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T-2 toxin-induced apoptosis involving Fas, p53, Bcl-xL, Bcl-2, Bax and caspase-3 signaling pathways in human chondrocytes*

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Abstract: Objective: To investigate the effects of T-2 toxin on expressions of Fas, p53, Bcl-xL, Bcl-2, Bax and caspase-3 on human chondrocytes. Methods: Human chondrocytes were treated with T-2 toxin (1~20 ng/ml) for 5 d. Fas, p53 and other apoptosis-related proteins such as Bax, Bcl-2, Bcl-xL, caspase-3 were determined by Western blot analysis and their mRNA expressions were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Results: Increases in Fas, p53 and the pro-apoptotic factor Bax protein and mRNA expressions and a decrease of the anti-apoptotic factor Bcl-xL were observed in a dose-dependent manner after exposures to 1~20 ng/ml T-2 toxin, while the expression of the anti-apoptotic factor Bcl-2 was unchanged. Meanwhile, T-2 toxin could also up-regulate the expressions of both pro-caspase-3 and caspase-3 in a dose-dependent manner. Conclusion: These data suggest a possible underlying molecular mechanism for T-2 toxin to induce the apoptosis signaling pathway in human chondrocytes by regulation of apoptosis-related proteins.

Key words: Apoptosis, Apoptosis-related proteins, Chondrocyte, T-2 toxin
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INTRODUCTION

T-2 toxin is one of the mycotoxins, a group of type A trichothecenes produced by several fungal genera including *Fusarium* species, and is detected in a number of field crops (wheat, maize, barley, oats, etc.) and processed grains (malt, beer, bread, etc.). T-2 toxin has been found to contaminate human foods, animal foods and agricultural products, which has been reported in many parts of the world (WHO, 1990). Although it is known that T-2 toxin induces lipid peroxidation, protein synthesis inhibition by interaction with ribosomes, DNA synthesis inhibition (Leal *et al*., 1999; Thompson and Wannemacher, 1990), and apoptosis by directly affecting cell membrane damage or activation of caspase-3 (Minervini *et al*., 2005; Ueno *et al*., 1995), the mechanism of T-2 toxin-induced toxicity is still unknown.

The Fas/Apo-1/CD95 molecule is a cell-surface receptor that mediates apoptotic signals upon its Fas ligand (FasL) engagement. Fas antigen expression is predominantly a feature of chondrocytes in the superficial and upper mid-zone of articular cartilage. Although the physiological role of its wide constitutive expression has not yet been clarified, Fas has been considered to be one of inducers of chondrocyte apoptosis (Hashimoto *et al*., 1997). The p53 protein has emerged as a key tumor suppressor protein by playing a central role in cellular stress response pathways. Through these pathways, one of its roles is to survey cellular stress and to induce apoptosis. p53 can promote apoptosis by several mechanisms (Hofseth *et al*., 2004). For example, p53 can directly engage each of the major apoptotic pathways in the cell,

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stimulating both death receptor signaling and mitochondrial perturbations, including cytochrome C release (Vousden, 2000). The Bcl-2 family has been shown to be a p53 target. Bax, the pro-apoptotic member, is up-regulated in a number of systems during p53-mediated apoptosis (Martin and Elkon, 2004). On the other hand, Bcl-2, the anti-apoptotic member, is down-regulated during p53-mediated apoptosis (Martin and Elkon, 2004). Recent studies have proposed that the alteration of the Bcl-xL, a pro-apoptotic member of the Bcl-2 family (Muchmore *et al*., 1996), to Bax ratio is one of the important factors to decide the fate of a cell (Gonzalez de Aguilar *et al*., 2000). However, the mechanism of T-2 toxin-induced apoptosis involved in the regulation of the balance between these pro- and anti-apoptotic proteins is not fully clear.

