Indian Journal of Experimental Biology
Vol. 51, April 2013, pp. 313-321

Effect of chinese traditional herb *Epimedium grandiflorum* C.Morren and its extract Icariin on osteoarthritis via suppressing NF-κB pathway

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Received 19 July 2011; revised 21 December 2012

Osteoarthritis (OA), which is also called degenerative arthritis, is the leading cause of disabilities in the old people. The Chinese traditional herb *Epimedium grandiflorum* had long been found to attenuate osteoarthritis process, but the detailed mechanism was not clear. To study the mechanisms of *E. grandiflorum* in the treatment of osteoarthritis, rabbit osteoarthritis model combined with D-galactose was used. After different treatments for 10 weeks, cartilage sections were analyzed by immunohistochemistry for uPA, uPAR and PAI expression level. *E. grandiflorum* could significantly attenuate OA condition and decrease uPA, uPAR and PAI expression. The extract of *E. grandiflorum*, icariin also had a similar effect when compared with *E. grandiflorum* treatment alone. Rabbit chondrocytes were further isolated to be stimulated by TNFα combined with different reagents treatment. Here, icariin treatment significantly reduced nuclear factor kappa B NF-κB (P65) activity, decreased uPA expression level and increased IκBα protein level. The results indicated that *E. grandiflorum* and its extract icariin could attenuate OA condition, reduce the expression of uPA and uPAR and increase PAI in experimental rabbit model and this effect may be conducted by suppressing NF-kB activity by increasing IκBα level.

Keywords: *Epimedium grandiflorum*, Icariin, Osteoarthritis, NF-κB (P65), uPA

Osteoarthritis (OA), which is also termed as degenerative arthritis, is the leading cause of disabilities in the aged people. OA is a common chronic clinical syndrome characterized by symptoms related to abnormalities of articular cartilage, degeneration of proteoglycan and collagen fiber network, inflammation of synovial membranes, and in some cases sclerosis of subchondral bone and osteophyte formation[1]. The disease associated changes in joint tissue matrices result to perturbations in tissue-remodeling activity, in which the family of matrix-degrading enzymes, the urokinase plasminogen activator (uPA) system, has been highly implicated[2].

uPA system comprises of uPA, uPA receptor (uPAR) and urokinase plasminogen activator inhibitor (PAI). The level of uPA expression is positively correlated with the damage extent of articular cartilage, meanwhile considering that uPA is mainly expressed in the superficial layer of articular cartilage, which is also severely damaged in OA, researchers just raise the idea that uPA system plays a key role in matrix degradation[3]. Moreover, studies have shown that uPA and matrix-degrading enzymes (MMPs) have cooperative interactions in inducing the degeneration of OA articular cartilage[4,5].

Upto now, few factors has been reported to regulate uPA expression, and NF-κB signaling pathway is one of these[6,7]. NF-κB is found basically in all cell types and involved in activation of an extremely large number of genes in response to infections, inflammation, and other stressful situations which require rapid reprogramming of gene expression. NF-κB signaling pathway is key for inflammatory reaction and placed in the center of signaling pathways network[8]. Exposure of cells to a variety of extracellular stimuli leads to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκB, which releases NF-κB to translocate to nucleus where it regulates downstream genes transcription.

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Epimedium grandiflorum (EG), known for kidney-reinforcing, bone-strengthening, blood activation and stasis dissolve, is an effective traditional Chinese medicine for treating bone loss[9] by inhibiting mouse osteoblasts apoptosis[10]. Chinese medicine *FuYuan capsule* which mainly contain *E. grandiflorum* could be used for treating OA by alleviating the pathological changes of articular cartilage, probably through inhibiting the expression of interleukin-6 (IL-6)[11], interleukin-1β (IL-1β), matrix metallo proteinase-13, induced nitric oxide synthase[12], tumor necrosis factor-alpha and vascular endothelial growth factor[13]. These information suggest that *E. grandiflorum* may attenuate OA by unclear mechanisms. Icariin is the main active ingredients of *E. grandiflorum* and have some effects on osteoblast cells and thus may have a role in treatment for OA[14,15]. The present study, based on experiments at both animal and cellular level, investigated the role of *Epimedium grandiflorum* and its main active ingredients, icariin, in protecting chondrocytes from OA damages and its potential mechanism.

