

Original Research Article

Benzoxime carbaldehyde prevents rheumatoid arthritis in a rat model by inhibition of oxidative damage

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Abstract

Purpose: To investigate the effect of benzoxime carbaldehyde (BXCD) on rheumatoid arthritis (RA) in a rat model.

Methods: Thirty male Sprague-Dawley rats were assigned randomly to 5 groups (6 rats per group): normal control, RA, and three treatment groups. Rats in the normal control and RA groups received normal saline, whereas those in the three treatment groups were given 2, 5 or 10 mg/kg of BXCD daily for 30 days by intraperitoneal injection. Pressure pain was analysed using electronic pressure pain detector, while the expressions of interleukin (IL)-6, interleukin (IL)-1 β , nuclear factor (NF)- κ B p65 and tumor necrosis factor (TNF)- α in serum were determined using enzyme-linked immunosorbent assay (ELISA) kits.

Results: Treatment of RA rats with BXCD for 30 days led to significant ($p < 0.05$) recovery in pain threshold. At a dose of 10 mg/kg, BXCD decreased pain threshold value to a level comparable to that in normal control rats, and decreased arthritis score to 1, relative to arthritis score of 16 in untreated animals. Malondialdehyde (MDA) level was 4-fold higher in untreated RA rats than in normal and BXCD-treated groups. BXCD treatment increased the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), and blocked increases in the blood levels of IL-6, IL-1 β , NF- κ B p65 unit, and TNF- α . Western blot assay showed that BXCD treatment prevented increase in the level of cyclooxygenase-2 (COX-2) in RA rat tissues.

Conclusion: These results indicate that BXCD prevents RA in a rat model via inhibition of expressions of inflammatory cytokines and decrease in oxidative stress. Thus, BXCD has a strong potential for the management of RA.

Keywords: Rheumatoid arthritis, Pain threshold, Antioxidant enzymes, Inflammation, Inflammatory cytokines

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INTRODUCTION

Rheumatoid arthritis (RA) is a joint disorder characterized by infiltration of inflammatory cells, hypertrophy and degeneration of cartilage and bone [1]. The mechanism underlying the

aetiology of RA is yet to be fully understood [1]. The main focus of treatment in patients suffering from joint disease is aimed at avoiding permanent disability associated with RA [2]. The initial stage of RA involves erosion of bone and cartilage tissues at the joints [2]. Thus, the arrest

of the growth and proliferation of synovial tissues is considered to be of significance in the treatment of RA [3]. Due to associated inflammatory disorder, RA is linked to increased expressions of inflammatory cytokines [4]. Indeed, IL-1 β and TNF- α are elevated in the tissues of RA patients [4]. In addition, COX-2 is expressed very markedly in the joints of RA patients [5,6]. It is also believed that antigen-catalysed, challenge-chained immune reaction plays an important role in the development and progression of RA [7, 8]. Moreover, genetic and environmental factors induce Th1 cells to produce higher content of IL-1, IL-6 and TNF- α [7,8]. The expressions of these cytokines facilitate B cell activation and ultimately lead to the formation of synovial lesions [7,8].

Thus, studies aimed at understanding the aetiology of RA, and developing efficient methods for its treatment are crucial. In the present study, the role of BXCD (Figure 1) in RA treatment in a rat model, and its mechanism of action were investigated.

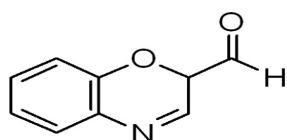


Figure 1: Chemical structure of benzoxime carbaldehyde

EXPERIMENTAL

Animals

Male Sprague-Dawley rats (weighing about 270 g) were used in this study. They were purchased from the Experimental Animal Centre of China Medical University (Shenyang, China). The animals were housed individually in cages under 12/12 h light and dark cycle at constant temperature of 23 °C. The housing atmosphere was humidity-controlled, and the rats were provided access to feed and water *ad libitum*. The study was approved by the Committee for Animal Care and Use of Sun Yat-Sen University, China (no. SYSU-206), and the experimental protocols were performed according to the guidelines of Care and Use of Laboratory Animals by the National Institute of Health, China [9].

Preparation of RA rat model

The RA rat model was prepared according to previous protocol [10]. The rats were kept in cages under 12/12 h light and dark cycle in an animal house with a humidity of ~85 % and

temperature 5 – 7 °C for 20 days. They were anesthetized with isoflurane (2 % in O₂) on day 21. Freund's complete adjuvant (10 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously into the right foot of each rat between 2nd and 3rd toes. Examination of the animals 72 h later revealed inflammation in the right ankles, and the appearance red coloured swellings in the forelimbs and contralateral limbs. These symptoms clearly indicated the development of arthritis in the rats.