Kashin-Beck disease is an endemic disabling osteoarticular disorder, a particular osteoarthritis, and affects about 2.5 million residents with over 100 millions at risk across China (Statistical Information Center of Heath Ministry of China, 2005). The disease attacks the growth plate of joint cartilage and leads to death of chondrocytes. Chondrocyte apoptosis and death in the middle and deep layers of the affected cartilage are the most important pathological symptoms of Kashin-Beck disease (Allander, 1994; Wang *et al*., 2006). T-2 toxin contamination has been suggested to contribute to the classical features of Kashin-Beck disease in human (Yang, 1995). Experimentally, it was shown that administration of T-2 toxin for 5 weeks to chicks provoked a syndrome with several features very similar to human Kashin-Beck disease (Yang, 1995). Further, apoptosis of chondrocytes can be induced by T-2 toxin in vitro (1~20 ng/ml) (Chen *et al*., 2006).

At present, the molecular modulation mechanism of the T-2 toxin-induced apoptosis remains unclear. Although the ability of T-2 toxin to produce necrosis and depletion of joint cartilages (Yang, 1995) including chondrocyte apoptosis is well documented (Chen *et al*., 2006), little is presently known regarding possible signaling pathways in human chondrocytes by this mycotoxin. Using human chondrocytes, we investigated the mechanism by which T-2 toxin induces apoptotic effects and how these effects are modulated by Fas, p53, some of Bcl-2 family proteins and caspases.

MATERIALS AND METHODS

Chemicals

Crystallized trypsin, collagenase type II and testicular hyaluronidase were purchased from Sigma (St. Louis, MO, USA). T-2 toxin was kindly provided by Prof. Jinsheng Yang and Chuanqing Peng at the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, China. Rabbit anti-Bax, caspase-3, p53, Fas and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies to Bcl-2 and Bcl-xL were from BD Biosciences (BD, CA, USA). Secondary goat anti-rabbit IgG and anti-mouse IgG conjugated with horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. (Bar Harbor, ME, USA). Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and Trizol reagent were purchased from Gibco (Grand Island, NY, USA). RT-PCR kits were products of Fermentas Life Sciences (Lithuania). SuperSignal Ultra Western blot chemiluminescence system was products of Pierce Life Science (Rockford, IL, USA). Polyvinylidene fluoride (PVDF) membrane was from Millipore Life Science (Arlington Heights, IL, USA). All other chemicals were of the highest grade available from commercial sources.

Chondrocyte isolation and culture

Fetal articular cartilage was obtained from informed donors who had to stop pregnancy and abort through therapy as approved by the Ethical Committee, School of Medicine, Xi'an Jiaotong University, Xi'an, China. Slices of cartilage were aseptically dissected and chondrocytes were obtained by sequential digestions with hyaluronidase, crystallized trypsin and collagenase type II as previously described (Wei *et al*., 1987). Cells were cultured to confluence at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2 in complete medium (DMEM/F12 with 15% (v/v) fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin). All experiments were performed using first passage chondrocytes to minimize any problems of de-differentiation.

Culture of chondrocytes with T-2 toxin

First passage chondrocytes were used. After 24 h

of culture, DMEM/F12 was replaced by the same medium containing various concentrations (0, 1, 10 and 20 ng/ml) of T-2 toxin. The concentrations of T-2 toxin used in this study were chosen based on results from previously reported 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Chen *et al*., 2006). Cultures were incubated from 1 to 5 d at 37 °C under 5% (v/v) CO_2.

Cell viability assay by MTT

Cells were seeded onto individual 96-well plates (5×10^3 well^{-1}). After 24 h of culture, complete medium (DMEM/F12 with 15% (v/v) fetal calf serum) with or without T-2 toxin (1~8000 ng/ml) was added and incubated for 1 to 5 d. Then, 10 μl MTT at a final concentration of 500 μg/ml was added into the culture medium. After 4 h, the medium containing MTT was aspirated and replaced by 100 μl dimethyl sulfoxide (DMSO) for 0.5 h to prepare cell lysate. The optical density (OD) was then measured in an enzyme linked immunosorbent assay (ELISA) plate reader at 490 nm.

