The protective effect of tetramethylpyrazine on cartilage explants and chondrocytes

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Aims of study: Ligusticum wallichi Franchat (\textit{chuanxiong}) is a very common traditional Chinese herbal medicine in China. Tetramethylpyrazine (TMP) is a major active ingredient extracted from \textit{Ligusticum wallichi Franchat}. We investigated the protective effect of TMP on interleukin-1β (IL-1β) induced proteoglycan (PG) degradation and apoptosis in rabbit articular cartilage and chondrocytes.

Materials and methods: Rabbit articular cartilage explants and chondrocytes were cultured with 10 ng/ml IL-1β for 72 h in the absence or presence of various concentrations of TMP (50, 100 or 200 μM). Cartilage and chondroprotective effects of TMP were determined by evaluating (1) the degree of PG degradation by measuring the amount of glycosaminoglycan (GAG) released into the culture media with 1,9-dimethylmethylen blue (DMMB) assay in cartilage explants; (2) gene expression of MMP-3 and TIMP-1 by real-time quantitative reverse transcription-polymerase chain reaction analysis in cartilage explants; (3) chondrocytes viability with MTT assay; (4) the production of intracellular reactive oxygen species (ROS) with laser scanning confocal microscopy (LSCM). Anti-apoptotic effects of TMP were determined by measuring (1) apoptosis with flow cytometric analysis; (2) mitochondrial membrane potential assay with LSCM; (3) caspase-3 activity with special assay kit.

Results: IL-1β treatment increased the level of GAG released into the culture media, and induced the gene expression of MMP-3 and inhibited the gene expression of TIMP-1 in cartilage explants. Moreover, IL-1β treatment decreased the cell viability and mitochondrial membrane potential, and enhanced the level of intracellular ROS, apoptosis rate, and caspase-3 activity in chondrocytes. However, simultaneous treatment with TMP attenuated the IL-1β-induced cartilage and chondrocyte destruction in a dose-dependent manner. TMP showed the decrease of GAG degradation and MMP-3 mRNA production, and the enhancement of TIMP-1 mRNA production in cartilage explants. TMP also increased the cell viability in chondrocytes. Furthermore, TMP inhibited the chondrocytes apoptosis through suppression of ROS production, maintaining of mitochondrial membrane potential and downregulation of caspase-3 activity.

Conclusion: These results demonstrate that TMP has the cartilage and chondroprotective effect, which suggest that TMP could act as an agent for pharmacological intervention in the progress of OA.

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1. Introduction

Many traditional Chinese medicines are used widely for treating various kinds of chronic diseases in China, Japan, and Korea. \textit{Ligusticum wallichi Franchat (\textit{chuanxiong})}, as a traditional Chinese medicine native to China, is a very common crude drug in traditional Chinese, Japanese and Korean folk medicines. It has been reported that \textit{Ligusticum wallichi Franchat} can promote blood circulation and relieve pain (Chen, 1997; Guo and Li, 2001), enhance the immune system and anti-inflammatory ability (Sinclair, 1998) so that it is widely prescribed for the treatment of various cardiovascular and cerebrovascular diseases in the clinic (Au et al., 2003; Liao et al., 2004). Moreover, \textit{Ligusticum wallichi Franchat} is one of the most important constituents of several Chinese herbal compounds which can efficiently cure arthritis (Sun, 2002; Liu et al., 2005). Because of its diverse therapeutic effects, several pure compounds have been isolated, such as tetramethylpyrazine (TMP), ligustilide, senkyunolide A and ferulic acid (Yan et al., 2005), and their biological functions studied. Until recently, among these ingredients, TMP is the most active ingredient whose biological functions involve anti-apoptotic activity, anti-inflammation, inhibition of NO and...
OA is one of the most common chronic diseases and is a major source of functional disability in elderly persons. With worldwide population aging, the incidence of OA is rapidly increasing, and it is anticipated that OA will become the fourth leading cause of disability in the coming decades (Urquhart et al., 2008). OA is a multifactorial disease characterized by progressive erosion of articular cartilage, proteoglycan (PG) degradation, disruption of the collagen network, and subsequent chondrocytes apoptosis and death (Das and Farooqi, 2008). Several studies have demonstrated that enzymatic cleavage by matrix metalloproteinases (MMPs) together with cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) play critical roles in the initiation and progression of articular cartilage destruction (Klatt et al., 2006; Pujol et al., 2008). Chondrocytes apoptosis has also been identified as a critical reason for cell loss in aging OA cartilage and is now considered as an important factor contributing to the breakdown of extracellular matrix in joint diseases (Johnson et al., 2008). Recently, some attention has been given to the suggestion that reactive oxygen species (ROS) participate in the pathogenesis of articular cartilage degradation (Kurz et al., 2004; Jallali et al., 2005). After exposure to various stimuli, chondrocytes have been shown to produce a variety of ROS including NO, superoxide, H2O2, and peroxynitrite (ONOO−) (Afonso et al., 2007). Excessive ROS production can cause damage to all matrix components of cartilage, either by a direct attack or indirectly by reducing matrix components synthesis, by inducing apoptosis or by activating latent MMPs (Henrotin et al., 2003).

