The Study of *GPX3* Methylation in Patients with Kashin-Beck Disease and its mechanism in chondrocyte apoptosis

Lixin Han[1§], Xiaoli Yang[1§], Wenyan Sun[1], Zhaofang Li[1], Hao Ren[1], Baorong Li[1], Rongqiang Zhang[1], Dandan Zhang[1], Ziyun Shi[1], Jifeng Liu[1], Junling Cao[1], Jianjun Zhang[2], Yongmin Xiong[1*]

1. Institute of Endemic Diseases and Key Laboratory of Trace Elements and Endemic Diseases, National Health Commission of the People's Republic of China, School of Public Health, Xi′an Jiaotong University Health Science Center, No.76 Yanta West Road, Xi′an, Shaanxi 710061, P.R. China.
2. Department of Epidemiology, Indiana University Richard M. Fairbanks School of Public Health, Indianapolis, 1050 Wishard Boulevard, IN 46202, USA

§ Co-first author

*Corresponding author: Yongmin Xiong. Institute of Endemic Diseases, School of Public Health, Xi'an Jiaotong University Health Science Center, No 76 Yanta West Road, Xi'an, Shaanxi 710061, P. R. China. Tel: 86-29-82655193, Fax: +86-29-82655032, Email address: xiongym@mail.xjtu.edu.cn.

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Abstract

Objective: Selenium deficiency is a risk factor for Kashin-Beck disease (KBD), an endemic osteoarthropathy. Although promoter hypermethylation of glutathione peroxidase 3 (GPX3) (a selenoprotein) has been identified in several cancers, little is known about promoter methylation and expression of *GPX3* and their relation to selenium in KBD. The present study was thus conducted to investigate this research question.

Methods: Methylation and expressions of *GPX3* in whole blood drawn from 288 KBD patients and 362 healthy controls and in chondrocyte cell line were evaluated using methylation-specific PCR and qRT-PCR, respectively. The protein levels of PI3K/Akt/c-fos signaling in the whole blood and chondrocyte cell line were determined with Western blotting. Chondrocytes apoptosis were detected by Hoechst 33342 and Annexin V-FITC/PI staining.

Results: *GPX3* methylation was increased, *GPX3* mRNA was decreased, and protein levels in the PI3K/Akt/c-fos signaling pathway were up-regulated in the whole blood collected from KBD patients as compared with healthy controls. Similar results were obtained for chondrocytes injured by oxidative stress. There was a significant, decreasing trend in *GPX3* expression across groups of unmethylation, partial methylation, and complete methylation for *GPX3,* in sequence. Compared with unmethylation group, protein levels in PI3K/Akt/c-fos pathway were enhanced in partial and complete methylation groups. Treatment of chondrocytes with sodium selenite resulted in reduced methylation and increased expression of *GPX3* as well as down-regulated level of PI3K/Akt/c-fos proteins.

Conclusions: The methylation and expression of *GPX3* and expression of PI3K/Akt/c-fos pathway are altered in KBD and these changes are reversible by selenium supplementation.

Key words: Kashin-Beck disease; glutathione peroxidase 3; DNA methylation; PI3K/Akt/c-fos signaling pathway; selenium

1. **Introduction**

Kashin-Beck Disease (KBD) is an endemic osteoarthropathy that primarily occurs among children and adolescents and is manifested by joint pain, arthritis, muscle atrophy, deformed joints, and even dwarfism (1). The main pathological features of KBD are cartilage necrosis and excessive apoptosis of chondrocytes in the deep layers of epiphysis and articular cartilage (2, 3). KBD is prevalent in a large belt extending from Southeast Siberia, North Korea, Northeast China, to Southwest China (4). In China, more than 104 million people reside in KBD-affected areas, and 567,637 people were diagnosed with the disease, including 12,730 patients under 13 years of age (5). The etiology and pathogenesis of KBD largely remain unclear. A growing body of epidemiological evidence suggests that KBD develops as a consequence of the interaction between genes and environmental factors (6, 7). Selenium deficiency was observed to be an environmental risk factor for KBD in most epidemiological studies (3, 8), but the biochemical mechanisms by which selenium deficiency induces cartilage damage and thereby leads to an elevated risk of KBD are yet to be elucidated.

Epigenetics provides an optimal framework for investigating the mechanisms of environmental factors in KBD development. It is well documented that gene expression is regulated by epigenetic modifications, especially DNA methylation, in response to environmental exposures in a variety of diseases (9, 10). DNA hypermethylation is considered an important gene modification that prevents the recruitment of transcription factors and consequently suppresses the transcription of various genes (11). Several studies have revealed that selenium is involved in epigenetic modifications (12, 13). Selenoproteins are the main form of selenium that are responsible for numerous biological functions. One of these selenoproteins is glutathione peroxidases 3 (GPX3), a primary antioxidant enzyme in the human body (14). It is possible that epigenetic modifications of *GPX3* induced by oxidative stress modulate antioxidant gene transcription and mRNA stability. *GPX3*

differential methylation has been linked to cervical cancer, breast cancer, and multiple myeloma (15-17). Therefore, the methylation status of *GPX3* may also play a role in the pathogenesis of KBD and other diseases related to oxidative stress.

Some studies have demonstrated that the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/c-fos signaling pathway is involved in celllular oxidative damage and apoptosis (18, 19). In addition, the differential level of PI3K/Akt pathway were found in KBD patients compared with controls and related to selenoprotein S (SEPS1) -105G>A polymorphisms (7). However, *GPX3* DNA methylation status and its potential influence on cartilage damage among KBD patients have not been investigated up to date. Therefore, the present study was conducted to evaluate the association between *GPX3* DNA methylation and KBD risk and to elucidate the mechanisms of *GPX3* DNA methylation in the development of cartilage damage.

2. Materials and methods

2.1. Study population

KBD was diagnosed using the criteria of National Health Commission of the People's Republic of China (WS/T207-2010). A total of 288 KBD cases and 362 controls were recruited from KBD endemic and non-endemic areas in Shaanxi province, respectively, which excluded osteoarthritis and other joint diseases. All subjects enrolled to the present study were Han Chinese. Cases and controls were comparable with regard to age and sex. The study protocol was approved by the Human Ethics Committee of Xi'an Jiaotong University, and a written informed consent was obtained from each of the study subjects prior to in-person interview and blood collection.

A peripheral venous blood sample (5 ml) procured from each of study subjects were extracted for DNA, total mRNA, and proteins, and stored in freezers (-80℃) until analysis.

Independent experiments for *GPX3* DNA methylation, *GPX3* mRNA expression, and protein level described below were each repeated at least three times.

