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Protective Effects of Hydrogen-Rich Water Against Cartilage Damage in a Rat Model of Osteoarthritis by Inhibiting Oxidative Stress, Matrix Catabolism, and Apoptosis

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Statistical Analysis C
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Manuscript Preparation E
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Background: The aim of this study was to investigate the mechanisms underlying the potential effects of hydrogen-rich water (HW) on articular cartilage in a rat osteoarthritis (OA) model.

Material/Methods: A rat model of OA was established using the modified Hulth method, and rats were forced to exercise for 30 min every day 1 week after surgery for 7 weeks. Mankin's method was used to score the severity of OA. The animals were assigned into the OA group, OA+HW group, and sham operation group. After 8 weeks, the animals in the OA group had a Mankin score >8 points, and HW was administered into the knee joint. After 2 weeks of treatment, articular cartilage was obtained for pathological examination, consisting of hematoxylin and eosin, toluidine blue, and Hoechst staining, as well as quantitative real-time PCR and Western blot analyses. This combination of pharmacological and molecular biological analyses was performed to examine the mechanism underlying the protective effect of HW on articular cartilage.

Results: The antioxidant effects of HW suppressed oxidative damage, which may have aided the inhibition of ECM-degrading enzymes (MMP3, MMP13, ADAMT4, and ADAMT5), the upregulation of Col II and aggrecan expression, and the downregulation of COX-2, iNOS, and NO expression. The results of HE staining indicated intra-articular treatment of HW attenuated cartilage degradation. However, Hoechst staining in the OA group indicated the nuclei of the fragmented chondrocytes were condensed compared to the sham operation group, and this effect was inhibited by HW.

Conclusions: HW showed a protective effect against the progression of OA in an animal model, which may have been mediated by its anti-oxidant and anti-apoptotic activities.

MeSH Keywords: Apoptosis • Chondrocytes • Osteoarthritis • Oxidative Stress

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Background

Osteoarthritis (OA) is a chronic degenerative joint disorder most commonly seen in elderly and obese people, which imposes heavy socio-economic burdens worldwide [1]. About 80% of people ≥65 years old have symptoms of OA [2], and medical costs and expenditures associated with OA globally increased from about $233.5 billion in 1997 to $321.8 billion in 2003. OA mainly damages articular cartilage and leads to functional decline. The clinical symptoms of OA are chronic pain, stiffness, and joint swelling, leading to joint deformity and low quality of life [3,4]. OA is characterized by erosion of the articular cartilage, chondrocyte death, and overexpressions of the pro-inflammatory factors IL-1β, TNF-α, prostaglandins, and NO [5]. Traditional therapy of bone, joint, and connective tissue diseases includes acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs). Nevertheless, conventional treatments have little effect on the progression of OA, leaving surgery as the only option. The essential effects of apoptotic mechanism in the process of OA have been investigated [6,7]. Oxidative stress, endoplasmic reticulum stress, and mechanical stress are considered risk factors that contribute to the induction of apoptosis in chondrocytes and the progression of OA. Therefore, a better understanding of the relevant molecular mechanisms underlying the process of OA is essential to development of new therapeutic strategies.

Oxidative stress is considered a main causative factor in the pathogenesis of OA. In addition, oxidative damage to genomic DNA and mitochondrial DNA (mtDNA) has been found in OA cartilage [8]. Reactive oxygen species (ROS) are mainly produced in mitochondria through the mitochondrial respiratory chain, but can also be produced by NADPH and xanthine oxidase, as well as by other sources (Figure 1) [9,10]. The level of oxidative damage to mtDNA is associated with markers of DNA damage, hypertrophy, and senescence. ROS can give rise to chondrocyte apoptosis and then accelerate articular cartilage dysfunction and degeneration (Figure 1) [11,12]. Oxidative damage plays a central role in OA and other aging-related diseases. Indeed, at the cellular level, oxidative damage to genomic and mtDNA triggers a DNA damage response and activation of the nuclear factor-κB (NF-κB) pathway [13], which is the master regulator of inflammation. Cells are exposed to oxidative stress due to an upregulation of oxidant generation or a downregulation of antioxidant protection. Continuous oxidant attack results in lipid peroxidation of membrane lipid constituents, which interrupts the functions of cell organelles and culminates in ultrastructural destruction. Nrf2 is a transcriptional activator that exerts essential effects in cellular responses to oxidative injury. The connection between oxidative stress and inflammation has been reviewed [14], and these conditions result in cell dysfunction at multiple levels. Oxidative stress causes senescence of chondrocytes, which is characterized by degradation of the extracellular matrix (ECM) proteins [15].

