5% CO_2. The first passage cell lines were used.

2.4. MTT assays

Cells were grown in a 96-well plate (5000/well), after incubated with various concentrations (0–80 μg/mL) of TMF for 24 h in a serum-free medium, MTT (5 mg/mL) was added in, with a final concentration at 20 μL/well. Cells were then incubated with MTT at 37 °C degrees centigrade for 4 h, culture medium was removed and DMSO (150 μL/well) was added subsequently. The absorbance was measured at 570 nm. This step was replicated for three times to get average results in order to reduce errors.

2.5. Quantitative analysis of apoptosis cells

Changes of cell apoptosis were quantified by loading FITC-annexin V/PI double – fluorescence labeling and using flow cytometry. Flow cytometry was performed according to the apoptosis detection kit (Nanjing KeyGEN Biological Technology Development Co. Ltd., Nanjing, China) procedures. After treated with 1 μM PGE_2 and TMF, cells (1×10^6/mL) were harvested by centrifugation and incubated in buffer containing FITC-annexin V and PI. Apoptosis cells were measured by a flow cytometer (FACSCalibur BD, San Jose, CA).

2.6. Gene expression analysis

Total RNA was extracted from chondrocytes using the Easy-spin™ total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea). For each sample, 2 μg of total RNA was reverse-transcribed using M-MLV (Promega, USA) to synthesize the first-strand of cDNA following standard protocols. To detect the expression level of EP2, EP4, β-catenin and COX-2 genes, EzOmics SYBR qPCR kits, purchased from Biomics in a Mastercycler (Eppendorf) were employed. Their respective primer sequences (listed in Table 1) were used. Amplification procedure was: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s, and finally at 72 °C for 10 min. The PCR reactions were performed by using the Ani-Cycler real time PCR system (Bio-Rad).

All of the PCR reactions were performed in sets of four. GAPDH was used as an internal standard control. Primer and template designs following the same criteria for each target, primers and Mg^{2+} concentrations had been optimized to render efficiency for each target near one per assumption underlying the $2^{-\Delta\Delta CT}$ method [16].

2.7. Luciferase reporter assays

Chondrocytes were resuspended in serum-free culture medium and plated on 48-well dishes (3.4×10^4 cells in 200 μL/well) and transfected with Wnt/β-catenin reporter plasmid (Upstate, Lake Placid, NY) (Topflash, encoding seven copies of LEF/TCF binding sites linked to firefly luciferase and reflects Wnt/β-catenin signaling activity) in the presence of Lipofectamine 2000. In all experiments, cells were co-transfected with renilla luciferase plasmid (pRL-CMV, Thermo Fisher Scientific) to control the transfection efficiency. Cultures were transfected for 4 h prior to addition of 200 μL FBS containing media and then incubated overnight. The next day, cells were administrated by TMF at different doses for 24 h. Cultures were then lysed with 1 × Passive Lysis Buffer (Promega, Madison, WI). The luciferase activities of both Topflash and pRL-TK-luc reporters were measured by using a dual luciferase assay kit (Promega, Madison, WI) in an L-max II microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.8. Western blot analysis

Cells were lysed in lysis buffer, and then subjected to immunoblot. Before sampling, the protein concentrations were measured by using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as a standard. After combined with gel loading buffer and boiled for 5 min, samples (50 μg) were electrophoresed on 10% SDS–PAGE gel for EP2, EP4, PKA, p-PKA, p-GSK-3β, β-catenin, COX-2, and anti-cleaved caspase-3. Proteins were Western-blotted onto PVDF transfer membranes, and blots were blocked with TBS containing 5% non-fat milk for 1 h, the incubated with EP2, EP4, PKA, p-PKA, p-GSK-3β, β-catenin, COX-2

Table 1
Primer sequences for different genes.