2.2. GPX3 DNA methylation

Genomic DNA were extracted from blood and chondrocytes using TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). Bisulfite conversion for methylation-specific polymerase chain reaction (MSP) was performed with EZ DNA Methylation Kit (ZYMO Research, Irvine, CA) according to the manufacturer's instructions. The methylation primers and unmethylation primers were used to amplify the 177-bp and 186-bp products from target sequences, respectively (20). The primer sequences are as follows: methylated primers (F: 5'-TATGTTATTGTCGTTTCGGGAC-3'; R: 5'-GTCCGTCTAAAATATCCGACG-3') and unmethylated primers (F: 5'-TTTATGTTATTGTTGTTTTGGGATG-3'; R: 5'-ATCCATCTAAAATATCCAACACTCC-3'). After bisulfite modification, a 50μL PCR was carried out in 5μL 10×PCR buffer, 4μL dNTP mixture, 1μL of each primer, 3μL bisulfite-modified DNA, 35.75μL ddH2O, and 0.25μL hot-start Taq-polymerase (Takara, Mountain View, CA). Thermal cycler conditions included an initial denaturation step of 95℃ for 10 min, followed by a three-step PCR program composed of 94℃ for 30 sec, 59℃ for 30 sec, and 72℃ for 30 sec for 40 cycles, and a final extension of 72℃ for 10 min. PCR products were identified by 2% agarose gels and visualized under the Light Transilluminator (UVP, Upland, CA).

2.3. GPX3 mRNA expression

Total RNA was isolated from the whole blood and chondrocyte samples using Trizol Kit (Life Technologies, Carlsbad, CA). cDNAs were synthesized with the RevertAidTM First Strand cDNA Synthesis Kit (MBI, Fermentas, Vilnius, Lithuania). Relative quantification of *GPX3* gene expression was performed using an iQe5 quantitative real-time PCR Detection Systems (Bio-Rad, Philadelphia, PA) and with β-actin as a reference gene. The primers used

for qRT-PCR were F: 5'-TTCACGACATCCGCTGGAA-3' and R: 5'-CATCTTGACGTTGCTGACCGT-3' for *GPX3* as well as F: 5'-GAACGGTGAAGGTGACAGCAG-3' and R: 5'-GTGGACTTGGGAGAGGACT-3' for *β-actin*. Amplification was run in a 20 μL reaction mixture containing 1.6 μL of cDNA, 0.8 μL of each primer, 10 μL 2×SYBR Premix Ex Taq Ⅱ (Takara, Mountain View, CA), and 6.8 μL ddH_2O. The reaction conditions were 94℃ for 2 min, 40 cycles at 94℃ for 10 sec, and 60℃ for 15 sec, with a final extension at 72℃ for 30 sec. All reactions were performed in duplicate. The data were normalized to the expression level of β-actin and analyzed by iQe5 software (version 2.0, Bio-Rad).

2.4. Protein level

Total proteins were extracted from whole blood and chondrocytes using Trizol Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Samples were separated by 10% SDS-PAGE and transferred to NC membranes (Millipore Ireland BV, Cork, Ireland). The membranes were blocked with 5% non-fat-milk at 37℃ for 1 hour. The primary antibodies against Gβγ, PI3Kp110, pAkt, c-fos, total Akt and β-actin were diluted with a ratio of 1: 500 (all purchased from Sigma, Fremont, CA) and incubated overnight at 4℃. After immunoreactive bands were detected with anti-rabbit IgG (1:10,000 dilution) (Pierce Company, WI), blots were developed using enhanced chemiluminescence (ECL) and visualized by gel imaging system (GDS-8000).

2.5. Oxidative damage induced by tBHP in C28/I2 human chondrocytes

The human C28/I2 chondrocytes (a human cell line provided by Dr. Mary Goldring from Cornell University) were cultured in DMEM/F-12 (Hyclone, Logan, UT) containing 12% (v/v) fetal bovine serum (SiJiQing Bio-Technique Co. Ltd., Zhejiang, China) and 1% (v/v) penicillin-streptomycin solution in a humidified incubator with 5% CO_2 at 37℃. When the cells grew and reached 90% confluence in dishes, they were seeded on 96-well culture plates.

The experiment was conducted in 6 groups: control group (C), purely Se group (S, 0.10 μg/mL), tert-butyl hydroperoxide (tBHP) injury group (O, tBHP 150 μmol/L), low Se group (OS1, 0.05 μg/mL Na_2SeO_3 + 150 μmol/L tBHP), medium Se group (OS2, 0.10 μg/mL Na_2SeO_3 + 150 μmol/L tBHP) and high Se group (OS3, 0.15 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS1, OS2 and OS3 were treated with different concentrations of Na_2SeO_3 (0.05, 0.10 and 0.15 μg/mL) for 24 hours, and then treated with 150 μmol/L tBHP for 24 hours.

2.6. Chondrocyte apoptosis

Chondrocytes were stained with 2% Hoechst 33342 in DMEM/F-12 containing 12% fetal bovine serum and then incubated at 37℃ for 30 min. Apoptotic chondrocytes with clear condensation and small bright nuclei were evaluated using a fluorescence microscope. The apoptotic rate of chondrocytes were measured using flow cytometry. Chondrocytes were collected and stained with Annexin V-FITC/PI according to the manufacturer's instructions (ROCHE, Basle, Switzerland). Cell fluorescence was detected by flow cytometry (BD, CA), and counts and percentages of live (annexin V-/PI-), early apoptotic (annexin V+/PI-), or late apoptotic/necrotic (annexin V+/PI+) cells were obtained. The apoptotic rates were analyzed using Cell Quest software version 1.0 (BD, Franklin Lakes, NJ).

2.7. Statistical analysis

The Kolmogorov-Smirnov test was used to evaluate the normality of the distribution. Continuous variables were denoted by mean ± standard deviation (SD), and differences in measured assays between two groups were assessed by two-tailed Student's t-test, as well as the differences among three or more groups were examined by one-way ANOVA. Categorical variables were presented as frequency counts, and differences in these variables were compared by Chi-square test. Odds ratio (OR) and 95% confidence interval (CI) were calculated to evaluate risk of KBD in relation to *GPX3* methylation. A p-value of <0.05

(two-sided) was considered statistically significant. All statistical analyses were performed with the SPSS 23.0 software (SPSS Inc., Chicago, IL).

3. Results

3.1. Demographic characteristics of study population

The baseline characteristics of the study population are shown as follows. Mean age (SD) was 51.9 (3.4) years for 288 KBD patients (151 men and 137 women) and 52.3 (3.8) years for 362 controls (168 men and 194 women). There were no significant differences in age (p = 0.16) and sex (p = 0.13) composition between the patients and controls.