Although much progress has been made on identifying the molecular events responsible for OA, there is still no effective drug that can reverse cartilage destruction or restore the functional integrity of a joint. Currently, most treatments for OA are directly targeted to symptoms of the disease rather than the underlying cause. As therapeutic agents, analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) represent the mainstay (Baker and Ferguson, 2005). These drugs generally decrease pain and stiffness and improve function, but beneficial effects to the underlying cartilage and bone changes have not been demonstrated.

The proinflammatory cytokine IL-1β can induce the enhanced production of MMPs and inhibit the level of tissue inhibitors of metalloproteinases (TIMPs) (Mengshoel et al., 2000; Fernandes et al., 2002) and also suppress synthesis of ECM and increase the apoptosis of chondrocytes thus inhibiting the repair process in cartilage (Pujol et al., 2008). It is always used on cartilage and chondrocyte to establish an OA model (Bau et al., 2002; Roman-Blas et al., 2009). Ligusticum wallichii Franchat is one of the main components of traditional Chinese herbal compounds treating osteoarthritis, and most of the foregoing biological functions of TMP are related to the etiological factors of OA. Thus, in the present study we investigated the potential of TMP to protect rabbit cartilage and chondrocytes in an IL-1β-mediated OA model.

2. Materials and methods

2.1. Drugs and reagents

TMP, pronase, collagenase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium (MTT), 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA), 1,9-dimethylmethylene blue (DMMB) were obtained from Sigma (St. Louis, USA); Dulbecco's Modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GibCO (Carlsbad, USA); 5,5′,6′-tetrachloloral-1,3,3-tetraethylbenzimidazol-carboxylic iodide (JC-1) was purchased from Molecular Probes (Leiden, Netherlands). IL-1β was purchased from R&D (Minneapolis, USA). RNA extraction kit was purchased from Invitrogen (Paisley, UK). The annexin-V fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Boehringer Mannheim (Mannheim, Germany). Caspase-3 assay kit was obtained from Promega (Madison, USA). All other reagents or drugs were of analytical grade.

2.2. Animals

New Zealand rabbits (5-week-old, 1200–1400 g) were provided by Peking University Experimental Animal Center. All rabbit experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996).

2.3. Cartilage explants culture

Articular cartilages were obtained from the knee joints of rabbits. Briefly, after the articular surfaces were exposed surgically under sterile conditions, articular cartilage was removed and steeped in complete medium (DMEM, supplemented with heat-inactivated 10% FBS; penicillin 100 U/ml; streptomycin 100 μg/ml) for 1 h before plate. Then the samples were rinsed several times with complete medium (basal medium supplemented with 10% FBS; penicillin 100 U/ml; streptomycin 100 μg/ml) and further incubated at 37°C in a humidified 5% CO2/95% air incubator for stabilization. The complete medium was replaced with a basal medium (DMEM, supplemented with heat-inactivated 1% FBS, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin).