Materials and Methods

Animals—Six- week-old New Zealand rabbits were used for culturing primary cartilage cells. New Zealand rabbits (54) with 4-month-old age (bisexual each half) weighing 2.0-2.3 kg were used for the establishment of OA model. All rabbits were obtained from Chongqing Medical Laboratory Animal Center. Licence：SCXK(yu) 20090001.

Drugs and reagents—Epimedium grandiflorum (EG) was purchased from Zhejiang Chemicals, China, it was prepared as powder and dissolved with ethanol (1 mL solution equals to 1.5 g drug powder). Rat serum containing *E. grandiflorum* was prepared as previously described[13]. Icariin (production lot number: 08101031, purity≧98%) was obtained from Shanghai TongTian Biotechnology Co., Ltd.). Glucosamine hydrochloride (lot number : Z20081011) was from Chongqing Xi Eran Pharmaceutical Company. TNF-α was obtained from Sigma. Rabbit uPA, uPAR, PAI, IKB antibodies were obtained from Wuhan Boster Company.

Establishment of OA model—Rabbit osteoarthritis model was established by combined treatment of D-galactose injection and plaster immobilization[16]. After being bred conventionally for 7 days, skin above the ankle joint of left fore of model rats was shaved. The left fore was wrapped up with soaked plaster bandage with 3 pieces of gauzes inside. The fore was immobilized in extension for 6 weeks. Meanwhile, D-galactose was given hypodermicly, 30 mg/kg, once a day[17,18].

New Zealand rabbits (54) were randomly divided into following 6 groups of 9 each: normal group, OA model group, positive medicine group (glucosamine hydrochloride, 0.09 g/kg/d), *E. grandiflorum* group (3 g/kg/d), low dose of icariin group (1 g/kg/d), and high dose of icariin group (6 g/kg/d), for a total of 10 weeks daily treatment. In the last five model groups, before establishment of OA model, one rabbit in each group was randomly sacrificed to detect all markers mentioned later. This procedure was to make sure the rabbit in model group were normal, and the 5 sacrificed rabbit's results were mentioned as model-control group.

Immunohistochemistry assay for the expressions of uPA, uPAR, PAI—Specimens from full-thickness articular cartilage of femoral condyle and tibial plateau cartilage were fixed in 4% PFA, decalcified in 10% EDTA for 2 weeks, made into paraffin sections routinely. These slices were deparaffined and hydrated, incubated with 0.3% H_2O_2 at room temperature for 10 min to reduce endogenous peroxidase activity, followed by antigen repair. After washing with PBS, the slices were blocked with normal goat serum at room temperature for 20 min, then incubated with uPA, uPAR and PAI primary antibody (dilution ratio: 1:100) overnight at 4 °C. After washing with PBS, a second biotin-labeled detection antibody was added, followed by horseradish peroxidase-labeled streptavidin working buffer and DAB colour development solution. Nucleus was counterstained using hematoxylin. Slices were dehydrated using graded ethanol, vitrificated by dimethylbenzene and observed under light microscope

Five high power images were selected per slide, percentage of uPA, uPAR, PAI positive cells were analyzed by counting the number of uPA, uPAR, PAI positive cells and total cells.

Isolation, culture and identification of rabbit cartilage cells—New Zealand rabbits were sacrificed by cervical dislocation, cartilage was obtained and cut into 0.5 mm^3 under sterile conditions. After centrifuged at 1200 r/min for 5 min, the precipate was digested with 0.25 % trypsin in a water bath at 37 °C

for 30 min, washed twice with benzyl-penicilling and streptomycin solution, once with 0.2% type 2 collagen, centrifuged at 1200 r/min for 5 min and the supernant was discarded. The precipitate was digested with 0.2% type-2 collagen at 37 °C until the tissue turned flocculent and the solution turned cloudy. The precipate was washed with PBS after centrifuged at 1200 r/min for 5 min, then single cell suspension was obtained by blowning the precipitate in DMEM culture media (supplemented with 15% FBS) and passed through a metal screen, followed by centrifugation at 1200 r/min for 5 min. After washing with PBS twice, the precipitate was added with 5 mL DMEM supplemented with 15% FBS; cells were planted in 25 cm^2 culture bottles with 5×10^5 cells in each bottle and were grown in a humidified atmosphere with 5% CO^2 at 37 °C, culture medium was changed 72 h later. The expressions of uPA, uPAR, PAI were determined by immunohistochemistry. Cartilage cells were identificated by collagen type 2 immunohistochemistry staining.

