

Original Research Article

Methylbenzoxime as a therapeutic agent for glucocorticoid-induced osteoporosis in rats

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Abstract

Purpose: To investigate the effect of methylbenzoxime on dexamethasone-induced rat model of osteoporosis.

Methods: Osteoporosis rat model was prepared by administration of dexamethasone to rats for sixty days. The rats were then divided into five groups of five animals each: normal control, untreated, and 2, 5 and 10 mg/kg treatment groups. All rats were administered dexamethasone for 60 days. Thereafter, rats in the three treatment groups received daily doses of 2, 5 or 10 mg/kg methylbenzoxime for 15 days, while rats in normal control and untreated groups were given equivalent volumes of normal saline in place of methylbenzoxime. After treatment, the rats were sacrificed, and the femur removed for histological assessment of pathological changes using H&E staining. Expressions of Wntn signalling pathway proteins in osteoblasts were assayed using reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot assays.

Results: Methylbenzoxime inhibited osteoblast proliferation, as revealed from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. It increased the expression of osteoprotegerin and downregulated receptor activator for nuclear factor-kappa B ligand. Dexamethasone decreased the expression of Wnt signalling pathway proteins in osteoblasts. However, treatment of the dexamethasone-exposed osteoblasts with methylbenzoxime reversed the inhibition of expressions of Wnt signalling pathway proteins. In vivo studies showed that methylbenzoxime treatment mitigated dexamethasone-induced pathological features in femur. In osteoporotic rats, methylbenzoxime significantly up-regulated the expression of osteocalcin but down-regulated the level of collagen-type I fragments, relative to the untreated group. The effect was significant in the 5 and 10 mg/kg treatment groups, when compared with 2 mg/kg group.

Conclusion: Methylbenzoxime prevents dexamethasone-induced osteoporosis in vitro and in rats. Therefore, it is a potential therapeutic agent for the management of osteoporosis.

Keywords: Glucocorticoids, Osteoporosis, Osteoprotegerin, Osteocalcin, Osteoblasts, Dexamethasone, Methylbenzoxime

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INTRODUCTION

Osteoporosis constitutes a medical as well as socio-economic problem characterised by decreased mechanical strength of bones which render them more prone to fracture [1]. Several factors including menopause and glucocorticoid administration are the main causes of osteoporosis [1]. Osteoporosis-associated necrosis in the femoral head leads to fractures which result in poor quality of life [2]. It has been recommended that administration of glucocorticoids should be incorporated in the treatment for osteoporosis [3]. Thus, the development of treatment for osteoporosis induced by glucocorticoids is of vital significance.

The growth and development of various tissues (including the bones) are regulated by the activation of Wnt/ β -catenin signalling pathway [4]. It has been reported that bone formation is maintained by the osteoblasts and osteoclasts [4]. Osteoprotegerin, the expression of which is induced in osteoblasts by the Wnt/ β -catenin pathway, inhibits differentiation in osteoclasts [5]. It has been reported that inhibition of β -catenin expression suppresses differentiation of osteoblasts and promotes differentiation of adipocytes [6]. These findings suggest that Wnt/ β -catenin signalling pathway plays an important role in osteoblast development.

Studies have also shown that non-functioning of low-density lipoprotein receptor-related protein 5 (LRP5) leads to reduction in bone mass [7]. Moreover, up-regulation of LRP5 has been found to promote bone density at certain locations [8]. These studies indicate the significance of Wnt/ β -catenin signalling pathway in the process of bone development since LRP5 is its main component. Therefore, up-regulation of Wnt/ β -catenin signalling pathway is of immense significance for treatment of osteoporosis. In the present study, the effect of methylbenzoxime on osteoporosis was investigated both *in vitro* and *in vivo*. The study demonstrated that methylbenzoxime effectively prevents dexamethasone-mediated osteoporosis in the rat model as well as *in vitro*.

EXPERIMENTAL

Animals

The male Sprague-Dawley rats 6-week old (160 - 210 g body weight) were provided by the Animal Resource Center of the First Affiliated Hospital of Dalian Medical University (Dalian, China). The animals housed in individual cages were provided free access to the laboratory food and water *ad libitum*. The rats were maintained

under standard laboratory conditions under 12-h light/dark cycles and humidity of around 60 % at a temperature of 24 °C. The animal experimental protocols were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Dalian Medical University (approval no. AS/17/145). All the experimental procedures were performed according to the guidelines for evaluation and treatment of glucocorticoid-induced osteoporosis of the Department of Veterans Affairs [9].