2.4. Glycosaminoglycan quantitation in cartilage explants

Approximately 50 mg cartilage pieces were placed in 24-well plates and treated with various concentrations of TMP. After 1 h of pretreatment, 10 ng/ml IL-1β was added to the culture media and further incubated at 37°C in a humidified 5% CO2/95% air incubator with or without various concentrations of TMP (50, 100 and 200 μM). The culture media were harvested 72 h later and stored at −20°C until assayed. The amount of GAG in the media at the end of reaction reflects the amount of PG degradation. GAG levels in the culture media were determined by the amount of polyanionic material reacting with DMBM, using shark chondroitin sulfate as the standard (Farnsdale et al., 1986). Samples were examined spectrophotometrically at 540 nm (Spectramax, Molecular Devices, Sunnyvale, CA, USA). Results were expressed as μg GAG released into the medium per mg wet weight of the cartilage.

2.5. MMP-3 and TIMP-1 mRNA quantitation expression

To investigate the gene expression of MMP-3 and TIMP-1 in cartilage, real-time quantitative RT-PCR was performed. 50 mg cartilage explants were lysed using Trizol and total RNA was extracted in accordance with the manufacturer’s instructions. 2 μg RNA was subjected to RT reaction for 60 min at 37°C and was stopped by incubation at 95°C for 5 min. Then 1 μl cDNA was subjected to real-time quantitative PCR to assess MMP-3 and TIMP-1 mRNA expression levels with an ABI 7700 Sequence Detection System, in accordance with the manufacturer’s instructions (Applied Biosystems, Warrington, UK). Obtained threshold cycle (CT) values of each sample were normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, and relative expression level was expressed mean value of control group as 1.

2.6. Isolation and culture of chondrocytes

Chondrocytes were obtained from articular of rabbit tibial plateaus and femoral condyle. Cartilage slices were digested in...
serum-free medium with sequential treatments of 0.2% pronase for 1 h, and then overnight with 0.025% collagenase type II in medium supplemented with 5% FBS in a humidified atmosphere of 5% CO2 at 37 °C with continuous agitation. After removing undigested cartilage using a 70 μm nylon sieve, the chondrocytes were collected by centrifugation, washed twice, and then cultured at 10^5 cells/ml in DMEM with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Chondrocytes were divided into five groups: a control group of chondrocytes; a model group of chondrocytes treated with 10 ng/ml IL-1β; and three TMP groups of chondrocytes treated with 10 ng/ml IL-1β and different TMP concentrations (50, 100 or 200 μM). Cells were treated with various concentrations of TMP and/or 10 ng/ml IL-1β for 72 h before analysis.

2.7. Cell viability assay in chondrocytes

Cell viability was determined by use of an MTT assay (Sladouski et al., 1993). Chondrocytes were seeded in 96-well plates at 1 × 10^4 cells/well and grown to 70% confluence in culture medium. The medium was replaced by medium containing various concentrations of TMP and/or 10 ng/ml IL-1β. A total of 5 mg/ml MTT was added to each well after 72 h, and the culture continued to incubate for another 4 h at 37 °C. After the medium had been removed, cells and dye crystals were solubilized with 200 μl dimethylsulfoxide (DMSO), and optical density was measured at 570 nm by use of a model ELX-800 microplate assay reader (One Lambda Inc.).

2.8. Determination of intracellular reactive oxygen species level in chondrocytes

Intracellular reactive oxygen species levels were measured with use of the fluorescent dye H2DCFDA staining method. H2DCFDA is a nonpolar compound that is converted into a nonfluorescent polar derivative (H2DCF) by cellular esterases after incorporation into cells. H2DCF is membrane impermeable and rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular reactive oxygen species (Sauer et al., 2001). After cells cultured on glass coverslips in every group were treated with TMP and/or IL-1β for 72 h, they were incubated for 30 min at 37 °C with 20 μM H2DCFDA dissolved in PBS. Coverslips were then washed three times with PBS and analyzed under a confocal laser microscope (Leica, Germany). DCF was excited at 488 nm, and the emission filter was a 510 nm barrier filter. The fluorescent intensities were compared between control and experimental cells.