Treatment strategy

Thirty rats were randomly assigned into 5 groups of 6 animals each. The five groups were normal control, RA group, and three treatment groups. The rats in the three treatment groups were injected intraperitoneally with BXCD at doses of 2, 5 or 10 mg/kg daily for 30 days. The rats in the normal control and RA groups received normal saline.

Determination of pain threshold

After 30 days of treatment, pressure pain was measured in rats in the five groups. Pressure pain was determined using an electronic pressure pain detector (Somedic AB, Hörby, Sweden) according to the manual protocol. The pressure pain for each animal was measured in triplicates, with an intervening period of 20 min in accordance with normal procedures [11].

Determination of RA scores

Rats in all the groups were examined for RA scores after 30 days of treatment. The RA scores were classified according to the degree of arthritis thus: arthritis in whole paw and digits was assigned scores of 11 – 15; arthritis in more than two joints was scored between 6 and 10, while arthritis in only two joints was scored between 1 and 5. Absence of arthritis was scored 0.

Determination of oxidative stress parameters in rat serum

At the end of treatments, blood samples were collected from each rat and centrifuged at 3,000 g at 4 °C for 20 min. The serum samples were then analysed for SOD, MDA, CAT and GSH-Px using commercially available kits (Nanjing Jiancheng Co Ltd) [12].

Assay of blood levels of cytokines

The rats were anaesthetized using 1% mebumal sodium (Sigma-Aldrich). Blood sample was

collected from the carotid artery of each rat and subjected to centrifugation for 20 min at 3,500 g. The supernatant was stored at -78 °C under liquid nitrogen prior to assay of IL-6, IL-1 β , NF- κ B p65 and TNF- α using enzyme-linked immunosorbent assay (ELISA) kits (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in accordance with the manufacturer's protocol.

Western blot assay for COX-2 expression

The expression of COX-2 in rat tissues was determined with western blot assay. The rats were sacrificed under 1 % pentobarbital anaesthesia, and 15 mg tissue sections were excised. The tissue samples were then incubated for 45 min under ice-cold conditions with RIPA buffer (Rockland, Gilbertsville, PA, USA). The tissue homogenates obtained were subjected to centrifugation at 4 °C for 15 min at 10,000 g. Protein concentration was determined with bicinchoninic protein assay kit (Pierce, Rockford, IL, USA). Resolution of the lysates (5 μ g) on 12.5 % SDS-PAGE gel was followed by transfer to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) using electroblotting. The membranes were incubated overnight at 4 °C with monoclonal primary antibodies against COX-2 (dilution 1: 1, 200; catalog no. sc-376861), and β -actin (dilution 1:1, 200; catalog no. sc-7210). Both antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). After incubation, the membranes were washed three times using Tris-buffered saline with Tween-20. The membranes were then subjected to incubation with polyclonal goat anti-mouse antibody (catalog no. 7076; dilution 1: 2, 000; Cell Signalling Technology, Inc.) for 1 h. Development of the blot was achieved using enhanced chemiluminescence kit (Intron Biotechnology Inc., Seongnam, Korea).

Statistical analysis

Data are presented as mean \pm SD ($n = 3$). Data analysis was performed with one-way analysis of variance (ANOVA) followed by the least significant difference (LSD), using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

BXCD treatment improved pain threshold in RA rats

Pain threshold was significantly decreased in the RA, when compared with rats in the normal control group ($p < 0.05$, Figure 2). However,

BXCD treatment for 30 days brought about significant recovery in pain threshold, with the 10 mg/kg BXCD group resulting in pain threshold comparable to that of normal control animals (Figure 2). These findings suggest that BXCD mitigates RA in the rat model.

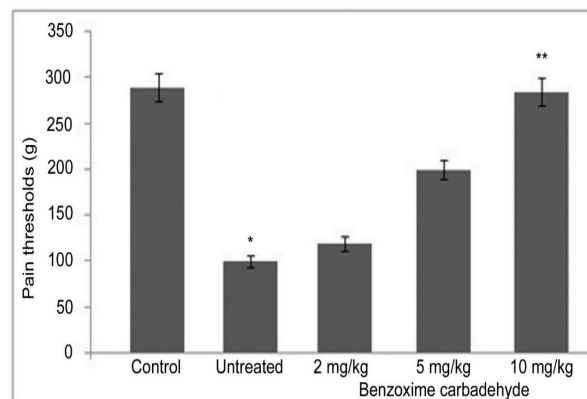


Figure 2: Effect of BXCD on pain threshold in rats with RA. Following induction of RA, the rats were treated with 2, 5 or 10 mg/kg BXCD for 30 days. * $p < 0.01$, compared with normal control rats; ** $p < 0.05$, compared with untreated RA rats

BXCD decreased RA scores in rats

Arthritis score was significantly higher in RA rats than in the normal control group (Table 1). On the other hand, arthritis score in the RA rats after 30 days of BXCD treatment was very low. In the 10 mg/kg BXCD-treated group, the arthritis score was 1, whereas untreated RA group had arthritis score of 16 (Table 1). These results are shown in Table 1.

