Osteoarthritis and Cartilage

Oxidative stress and status of antioxidant enzymes in children with Kashin–Beck disease

W. Wang†, S. Wei‡, M. Luo†, B. Yu†, J. Cao†, Z. Yang†, Z. Wang†, M.B. Goldring§, J. Chen†,*

†Institute of Endemic Diseases, Xi’an Jiaotong University College of Medicine, Key Laboratory of Environment and Genes Related to Diseases (Xi’an Jiaotong University), Ministry of Education, Xi’an 710061, Shaanxi, PR China
‡Yan’an University Affiliated Hospital, Yanan 716000, Shaanxi, PR China
§Research Division, Hospital for Special Surgery and Department of Cell and Developmental Biology, Weill Cornell Medical College, Caspary Research Building, 535 E. 70th Street, New York, NY 10021, USA

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SUMMARY

Objectives: To clarify whether there is oxidative stress in Kashin–Beck disease (KBD) and if cartilage damage from reactive oxygen species (ROS) and oxidative stress mediate the chondral necrosis in articular cartilage of KBD.

Methods: We recruited 64 KBD patients, 46 healthy children from severely affected KBD regions, 81 healthy children from a non-severely affected KBD endemic region, and 91 healthy control children from a non-KBD region. Ten patients with KBD from the non-severely affected KBD regions were included in the experiment. The 2,3-DAN fluorescence technique was used to test selenium in the hair and blood. The biochemical techniques used to test the indicators of oxidative stress included thiobarbituric acid reactive substances (TBARS) levels, and antioxidant enzyme activities in serum samples. Histochemical staining was used to detect proteoglycans in cartilage sections. The 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxydeoxyguanosine (8-OHdG) were localized by immunohistochemistry.

Results: The levels of TBARS in serum were significantly increased in KBD children. The levels of antioxidants in serum were significantly higher in both KBD and normal children from KBD regions than in the normal children from non-KBD regions. The percentage of chondrocytes staining for 4-HNE and 8-OHdG in KBD patients was significantly higher than in controls. Staining for 4-HNE and 8-OHdG in KBD patients was prominent in all zones of articular cartilage, especially in the necrotic chondrocytes of the deep zone.

Conclusion: KBD is an oxidative stress-related disease, and the oxidative stress in cartilage contributes to the pathology of cartilage damage in KBD.

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Introduction

Kashin–Beck disease (KBD) is an endemic deform osteoarthropathy mainly occurring in China, Russia and North Korea. It was reported in China that there are 106 million people at risk, and 0.69 million patients with KBD in 366 counties of 14 provinces or autonomous regions were reported in 2009 by the Ministry of Health of the People’s Republic China. Until now, the precise pathogenesis and etiological factors are still unclear; however, three major environmental hypotheses have been proposed: selenium deficiency, serious cereal contamination by mycotoxin-producing fungi, and high humic acid levels in drinking water1–3. Moreover, chondronecrosis develops in knee articular cartilage in a rat model of KBD using T-2 toxin and selenium deficiency conditions4. Thus, we proposed that T-2 toxin in food under selenium deficiency maybe be among the etiological factors for occurrence of KBD. In addition recent findings of differential expression of glutathione peroxidase (GPX) and other genes indicative of metabolic dysfunction suggest the potential importance of interactions between genetic and environment factors5.

Distinct from other degenerative joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), the characteristic...
features of KBD include (1) the onset in affected zones of articular cartilage and growth plates, in which chondrocyte necrosis (chondronecrosis) occurs in deep-zone cartilage near the adjacent subchondral bone of peripheral joints, ribs, and vertebrae; (2) onset in children as early as 2–3 years and evidence of osteoarthropathy between the ages of 5 and 13; and (3) involvement of all hyaline cartilage arising from endochondral ossification throughout the body, including that in loaded and non-loaded joints, but the most frequently affected sites are the distal limbs, especially the hand. Other known features of KBD include dysregulation of the extra-cellular matrix (ECM), and activation of matrix metalloproteinases (MMPs), such that the mechanical integrity of the tissue is impaired. The cause and the mechanisms underlying perpetuation of cartilage necrosis and destruction are still unknown.