Furthermore, 50 cases and 50 controls were randomly selected from the above population for detection of GPX3 methylation and gene expression. The demographic characteristics of the samples were shown in Table 1. No statistically significant differences were observed between both groups in age (P=0.202) and sex (P=0.841).

3.2. GPX3 methylation in whole blood

The *GPX3* methylation status of peripheral blood mononuclear cells (PBMCs) procured from 50 KBD patients and 50 healthy controls were examined using MSP. These patients and controls were randomly selected from all patients and controls enrolled to the present study. Complete methylation of *GPX3* was identified in 16% of KBD patients, but only in 2% of controls (p = 0.036). Results showed that the *GPX3* hypermethylation were associated with an increased risk of KBD [OR (95% CI): 9.33, 95% (1.12~77.7)].

3.3. mRNA expression of GPX3 in KBD patients

GPX3 mRNA level in whole blood was determined by qRT-PCR, and β-actin cDNA was the reference in the experiments. Results showed that *GPX3* expression levels were significantly reduced in KBD patients compared with those of health individuals (p = 0.001) (Figure 1A).

3.4. Protein level of PI3K/Akt/c-fos signaling pathway in KBD patients

The levels of Gβγ (11.2 fold), PI3Kp110 (1.89-fold), pAkt (3.27-fold), and c-fos (16.5-fold) were significantly increased in KBD group compared with those of control group (all $p < 0.001$) (Figure 2), suggesting that PI3K/Akt/c-fos signaling pathway was overexpressed in KBD patients.

3.5. Alterations in oxidatively injured chondrocytes

tBHP was used to induce the oxidatively damaged chondrocyte model. The effects of oxidative stress on cell apoptosis, methylation and mRNA level of *GPX3*, and protein levels of PI3K/Akt/c-fos were evaluated in human C28/I2 chondrocytes. Results with hoechst33342 staining showed that the number of chondrocyte apoptosis in tBHP injury group (65.96 ± 3.98) was significantly increased compared with that of the control group (1.30 ± 0.32). However, the number of chondrocyte apoptosis was decreased in all three selenium supplement groups (OS1: 14.54 ± 2.02, OS2: 4.15 ± 0.19, OS3: 14.74 ± 2.56) compared with tBHP injury group ($p < 0.05$) (Figure 3). The late apoptotic rate of chondrocytes in C, S, O, OS1, OS2, and OS3 groups were 0.09%, 7.05%, 41.01%, 39.47%, 36.75% and 29.36%, respectively (Figure 4). The late apoptotic rate of chondrocyte in O group was increased compared with C group, but it was reduced in OS1, OS2, OS3 groups, suggesting that Se supplement at concentrations from 0.05 μg/mL to 0.15 μg/mL could decrease apoptotic rate in human C28/I2 chondrocytes and exert a dose-dependent trends.

Complete methylation and unmethylation of *GPX3* was observed in the oxidative injury group and control group, respectively, indicating that oxidative damage resulted in methylation of this gene (Table 2). *GPX3* expression was lower in the tBHP injury group than in the control group ($p < 0.001$) (Figure 1B). Protein levels of Gβγ, PI3Kp110, pAkt and c-fos were detected in chondrocytes. As shown in Figure 5, the protein levels of Gβγ,

PI3Kp110, pAkt, and c-fos were increased in the tBHP group compared with those of the control group (all $p < 0.0001$).

3.6. GPX3 expression under different methylation status in chondrocytes

The results revealed that there was a significant difference on mRNA levels of *GPX3* among the three groups ($p < 0.0001$). The mRNA level of *GPX3* in unmethylation group was higher than that in partial methylation group and in complete methylation group (all $p < 0.01$), while mRNA level of *GPX3* was higher in the partial methylation group than in complete methylation group ($p < 0.01$). These results indicate that reduced *GPX3* gene expression was mediated through by its increased methylation (Figure 1C).

3.7. Protein levels of PI3K/Akt/c-fos signaling in relation to methylation status in chondrocytes

Compared with unmethylation group, protein levels of Gβγ, PI3Kp110, pAkt, and c-fos were increased in partial methylation group (all $p < 0.01$) and complete methylation group (all $p < 0.01$). The protein levels of Gβγ, PI3Kp110, and c-fos in partial methylation group were significantly higher than those in complete methylation group (all $p < 0.01$) (Figure 6), suggesting that the down-regulation of PI3K/Akt/c-fos protein level was correlated with a decreased *GPX3* methylation.

3.8. Effects of Selenium on expression and methylation of GPX3 and PI3K/Akt/c-fos signaling in chondrocytes

It was found that *GPX3* was completely methylated in the tBHP injury group, partially methylated in low Se and medium Se groups, and unmethylated in high Se group (Table 2), suggesting that selenium supplementation reduced *GPX3* methylation. The *GPX3* mRNA expression was significantly up-regulated in Se supplementation group than in the tBHP injury group (all $p < 0.01$) (Figure 1B). Our results revealed that down-regulated *GPX3*

expression was related to *GPX3* hypermethylation in chondrocytes and Se treatment could up-regulate the mRNA expression levels of *GPX3.*

Compared with the O group, a significant reduction in protein levels were found for PI3Kp110 in OS2 and OS3 groups, Gβγ in OS1 and OS2, pAkt in OS3 group, and c-fos in three Se supplementation groups (all $p < 0.01$) (Figure 5). These results suggest that there are a significantly up-regulated protein levels of PI3K/Akt/c-fos when chondrocytes are damaged by oxidative stress and that selenium supplementation can down-regulate protein levels of PI3K/Akt/c-fos signaling.

4. Discussion

The characteristics of pathological changes in KBD are the necrosis and excessive apoptosis in articular and epiphyseal plate cartilages. Oxidative stress has been identified to contribute to the pathogenesis of KBD (3). The serum levels of thiobarbituric acid were higher and GPX levels were lower in KBD patients than in controls, suggesting that selenium deficiency may alter oxidative stress in cartilage tissues (2). Recent studies have revealed that oxidative stress induced epigenetic dysregulation (10, 21), and accumulation of excess cellular reactive oxygen species resulted in increased DNA mutations and subsequent carcinogenesis (22). An increasing body of evidence has demonstrated that *GPX3* promoter hypermethylation are present in several cancers (21-26). In addition, an *in vitro* reduction in *GPX3* expression was associated with an elevated generation of reactive oxygen species and an increased formation of DNA damage in complex diseases or abnormal health conditions such as cancer and metabolic syndrome (10, 23). To date, however, little is known about the patterns of *GPX3* promoter methylation in KBD.

