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Anti-Osteoarthritic and Anti-Inflammatory Activities of Diazine: *In Vitro* and *In Vivo* Studies

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG **Binjie Gui**
BCD **Jinling Zhang**
CDF **Sisheng Wang**
DF **Genxiang Rong**

Department of Joint Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, P.R. China

Corresponding Author: Binjie Gui, e-mail: binjiegui12@hotmail.com
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Background: The present study evaluated the effects of diazine (DZN) on collagenase-induced osteoarthritis (OA) in rats.

Material/Methods: OA was produced via intra-articular injections of collagenase type II into the knee joint. The rats were then treated with DZN (25, 50, or 100 mg/kg, p.o.) for three weeks. At the end of the protocol, all rats were evaluated for paw latency, paw edema, and knee swelling. Additionally, serum concentrations of glycosaminoglycan (GAG), alkaline phosphatase (ALP), and C-reactive protein (CRP) were determined. X-rays were performed to estimate radiological and histopathological changes in the knee joint. The expressions of antioxidant enzymes, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) were estimated in the synovial tissues.

Results: DZN treatment attenuated inflammation in osteoarthritic rats, as evidenced by decreases in paw edema and knee swelling and enhanced paw latency compared to the negative control group. Additionally, there were significant decreases in the serum levels of CRP and GAG and increases in ALP in the DZN-treated groups compared to the negative control group. The radiological and histopathological results showed that DZN protected against cartilage damage in the knee joint. Additionally, MMP levels decreased and there were significant reductions in the expressions of antioxidant enzymes and TIPMs in the DZN-treated groups compared to the negative control group.

Conclusions: The present findings demonstrated the chondroprotective effects of DZN via its modulation of the expressions of TIMP-1 and MMPs in the synovial tissues of osteoarthritic rats.

MeSH Keywords: **Anti-Inflammatory Agents • Matrilin Proteins • Osteoarthropathy, Primary Hypertrophic**

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Background

Osteoarthritis (OA) is an inflammatory joint disorder that commonly affects elderly individuals [1]. It is characterized by chronic pain, the formation of osteophytes, remodeling of subchondral bone, and a loss of cartilage [2,3]. In patients with OA, the loss of cartilage occurs due to an imbalance between anabolic and catabolic activities within the joint that ultimately results in the death of cartilage and decreases in proteoglycans. Additionally, the release of inflammatory cytokines, such as interleukin (IL)-1β, and other enzymes is enhanced due to altered metabolism levels in joint cells [4] that result from enhanced mechanical load on the joints [5,6]. In OA, matrix metalloproteinase (MMP)-13 expression is enhanced and results in the degradation of aggrecan and type II collagen [7], which are constituents of the cartilaginous extracellular matrix (ECM). Subsequently, macrophages and synoviocytes are activated due to the release of aggrecan fragments [8]. Thus, MMP and cytokine levels increase in the synovial fluid and inflammation is further enhanced by catabolic molecules. Moreover, in patients suffering from OA, there is an increase in the production of reactive oxygen species (ROS) in the joints and this further disturbs the balance of the ECM in articular cartilage. Enhanced oxidative stress causes damage in mitochondria, which, in turn, leads to cartilage damage [9].

CXC chemokine receptor type 4 (CXC4) has a proven role in the development of OA [10]. This receptor is activated in conjunction with proinflammatory cytokines and aids in the degeneration of chondrocytes [11,12]. Diazine (DZN) is a phytoestrogen that has the chemical structure of an isoflavone; it is isolated from *Pueraria lobata* (Fabaceae) and inhibits the activation of CXCR4 [13]. Phytoestrogens possess anti-inflammatory and antioxidant properties [14,15] and DZN is known to inhibit the formation of proinflammatory mediators such as signal transducer and activator of transcription 1 (STAT1) and nuclear factor (NF)-κB [16]. Thus, the present study evaluated the chondroprotective effects of DZN in a rat model of OA induced by intra-articular injections of collagenase.

Material and Methods

Chemicals

Diazine, TRIzol reagent and collagenase type II were procured from Sigma Aldrich Ltd., USA. Other solvent and chemicals were procured from Shanghai chemical supplier Ltd., China.

