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Promotion of the articular cartilage proteoglycan degradation by T-2 toxin and selenium protective effect*

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Abstract: Objective: To identify the relationship between T-2 toxin and Kashin-Beck disease (KBD), the effects of T-2 toxin on aggrecan metabolism in human chondrocytes and cartilage were investigated in vitro. Methods: Chondrocytes were isolated from human articular cartilage and cultured in vitro. Hyaluronic acid (HA), soluble CD44 (sCD44), IL-1β and TNF-α levels in supernatants were measured by enzyme-linked immunosorbent assay (ELISA). CD44 content in chondrocyte membrane was determined by flow cytometry (FCM). CD44, hyaluronic acid synthetase-2 (HAS-2) and aggrecanases mRNA levels in chondrocytes were determined using reverse transcription polymerase chain reaction (RT-PCR). Immunocytochemical method was used to investigate expressions of BC-13, 3-B-3(−) and 2-B-6 epitopes in the cartilage reconstructed in vitro. Results: T-2 toxin inhibited CD44, HAS-2, and aggrecan mRNA expressions, but promoted aggrecanase-2 mRNA expression. Meanwhile, CD44 expression was found to be the lowest in the chondrocytes cultured with T-2 toxin and the highest in control plus selenium group. In addition, ELISA results indicated that there were higher sCD44, IL-1β and TNF-α levels in T-2 toxin group. Similarly, higher HA levels were also observed in T-2 toxin group using radioimmunoprecipitation assay (RIPA). Furthermore, using monoclonal antibodies BC-13, 3-B-3 and 2-B-6, strong positive immunostaining was found in the reconstructed cartilage cultured with T-2 toxin, whereas no positive staining or very weak staining was observed in the cartilage cultured without T-2 toxin. Selenium could partly inhibit the effects of T-2 toxin above. Conclusion: T-2 toxin could inhibit aggrecan synthesis, promote aggrecanases and pro-inflammatory cytokines production, and consequently induce aggrecan degradation in chondrocytes. These will perturb metabolism balance between aggrecan synthesis and degradation in cartilage, inducing aggrecan loss in the end, which may be the initiation of the cartilage degradation.

Key words: T-2 toxin, Kashin-Beck disease (KBD), Aggrecan, IL-1β, TNF-α, Aggrecanase, Hyaluronic acid (HA), CD44
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INTRODUCTION

Kashin-Beck disease (KBD) is an endemic, chronic, degenerative osteoarthropathy, which occurs primarily in agricultural regions in China. KBD was found to cover a long, narrow section from the Northeast to Southwest regions such as Sichuan and Tibet. From a grand view, the section is located exactly at the intersection where cold and dry continental climate meets with warm and damp oceanic climate. Population of the section is over 30000000, and KBD patients are about 2500000 (Wang, 1999).

KBD often occurs in children, and the younger

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the patients, the more serious the patient's conditions. In early stages of KBD, flexion of the terminal parts of fingers is present. As a result, the fingers may become crooked. About two thirds of all patients have stiffness of the joints after getting up in the morning, and most patients have characteristic flexion and extension disturbances in the elbows and enlarged interphalangeal joints as well as joint pains. Patients with advanced cases of KBD develop increasingly limited motion in the mid-sized and large joints, which could slowly progress to complete disability as age advanced. Hyaline cartilage in the epiphysis plate is the primary target of the disease. Pathologically, the epiphysis plate, the growth center of the bone, shows necrosis in the hypertrophied layer near the adjacent subchondral bone. That is the reason why KBD patients often have joints development deformity (Yin and Guo, 1992).

The etiology of KBD is unclear. In China, the risk factors seem to include selenium deficiency, fungal contamination of staple grains, and iodine deficiency. After about 20 years research, it has been confirmed that selenium deficiency does not cause KBD, but is closely related (Mo *et al*., 1997). Because the T-2 toxin generated by fusarium fungus contained in endemic grain remains at high levels (2.0~1549.9 ng/g) (Sun *et al*., 1997), and T-2 toxin-containing food can lead to some pathologic changes in the cartilage of Guinea pig that are similar to the changes observed in KBD patients (Xiong *et al*., 1997), T-2 toxin contamination probably contributes to the classical features of KBD (Yang, 1998).

Cartilage is composed of collagen fibers within which are trapped proteoglycan molecules that pull water into the tissue. The major proteoglycan in cartilage is the large aggregating aggrecan, which consists of an extended core protein to which many chondroitin sulfate and keratan sulfate glycosaminoglycans chains are attached (Ratcliffe *et al*., 1993; Hardingham and Fosang, 1992). Aggrecan is bound to hyaluronic acid (HA), which is stabilized around chondrocytes by CD44. Therefore, a number of aggregating aggrecans around chondrocytes can provide a microenvironment for cell survival and metabolism. According to these, there were close relationships between CD44 as well as HA and aggrecan metabolism. Aggrecan is synthesized and secreted to matrix by chondrocyte. Many enzymes are responsible for aggrecan degradation such as aggrecanases and matrix metalloproteinases (MMPs) (Little *et al*., 2007; Song *et al*., 2007; Fortier *et al*., 2007). The primary roles of MMPs in cartilage degradation are metabolism of collagen, another main component of cartilage. Aggrecanases are considered to be the main enzymes to cleave aggrecan in the early stage of arthritis (Little *et al*., 2002). In normal cartilage, where a steady state exists, synthesis rates of matrix components such as aggrecan are equal to their degradation rates, and any disturbance in this steady state can affect cartilage function. Accelerated proteolytic cleavage of the aggrecan core protein, and the consequent loss of glycosaminoglycan-bearing fragment from articular cartilage, both are an early and persistent process following a joint injury and in the development of a degenerative joint disease, such as osteoarthritis (OA) or rheumatoid arthritis (RA) (Flannery *et al*., 1999).

