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RESEARCH PAPER

Down-regulation of ATF2 in the inhibition of T-2-toxin-induced chondrocyte apoptosis by selenium chondroitin sulfate nanoparticles

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Abstract Selenium chondroitin sulfate nanoparticles (SeCS) with a size range of 30–200 nm were obtained in our previous study. Meanwhile, the up-regulated expression of ATF2 mRNA and protein levels could be observed in the cartilage from Kashin–Beck disease (KBD) patients. In this paper, we investigated the inhibition effect of SeCS on T-2-toxin-induced apoptosis of chondrocyte from KBD patients. Here, we found that when the chondrocytes were treated with T-2 toxin, the chondrocyte apoptosis performed in a concentration-dependent manner. The apoptosis of chondrocyte induced by T-2 toxin involved the increased levels of ATF2, JNK and p38 mRNAs and related protein expression. SeCS could partly block the T-2-toxin-induced chondrocyte apoptosis by decreasing the expression of ATF2, JNK and p38 mRNAs and p-JNK, p-38, ATF2 and p-ATF2 proteins. JNK and p38 pathways involved in the apoptosis of chondrocyte induced by T-2 toxin, and SeCS was efficient in the inhibition of chondrocyte apoptosis by T-2 toxin. These results suggested that SeCS had a potential for further prevention and treatment for KBD as well as other selenium deficiency disease.

Keywords Apoptosis · T-2 toxin · Kashin–Beck disease · Selenium · ATF2 · Nanobiotechnology

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Introduction

Kashin–Beck disease (KBD) is an endemic osteochondropathy manifested by chondrocyte necrosis and apoptosis, cartilage degeneration and matrix degradation (Mo 1979; Yamamuro 2001). In China, approximately 0.7 million patients suffer from the disease, and the 105 million residents who are living in the endemic provinces are at risk (An et al. 2010). By genome screening gene chip analysis, there were different expressions of certain genes in the pathological process of KBD patients related to apoptosis (Wang et al. 2009, 2012).

Selenium deficiency is proposed as one of the risk factors for KBD, and compromised function of selenoproteins would lead to oxidative stress and apoptosis, which has been manifested in KBD (Tan et al. 2002). For the prevention and treatment for KBD, the commercial sodium selenite products which contained vitamin E were used in clinical practice; however, the maximum safety dose of selenium is

400 μg per day (Wilber 1980), so the toxic effect of sodium selenite as an inorganic compound needs to be alleviated for the further application in the treatment for KBD.

T-2 toxin is one of the A-type trichothecenes produced by several fungal genera including Fusarium species, which is proposed as one of the environmental factors for KBD (Guo 2008). T-2 toxin can be detected with a high level (2.0–1,549.9 ng/g) in endemic grain (Yang et al. 1995). Previous studies also demonstrated that T-2 toxin was one of the important etiological factors for KBD, and supplementation of selenium salt could partly prevent KBD (Guan et al. 2013; Zou et al. 2009). However, the mechanism of T-2 toxin in KBD cartilage damages as well as the role of selenium in counteracting the process remained unclear.

Our previous study showed the synthesis and characterization of novel selenium chondroitin sulfate (SeCS) nanoparticles. SeCS nanoparticles were obtained by ultrasonic and dialysis method. Characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and transmission electron microscopy (TEM), SeCS was found to form nanoparticles in distilled water through self-aggregation progress. The SeCS nanoparticles with sizes of 30–200 nm showed selenium entrapment efficiency of about 10.1 %. The antitoxin capacity of SeCS nanoparticles was demonstrated through MTT and apoptosis assays in vitro. SeCS nanoparticles showed a potential for the treatment for KBD in the preliminary studies (Han et al. 2012, 2013a). In order to understand how the SeCS nanoparticles functioned in the alleviation of chondrocyte apoptosis during cell culture with T-2 toxin, we compared the expression of certain mRNAs and proteins of JNK and p38 signal transduction pathways.

Materials and methods

Chondrocyte isolation and cultures

Samples of articular cartilage were collected from a total of 6 KBD patients (3 males and 3 females, 50–62 years of age) who were diagnosed as the third degree of KBD, based on the diagnosis criteria of Kashin–Beck disease in China (Guo 2001). The study was approved by the Human Ethics Committee, Medical School of Xi'an Jiaotong University. All donors provided a written informed consent for participation in the study. The health status of the samples was examined to exclude genetic bone and cartilage diseases, osteoarthritis (OA) and rheumatoid arthritis (RA).

