

## Original Research Article

# Benzoxime inhibits matrix metalloproteinase-13 activation and cartilage damage in osteoarthritis rats via inhibition of NF- $\kappa$ B pathway

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### Abstract

**Purpose:** To investigate the effect of benzoxime on degradation of articular cartilage in a rat model of osteoarthritis (OA), and the mechanism involved.

**Methods:** The OA rat model was prepared by injecting monosodium iodoacetate (MIA) intra-articularly to Wistar rats. Rats in the treatment group were given benzoxime (5 mg/kg) daily for 21 days through the intra-articular route. The animals were then examined for behavioral changes by assessment of asymmetry in bearing weight and paw withdrawal threshold of the hind limb. Western blot assay was used for the analysis of inflammatory cytokine expressions.

**Results:** The expression of P2X purinoceptor 7 receptor (P2X7R) mRNA was significantly elevated in the OA rats ( $p < 0.02$ ). However, benzoxime treatment caused a marked decrease in the level of P2X1-8R mRNA. Benzoxime treatment also prevented asymmetry in bearing weight, decreased paw withdrawal threshold, and inhibited the expressions of interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$  in plasma and cartilage. Moreover, benzoxime exhibited significant inhibitory effects on the expressions of P2X7R, matrix metalloproteinase (MMP)-13 and prostaglandin E2 (PGE2) in cartilage tissue. It also significantly suppressed OA-induced increases in the levels of inhibitor of nuclear factor- $\kappa$ B (NF- $\kappa$ B) kinase (IKK) $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65, and blocked OA-induced increases in the expressions of P2X7R, MMP-13 and PGE2.

**Conclusion:** These results demonstrate that benzoxime prevents cartilage degradation in OA rats by targeting NF- $\kappa$ B signaling pathway. Thus, benzoxime possesses clinical and therapeutic potentials for the prevention of cartilage degradation in OA.

**Keywords:** Interleukin-1 $\beta$ , Purinoceptor-7, Benzoxime, Osteoarthritis, Prostaglandin, Matrix metalloproteinases

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## INTRODUCTION

Osteoarthritis (OA), which is characterized by alteration in the architecture and composition of

joints has adverse impact on socio-economic development [1, 2]. The symptoms of early-stage OA include joint inflammation, edema, stiffness and pain, while the advanced stage is

characterized by deformity of the joints [1,2]. The pathological alterations associated with OA are articular cartilage degradation, damage to subchondral bone and hyperplasia of the synovium [2]. Changes in the equilibrium between formation and degradation of cartilage matrix components are linked to etiology of bone tissue damage [3].

The current treatment for OA involves conservative measures like modification of lifestyle, physiotherapy, chemotherapy and surgery [4]. At the early stage of OA, treatment involves symptomatic relief using available chemotherapeutic agents, while the advanced stage (characterized by irreversible disability of joints) requires surgery to relieve pain and improve joint function [5]. The development and progression of OA are induced by the expression of cytokines through alteration in the equilibrium between formation and degradation of chondrocytes [6]. Up-regulation of interleukin-13 leads to the breakdown of cartilage matrix and affects chondrocyte function [6,7]. The chondrocytes and synovial membrane of OA patients express higher level of interleukin-1 $\beta$ , interleukin-6, tumor necrosis factor- $\alpha$  and prostaglandin E2 [8].

The expression of matrix metalloproteinases (which degrade cartilage tissues) is enhanced by prostaglandin E2 [9]. The currently-used non-steroidal anti-inflammatory drugs for OA treatment are associated with development of complications, while steroidal agents do not show satisfactory results [9]. Thus, the need for identification of newer and more effective chemotherapeutic agents for OA has continued to be a challenge to clinicians. Purinergic membrane receptor P2X expression in cartilage tissues has been shown to be associated with the onset of pain in OA patients [10]. The P2X7 receptor (P2X7R) plays an important role in the regulation of joint pain and edema [11]. It has been reported that damage to peripheral nerves causes up-regulation of P2X7R in macrophages [12].