2.9. Mitochondrial membrane potential in chondrocytes

The mitochondrial membrane potential of cells was measured with use of the probe JC-1 (Cosarizza et al., 1995). JC-1 is able to enter mitochondria selectively, which appear green at low concentrations or at low membrane potential as a monomer. However, at high concentrations, mitochondria show red fluorescent aggregates. JC-1 is sensitive to mitochondrial membrane potential and the changes in the ratio between red and green fluorescence can provide information regarding the mitochondrial membrane potential. After the treated cells were loaded with 1 μmol/l JC-1 for 10 min at 37 °C, the fluorescent dye was excited at 490 nm, and the fluorescence intensities of both monomer and aggregated molecules were recorded at 590 nm under a confocal scanning laser microscope. The results of mitochondrial membrane potential were expressed by red/green ratio.

2.10. Chondrocytes apoptosis

We used annexin-V-FITC apoptosis detection kit to bind annexin-V, which has a strong affinity for phosphatidylserine and can probe for apoptosis (Liu et al., 2002). In brief, chondrocytes were harvested and suspended in binding buffer at a final cell concentration of 10^5 cells/ml. Approximately 10^5 cells were incubated in the dark with annexin-V and propidium iodide for 15 min. Then the suspension was analyzed with use of a FACS scan flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin-V FITC and propidium iodide-related fluorescence was recorded on FL1-H (525 nm) and FL2-H (575 nm) filters, respectively.

2.11. Caspase-3 activity in chondrocytes

Caspase-3 activity was detected by use of the Apo-ONE TM Homogeneous caspase-3 assay kit, according to the manufacturer instructions. Briefly, chondrocytes were seeded into 96-well plates at 10^4 cells/well. After being exposed to various concentrations of TMP and/or 10 ng/ml IL-1β for 72 h, cells were washed with ice-cold PBS. Then, 1 μl Z-DEVD-R110 and 99 μl caspase buffer were mixed to make the homogeneous caspase-3 reagent. A total of 100 μl homogenate caspase-3 reagent was added to each well. The contents were gently mixed at 300–500 rpm for at least 30 s and incubated for 4 h at room temperature in the dark. The intensity of the fluorescence of the Z-DEVD-R110 substrate was measured at an excitation wavelength of 498 nm and an emission wavelength of 521 nm with use of a microplate spectrophotometer (Wallac Victor2TM 1420 Multilabel Counter, USA).

2.12. Statistical analysis

Results are expressed as the mean ± S.E.M. of triplicate values for each experiment. The significance of statistical differences among groups was determined using Student’s t-test. Values at p < 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of TMP on proteoglycan degradation in cartilage explant cultures

Rabbit cartilage explants were cultured for 72 h with 10 ng/ml IL-1β in the absence or presence of various concentrations of TMP to examine the protective effects on PG degradation. After incubation with 10 ng/ml IL-1β for 72 h, the amount of released GAG into the culture medium increased significantly compared to the control group (6.32 ± 0.71 μg/mg vs 1.13 ± 0.18 μg/mg). And TMP treatment caused a significant, dose-dependent reduction in IL-1β-mediated GAG release into the media (Fig. 1).

3.2. Effect of TMP on the gene expression of MMP-3 and TIMP-1 in cartilage explants

As shown in Fig. 2, real-time quantitative RT-PCR analysis indicated that IL-1β enhanced the gene expression of MMP-3 (2.8-fold), and inhibits the gene expression of TIMP-1 (0.7-fold). TMP suppressed the IL-1β-mediated gene expression of MMP-3 in a dose-dependent manner. In addition, TMP enhanced the production of TIMP-1 in a dose-dependent manner, and 2.1-fold increases was observed at 200 μM.

3.3. Effect of TMP on cell viability in chondrocytes

After incubation with IL-1β, approximately 51.3% of the chondrocyte underwent death. Treatment with TMP (50, 100 or 200 μM)
Fig. 1. Effect of TMP on proteoglycan degradation in cartilage explant cultures. GAG concentration in culture media was determined by the DMMB assay. Results were expressed as μg GAG released into the medium per mg wet weight of the cartilage. n = 8. Data are mean ± S.E.M. **p < 0.01 as compared to control cells; *p < 0.05, **p < 0.01 as compared to IL-1β.

decreased the cell death rate in a dose-dependent manner (cell death rate was 36.8%, 23.5% and 7.2%, respectively; Fig. 3). And TMP treatment alone did not affect chondrocyte viability at any of these concentrations (data not shown). Thus, TMP has a good chondrocyte protective effect on IL-1β-induced cell death.