EP2	Forward Reverse	5′-CCTGCCGCTGCTCAACTACG-3′ 5′-GTCTCCTCTGCCATCGAAGTCCTC-3′	Ref. [13]
EP4	Forward Reverse	5′-CTGCTGATCTCCTTTAACTCCC-3′ 5′-CTGCTGATCTCCTTTAACTCCC-3′	Ref. [13]
β-Catenin	Forward Reverse	5′-ACAGCACCTTCAGCACTCT-3′ 5′-AAGTTCTTGGCTATTACGACA-3′	Ref. [14]
COX-2	Forward Reverse	5′-GCACAAATATGATGTTCGCATTC-3′ 5′-CAGGTCCTCGCTTCTGATCTG-3′	Ref. [15]
GAPDH	Forward Reverse	5′-CAGTGGCAAAGTGGAGATTG-3′ 5′-AATTTGCCG-TGAGTGGAGTC-3′	Ref. [16]

and anti-cleaved caspase-3 at 4 °C over night respectively. The blots were then rinsed and incubated with HRP-conjugated IgG goat anti-rat for 1 h. After washing, the blots were developed by use of a Super Enhanced chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China) and the protein bands were visualized after exposure of the membranes to Kodak film (USA). GAPDH was used as the internal control in all Western blot analyses.

2.9. cAMP immunoassay

PGE_2 induced cells were treated with TMF at different doses for 24 h at 37 °C. Therefore, cells were washed with cold PBS, the levels of intracellular cAMP were determined by using a cAMP ELISA kit following the manufacturer's protocol.

2.10. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis of gene expression data were analyzed by a paired *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. TMF down regulated the expressions of the inflammatory cytokines in knee OA SF lavages

The intragastric dosing of TMF was 100, 50, and 25 mg/kg, which were recommended by our previous studies. No rats were died during the experiment. The contents of IL-1β, TNF-α, and PGE_2 in rats SF were decreased greatly in TMF groups, in a dose-dependent manner. In the 100 mg/kg TMF treating group, the contents of IL-1β, TNF-α, and PGE_2 were 41.9 ± 15.3 pg/mL ($p < 0.05$), 38.4 ± 10.5 pg/mL ($p < 0.05$), and 18.6 ± 3.8 pg/mL ($p < 0.05$), respectively (Fig. S1). In contrast, the model group showed the contents of IL-1β, TNF-α, and PGE_2 as 92.4 ± 16.8 pg/mL, 82.6 ± 11.4 pg/mL, and 32.8 ± 4.9 pg/mL, respectively.

3.2. TMF exhibited chondroprotective activities with down regulation of β-catenin in vivo

Six weeks after the OA rat models established, rats were sacrificed. Gross observation and histomorphological examination (Fig. 1) were investigated, some degrees of hyperplasia, hypertrophy, congestion in femoral condyle and the rough surface of cartilage with erosion in the model group were observed, indicating the successful establishment of knee OA model. No obvious damage was found on gross observation in TMF groups, which exhibiting significant effect of preventing knee osteoarthritis of TMF. Histomorphological examination by hematoxylin-eosin staining found that the chondrocytes in the model group were hypertrophic lined in disorder way. TMF dose-dependently inhibited chondrocytes hypertrophy and decreased the cartilage thickness. The immuno-fluorescence assay for β-catenin *in situ* detection (Fig. 1) demonstrated that the expression of β-catenin was significantly increased in the model group. In contrast, TMF exhibited down regulation of β-catenin in a dose-dependent manner. At the dose of 100 mg/kg, the fluorescence intensity of β-catenin *in situ* was 0.28 ± 0.07 ($p > 0.05$) compared with that in the model group. However, it did not reach a statistically significant difference.

3.3. Effects of TMF on cell viability

The chondrocytes toxicities at 80, 40, 20, 10, 5 and 0 μg/mL of TMF were assessed by MTT assay (Fig. S2). TMF with concentrations higher than 80 μg/mL had toxic effects on chondrocytes. However, the viability of the chondrocytes incubated with 40 μg/mL TMF was much less than that with 20 μg/mL TMF. As a result, 20, 10 and 5 μg/mL TMF were selected as the high, medium and low concentrations, respectively. The criterion was employed in subsequent experiments.

3.4. TMF decreased the apoptotic rate of chondrocytes in vitro

To determine the effect of TMF on cell death, flow cytometry using the FITC-Annexin V/PI double staining method was employed. Treatment of 1 μM PGE_2 resulted in significant cell death and TMF exhibited chondroprotective activity (Fig. 2). As showed in Fig. 2, at the doses of 20 μg/mL of TMF, the situations were almost as moderate as that in the control group. In contrast, the model group showed the apoptosis rate as 29.46 ± 3.65% (Fig. 2).