Although its etiology differs from OA and RA, the central pathological feature of KBD is still a degradation of cartilage, similar to OA and RA. Therefore, there must be an aggrecan degradation involved in KBD articular cartilage. Guo *et al*.(1997) found that the serum from children with KBD significantly inhibited ^{35}S incorporation rate in cultured chondrocytes. Lin *et al*.(2001) reported that glycosaminoglycan in the head of femur, tibial plateau and costal cartilage from the Rhesus monkey fed with grains and water from KBD endemic area was undersulfated. Decreased unsaturated 4-sulphated disaccharide (ΔDi-4S) from the glycosaminoglycans in the head of femur and tibial plateau and decreased unsaturated 6-sulphated disaccharide (ΔDi-6S) from the glycosaminoglycans in the costal cartilage were also discovered. All these results suggest that the pathogenic factors in KBD endemic area could influence aggrecan metabolism. In our previous studies, T-2 toxin was suggested to decrease aggrecan synthesis in human chondrocytes (Chen *et al*., 2006a). However, few studies have been done in this filed.

In this study, we report that T-2 toxin can disturb some biomacromolecules related to aggrecan metabolism such as CD44, HA, aggrecanases metabolism, thus causing aggrecan degradation. Moreover, we also investigated these molecules expression under selenium exposure. All these results give us valuable evidence to understand pathogenesis and the etiology of KBD.

MATERIALS AND METHODS

All reagents were obtained from Sigma (USA) unless otherwise specified.

Human fetal articular cartilage sources

Human fetal articular cartilage was obtained from informed donors who had to stop pregnancy and abort through therapy as approved by the Humane and Ethical Committee, School of Medicine, Xi'an Jiaotong University, China. The fetuses were about 5-month old and all of the samples were obtained from knee, coxa and shoulder joints within 4 h after abortion.

Cell monolayer culture

Chondrocytes were isolated from fetal cartilage as described previously (Chen *et al*., 2006b). Briefly, full thick pieces of articular cartilage were removed aseptically from the subchondral bone with a scalpel and cut into small pieces. These pieces were digested with 0.25% trypsin in D-Hank's buffer at 37 °C for 30 min. Then the samples were digested with 0.2% clostridia collagenase type II in D-Hank's buffer at 37 °C for 60 min. Chondrocytes were collected and counted. The cells were then seeded at a density of 4×10^5 cells/flask and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, China), 100 U/ml of penicillin, 100 μg/ml of streptomycin at 37 °C under 5% CO_2 in air for 24 h. Then, 0.01 μg/ml T-2 toxin (kindly provided by Prof. Jinsheng Yang, Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences of China) and 0.1 μg/ml selenium were supplemented into culture medium according to group, respectively. The medium was replaced every other day, and collected and stored at −30 °C for further analysis.

Cartilage reconstructed in vitro

Chondrocytes were isolated from cartilages as described previously (Chen *et al*., 2006b). Bones were also obtained from thighbone, humerus, tibia and etc. of the same fetus, and made into bone matrix gelatin (BMG) after decalcification, defat and other steps; and the BMGs were shaped into round pieces. Chondrocytes were transplanted on the BMG at a density of 4×10^5 cells/tube and grown in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin at 37 °C under 5% CO_2. 0.01 μg/ml T-2 toxin and 0.1 μg/ml selenium were also supplemented into culture medium according to group, respectively. The medium was replaced every two days, and the replaced medium was collected and stored at −30 °C for further analysis. The reconstructed cartilages were cultured for 18 d, and then the cartilages were fixed with 4% paraformaldehyde (pH 8.0).

Immunohistochemistry

Paraformaldehyde-fixed paraffin-embedded reconstructed cartilages were cut into 6-μm sections and placed on poly-L-lysine-coated glass slides. Immunohistochemical staining was performed with the strept-avidin-biotin-peroxidase complex (SABC) method using SABC kits (Boster Co., Wuhan, China). Briefly, after deparaffinization, the sections for NITEGE immunostaining were digested with 0.1% chondroitinase ABC at 37 °C for 25 min, then endogenous peroxidase was blocked with 3% H_2O_2 for 30 min. The slides were washed with 0.01 mol/L phosphate-buffered saline (PBS) 3 times for 5 min and incubated with 10% normal goat serum to block non-specific binding. The sections were then incubated with primary antibody BC-13, 3-B-3 and 2-B-6 (diluted 1:100) or control mouse serum at 4 °C for 18 h, exposed to biotinylated goat anti-mouse IgG (Boster Co., Wuhan, China), followed by treatment with the SABC and stained with 3-amino-9-ethylcarbozole (AEC).

Total RNA isolation

Total RNA was extracted using Trizol kits (GIBCOBRL, MD, USA) method according to manufacturer's instructions. Briefly, cells were lysed by adding 1 ml of Trizol reagent. The homogenized samples were incubated for 5 min at 20 °C, followed by adding 0.2 ml of chloroform per 1 ml of Trizol reagent. The samples were centrifuged at no more than 12000×g for 15 min at 8 °C. Then the aqueous phase was transferred to a fresh tube, and the organic phase was saved for isolation of DNA and protein. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, followed by centrifugation at 12000×g for 10 min at 4 °C. RNA pellet was washed twice with 75% ethanol, and dissolved in diethylpyrocarbonate-treated water. RNA

concentration and purity were determined on a spectrophotometer (UV-1201, Shimadzu, Japan) by calculating the ratio of optical density at wavelengths of 260 and 280 nm.