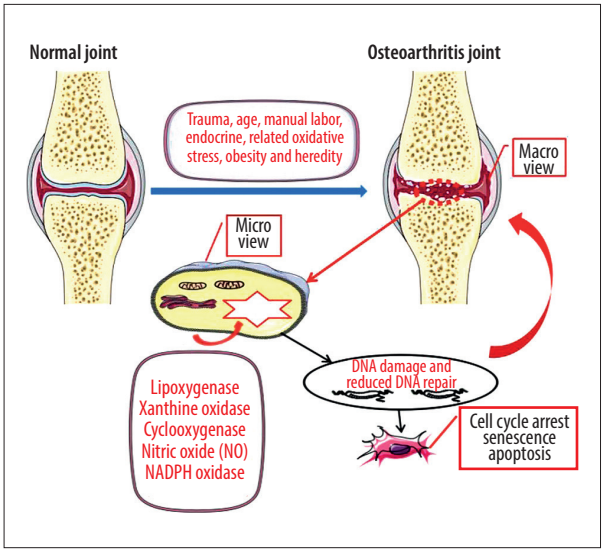

Figure 1. Macro- to micro-views of changes occurring in articular cartilage during OA onset and development. As a consequence of different risk factors, profound changes occurred in all the joint tissues. The major sources of ROS are the mitochondria during oxidative phosphorylation. Deranged metabolic factors together with aging contribute to mitochondrial dysfunction, accumulation of ROS and RNS, which increase the level of protein misfolding and aggregation, and accumulation of DNA damage cannot be efficiently corrected because mitochondrial dysfunction leads to failure of the energy supply required by the DNA damage response.

The administration of hydrogen in different forms, such as hydrogen-rich water (HW) and hydrogen-saturated saline, has been shown to be an efficacious treatment for many ROS-induced diseases, including cardiac and hepatic hypoxia/ischemia injury, neonatal hypoxia/ischemia injury, cisplatin-induced nephrotoxicity, and Parkinson's disease [16,17]. Molecular hydrogen is a novel antioxidant with protective effects against neonatal cerebral hypoxia/ischemia injury [18], transient cerebral ischemia [19], inflammation-associated deterioration in cardiac and aortic allograft recipients [20], and cold ischemia-reperfusion injury [21]. Hydrogen-rich saline and HW are economical, safe, and more readily effective than hydrogen gas. Injection of hydrogen-rich saline may be more beneficial for long-term therapy of OA in animal models. Therefore, we investigated the effects of HW injection on inflammatory reactions in articular cartilage, oxidative stress, and damage to articular cartilage in OA rats. We also examined whether HW inhibited apoptotic level of chondrocytes induced by oxidative stress, and investigated the potential mechanism.

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Material and Methods

Chemical reagents and animals

Antibodies (anti-caspase-3, anti-Bcl-2, and anti-Bax) were purchased from Abcam (Cambridge, UK). All other experimental reagents were acquired from Beyotime Institute of Biotechnology unless otherwise specified. SD rats were acquired from Shanghai Kelaisi Experimental Animal Co. The rat experiments were performed according to the guidelines of the Animal Care and Use Committee of Hainan Medical College.

Preparation of hydrogen water

Hydrogen was dissolved in water for 4 h at 0.4 MPa and was stored in an aluminum bag according to a previously described method [22]. Saturated HW (1.3 mg/L) was freshly prepared to achieve a continuous concentration and was sterilized by gamma radiation before use. The hydrogen content was confirmed using a hydrogen electrode. Each day, HW was put into a closed glass vessel, which maintained the hydrogen concentration at >0.8 mg/L after 24 h. HW that had been degassed by gentle stirring was used for the experimental treatment groups.

Experimental design

Twenty male SD rats weighing 200–250 g were assigned into 3 groups: the OA group, in which OA was induced surgically according to the Hulth method as described below; the OA+HW group, with surgically induced OA plus HW treatment; and a sham operation group. All animals were anesthetized with trichloroacetaldehyde hydrate (ip, 300 mg/kg). Then, transection of the anterior cruciate ligament and resection of the medial meniscus of the right knee joints were performed in the OA group according to a previously described method [23]. At 4 weeks after surgery, the sham and OA groups were injected with PBS into the abdominal cavity, while the OA+HW group received injection of HW. At 10 weeks after surgery, all animals were killed by cardiac exsanguinations for further experiments.