3.5. Treatment with TMF inhibited the expressions of EP2, EP4, β-catenin, and COX-2 gene

PGE_2, a pro-inflammatory cytokine, is the product of COX-2, which is the down stream gene regulated by *β-catenin*. To determine whether PGE_2 exhibits positive feedback to promotion of β-catenin signaling pathway associated with induction of apoptosis, the mRNA expressions of β-catenin signaling-associated genes *β-catenin*, *COX-2*, and the PGE_2 receptor genes *EP2* and *EP4* were assessed using qRT-PCR (Fig. 3A). Treatment of chondrocytes with TMF, the mRNA levels of *β-catenin*, *COX-2*, *EP2*, and *EP4* were significantly decreased by a dose-dependent way, comparing to those in the model group. When exposed to 20 μg/mL of TMF, cells showed a significant difference from the model group. To further demonstrate that PGE_2 activated β-catenin signaling pathway, chondrocytes were transfected with Fopflash/Topflash reporters to determine β-catenin regulated reporter activity (Fig. 3B). The results showed that treatment with 20, 10 and 5 μg/mL TMF for 24 h caused a significant decrease in Topflash activity comparing to Fopflash activity.

3.6. Treatment with TMF inhibited the protein expressions in EP/cAMP/PKA signaling pathway and β-catenin signaling pathway

We used PGE_2 to further determine whether inflammatory mediators mediate the activation of EP/cAMP/PKA signaling pathway and β-catenin signaling pathway in chondrocytes. The cells were treated by 1 μM PGE_2 and various concentrations of TMF for 48 h, then harvested and cell lysates prepared for Western blot analysis. As showed in Fig. 4, Western blot data revealed that PGE_2 significantly activated the protein expressions of receptors PKA, p-PKA, EP2, EP4, together with the down stream factors cAMP (Fig. S3). Similarly, the β-catenin signaling pathway was also activated, β-catenin and its down stream factor COX-2 were also up-regulated, but p-GSK-3β was down-regulated. However, TMF dose-dependently inhibited the protein expressions in EP/cAMP/PKA signaling pathway and β-catenin signaling pathway.

4. Discussions

There is excess production of pro-inflammatory mediators, such as IL-1β, TNFα, NO, and PGs, in chondrocytes, which have been demonstrated to play a pivotal role in the development of OA process. In particular, IL-1β and TNFα seem prominent to cartilage destruction [17], by stimulating their own production and inducing to produce other cytokines, such as PGE_2. Current treatments for OA mainly depend on the use of NSAIDs, which targeting down-regulation of PGE_2. Over-expression of COX-2 and prostaglandins