Recent investigations have revealed oxidative stress and oxidative damage in the pathogenesis of articular cartilage degradation in OA and RA. The over-production of reactive oxygen species (ROS) may contribute to the degradation of cartilage matrix with an important role in cartilage destruction in arthritis. Antioxidant enzyme [superoxide dismutase (SOD), catalase (CAT), GPX, Glutathione S-Transferase (GST)] activity and lipid peroxide [malondialdehyde (MDA)] levels are increased in knee joint synovial fluids of OA patients, while vitamin E levels are decreased. Total antioxidant capacity (T-AOC) in the plasma and GPX and CAT in red cells of RA patients are significantly lower than in controls, MDA levels in plasma of RA patients are significantly higher than in control group.

Interleukin (IL)-1β, IL-6, Tumor necrosis factor alpha (TNFα) levels are significantly higher in joint fluids and the cartilage tissues of OA patients, and these cytokines have key roles in the pathogenesis of RA. In addition, MDA, 8-hydroxydeoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (4-HNE) are oxidative damage biomarkers that are produced by synovial cells of patients with OA, while vitamin E levels are decreased. Total antioxidant capacity (T-AOC) in the plasma and GPX and CAT in red cells of RA patients are significantly lower than in controls, MDA levels in plasma of RA patients are significantly higher than in control group.

Moreover, our previous studies showed that addition of T-2 toxin to selenium deficiency caused severe oxidative stress. Therefore, our hypothesis is that increased oxidative stress mediates articular cartilage destruction in KBD. In this study, we first measured the levels of MDA in serum to confirm oxidant damage in children with KBD. Then oxidative damage biomarkers (8-OHdG and 4-HNE) were demonstrated in areas of chondral destruction in deep-zone articular cartilage from children with KBD cartilages. Levels of T-AOC, SOD, CAT, GPX, and MDA in serum in children with KBD were determined in order to link these ROS-related enzymes or oxidant damage markers to the pathogenesis of KBD.

Materials and methods

Serum and hair samples and patient groups

Serum and hair samples were collected from two KBD regions, including Xinhan county in Qinghai province and Changu county in Shaanxi province, and from one non-KBD region, Changan county in Shaanxi province. Patients with KBD were diagnosed on the basis of the clinical criteria for the diagnosis of KBD in China (diagnostic code GB16395-1996). All serum and hair samples were obtained after consents approved by the patients or guardians as authorized by the Ethics Committee of the Ministry of Health, Shaanxi Province. The details of samples are as follows: Sixty-four KBD children and 46 healthy subjects were chosen from severely affected KBD regions, including primary schools of Tangnaihai, Xialujuan and Qushian of Xinhan county in Qinghai province. Ten KBD and 81 healthy subjects were chosen from a less severely affected KBD endemic region, including primary schools of Ran dian of Changwu county in Shaanxi province. Fifty-one healthy control subjects were from a non-KBD region, the Nanfan primary school of Chang’an county in Shaanxi province. All the children aged between 5 and 13 years old were without other diseases, such as cold, headache, diarrhea, etc. All subjects were divided into three groups: KBD children, healthy children in KBD region, and healthy children in non-KBD region. The number of people selected for analysis from KBD and non-KBD regions were extracted by simple random sampling method into the groups as experiment subjects (Tables I and II).

KBD onset in children is as early as 2–3 years and osteoarthropathy usually becomes evident between the ages of 5 and 13. Therefore, we have collected samples of KBD and non-KBD children from the same primary school at 5–13 years old (same number of KBD and healthy students from same class), and healthy samples from primary schools of non-KBD areas at 5–13 years old. Within this narrow age range, chi-square test analysis showed that subjects from the three groups are not statistically significant as shown in Tables I and II (P > 0.05) in distribution of sex. Therefore, age and sex were controlled in this experiment among the three groups, the different of sex and age among the three groups is comparable and acceptable.