Reverse transcription-polymerase chain reaction (RT-PCR)—1×10^6 cells from each group cells were used for one experiment. After culturing in incubator for 2 h, cell were treated with TNFα for 1 h and then collected for extracting mRNA. Cells from different groups were centrifuged and total RNA was extracted using the RNAiso Plus kit, as recommended by the manufacturer. The quality and concentration of extracted RNA were confirmed by agarose gel. Reverse transcription reaction was performed followed the manual instructions. Primer pairs used for RT-PCR including NF-κB (P65), uPA, β-actin were as follows:

NF-κB (P65) forward primer : 5′- GGATTTCGTTTC CGTTATGTCT -3′, reverse primer: 5′- CTCAGGTC CATCTCCTTTGTCT -3′, amplified fragment: 470 bp;

uPA forward primer: 5′-TCACTCTTATCGTGG AAAGGC-3', reverse primer: 5′- CGACCCAGGT AGACAATGTAG -3′, amplified fragment: 498 bp;

β-actin forward primer: 5′-TGCTGTCCCTGTAC GCCTCTGG-3′, reverse primer: 5′- TCTCCTTGAT GTCCCGCACGAT-3′, amplified fragment: 228bp.

Western blot for IκB-α level—Proteins were extracted from each group with pre-cooled RIPA lysis buffer [50m*M* Tris-HCl (*p*H7.6), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1m*M* PMSF, 21 μg/mL Aprotinin, 0.5 μg/mL Leupeptin] on ice for 10 min after drug treatment, followed by centrifugation at 12000 r/min for 10 min. Supernant was collected and protein concentration was measured using Lowry's method. The protein was suspended in 2×SDS loading buffer (125 m*M* Tris-HCl (*p*H6.8), 20% glycerol, 0.01% bromophenol blue, 4% SDS, 200 m*M* DTT), boiled for 5 min, separated by SDS polyacrylamide gel electrophoresis with 30 μg total protein in each lane. After electrophoresis, proteins were transferred to a PVDF membrane and detected with anti-β-actin antibody or anti-phosphorylated IκB antibody, employing enhanced chemiluminescence. Density of the protein bands were analyzed by Quantity One software and the ratios of targeted protein bands to the β-actin bands were calculated.

Electrophoretic mobility shift assay for NF-κB (p65) DNA binding activity—After drug treatment, cells from different groups were digested, collected by centrifugation at 2000 r/min for 10 min, washed with PBS twice and recollected by centrifugation. Nuclear protein were extracted using NE-PER nuclear and cytoplasm extraction kit (Pierce) according to the instructions. Double chain oligonucleotides probes were synthesized by Shanghai Saibaisheng bio-company.

NF-κB (p65) activity was detected using Light-Shift Chemiluminescent EMSA kit (Pierce) as follows: Binding reaction was performed in a reaction mixture containing 50 mg/L Poly (dI - dC), 0.05% NP40, 50 mM KCl, 5 mM $MgCl_2$, 10 mM EDTA. After separating by 5% nondenaturing polyacrylamidegel electrophoresis at 100 V for 30 min, the binding mixture was transferred to nylon membrane at 100 V for 1 h, cross-linked using 254 nm UV light and detected using ECL method. The bands were scanned using UVP-GDS 8000 system and the intensities of bands were analyzed.

Sequences of the probes used for EMSA were (x represented biotin): 5′ -xGccTGGGAAAGT ccccTcAAc-3′ (a). 5′-xAGTTGAGGGGACTTTCCC AGGC-3′ (b).

Statistical analysis—All data are presented as mean±SD. Data were statistically analyzed by Student's *t* test with the use of the SPSS11.0 program. $P<0.05$ was considered statistically significant.