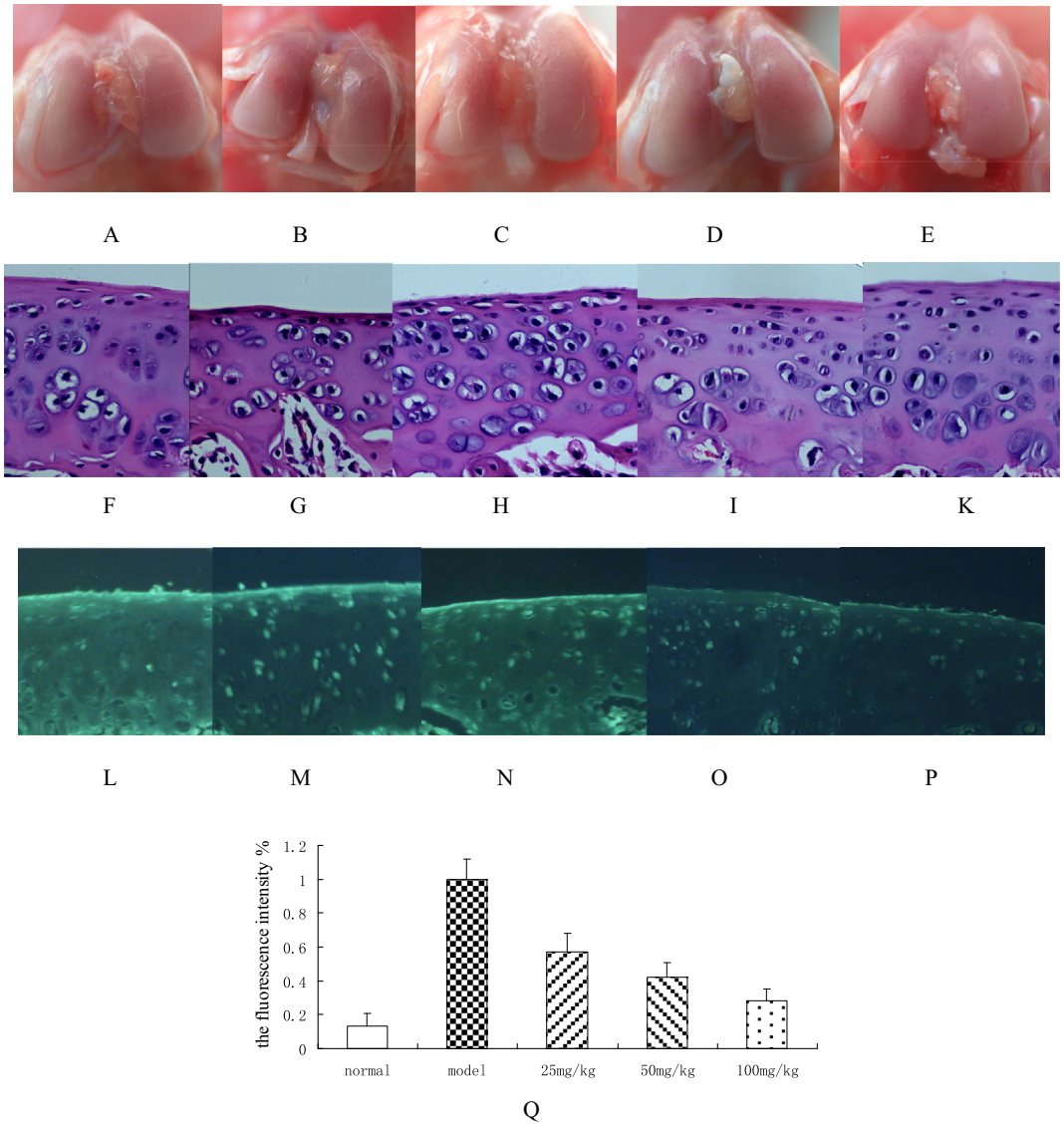

Fig. 1. The gross observation, histomorphological examination, and the immuno-fluorescence assay in the cartilage. (A–E) were control group, model group, and the TMF groups at the dose of 25, 50, and 100 mg/kg, respectively. Similarly, the figures in H & E staining (F–K) and the immuno-fluorescence assay (L–P) were in the same order. (Q) was the fluorescence intensity of β-catenin *in situ* compared to that in the model group. Data were presented by mean ± standard deviation of 10 replicates. $^{*}p < 0.05$ as compared with the model.

are considered to be potent regulators and biomarkers of inflammation and, in osteoarthritis, over-expression of COX-2 and PGE_2 has been associated with chondrocytes in apoptosis. In the rat OA models, TMF, a potent anti-inflammatory natural product, were found to exhibit protective activity with down-regulating the expressions of inflammatory cytokines.

Chondrocyte is the unique cell present in cartilage, responsible for maintaining the integrity of the extracellular matrix (ECM). Growing evidences indicate that cartilage degradation and chondrocytes apoptosis have many associations [18], which promote the pathological process of OA. By histomorphological examination, TMF significantly prevented the decrease of the cartilage thickness and the cartilage degradation. β-Catenin is a key molecule in the canonical Wnt signaling pathway which plays a critical role in multiple steps during chondrocyte formation and maturation. Both excessive and insufficient β-catenin levels may therefore impair the homeostasis of articular chondrocytes by enhancing pathological maturation and apoptosis, respectively [19]. Fluorescent immunohistochemical staining showed that the expression of β-catenin was significantly up regulated in rat OA cartilage. It indicates that β-catenin might be the potential target for novel therapeutical strategies. TMF could significantly decrease the expression of β-catenin *in vivo*. The expressions of pro-inflammatory mediators and β-catenin had a relative tendency. However, the underlying mechanism remains unclear.

Proinflammatory mediators, IL-1β and TNFα, induce the synthesis of PGE_2 by promoting the expression of COX-2. The synthesized PGE_2 promotes IL-1 expression as a positive feedback mechanism, degrades the ECM, and induces apoptosis of chondrocytes [20]. Chondrocytes apoptosis can be induced by various stimuli, such as inflammatory mediators [21]. In this study, PGE_2 was used as prototype pro-inflammatory cytokine to reproduce the "OA-like effect" in the cells. *In vitro*, the chondrocytes apoptosis with FITC annexin V/PI double staining were evaluated by flow cytometry. It showed that TMF could significantly protect chondrocytes from initiating apoptotic processes induced by PGE_2 dose dependently. It further demonstrated that the pro-inflammatory mediators could cause a vicious cycle in cartilage *in vivo*.