Within 2 h after surgery, the cartilage tissue was collected and washed with phosphate-buffered saline (PBS) three times and then cut into small pieces and incubated at 37 °C with trypsin for 10 min. After removing the trypsin solution, the cartilage was digested with type II collagenase using 1 ml of digestion solution per 100 mg tissue (Gibco, Grand Island, NY, USA) at 37 °C. Every 3 h, the chondrocytes were collected by removing undigested cartilage fragments through the gauze and centrifugation. We repeated the protocol three times until we got enough chondrocytes. Chondrocytes were plated in cell culture flasks in DMEM/F12 (1:1) (HyClone, Thermo Scientific, Logan, UT, USA), supplemented with 10 % fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and maintained in a humidified atmosphere at 5 % CO_2 and 37 °C. The medium was replaced two or three times a week according to the cell growth state. Primary cells were used in all experiments. The chondrocytes from individual donors were kept strictly separated in all experiments.

Inhibition effect of T-2 toxin on cellular proliferation by MTT assay

Cells (1×10^4/well) were seeded into 96-well plates and incubated under the above chondrocyte culture condition. After 24 h of incubation, DMEM medium with 0–500 ng/ml T-2 toxin was added. The measurements of viability were taken after 24 h of incubation for 5 days. Everyday, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Amresco, USA) was used and the absorbance of each well at 490 nm was recorded using a 96-well plate reader (Bio-Rad Laboratories). Chondrocytes from three donors were used, and this in vitro experiment was performed in triplets.

The effect of SeCS on the T-2-toxin-inhibited cellular proliferation by MTT assay

Cells (1×10^4/well) were seeded into 96-well plates and incubated overnight. The next day, SeCS

nanoparticles were dispersed by DMEM medium to form a serial of solutions with 0–1,000 ng/ml SeCS concentrations. Meanwhile, the above SeCS solutions were used to culture the chondrocytes, and 30 min later, the chosen concentration of T-2 toxin (based on the MTT study of inhibition effect of T-2 toxin on cellular proliferation) was added. Finally, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Amresco, USA) was used and the absorbance of each well at 490 nm was recorded by a 96-well plate reader (Bio-Rad Laboratories).

The concentration influence on SeCS aggregation

The aggregation of the SeCS nanospheres was characterized using zeta potential–particle size analyzer (Nano-ZS, England). SeCS nanospheres were dispersed in distilled water with concentrations of 0–1,000 ng/ml, ultrasonicated for 1 min and then transferred into cuvette and observed by Nano-ZS.

The function of JNK and p38 pathways in the inhibition effect of SeCS nanoparticles on T-2-toxin-induced apoptosis

Chondrocytes were cultured in 50-ml cell culture flasks (BD, USA) and divided into 3 groups: (1) control, (2) SeCS and T-2 toxin and (3) T-2 toxin alone. Meanwhile, SeCS were added 30 min before T-2 toxin. Finally, flow cytometry (FCM) was used to analyze the early apoptosis rates; DAPI staining was used to show the apoptotic morphological changes; total RNA and protein were extracted, and then, real-time quantitative polymerase chain reaction (qPCR) and western blotting analysis were used to study the changes in JNK, p38 and ATF2 gene and protein levels.

Early apoptosis rates by FCM

Three days later, the apoptosis rates were determined by Annexin V-FITC Apoptosis Detection kit according to the manufacturers' instructions (KenGEN, Nanjing, China). For the analysis, the cells were detached from the culture plate with trypsin and harvested by centrifugation, washed twice with PBS and dispersed in 400 μl of binding buffer at a concentration of at least 1×10^6 cells/ml. Then, 4 μl of Annexin V-FITC and 4 μl of propidium iodide were added. The solution was incubated in the dark for 15 min before being transferred into a 5-ml flow cytometry tube. Within an hour, quantitative analysis of apoptotic level was performed using a flow cytometer (Becton–Dickinson, Mountain View, CA, USA). The percentage of apoptotic cells in 10,000 cells was determined. All these experiments were performed in triplicate.