The present study was undertaken to investigate the effect of benzoxime on cartilage tissue degradation in OA rats and the mechanism involved.

## EXPERIMENTAL

### Animals

Thirty, 8 – 10-week old male Wistar rats were obtained from the Shanghai SLAC Animal Co., Ltd., Shanghai. The rats were maintained singly

in plastic boxes under 12 h light - 12h dark cycle at a constant temperature of 24 °C and humidity of 55  $\pm$  10 %, with *ad libitum* access to fresh water and standard laboratory feed. The rats were acclimatized to the laboratory conditions for one week before the start of the study. All animal experiments carried out according to the guidelines of Principles of international laboratory animal care [13]. The approval for the study was provided by the Research Ethical Committees of the Provincial Hospital Affiliated to Shandong University (approval no. TKY03331).

### Preparation of OA rat model and treatment

Twenty rats exposed to volatile isoflurane anesthesia were administered 5 mg/kg MIA through the intra-articular route. The rats were then assigned to two groups of 10 animals each: benzoxime treatment group and model control group. The rats in the treatment group were intra-articularly injected 5 mg/kg benzoxime daily for 21 days. After analysis of behavioral changes for 21 days, the rats were sacrificed for western blot, enzyme-linked immunosorbent assay (ELISA) and quantitative reverse transcription polymerase chain reaction (RT-qPCR).

### RT-qPCR

After treatment with benzoxime for 21 days, the OA rats were sacrificed by spinal dislocation. The limb skin was disinfected to intercept the femur 5cm from femoral condyle. Subsequently the tibia was also dissected below the tibia plateau to isolate the knee joint. The tissues around the joint were cleaned to expose the articular cartilage which was then carefully cut off from the joint. The RNA present in the cartilage was then isolated by TRIzol reagent (BD Biosciences) in accordance with the instructions on the kit manual. Samples of the RNA (5  $\mu$ g samples) were subjected to reverse transcription using the PrimeScript RT reagent kit (Sigma-Aldrich, St. Louis, MO, USA). A mixture of 5X PrimeScript buffer (2  $\mu$ L), PrimeScript RT enzyme mix (0.5  $\mu$ L), total RNA (2  $\mu$ L) and RNase-free dH<sub>2</sub>O (5  $\mu$ L) was incubated at room temperature for 20min, followed by heating to 85 °C for 20 s.

SYBR Premix Ex Taq TM II kit (RR041A; Sigma-Aldrich) was used for quantitative polymerase chain reaction. The cDNA (2  $\mu$ L) was combined with SYBR Premix Ex Taq (12.5  $\mu$ L), 1  $\mu$ L each of forward and reverse primers, and dH<sub>2</sub>O (8.5  $\mu$ L). The mixture was subjected to 40 cycles: initially 5 min at 95 °C, then denaturation for 30 sec at 95 °C, annealing for 40 sec at 60 °C, and extension for 30 sec at 72 °C. After completion, 71 more cycles were performed at 80 °C for 30 sec.

Subsequently, the temperature was increased by 0.5 °C for each repetition. Protein expression was normalized to GAPDH mRNA. The expressions of genes were quantified using SYBR Green Master mix (Life Technologies, Carlsbad, CA, USA).

### Behavioral assessments

Following benzoxime treatment for 21 days, the OA rats were examined for asymmetry in bearing weight for the hind limb, and their paw withdrawal thresholds were evaluated. Asymmetry in bearing weight is expressed as the percentage of the distribution of weight between left and right hind limbs. The reference level was taken as the value obtained at the start of treatment. In the determination of paw withdrawal threshold, the animals were put on a steel wire mesh in an organic transparent glass box of dimensions 20 cm x 20 cm x 30 cm. Von Frey monofilaments (58011, Stoelting Co., USA) were employed for the measurement of paw withdrawal threshold. For each animal, three readings were recorded for both hind limbs, and the mean value was calculated. The difference between the two hind limbs was used to determine paw withdrawal threshold.