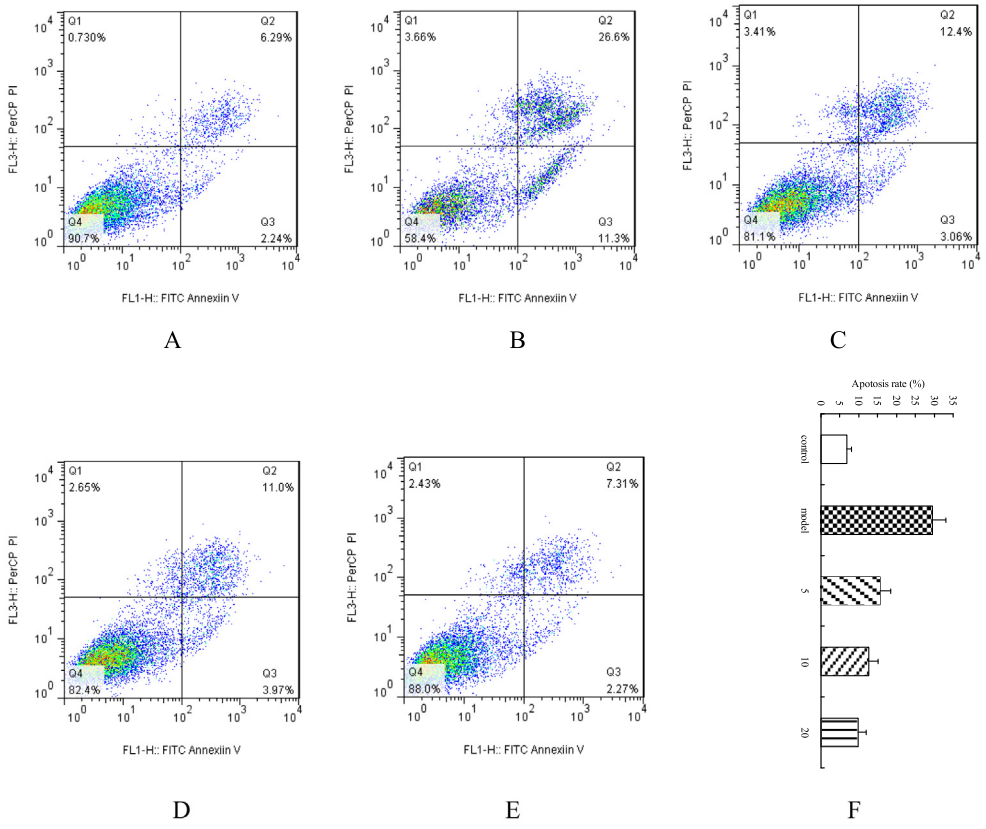

Fig. 2. Inhibition of apoptosis by TMF. The healthy chondrocytes were incubated for 24 h. Chondrocytes in the control group (A) was incubated without adding any medicines. Model group (B) was the healthy chondrocytes incubated with 1 μM PGE_2. (C–E) were groups incubated with 1 μM PGE_2 and 5, 10, and 20 μg/mL TMF, respectively. (F) Was the summarized data indicating the rate of apoptosis cells, as detected by flow cytometry. Data were presented by mean ± standard deviation of 4 replicates. $^*p < 0.05$ as compared with control.