Fig. 2. Effect of TMP on the gene expressions of MMP-3 and TIMP-1 in IL-1β-induced cartilage explants. Relative expression levels of mRNA were expressed mean value of control group as 1. n = 8. Data are mean ± S.E.M. *p < 0.05, **p < 0.01 as compared to control cells; *p < 0.05, **p < 0.01 as compared to IL-1β.

3.4. Effect of TMP on ROS formation in chondrocytes

The level of ROS generated in IL-1β-induced chondrocytes increased markedly compared with controls. However, the fluorescent intensity in TMP-treated chondrocytes decreased by 31.2%, 63.6% and 86.5% with 50, 100 or 200 μM TMP treatment, respectively (Fig. 4). Thus, TMP can inhibit the generation of ROS in chondrocytes induced by IL-1β.

3.5. Effect of TMP on mitochondrial membrane potential in chondrocytes

Control cells exhibited numerous staining mitochondria that emitted red-orange fluorescence and red/green-ratio is 4.36 ± 0.47, which was indicative of normal high membrane potential. IL-1β treatment induced a transition in mitochondria permeability and a significant loss of membrane potential (red/green-ratio is 0.38 ± 0.04) (Fig. 5). However, TMP treatment inhibited the collapse of mitochondrial membrane potential induced by IL-1β in chondrocytes with increasing dosage. TMP gradually resumed the mitochondrial membrane potential in a dose-dependent manner.

3.6. Effect of TMP on apoptosis in chondrocytes

The anti-apoptotic action of TMP was also confirmed by measuring the presence of phosphatidylserine on the outer cell membrane. Fig. 6 shows the results of annexin-V binding experiments to
3.7 Effect of TMP on caspase-3 activity in chondrocytes

Caspase-3 activity was increased by 9.2-fold compared with controls after IL-1β incubation. In contrast, chondrocytes which were simultaneously treated with 50, 100 or 200 μM TMP showed a significant decrease in caspase-3 activity compared with IL-1β-treated cells at the same time point (Fig. 7). The data showed that treatment with TMP resulted in the inhibition of IL-1β-induced activation of caspase-3.

4. Discussion

The etiology of OA, a degenerative joint disease, is still not fully understood (Brandt et al., 2009). The main objectives in the management of OA are to reduce symptoms, to minimize functional disability and to limit disease progression. Until now, several drugs used in the treatment of OA have been demonstrated to be symptom-modifying drugs that induce symptomatic relief, but few of these drugs have been investigated for their effects on the structural changes of the tissue. Therefore, more suitable therapies that modify the pathophysiology of OA are needed. At this point, cartilage and chondrocyte protection is an essential target in developing treatment of OA. TMP, as a major component extracted from *Ligusticum wallichi Franchat*, is the very important candidate for the foregoing objectives. In the present study, we found that TMP prevented rabbit articular cartilage degradation through the antioxidative effects, the increase of TIMP-1 activity and decrease of MMP-3 activity, and inhibited the apoptosis through the maintaining the mitochondria function and downregulation of caspase-3 activity. This study provided the evidence of a beneficial effect of TMP on OA for the first time.

Excessive MMPs production is a key mechanism by which cartilage matrix destruction occurs during the development of OA (Bluteau et al., 2002). Imbalance between MMPs and TIMPs is considered to be of importance in the degradation of ECM (Bluteau et al., 2001). MMP-3 (Stromelysin-1) is one main member of the MMPs superfamily, which can degrade several ECM molecules, including GAG and type II collagen (Aigner et al., 2001). In addition, MMP-3 is known to be essential for the full activation of proMMP-1 (Saito et al., 1998). The activity of MMP-3 is controlled by a tissue inhibitor of metalloproteinase (TIMP) which inhibits MMP-3 at a stoichiometry of 1:1 by forming high-affinity complexes. On basis of such result, we investigated the inhibitory effects of TMP on IL-1β-induced GAG release in cartilage explant cultures. In this study, TMP dose-dependently reduced IL-1β-induced GAG into the culture medium. These results suggest that TMP can be effective for reduction of GAG degradation in cartilage. Moreover, treatment with TMP significantly suppresses the gene expression of MMP-3 in chondrocytes. By contrast, TMP increased the gene expression of TIMP-1, indicating that TMP has an anti-catabolic action, not only via MMP-3 suppression, but also via TIMP-1 induction.