Human tissue preparation

Cartilage samples were obtained from metacarpophalangeal joints of the third or little finger of five KBD patients and five normal children (ranging from 3 to 7 years old). The control subjects from non-KBD regions had died from clinical problems not involving joint pathology. KBD subjects were from the diseased regions, as shown in Table III, and had died from accidents or other diseases such as bacillary dysentery, acute pneumonia, or acute diarrhea. KBD children were diagnosed based on the national diagnosing criteria of KBD in China (diagnostic code GB16395-1996) by X-ray films of the right hand, and cartilage sections after hematoxylin and eosin (H&E) staining. The health status of control children was diagnosed by histological examination of cartilage

<table>
<thead>
<tr>
<th>Sample set</th>
<th>KBD children</th>
<th>Normal children in KBD region</th>
<th>Normal children in non-KBD region</th>
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<tr>
<td>20</td>
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<td>7</td>
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</table>

Mean 10.78 – 9.05 – 8.85 –
sections after H&E staining. Both KBD and normal cartilage samples were obtained within 2–4 h of death and fixed in 4% paraformaldehyde. For all samples, we obtained the patient’s or guardian’s consent and approval of the Human and Ethical Committee for Medical Research at Xi’an Jiaotong University, School of Medicine (Dr. Yong Liu, Director).

Determination of selenium levels

Serum selenium and hair selenium levels were measured by 2,3-diaminonaphthalene (DAN) fluorescent atomic absorption spectrometry26, with a limitation of sensitivity of 5 ng/ml (64 nM); undetectable concentrations were assigned a value of 5 ng/ml.

Antioxidant enzyme activity

GPX activity

GPX activity in serum was measured using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the method of Hafeman et al.26 by a coupled assay using H2O2 and dithio-bis-nitrobenzoic acid (DTNB). One unit was defined as the amount of enzyme that catalyzes the decomposition of 1 mMol of hydrogen peroxide per minute.

SOD activity

Total SOD activity in serum was measured using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the nitroblue tetrazolium method described by Sun et al.27 This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine to form a red formazan dye, measured at the optical density of 550 nm using a spectrophotometer. The SOD activity was then calculated as the degree of inhibition of this reaction. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The SOD assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). Data are expressed as U/mg of protein.

CAT activity

CAT activity in serum was determined spectrophotometrically by Goth’s colorimetric method28, in which supernatant was incubated in H2O2 substrate, and the enzymatic reaction stopped by the addition of ammonium molybdate. In brief, after incubation of 0.5 ml of hydrogen peroxide and 0.1 ml of pancreatic homogenate in a water bath at 37 °C for 60 s, the reaction was terminated by adding 0.5 ml of ammonium molybdate solution. The absorbance of the yellow color of the complex of ammonium molybdate and hydrogen peroxide was measured at 405 nm using a spectrophotometer. One unit of CAT was defined as the amount of enzyme that catalyzes the decomposition of 1 mMol of hydrogen peroxide per minute.

T-AOC activity

T-AOC in serum was measured using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the method of Benzie and Strain28 with minor modifications. This assay measures the ferric reducing ability of the supernatant. The stable color of the Fe2⁺-O-phenanthroline complex (produced by the reducing agents in plasma reducing Fe3⁺ to Fe2⁺, which reacts with the substrate O-phenanthroline) was measured at 520 nm. T-AOC was expressed in U/ml where 1 U is defined as an increase in absorbance (A520) of 0.01/min at 37 °C.

Lipid peroxidation (LPO) assay

For evaluation of the MDA production rate, a measure of LPO, the thiobarbituric acid reactive substances (TBARS) assay was used. The concentration of TBARS was determined using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the method of Ohkawa et al.29 Briefly, 100 ml of serum from each subject, homogenates of cartilage, or MDA standards were pipetted into test tubes containing 1.5 ml of 20% (w/v) glacial acetic acid (pH 3.5), 200 ml of 8.1% (w/v) sodium dodecyl sulfate (SDS), 1.5 ml of 0.8% (w/v) thiobarbituric acid (TBA), and 700 ml of distilled water. The test tubes were incubated at 95 °C for 60 min with a marble on top of each test tube. After incubation, the test tubes were cooled and then centrifuged at 3000g for 10 min. The amount of MDA formed was measured spectrophotometrically at 532 nm, 1,1,3,3-Tetraethoxypropane (TEP), a form of MDA, was used as standard in this assay. TBARS concentration was expressed as pg/ml.

Histology

Cartilage tissue samples were fixed in 4% (w/v) paraformaldehyde for 2–3 days and decalcified in 10% (w/v) ethylene-diamine tetraacetic acid (EDTA) for 4 weeks. The samples were then embedded in paraffin and serial sections of 5 mm were cut in the coronal plane and stained with H&E for histological examination.
Toluidine blue staining

After deparaffinizing, sections were stained in 0.04% toluidine blue dye in 0.1 M sodium acetate at pH 4 for 30 min, washed in running distilled water for 10 min, and rinsed quickly in ammonia water. After the water wash, slides were dehydrated in two changes each of 95% ethanol, absolute ethanol, and xylene for 3 min in each solution, and mounted in Permount™ (Fisher Scientific, Beijing, China).