Results

Mankin's evaluation standard for OA—According to Mankin's evaluation standard: the score of 1~5 represented early phase of OA, while score of 6~9 and

10~14 represented metaphase and late phase of OA, separately. Cartilage cells in control and model control group showed clear construction and arranged orderly. Score of OA model group was significantly higher compared with control group, treatment with glucosamine hydrochloride markedly decreased the score, while *EG* and Icariin administration both exhibited similar effects as glucosamine hydrochloride (Fig. 1).

uPA, uPAR and PAI expression in different groups—The positive staining for uPA, uPAR and PAI was in orange or brown colour, and was scattered or mottled within the cytoplasm of chondrocyte cells, only a few positive cells which were brightly stained were found in control and model control groups. OA model groups showed increase of both uPA, uPAR and PAI positive cell number (Figs 2-4) with deeper in colour. Statistical results demonstrated that compared with control, the positive ratio of uPA, uPAR and PAI was both increased significantly in model control groups, which indicated the model was successful, while glucosamine hydrochloride treatment significantly abolished this increase (P<0.01). It was further observed that *EG* or icariin administration could also significantly inhibit the increase of uPA, uPAR and PAI stimulated by D-galactose treatment in OA group. Indeed, for uPAR, the *EG*'s effect was weaker than glucosamine hydrochloride, while icariin alone exhibited a dose-dependent effect; and for PAI, the highest dose of Icariin has the best effect and which was equally to the effect of glucosamine hydrochloride (Figs 2-4).

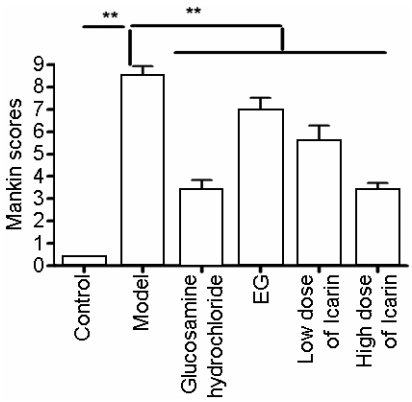

Fig. 1—Mankin scores in different groups. **P<0.01.

Culture and identification of rabbit chondrocyte cells—Isolated chondrocyte cells were small and granular, adhered to culture dishes 12 h after dissection. The mature cells showed a typical appearance with oblate, trilateral or polygon, while having large and bright cellular plasma and clear cell outline, forming a well "pavement" shape, which was identified in the earlier report[19]. To detect the purity of primary chondrocytes, type-2 collagen staining was performed and positive cells showed a brown plasma staining. The morphology and type-2 collagen immunocytochemistry staining indicated that the isolated and cultured cell were actually chondrocyte cells and was pure enough for the following experiments (Fig. 5).

IκB-α and NF-κB (p65) expression and activity in different groups—NF-κB signaling pathway is a key pathway for inflammatory reaction and plays an

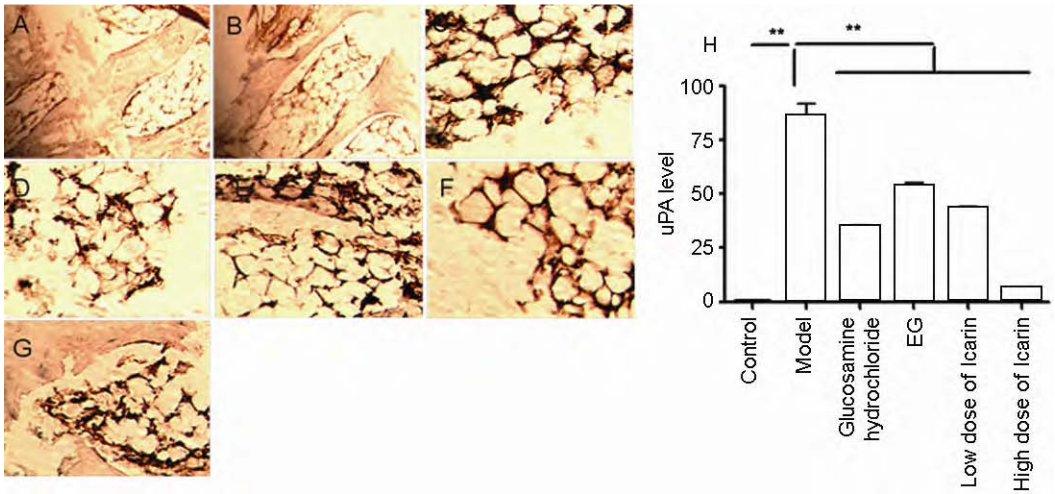

Fig. 2—uPA expression in different groups. (a): control (X100); (b): model control (X100); (c): model (X400); (d): glucosamine hydrochloride (X400); (e) GE group (X400); (f) low dose of Icariin (X400); (g) High dose of Icariin (X400); (h) the statistic results. **P<0.01.