RT-PCR analyses

Total RNA was isolated using an RNA isolation kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocols. The purity of the isolated mRNA was tested by the ratio of absorbance at 260–280 nm. RT-PCR was examined with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) using an RT-qPCR system (Agilent Technologies, Tokyo, Japan). We used RT-PCR to evaluate the mRNA expressions (GAPDH, COX-2, iNOS, NO, aggrecan, Coll II, MMP3, MMP4, ADAMT4, and ADAMT5). The results were quantified using comparative cycle threshold method [24,25].

Western blot analysis

Total protein was extracted by an extraction kit following the manufacturer's protocols. Protein specimens were loaded onto SDS-gel and transferred to PVDF membranes. The membranes were then incubated with 5% nonfat milk for 1.5 h, incubated with primary antibodies (anti-caspase-3, anti-Bax) overnight at 4°C, and treated with secondary antibodies for another 1 h. Bands were imaged and quantified using the ChemiDic™ XRS+ Imaging System (Bio-Rad).

Histological assay

The right knee joints of all animals were acquired and incubated in 4% PFA for 2 days. Calci-Clear slow solution was administrated for decalcification of joints for approximately 3 weeks, and all specimens were then embedded in paraffin wax. The slides were then incubated with hematoxylin and eosin (HE), Hoechst, and toluidine blue stains, and imaged under a fluorescence microscope. Finally, Mankin's method was used to score the severity of OA in the knee joints of the rats.

Results

Hydrogen-rich water attenuated cartilage damage by inhibiting oxidative stress

To test whether ROS are involved in the protective effects of HW in OA group, we tested the levels of COX-2, iNOS, and NO by PCR. PCR results showed that the levels of COX-2, iNOS, and NO expression were lower in the sham group, while the expressions of COX-2, iNOS, and NO were higher in the OA group (Figure 2). However, HW treatment attenuated these effects. Therefore, we demonstrated that hydrogen-rich water attenuates cartilage damage by reducing oxidative stress.

Hydrogen-rich water reduced apoptotic in the OA group

To test whether the HW exerts anti-apoptotic effects of HW in the OA group, we tested the levels of cleaved-caspase3 and Bax by Western blot analysis. The results showed higher apoptotic levels in the OA group than in the sham group, which were attenuated by HW treatment (Figure 3). Therefore, we demonstrated that hydrogen-rich water attenuates apoptotic levels in the OA group.

Hydrogen-rich water reduced ECM degradation by inhibiting matrix-degrading enzyme

In the OA group, PCR results showed that the levels of Col II and aggrecan expression were lower and were improved by HW treatment (Figure 4). The main matrix-degrading enzymes

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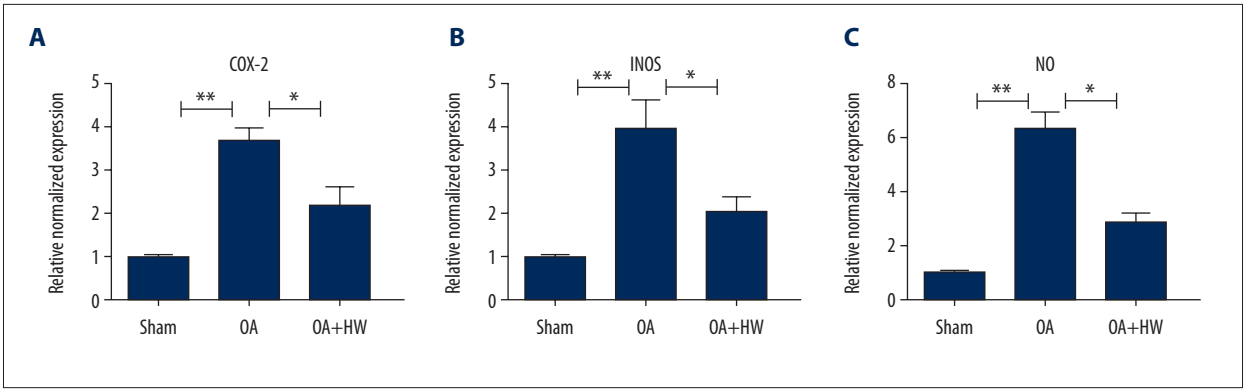

Figure 2. (**A–C**) Expressions of target genes COX-2, iNOS, and NO in each group. Data were represented as mean±SEM (n=5). *** P<0.001, ** P<0.01; * P<0.05.

