

Effects of sodium selenite on c-Jun N-terminal kinase signalling pathway induced by oxidative stress in human chondrocytes and c-Jun N-terminal kinase expression in patients with Kashin–Beck disease, an endemic osteoarthritis

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Abstract

The c-Jun N-terminal kinases (JNK) are members of the mitogen-activated protein kinase family and are activated by environmental stress. Se plays an important role in the biological pathways by forming selenoprotein. Selenoproteins have been shown to exhibit a variety of biological functions including antioxidant functions and maintaining cellular redox balance, and compromise of such important proteins would lead to oxidative stress and apoptosis. We examined the expression levels of JNK in Kashin–Beck disease (KBD) patients, tested the potential protective effects of sodium selenite on tert-butyl hydroperoxide (tBHP)-induced oxidative injury and apoptosis in human chondrocytes as well as its underlying mechanism in this study. We produced an oxidative damage model induced by tBHP in C28/I2 human chondrocytes to test the essential anti-apoptosis effects of Se *in vitro*. The results indicated that the expression level of phosphorylated JNK was significantly increased in KBD patients. Cell apoptosis was increased and molecule expressions of the JNK signalling pathway were activated in the tBHP-injured chondrocytes. Na₂SeO₃ protected against tBHP-induced oxidative stress and apoptosis in cells by increasing cell viability, reducing reactive oxygen species generation, increasing Glutathione peroxidase (GPx) activity and down-regulating the JNK pathway. These results demonstrate that apoptosis induced by tBHP in chondrocytes might be mediated via up-regulation of the JNK pathway; Na₂SeO₃ has an effect of anti-apoptosis by down-regulating the JNK signalling pathway.

Key words: c-Jun N-terminal kinases signalling pathway; Kashin–Beck disease; Apoptosis; Chondrocytes; Selenium

Kashin–Beck disease (KBD) is a chronic, endemic osteoarthritis (OA) that occurs in a limited endemic area in China, Central China, from Southeastern Siberia to North China and North Korea⁽¹⁾. Degradation of the matrix and cell necrosis in the growth plate and articular surface are the basic pathological features, and this can result in growth retardation, secondary osteoarthritis and disability in the advanced stages of the disease^(2,3). The cause of KBD remains unknown⁽⁴⁾, but endemic deficiency of Se⁽⁵⁾, serious cereal contamination by mycotoxin-producing fungi⁽⁶⁾ and high humic acid levels in drinking water⁽⁷⁾ are considered to contribute to KBD. Low dietary levels of Se are thought to be the most important environmental factor causing the disease⁽⁸⁾, and populations from KBD-affected areas often show a deficiency of Se^(9,10). Researchers believe that multiple heterogeneous factors are involved in the aetiology of KBD⁽¹¹⁾. Recent findings of differential expressions of genes between KBD and normal controls

mainly in chondrocyte metabolism and apoptosis, signal transduction, oxidative stress and cytokines have been reported^(12–14). Furthermore, the chondrocyte necrosis mediated by oxidative stress in KBD cartilage damage has also been reported⁽¹⁵⁾.

The c-Jun N-terminal kinases (JNK) are members of a larger group of serine/threonine protein kinases known as the mitogen-activated protein kinase (MAPK) family⁽¹⁶⁾. JNK are involved in cell proliferation, differentiation and apoptosis^(17,18). Activated JNK phosphorylates nuclear substrates such as c-Jun, a component of the activator protein 1 (AP-1) transcription factor family, which mediate nuclear events that lead to cell death. Thus, a blockade of JNK activation prevents cell death⁽¹⁹⁾. Similar to other degenerative articular cartilage diseases such as OA and rheumatoid arthritis (RA), apoptosis is also a possible pathogenic mechanism in KBD. Chondrocytes play a central role in maintaining cartilage homeostasis. The vitality of articular cartilage is

Abbreviations: ATF2, activating transcription factor 2; Bcl-2, B-cell lymphoma 2 (Bcl-2); GPx, glutathione peroxidase; JNK, c-Jun N-terminal kinase; KBD, Kashin–Beck disease; MEKK1, mitogen-activated protein kinase kinase 1; MTT, methylthiazolyl tetrazolium; OA, osteoarthritis; RA, rheumatoid arthritis; ROS, reactive oxygen species; tBHP, tert-butyl hydroperoxide.

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also critical and it can be judged on the basis of the capacity of chondrocytes to resist apoptosis⁽²⁰⁾. In addition, investigations have revealed that the JNK pathway is involved in the pathogenesis of articular cartilage degradation in OA and RA. JNK and the key upstream activators of JNK – mitogen-activated protein kinase kinase 4 (MKK4) and 7 (MKK7) – are expressed and activated in OA synovial tissues⁽²¹⁾. JNK are also expressed and activated in OA chondrocytes^(22–24). JNK and the upstream kinases (MKK4 and MKK7) are highly activated in isolated RA fibroblast-like synovio-cytes and in the rheumatoid synovial lining layer and synovial mononuclear cell infiltrates^(25–27).

In the present study, we examined the expressions of the JNK signalling molecules in KBD patients, evaluated the protective effect of Na₂SeO₃ in tert-butyl hydroperoxide (tBHP)-induced chondrocyte apoptosis and investigated the potential mechanisms underlying these effects.