Immunohistochemistry of oxidative damage markers

Immunohistochemical staining of HNE and 8-OHdG in the sections of five different cartilage tissues was performed with the following rabbit polyclonal antibodies: anti-4-HNE (Alpha Diagnose, USA) anti-8-OHdG (Biosynthesis Biotechnology, China). For control sections, the primary antibody was omitted or irrelevant immunoglobulins were applied. For the positive control, the primary antibody was MMP-13. Briefly, five paraffin-embedded sections (5 μm) from each group were dewaxed and dehydrated in a gradient of alcohols. Endogenous peroxidase activity was quenched with 0.3% H2O2 and 0.1% sodium azide in phosphate-buffered saline (PBS). 10% normal goat serum was added for 15 min at 37°C in PBS. The primary antibodies were diluted in PBS at the following dilutions: anti-4-HNE (1:1000), anti-8-OHdG (1:50) and incubated overnight at 4°C. Thereafter, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat HRP (1:500, Pierce Biotechnology, USA) in PBS/1% Bovine serum albumin (BSA) for 30 min at room temperature. The sections were washed between all steps with PBS. HRP activity was detected using hydrogen peroxide as substrate and 3-amino-9-ethylcarbazole (AEC) as dye. Sections were briefly counterstained with hemalum solution.

Classification of cartilage zones

In the articular cartilage, chondrocytes were divided into three cell morphologies by light microscopy criteria located in the upper, middle, or deep zone. Chondrocytes in the upper zone were relatively small and flat, and oriented with the long axis parallel to the surface; larger and rounded cell profiles in the middle zone were randomly distributed in a matrix with fibers running in oblique directions; cells in the deep zone were of increasing size and were arranged in columnar manner perpendicular to the surface.

Positive staining analysis

Positive staining in cytoplasm was quantitatively analyzed. Briefly, the positive- and negative-stained cells in the whole depth of three articular cartilage zones from each image were labeled and counted using Image J software (NIH, USA). Immunostained slides were examined by two independent reviewers. The percentage of positive cells was then calculated using the equation below. Six randomly chosen fields in each zone were counted at 400× magnification. The average rate in different zones was calculated for each subject sample, and then for each group.

\[
\text{The percentage of positive cells} = \frac{\text{positive stained cells}}{\text{positive stained cells} + \text{negative stained cells}} \times 100\%
\]

Statistical analysis

Statistical analyses were performed independently by a non-clinical research assistant and an outside party to ensure objectivity, using SPSS Version 18.0 software (SPSS, Inc., USA). The data were expressed as mean ± standard deviation (SD), and were analyzed using one-way analysis of variance (ANOVA) followed by LSD-q test. All the data were submitted the Gaussian distribution and homogeneous variance before one-way ANOVA. Categorical variables were analyzed using chi-square analysis. Results were considered statistically significant if the P-value was <0.05 for all variables.

Results

Selenium levels in hair and blood of KBD

We first compared the selenium levels in hair and blood assayed from KBD children and healthy controls from KBD and non-KBD regions as reported in Table IV. The results showed that the selenium levels in hair and blood of KBD children patients were significantly lower than in normal children from the same KBD region and from the non-KBD region (P < 0.05). Selenium levels in hair and blood in KBD children were the lowest among those of the three groups, whereas the highest selenium levels were detected in normal children from a non-KBD region (Table IV).

We then compared the selenium levels in hair and blood between the severely affected and non-severely affected KBD regions. The results showed that the selenium levels in blood of KBD children and normal children from KBD regions in Xianghai county were significantly lower than in normal children from Changwu county.

TBARS in serum of KBD

The biomarker of oxidative stress, namely MDA, which measures the extent of TBARS levels is reported in Fig. 1. The serum levels of TBARS in KBD children from both Xinghai and Changwu endemic regions were significantly (P < 0.05) higher than in the normal children in the same KBD region and in a non-KBD region. There was no significant difference in the serum TBARS levels between the normal children from the KBD region and the non-KBD region (Fig. 1). The accumulation of MDA in KBD patients was high compared to values generally observed in the two healthy populations.