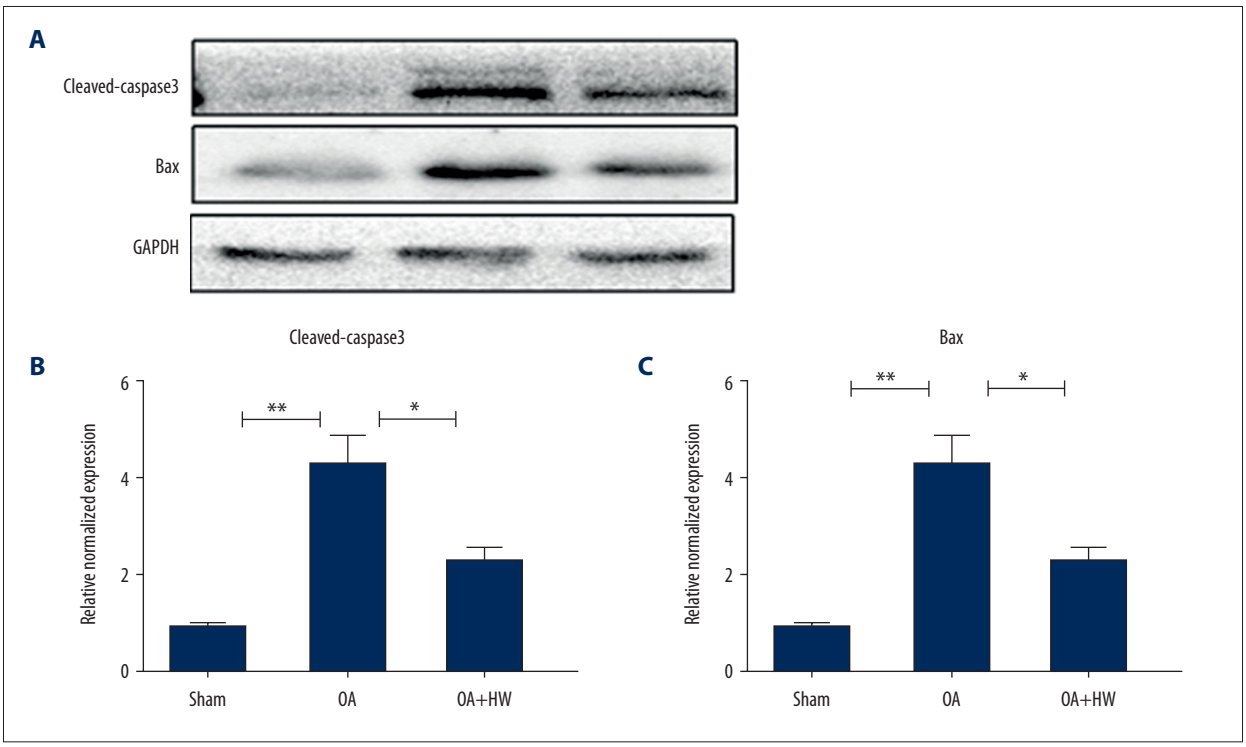

Figure 3. (**A–C**) The protein levels of cleaved caspase-3 and Bax for the sham, OA, and HW groups. Data are presented as mean±SEM (n=5). *** P<0.001; ** P<0.01; * P<0.05.

(MMP3, MMP13, ADAMT4, and ADAMT5) were increased in the OA group, and this was attenuated by HW treatment. These results indicated that HW can reduces ECM degradation by inhibiting matrix degrading enzyme.

Effects of hydrogen-rich water on OA cartilage histology

Histological changes were evaluated for matrix content and the cartilage structure in each group. The results of HE staining (Figure 5A) indicated irregular degradation of the articular cartilage in the OA rats compared to the normal rats. Cartilage significantly increased after intra-articular treatment with HW, and cartilage degradation was markedly attenuated. The nuclei were densely stained or showed fragmented dense staining when chondrocytes were apoptotic. Hoechst staining indicated that there were few apoptotic chondrocytes in the sham operation group (Figure 5A). In the OA group, however, the nuclei of the fragmented chondrocytes were condensed compared to those in the sham operation group, and this effect was inhibited by intra-articular injection of HW. Toluidine blue staining and Mankin's score were consistent with the results of HE and Hoechst staining (Figure 4B, 4C). Taken together, these observations indicate that HW had a protective effect, and significantly enhanced cartilage recovery in this rat model of OA.