In the present study, we investigated the status of *GPX3* promoter methylation between KBD patients and healthy controls. It was found that the frequency of *GPX3* DNA methylation was significantly higher in KBD patients than in controls. In addition, *GPX3*

hypermethylation was associated with a more than 9-fold increased risk of KBD. Our study offers novel evidence supporting that *GPX3* promoter hypermethylation might be partially involved in the pathogenesis and etiology of KBD. DNA methylation and tissue-specific gene expression are the most common epigenetic alterations. DNA methylation has been used as biomarkers for early diagnosis, progression, and prognosis of various cancers (22-24). Our results showed that the expression of *GPX3* was significantly down-regulated in KBD patients compared with healthy controls, indicating that DNA methylation might suppresses the expression of *GPX3*. Sabrina et al. (10) reported that GPX activity depended on the transcription levels of its related genes, which were themselves subject to regulation by methylation of their promoter regions. Therefore, it is possible that *GPX3* expression was silenced in KBD patients related to *GPX3* hypermethylation, resulting in reduced GPX3 activity and subsequent compromised antioxidative and antiapoptotic functions.

Although *GPX3* promoter hypermethylation induced by oxidative stress has been observed to down-regulate its expression, other mechanisms modulating *GPX3* expression are not well understood. DNA methylation is involved not only in transcriptional regulation and DNA damage repair, but also in intracellular signal transduction (10). PI3K/Akt/c-fos is the predominant signaling pathway of chondrocyte proliferation, growth, and apoptosis (27, 28). Similarly, GPX3 methylation is related to cell apoptosis (29). Therefore, we attempted to explore the relationship between GPX3 methylation and PI3K/Akt/c-fos signaling pathway. Of note, we found that the protein levels of Gβγ, PI3Kp110, pAkt and c-fos in KBD patients were significantly elevated in KBD patients, indicating that PI3K/Akt/c-fos signaling is substantially enhanced in KBD patients.

Several studies have demonstrated that the excessive apoptosis of chondrocytes and oxidative stress plays a critical role in the pathophysiology of KBD (30, 31); however, the underlying mechanisms remain unclear. The present study explored the mechanisms of *GPX3*

methylation in oxidatively damaged chondrocytes. We found that *GPX3* was completely methylated in oxidative damage group, but was unmethylated in control group and purely Se group. Furthermore, downregulated *GPX3* expression was observed in oxidative damage group. These results indicated that oxidative stress could result in epigenetic dysregulation in chondrocytes. We also investigated the mRNA levels of *GPX3* and protein levels of PI3K/Akt/c-fos signaling molecules under different degrees of *GPX3* DNA methylation. It was found that the mRNA levels of *GPX3* was significantly decreased in the complete methylation group compared with unmethylation group. The protein levels of PI3K/Akt/c-fos were higher in complete methylation group than in unmethylation group. Taken together, our study suggests that *GPX3* promoter methylation might be associated with the downregulation of *GPX3* and that the PI3K/Akt/c-fos signaling pathways is up-regulated by hypermethylation of *GPX3*. Hypermethylation status was often accompanied by an increased apoptosis of chondrocytes. Of particular interest is that aforementioned findings were similar to those obtained from KBD patients. Our study provides additional evidence for a role of *GPX3* hypermethylation in chondrocyte apoptosis. Potential mechanisms for this observation might be that chondrocyte apoptosis arises as a consequence of *GPX3* inactivation mediated through its hypermethylation and elevated levels of proteins involved in PI3K/Akt/c-fos signaling pathway. Similar findings were reported in several studies of multiple cancers in which *GPX3* expression was down-regulated by its promoter hypermethylation, leading to reduced GPX3 capacity against oxidative damage (14, 20, 24, 25, 32).

Epigenetics is a biological process that is modulated by environmental factors, especially nutrition (10). Selenium is a micronutrient that serves as a co-factor of GPX, a primary antioxidant enzyme. As mentioned above, selenium deficiency is a main risk factor of KBD, but its effects on cartilage damage are not fully understood. Therefore, it is important to elucidate biological mechanisms by which selenium deficiency is implicated in the etiology

and molecular pathogenesis of KBD. Inadequate selenium nutritional status has also been linked to a variety of human diseases, including cancer and cardiovascular disease (33, 34). Recent studies have demonstrated that selenium plays an important role in epigenetic transcriptional regulation. Selenium can modulate DNA and histones to activate methylation-silenced genes (13) and regulate inflammatory gene expression at the epigenetic level (12). As the methylation is a reversible process, removal of methylation could restore *GPX3* gene function (22). Our study showed that selenite treatment resulted in promoter DNA demethylation and reexpression of *GPX3* as well as downregulation of protein levels of PI3K/Akt/c-fos. All of these changes might promote anti-apoptosis. Our study demonstrates that methylation status of *GPX3* could be reversed by selenium supplementation. Specifically, selenium could activate methylation-silenced *GPX3* gene by modifying DNA methylation and regulating PI3K/Akt/c-fos signaling pathway in chondrocytes.

In conclusion, our results showed that *GPX3* promoter hypermethylation and down-regulated expression of *GPX3* gene in KBD patients. Furthermore, *GPX3* hypermethylation down-regulates its expression and up-regulates the proteins in the PI3K/Akt/c-fos signaling pathway. Selenium significantly protected against tBHP-induced chondrocyte apoptosis, possibly mediated through reduced *GPX3* DNA methylation, increased *GPX3* gene expression, and decreased PI3K/Akt/c-fos signaling. The findings of the present study will provide a new insight into the pathogenesis, etiology, and prevention of KBD.

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Conflict of interests

The authors declare no conflict of interest.

Authors' contributions

All authors were involved in drafting the manuscript or critically revising it for important intellectual content and approved the final version of submitted manuscript. Yongmin Xiong takes final responsibility for the integrity of the work. Study conception and design: Lixin Han, Xiaoli Yang, Yongmin Xiong. Acquisition of data: Lixin Han, Xiaoli Yang, Zhaofang Li, Hao Ren, Baorong Li, Rongqiang Zhang, Dandan Zhang, Ziyun Shi, Jifeng Liu, Junling Cao. Analysis and interpretation of data: Yongmin Xiong, Lixin Han, Xiaoli Yang, Hao Ren, Jianjun Zhang, Wenyan Sun.