Animals

Female Wistar rats (180–200 mg/kg), six weeks old, were procured from SLAC Laboratory Animal, Shanghai. All the rats were housed under a controlled condition specified as per guidelines. All the experiments used in the study were approved by the Animal Ethical Committee of The First Affiliated Hospital of Anhui Medical University, China (Approval No: AMU/IAEC/2016/09) and the study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use.

Experimentation

All rats were anesthetized with diethyl ether and the fur on the right knee was shaved off. Next, the rats were divided into four groups. On the first and fourth days of the protocol, the control group received injections of a saline solution and the three remaining groups received intra-articular injections of collagenase type II (50 µL) in the knee joint. From the fourteenth to the thirty-fifth day of the protocol, the negative control group received no treatment and the DZN groups received either 25, 50, or 100 mg/kg (p.o.) administrations of DZN.

Estimation of paw latency, paw volume, and knee diameter

A Vernier caliper was used to measure knee diameter after a specific amount of time, a tail flick apparatus was used to determine paw latency, and a digital plethysmometer was used to determine paw volume and calculate the percentage inhibition of paw edema.

Estimation of biomarkers of OA

Kind and King's method was used to determine the serum concentrations of alkaline phosphatase (ALP), the method of Hoemann et al. was used to perform the glycosaminoglycan (GAG) assay, and a quantitative turbidometric test was performed to estimate the concentrations of C-reactive protein (CRP).

X-ray analysis

All rats were anesthetized at the end of protocol with diethyl ether. Changes in the knee joint and cartilage were estimated using AGFA thermal laser digital photo films to take x-rays of the knee joint.

Histopathological procedures

At the end of treatment protocol, the knee joints of each animal were dissected out after scarifying the rats. The isolated knee joints were fixed with formalin, the tissues were sectioned, and the sections were stained with Masson's trichrome, Safranin-O, and hematoxylin and eosin (H&E) staining. All tissue sections were examined under a trinocular microscope.

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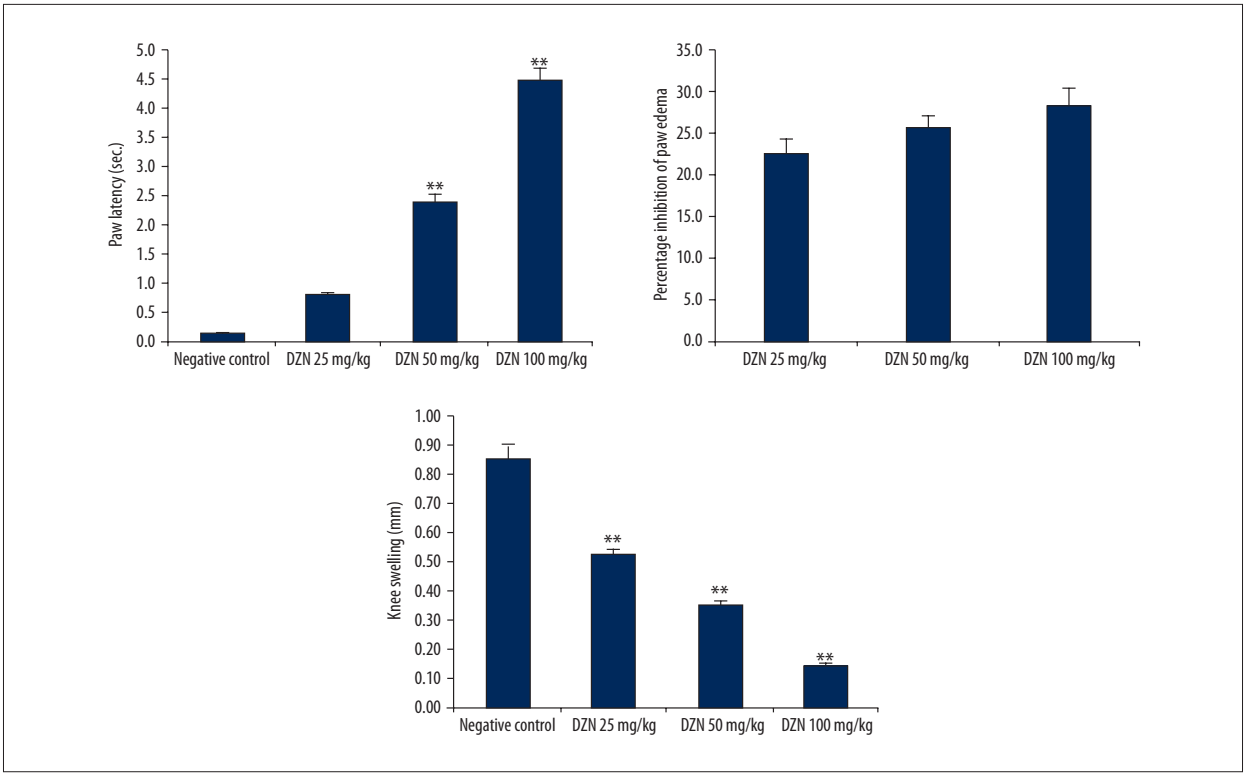