Besides its direct beneficial effect on cartilage tissue, TMP is also able to protect chondrocytes in several aspects. Chondrocytes play a crucial role in maintaining cartilage homeostasis, and can become a main source of various catabolic factors (Mazzetti et al., 2001; Chakraborti et al., 2003; Diaz et al., 2005). In the present study,
TMP significantly reversed the reduction in chondrocyte viability in an OA model in a dose-dependent manner. This protective action may be related to the antioxidative effect and inhibition of ROS production of TMP. Previous researches showed that high ROS concentrations can be detected in synovial fluid and cartilage of OA patients (Loeser et al., 2002). Chondrocytes can be induced to produce ROS by cytokines such as IL-1β. Excessive ROS have various catabolic effects in OA, including suppressing the synthesis of collagen and proteoglycan, and activation of MMPs and other catabolic factors (Mathy-Hartert et al., 2008). On the other hand, inhibition of ROS production can attenuate the degeneration of OA cartilage, which supports the role of ROS in the OA process (Matsushita et al., 2004).

Ligusticum wallichi Franchat has been widely used because of various pharmacological effects. In recent years, more attention has been focused on its antioxidative effects and scavenging of free radicals. Studies by Hou et al. (2004) suggested that Ligusticum wallichi Franchat had a protective capacity on endothelial cells against hydrogen peroxide damage by suppressing the production of ROS. Furthermore, Li et al. (2010) found that TMP, the major component extracted from Ligusticum wallichi Franchat can protect against oxidative brain injury through stabilization of mitochondrial function by quenching of ROS in the rat hippocampus. In this study, our results also showed that TMP had a similar effect of significant downregulation of ROS production in a dose-dependent manner in IL-1β-induced chondrocytes and displayed high antioxidative potential against oxidative stress, just as in cardiovascular and nerve tissues and cells. Due to the vital effect of ROS on the pathogenesis of OA, the effect of TMP against ROS suggests that TMP not only has potential OA therapeutic action, but may also prevent the pathological progression of OA. We believe that the effect of ROS may be the important mechanism involved in TMP effects on OA.

On the basis of the above results, we intended to investigate the inhibitory effect of TMP on chondrocyte apoptosis. Much evidence suggests that several biochemical events, including reduced mitochondrial membrane potential and hyperproduction of ROS have been proposed to be necessary for apoptosis (Heiden et al., 1997; John and Orrenius, 2002). Outer stimuli, such as IL-1β can initiate apoptosis through excessive ROS and mitochondria dysfunction and may converge on the caspase pathway to execute the final phase of the apoptotic process (Thornberry and Lazebnik, 1998). Stefanis et al. (1999) demonstrated that caspase inhibitors rescue cortical neurons from apoptotic death after exposure to the damaging agent. Thus, substances that can inhibit the activity of caspase-3 might protect cells from apoptosis. The present results showed that TMP treatment dose-dependently decreased the enhanced level of ROS and caspase-3, and resumed the mitochondrial membrane potential in the IL-1β-treated chondrocytes. Simultaneously, TMP reduced the percentage of chondrocytes apoptosis induced by IL-1β. So TMP might protect chondrocytes against apoptosis by directly scavenging intracellular ROS, maintaining the mitochondria function and inhibiting the activity of caspase-3.

In conclusion, our study proved the protective effects of TMP on cartilage and chondrocytes. However, some researches showed that MAPK pathways (p38 and JNK) as well as the transcription factor NF-κB pathway are involved in IL-1-induced MMPs expression and apoptosis in chondrocytes (Mengshol et al., 2000). So, further investigation will be conducted to assess the molecular mechanism of action of TMP on MMPs and TIMPs expression, apoptosis as well as anti-inflammation. Besides, in vivo test using animal OA model is in process at present. We suggest that TMP could act as an agent for pharmacological intervention in the progress of OA.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 30973043, 30700906), Beijing Municipal Natural Science Foundation (No. 7102159), and Science and Technology New Star Funds of Beijing (No. 2007A008).

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