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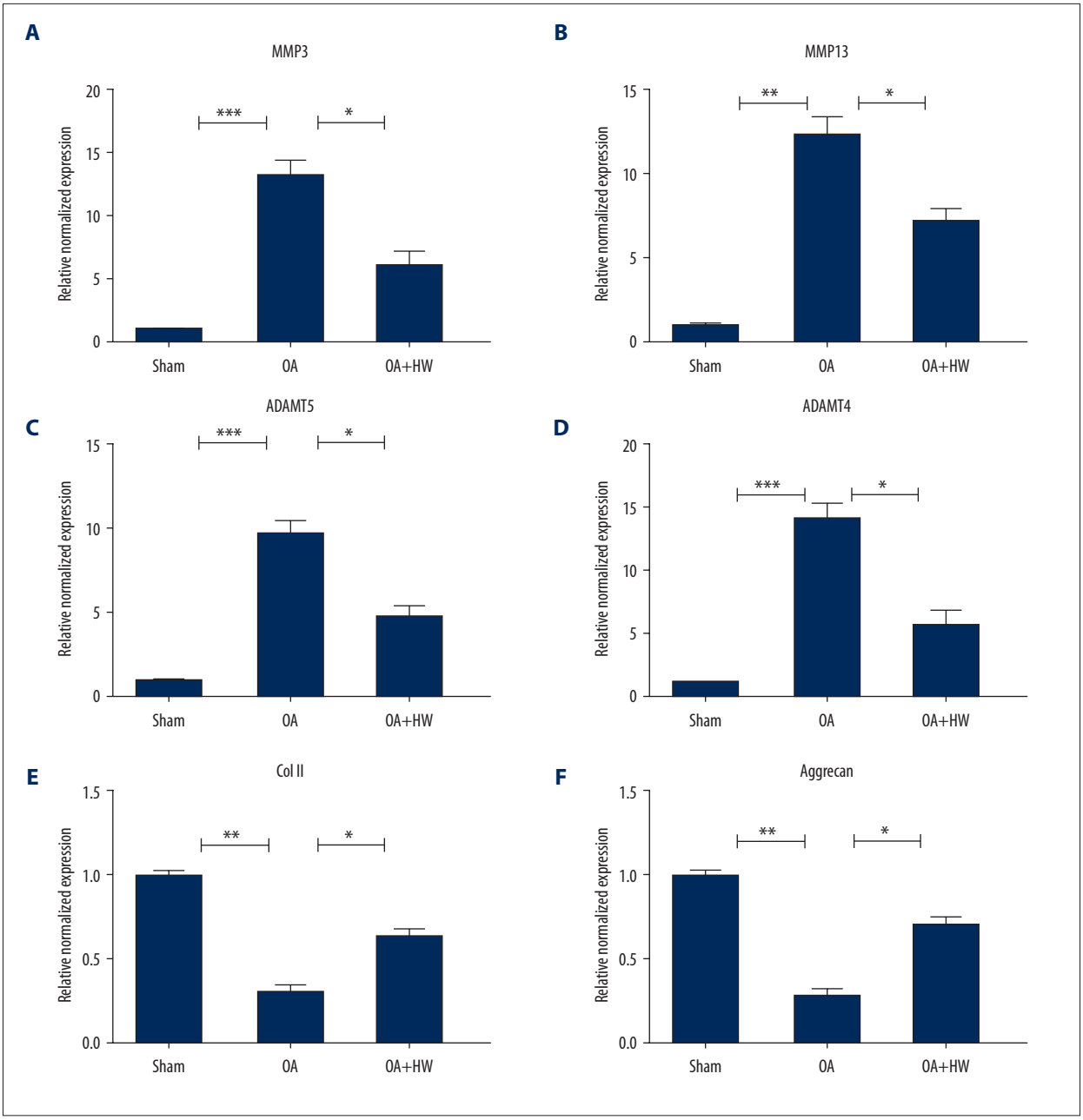

Figure 4. (**A–F**) Expressions of target genes MMP3, MMP13, ADAMT5, ADAMT4, Col II, and Aggrecan in each group. Data are presented as mean±SEM (n=5). *** P<0.001; ** P<0.01; * P<0.05.