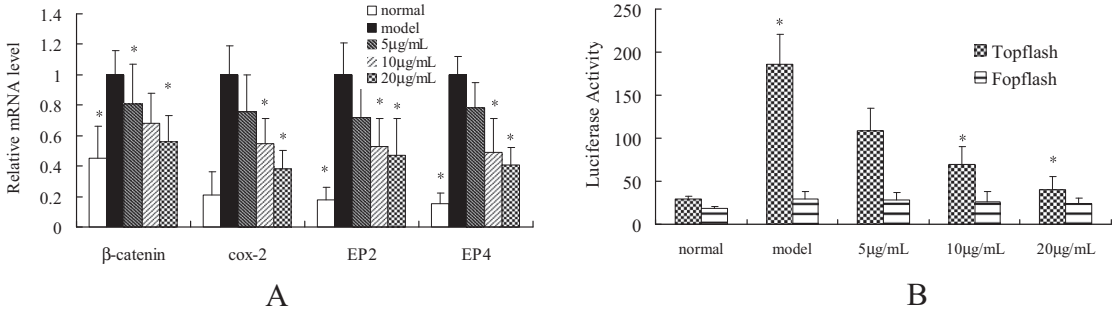

Fig. 3. (A) Changes in *β-catenin*, *COX-2*, *EP2*, and *EP4* mRNA expression in the control, model groups and in the groups treated for 24 h with 20, 10 and 5 μg/mL TMF. qRT-PCR was used to detect changes in mRNA expression of these genes. GAPDH was used as internal control. These data were representative of results obtained from the analysis of three independent experiments. Data were presented by mean ± standard deviation of 4 replicates. $^*p < 0.05$ as compared with the model. (B) Chondrocytes were transfected with Fopflash or Topflash luciferase reporters. Transfected cultures were treated with 20, 10 and 5 μg/mL TMF for 24 h. Data were ratios of firefly luciferase units from the respective reporters to constitutive CMV-regulated renilla luciferase units normalized to their respective model group cultures. Data were presented by mean ± standard deviation of 4 replicates. $^*p < 0.05$ as compared with the control.

The biologic effect of PGE_2 in articular cartilage depends on the expression of EP receptors, EP2 and EP4 [22], which are coupled to the G protein Gs that causes accumulation of cAMP and activates PKA and Akt pathways. The biological effects of PKA are mediated through the phosphorylation, which can directly phosphorylate GSK-3β, thereby inhibiting its kinase activity and stimulating canonical Wnt/β-signaling [23]. However, little is known about the potential link between the COX-2/PGE_2 axis and β-catenin signaling. Our previous studies indicated that β-catenin signaling was activated and COX-2/PGE_2 was also up regulated in osteoarthritis [7].

As for EP, the mRNA expressions and the protein synthesis of both EP2 and EP4 were significantly up-regulated by PGE_2. It has been demonstrated that PGE_2 increases the stabilization of *COX-2* mRNA and the synthesis of COX-2 protein [24]. Similarly, we also found the mRNA expression and the protein synthesis of COX-2 was up-regulated. The activity of *COX-2* gene is mediated by β-catenin signaling [7], which indicates activation of β-catenin signaling. To support it, the mRNA expression and the protein synthesis of β-catenin and the β-catenin regulated reporter gene activity were determined. PGE_2 dose-dependently increased Top-