DNA isolation and determination

DNA was obtained from the interphase and organic phase with ethanol after RNA extraction. 0.3 ml of 100% ethanol per 1 ml of Trizol reagent was mixed with samples, and the mixture was stored at 20 °C for 3 min, followed by centrifugation at 2000×g for 5 min at 8 °C. The DNA pellet was washed twice in a solution containing 0.1 mol/L sodium citrate in 10% ethanol. At each wash, DNA pellet was stored in the washing solution for 30 min at 20 °C (with periodic mixing), followed by centrifugation at 2000×g for 5 min at 8 °C. Then the DNA pellet was resuspended in 75% ethanol (2 ml of 75% ethanol per 1 ml Trizol reagent) and stored for 20 min at 20 °C, followed by centrifugation at 2000×g for 5 min at 8 °C. DNA was air dried for 15 min in an open tube and its content was determined.

Determination of mRNA levels by semi-quantitative RT-PCR

RT-PCR was performed on PTC-100 programmable thermal controller (MJ Research Inc., USA) using one-step AccessQuick™ RT-PCR System (Progema, San Luis, USA), with the final 50 μl RT-PCR mixture containing 2 μl RNA, 1 μl AMV reverse transcriptase, 20 pmol/μl primers and 18 μl nucleic acid-free water. The reaction protocols were: 48 °C for 50 min; 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s; 62 °C (CD44), 60 °C (hyaluronic acid synthetase-2 (HAS-2)), 63 °C (aggrecan), 50 °C (ADAMTS-4), 62 °C (ADAMTS-5) for 60 s; 70 °C for 2 min; at the end of the final cycle, samples were incubated at 72 °C for 7 min to stop the reaction. Primer pairs used to amplify CD44, HAS-2, aggrecan, ADAMTS-4, ADAMTS-5 and GAPDH fragments were respectively in Table 1.

After reaction, 5 μl of RT-PCR products with 1 μl of 6×PCR loading buffer were then loaded into a 2% agarose gel to separate the amplified DNA species. The bands corresponding to the amplified DNA were then visualized by 0.5% ethidium bromide-stained agarose gels, and resultant gels were photographed with UVP ImageStore 7500.

Flow cytometry (FCM)

The expression of CD44 in chondrocytes was assessed by FCM. Cells were washed with PBS and removed from the culture flask by scraping. After centrifugation, cell pellet was resuspended in ice-cold PBS, followed by incubation with a monoclonal FITC-conjugated anti-CD44 antibody (Progema, San Luis, USA) for 30 min at 37 °C avoiding light. The cells were then washed twice with ice-cold PBS, and analysis was performed on a FACS Calibur FCM (Biorad Co., USA) equipped with an argon laser (488 nm). Ten thousand events were collected. Irrelevant mouse serum was used as negative control.

Enzyme-linked immunosorbent assay (ELISA)

To determine IL-1β, TNF-α and soluble CD44 (sCD44) levels in culture medium secreted by chondrocytes, IL-1β and TNF-α (Genzyme Co., USA) as well as sCD44 (R & D Systems, UK) ELISA analysis was performed. The sandwich ELISA was carried out according to the supplier's protocols and optical densities were determined using an automated reader (Biorad Co., USA). Concentrations of IL-1β, TNF-α and sCD44 were determined by the Microman2-reader software (Biorad Co., USA).

Radioimmunoprecipitation assay (RIPA)

HA levels in culture media were investigated using HA RIPA analysis kit (Haiyan Co., Shanghai, China). The RIPA was carried out according to supplier's protocols and optical densities were determined using FM-2000 Gamma Counter (Kaipu Co., Xi'an, China).

Table 1 Primers for semi-quantitative RT-PCR

Primer	Upstream primer	Downstream primer	Size (bp)
CD44	5′AACCGCGAGAATCAAAGCCAAGGCC3′	5′GATCCACCCCAATTCCATCTGTGC3′	602
HAS-2	5′TTTCTTTATGTGACTCATCTGTCTCACCGG3′	5′ATTGTTGGCTACCAGTTTATCCAAACGG3′	409
Aggrecan	5′CTCCACTGCCTGTGAAGTCACCAC3′	5′GCACCATGCCTTCTGCTTCCGAG3′	620
ADAMTS-4	5′ACCACTTTGACACAGCCATTC3′	5′ACCCCCACAGGTCCGAGAGCA3′	692
ADAMTS-5	5′TGTGCTGTGATTGAAGACGAT3′	5′GACTGCAGGAGCGGTAGATGG3′	673
GAPDH	5′GCCTCAAGATCATCACTCAAT3′	5′AGGTCCACCACTGACACGTT3′	465

Statistic analysis

Data were presented as mean±*SEM* and checked for normal distribution and equal variance. Student *t*-test or one-way analysis of variance (ANOVA) was carried out by SPSS 12.0 software. Differences were considered significant at $P<0.05$.

RESULTS

Hematoxylin and eosin (H & E) staining

H & E staining of reconstructed cartilage cultured without T-2 toxin in vitro was shown in Figs.1a and 1b (shown later in this paper). The BMG staining was purple red, on which 5~10 layers of chondrocytes were grown (Fig.1a). Chondrocytes on BMG without T-2 toxin were morphologically fusiform, triangle, varying in size and spreading out. Cytomembrane was integral, cytoplasm was distinct and nuclei staining was blue (Fig.1b). Staining of reconstructed cartilage cultured with T-2 toxin in vitro was shown in Figs.1c and 1d. Chondrocytes on BMG were only 1~5 layers (Fig.1c), nuclei staining became lighter, and nuclear fragmentations and many dots as well as pellets of necrosis could be seen in cartilage. Cytomembrane was not integral, and there were many cell ghosts (Fig.1d).