Methods

Patients and blood sample collection

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of the Xi'an Jiaotong University College of Medicine. Written informed consent was obtained from all the subjects. Patients with KBD were selected on the basis of the clinical criteria for the diagnosis of KBD in China (diagnostic code GB16395-1996)⁽²⁸⁾. In all, 110 KBD patients aged between 40 and 70 years were from the KBD-endemic areas of Linyou, Changwu and Yongshou counties, Shaanxi province, China. In all, 160 healthy control subjects aged between 35 and 70 years were from non-KBD areas and had no history of joint diseases such as genetic bone and cartilage diseases, OA and RA. In all, twenty people from each group were selected by simple random sampling method as experimental subjects. In all the comparisons mentioned, the groups were age and sex matched. Blood samples were drawn from the antecubital vein of all subjects following an overnight fast into tubes containing EDTA for immediate protein extraction or storage at –20°C. Protein extracts were prepared, and protein expression levels of JNK and phosphorylated JNK (p-JNK) in whole blood from KBD patients and healthy controls were detected by Western blot.

Cell culture

C28/I2 chondrocytes, a human cell line (kindly provided by Dr Mary B. Goldring from the Harvard Institutes of Medicine, Boston, MA, USA), were cultured in Dulbecco's modified Eagle's medium/F12 with a ratio of 1:1 (v/v) (Hyclone) containing 10% fetal calf serum (Hyclone) in 5% CO₂ and 95% humidified air atmosphere at 37°C.

Cell viability and proliferation assay

The methylthiazolyl tetrazolium (MTT) assay was used to assess cell viability by the mitochondrial-dependent reduction to formazan. Absorbance at 490 nm was used to quantify the amount

of MTT, which was assumed to correlate to the number of viable cells. C28/I2 chondrocytes seeded on ninety-six-well culture plates at a density of approximately 5×10^4 cells/well were pre-protected with various concentrations of Na₂SeO₃ (0.05, 0.1 and 0.15 µg/ml) for 24 h. To evaluate the protective effect of Na₂SeO₃, cells were subsequently treated with 300 µmol/l tBHP for 24 h. The protective effect against oxidative stress was measured using the MTT assay. MTT reagent (0.5 mg/ml MTT in PBS) was diluted into complete medium (dilution ratio of 1:10) and added to each well. After incubating in a CO₂ incubator for 4 h, the medium was aspirated from each well and 200 µl dimethyl sulphoxide (DMSO) (Sigma) was added. The absorbance was measured in a microplate reader (Thermo Electron Corporation) at 490 nm. Cell viability was expressed as a percentage of the control (0.1% DMSO). Assays were performed in triplicate in three independent experiments and the results are presented as mean values and standard deviations. A preliminary study with increasing concentrations of tBHP (100–500 µmol/l) for different time points (6–24 h) was performed, and the results showed that the appropriate concentration and tBHP treatment time for further studies were 300 µmol/l and 24 h, respectively.

Hoechst staining

C28/I2 chondrocytes were plated onto 60-mm-diameter dishes (1.0×10^6 cells/dish) and allowed to grow for 24 h. After incubation with various concentrations of Na₂SeO₃ (0.00, 0.05, 0.1 and 0.15 µg/ml) for 24 h and then tBHP for 24 h, chondrocytes were fixed in 2% glutaraldehyde for 4 h. Cells were washed twice with PBS and stained with 1 mg/ml Hoechst 33342 (Sigma-Aldrich) diluted in PBS (137 mM-NaCl, 12 mM-phosphate, 2.7 mM-KCl, pH 7.4) for 30 min under ice-cold conditions in the dark. Cells were then washed twice with PBS. Apoptosis, with condensed and fragmented nuclei, was observed with a fluorescence microscope using appropriate filters for blue fluorescence at 200× magnification (BX51T-32H01; Olympus).

Determination of reactive oxygen species

Cellular reactive oxygen species (ROS) levels were measured using a 5-(and-6)-chloromethyl-2,7-dichlorofluorescein diacetate (DCHF-DA) fluorescent probe (Sigma)⁽²⁹⁾. C28/I2 chondrocytes were pre-treated or untreated with various concentrations of Na₂SeO₃ (0.05, 0.1 and 0.15 µg/ml) for 24 h. After 24 h, cells were treated with the 300 µmol/l tBHP for 24 h. At the end of the treatment, cells were incubated in the presence of 25 M-DCFH for 50 min. After incubation, dichlorofluorescein was measured using a fluorescence spectrophotometer (PerkinElmer) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Glutathione peroxidase activity

Glutathione peroxidase (GPX) activity in cell lysate was measured using a kit (Nanjing Jiancheng Bioengineering Institute) based on the method of Hafeman *et al.*⁽³⁰⁾ – a coupled assay using H₂O₂ and dithio-bis-nitrobenzoic acid. One unit of GPX is defined as the amount of enzyme that catalyses the oxidation of 1 nmol NADPH/min at 37°C, whereas heated samples with

inactivated enzymes were used as non-enzymatic controls to eliminate interference from endogenous reduced GPX. GPX activity was assessed by dinitrobenzoic acid colourimetry and read at a wavelength of 412 nm.