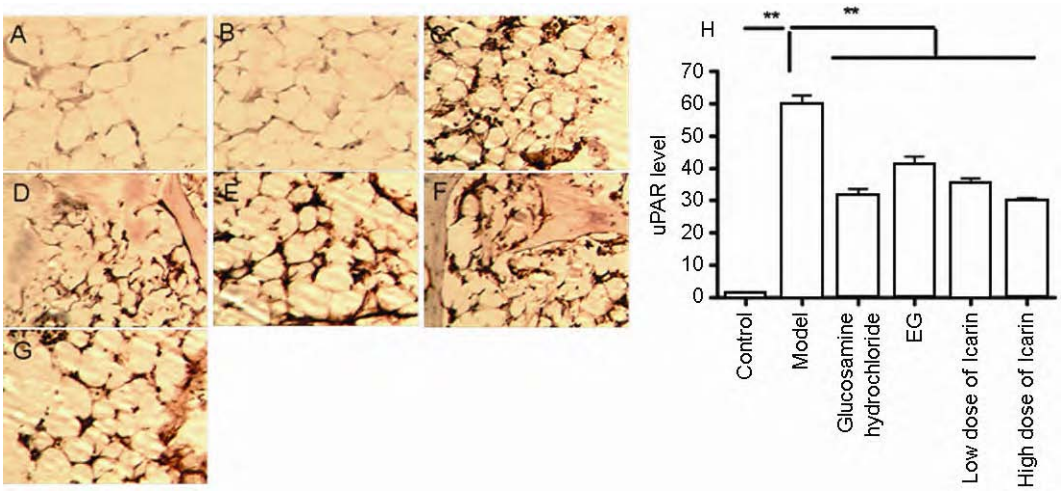

Fig. 3—uPAR expression in different groups. (a): control (X400); (b): model control (X400); (c): model (X400); (d): glucosamine hydrochloride (X400); (e) GE group (X400); (f) low dose of Icariin (X400); (g) High dose of Icariin (X400); (h) the statistic results. ***P*<0.01.

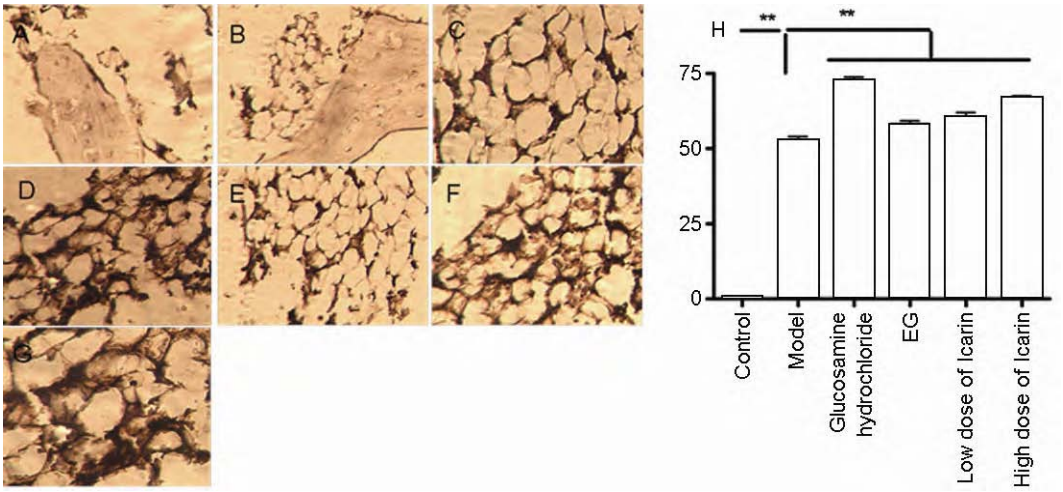

Fig. 4—PAI expression in different groups. (a): control (X400); (b): model control (X400); (c): model (X400); (d): glucosamine hydrochloride (X400); (e) GE group (X400); (f) low dose of Icariin (X400); (g) High dose of Icariin (X400); (h) the statistic results. ***P*<0.01.