Figure 1. Effects of diazine on paw latency, percentage inhibition of paw edema, and knee swelling in a rat model of osteoarthritis. Mean ±SD (n=6), ** $p<0.01$ compared to the negative control group.

Real-time polymerase chain reaction

Synovial tissues were isolated from the knees of the sacrificed rats and stored at –80°C until further use. The TRIzol method was used to isolate total RNA from the synovial tissue with a PureLink RNA mini kit. Agarose gel electrophoresis was performed to assess the quality of the isolated RNA by determining absorbance at 260 nm and the Super Script first-strand synthesis system was used to synthesize first strand cDNA from the total RNA. TaqMan gene expression assays were used for the quantitative polymerase chain reaction (qPCR) analyses to determine the levels of TIMP-1, MMP-9, MMP-3, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) in the synovial tissue.

Statistical analysis

All data are presented as a mean ± standard deviation (SD; n=6) and were analyzed with one-way analysis of variance (ANOVA) tests and Dunnett's post hoc tests. A p value <0.05 was considered to indicate statistical significance.

Results

Effects of DZN on paw latency, paw volume, and knee diameter

Paw latency was significantly ($p<0.01$) enhanced in the DZN-treated groups compared to the negative control group. Paw volume was defined as the percentage inhibition of paw edema; DZN treatment increased the percentage inhibition of paw edema in a dose-dependent manner. Additionally, DZN treatment significantly attenuated the increased diameter of the knee (Figure 1).

Effects of DZN on biomarkers of OA

The effects of DZN on serum concentrations of biomarkers of OA are presented in Figure 2. The serum level of CRP was enhanced in the negative control group but DZN treatment significantly reduced this concentration. The serum level of ALP was significantly higher in the DZN-treated groups relative to the negative control group. The serum level of GAG was higher in the negative control group compared to the control group but DZN treatment significantly reduced this concentration compared to the negative control group.