Western blotting

Total protein from blood samples in KBD patients and healthy controls was extracted using a Western and IP Cell Lysis Kit (Beyotime). C28/I2 chondrocytes were scraped in RIPA lysis buffer plus a protease inhibitor cocktail (Sigma) (50 mM-TRIS, 150 mM-NaCl, 1 mM-EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM-PMSF, 1 mM- Na_3VO_4 , 1 mM-NaF, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ pepstatin). Protein concentration of samples was determined with the bicinchoninic acid assay method. Equivalent amounts of sample protein were separated in 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Millipore) using a semi-dry transfer method. The membrane was then blocked with 5% milk in TRIS-buffered saline solution (TBS) with 0.1% Tween-20 and incubated with primary antibodies at 4°C overnight (JNK 1:1000; p-JNK 1:1000; α -tubulin 1:5000; c-jun 1:1000; activating transcription factor 2 (ATF2) 1:1000; p-c-jun 1:1000; mitogen-activated protein kinase kinase 1 (MEKK1) 1:500; B-cell lymphoma 2 (Bcl-2) 1:1000; and β -actin 1:1000, all from Santa Cruz), followed by incubation with the matching horseradish peroxidase-conjugated IgG antibody (1:8000; Pierce Company) at room temperature for 1 h. Bands were developed with enhanced chemiluminescence (ECL) (Amersham ECL Prime Western Blotting Detection Reagent, RPN2232; GE HealthCare) and exposure to X-ray film. The immunoblots were visualised and quantified by gel imaging system (GDS-8000 type; UVP).

Statistical analysis

Data are presented as mean values and standard deviations unless otherwise indicated. Student's *t* test was used to compare two groups. $P \leq 0.05$ was considered to be statistically significant.

Results

Phosphorylated c-Jun N-terminal kinase protein expression was greater in Kashin–Beck disease patients compared with healthy controls

The protein expression levels of JNK and p-JNK in whole blood from KBD patients and healthy controls were detected by Western blot. Western blotting demonstrated that JNK phosphorylation was significantly increased (normalised to α -tubulin) in KBD patients compared with that in the healthy controls ($P < 0.05$) (Fig. 1, right panel), but the levels of total JNK protein in KBD patients remained unaltered (Fig. 1, left panel).

Effect of tert-butyl hydroperoxide on chondrocyte survival

Chondrocytes were exposed to increasing concentrations of tBHP (100–500 $\mu\text{mol}/\text{l}$) at different time points (6–24 h), and cell viability was determined by the MTT assay (Fig. 2(a) and (b)).

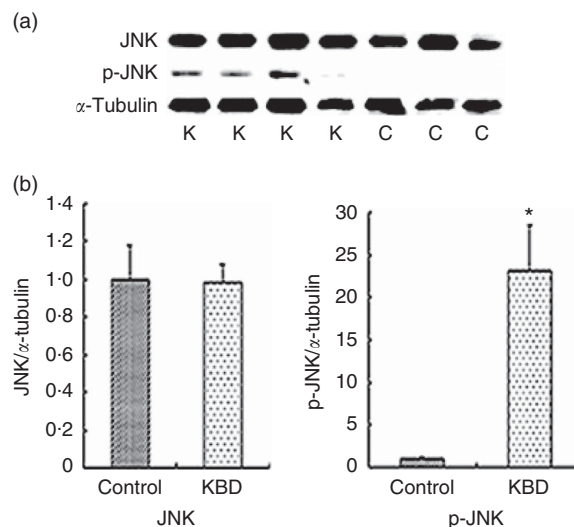


Fig. 1. Increased protein expression of phosphorylated c-Jun N-terminal kinase (p-JNK) in whole blood of Kashin–Beck disease (KBD) patients compared with healthy control subjects. (a) Protein extracts from patient and healthy control whole blood samples were prepared and analysed by immunoblotting with JNK, p-JNK and α -tubulin antibodies. (b) Signal intensity was then quantified, and the results of the densitometric analysis are shown as mean values and standard deviations represented by vertical bars for p-JNK expression relative to α -tubulin (right panel) and mean values and standard deviations represented by vertical bars for JNK expression relative to α -tubulin (left panel). One experiment is representative of three independent experiments. p-JNK protein expression levels in the KBD group were significantly higher than that in the control group ($P < 0.05$). K, samples from KBD patients; C, samples from control subjects. * $P < 0.05$.

tBHP induced a time- and dose-dependent inhibition of cellular proliferation in chondrocytes. We chose the exposure time of 24 h and the concentration of 300 $\mu\text{mol}/\text{l}$ for the subsequent experiment because the cell survival rate reached 40–60% of the normal control group at this point.

Protective effect of Na_2SeO_3 on tert-butyl hydroperoxide-induced cytotoxicity

Exposure to tBHP induced about 50% decrease in cell viability (Fig. 3). However, pre-treatment with 0.05 and 0.1 $\mu\text{g}/\text{ml}$ Na_2SeO_3 for 24 h significantly increased cell viability, indicating that pre-treated cells were protected against oxidative injury.

We next addressed whether tBHP reduces cell viability of chondrocytes by enhancing apoptosis. We first measured apoptosis induced by tBHP by Hoechst 33342 staining. Fig. 4 shows the chondrocyte apoptosis detected by Hoechst 33342 staining and the protective effects of different Na_2SeO_3 concentrations in C28/I2 cells. Compared with the control group, apoptotic nuclei were obviously increased in the tBHP injury group. Pre-treatment with different concentrations of Na_2SeO_3 for 24 h decreased apoptotic nuclei in the three Se treatment groups. The middle Se pre-protection group (0.1 mg/ml Na_2SeO_3) exhibited the lowest apoptotic nuclei numbers among the three groups. Our results indicate that tBHP injury could induce apoptosis, whereas Na_2SeO_3 could reduce apoptosis in chondrocytes.