We then compared the serum levels of TBARS assayed in KBD children from the severely affected and non-severely affected KBD regions with healthy children from two KBD regions. The results showed that the TBARS levels in serum of KBD children patients were not different between the two KBD regions of Xinghai county and Changwu county. However, serum TBARS levels in normal children from KBD regions in Xinghai county were significantly higher than in normal children from Changwu county.

Antioxidant enzyme in serum of KBD

To determine whether alterations in the expression of these antioxidant enzymes contribute to the oxidative stress in KBD, we examined T-AOC as well as the levels of antioxidant enzymes, SOD, CAT and GPX, in serum of KBD (Fig. 2). The activities of antioxidant enzymes SOD and CAT as well as T-AOC in both KBD and normal children from Xinghai endemic regions were significantly increased compared to normal children in a non-KBD region, while the activities of GPX were significantly decreased in patients with KBD compared to normal children from both KBD regions and non-KBD
Table IV  
Selenium levels in hair and blood of KBD in healthy controls and KBD patients from Xinghai county in Qinghai province and Changwu county in Shannxi province

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Selenium levels in hair (µg/g)</th>
<th>Selenium levels in blood (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xinghai county in Qinghai province</td>
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<td></td>
</tr>
<tr>
<td>Normal children in non-KBD region</td>
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<td>242.35 ± 38.56</td>
<td>98.93 ± 17.18</td>
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<tr>
<td>Normal children in KBD region</td>
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<td>153.32 ± 24.31</td>
<td>63.06 ± 13.66</td>
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<tr>
<td>KBD children</td>
<td>20</td>
<td>67.64 ± 17.28</td>
<td>36.27 ± 13.29</td>
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<tr>
<td>Changwu county in Shannxi province</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal children in non-KBD region</td>
<td>18</td>
<td>242.35 ± 38.56</td>
<td>98.93 ± 17.18</td>
</tr>
<tr>
<td>Normal children in KBD region</td>
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<td>164.47 ± 32.64</td>
<td>84.62 ± 14.71</td>
</tr>
<tr>
<td>KBD children</td>
<td>10</td>
<td>119.58 ± 30.92</td>
<td>74.64 ± 13.66</td>
</tr>
</tbody>
</table>

Note: Values are means ± S.D.

* P < 0.05, KBD and normal children in a KBD region compared to normal children in a non-KBD region.

† P < 0.05, KBD children in a KBD region compared to normal children in a KBD region.

‡ P < 0.05, normal children from KBD regions in severely affected Xinghai county compared to a KBD region in Changwu county.

region (Fig. 2). Children from the KBD regions presented with increased T-AOC, SOD and CAT activities as compared to values generally observed in the healthy population from non-KBD regions in Chang’an.

Histochemical analysis

H&E staining of cartilage from normal and KBD patients is shown in Fig. 3. Chondral necrosis was indicated by disappearance of cartilage cells, presence of red nuclear cell outlines (nuclei without cytoplasm) where only the red “ghost” outlines of the chondrocytes remain (Fig. 3), and loss of alkalinity in the ground substance, which appeared as a lighter blue color of cytoplasm in the H&E sections. Chondral necrosis occurred focally, in patches or in banana-like areas mainly in the deep zone close to the bone edge of articular cartilage from KBD patients [Fig. 3(B)]. Usually, multiple bones were involved in one hand and wrist. The secondary histopathologic changes were clusters of surviving cartilage cells around the necrotic area [Fig. 3(B) and (C)]. The surface of the control cartilage was smooth, and chondrocytes in the hypertrophic layer were prevalent with territorial matrix staining around the chondrocyte, and the tide-mark can be clearly seen [Fig. 3(A)].

Expression of oxidative damage markers in articular cartilage in KBD

To identify and quantify the extent of oxidative damage, 4-HNE and 8-OHdG staining in cartilages of KBD children was performed by immunohistochemistry. Strong 4-HNE and 8-OHdG staining was present in the cytoplasm of chondrocytes throughout the depth of the articular cartilage from KBD children (Fig. 4), while there was no 4-HNE staining or lesser amounts of 8-OHdG in the deep-zone chondrocytes of the articular cartilage of normal children. Especially, KBD cartilage showed an accumulation of 4-HNE and 8-OHdG staining in the necrotic cells of the deep zone, which coincided with the regions of chondrocyte necrosis, as shown in Fig. 4(D) (TB staining) and Fig. 3(C). In addition, 4-HNE and 8-OHdG staining was increased in the cytoplasm of clusters of cartilage cells above the necrotic zones [Fig. 4(E) and (F), Table V].