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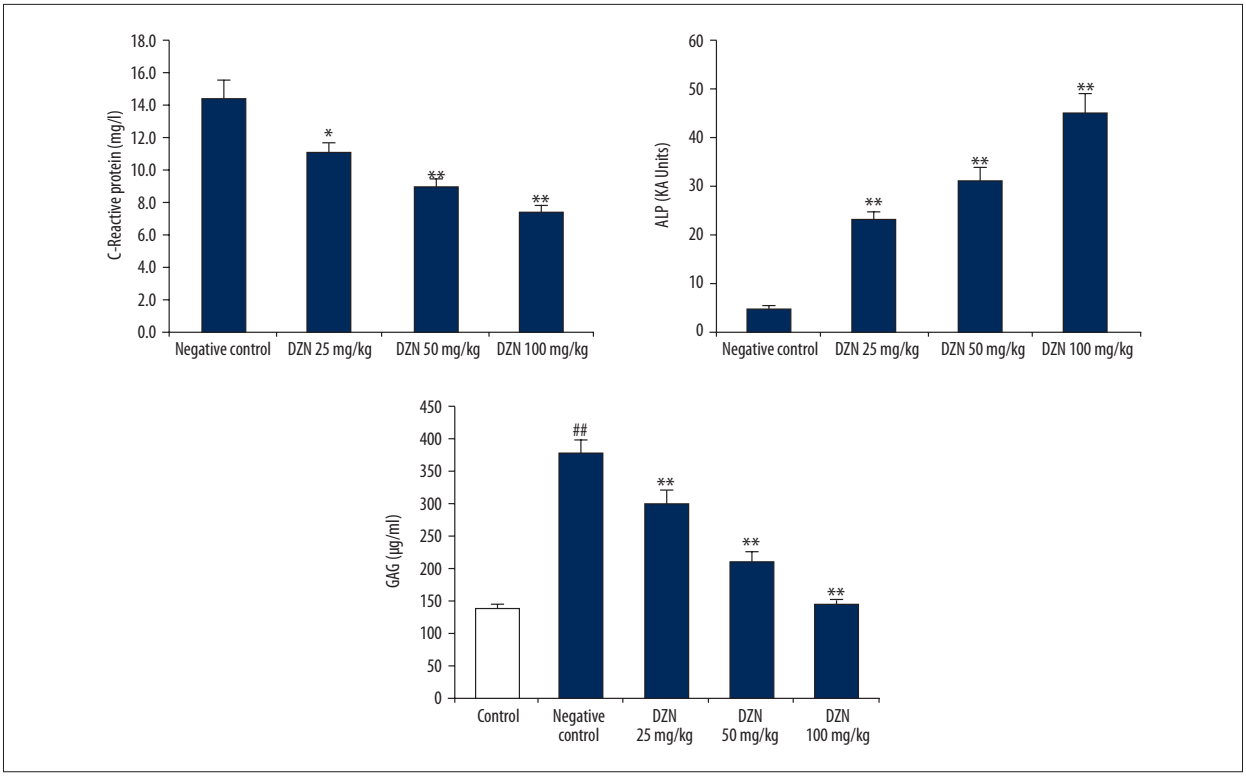

Figure 2. Effects of diazine on the serum concentrations of C-reactive protein, alkaline phosphatase, and glycosaminoglycan in a rat model of osteoarthritis. Mean ±SD (n=6), ## $p<0.01$ compared to the control group; ** $p<0.01$ compared to the negative control group.

Histopathological and radiological results of DZN treatment in the knee joint

Radiological assessments of the effects of DZN on the structure of the knee joint were made by comparing the left and right knee joints for osteophyte formation, spaced joints, and reticular cartilage (Figure 3). These examinations revealed normal surrounding tissue in the knee in the control group and reduced joint space due to loss of articular cartilage in the negative control group. On the other hand, the DZN-treated groups showed normal articular surfaces and improved joint spaces.

The effects of DZN treatment on the cartilage and synovial membrane in OA rats were histopathologically assessed using Masson's trichrome, Safranin-O, and H&E staining (Figure 4). In the control group, cellular filtration was absent in synovium that contained 1–2 cell layers. The negative control group exhibited mononuclear infiltration and the bone lining cells showed an increased number of layers. However, DZN treatment resulted in normal synovium in a dose-dependent manner. Histopathology of the cartilage revealed that the matrix was stained red with Safranin-O and the presence of normal chondrocytes. In the negative control group, there were clusters of chondrocytes and the erosion of cartilage with fibrillation and an irregular surface but DZN protected against the degeneration of the cartilage and the chondroid matrix.

Effects of DZN on the expressions of TIMPs and MMPs

The effects of DZN on the expressions of TIMP1, MMP-3, and MMP-9 in the synovial tissues of OA rats are shown in Figure 5. The expressions of MMP-3 and MMP-9 significantly increased in the synovial tissues of the negative control group compared to the control group but DZN treatment significantly reduced the expressions of MMP-3 and MMP-9 compared to the negative control group. The expression of TIMP-1 was significantly enhanced in the synovial tissues of the DZN-treated groups compared to the negative control group.

Effects of DZN on the expressions of antioxidant enzymes

The effects of DZN on the expression of antioxidant enzymes in the synovial tissues of OA rats are shown in Figure 6. The expressions of SOD, CAT, and GPx were significantly lower in the synovial tissues of the negative control group compared to the control group. However, DZN treatment significantly enhanced these expressions relative to the negative control group.