Semi-quantitative RT-PCR

mRNA levels of aggrecan metabolism related macromolecules were investigated using semi-quantitative RT-PCR. The specific products generated by RT-PCR were normalized to housekeeping gene GAPDH. T-2 toxin inhibited CD44, HAS-2 and aggrecan mRNA expression but promoted aggrecanase-2 mRNA levels (ANOVA, P=0.00, 0.04, 0.05 and 0.045, respectively). Selenium had an antagonism for T-2 toxin effects on chondrocyte with slightly increased CD44H, HAS-2 and aggrecan mRNA levels. However, selenium did not increase aggrecanase-2 mRNA level (Fig.2).

CD44 expression in chondrocyte analysis using FCM

CD44 expression in chondrocytes was detected by FCM. The results are shown in Table 2. After cultured for 5 d, CD44 content was the lowest in T-2 group, and the highest in control+Se group among all groups, suggesting that T-2 toxin can inhibit CD44 expression in human chondrocyte. There was a slight increased CD44 expression in T-2+Se group, which illustrates that selenium can reverse T-2 toxin inhibitory effect on CD44 expression in chondrocyte.

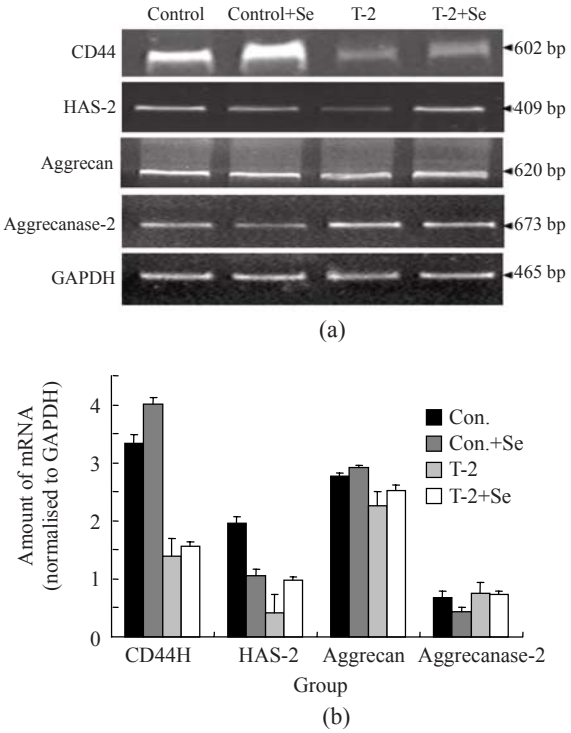

Fig.2 RT-PCR analysis of T-2 toxin effects on the mRNA expression of aggrecan metabolism related biomacromolecules in human chondrocytes. (a) Representative RT-PCR results. Total RNA was extracted from human chondrocytes which had been cultured for 5 d with or without T-2 toxin and selenium; (b) Relative mRNA levels of CD44H, HAS-2, aggrecan, aggrecanase-2 and GAPDH. Results were normalized to GAPDH level. Results are presented as mean±*SEM* (*n*=4)

Table 2 The results of CD44 expression

Group	CD44 positive rate (%)
Control	$43.97^{\S\ddagger}$
Control+Se	$47.60^{\dagger\ddagger}$
T-2	$27.21^{\S\Delta}$
T-2+Se	$38.90^{\dagger\Delta}$

§Control group vs T-2 group, $P<0.01$; ‡Control group vs control+Se group, $P<0.01$; †Control+Se group vs T-2+Se group, $P<0.01$; $^{\Delta}$T-2 group vs T-2+Se group, $P<0.01$

DNA content determination

DNA content was determined to normalize cells number of different groups after 5 d culture with or without T-2 toxin and selenium. The results in Fig.3

indicate that DNA content in T-2 toxin group was lower than that in control group (P=0.002), and DNA content in T-2+Se group was lower than that in control+Se group. These suggest that T-2 toxin can inhibit chondrocyte proliferation. DNA content in T-2+Se group seemed higher than that in T-2 group, although no significant difference arrived (P>0.05).

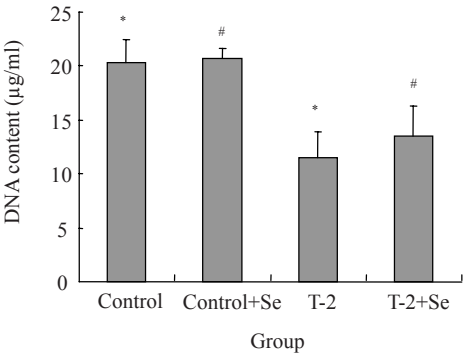

Fig.3 DNA content in different groups. Chondrocytes were cultured for 5 d with or without T-2 toxin and selenium. DNA content in T-2 toxin group was significantly lower than that in control group. $^{*}P$=0.002, T-2 toxin group vs control group; $^{\#}P$=0.003, T-2 toxin+Se group vs control+Se group (mean±*SEM*, n=6)

Investigation of HA levels in culture media using RIPA

To investigate whether T-2 toxin regulates HA synthesis on post-transcription level in human chondrocyte, HA levels in culture media were determined by RIPA. In order to remove the errors caused by inequality in chondrocytes number in different groups after cultured for 5 d with or without T-2 toxin and selenium, HA levels in culture media were normalized to DNA content. As shown in Fig.4, HA level in T-2 toxin group was higher than that in control group (P=0.001), and higher HA level in T-2 toxin+Se group was observed when compared with control+Se group (P=0.01).