Discussion

Elevated levels of oxidative stress occur in OA and aged cartilage. Although ROS are involved in the control of various aspects of biological processes in chondrocytes as intracellular second messenger molecules, elevated production of ROS leads to (1) telomere instability and downregulation of chondrocyte function, (2) increased inflammatory response, (3) cartilage degradation, by cleaving collagen and aggrecan and activating MMPs, and (4) cell death. Oxidative stress also results in the degeneration of mitochondria, the main source of ROS, leading to a leakage of oxidative chain, significant damage to the mitochondrial genome, and reduced mtDNA capacity for repair. In the present study, the LPO product, MDA, increased significantly in serum of patients with KBD. It is interesting that the TBARS levels were not different in serum of KBD children patients between the severely affected and non-severely affected KBD regions. However, serum TBARS levels in normal children from KBD regions in severely affected Xinghai county were significantly higher than in normal children from non-severely affected Changwu county. The results demonstrate that...
oxidative stress is the common pathological change in KBD children patients from both the severely affected and non-severely affected KBD regions. Severity of oxidative stress is associated with occurrence and development of KBD.

In order to answer whether the rise in MDA is due to increased generation of ROS due to the excessive oxidative damage generated in cartilages of KBD patients, immunohistochemical staining was used to identify 4-HNE and 8-OHdG. The results showed that strong 4-HNE and 8-OHdG staining was present in the cytoplasm of chondrocytes throughout the depth of the KBD articular cartilage including necrotic chondrocytes. Therefore, we speculate that the etiological factor of KBD may elevate production of ROS in cartilage, which in turn could oxidize many important biomolecules, including membrane lipids, proteins, and DNA, and finally lead to death in deep-zone chondrocytes. Because KBD patients present with pathologic changes as early as 5–13 of age7, it is necessary to

Fig. 3. H&E staining of articular cartilage in metacarpophalangeal joints of the little finger from a control patient (5-year-old male from a non-KBD regions) and a KBD patient (4-year-old with clinical manifestations of first degree KBD). (A) Control cartilage shows no chondronecrosis; (B) KBD cartilage shows chondronecrosis with red “ghost” outlines of the chondrocytes in the deep zone (arrowheads); (C) KBD deep-zone cartilage shows large chondronecrotic areas, where the presence of cells with red nude nucleus as red “ghost” outlines of the chondrocytes (arrowheads), and loss of alkalinity in the matrix without cells (asterisk). Bar = 20 μm.

Fig. 4. Increased 4-HNE and 8-OHdG in KBD cartilage. Representative sections of articular cartilage from metacarpophalangeal joints of the fingers of healthy controls and KBD patients. Positive staining (arrowheads) appears in red color. (A and D) TB staining; (B and E) staining for 8-OHdG; (C and F) staining for 4-HNE. (A) Control cartilage shows TB staining in whole zone of the cartilage. (D) KBD cartilage shows no TB staining in the deep zone. (B and C) Control cartilage shows less 8-OHdG positive staining and no 4-HNE positive staining in chondrocytes, respectively. (E and F) KBD cartilage shows strong staining for 4-HNE and 8-OHdG with intensity in the deep zone (arrow) of necrotic cells and adjacent cells of necrotic zones. Bar = 20 μm.
emphasize that the pathogenesis of KBD likely begins from early childhood, although such samples are difficult to obtain.