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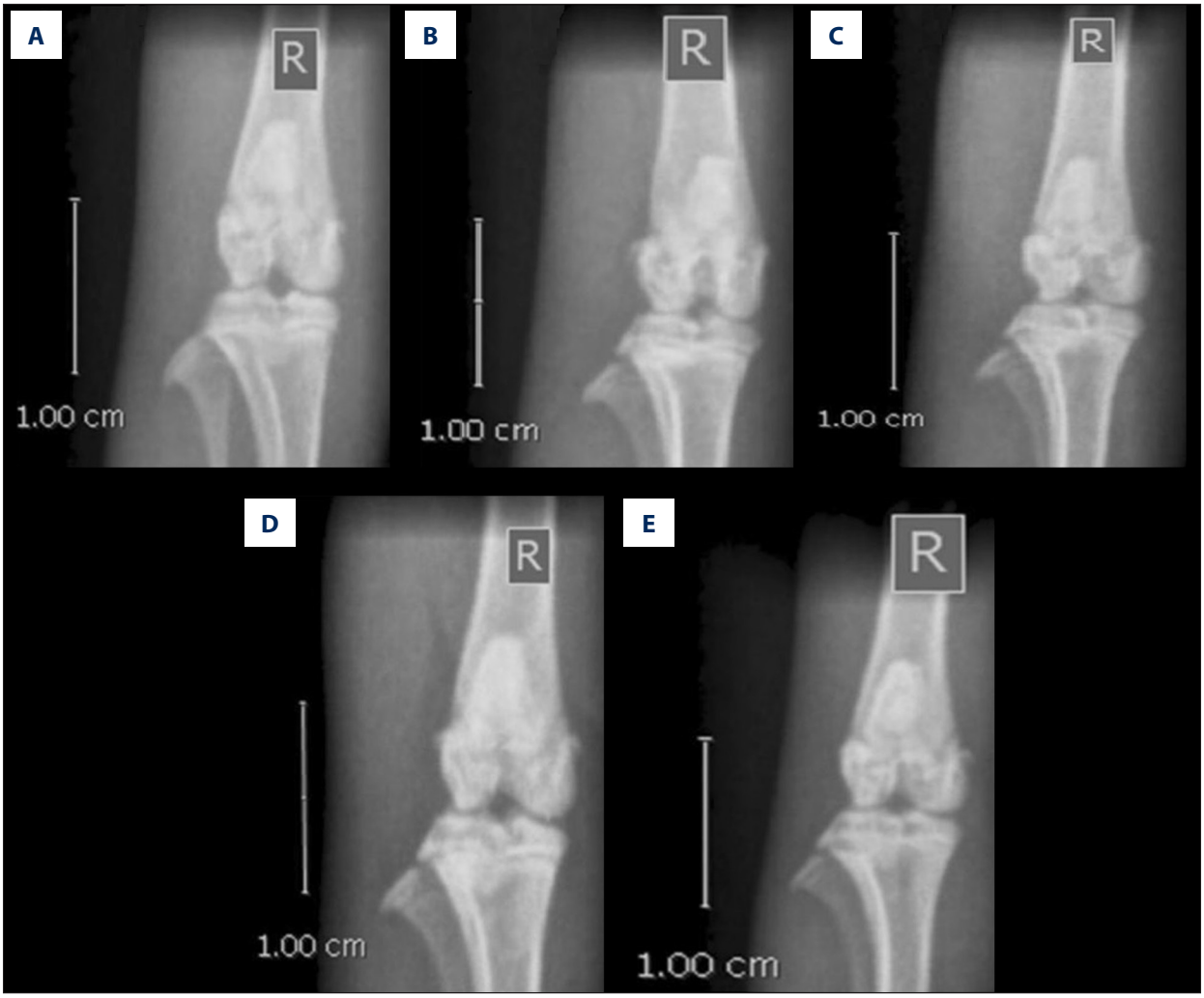

Figure 3. Radiography of the knee joint with x-rays. (**A**) Control; (**B**) Negative control; (**C**) DZN 25 mg/kg; (**D**) DZN 50 mg/kg; (**E**) DZN 100 mg/kg.