Western blotting

Western blot analyses for Bax, Bcl-2, Bcl-xL, p53, Fas, caspase-3 and β-actin expressions were performed as previously described with minor modifications (Tian *et al*., 2000). Cells were harvested and protein was obtained after total RNA was extracted from cell cultures by using Trizol reagent according to the manufacturer's instructions. Equal amounts of total proteins were subjected to 8% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membrane. After being blocked with Tween 20 Tris-buffered saline (TTBS; 20 mmol/L Tris HCl pH 7.6, 137 mmol/L NaCl, and 0.05% (v/v) Tween 20) containing 5% (w/v) nonfat milk for 1 h at room temperature, the membrane was incubated with primary antibody overnight at 4 °C. After being washed three times for 10 min each in TTBS, the membrane was incubated with an appropriately diluted horseradish peroxidase-labeled secondary antibody (1:5000) in blotting buffer for 1 h at room temperature. The membrane was washed three times, then reacted with SuperSignal Ultra Western blot chemiluminescence system according to the manufacturer's protocol, and finally subjected to autoradiography. The strength of the signal was analyzed by using densitometry, and the results were expressed as arbitrary units. Protein levels were standardized by comparison with anti-β-actin antibody.

RNA extraction and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cell cultures using Trizol reagent according to the manufacturer's instructions, and the RNA concentration was determined spectrophotometrically at 260 nm. RNA was diluted to 2 μg/ml with water pretreated with diethylpyrocarbonate, containing 1 U/μl RNase inhibitor and 3 mmol/L dithiothreitol (DTT). The following substracts were placed into a tube: 1 μl RNA (containing 2 μg total RNA), 1 μl oligo(dT) 12~18, 1 μl reverse transcriptase, 2 μl 10 mmol/L deoxynucleoside triphosphate (dNTP), 2 μl 0.1 mol/L DTT, 4 μl 5× buffer, and sterilized distilled water up to a total volume of 20 μl. The mixture was incubated at 37 °C for 60 min. After reverse transcription, the sample was heated at 95 °C for 5 min to denaturate the reverse transcriptase, and then stored at −30 °C for PCR.

Table 1 shows the synthetic oligonucleotide primers used for RT-PCR and the product sizes. All primers were synthesized by Boya Biotechnology Co., Ltd. (China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize all samples for potential variations in mRNA content. Single-stranded cDNA was used as a template for PCR amplification by using Taq polymerase. After 2 min at 94 °C, amplifications were preformed for all samples under the following conditions: 94 °C for 30 s, 52 °C for 60 s, and 72 °C for 2 min for 40 cycles, with a final incubation at 72 °C for 7 min. Following PCR, 5 μl samples aliquots were electrophoresed on a 2% (w/v) agarose gel for 35 min at 120 V, and then stained with ethidium bromide and photographed. Densitometry was performed using WorkLab software. The data were recorded as the ratio of sample to internal standard.

Statistical analysis

All data were expressed as mean±*SD*. Means of different groups were compared by using analysis of variance (ANOVA) with post-hoc testing. A P value less than 0.05 was considered statistically significant.

Table 1 Primers for semiquantitative RT-PCR

Apoptosis modulator	Upstream primer (5′−3′)	Downstream primer (5′−3′)	Size (bp)
Bax	ACCAAGAAGCTGAGCGAGTGT	ACAAACATGGTCACGGTCTGC	332
Bcl-2	AGATGTCCAGCCAGCTGCAC	TGTTGACTTCACTTGTGGCC	388
Bcl-xL	GGAGCTGGTGGTTGACTTTCT	CCGGAAGAGTTCATTCACTAC	379
p53	CAGCCAAGTCTGTGACTTGCACGTAC	CTATGTCGAAAAGTGTTTCTGTCATC	293
Fas	CAAGTGACTGACATCAACTCC	CCTTGGGTTTTCCTTTCTGTGC	549
Caspase-3	CTCGGTCTGGTACAGATGTCGATG	GGTTAACCCGGGTAAGAATGTGCA	540
GAPDH	TTACTCCTTGGAGGCCATGTGGGCC	ACTGCCACCCAGAAGACTGTGGATGG	465