Determination of IL-1β and TNF-α levels

IL-1β and TNF-α levels in culture media were investigated using ELISA. In order to remove the errors caused by inequality in chondrocytes number in different groups after cultured for 5 d with or without T-2 toxin and selenium, IL-1β and TNF-α levels in culture media were normalized to DNA content. As shown in Fig.5, IL-1β and TNF-α levels in T-2 toxin group were higher than those in control group (P=0.001 and 0.021, respectively); meanwhile, higher IL-1β and TNF-α levels were observed in T-2 toxin+Se group when compared with control+Se group (P=0.031 and 0.009, respectively).

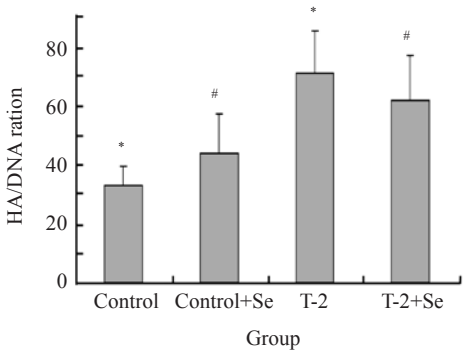

Fig.4 HA levels in culture media. The chondrocytes were cultured for 5 d and the culture medium was collected. HA levels were normalized to DNA content. HA level in T-2 group was the highest and the lowest in the control group. $^{*}P$=0.001, T-2 toxin group vs control group; $^{\#}P$=0.01, T-2 toxin+Se group vs control+Se group (mean±*SEM*, n=5)

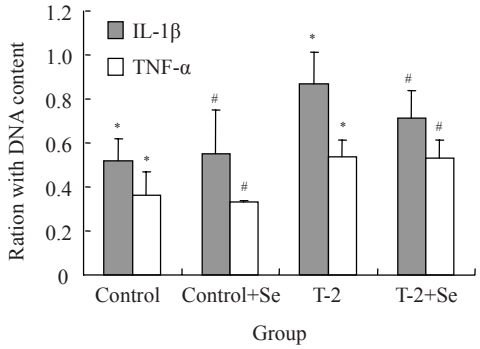

Fig.5 IL-1β and TNF-α levels in culture media. Chondrocytes were cultured for 5 d, and the culture media were collected to investigate IL-1β and TNF-α levels by ELISA. The samples were normalized to DNA content of the same flask in order to remove the errors caused by inequality in chondrocytes number. *Control group vs T-2 group, P<0.05; $^{\#}$Control+Se group vs T-2+Se group, P<0.05 (mean±*SEM*, n=5)

Determination of sCD44 levels

With the same method used above, sCD44 levels in culture media were also determined. Similarly, sCD44 levels were normalized to DNA content. As shown in Fig.6, sCD44 level in T-2 toxin group was higher than that in control group (P=0.002); however, there was no significant difference in sCD44 level between control+Se group and T-2 toxin+Se group (P>0.05).

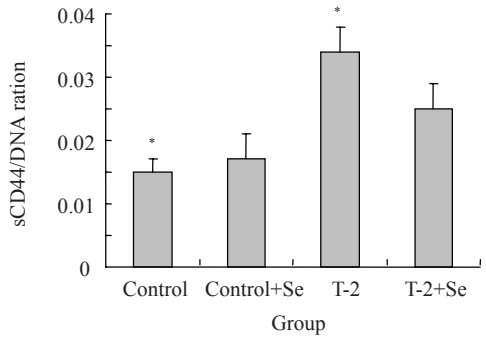

Fig.6 sCD44 levels in culture media. Chondrocytes were cultured for 5 d, and the culture media were collected to investigate sCD44 levels. sCD44 levels were normalized to DNA content to remove the errors caused by inequality in chondrocytes number. *Control group vs T-2 group, $P<0.05$ (mean±*SEM*, $n=5$)

Immunostaining for neoepitope …NITEGE

The aggrecanase generated neoepitope ...NITEGE in cartilage reconstructed in vitro was investigated using antibody BC-13. As shown in Fig.7, there is weak positive staining in cartilage cultured without T-2 toxin (Figs.7a and 7b). The positive staining in cartilage cultured with T-2 toxin is the strongest (Fig.7c), and the staining in cartilage cultured with T-2+Se is stronger than that in control and control+Se groups but weaker than that in T-2 group (Fig.7d).

Immunohistochemical analysis with monoclonal antibody 3-B-3(−)

In this study, immunolocation with monoclonal antibody 3-B-3 was preformed without chondroitinase predigestion. Under this condition, antibody 3-B-3(−) was used to identify a "native" mimotope that occurs at the non-reducing terminal of some of the chondroitin sulfate (CS) chains (Geysen *et al*., 1988; Caterson *et al*., 1990; Slater *et al*., 1995). Fig.8 indicates that there is weak staining in cartilage cultured without T-2 toxin (Figs.8a and 8b). The staining in cartilage cultured with T-2 toxin is the strongest (Fig.8c) and denser 3-B-3(−) staining is observed in T-2+Se group when compared with that in control and control+Se groups (Fig.8d).