Apoptotic morphological changes by DAPI staining

Apoptotic morphological changes in the nuclear chromatin of cells were detected by DAPI staining. Chondrocytes were seeded on sterile glass coverslips placed in 6-well dishes. The next day, the chondrocytes were intervened according to the divided three groups (control, SeCS + T-2 toxin and T-2 toxin alone). Three days later, cells were washed with PBS and fixed with 4 % paraformaldehyde for 10 min, followed by incubation with DAPI staining solution for 10 min (Beyotime, Haimen, China). After three washes with PBS, the cells were viewed under a fluorescence microscope (Olympus, IX-70, Japan).

RNA extraction and qPCR analysis

Total RNAs were extracted using RNAfast200 kit (Fastagen, Shanghai, China), tested for quality and concentration by a NanoDrop spectrophotometer (Thermo Scientific, USA) and then subjected to reverse transcription using RevertAidTM First Strand cDNA Synthesis kit (Fermentas, MBI, Vilnius, Lithuania) by Eppendorf gradient type mastercycler (Eppendorf, Hamburg, Germany). Reverse transcription products were used for quantitative real-time PCR analysis performed with iQTM5 Real-Time PCR Detection Systems device (Bio-Rad, Philadelphia, PA, USA) using BioEasy SYBR Green I Real-Time PCR Kit (Bioer, Hangzhou, China), with oligonucleotide pairs specific for human JNK, p38, ATF2 and GAPDH with the following cycling conditions: 94 °C for 2 min, 94 °C for 10 s, 58 °C for 30 s and 72 °C for 30 s for 40 cycles, followed by a melting curve analysis. The forward and reverse primer pairs designed to generate a 102-bp fragment of JNK, a 107-bp fragment of p38, a 104-bp fragment of ATF2 and a 226-bp fragment of GAPDH (an internal control) are presented in Table 1.

Table 1 The primers used for the real-time reverse transcription PCR

Gene	Forward primer	Reverse primer
JNK (NM_002750)	5′-CAA GCA CCT TCA TTC TGC TG-3′	5′-GCC AGA CCG AAG TCA AGA AT-3′
p38 (NM_001315)	5′-CGA GCG TTA CCA GAA CCT GT-3′	5′-TGG AGA GCT TCT TCA CTG CC-3′
ATF2 (NM_001880)	5′-GGT GCT TTG TAA ACA CGG CT-3′	5′-GCA GTC CTT TCT CAA GTT TCC-3′
GAPDH	5′-GAA GGT GAA GGT CGG AGT C-3′	5′-GAA GAT GGT GAT GGG ATT TC-3′

Western blotting analysis

The proteins were extracted with RIPA Cell Lysis Solution (Beyotime, Jiangsu, China) supplemented with PMSF and PhosSTOP alkaline phosphatase inhibitors (Hoffmann-La Roche Ltd, Basel, Switzerland). Protein concentration was assayed to adjust an equivalent loading dose of 50 μg. The antihuman p-JNK, p-p38, ATF2, and p-ATF2 antibodies were purchased from Bioworld Technology (St. Louis Park, MN, USA; Cat No: BS4763, BS4766, BS1021, BS4018). The immunoreactive protein was visualized using an enhanced chemiluminescence detection kit (ECL, Thermo Scientific, USA). To show equal loading of the protein samples, β-actin was used as an internal control (Han et al. 2013b).

Statistical analysis

Every sample was studied in triplicate. Data are presented as mean ± SD. Comparisons between the groups were carried out using the Student's two-tailed *t* test. *P* values less than or equal to 0.05 were considered significant.

Results

The inhibition of T-2 toxin on the cellular proliferation

The MTT results of chondrocyte apoptosis caused by T-2 toxin exposure are shown in Fig. 1a. T-2 toxin could induce a time-dependent and dose-dependent inhibition of cellular proliferation in chondrocytes. While in the first day, the inhibition role of T-2 toxin was not demonstrated; instead, a promoting effect on chondrocytes' growth could be seen. From the second day, the chondrocytes proliferation was inhibited by T-2 toxin at concentrations of 2–500 ng/ml. After 3 days of exposure to every concentration of T-2 toxin, the cellular proliferation in every group decreased as well, and the inhibitory effects of T-2 toxin peaked after 5 days of exposure to T-2 toxin.

