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# T-2 toxin enhances catabolic activity of hypertrophic chondrocytes through ROS-NF-κB-HIF-2α pathway

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ABSTRACT

T-2 toxin (T-2), one of the most important and toxic trichothecene mycotoxins, can cause many medical problems, such as diarrhea, nervous disorders, immunodepression and death, and is also believed as an etiological factor of Kashin-Beck disease, an endemic osteochondropathy prevailing in North China. However, the molecular mechanisms underlying T-2 effects on tissue damage remain elusive. We differentiated ATDC5 chondrogenic cells into hypertrophic chondrocytes, and found that T-2 reduced the expression of anabolic genes, and increased the expression of catabolic genes. To uncover the mechanism that T-2 influenced metabolic homeostasis of hypertrophic chondrocytes, we observed that T-2 increased the production of reactive oxygen species (ROS) and the degradation of IκB-α, and up-regulated the expression of hypoxia-induced factor-2α (HIF-2α). Bay11-7085 (an inhibitor of NF-κB pathway) inhibited the up-regulation of HIF-2α, and *N*-acetyl-L-cysteine (a ROS scavenger) inhibited both the decrease of IκB-α and the up-regulation of HIF-2α. Our results demonstrate that ROS-NF-κB-HIF-2α pathway participates in the effects of T-2 on hypertrophic chondrocytes, and HIF-2α plays an important role as a key mediator in this process.



## 1. Introduction

Trichothecene mycotoxins can cause a number of medical problems, such as skin irritation, diarrhea, nervous disorders, cardiovascular alterations, immunodepression, hemostatic derangements and death (Hussein and Brasel, 2001; Pestka et al., 2008; Steyn, 1995). T-2 toxin (T-2), one of the most important and toxic trichothecene mycotoxins, is produced by *Fusarium* which contaminates grain crops during harvest and storage under cold-damp conditions (Li and Pestka, 2008).

T-2 had been believed responsible for a fatal human disease known as "alimentary toxic aleukia" in the USSR during the Second World War (Lutsky et al., 1978). It was also detectable in the sample of "Yellow Rain" which was used as a chemical weapon during the war of Afghanistan and South Asia (Watson et al., 1984). Nowadays, T-2 still jeopardizes human health, and 20% of food samples from 12 European Union countries were detected to have contained it (Schothorst and van Egmond, 2004). Epidemiological data have supported that T-2 exposure may be implicated in the etiology of Kashin-Beck Disease (KBD), an endemic, chronic osteoarthritic disease (Li et al., 2008). Clinically, KBD manifests in arthritic pain, deformed and enlarged joints, shortened fingers, and limited motion of extremity joints, even dwarfism and disability (Guo, 2001). It was reported that the wheat flour of KBD families contained high level of T-2 (range 2.0–1549.4 μg/kg, mean 468.7 μg/kg) (Yang et al., 1995). The basic pathological features of KBD include extracellular matrix (ECM) degradation and cell necrosis, mainly in the growth plate and in the deep layer of articular cartilage (Allander, 1994).

Considering the correlation of T-2 and KBD, some researches have focused on the effects of T-2 on chondrocytes. T-2 can reduce DNA synthesis in rabbit articular chondrocytes (Wright et al., 1987), and induce apoptosis of human chondrocytes (Chen et al., 2008, 2006). T-2 also reduces expression of aggrecan in Zelanian rabbit chondrocytes (Liu et al., 2008), and up-regulates matrix metalloproteinases (MMP-1, MMP-13), α2-macroglobulin (α2-M) and TIMPs (Chen et al., 2011). T-2 disrupts the expression of chondroitin sulfate sulphation motifs in articular cartilage from an animal model of KBD (Li et al., 2011). These researches have provided evidence that T-2 could cause cell apoptosis and ECM degradation in cartilage. However, neither the exact changes of catabolic and

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anabolic activity nor the molecular mechanisms to decipher the metabolic changes of chondrocytes exposed to T-2 are clear.

During endochondral ossification, chondrocytes undergo a successive process of proliferation, pre-hypertrophy, hypertrophy and mineralization. Among pathologic changes of cartilage from KBD patients, a striking feature is that the growth plate chondrocytes especially hypertrophic chondrocytes in the deep portion are impaired (Kolsteren, 1992). Nevertheless, up to now, effects of T-2 on hypertrophic chondrocytes are still lacking. So the objective of the present study is to reveal the role of T-2 in hypertrophic chondrocytes and to unravel the signal pathway involved in this process.

This study suggests that T-2 induces the production of ROS, then activates nuclear factor-κB (NF-κB) pathway, and up-regulates hypoxia-induced factor-2α (HIF-2α) expression. HIF-2α, as a key mediator, transactivates a series of catabolic gene expression in hypertrophic chondrocytes.