Western blot analysis of the apoptosis-related proteins further confirmed the occurrence of apoptosis. As shown in Fig. 5, quantification of band intensity showed that the chondrocytes

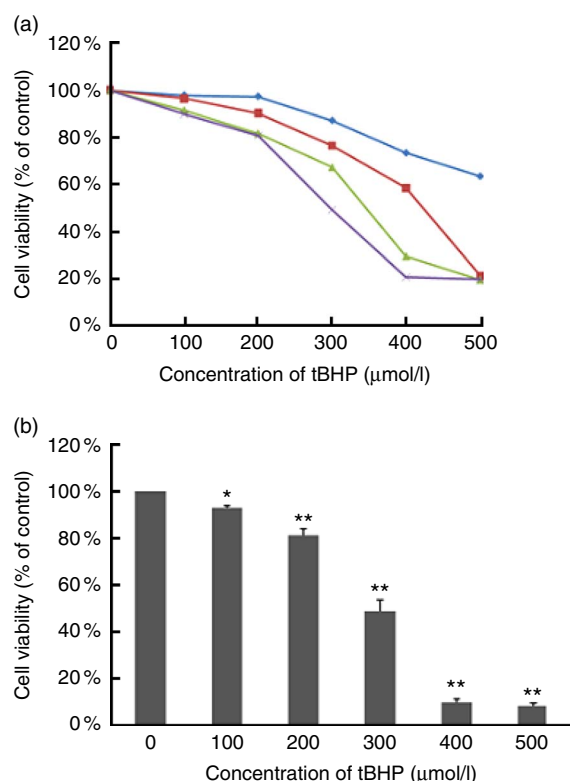


Fig. 2. Effects of different concentrations of tert-butyl hydroperoxide (tBHP) on the cellular viability of chondrocytes were estimated by methylthiazolyl tetrazolium (MTT) reduction. (a) Cells were incubated in absence or presence of several tBHP concentrations for different time periods (6–24 h: —◆—, 6 h; —■—, 12 h; —▲—, 18 h; —×—, 24 h). (b) Chondrocytes were treated with various doses of tBHP for 24 h and viability was determined by MTT. Values are means and standard deviations represented by vertical bars. One experiment is representative of four independent experiments. Mean value was significantly different from that of the control group: * $P < 0.05$, ** $P < 0.01$ (chondrocytes untreated with tBHP).

treated with tBHP (300 μmol/l) for 24 h enhanced the expression levels of pro-apoptotic proteins p-c-jun, c-jun and ATF2 compared with the control group ($P < 0.01$ or 0.05) and produced a decrease in the anti-apoptotic protein Bcl-2 ($P < 0.01$). Compared with the tBHP injury group, the expression levels of p-c-jun, c-jun and ATF2 protein in the three Na_2SeO_3 pre-protection groups showed a down-regulated trend. The expression levels of p-c-jun and ATF2 in the high Se pre-protection group significantly decreased ($P < 0.01$). The expression levels of p-c-jun, c-jun and ATF2 in the middle Se pre-protection group significantly decreased ($P < 0.05$). The expression levels of p-c-jun and ATF2 in the low Se pre-protection group significantly decreased ($P < 0.05$). The expression level of Bcl-2 in the low Se pre-protection group significantly increased ($P < 0.05$).

Effect of Na_2SeO_3 on reactive oxygen species generation

To evaluate the cellular oxidative stress generated from tBHP, the intracellular ROS production was measured. Cells treated with tBHP showed a significant increase in ROS generation compared with untreated controls (Fig. 6(a), $P < 0.05$). However, pre-treatment of chondrocytes with Na_2SeO_3 significantly

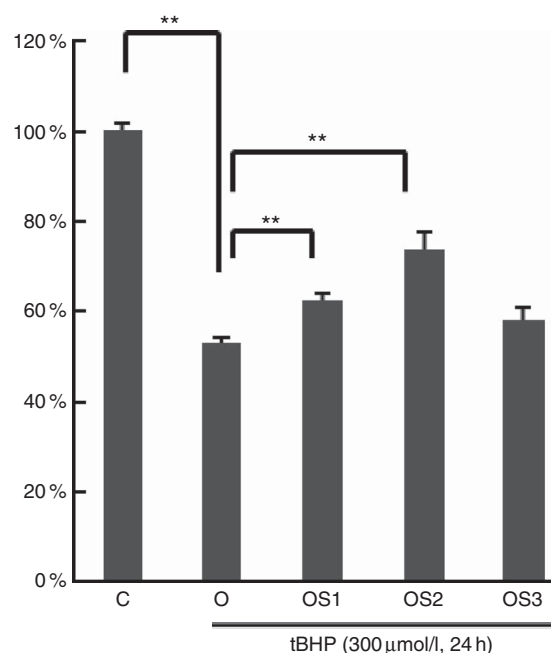


Fig. 3. Protection against tert-butyl hydroperoxide (tBHP)-induced oxidative stress by Na_2SeO_3 . Cell viability was determined by the methylthiazolyl tetrazolium (MTT) assay 24 h after exposure to tBHP, following a 24 h pre-treatment with Na_2SeO_3 . Treatment with tBHP alone was seen to significantly decrease cell viability. Na_2SeO_3 showed protection against tBHP-induced cell toxicity. Values are means and standard deviations represented by vertical bars. One experiment is representative of four independent experiments. C, control group; O, tBHP injury group (tBHP 300 mmol/l); OS1, low Se pre-protection group (0.05 mg/ml Na_2SeO_3 + 300 mmol/l tBHP); OS2, middle Se pre-protection group (0.1 mg/ml Na_2SeO_3 + 300 mmol/l tBHP); OS3, high Se pre-protection group (0.15 mg/ml Na_2SeO_3 + 300 mmol/l tBHP). ** $P < 0.01$.

reduced ROS generation in the presence of tBHP compared with the tBHP injury group ($P < 0.05$ or 0.01).