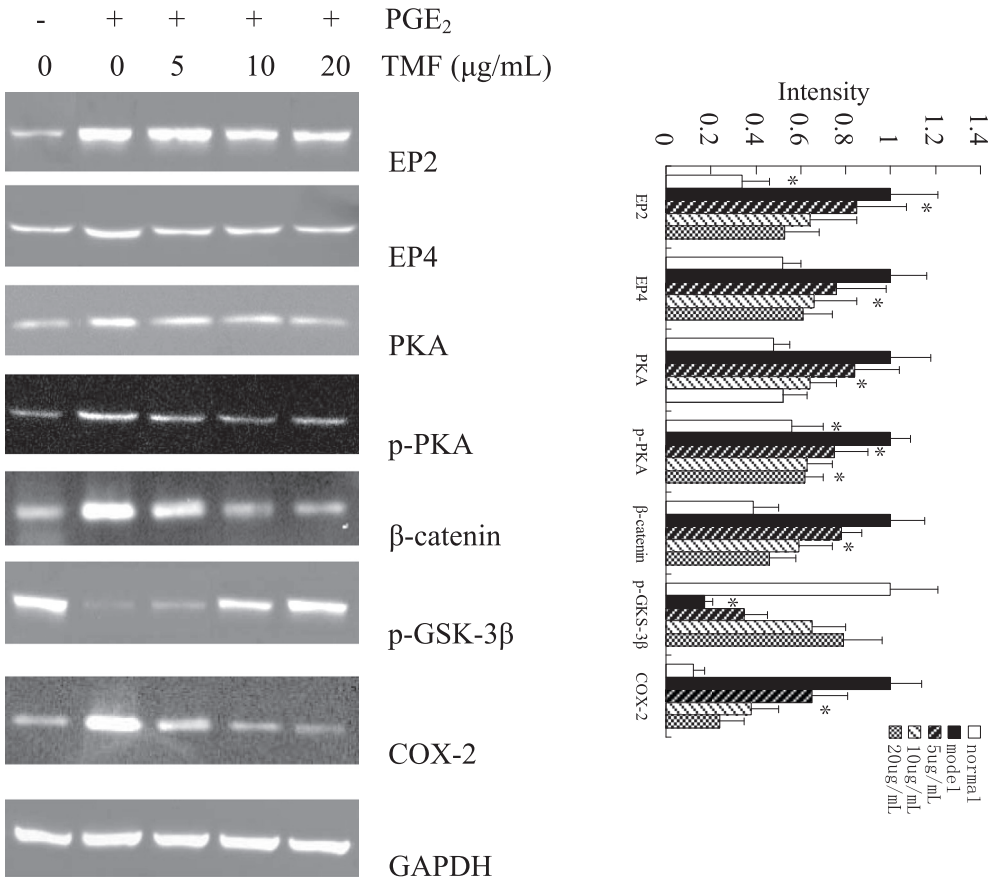

Fig. 4. Changes in protein expression of EP2, EP4, PKA, p-PKA, β-catenin, p-GSK-3β, and COX-2 in the model, control groups and in the groups treated for 48 h with 1 μM PGE2 and 20, 10 and 5 μg/mL TMF. Western blot was used to determine changes in protein expression. These data were representative of results obtained from the analysis of three independent experiments. Data were presented by mean ± standard deviation of 4 replicates. $^{*}p < 0.05$ as compared with the model.

flash activity comparing to Fopflash activity and activated β-catenin signaling pathway, which was consistent with that in rat OA model.

PGE_2-mediated regulation of Wnt/β-catenin signaling has been demonstrated in cell lines. The underlying mechanism of this interaction might be different, depending on the particular cell lines studied [25]. In HEK293 cell line, EP2 and EP4 activate TCF/LEF signaling through activation of PKA-dependent pathway and PI3K-dependent pathway [26]. In hematopoietic stem cells, PGE_2 modulates Wnt/β-catenin signaling at the level of β-catenin degradation through cAMP/PKA-mediated stabilizing phosphorylation [27]. Our studies demonstrated that PGE_2 induced phosphorylations of PKA and GSK-3β in chondrocytes, leading to activation of both cAMP/PKA signaling pathway and β-catenin signaling pathway.

Several drugs and synthetic or natural compounds have been reported to inhibit and/or modulate β-catenin signaling [28]. However, their detailed mechanisms are poorly understood. These small-molecule inhibitors may act by reducing β-catenin stability, blocking β-catenin-TCF interaction or β-catenin-CREB binding protein interaction, stabilizing Axin2 level, preventing dishevelled-Frizzled interaction, or other indirect inhibition [28]. For instance, inhibitor of β-catenin and T cell factor (ICAT) is an 82-amino-acid small molecule [29] whose crystal structure reveals binding capacity to the armadillo repeats of β-catenin. This binding disrupts the complex formation of β-catenin with TCF/LEF [29] and thus leads to inhibition of signaling in this pathway.

Roles of PMFs in prevention and treatment of diseases have received considerable attention recently. Nobiletin, the most abundant and studied PMF in orange peel extract, has been found to induce neurite outgrowth through cAMP and MAPK-dependent mechanism to stimulate CRE transcription activity [30]. Quercetin, one of the important components in *M. exotica*, has been demonstrated to antagonize the Wnt signaling pathway via disrupting the association of β-catenin with TCF/LEF-1 [31]. Our previous study found that *M. exotica* down regulated the expression of β-catenin and reduced chondrocytes apoptosis [7]. In current studies, TMF, one of the most abundant flavone compounds found in *M. exotica* [11], was demonstrated to down regulate the activities of cAMP, PKA, and β-catenin signaling in chondrocytes. But whether TMF plays an effect on CREB still needs further investigation.

In conclusion, TMF is a potential PMF compound with chondroprotective activity by inhibiting EP/cAMP/PKA signaling pathway and β-catenin signaling pathway.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was financially supported by the National Science Foundation of China (81360277 and 81102797), the National Science Foundation of Jiangxi Province (20142BAB215047 and 20142BAB215069), and Scientific Research Fund of Jiangxi Provincial Education Department (GJJ13669) award to LH Wu.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.129.

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