Table 1: Effect of BXCD on arthritis score in rats with RA

Group	RA score
Control	0
Untreated	16
2 mg/kg BXCD	12
5 mg/kg BXCD	7
10 mg/kg BXCD	1

BXCD treatment prevented increase in MDA and decreases in activities of SOD, CAT and GSH-px in the blood of RA rat

There was a significant increase in MDA level in RA group, relative to normal control group. The level of MDA was 4-fold higher in untreated RA rats than in normal rats. However, BXCD treatment of the RA rats for 30 days inhibited the RA-induced increase in MDA level (Figure 3). The activities of SOD, CAT and GSH-Px in the blood of RA rats were significantly lower than the corresponding activities in normal control rats. However, the expressions of these enzymes in

RA rats were significantly increased by treatment with 10 mg/kg BXCD (Figure 3).

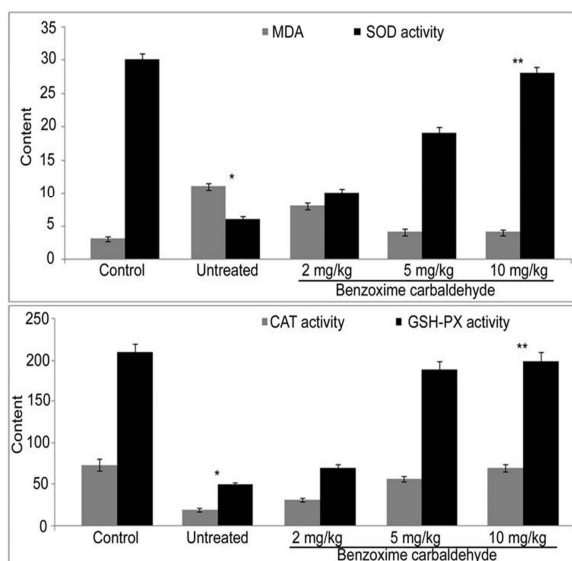


Figure 3: Effect of BXCD on levels of MDA and the expressions of SOD, CAT and GSH-Px in the blood of RA rats. The rat model of RA was treated for 30 days with 2, 5 or 10 mg/kg BXCD. * $p < 0.01$, compared to normal control rats, and ** $p < 0.01$, compared to untreated rheumatoid arthritis rats

BXCD down-regulated the expressions of IL-6, IL-1 β , NF- κ B p65 unit, and TNF- α in RA rat blood

The levels of IL-6 and IL-1 β were markedly elevated in untreated RA rats. In addition, NF- κ B p65 unit and TNF- α in the blood of untreated RA rats were significantly higher than their corresponding levels in normal rats (Figure 4). However, treatment of RA rats with 10 mg/kg BXCD for 30 days blocked increases in the blood levels of these inflammation-related factors (Figure 4).

BXCD treatment reduced COX-2 in RA rats

Western blot assay showed that the level of COX-2 was markedly higher in RA rat tissues than in normal control rats. However, COX-2 level was significantly lowered in the RA group by BXCD treatment (Figure 5).

DISCUSSION

Rheumatoid arthritis, a chronic joint disease affecting many small and peripheral joints, is characterised by infiltration of inflammatory cells, leading to damage to cartilage and bone [13]. The present study has demonstrated the role of BXCD in the treatment of RA in a rat model, and its mechanism of action. The common symptoms of RA include joint pain, swelling, difficulties in