Effect of Na_2SeO_3 on glutathione peroxidase activity

The cellular antioxidant enzyme system plays an important role in the defence against oxidative stress, and changes in the activity of antioxidant enzymes can be used as biomarkers of antioxidant response. Therefore, the effect of Na_2SeO_3 on GPX activity in C28/I2 chondrocytes was evaluated (Fig. 6(b)). Treatment with tBHP induced a remarkable decrease in GPX activity compared with the control ($P < 0.05$). However, pre-treatment with Na_2SeO_3 increased GPX activity. The low Se pre-protection group prevented GPX activity depletion to near control levels. The middle Se pre-protection group and the high Se pre-protection groups showed significantly higher GPX activity.

Effect of Na_2SeO_3 on c-Jun N-terminal kinase signalling pathway

The JNK pathway plays important roles in the stimulation of apoptotic signalling as well as inflammatory diseases. As shown in Fig. 7(a) and (c), both p-JNK and MEKK1 protein levels were significantly increased in tBHP-treated cells. Densitometry analysis showed that p-JNK and MEKK1 levels, when normalised to β -actin levels, were significantly increased in the tBHP

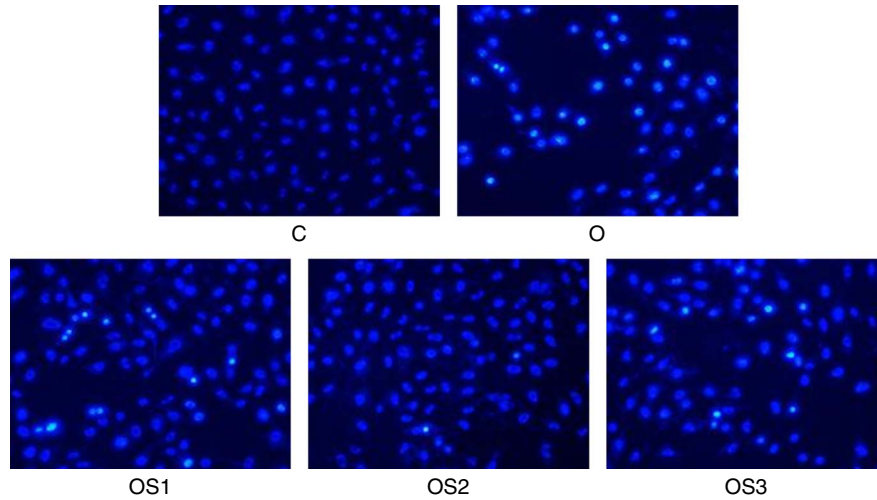


Fig. 4. The apoptosis of C28/I2 human chondrocytes detected by Hoechst 33342 staining and the protective effects of different Na_2SeO_3 concentrations on C28/I2 cells. Tert-butyl hydroperoxide (tBHP) injury obviously increased apoptosis of C28/I2 chondrocytes, whereas pre-treatment with Na_2SeO_3 reduced apoptosis. One experiment is representative of three independent experiments. C, control group; O, tBHP injury group (tBHP 300 $\mu\text{mol/l}$); OS1, low Se pre-protection group (0.05 mg/ml Na_2SeO_3 + 300 $\mu\text{mol/l}$ tBHP); OS2, middle Se pre-protection group (0.1 mg/ml Na_2SeO_3 + 300 $\mu\text{mol/l}$ tBHP); OS3, high Se pre-protection group (0.15 mg/ml Na_2SeO_3 + 300 $\mu\text{mol/l}$ tBHP).