Discussion

OA is a joint disorder in which collagen fiber is lost, osteophytes form, synovitis develops, and articular cartilage degenerates [1]. Presently available treatment options for the management of OA primarily treat the inflammation but have only a small effect on the progression of the disease. Moreover, several adverse reactions are associated with these drugs following their chronic use and, thus, more emphasis has recently been given to alternative therapies for the management of OA. Mechanical and biochemical factors contribute to the degeneration of cartilage in OA and several herbal medicines have been shown to manage this disease due to their anti-inflammatory and antioxidant activities that restore the MMP/TIMP balance in synovial tissues [17].

The present study used a rat model to evaluate the effects of DZN on OA induced by intra-articular injections of collagenase by analyzing serum concentrations of biomarkers of OA and assessing swelling. Additionally, histopathological and radiological evaluations of the knee and its associated tissues were performed and the expressions of MMPs, TIMPs, and antioxidant enzymes were estimated in the synovial tissues. The present results show that DZN treatment effectively attenuated the inflammation of paw edema, knee swelling, and paw latency in OA rats and that the levels of biomarkers of OA were significantly reduced by DZN.

The degeneration of cartilage is enhanced by catalytic enzymes such as MMP-3 and MMP-9, which are enhanced following the secretion of cytokines and the generation of ROS in OA [18]. The present results showed that DZN-treated rats had a significant decrease in the expressions of MMP-3 and MMP-9 in the synovial tissues compared to the negative control group. ROS control the secretion of proinflammatory cytokines such as NF-κB and IL, and NF-κB controls the regulation of MMPs

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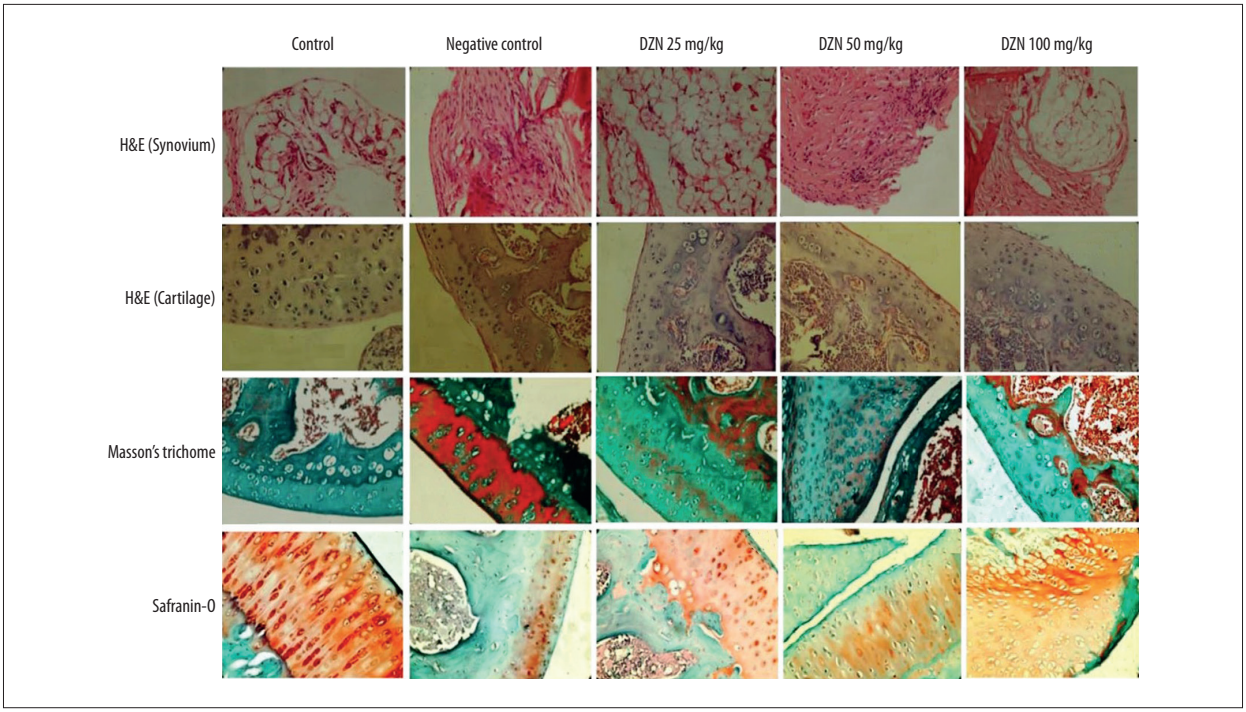

Figure 4. Histopathology of the cartilage and synovial membrane in osteoarthritic rats using Masson's trichrome, Safranin-O, and hematoxylin and eosin staining.