Cell culture

The primary osteoblasts were cultured from the calvarial bones of the neonatal rats of 36 h age. The animals were sacrificed using euthanasia to extract the calvarial bones which were subsequently sliced into 2-mm³ sections. The bone sections were subjected to digestion in a mixture of trypsin (1 %) and collagenase type II (0.2 %) for 40 min at 37 °C. Following digestion, the tissue mixture was subjected to centrifugation at 300 x g for 15 min to obtain the cells after discarding the supernatant. Tris-buffered saline (PBS) was used to wash the cells two times which were subsequently put into Dulbecco's modified essential medium (DMEM, Gibco Life Technologies, Carlsbad, CA, USA). The medium contained fetal bovine serum (FBS; HyClone, Logan, UT, USA; 10 %), l-glutamine (1 mM) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cells were then cultured at 37 °C temperature under a humidified atmosphere of 5 % CO₂.

MTT assay

The cells placed into the culture plates at 4 x 10⁴ cells/well density were exposed to 100 nM doses of dexamethasone for 24 h. The treatment cell cultures were incubated with 5, 10, 15, 20, 25 and 30 μ M concentrations of methylbenzoxime for 48 h before 24 h of exposure to 100 nM doses of dexamethasone. After completion of treatment, each well was exposed to 0.2 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) and incubation was continued for 4 h more at 37 °C. From the plates supernatant was discarded followed by addition of 200 μ l dimethyl sulfoxide to dissolve any precipitate formed. The absorbance for each well was measured at 570 nm using a microplate reader (ELX-800, Bio-Tek Instruments).

Osteoporosis induction in rats and treatment

The rats were divided into five groups of 5-animals each: dexamethasone, control and three treatment groups (2, 5 and 10 mg/kg). The

animals in the dexamethasone group were injected subcutaneously 0.1 mg/kg doses daily for 60 days. Control group of rats received equal volume of normal saline and those in the treatment groups were injected subcutaneously 0.1 mg/kg dexamethasone and 2, 5 or 10 mg/kg doses of methylbenzoxime daily for 60 days. Establishment of osteoporosis rat model was confirmed by the measurement of density of minerals at proximal tibia. After treatment the rats were sacrificed by injecting 50 mg/kg doses of sodium pentobarbital intraperitoneally. The blood sample from the inferior vena cava of the rats was collected for analysis. The femurs were extracted by removing the muscles around it carefully using surgical scissors.

Determination of alkaline phosphatase activity in serum

Analysis of the activity of alkaline phosphatase in rat serum was performed using the commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of manufacturer. The alkaline phosphatase kit solution was added to the serum samples at 37 °C and kept at standing for 45 min. The activity of alkaline phosphatase was determined by the measurement of absorbance at 530 nm using microplate reader (ELX-800; Bio-Tek Instruments, Winooski, VT, USA).

Analysis of osteocalcin and collagen type I fragment levels in serum

Determination of the expression of osteocalcin and collagen type I fragment in rat serum was performed commercially available kits (USCN Life Science, Wuhan, China) in accordance with the instructions of manufacturer. The osteocalcin and collagen type I fragment antibody solutions were mixed with the serum samples for 45 min at 37 °C. After incubation, the samples were treated with streptavidin-HRP for 40 min at 37 °C. The microplate reader (ELX-800; Bio-Tek Instruments) was used for measurement of absorbance at 455 nm.

Reverse transcription-quantitative PCR (RT-qPCR)

From the femoral bone or primary osteoblasts total RNA was extracted using TRIzol reagent, in accordance with the instructions of manufacturer. The DNA strand was synthesized using the Reverse Transcriptase M-MLV (RNase H) kit containing random hexamer primers. Gene expression analysis was determined using touchdown PCR as follows: one cycle at 95 °C x 6 min, 12 cycles at 95 °C x 40 s, 68 °C x 30 s

(with a decrease of 1 degree/cycle) and 72 °C x 30 sec and 25 cycles at 95 °C x 30 sec, 55 °C x 30 s, and 72 °C x 40 s, one cycle 72 °C x 15 min. Separation of the PCR products was carried out on 2 % agarose gel by electrophoresis. The mRNA expression level was compared to the GAPDH. Rotor-Gene 6000 Real-Time PCR machine (Corbett Research, Sydney, Australia) combined with SYBR Premix Ex Taq (Thermo) was used for RT-qPCR analysis. The procedure involved HotStar Taq DNA polymerase activation at 95 °C for 15 min, then 40 cycles of 95 °C for 10 s and 60 °C for 20 s. the experiments were performed in triplicates independently.