RESULTS

Effect of T-2 toxin on cellular proliferation of chondroctyes

Effects of T-2 toxin on chondrocyte viability were quantified with the MTT reduction assay (Fig.1). T-2 toxin induced a time- and dose-dependent inhibition of cellular proliferation in chondrocytes. All the concentrations (1~8000 ng/ml) significantly decreased the cell viability after 3 d of exposure to T-2 toxin. Maximum suppression of cell viability was evident at Day 5. At Day 5, 10 ng/ml of T-2 toxin produced a 50% reduction in cell viability; this concentration, as well as 1 and 20 ng/ml, was therefore selected for subsequence studies.

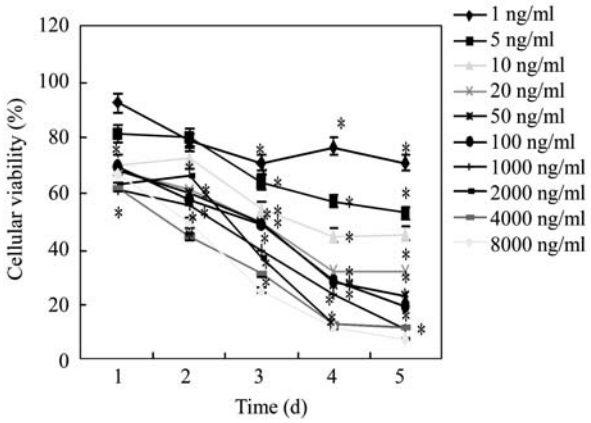

Fig.1 Effects of different concentrations of T-2 toxin on the cellular viability of chondrocytes estimated by MTT reduction. Cells were incubated in absence or presence of T-2 toxin at different concentrations for different time periods (1~5 d). MTT reduction was then calculated to indicate cellular proliferation. Results are expressed as mean±*SD* of six independent determinations. *$P<0.05$, significantly different from control values

Protein expression on T-2 toxin-induced apoptotic chondrocytes

As prototypes of the regulatory proteins of apoptosis, we examined the levels of the Bcl-2, Bcl-xL, p53, caspase-3, Fas and Bax proteins in chondrocytes. Cells were treated with T-2 toxin (1, 10 and 20 ng/ml) for 5 d followed by Western blot analysis for those proteins (Fig.2). The results show that Bax protein levels increased in a dose-dependent manner in T-2 toxin-treated cells, whereas Bcl-xL levels decreased slightly and Bcl-2 remained unchanged, resulting in an increase of the Bax/Bcl-2 and Bax/Bcl-xL ratios (Fig.3). Moreover, increases in Fas and p53 proteins were also observed in a dose-dependent manner following treatment with T-2 toxin (10 and 20 ng/ml), as shown in Fig.4.

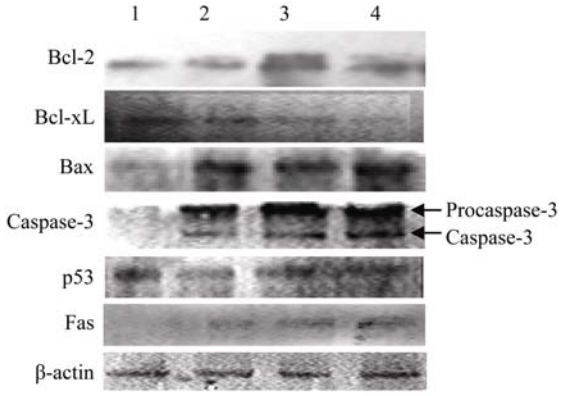

Fig.2 Western blotting analyses of T-2 toxin-induced expressions of Bcl-2, Bcl-xL, Bax, caspase-3, p53 and Fas proteins in human chondrocytes. The intensities of Bcl-2, Bcl-xL, Bax, caspase-3, p53 and Fas bands were normalized with respect to the intensities of β-actin bands detected on the same blots