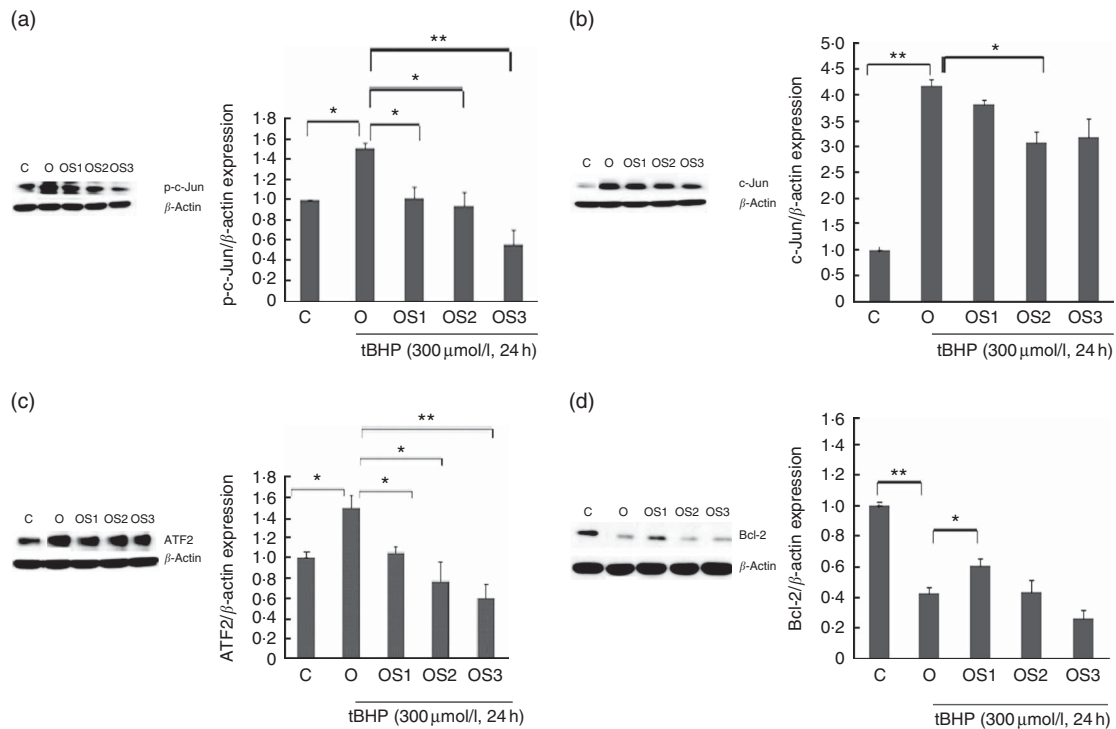


Fig. 5. The expressions of apoptosis-related proteins in C28/I2 cells using Western blots. (a) Protein extracts were prepared and analysed by immunoblotting with antibodies recognising phosphorylated (p)-c-Jun and β -actin. Signal intensity was then quantified and the results of the densitometric analysis are shown as mean values and standard deviations represented by vertical bars for p-c-Jun expression relative to β -actin (right panel). (b) Protein extracts were prepared and analysed by immunoblotting with c-Jun and β -actin antibodies. Signal intensity was then quantified and the results of the densitometric analysis are shown as mean values and standard deviations represented by vertical bars for c-Jun expression relative to β -actin (right panel). (c) Protein extracts were analysed by immunoblotting with activating transcription factor 2 (ATF2) and β -actin antibodies. The results of the densitometric analysis are shown as mean values and standard deviations represented by vertical bars for ATF2 expression relative to β -actin (right panel). (d) The results of immunoblotting with antibodies recognising B-cell lymphoma 2 (Bcl-2) and β -actin and densitometric analysis are shown as mean values and standard deviations represented by vertical bars for Bcl-2 expression relative to β -actin (right panel). For all, one experiment is representative of four independent experiments. C, control group; O, tBHP injury group; OS1, low Se pre-protection group; OS2, middle Se pre-protection group; OS3, high Se pre-protection group. * $P < 0.05$, ** $P < 0.01$.

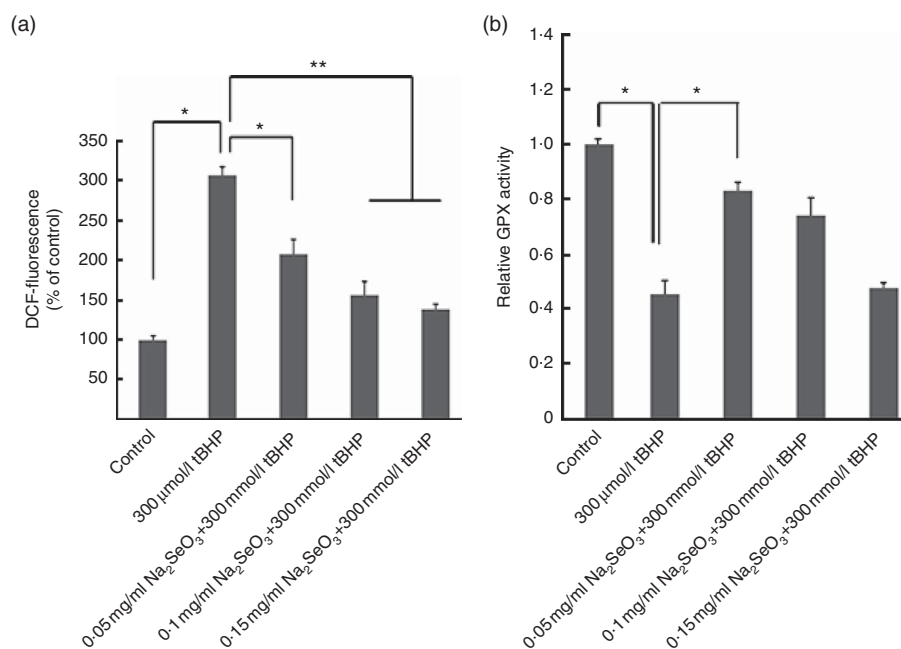


Fig. 6. Intracellular reactive oxygen species (ROS) levels and activity of glutathione peroxidase (GPX). (a) ROS levels after pre-treatment with various concentrations of Na₂SeO₃ for 24 h followed by treatment with tert-butyl hydroperoxide (tBHP) (300 mmol/l) for 24 h. Treatment with tBHP significantly increased the ROS levels. Pre-treatment with Na₂SeO₃ significantly decreased the ROS levels. Values are means and standard deviations represented by vertical bars. One experiment is representative of four independent experiments. * $P < 0.05$, ** $P < 0.01$. (b) Activity of GPX. Values are means and standard deviations represented by vertical bars. * $P < 0.05$. DCF, 5-(and-6)-chloromethyl-2,7-dichlorofluorescein.

injury group ($P < 0.01$) compared with the control group. tBHP treatment for 24 h did not significantly affect JNK protein expression in chondrocytes (Fig. 7(b), $P > 0.05$). Compared with the tBHP injury group, the expression levels of p-JNK and MEKK1 in the Se pre-protection group significantly decreased ($P < 0.01$ or 0.05).