### Analysis of knee edema size

Edema sizes of the knee in the treatment, OA and vehicle control groups were determined after 21 days of treatment. Knee diameter was determined with a digital caliper and recorded for each rat. The diameter of the left knee was then subtracted from that of right knee to obtain the knee edema [14].

### Determination of cytokine expressions

*Blood samples were collected from the carotid artery of the animals under isoflurane anesthesia in EDTA phlebotomy blood sample bottles (Jiangsu Yuli Medical instruments Co., Ltd, China). The blood samples were centrifuged for 20 min at 3,500 x g to obtain plasma samples which were stored under liquid nitrogen atmosphere. Cytokine levels in the plasma samples were determined using ELISA kits (Santa Cruz Biotechnology Inc., CA, USA) as per the manufacturer's protocol.*

### Western blot analysis

Sliced cartilage tissues from the treatment, OA and vehicle groups of rats were subjected to homogenization in TRIzol® (50mg fresh weight/mL; Invitrogen, Paisley, UK) in liquid

nitrogen using a dismembrator (Sartorius, Epsom, UK). The tissue homogenates were frozen, treated with lysis buffer and centrifuged for 10min at 12,000 x g at 4 °C to remove the sediments. The protein contents of the supernatant fractions were determined with BCA protein assay kits. Thereafter, protein samples (25 - 35 µg) were subjected to electrophoresis on 12 % sodium dodecyl sulfate-polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. Membrane blocking was performed for 2h at 36 °C using 3 % non-fat milk.

The membranes were incubated overnight with primary anti-bodies at a temperature of 4 °C. The following primary antibodies were used: P2X7R (dilution 1:800; catalog no. P8232; Sigma-Aldrich), MMP-13 (dilution 1: 1, 000; catalog no. sc-2119; BD Biosciences), PGE2 (dilution 1: 1, 000; catalog no. ab176140; Abcam), IL-1β (dilution 1: 500; catalog no. KAC1211; Thermo Fischer Scientific), IL-6 (dilution 1: 500; catalog no. PHC0066; Thermo Fischer Scientific), TNF-α (dilution 1: 500; catalog no. KAC1751), IKKα (dilution 1: 600; catalog no. 14A231; Merck), p-IKKα (dilution 1: 500; catalog no. 16A6; Cell Signaling Technology), IKKβ (dilution 1: 1, 000; catalog no. SC8330; Santa Cruz), p-IKKβ (dilution 1: 400; catalog no. AB55341; Abcam), IκBα (dilution 1: 800; catalog no. 2118S; Cell Signaling Technology), p-IκBα (dilution 1:500; catalog no. 2859; Cell Signaling Technology), NF-κBp65 (dilution 1: 600; catalog no. KHO0371; Thermo Fischer Scientific), p-NF-κBp65 (dilution 1: 500; catalog no. 3033; Cell Signaling Technology) and GAPDH (dilution 1: 2, 000, BD Biosciences). The membranes were washed two times with PBS, and incubated for 2h at ambient temperature with secondary antibodies conjugated with HRP. Visualization of the protein bands was achieved using an enhanced chemiluminescence detection system. Analysis of the densitometric values was done by the use of Image-ProPlus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Normalization was done with GAPDH.

### Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA) and Bonferroni tests for comparison among more than two groups. Comparison between two groups was performed using Student's *t*-tests, with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### Effect of benzoxime on OA rats

Rats in the control group showed no changes in asymmetry in bearing weight and paw withdrawal thresholds during the 21 days of study (Figures 1 A and B). On the other hand, MIA administration significantly increased asymmetry in bearing weight to 32 % on day 21 (Figure 1 A;  $p < 0.05$ ), and significantly decreased paw withdrawal threshold ( $p < 0.05$ ) on day 21, when compared to the control group (Figure 1B). The MIA administration also led to a significant increase in knee edema within the same period (Figure 1 C;  $p < 0.05$ ). Administration of benzoxime to OA rats reversed the effects produced by MIA (Figure 1A). Benzoxime administration blocked the MIA-induced increases in weight bearing asymmetry and increased paw withdrawal threshold ( $p < 0.05$ ). Moreover, benzoxime significantly reduced knee edema in the OA rats, relative to rats administered MIA alone ( $p < 0.05$ , Figure 1C).