Discussion

Articular cartilage includes chondrocytes that control the metabolism of the ECM, an avascular tissue consisting of collagen II, and other small hydrophilic macromolecules [26]. OA is now considered a major chronic public health problem worldwide [27]. Chondrocytes are the only cell type in cartilage, and chondrocytes respond to injury, maintain tissue homeostasis, and maintain cartilage homeostasis by regulating the synthesis and enzymatic breakdown of the ECM [28,29]. There is accumulating evidence of an association between cartilage degeneration and chondrocyte apoptosis [30,31]. Chondrocyte apoptosis leads to the destruction of articular cartilage, which is characteristic of OA [32,33] and plays a critical role in the onset and progression of OA [34]. Thus, the suppression of chondrocyte apoptosis could represent a potent therapeutic strategy for OA.

The pathogenesis of OA is closely related to ROS production and oxidative stress [11]. Chondrocyte apoptosis is involved

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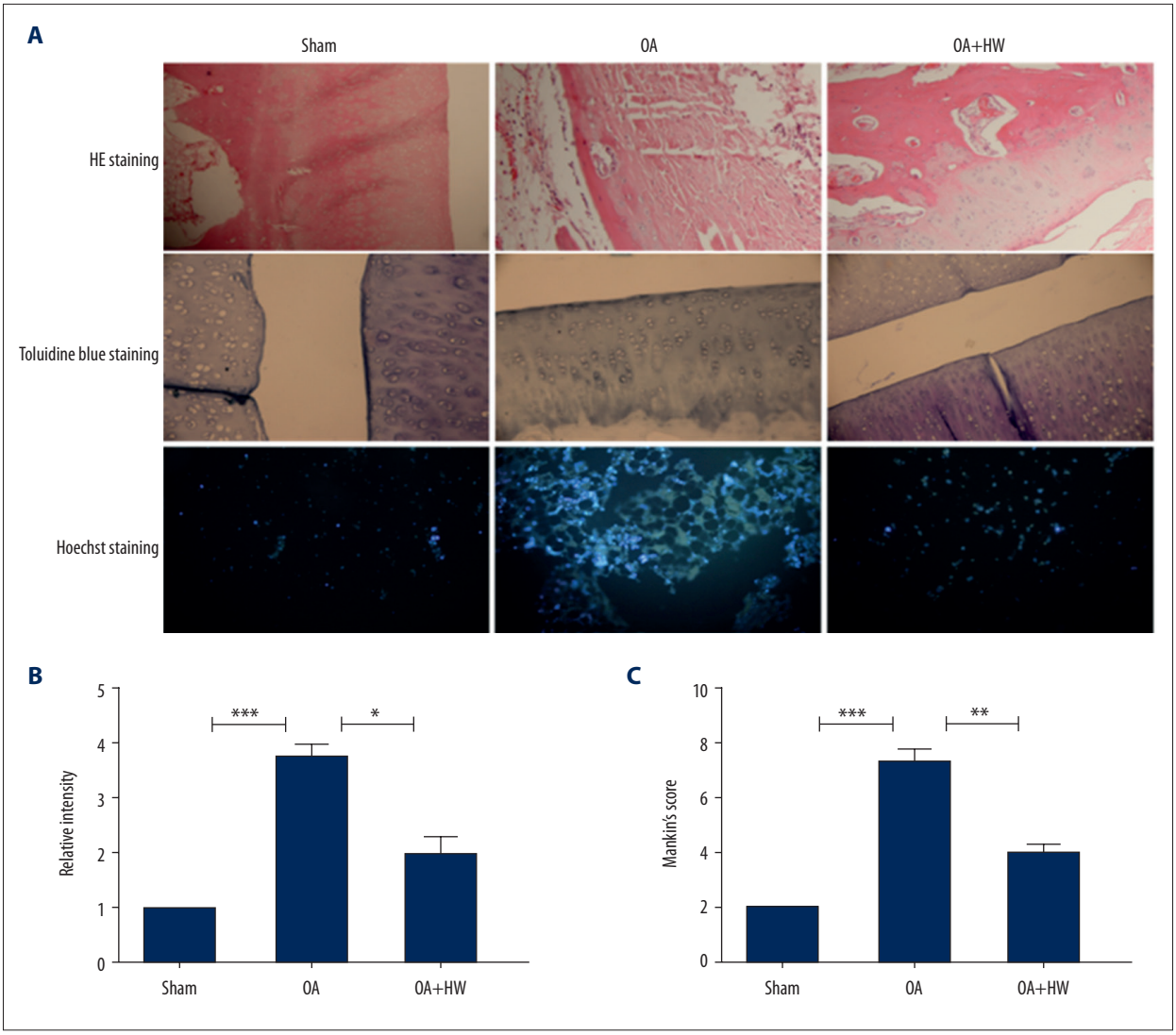