Discussion

In this study, we demonstrated that JNK phosphorylation was up-regulated in KBD patients and tBHP-treated chondrocytes; tBHP treatment reduced cell viability, proliferation of chondrocytes, induced apoptosis and produced oxidative stress. Moreover, we showed that tBHP decreased anti-apoptotic protein Bcl-2 and increased levels of phosphorylated p-c-jun, c-jun and ATF2. tBHP treatment also up-regulated JNK signalling. In the parallel experiments, Se supplementation elicited protective effects on chondrocytes, including the inhibition of cell viability, apoptosis and oxidative injury, the attenuation of JNK signalling pathways, the reduction of phosphorylated c-jun, c-jun and ATF2 protein expressions and an increase in Bcl-2 protein expression. These findings are in accordance with a previous report⁽¹⁴⁾ indicating the involvement of JNK pathway in KBD via ATF2 on cartilage and chondrocytes.

The mammalian JNK are encoded by three distinct genes (*Jnk1*, *Jnk2* and *Jnk3*)⁽¹⁶⁾. The classical JNK pathway is activated following the exposure of cells to extracellular stresses such as UV irradiation, hyperosmolarity and heat shock⁽³¹⁾. The range of initiating signals has been expanded to include a diversity of stimuli. After JNK are activated, they subsequently phosphorylate a variety of substrates that regulate a wide range of cellular

functions⁽³²⁾. The JNK were originally identified by their ability to phosphorylate both Ser63 and Ser73 within the transactivation domain of the transcription factor c-Jun, which potentiates its transcriptional activity⁽³³⁾. JNK phosphorylates and regulates the activity of transcription factors other than c-Jun, including ATF2, Elk-1, p53 and c-Myc as well as non-transcription factors such as members of the Bcl-2 family⁽³²⁾. Several reports suggest a critical role of JNK in arthritis^(25,34,35). The inflammatory process in patients with OA and RA involves activation of the JNK signalling pathway. In thermal stress-induced activation of chondrocytes, the suppression of JNK activity helps maintain the capacity of chondrocytes for proteoglycan synthesis⁽³⁶⁾. Notably, JNK signalling in RA synovial tissue is activated early in the course of disease⁽³⁷⁾. Furthermore, studies have shown that JNK inhibitors are potential therapeutic agents for the management of a variety of inflammatory disorders^(38,39). In this study, we found that the expression level of p-JNK was significant higher in KBD patients compared with controls. Accordingly, the major pathological changes in KBD patients are chondrocyte apoptosis and death in the deep layers of the affected cartilage and the apoptosis and necrosis mediated by oxidative stress, which could be ameliorated by Se supplementation^(5,15,40). In order to further explore the mechanism of JNK pathway up-regulation in chondrocyte apoptosis and the protective effects of Se, we conducted a C28/I2 chondrocytes experiment. Our results demonstrated that chondrocyte apoptosis increased and the JNK pathway was up-regulated in the tBHP treatment group, which is similar to observations in KBD patients. Chondrocyte apoptosis decreased and the JNK pathway was down-regulated in the Se treatment group. These findings suggest that up-regulation of the JNK pathway may