Western blot analysis

The cells or bone samples after PBS washing were subjected to homogenization with RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1 % SDS, 1 % N P-40 and 0.5 % sodium deoxycholate] containing phenylmethanesulfonyl fluoride. The samples were then centrifuged for 20 min at 12,000 x g at 4 °C to collect the supernatant for determination of proteins by bicinchoninic acid (BCA) method. The protein separation was achieved using 10 % sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel followed by electrophoretic transfer onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was blocked by 5 % non-fat milk for 50 min at room temperature. Incubation of the membranes was carried out for overnight at 4 °C with p-glycogen synthase kinase (p-GSK-3 β ; dilution 1: 200, catalog no. sc-11757; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), GSK-3 β (dilution 1: 200, catalog no. sc-9166; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), OPG (dilution 1: 500, catalog no. bs-0431R; Bioss, Beijing, China) and receptor activator for nuclear factor-kappa B ligand (RANKL; dilution 1: 500, catalog no. bs-0747R; Bioss, Beijing, China) primary antibodies.

Following incubation the membranes were washed three times with TBS Tween-20 and then incubated with HRP-conjugated secondary antibodies (dilution 1:5,000, Beyotime Institute of Biotechnology) at room temperature for 1 h. For visualization of the protein blots enhanced chemiluminescence (7 Sea Pharmtech, Shanghai, China) was used. The internal loading control taken was β -actin.

Statistical analysis

The data presented are the mean \pm SE of experiments performed in triplicate. One-way ANOVA followed by Fisher's least significant

difference (LSD) test was used for determination of significant difference between the groups. Data analysis was carried out using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of dexamethasone and methylbenzoxime on viability of primary osteoblasts

Incubation of primary osteoblasts with dexamethasone at 100 nM for 24 h caused significant reduction in their viability, when compared with the control cells. Dexamethasone-exposed cells were treated with methylbenzoxime at doses of 5, 10, 15, 20, 25 and 30 μM for 48 h and then viability was analysed by MTT assay (Figure 1). Treatment of the primary osteoblasts with methylbenzoxime after incubation with 100 nM dexamethasone prevented reduction in viability. Although inhibition of dexamethasone-induced reduction of primary osteoblast viability was significant as from 15 μM methylbenzoxime, the effect was maximum at 30 μM (Figure 1). These findings suggest that methylbenzoxime prevents dexamethasone-induced inhibition of primary osteoblast viability.

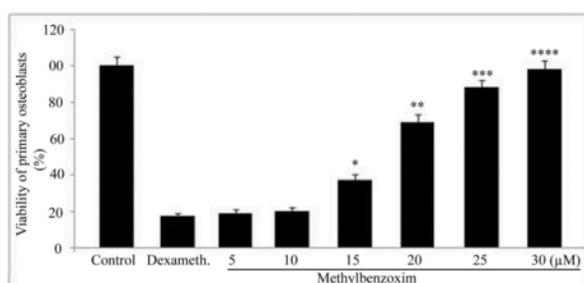


Figure 1: Effect of dexamethasone and methylbenzoxime on primary osteoblast viability. The cells were exposed to dexamethasone (100 nM) for 24 h and then treated with 5, 10, 15, 20, 25 and 30 μM methylbenzoxime for 48 h. Cell viability was assessed with MTT assay and data are expressed as mean \pm SD of experiments performed in triplicates independently; * $p < 0.05$, ** $p < 0.02$ and *** $p < 0.01$, compared to the dexamethasone-treated cells. (Dexameth. = dexamethasone)

Effect of methylbenzoxime on the differentiation and maturation of primary osteoblasts

The osteoblasts were treated simultaneously with methylbenzoxime and dexamethasone prior to determination of alkaline phosphatase activity. It was observed that dexamethasone treatment at a concentration of 100 nM led to a significant reduction in alkaline phosphatase activity in osteoblasts. However, there was no significant decrease in the activity of alkaline phosphatase

in osteoblasts on treatment with combination of 100 nM dexamethasone 30 μM methylbenzoxime (Figure 2 A). On the other hand, a significant decrease was observed in the activity of alkaline phosphatase on treatment of osteoblasts with combinations of dexamethasone (100 nM) and methylbenzoxime (20, 15, 10 and 5 μM) (Figure 2 A). Dexamethasone treatment of osteoblasts led to down-regulation of mRNAs corresponding to osteoclastin, collagen, alpha 1 (Col1A1), osteonectin, runt-related transcription factor-2 (Runx2) and osterix (Figure 2 B). However, treatment of osteoblasts with methylbenzoxime (30 μM) reversed the inhibitory effect of dexamethasone on these mRNAs (Figure 2 B). Dexamethasone treatment also decreased the expressions of OPG and RANKL in osteoblasts. However, treatment of osteoblasts with 30 μM methylbenzoxime reversed the effect of dexamethasone on OPG and RANKL expressions (Figure 2 C).