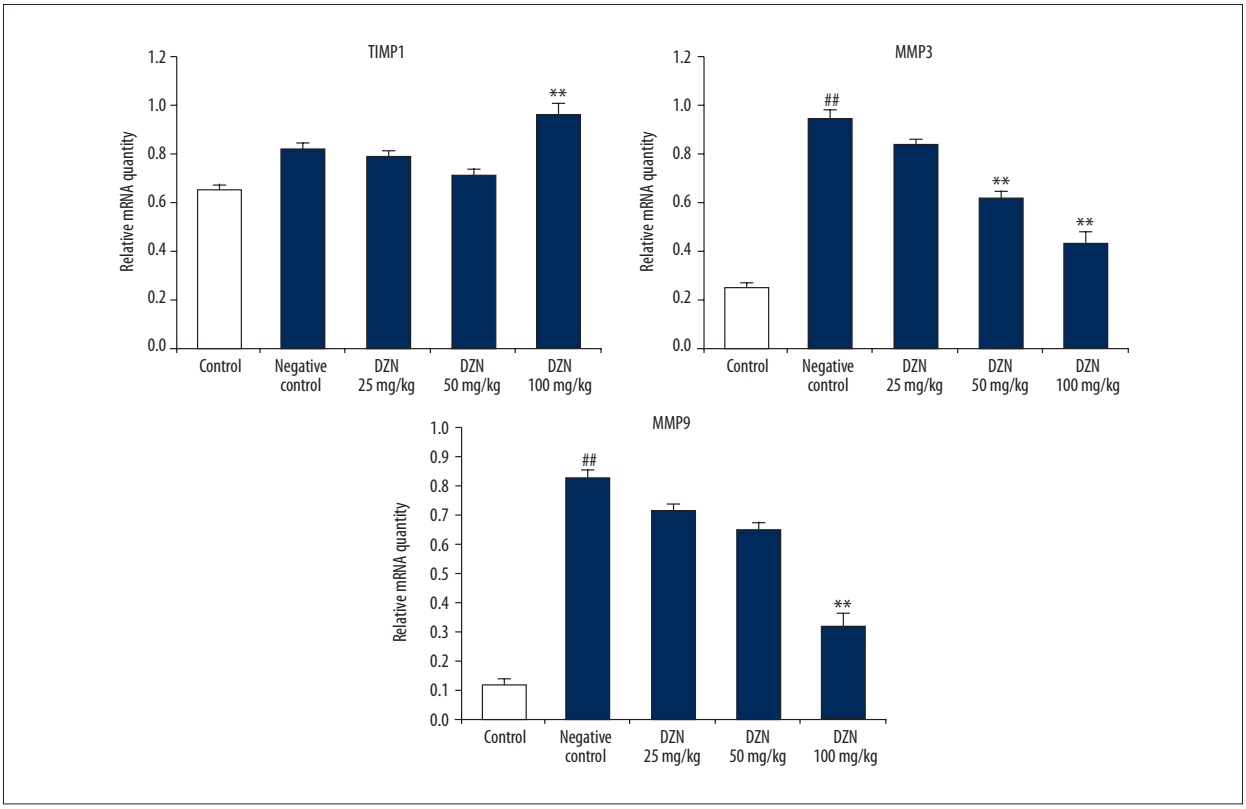

Figure 5. Effects of diazine on the expressions of TIMP-1, MMP-3, and MMP-9 in the synovial tissues of osteoarthritic rats. Mean ±SD (n=6), ## $p<0.01$ compared to the control group; ** $p<0.01$ compared to the negative control group.