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Figure legends

Figure 1. The *GPX3* expression in KBD patients and chondrocytes. (A) The expression of *GPX3* in whole blood among KBD patients and controls. The mRNA levels of *GPX3* were significantly down-regulated in KBD group as compared with control group ($p = 0.001$). (B) The *GPX3* expression in cultured human C28/I2 chondrocyte. The expression levels of *GPX3* were lower in the O group than in the C group ($p < 0.001$). Compared with the O group, the *GPX3* expressions in three Se supplement group were up-regulated significantly ($p < 0.05$). C: control group. S: purely Se group (0.05 μg/mL Na_2SeO_3). O: tBHP injury group (tBHP 150 μmol/L). OS1: low Se group (0.05 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS2: medium Se group (0.1 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS3: high Se group (0.15 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). (C) The *GPX3* expressions in chondrocyte under different methylation status. The mRNA level of *GPX3* in unmethylation group was higher than that in partial methylation group and in complete methylation group (all $p < 0.01$), while mRNA level of *GPX3* was higher in the partial methylation group than in complete methylation group ($p < 0.01$). UM: unmethylation CM: complete methylation. PM: partial methylation.

* represents significance at $p < 0.05$.

Figure 2. Protein levels of PI3K/Akt/c-fos in whole blood between the KBD patients and controls. (A) The protein level of Gβγ. (B) The protein level of PI3Kp110. (C) The protein level of pAkt. (D) The protein level of c-fos. All protein levels in KBD group were significantly higher than that in control group ($p < 0.001$). K1, K2 and K3: samples from KBD patients; C1, C2 and C3: samples from control subjects. *represents significance at $p < 0.05$.

Figure 3. The apoptosis numbers were detected by Hoechst 33342 staining in human C28/I2 chondrocyte. Compared with C and S groups, the apoptosis numbers were obviously increased in O group, while the apoptosis numbers were reduced by selenite treatment. C: Control group. S: purely Se group (0.05 μg/mL Na_2SeO_3). O: tBHP injury group (tBHP 150 μmol/L). OS1: low Se group (0.05 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS2: medium Se group (0.1 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS3: high Se group (0.15 μg/mL Na_2SeO_3 + 150 μmol/L tBHP).

Figure 4. The apoptosis rate were detected by Annexin V-FITC/PI double staining in cultured human C28/I2 chondrocyte. (A) Annexin V-FITC/PI staning in chondrocytes. (B) The late apoptosis rate in chondrocytes. The late apoptotis rate of chondrocyte in O group was increased compared with C group, but it was reduced in OS1, OS2 and OS3 groups. Numbers in each quadrant indicated the percentage of chondrocytes labeled with annexin V-FITC (top left), PI (bottom right), annexin V-FITC and PI (top right), and untaged (bottom left). C: Control group. S: purely Se group (0.05 μg/mL Na_2SeO_3). O: tBHP injury group (tBHP 150 μmol/L). OS1: low Se group (0.05 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS2: medium Se group (0.1 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS3: high Se group (0.15 μg/mL Na_2SeO_3 + 150 μmol/L tBHP).

Figure 5. Protein levels of PI3K/Akt/c-fos signaling in human C28/I2 chondrocytes. (A) The level of Gβγ. (B) The protein level of PI3Kp110. (C) The protein level of pAkt. (D) The protein levels of c-fos. Compared with the C group, the protein level of Gβγ, PI3Kp110, pAkt and c-fos protein were increased in the O group than those of the C group (all $p < 0.0001$). Compared with the O group, a significant reduction in protein levels were found for PI3Kp110 in OS2 and OS3 groups, c-fos in three Se supplementation groups, Gβγ in OS1

and OS2 (all $p < 0.01$), and pAkt OS3 groups ($p < 0.01$). C: control group. S: purely Se group (0.05 μg/mL Na_2SeO_3). O: tBHP injury group (tBHP 150 μmol/L). OS1: low Se group (0.05 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS2: medium Se group (0.1 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). OS3: high Se group (0.15 μg/mL Na_2SeO_3 + 150 μmol/L tBHP). *represents significance at $p < 0.05$.

Figure 6. The protein levels of PI3K/Akt/c-fos signaling under different methylation status of *GPX3* in chondrocytes. (A) The protein level of Gβγ with *GPX3* methylation status. (B) The protein level of PI3Kp110 with *GPX3* methylation status. (C) The protein level of pAkt with *GPX3* methylation status. (D) The protein level of c-fos with *GPX3* methylation status. Compared with unmethylation group, protein levels of Gβγ, PI3Kp110, pAkt, and c-fos were increased in partial methylation group (all $p < 0.01$) and complete methylation group (all $p < 0.01$). The protein levels of Gβγ, PI3Kp110, and c-fos in partial methylation group were significantly higher than those in complete methylation group (all $p < 0.01$). UM: unmethylation. CM: complete methylation. PM: partial methylation.

* represents significance at $p < 0.05$.

Table 1 Demographic characteristics of the study population

Characteristics	Cases (n=50)	Control (n=50)	Values	*P*
Sex, male/female	26/24	27/23	0.04	0.841
Age, mean ± SD	51.90 ± 3.39	52.85 ± 3.98	1.285	0.202

[a] The difference of age between KBD and controls was performed using t-test and no statistical significance.

[b] The frequency of sex between KBD and controls was performed using x^2-test and no statistical significance.

Table 2. *GPX3* gene methylation in chondrocytes

Groups	*GPX3* methylation status			*p-value*
	Complete Methylation n (%)	Partial methylation n (%)	Unmethylation n (%)	
C	0	0	24(100.00)	
S	0	0	24(100.00)	
O	23(95.83)	1(4.17)	0	<0.001[a]
OS1	3(13.04)	20(86.96)	0	<0.001[b]
OS2	2(8.70)	21(91.30)	0	<0.001[b]
OS3	0	1(4.35)	22(95.65)	<0.001[b]

C: control group; S: purely selenium group (0.05 μg/mL Na_2SeO_3); O: tBHP injury group (tBHP 150 μmol/L); OS1: low selenium group (0.05 μg/mL Na_2SeO_3 + 150 μmol/L tBHP); OS2: medium selenium group (0.1 μg/mL Na_2SeO_3 + 150 μmol/L tBHP); OS3: high selenium group (0.15 μg/mL Na_2SeO_3 + 150 μmol/L tBHP).

a p-value for comparing O group with C groups, b p-value for comparing O group with OS1, OS2, OS3.

Hightlights:

1. GPX3 DNA hypermethylation and low expression of gene existed in KBD patients.
2. Up-regulation of PI3K/Akt/c-fos in KBD patients and oxidative damage chondrocytes.
3. Hypermethylation and low gene expression of GPX3 found in apoptotic chondrocyte.
4. GPX3 hypermethylation related to changes in gene expression and PI3K/Akt/c-fos.
5. Methylation, gene expression of GPX3 and PI3K/Akt/c-fos improved by Se supplement.