### Benzoxime prevented up-regulation of P2X7R expression in OA rats

In the normal rats, the expression of P2X1-8R mRNA was slightly higher in cartilage tissues when compared to other members of P2X family (Figure 1 D). However, the expression of P2X1-8R mRNA was significantly higher in the OA rats, relative to the other members of this family ( $p < 0.05$ , Figure 1 D). Benzoxime treatment of the OA rats caused significant decreases in the levels of P2X1-8R mRNA, when compared to the untreated control group.

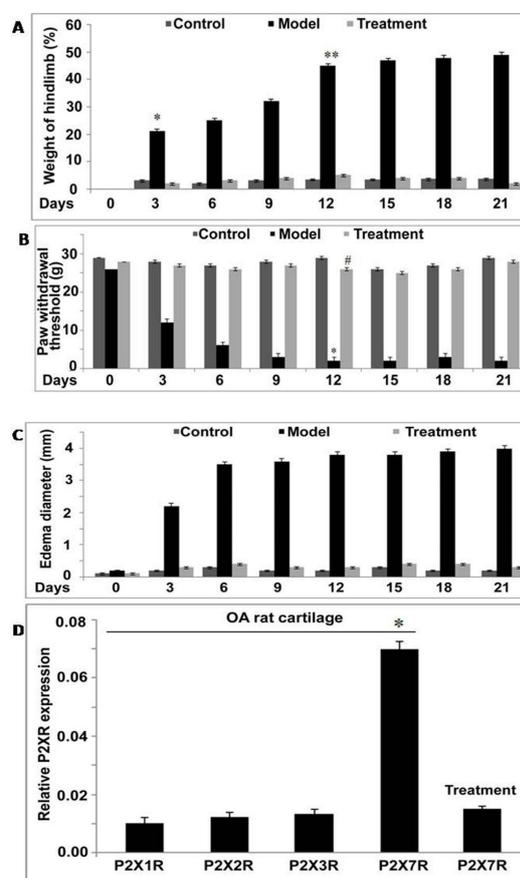
### Benzoxime inhibited interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$ expressions in OA rat plasma

Analysis of cytokine expression in OA rat plasma showed significantly higher levels of interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$  ( $p < 0.02$ ). However, intra-articular administration of benzoxime to the OA rats prevented the MIA-induced increases in the expressions of interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$  (Figure 2).

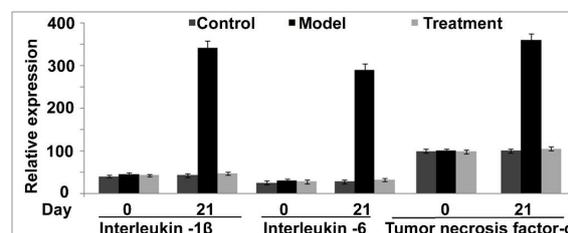
### Benzoxime inhibited expressions of cytokines in the cartilage tissues of OA rats

Western blot analysis showed significantly higher expressions of interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$  in cartilage tissues of OA rats, when compared to the normal rats (Figure 3). The expressions of interleukin-1 $\beta$ , interleukin-

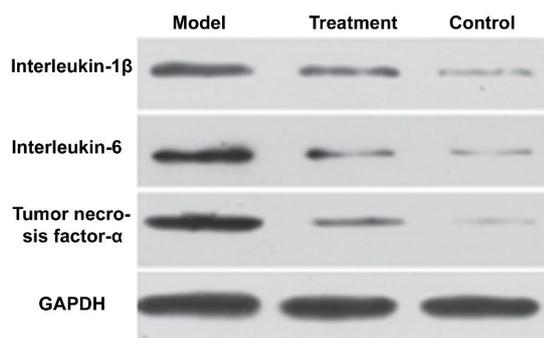
6 and tumor necrosis factor- $\alpha$  in the cartilage tissues of OA rats were markedly down-regulated by administration of benzoxime ( $p < 0.01$ , Figure 3).