Immunohistochemical analysis with monoclonal antibody 2-B-6

Immunostaining with monoclonal antibody 2-B-6 in cartilage reconstructed in vitro is shown in Fig.9. There is no positive or very weak staining in cartilage cultured without T-2 toxin (Figs.9a and 9b). The staining in cartilage cultured with T-2 toxin is the strongest (Fig.9c) and a weaker staining in T-2+Se group (Fig.9d) is observed when compared with T-2 toxin group.

DISCUSSION

In this study, we investigated the effects of T-2 toxin and selenium on metabolism of aggrecan and related biomacromolecules using cartilage reconstructed with BMG in vitro to mimic cartilage metabolism environments in vivo. BMG is a kind of amorphous matrix that lost its fibril construction. The antigenicity of BMG is very low, with bone morphogenetic protein (BMP) in it, which would induce activities of chondrocyte and promote cartilage growing. In the experiment, chondrocytes were seeded on the BMG and cultured for 1 d, then 0.01 μg/ml T-2 toxin and 0.1 μg/ml selenium were incorporated into culture media and continued to culture for 18 d. In the end we succeeded in getting the cartilage necrosis model, which was widely used in our lab to detect T-2 toxin damage effects on cartilage and relationship between T-2 toxin and KBD (Xie *et al*., 2003; Song *et al*., 2006).

T-2 toxin is one of the mycotoxins, a group of type A trichothecenes produced by several fungal genera including *Fusarium* sp., which is detected in a number of field crops (wheat, maize, barley and oats) and processed grains (malts, beer and bread) (Chen *et al*., 2006c). It has been more than ten years since T-2 toxin pollution in environment is regarded as the etiology of KBD. There are still several pivotal questions which cannot be explained by it. For example, why does not KBD occur in some areas in which grains are also polluted by T-2 toxin, such as two villages adjacent to each other, one of them is KBD area but the other not? If T-2 toxin is the real etiology of KBD, what is the mechanism of the pathological process, and how does T-2 toxin cause chondrocyte necrosis and cartilage degradation? Actually, the knowledge about the mechanism of T-2 toxin on chondrocyte and cartilage is poorly understood. Previous studies have shown that T-2 toxin could disturb proteoglycan metabolism in chondrocytes cultured in vitro by decreasing proteoglycan synthesis (Lin *et al*.,

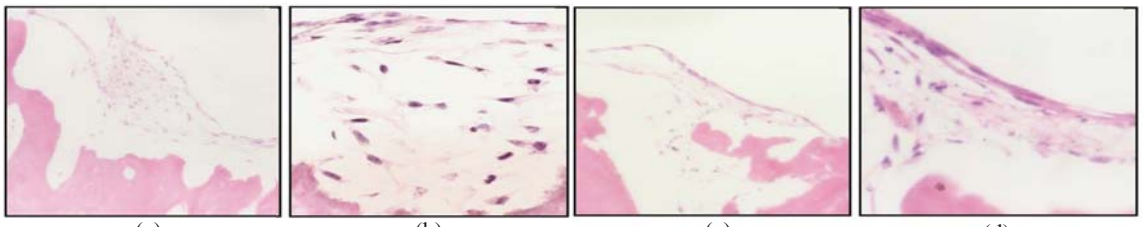

Fig.1 H & E staining of cartilage reconstructed in vitro. (a) and (b) Staining of cartilage reconstructed without T-2 toxin. 5~10 layers of chondrocytes were grown on BMG, and the cell was intact with clear red in cytoplasm and blue in nuclei; (c) and (d) Staining of cartilage reconstructed with T-2 toxin. Only 1~5 layers of cells were grown on BMG. Nuclei staining became lighter, nuclear fragmentations and many dots as well as pellets of necrosis could be seen. The magnification of (a) and (c) was 10× by light microscope and (b) and (d) was 40×

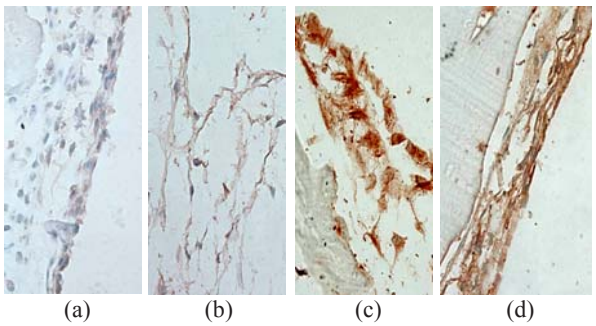

Fig.7 Immunostaining for neoepitope NITEGE on the cartilage reconstructed in vitro. Chondrocytes were cultured for 18 d after seeded on the BMG. (a) Control group; (b) Control+Se group; (c) T-2 group; (d) T-2+Se group. NITEGE epitope was stained red and nuclei were blue. The magnification was 20×

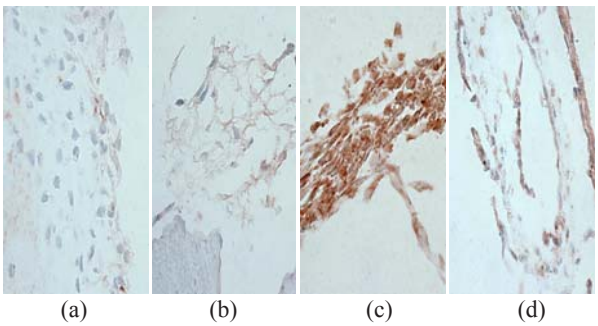

Fig.8 Immunostaining with monoclonal antibody 3-B-3(−) on cartilage reconstructed in vitro. Chondrocytes were cultured for 18 d after seeded on the BMG. (a) Control group; (b) Control+Se group; (c) T-2 group; (d) T-2+Se group. NITEGE epitope was stained red and nuclei were blue. The magnification was 20×