Lane 1: Untreated control; Lanes 2~4: Treatment with 1, 10 and 20 ng/ml of T-2 toxin for 5 d, respectively

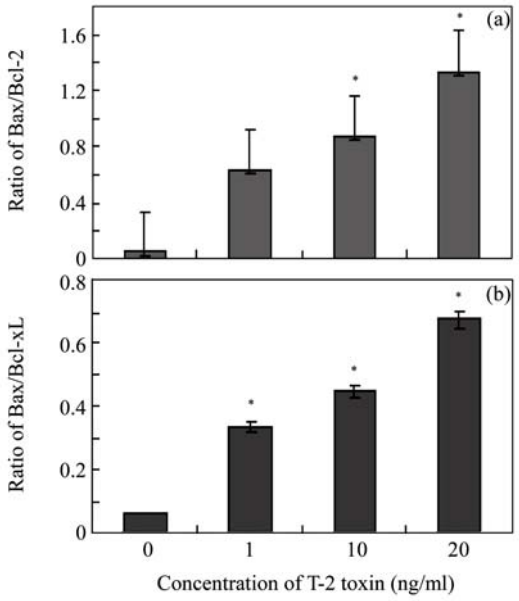

Fig.3 Ratios of (a) Bax/Bcl-2 and (b) Bax/Bcl-xL
*P<0.05 vs control

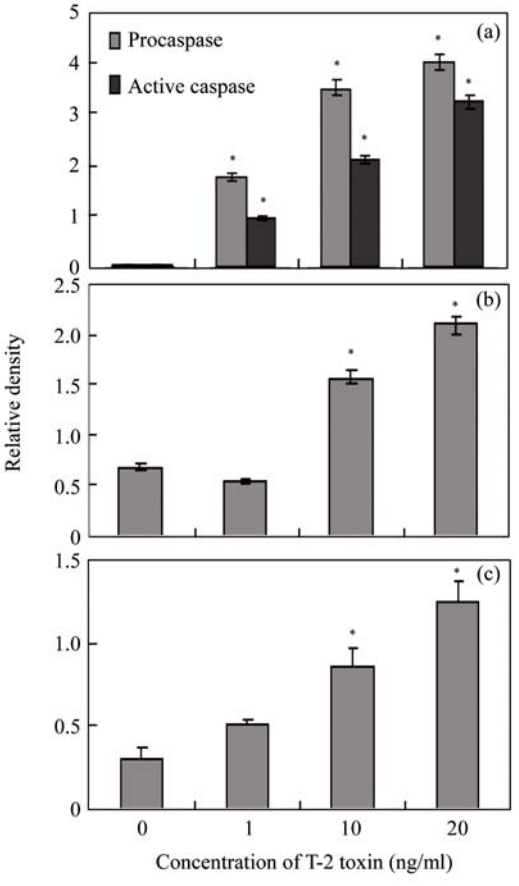

Fig.4 The levels of (a) caspase-3, (b) p53 and (c) Fas quantified by densitometric analysis of the three autoradiographs
*P<0.05 vs control

To determine whether the signature apoptotic effecter caspase-3 was activated by T-2 toxin, caspase-3 protein was examined. The results show that both procaspase-3 and caspase-3 proteins were increased in a dose-dependent manner following treatment with T-2 toxin (1, 10 and 20 ng/ml) for 5 d (Fig.4), suggesting that capase-3 is a downstream mediator in T-2 toxin-induced chondrocyte apoptosis.