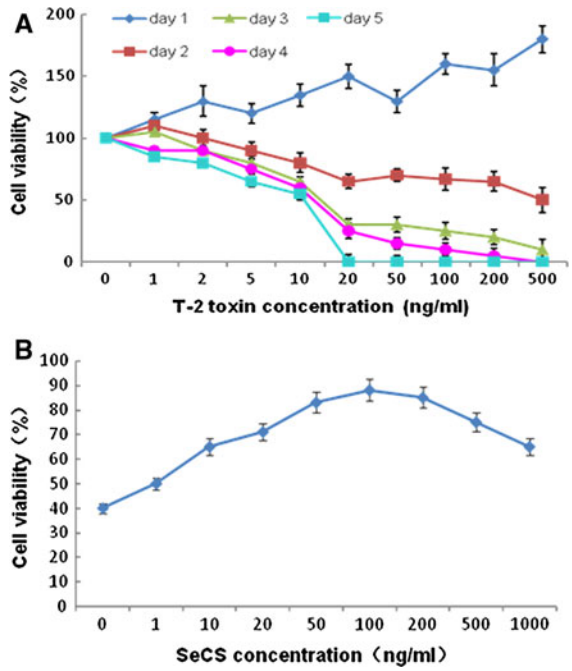

Fig. 1 **a** MTT results of T-2 toxin on the proliferation of KBD chondrocytes. **b** MTT results of SeCS on the T-2-toxin-inhibited cellular proliferation

The effect of SeCS on T-2-toxin-inhibited cellular proliferation

Based on the above study, 20 ng/ml was the chosen concentration for T-2 toxin in the following experiments. As shown in Fig. 1b, the SeCS inhibited the T-2-toxin-induced chondrocyte apoptosis (horizontal axis represented the concentration of SeCS nanoparticles) and as a result increased the cell viability. When the concentrations of SeCS nanoparticles were at 50–200 ng/ml, SeCS could effectively inhibited the apoptotic effect of T-2 toxin on chondrocyte damages

(cell viability >80 %). As the concentration increased, from 500 to 1,000 ng/ml, the inhibitory effect of SeCS on T-2 toxin showed a slight decline, and the cell viability was about 75 and 65 %, respectively.

The concentration influence on SeCS aggregation

The changes in the SeCS concentrations could alter the distribution of the nanosphere size. The size distribution of SeCS nanospheres increased with the increase in SeCS concentrations. The average size of SeCS (20 ng/ml) was about 40 nm with the polydispersity index (PDI) of about 0.03, and when the concentrations of SeCS were at 100 ng/ml, the average size and PDI were about 60 nm and 0.05. The results indicated that the nanospheres showed relative uniform particle size distribution at the above concentrations. However, the average size of the nanoparticles was 150 nm, and the PDI was about 0.18 when the SeCS concentration was at 500 ng/ml, which showed the aggregates of nanoparticles.

The effect of SeCS nanoparticles on the chondrocyte apoptosis cultured with T-2 toxin

The concentration at 20 ng/ml was chosen as the effective dose of T-2 to induce chondrocyte apoptosis, and the effective inhibition dose of SeCS for the prevention of T-2 toxin was 100 ng/ml based on the MTT study. Meanwhile, SeCS was added 30 min before T-2 toxin was added.

By FCM analysis, the early apoptosis rate of control group was 4.5 ± 1.1 %, while for T-2 toxin group, the early apoptosis rate was much higher with 21.6 ± 1.3 % ($P < 0.01$). The addition of SeCS could significantly decrease the T-2-toxin-induced apoptosis rate from 21.6 ± 1.3 to 8.4 ± 1.2 % ($P < 0.05$) (Han et al. 2013a).

As shown in Fig. 2, DAPI-stained nuclei from chondrocytes of control group did not show any cytoplasmic signs of apoptotic cell death. In contrast, KBD nuclei with T-2 toxin alone were less, smaller and brighter than that in control group, and nuclear fragmentation and condensation were evident. While for the group of T-2 toxin and SeCS, the apoptosis was alleviated to some extent, which was accordance with the results of FCM analysis.

Figure 3 showed the results of mRNA expression. The expression levels of the p38, JNK and ATF2 mRNAs were higher in the T-2 toxin group than the control group with about 3.5 ± 0.2-fold, 3.4 ± 0.7-fold and 4.3 ± 0.8-fold, respectively. While for the SeCS, we found that the JNK, p38 and ATF2 mRNA levels were all decreased with about 1.7 ± 0.9-fold, 1.2 ± 0.5-fold and 1.4 ± 0.8-fold when compared with which in the control group, respectively ($P < 0.05$). The results showed that the addition of SeCS could decrease the mRNA expression of p38, JNK and ATF2 significantly.