## 2. Methods

### 2.1. Cell culture

The murine pre-chondrogenic ATDC5 cells were purchased from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). ATDC5 cells were cultured and maintained in Dulbecco's modified Eagle medium and Ham's F-12 medium (DMEM/F-12) (HyClone, Logan, Utah, USA) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, Utah, USA) at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. Inoculum size of the cells was $8 \times 10^3$ cells/well in a 96-multiwell plate, $2 \times 10^4$ cells/well in a 24-multiwell plate, $4 \times 10^4$ cells/well in a 12-multiwell plate, or $6 \times 10^4$ cells/well in a 6-multiwell plate (Nunc, Roskilde, Denmark). For induction of differentiation, ATDC5 cells were cultured in chondrogenic medium in which DMEM/F-12 contained 5% FBS supplemented with 10 μg/ml human transferrin, $3 \times 10^{-8}$ M sodium selenite and 10 μg/ml bovine insulin (Sigma–Aldrich, St. Louis, MO, USA) (Shukunami et al., 1996), and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. The medium was replaced every other day. Cells were cultured in 96-multiwell plates for MTT assay, in 24-multiwell plates for Alcian blue staining and measurement of ROS production, in 12-multiwell plates for RNA isolation and in 6-multiwell plates for protein extraction. For the inhibitor assay, BAY11-7085 (ALEXIS Biochemicals, San Diego, CA, USA) and *N*-acetyl-L-cysteine (NAC) (Sigma–Aldrich, St. Louis, MO, USA) were used at the concentration of 5 μM and 10 mM, respectively. After treatment with the inhibitors for 30 min, hypertrophic chondrocytes were co-treated with T-2 and the inhibitors for 24 h.

### 2.2. Alcian blue staining

Cells were cultured in 24-well plates. After aspirating culture medium and rinsing twice with PBS, the cells were fixed in 4% paraformaldehyde for 30 min, and then rinsed with 0.1 N HCl once. The cells were stained with 0.1% Alcian blue 8 GS (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 N HCl for 1 h. Lastly, the cells were rinsed, dried and rehydrated with 0.1 N HCl. Results were recorded with a digital camera (Canon, Tokyo, Japan).

### 2.3. Cell viability measured by MTT assay

Cells were incubated with T-2 or inhibitors. At the end of the designed reaction time, the culture medium was replaced by medium containing 0.5 mg/ml MTT (Solarbio, Beijing, China) and the cells were incubated 4 h at 37 °C. The supernatant was discarded and DMSO was used to dissolve the purple crystal in the wells. The absorbance at wavelength of 550 nm was measured by a microplate reader (Thermo Electron Corporation, Vantaa, Finland).

### 2.4. Measurement of ROS production by fluorescent microscopy and flow cytometry

Cells were incubated with different concentrations of T-2 for different time. The supernatant was discarded and the cells were rinsed with PBS for three times. DMEM/F12 containing 10 μM DCDHF was added and the cells were incubated for 30 min at 37 °C under protection from light, and then rinsed with PBS for three times. The cells were observed by using a fluorescent microscopy (Olympus, Tokyo, Japan). For measurement of ROS production by FACS, the cells were digested with trypsin, and re-suspended in PBS. Formation of DCF was monitored at an excitation wavelength of 502 nm and emission wavelength of 523 nm by a FACScanto (Becton Dickinson, Franklin Lakes, NJ, USA). Data were then analyzed by using FlowJo software (Tree Star, Ashland, OR, USA).

### 2.5. RNA isolation and RT-qPCR

Total RNA was isolated with Trizol® Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas life Sciences, St. Leon-Rot, Germany) according to the manufacturer's instructions. Real-time quantitative-polymerase chain reaction (RT-qPCR) was performed by iQ5 (BIO-RAD, Richmond, CA, USA) with SYBR® Premix *Ex Taq*™ II (TaKaRa Bio Inc., Otsu, Japan). The information of primers is depicted in Table 1. Gene expression analyses were performed relative to β-actin and calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### 2.6. Protein extraction and Western blot

Cells were lysed in RIPA solution (Beyotime, Shanghai, China) containing a protease inhibitor cocktail (Roche Diagnostics Ltd., Mannheim, Germany). The concentration of total protein was measured by BCA protein assay kit (Beyotime, Shanghai, China). After degenerating and separating in 10% SDS–PAGE, protein was transferred onto PVDF membranes. The membrane was blocked in a solution of TBS-T containing 5% nonfat dry milk for 1 h at 25 °C with constant agitation, and then was incubated in the primary antibody solution overnight at 4 °C. The primary antibodies were rabbit anti-HIF-2α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:400 dilution, rabbit anti-IκB-α (Bios, Beijing, China) at 1:200 dilution and mouse anti-β-actin (Beyotime, Shanghai, China) at 1:1000 dilution. After washing with TBS-T, the membranes were incubated with the corresponding HRP-conjugated anti-IgG antibody (Beyotime, Shanghai, China) for 1 h at room temperature. The bands were visualized by using the ECL–Western blotting detection reagents (Pierce, Rockford, USA). The product bands were analyzed using GeneSnap software (Syngene, NJ, USA). The relative intensity was calculated by normalizing with β-actin.

### 2.7. Statistics

Quantitative data are represented as mean ± SD from three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) among groups, and then the least significant difference *t* test was employed to analyze the significant differences between groups. The statistical analysis of differences between two experimental groups was performed by Student's *t*-test. Pearson's correlation test was used to evaluate the correlation coefficient. $P < 0.05$ was considered statistically significant.