RT-PCR detection of Bax, Bcl-xL, Bcl-2, p53, Fas and caspase-3 mRNA of T-2 toxin-induced apoptotic chondrocytes

To determine whether regulation of the Bax, Bcl-xL, p53, Fas and caspase-3 proteins was mediated via modulating expression of their respective mRNA, we analyzed the expression of the transcripts encoding these proteins by semiquantitative RT-PCR procedures. The Bax, Bcl-xL, p53, Fas and caspase-3 specific products generated by RT-PCR were normalized with respected to GAPDH RT-PCR bands generated by amplification of the same cDNAs (Fig.5). Fig.6 shows that the chondrocytes express mRNA for the Bax, Bcl-xL, p53, Fas and caspase-3 at the 5th day as mediator of apoptosis, in agreement with their protein levels.

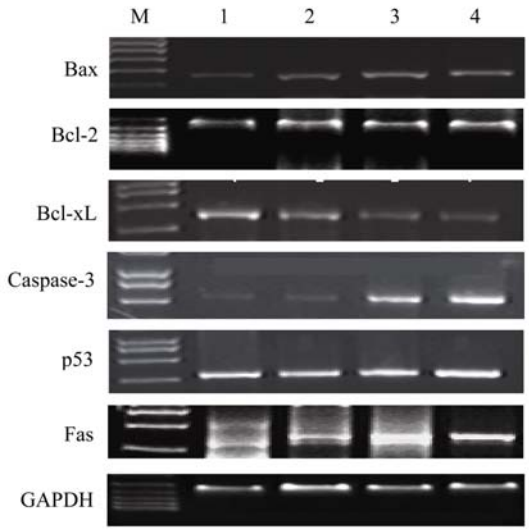

Fig.5 RT-PCR analysis of T-2 toxin-induced expressions of Bcl-2, Bcl-xL, Bax, caspase-3, p53 and Fas mRNA in human chondrocytes. Results were normalized according to GAPDH levels (n=3)
Lane 1: Untreated control; Lanes 2~4: Treatment with 1, 10 and 20 ng/ml of T-2 toxin for 5 d, respectively