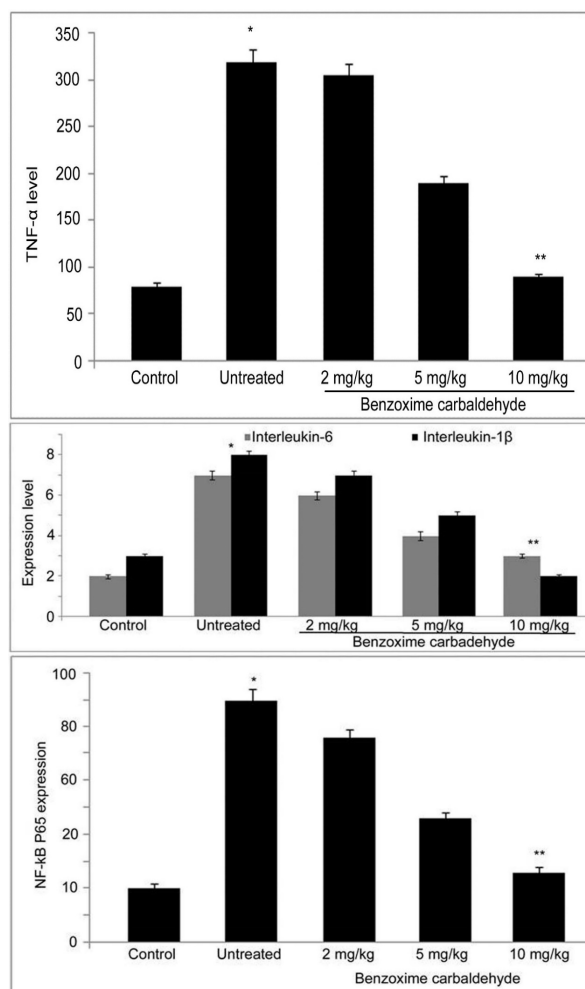


Figure 4: Effect of BXCD on IL-6, IL-1 β , NF- κ B p65 and TNF- α levels in RA rat blood. RA rats were treated for 30 days with 2, 5 and 10 mg/kg BXCD and then the levels of IL-6, IL-1 β , NF- κ B p65 unit, and TNF- α were determined

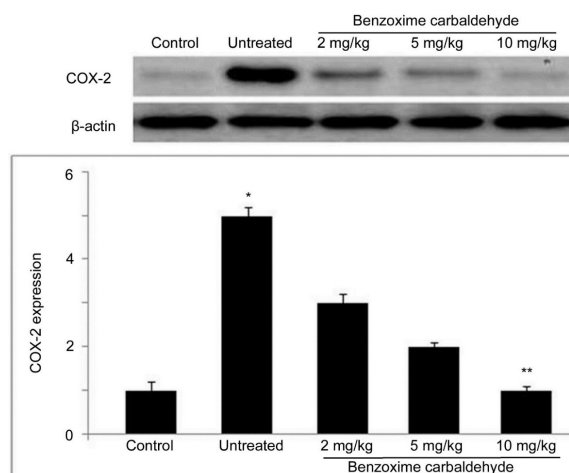


Figure 5: Effect of BXCD on COX-2 levels in RA rat tissues. The level of COX-2 in rheumatoid arthritis rats was analysed by western blot assay after 30 days treatment with BXCD

flexibility of joints, and weight gain [14]. The present study has revealed that BXCD treatment increases the pain threshold of RA rats. This suggests that BXCD can play a role in the treatment of RA. Treatment of RA with BXCD resulted in clinical arthritis score much lower than in untreated animals. In addition, symptoms of RA such as inflammation of joints and ear were markedly suppressed in rats after treatment with BXCD. In animal systems, accumulation of oxidants due to malfunctioning of anti-oxidant system leads to tissue damage [15,16]. Decrease of antioxidant enzymes in the expression of SOD have been reported to be responsible for onset of oxidative stress and development of RA [17]. The results from the current study showed that the level of MDA was higher in RA rats. However, treatment of the RA rats with BXCD inhibited the increase in MDA level.

The RA-induced decreases in the activities of SOD, CAT and GSH-Px in the blood of RA rats were reversed by BXCD treatment. It has been reported that expressions of interleukin-1 β and TNF- α are higher in RA patients [18]. Activation of macrophages and expression of inflammatory cytokines are thought to be associated with the degree of RA [19]. The secretion of interleukins by macrophages and chondrocytes is induced by TNF- α [20]. Previous studies have reported high levels of IL-6 and IL-1 β in RA rats. In the present study, the RA-induced increases in blood levels of NF- κ B p65 unit and TNF- α were blocked by BXCD treatment.

The production of prostaglandins, inflammation and damage to tissues is catalysed by COX-2 [21]. It has been reported that the level of COX-2 in the joints of RA rats is markedly elevated [5]. Inhibition of COX-2 expression plays an important role in the suppression of arthritis [22]. The COX-2 level in the untreated RA group in the present study was significantly elevated, but was markedly lowered by BXCD treatment, suggesting a therapeutic potential of BXCD for RA.

CONCLUSION

The findings of this study demonstrate that benzoxime carbaldehyde mitigates rheumatoid arthritis by increasing pain threshold, inhibiting tissue inflammation, and blocking the expressions of inflammatory cytokines. Thus, benzoxime carbaldehyde possesses a potential for use as a therapeutic agent for the treatment of rheumatoid arthritis.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

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.

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