**Figure 1:** Effect of benzoxime on behavioral changes and expression of P2X1-8R mRNA in OA rats. (A) OA rats showed increase in asymmetry of bearing weight which was prevented by benzoxime administration. (B) In OA rats, benzoxime increased paw withdrawal thresholds and (C) inhibited edema formation. \* $p < 0.02$ , untreated vs. normal; # $p < 0.05$ , treatment group vs. untreated. (D) Benzoxime treatment for 21 days inhibited P2X1-8R mRNA expression in OA rats



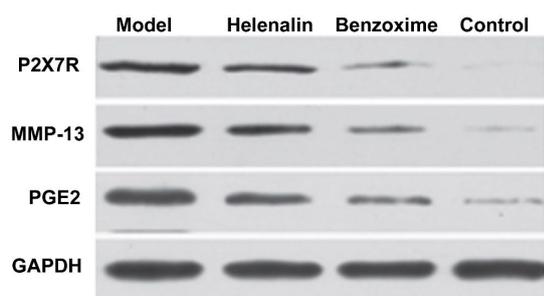
**Figure 2:** Effect of benzoxime on cytokine expression in OA rats. After 21 days of treatment, the levels of interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$  in the rat plasma were examined by ELISA. Benzoxime treatment inhibited the OA-induced up-regulation of cytokines in the plasma. \* $p < 0.02$ , untreated vs. normal; # $p < 0.02$ , treatment vs. untreated



**Figure 3:** Effect of benzoxime on the expressions of pro-inflammatory factors in the cartilage tissues of OA rats. The cartilage tissues of OA rats contained higher levels of interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$ . Treatment of OA rats with benzoxime for 21 days inhibited OA-mediated up-regulation of these cytokines

### Benzoxime inhibited P2X7R, matrix metalloproteinase-13 and PGE2 expressions in OA rats

The expressions of P2X7R, matrix metalloproteinase-13 and PGE2 in the cartilage tissue were significantly higher in OA rats than in normal control rats ( $p < 0.05$ , Figure 4). However, benzoxime treatment exerted inhibitory effects on the expressions of these proteins (P2X7R, matrix metalloproteinase-13 and PGE2) in the cartilage tissues of OA rats (Figure 4). Significant decreases in the expression of P2X7R, MMP-13 and PGE2 were also observed in OA rats administered helenalin, a nuclear factor- $\kappa$ B (NF- $\kappa$ B) inhibitor.

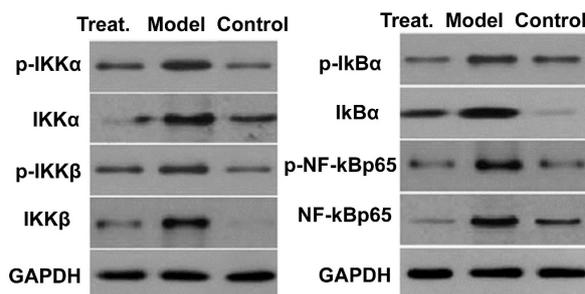


**Figure 4:** Effect of benzoxime treatment on the expressions of PGE2, MMP-13 and P2X7R expression in cartilage tissues of OA rats

### Effect of benzoxime on the expressions of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65 in OA rats

Results from western blot assay showed significant increases in the expressions of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , and NF- $\kappa$ B p65 in the cartilage tissues of OA rats (Figure 5;  $p < 0.05$ ). The expressions of activated IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 were significantly increased in the OA rats, when compared to normal rats. However,

benzoxime administration to OA rats suppressed the increases in the levels of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 in the OA rats (Figure 5).