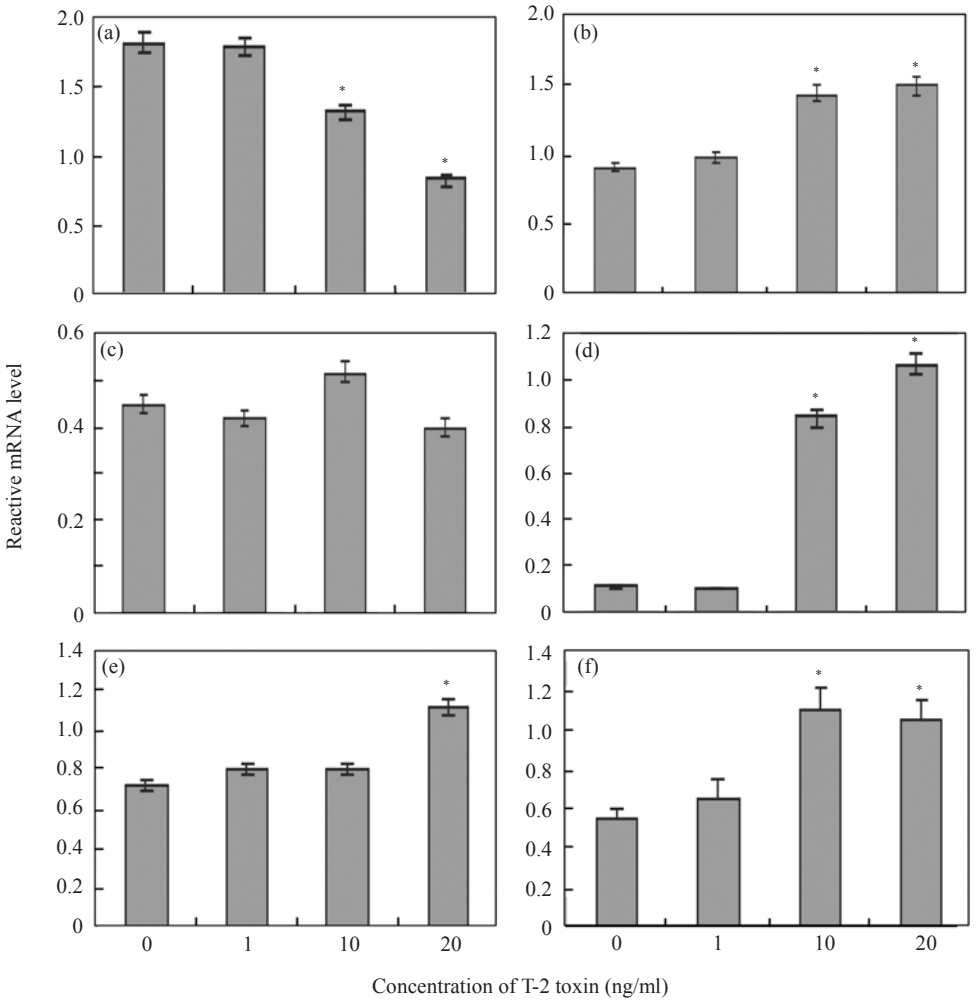

Fig.6 Relative mRNA levels of (a) Bcl-xL, (b) Bax (c) Bcl-2, (d) caspase-3, (e) p53 and (f) Fas
*$P<0.05$ vs control

DISCUSSION

T-2 toxin belongs to a group of mycotoxins that are widely encountered as natural contaminants known to elicit toxic responses in human cell lines in vivo, such as inhibition of DNA, RNA and protein syntheses in several cellular systems (Ji *et al*., 1994). A number of mechanisms of cell growth inhibition and apoptosis induction by T-2 toxin have been demonstrated (Holme *et al*., 2003). Our previous results show that T-2 toxin can inhibit the proliferation of human chondrocytes and induce apoptosis of chondrocytes (Chen *et al*., 2006). However, the signaling pathways governing apoptosis in mammalian cells are complex and the pro- and anti-apoptotic variations regulate cell survival change according to cell type (Cory and Adams, 2002). The cellular and molecular mechanisms underlying T-2 toxin-induced apoptosis in chondrocytes have not been well defined. Therefore, in this study, we focused on characterizing the T-2 toxin-induced apoptotic signaling pathways in human chondrocytes.

Studies of Fas/FasL expression in isolated human chondrocytes indicate that agonistic Fas antibody is able to induce apoptosis in approximately 20% of chondrocytes in vitro (Hashimoto *et al*., 1997). The expression of functional Fas antigen on osteoarthritis chondrocytes has shown that these cells undergo Fas-mediated apoptosis both in vivo and in vitro (Hashimoto *et al*., 1997). In our present study, we demonstrated that high levels of Fas protein and mRNA were induced by T-2 toxin in chondrocytes,

which suggests that the Fas/FasL apoptotic pathway may also contribute to T-2 toxin-induced apoptosis in chondrocytes.

In a study of the mechanism of action of T-2 toxin on the liver, placenta and fetal liver, it was reported that mechanism of T-2 toxin-induced toxicity in pregnant rats is due to oxidative stress followed by the activation of the mitogen-activated protein kinase (MAPK) pathway, finally inducing apoptosis, and that the c-Jun gene was suggested to play an important role in T-2 toxin-induced apoptosis (Sehata *et al*., 2005). In the present study, we propose another possible underlying molecular mechanism of T-2 toxin-induced chondrocyte apoptosis via the activation of p53 and the regulation of apoptosis-related proteins. p53 plays an important role in mitochondrial membrane stability and promotes apoptosis (Rich *et al*., 2000; Vousden, 2000). p53 is a tightly regulated transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress, such as DNA damage (Evan and Littlewood, 1998). Despite its central role in cell apoptosis, the mechanism of p53-mediated apoptosis after cellular stress remains unclear. It is now clear that a number of phosphorylation sites on p53 are altered after DNA damage, and such phosphorylation events have been shown to result in alterations in p53 that make it more stable and more active (Vousden, 2000; Vogelstein *et al*., 2000). Moreover, current evidence indicates that the mode of action of p53-mediated apoptosis involves transactivation of target genes and direct signaling events that may induce two sets of genes upon stress signals. One set, such as p21/*waf-1* and GADD45, mainly functions in cell growth control, and the other, such as Bax and Bcl-2, acts on apoptosis (Agarwal *et al*., 1998). In the present study, we observed that p53 protein and mRNA were highly expressed in T-2 toxin-induced chondrocytes. The activation of p53 by T-2 toxin would then affect the expression of its downstream effectors, such as the Bcl-2 family proteins.