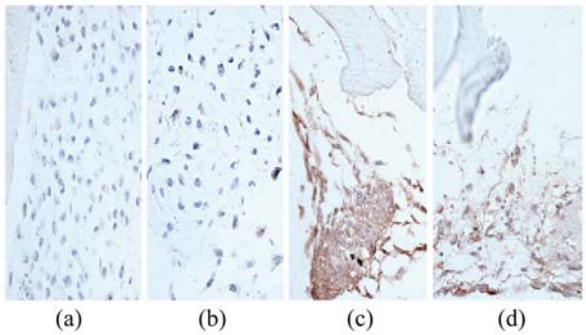

Fig.9 Immunostaining with monoclonal antibody 2-B-6 on cartilage reconstructed in vitro. Chondrocytes were cultured for 18 d after seeded on the BMG. (a) Control group; (b) Control+Se group; (c) T-2 group; (d) T-2+Se group. NITEGE epitope was stained red and nuclei were blue. The magnification was 20×

2001; Cao *et al*., 1998), which may play a pivotal role during pathogenesis of KBD. Based on this, we explored the mechanism of T-2 toxin disturbing aggrecan mechanism in cartilage, which would be valuable for understanding the pathogenesis of KBD.

Aggrecan is the major proteoglycan present in articular cartilage, and it is the molecule that endows cartilage with its intrinsic capacity to bear load and resist compression. One of the central pathophysiological features contributing to cartilage erosion during the progress of a degenerative joint disease is the catabolism and loss of aggrecan (Roughley, 2006). CD44, HA, IL-1β, TNF-α and aggrecanases are biomacromolecules which are structurally and functionally involved in aggrecan metabolism, and their expressions directly influence aggrecan synthesis and degradation equilibration. According to their relationships with aggrecan metabolism, we divided them into three categories: synthesis-related, degradation-related and control-related molecules.

Aggrecan consists of an extended core protein to which many chondroitin sulfate and keratan sulfate glycosaminoglycan chains are attached, so core protein mRNA expression is a direct marker responding to aggrecan synthesis. Our RT-PCR results show that

T-2 toxin could significantly inhibit human chondrocyte aggrecan core protein transcription, and selenium could antagonize this effect of T-2 toxin partially, which illustrates that, at least at mRNA level, aggrecan synthesis would be inhibited by T-2 toxin. The mechanism of this effect is not clear to date, although some experiments have revealed that T-2 toxin can inhibit DNA, RNA and protein synthesis in several cellular systems (Cao *et al*., 1998; Ji *et al*., 1994). Aggrecan is lost from cartilage following proteolysis of the core protein. Aggrecanases are thought to be the enzymes primarily responsible for this proteolysis, and their activities and amounts directly influence aggrecan degradation rate. Our studies show that aggrecanases-2 mRNA transcription was promoted by T-2 toxin in chondrocyte cultured in vitro, and selenium could inhibit its transcription, which illustrates that aggrecanase-2 may be involved into T-2 toxin inducing cartilage aggrecan degradation. These results are consistent with the earlier research results from cartilage treated with IL-1 (Ishiguro and Kojima, 2004). Unexpectedly, however, no detectable aggrecanase-1 mRNA expression was found in chondrocytes cultured with T-2 toxin in vitro. Previous studies have shown that aggrecanase-1 usually is an up-regulated expression in the cartilage induced by IL-1 (Liacini *et al*., 2005; Tsuzaki *et al*., 2003; Pratta *et al*., 2003), whereas aggrecanse-2 mRNA is expressed constitutively in cartilage, and the aggrecanase activity of aggrecanase-2 was at least 1 000-fold greater than that of aggrecanase-1 (Gendron *et al*., 2007). Therefore, aggrecanase-2 is a major aggrecanase in cartilage metabolism and pathology. Our results indicate that aggrecanase-2 may play a pivotal role in aggrecan degradation induced by T-2 toxin. However, it is not clear to date whether the increased aggrecanase-2 mRNA expression in cartilage is an initial response for T-2 toxin treatment or just a result caused by other cytokines.

BC-13 is a monoclonal antibody to identify "catabolic neoepitopes" (new epitopes created as specific N- or C-terminal amino acid sequences of a proteolytic cleavage product) NITEGE generated after aggrecanases catabolism within the interglobular domain (IGD) of aggrecan core protein (Hughes *et al*., 1992; 1995). NITEGE is usually regarded as a marker of aggrecanases cleavage aggrecan in cartilage, in another word, a marker of aggrecan degradation. In this study, there was an intense NITEGE staining in the reconstructed cartilage cultured with T-2 toxin but a weak staining in the cartilage cultured without T-2 toxin, which illustrates that T-2 toxin can promote aggrecanases proteolytic cleavage activities in cartilage. Selenium could partially antagonize this effect of T-2 toxin. Furthermore, using monoclonal antibody 3-B-3, we also investigated newly synthesis proteoglycan in cartilage. Immunohistochemistry staining of monoclonal antibody 3-B-3 usually is carried out under two different conditions. The first is that the tissue sections are predigested by chondroitinase ABC or hyaluronanase before immunostaining, which is named 3-B-3(+). 3-B-3(+) specifically recognizes the chondroitinase hyaluronidase-generated epitope on chondroitin-6-sulfate, on which unsaturated chondroitin sulfate disaccharide is removed by digestion and leaves as a product of proteoglycan core protein containing numerous linkage region oligosaccharide stubs with a terminal unsaturated chondroitin sulfate residue (Caterson *et al*., 1985). If there was no pre-digestion by enzymes before the incubation with 3-B-3, we usually call it 3-B-3(−). Under this experimental condition, reactivity of 3-B-3(−) with native chondroitin sulfate usually occurs in cartilage undergoing osteoarthritic changes. Expression of this disaccharide epitope indicates the presence of the newly synthesized proteoglycan, illustrating the repairing or remodeling activities of chondrocytes for the injured cartilage extracellular matrix (Hughes *et al*., 1992). In our study, 3-B-3(−) was preformed for the immunostaining. The results show that there was a strong positive staining in the cartilage cultured with T-2 toxin in vitro, which illustrates that chondrocytes indeed synthesized new aggrecan, trying to repair or remodel proteoglycan injured by T-2 toxin or other damaged factors caused by T-2 toxin. In addition, we also investigated the chondroitin-4 and dermatan sulfate glycosaminoglycan chains expression in the reconstructed cartilage using monoclonal antibody 2-B-6. Usually, this antibody only recognizes the epitope on chondroitin-4 and dermatan sulfate glycosaminoglycan chains generated by chondroitinase ABC digestion; however, no chondroitinase ABC predigestion before immunostaining was preformed in our study, and a strong positive staining in the cartilage cultured with T-2 toxin was observed but there was no positive immunostaining in the control