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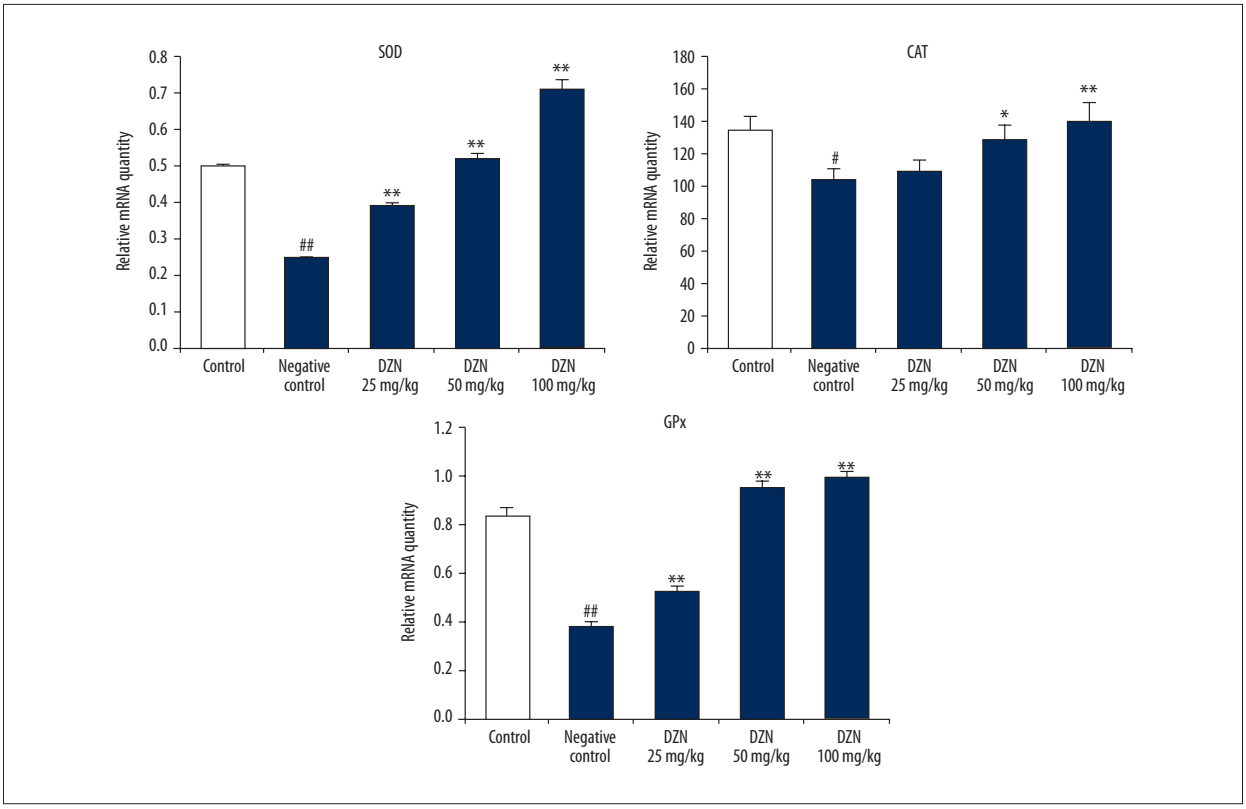

Figure 6. Effects of diazine on the expressions of antioxidant enzymes in the synovial tissues of osteoarthritic rats. Mean ±SD (n=6), ## $p<0.01$ compared to the control group; * $p<0.05$ and ** $p<0.01$ compared to the negative control group.

as well as the degradation of ECM turnover [19]. On the other hand, TIMPs maintain the regulation of MMPs in synovial tissues; thus, both MMPs and TIMPs play vital roles in the progression of OA [20]. The present findings indicate that DZN improved the expression of TIMP-1 in synovial tissue and that the expression of antioxidant enzymes increased in a dose-dependent manner in the DZN-treated groups compared to the negative control group. Moreover, the present histopathological results suggest that DZN treatment prevented the degradation of the ECM and the erosion of cartilage.

Conclusions

In the present study, DZN exerted chondroprotective effects by modulating the expressions of TIMPs and MMPs in the synovial tissues of OA rats. Additionally, DZN reduced the OA-induced expressions of antioxidant enzymes. Thus, DZN may be useful as an alternative therapy for the management of OA.

Acknowledgements

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Conflicts of interest

None.

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