It is well recognized that the Bcl-2 family proteins are central regulators of apoptosis and that the Bcl-2 family members act like checkpoints through which survival and death signals pass before they determine the fate of the cell (Cory and Adams, 2002). In our study, T-2 toxin was shown to be capable of both down-regulating the anti-apoptotic factor Bcl-xL and up-regulating the pro-apoptotic factor Bax, thereby increasing the Bax/Bcl-xL ratio and disposing to apoptosis. Interestingly, T-2 toxin induced apoptosis with an increased level of p53, which transactivates Bax expression. But in these cells, Bcl-2 levels remained almost unchanged, thereby shifting the Bax/Bcl-2 ratio towards apoptosis. The activation of the p53-mediated apoptotic signaling pathway may play an important role in apoptosis by modulating the Bax/Bcl-2 or Bax/Bcl-xL ratio.

Caspases, represented by a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9. These activated caspases cleave many cellular substrates, ultimately leading to cell death (Earnshaw *et al*., 1999; Wang *et al*., 2007). We found that T-2 toxin could up-regulate caspase-3 protein and mRNA expression in a dose-dependent manner on the human chondrocytes. Thus, T-2 toxin-induced chondrocyte death was accompanied by an increase in the caspase-3, which then stimulates the molecular cascade of apoptosis.

Tumour necrosis factor α (TNF-α) is also one of mediators that induce chondrocyte apoptosis in vitro (Yoshimura *et al*., 2006). In vivo, however, it is likely that TNF-α as one of catabolic cytokines is produced by cartilage in osteoarthritis that could function as effectors of chondrocyte apoptosis (Fernandes *et al*., 2002). Production of TNF-α results in high level of nitric oxide (NO), which in turn induces chondrocyte apoptosis (Clancy *et al*., 2004). Our previous study showed that T-2 toxin increased the production of TNF-α (Li *et al*., 2008). Therefore, TNF-α could play an important role in triggering and modulating of T-2 toxin-induced apoptosis.

Our results suggest that T-2 toxin creates a pro-apoptotic environment by inducing Fas up-regulation on the chondrocyte surface, and then up-regulates p53 proteins, which in turn increases both the Bax/Bcl-2 and the Bax/Bcl-xL ratios, activates caspase-3, and induces apoptosis. Thus, Fas, an apoptosis inducer, and p53, a pleiotropic mediator in Kashin-Beck disease, play a crucial role in chondrocyte death. Previous work from our laboratory has shown that a feature of Kashin-Beck disease is the apoptosis of chondrocytes in the middle layer of cartilage and chondrocyte death in the deep layer of

affected cartilage, and that expressions of Bcl-2, Bax and Fas changed in articular cartilage in Kashin-Beck disease (Wang *et al*., 2006). Therefore, this phenomenon of T-2 toxin-induced apoptosis and its signal pathway in vitro might occur and be significant in the progression of Kashin-Beck disease, and ultimately lead to pathological cartilage destruction in Kashin-Beck disease. It is now established that Bcl-2, Bax, Bcl-xL, p53 and Fas are expressed on chondrocytes, and that alterations in the susceptibility of the cells to T-2 toxin-induced cell death have been strongly implicated in the pathogenesis of Kashin-Beck disease. Results of this study may provide an explanation for effects of T-2 toxin on pathogenesis of Kashin-Beck disease.

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