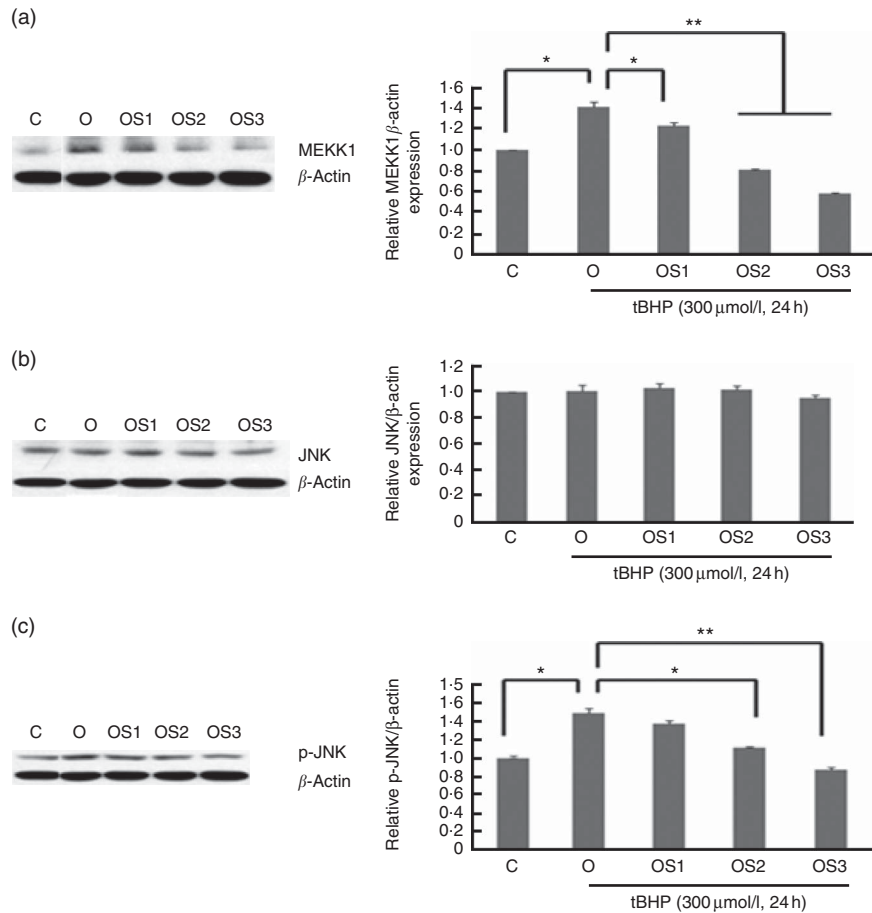


Fig. 7. Effect of Na_2SeO_3 on the c-Jun N-terminal kinase (JNK) signalling pathway. (a–c) Western blotting and densitometric analysis results of mitogen-activated protein kinase kinase 1 (MEKK1), JNK and phosphorylated JNK (p-JNK). It showed that tert-butyl hydroperoxide (tBHP) injury for 24 h increased MEKK1 and p-JNK levels but not JNK expression in chondrocytes. Pre-treatment with various concentrations of Na_2SeO_3 for 24 h significantly decreased the expression levels of MEKK1 and p-JNK. One experiment is representative of four independent experiments. Values are means and standard deviations represented by vertical bars. C, control group; O, tBHP injury group; OS1, low Se pre-protection group; OS2, middle Se pre-protection group; OS3, high Se pre-protection group. Values are statistically significant at * $P < 0.05$ and ** $P < 0.01$.

play an important role in chondrocyte apoptosis and necrosis. Furthermore, Se supplementation can ameliorate cell apoptosis by regulating the JNK pathway.

In addition, MEKK1 protein levels were significantly increased in the tBHP injury group of chondrocytes. Of the three MAPK families, JNK is particularly interesting because it efficiently phosphorylates c-Jun. Among the components of the MAPK family, MEKK1 is a 196-kDa serine–threonine kinase that is activated in response to cytokines and various stresses. MEKK1 preferentially activates the JNK pathway and also influences the activity of the extracellular-signal-regulated kinases (ERK) pathway^(41–43). The roles of MEKK1 and downstream JNK have been extensively studied in many research areas. For instance, MEKK1 is required for JNK and c-Jun activation in the corneal epithelia of MEKK1–/– mice⁽⁴⁴⁾. MEKK1 is also critical for JNK activation in response to pro-inflammatory stimuli and cell migration in MEKK1–/– mouse embryo fibroblasts (MEF)⁽⁴⁵⁾. On the basis of our studies using tBHP-injured chondrocytes, MEKK1 protein level was significantly increased during phosphorylated JNK activation in cultured chondrocytes. This study suggests that MEKK1 is a crucial activator of the JNK pathway in chondrocytes.

Se has been supplied for the past few decades to the residents of affected areas in China for the prevention of KBD. Se is an essential element for humans, animals and other species. Se is incorporated into proteins not simply through ionic association, as most metals are, but is covalently bonded within the amino acid selenocysteine, the twenty-first amino acid⁽⁴⁶⁾. Se deficiency induces dysfunction of selenoproteins. Selenoproteins have been shown to exhibit a variety of biological functions including antioxidant functions, maintaining cellular redox balance and heavy metal detoxification, and compromise of such important proteins would lead to oxidative stress and apoptosis^(47,48). Moreover, there is a significantly lower Se level in the grains in KBD areas when compared with non-KBD areas, suggesting a close relationship between Se and KBD occurrence⁽⁴⁹⁾. In this study, we found that tBHP induced the inhibition of cellular proliferation, excessive oxidative stress in chondrocytes, leading to chondrocyte apoptosis. The protein expression levels of p-JNK, MEKK1, p-c-Jun, c-Jun and ATF2 in the tBHP treatment group were higher than that in the control group and Bcl-2 was lower than that in the control group. Supplementation with Se caused a large increase in GPX activity

and level of Bcl-2 in the low Se protection treatment and an obvious decrease in p-JNK, MEKK1, p-c-jun, c-jun and ATF2 in the low and middle Se protection groups. It is likely that tBHP-induced oxidative stress, which in turn led to tissue degeneration similar to that observed in KBD patients. On the contrary, Se can partly block tBHP-induced oxidative and chondrocyte apoptosis because of its antioxidant functions. These findings suggest that oxidative damage could lead to activation of the JNK signalling pathway, whereas Na₂SeO₃ supplementation could inhibit the activation and minimise oxidative damage. Therefore, Na₂SeO₃ exerts its anti-apoptosis effect to protect cartilage cells from oxidative stress injury by the inhibition of JNK activation.

In conclusion, elevated expression levels of p-JNK were observed in KBD patients. Chondrocyte apoptosis induced by oxidative stress might be mediated via activation of the JNK signalling pathway. Thus, the activation of the JNK pathway may play an important role in chondrocyte apoptosis. Se exhibits anti-apoptotic effects by down-regulating the JNK signalling pathway. These findings provide the experimental evidence to elucidate the role of JNK pathway in the pathogenesis of KBD.

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The authors declare that they have no conflicts of interest.

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