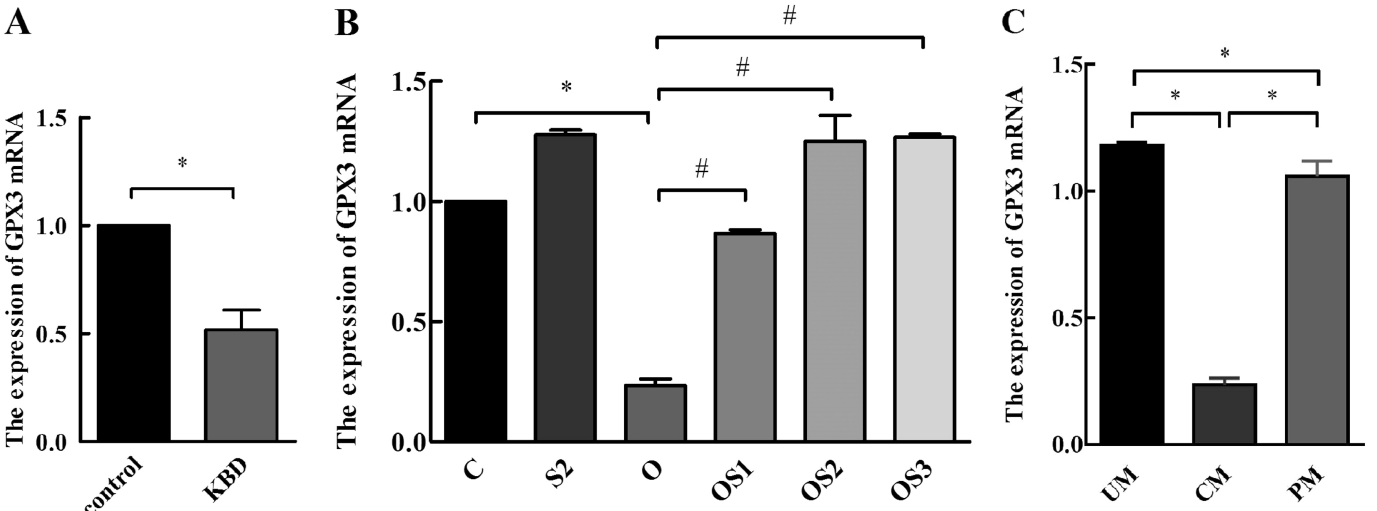

A
The expression of GPX3 mRNA
1.5
1.0
0.5
0.0
*
control
KBD
B
The expression of GPX3 mRNA
1.5
1.0
0.5
0.0
*
#
#
#
C
S2
O
OS1
OS2
OS3
C
The expression of GPX3 mRNA
1.5
1.0
0.5
0.0
*
*
*
UM
CM
PM

Figure 1

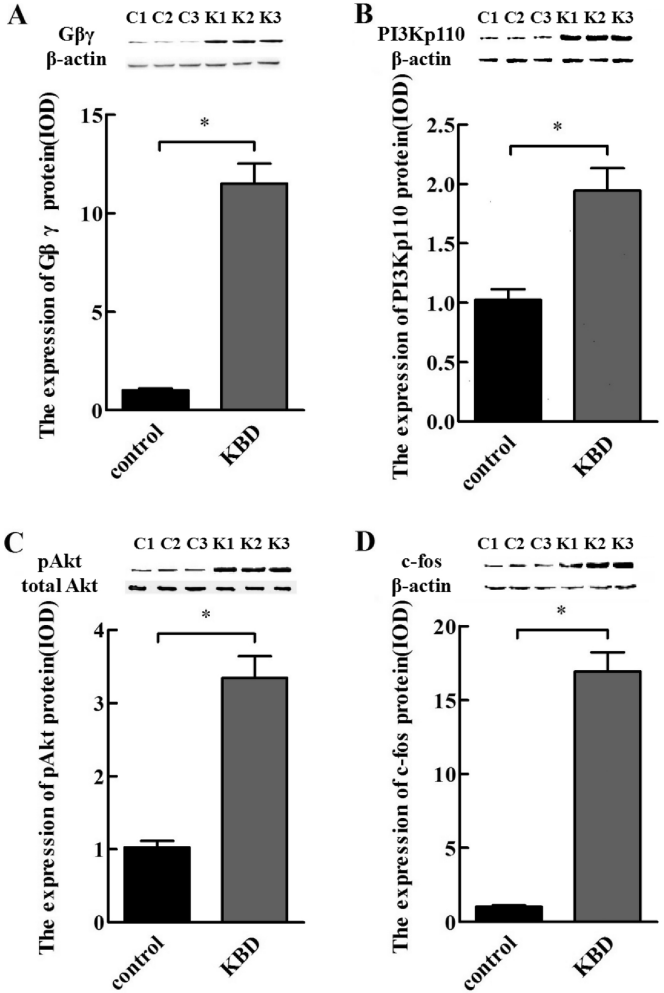
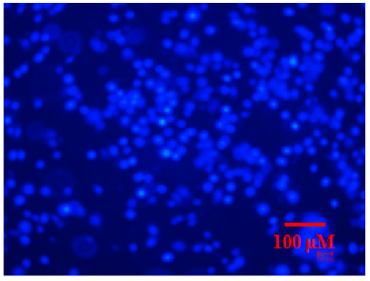
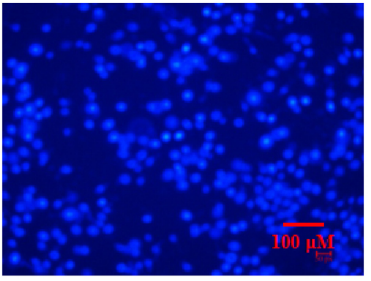

A
C1 C2 C3 K1 K2 K3
Gβγ
β-actin
The expression of Gβ γ protein(IOD)
15
10
5
0
*
control
KBD
B
C1 C2 C3 K1 K2 K3
PI3Kp110
β-actin
The expression of PI3Kp110 protein(IOD)
2.5
2.0
1.5
1.0
0.5
0.0
*
control
KBD
C
C1 C2 C3 K1 K2 K3
pAkt
total Akt
The expression of pAkt protein(IOD)
4
3
2
1
0
*
control
KBD
D
C1 C2 C3 K1 K2 K3
c-fos
β-actin
The expression of c-fos protein(IOD)
20
15
10
5
0
*
control
KBD