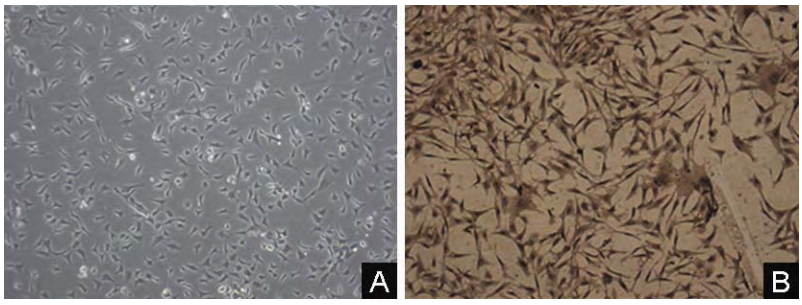

Fig. 5—Culture and identification of rabbit chondrocyte cells. (a), Adherent rabbit chondrocyte cells had a typical appearance of oblate, trilateral or polygon (X100). (b), Type Ⅱ collagen staining indicated that the isolated cells were chondrocyte cells with high purity (X100).

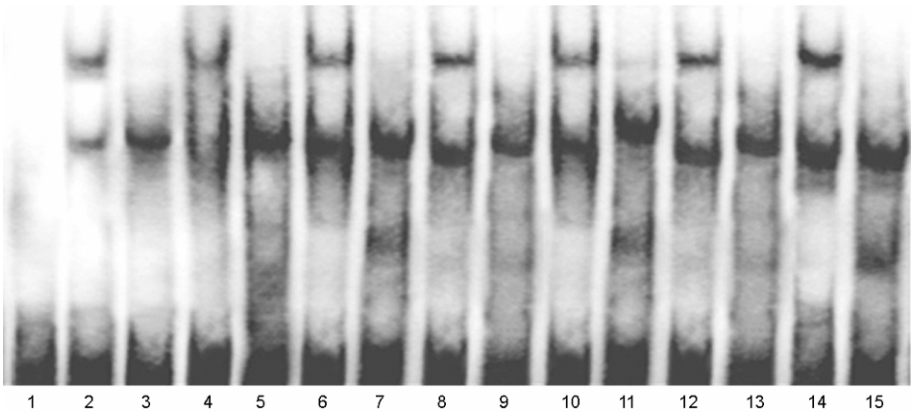

Fig. 6—NF-kB(P65) mRNA expression in different groups (a) showed the result of NF-kB mRNA level, and (b) was the statistic result of it. *P<0.05.

important role in OA. The major form of NF-κB is a heterodimer composed of 50- and 65-kDa subunits (p50 and p65, respectively), RT-PCR and EMSA were performed to evaluate the expression of NF-κB mRNA (p65) and its activity. TNF-α (10 μg/L) treatment for 1 h significantly increased NF-κB mRNA while glucosamine hydrochloride, *EG* and icariin treatment could both notably decrease this enhanced NF-kB mRNA expression which was stimulated by TNF-α administration, meanwhile, no significant differences was observed among those drug treatment groups (Fig. 6). Then NF-κB DNA binding activity was tested using EMSA. Results showed NF-κB DNA binding activity increased after TNF-α treatment, while glucosamine hydrochloride, *E. grandiflorum* and icariin treatment could both abolish this increase (Fig. 7).

IκB-α is the inhibitor of NF-κB, after phosphorylated by kinases, which could bind with NF-κB and inhibit its activity, so the level of IκBα could also indicate NF-κB activity. Western blotting was performed to evaluate the IκBα level, and the results were constant with previous RT-PCR and EMSA results, IκBα decreased after 10 ug/L TNF-α treatment while glucosamine hydrochloride, *E. grandiflorum* or icariin treatment could significantly increase IκB-α extent (Fig. 8).

uPA mRNA expression in different groups— Because previous studies have reported that NF-κB could regulate uPA expression and the latter one plays a very important role in OA occurrence and development, it is speculated that therapeutic effect of *E. grandiflorum* came from reducing uPA mRNA expression by decreasing NF-κB activity. RT-PCR was performed to evaluate the mRNA level of uPA.