cartilage. Although, the mechanism of this positive staining is not clear yet, we speculate that it illustrates that T-2 toxin may initialize the epitope exposure process through some unknown mechanisms, which is generally completed by cleavage chondroitin-4 and dermatan sulfate glycosaminoglycan chains using chondroitinase ABC in previous experiments. If this was the case, our results would give some valuable clues about how T-2 toxin injures cartilage. However, more work is needed to confirm it.

CD44, HA, IL-1β and TNF-α are all aggrecan metabolism related molecules, and their expression levels in cartilage regulate aggrecan metabolism equilibrium. HA is the main component of aggregating aggrecan and many aggrecan monomers attach to HA by link proteins forming aggregating aggrecan. In cartilage, HA is mainly synthesized by HAS-2. Our experiments show that T-2 toxin significantly inhibits HAS-2 mRNA transcription and decreases the amount of HA in cartilage, which will profoundly influence the aggrecan aggregating. Meanwhile, we also investigated HA levels in culture media, and found that T-2 toxin increases HA levels in culture media prominently. There are two possible sources of free HA in culture media, and the first one is a direct secretion by chondrocyte and the second is the HA dissociated from the matrix around chondrocytes. RT-PCR results show that T-2 toxin inhibited HAS-2 synthesis, suggesting HA synthesis in chondrocytes was inhibited by T-2 toxin. Therefore we can infer that the increased HA levels in culture media were caused by its dissociation from the matrix. T-2 toxin may dissociate and free HA from aggregating aggrecan, seriously damage aggrecan biological functions, initiate aggrecan degradation process and eventually lead to cartilage degradation. While its biochemical and molecular biological mechanism is not clear to date, previous study indicated that the increasing aggrecanase level in cartilage may be involved (Chockalingam *et al*., 2004).

One main function of CD44 is to maintain aggrecan around chondrocyte and act as a bridge of matrix and chondrocyte communications. Our experiments show that T-2 toxin could inhibit CD44 transcription in chondrocytes, suggesting that T-2 toxin could inhibit chondrocyte maintaining aggrecan around itself and thus inhibit matrix synthesis. Meanwhile, FCM as well as ELISA results show that T-2 toxin can inhibit CD44 expression in chondrocytes and promote CD44 shedding from chondrocyte into culture media, both of which would inhibit aggrecan synthesis and initiate aggrecan degradation. sCD44 determination results show there was no significant difference between control+Se and T-2+Se groups, which indicates that selenium could inhibit CD44 shedding from chondrocytes.

IL-1β and TNF-α are cytokines which could induce cartilage degradation. Increased IL-1β and TNF-α levels are considered important influence factors in OA and RA and many therapeutic target (Cawston *et al*., 2003; Fan *et al*., 2007). Previous studies have shown that IL-1β and TNF-α can up-regulate aggrecanase expressions in OA and RA (Fan *et al*., 2007; Arner *et al*., 1998), which would predominantly increase aggrecan degradation and lead to glycosaminoglycan loss from the cartilage. Similar to OA and RA, it has been confirmed that IL-1β and TNF-α levels are also increased in adult KBD patients' synovial fluid (Tong and Yang, 2000). As KBD suspected etiology, T-2 toxin is supposed to promote chondrocyte expressing IL-1β and TNF-α, and our experiments also have confirmed this deduction. ELISA results show that IL-1β and TNF-α levels in T-2 toxin group significantly increased, although the mechanism of the increase is not clear yet. Therefore, T-2 toxin can promote aggrecan degradation through up-regulating the pro-inflammatory cytokines in human cartilage. In addition, no significant differences in IL-1β and TNF-α between T-2 toxin and T-2+Se groups were observed, which illustrates that selenium cannot or just slightly inhibit the IL-1β and TNF-α increased expressions in chondrocytes caused by T-2 toxin.

In general, our study results demonstrate that T-2 toxin can inhibit aggrecan synthesis in chondrocyte and promote aggrecanases and pro-inflammatory cytokines expression and aggrecan degradation. All these effects will perturb the balance between aggrecan synthesis and degradation, inducing aggrecan loss from cartilage, which may be the initiation of the cartilage degradation. An improved understanding of these biochemical and molecular biological changes may provide new insights into the mechanisms that regulate chondrocyte pathology in KBD.

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