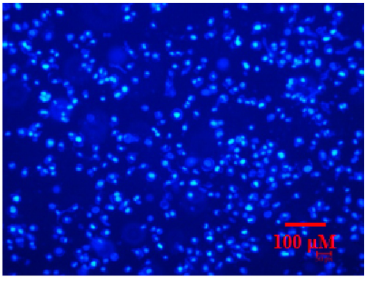
Figure 2



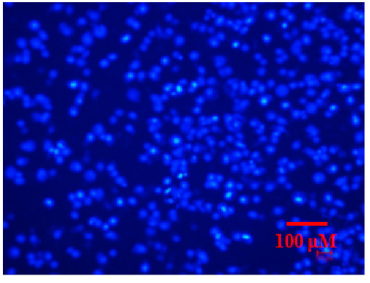
C group



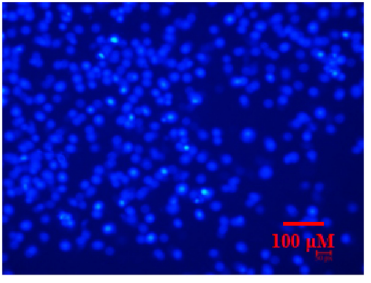
S2 group



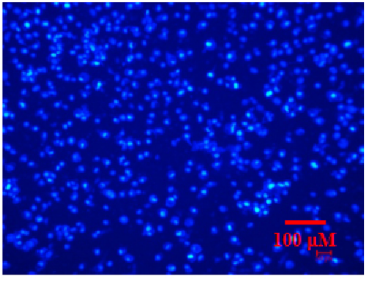
O group



OS1 group

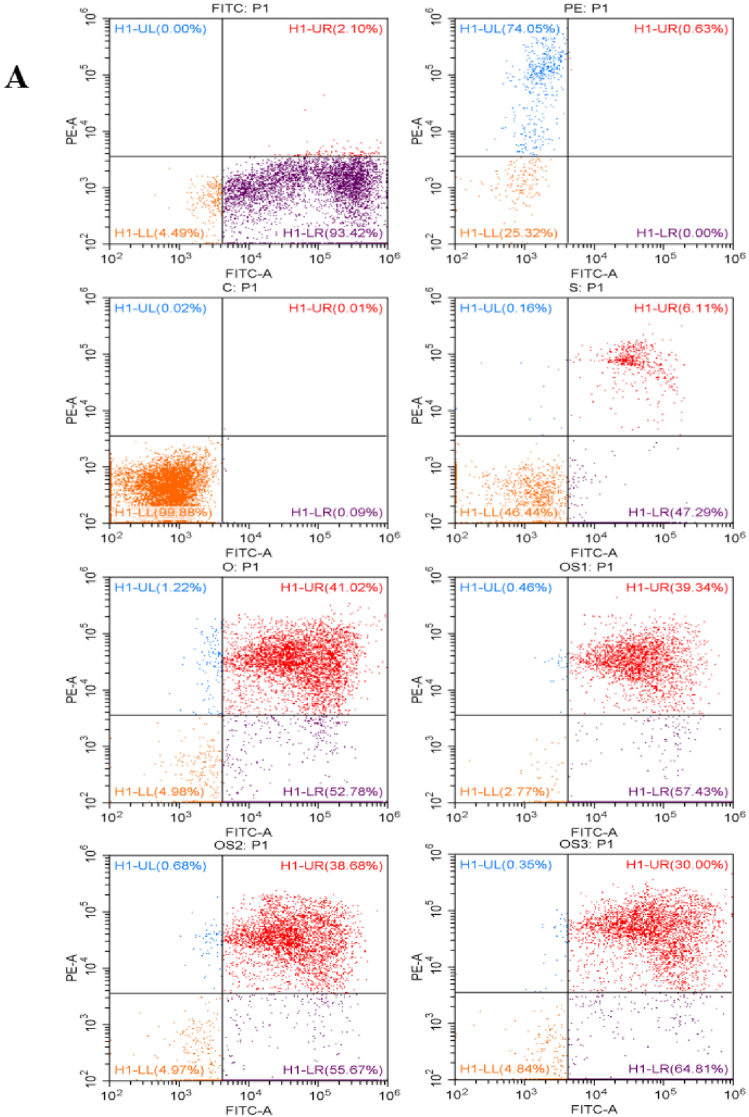


OS2 group



OS3 group

Figure 3


A
FITC: P1
H1-UL(0.00%)
H1-UR(2.10%)
H1-LL(4.49%)
H1-LR(93.42%)
PE: P1
H1-UL(74.05%)
H1-UR(0.63%)
H1-LL(25.32%)
H1-LR(0.00%)
C: P1
H1-UL(0.02%)
H1-UR(0.01%)
H1-LL(99.88%)
H1-LR(0.09%)
S: P1
H1-UL(0.16%)
H1-UR(6.11%)
H1-LL(46.44%)
H1-LR(47.29%)
O: P1
H1-UL(1.22%)
H1-UR(41.02%)
H1-LL(4.98%)
H1-LR(52.78%)
OS1: P1
H1-UL(0.46%)
H1-UR(39.34%)
H1-LL(2.77%)
H1-LR(57.43%)
OS2: P1
H1-UL(0.68%)
H1-UR(38.68%)
H1-LL(4.97%)
H1-LR(55.67%)
OS3: P1
H1-UL(0.35%)
H1-UR(30.00%)
H1-LL(4.84%)
H1-LR(64.81%)
PE-A
FITC-A

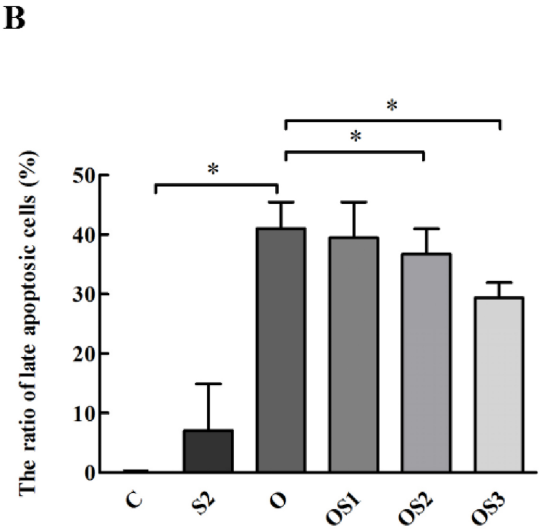

B
The ratio of late apoptosis cells (%)
0
10
20
30
40
50
*
*
*
C
S2
O
OS1
OS2
OS3