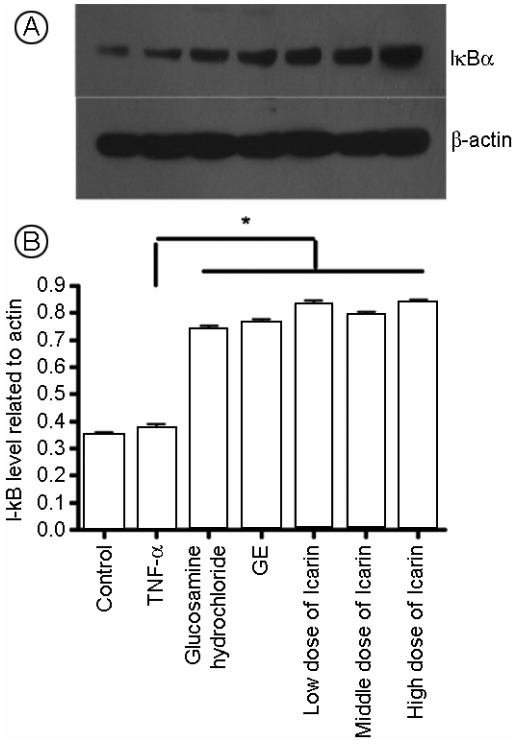

Fig. 7—NF-κB DNA binding activity in different groups. From left to right: control, blank (experimental group, competitive group), glucosamine hydrochloride (experimental group, competitive group), GE (experimental group, competitive group), low dose of icariin (experimental group, competitive group), middle dose of Icariin (experimental group, competitive group), high dose of Icariin (experimental group, competitive group), TNF-α(experimental group, competitive group).

Results showed uPA mRNA expression level increased after TNF-α treatment, while administrated with both glucosamine hydrochloride, *E. grandiflorum* and icariin could significantly decrease it (Fig. 9). These results indicated that therapeutic effect of

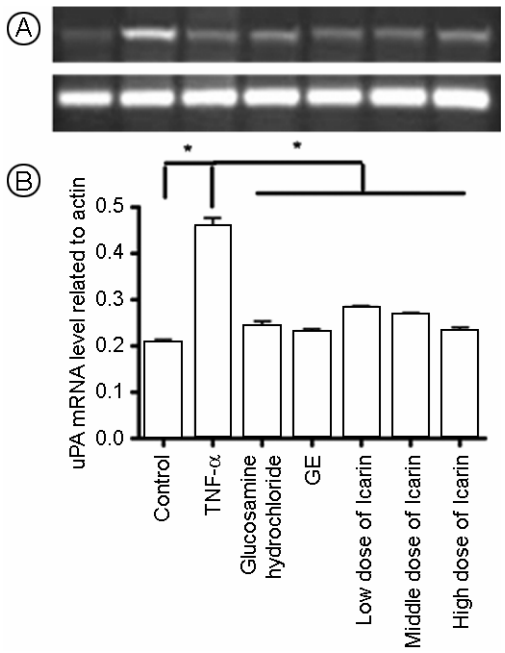

Fig. 8 — IκB-α level in different groups. (a) showed the result of I-kB mRNA level, and (b) was the statistic result of it. **P*<0.05.

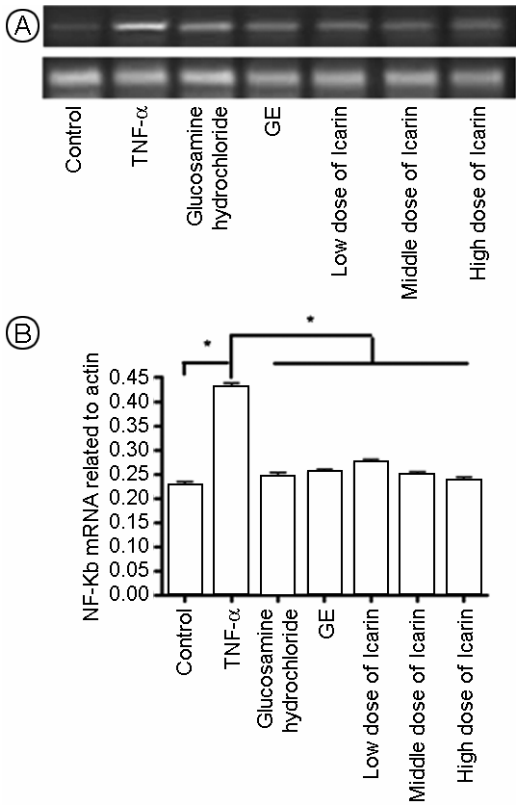

Fig. 9 — uPA mRNA expression in different groups (a) showed the result of uPA mRNA level, and (b) was the statistic result of it. **P*<0.05.