Figure 5. (**A**) Histopathological assay of rat articular cartilage by HE staining, Toluidine blue staining, and Hoechst staining in each group. (**B**) Relative intensity. Data are presented as mean±SEM (n=3). *** P<0.001; ** P<0.01; * P<0.05. (**C**) Mankin's grade. Data are presented as mean±SEM (n=5). *** P<0.001; ** P<0.01; * P<0.05.

with the process of ECM degradation and cartilage erosion in osteoarthritic cartilage. Oxidative stress-mediated post-translational modifications of redox-sensitive proteins appear to be key mechanisms underlying cellular dysfunction and disease progression. Here, we focused on the intrinsic signaling of the apoptotic pathway controlled by mitochondrial parameters [35]. NO is a highly reactive cytotoxic free radical, which can suppress the production and enhance degradation of collagen II by decreasing the level of Col2a1 [36]. iNOS is the essential enzyme involved in the production of NO, and the expression of iNOS was increased in OA chondrocytes, resulting in the production of large amounts of NO. Selective COX-2 inhibitor ameliorates osteoarthritis by repressing apoptosis of chondrocytes [37]. In addition, we observed a marked increase of NO in OA chondrocytes, with reduced Col2a1 expression and increased iNOS expression, indicating that catabolism of the ECM dominated under these conditions. The major antioxidant enzyme SOD plays important roles in this balance. RT-PCR and Western blot analyses showed that hydrogen-rich water attenuates cartilage damage by reducing oxidative stress; this is evidenced by the significantly higher expressions of apoptotic protein (caspase-3 and Bax) and ROS (COX-2, INOS, and NO) in the OA rats compared to the normal rats. OA model rats treated with HW showed significantly lower levels of apoptotic proteins (caspase-3 and Bax) and ROS (COX-2, INOS and NO) compared to the OA rats. All results showed that HW inhibits oxidative stress by downregulating the activity of antioxidant enzymes, including COX-2, iNOS, and NO.

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The therapeutic application of antioxidant gas may be a reasonable method for treatment of oxidative stress. There has been a great deal of research interest in the use of hydrogen for this purpose. In fact, increasing evidence shows that injection of HW attenuates oxidative injury in various diseases. HW has been shown to have an anti-apoptotic effect in a neonatal hypoxia/ischemia rat model. To determine whether HW exhibits the same inhibitory effect on apoptosis in a rat model of OA, we examined chondrocyte apoptosis by toluidine blue, HE, and Hoechst staining. HW significantly inhibited chondrocyte apoptosis, and the morphology of chondrocytes was mostly regular. In addition, there were fewer nuclei of fragmented chondrocytes clustered into a mass compared to the OA group, and the extent of such lesions was less in the OA+HW group, consistent with previous findings.

Preclinical experimental models of diseases have indicated that hydrogen resuscitation is effective for ischemia, hypoxia, Parkinson's disease, sepsis, diabetes, and cancer. Hydrogen is a newly-explored antioxidant and anti-inflammatory gas that offers a number of advantages compared to traditional antioxidants. First, unlike most known antioxidants, which are unable to target organelles, hydrogen can easily penetrate biomembranes and diffuse into the cytosol, mitochondria, and nucleus due to its low molecular weight. Its high gaseous diffusion rate makes it effective in decreasing cytotoxic free radicals. Hydrogen may be useful in treatment of various diseases. Furthermore, it selectively decreases OH^-, the most reactive ROS, resulting in the formation of water. All of these properties of hydrogen result in reduced oxidative stress by treatment using HW, which makes it a promising treatment for OA.

Conclusions

HW has protective effects against the progression of OA in an animal model, which may be mediated by its antioxidant and anti-inflammatory activities. Further studies are needed to elucidate the precise mechanism underlying the protective effects of HW against the cartilage damage associated with OA.

Conflict of interest

None.

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