To prevent an accumulation of ROS-mediated damage, chondrocytes possess a well-coordinated enzymatic antioxidant system formed principally by SODs, CAT and GPX. SOD2 and SOD3 are downregulated in OA cartilage and CAT activity is increased in OA patients compared with healthy controls. In our study, the activities of antioxidant enzymes SOD and CAT, as well as T-AOC, in both KBD and normal children from Xinghai endemic regions were significantly increased compared to normal children in a non-KBD region. SOD and CAT are important antioxidant enzymes having anti-toxic effects against superoxide anion. The over-expression of SOD might be an adaptive response, as it results in increased dismutation of superoxide to hydrogen peroxide. The rise in the levels of SOD, CAT and T-AOC may be part of the response to counter the effect of increased oxidative stress. A recent epidemiological investigation of the prevalence of KBD in China from 1990 to 2007 showed that the incidence of this disease was increased in some endemic areas but then dropped gradually, but considerably, in the past 18 years. With the improved life conditions in KBD endemic areas, the prevalence of KBD has greatly decreased recent years. The rise in the levels of SOD, CAT and T-AOC in both KBD and healthy children in the KBD endemic area suggests that an unknown etiologic factor may still exist in KBD endemic areas and affect all the children. The over-expression of antioxidant enzymes with no MDA elevation may be an adaptive response in normal children with increased turnover for preventing oxidative damage. Despite increased defenses against oxidant damage, there is still oxidative stress with MDA elevation in KBD children.

However, GPX, a selenoprotein that is another oxidative stress inducible enzyme, was significantly decreased in patients with KBD compared to normal children from both the KBD region and non-KBD region. We observed significant decreases in the levels of hair and blood selenium, associated with decreased GPX activities in KBD. The present findings indicate a positive correlation between selenium concentration and the activity of GPX. GPX contains selenocysteine and the production of the active GPX is maintained principally by SODs, CAT and GPX. SOD2 and SOD3 are downregulated in OA cartilage and CAT activity is increased in OA patients compared with healthy controls. In our study, the activities of antioxidant enzymes SOD and CAT, as well as T-AOC, in both KBD and normal children from Xinghai endemic regions were significantly increased compared to normal children in a non-KBD region. SOD and CAT are important antioxidant enzymes having anti-toxic effects against superoxide anion. The over-expression of SOD might be an adaptive response, as it results in increased dismutation of superoxide to hydrogen peroxide. The rise in the levels of SOD, CAT and T-AOC may be part of the response to counter the effect of increased oxidative stress. A recent epidemiological investigation of the prevalence of KBD in China from 1990 to 2007 showed that the incidence of this disease was increased in some endemic areas but then dropped gradually, but considerably, in the past 18 years. With the improved life conditions in KBD endemic areas, the prevalence of KBD has greatly decreased recent years. The rise in the levels of SOD, CAT and T-AOC in both KBD and healthy children in the KBD endemic area suggests that an unknown etiologic factor may still exist in KBD endemic areas and affect all the children. The over-expression of antioxidant enzymes with no MDA elevation may be an adaptive response in normal children with increased turnover for preventing oxidative damage. Despite increased defenses against oxidant damage, there is still oxidative stress with MDA elevation in KBD children.

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In conclusion, we propose a mechanism in the development of KBD, in which oxidative stress causes pathologic hypertrophic death of articular chondrocytes. We have previously demonstrated that altered CD44, IL-1β and TNFα metabolism occurs in the pathogenesis of KBD and there is increased aggrecanase-generated proteoglycan loss in cartilage from KBD adults and children. These primary metabolic changes are likely to be significant contributing factors causing pathological joint formation and instability that lead to secondary OA in KBD patients. Furthermore, we have recently observed upregulation of MMP-1 and MMP-13 expression in KBD chondrocytes in vitro. Taking into account the incidence of oxidative stress in the KBD process, as well as the incidence of the antioxidant enzymes in the KBD disease, the results suggest that oxidative stress may play a major role in the initiation and progression of KBD disease and lead to necrosis of chondrocytes and the degeneration of ECM. Therapies might be developed that interfere with oxidative stress and are able to postpone or prevent KBD. In addition, although the prevalence of KBD is greatly reduced, the unknown etiologic factor likely still exists and could affect all the children in KBD endemic regions. Etiologic research is still necessary in case there is recurrence of the outbreak of KBD as happened 30 years ago.

**Contributions**

Jinghong Chen takes responsibility for the integrity of the work as a whole, from inception to finished article. Wei Wang, Shuling Wei, Mingxiu Luo and Boquan Yu were responsible for acquisition of data, analysis and interpretation of data. Mary B Goldring, Zhijun Wang, and Junling Cao were responsible for parts of acquisition of data and critical revision of the article for important intellectual content.

**Conflicts of interest**

We, all authors, state that we do not have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) this work. We also do not have any potential conflicts of interest due to employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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