Western blotting results were shown in Fig. 4, and T-2 toxin treatment for 24 h enhanced the phosphorylation of p38, JNK ($P < 0.05$) and ATF2 ($P < 0.01$), and the total protein level of ATF2 ($P < 0.01$). While for the SeCS group, the addition of SeCS could not only inhibit the phosphate of JNK ($P < 0.05$) and p38 proteins, but also decrease the expression of total ATF2 protein ($P < 0.05$) and its phosphate type ($P < 0.05$) when compared with T-2 toxin group. Thus, the down-expression of p-ATF2 by SeCS could be the result of down-regulate of p38 and ATF2 mRNAs and their phosphate protein types.

Discussion

In this paper, we presented the evidences that T-2 toxin induces apoptosis in KBD chondrocytes in vitro. The p38, JNK and ATF2 proteins were participants in this process. Furthermore, we showed that SeCS nanoparticles could partially block the T-2-toxin-induced apoptosis; meanwhile, the down-regulation of ATF2 mRNAs and proteins by the down-regulation of JNK and p38 mRNAs and proteins involves in the inhibition function of SeCS against T-2 toxin.

This investigation demonstrated that T-2 toxin significantly induced a loss of cell viability in KBD chondrocyte cultures in a concentration- and time-dependent manner. The loss of cell viability caused by cell toxic injury might involve cell apoptosis. SeCS could inhibit the apoptosis induced by T-2 toxin. However, in the study of inhibition effect of SeCS on T-2-toxin-inhibited cellular proliferation, the cell viability dropped with the increase in concentration of SeCS. We found that the concentration of SeCS smaller than 500 ng/ml did not generate the particle aggregates, and SeCS nanoparticles at the concentration of

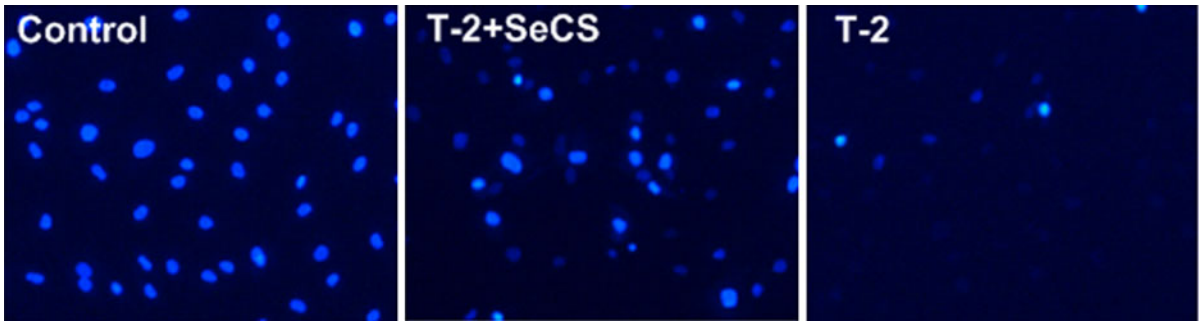

Fig. 2 Apoptotic morphological changes in the nuclear chromatin of cells were detected by DAPI staining (×200). In control groups, the majority of cells had uniformly stained nuclei, in T-2 toxin groups, cell became less, the remained cells showed fragmentation and condensation, while in T-2 and SeCS groups, the apoptotic morphology changes were reduced to some extent)

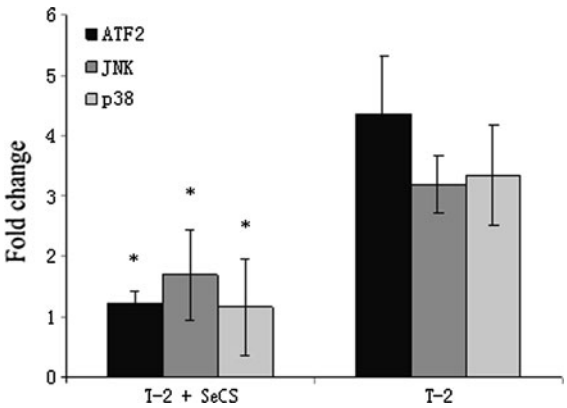

Fig. 3 qPCR of ATF2, JNK and p38 in chondrocytes of different treatment normalized to GAPDH and normalized to control. SeCS decreased the fold changes of ATF2, JNK and p38 to 1.7 ± 0.9-fold, 1.2 ± 0.5-fold and 1.4 ± 0.8-fold, respectively, $P < 0.05$