**Figure 5:** Effect of benzoxime on the expressions and phosphorylation of IKK $\alpha$ , I $\kappa$ B $\alpha$ , NF- $\kappa$ Bp65 and IKK $\beta$  in OA rats. The expressions and activation of these molecules were significantly enhanced in OA rats, but were inhibited by benzoxime treatment

## DISCUSSION

It has been reported that inhibition of the expression of inflammatory cytokines prevents the decomposition of cartilage tissues [15]. The characteristic features of OA include knee joint edema, decomposition of cartilage, hyperplasia of synovium and degradation of sub-chondral bones [16]. Various cytokines such as nitric oxide, PG, interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , interleukin-6 and interleukin-8 are expressed in higher proportions in the chondrocytes of OA patients [6,9]. Chondrocytes play an important role in the onset of pain during inflammation by activating the neurons [7,16]. Studies have demonstrated that the expressions of interleukin-4, tumor necrosis factor- $\alpha$ , interleukin-6, and interleukin-13 are promoted by P2X7R activation [17].

Down-regulation of tumor necrosis- $\alpha$  also leads to longer inhibition of inflammation [18]. It has been observed that P2X7R is linked to inflammation of knee joints and development of OA [10]. The current study has demonstrated that pain associated with OA in rats can be successfully suppressed by treatment with benzoxime. In addition, edema formation in the OA was prevented by treatment with benzoxime through inhibition of inflammatory processes. It was also demonstrated that in OA rats the expression of P2X1-8R mRNA was significantly higher and that of other members of P2X receptors.

Benzoxime administration blocked MIA-induced increases in asymmetry in bearing weight and decreased paw withdrawal threshold. In addition, benzoxime administration to OA rats significantly prevented knee edema, when compared to rats

administered MIA alone. In OA patients benzoxime exhibits inhibitory effect on inflammation, and PGE2 upregulates degradation of cartilage by promoting the expressions of matrix metalloproteinases [9]. The level of matrix metalloproteinase-13 expression in OA patients is considered to be a reliable indicator of the extent of degradation of cartilage [19].

The expression of metalloproteinase-13 was significantly higher in the OA but was inhibited by treatment with benzoxime. It has been reported that the expression of interleukin-1 $\beta$  in the chondrocytes causes activation of NF- $\kappa$ B pathway [20]. Studies have also revealed that the NF- $\kappa$ B signaling pathway is activated by expression of P2X7R in OA patients [21]. In the present study, the activation of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65 was promoted in OA rats. However, treatment of the OA rats with benzoxime prevented up-regulation of these molecules. The inactive form of NF- $\kappa$ B exists in the cytoplasm and on activation by P2X7R the molecule enters the nucleus [22]. This study has demonstrated that expression of P2X7R, matrix metalloproteinase-13 and PGE2 in the cartilage tissues of OA rats were significantly higher than their corresponding levels in normal rats.

Administration of benzoxime exhibited inhibitory effects on the expression of P2X7R, matrix metalloproteinase-13 and PGE2 in the cartilage tissues of OA rats. Western blot assay showed that the expression of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65 were increased in the cartilage tissues of OA rats. The expressions of activated IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 were increased significantly in the OA rats, relative to normal rats. However, benzoxime administration suppressed the increased levels of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 in the OA rats.

## CONCLUSION

The findings of the current study demonstrate that benzoxime prevents cartilage degradation in OA rats through targeting NF- $\kappa$ B signaling pathway. This implies that benzoxime possesses clinical and therapeutic potential for the management of cartilage degradation associated with OA.

## DECLARATIONS

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### Conflict of interest

The authors declare that no conflict of interest is associated with this study.

### Author contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tao He and Zhi-ming Li performed the experimental work and Ming Li designed the study. Ting-bin Yan compiled the results and did literature study. Ming Li and Ting-bin Yan wrote the paper. All the authors read and approved the manuscript before its submission for publication.

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