E. grandiflorum and icariin may result from decreased uPA mRNA expression.

Discussion

Cartilage degeneration is one of the characteristic pathological changes of osteoarthritis. Protease, which causes damage to articular cartilage tissue, has become the focus on OA researching field for a long time[20]. Activated protease could accelerate cartilage degeneration and tissue death due to a loss of protection and nutrition from cartilage matrix. uPA system was focused as one target to investigate the potential mechanisms under the therapeutically effect of *E. grandiflorum* in treatment of OA. On the other hand, linkages have been found between osteoarthritis and aging, as articulation degeneration occurred more often in aging organisms. Therefore, establishment of aging model by hypodermic or intraperitoneal injection of D-galactose is a rapid, easy and repeatability way to research OA, and this has actually been widely used since it was first proposed in 1991. Here combined treatment of joint immobilization and aging promotion was utilized to establish osteoarthritis model, avoided the operation injury and was consistent with degenerative changes and natural course of clinical osteoarthritis.

uPA system comprises of uPA, uPAR and PAI. After binding with uPAR, uPA activity will increase and thus accelerate the transformation plasmin from plasminogen, which could further accelerate extracellular matrix degeneration. Further, the binding of uPA and uPAR could also active the generation of collagenase and cytokine, which had coordinated effect in degenerating extracellular matrix. PAI could downregulate uPA activity by binding and forming a stable complex with uPA, which process could attenuate extracellular matrix degeneration. uPA and uPAR are mainly synthesized and expressed by synovial cell, chondrocyte and endothelial cells. The expression level of uPA is positively correlated with the damage extent of articular cartilage and is mainly expressed in the superficial layer of articular cartilage tissue, which is also severely damaged in OA, suggesting that uPA system play a key role in matrix degradation. The present results indicated that *E. grandiflorum* significantly raised PAI expression while decreased uPA and uPA in rabbit OA models cartilage tissue, suggesting that the beneficial effect of *E. grandiflorum* in attenuating the

symptoms of experimental OA rabbit may be through uPA system.

TNF-α, which is synthesized and secreted by macrophage, is pivotal to the pathogenesis of OA. TNF-α could be detected in synovial fluid at early phase of experimental OA condition while at late phase of OA, the concentration of TNF-α in synovial fluid would dramatically increased[21]. Exogenous TNF-α treatment could increase the mRNA level of collagenase in cultured rabbit chondrocytes. Injection of TNF-α to the joint cavity of the knee exhibited cytotoxic effect to chondrocytes, which could increase collagenase generation, inhibited proteoglycan activity and damage resorption of cartilage and matrix. TNF-α and IL-1 also had a coordinate role to make cartilage more fragile than usual. Antibodies and inhibitors of TNF-α could notably alleviate the symptoms of osteoarthritis and inhibited angiogenesis.

Chondrocyte is the only kind of cell in cartilage tissue and is impaired in OA condition. Chondrocytes could synthesize and secrete cartilage matrix, meanwhile biochemical and mechanical factors could also stimulate them to secrete different kinds of factors to facilitate cartilage tissue degradation. Further, chondrocytes abnormalities also take an important role in OA occurrence. Therefore in the present study, chondrocytes was focused to investigate the molecular mechanisms of the therapeutic effects of *E. grandiflorum* and its main ingredients, icariin. Here both *E. grandiflorum* and icariin were found could reduce TNF-α induced activation of uPA and NF-κB signal systems.

In the present investigation, *E. grandiflorum* was found could reduce the expression of uPA and uPAR and increase PAI in cartilage tissue in experimental osteoarthritis rabbits model, which may be potential mechanisms underlying the treatment effect of *E. grandiflorum* for OA. Moreover, *E. grandiflorum* and icariin could significantly reduce NF-kB activities and uPA mRNA expression in primary cartilage cell inflammatory model induced by TNF-α treatment, as well as increases the inhibitory molecule IκB-α. These results provided the cellular and molecular mechanisms for the effect of *E. grandiflorum* and icariin in treating OA.

Acknolegement

This work was supported by Health Bureau of Chongqing No.[2004]12-B-55.

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