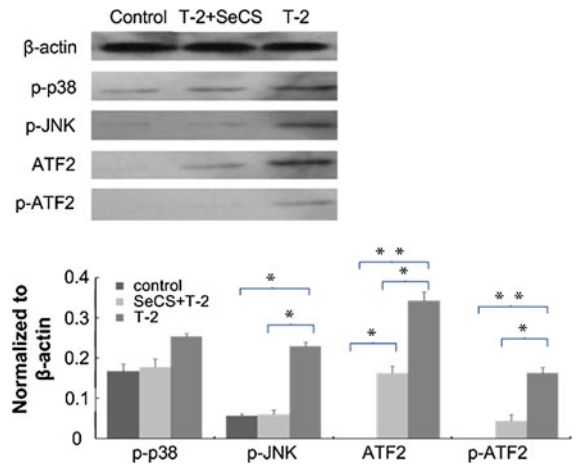

Fig. 4 Western blotting of p-p38, p-JNK, ATF2 and p-ATF2 in chondrocytes with different treatments. (The levels of p-p38, p-JNK, ATF2 and p-ATF2 were lower in chondrocytes cultured with SeCS, and p-ATF2 was more likely to be influenced by SeCS.) $*P < 0.05$; $**P < 0.01$

500 ng/ml started to aggregate heavily with the increase in concentration. It is reported that aggregated nanoparticles are less internalized into the cells than non-aggregated particle (Chithrani et al. 2006), which could explain that the inhibition effect of SeCS was alleviated due to the particles aggregated with the high concentration.

Under our experimental conditions, the levels of p38, JNK and ATF2 mRNAs and proteins were up-regulated, which indicated the loss of cell viability after the treatment of chondrocyte with T-2 toxin was due mainly to the induction of apoptosis. Comparison of changes in JNK, p38 and ATF2 mRNA and their protein levels during the induction process suggested that the increase in ATF2 protein expression was modulated via an obvious transcriptional up-regulation of JNK mRNA consistent with p38 mRNA in T-2 toxin group. By striking contrast, ATF2 protein levels in SeCS group decreased by the down-regulation of p38 mRNA and JNK mRNA, accordingly with the decrease in total p38 and JNK protein levels as well. Furthermore, the findings that the phosphate types of p38, JNK and ATF2 played an important role in the T-2-toxin-induced apoptosis strongly suggested that ATF2 expression increases via a post-transcriptional control mechanism.

In our previous study, the up-regulated levels of ATF2 mRNA and protein were observed in the cartilage of KBD patients (Han et al. 2013b). ATF2 is a member of a family of genes that regulated the

apoptosis process (Raman et al. 2007). Typically, it binds to the cAMP-responsive element (CRE), a sequence present in many cellular promoters. However, depending on the heterodimeric partner, ATF2 binds to different response elements on target genes. ATF2 is activated by stress kinases, including JNK and p38. ATF2 is implicated in transcriptional regulation of immediate early genes, which regulate stress and DNA damage responses and cell cycle control under normal growth conditions (Gupta et al. 1995). Many drugs were currently being tested for their ability to inhibit cell proliferation and induce apoptosis through various pathways including ATF2 (Kumar et al. 2003).

Many studies on selenium have revealed that the alteration of selenium–enzyme activities triggers apoptosis (Bellinger et al. 2009). In the present study, when incubated with T-2 toxin, SeCS could partly block the T-2-toxin-induced apoptosis in chondrocyte and result in the decline of p38, JNK and ATF2 proteins as well as their phosphate types through decreasing the level of their mRNAs. The phenomenon that the treatment of SeCS against T-2 toxin could affect the apoptosis process was difficult to be explained clearly, and maybe it was related to the function of selenium. We will pay further attention in later experiments about the SeCS.

In this paper, the possible mechanism of T-2 toxin and SeCS on KBD chondrocytes was studied. In summary, our results showed that T-2 toxin could induce apoptosis in cultured KBD chondrocytes. Meanwhile, JNK and p38 pathways played an important role in the apoptosis process. Furthermore, SeCS could partly block T-2-toxin-induced apoptosis in chondrocytes via JNK and p38 pathways, and this might result from its effective down-regulation of ATF2 expression level. The capability in alleviating chondrocyte apoptosis by T-2 toxin suggested that SeCS might be provided as a candidate for the prevention and treatment for KBD.

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Conflict of interest The authors declare that they have no competing interests.

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