Figure 4

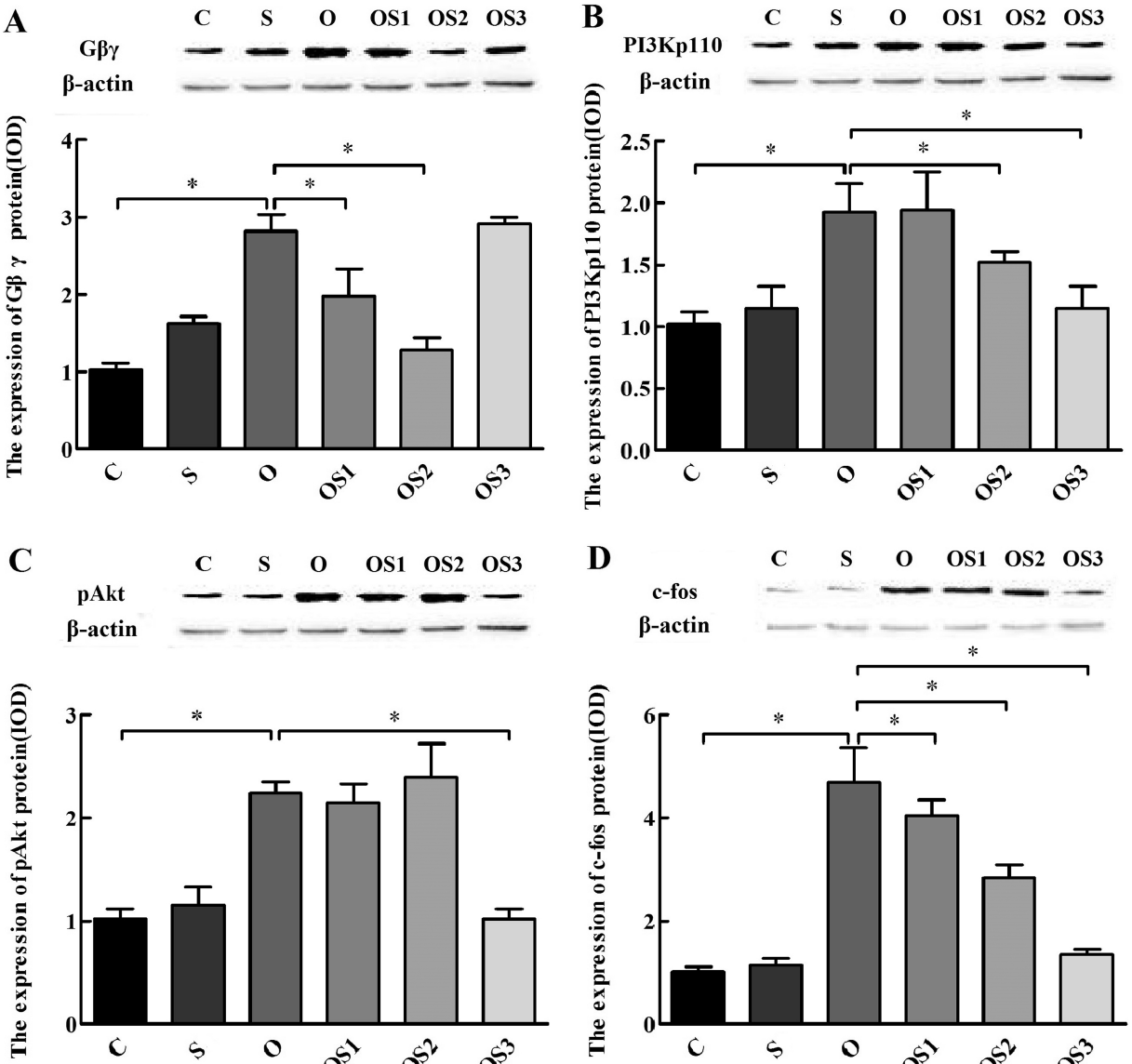

A
C S O OS1 OS2 OS3
Gβγ
β-actin
The expression of Gβ γ protein(IOD)
B
C S O OS1 OS2 OS3
PI3Kp110
β-actin
The expression of PI3Kp110 protein(IOD)
C
C S O OS1 OS2 OS3
pAkt
β-actin
The expression of pAkt protein(IOD)
D
C S O OS1 OS2 OS3
c-fos
β-actin
The expression of c-fos protein(IOD)

Figure 5

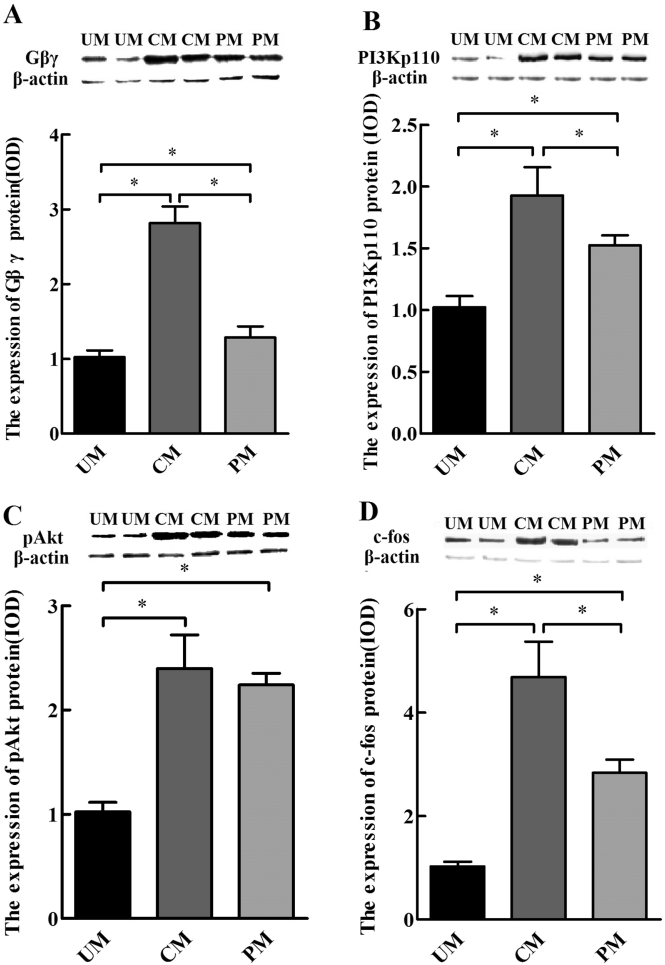

A
UM UM CM CM PM PM
Gβγ
β-actin
The expression of Gβ γ protein(IOD)
UM
CM
PM
B
UM UM CM CM PM PM
PI3Kp110
β-actin
The expression of PI3Kp110 protein (IOD)
C
UM UM CM CM PM PM
pAkt
β-actin
The expression of pAkt protein(IOD)
D
UM UM CM CM PM PM
c-fos
β-actin
The expression of c-fos protein(IOD)

Figure 6