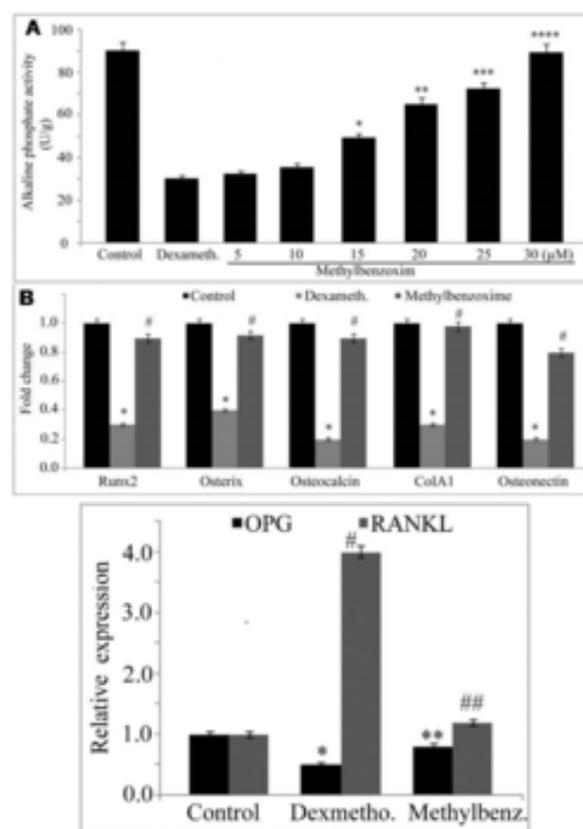


Figure 2: Effect of methylbenzoxime on alkaline phosphatase activity and other differentiation-related factors. Primary osteoblasts were exposed for 24 h to dexamethasone and then incubated with the indicated doses of methylbenzoxime for 48 h. Alkaline phosphatase activity (A), and osteoclastin, collagen, type 1, alpha 1 (Col1A1), osteonectin, Runx2, osterix (B), osteoprotegerin and RANKL (C) expressions were assayed. The results are expressed as mean \pm SD of experiments performed in triplicates; * $p < 0.05$, ** $p < 0.02$ and *** $p < 0.01$, compared to dexamethasone-treated cells

Effect of methylbenzoxime on the Wnt, β -catenin and LRP5 expressions in primary osteoblasts

Dexamethasone administration caused marked down-regulations of Wnt, β -catenin and LRP5 mRNAs in the rats (Figure 3). However, treatment of the dexamethasone-administered rats with methylbenzoxime at doses of 2, 5 and 10 mg/kg prevented down-regulations of Wnt, β -catenin and LRP5 mRNA (Figure 3). The inhibition of Wnt, β -catenin and LRP5 mRNA down-regulation was significant on treatment with methylbenzoxime at doses of 5 and 10 mg/kg (Figure 3).

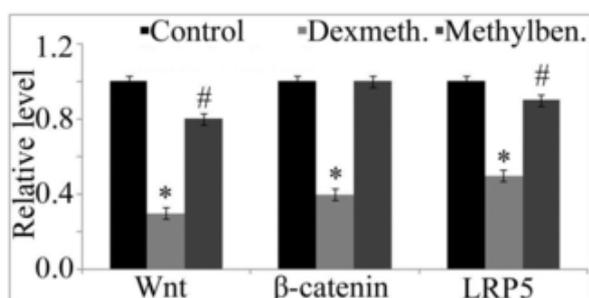


Figure 3: Effect of methylbenzoxime on Wnt, β -catenin and LRP5 expressions in primary osteoblasts. The Wnt, β -catenin and LRP5 mRNA expressions in primary osteoblasts were determined after 24 h of exposure to dexamethasone and 48 h of treatment with methylbenzoxime. The results are presented as mean \pm SD of triplicate experiments; * p < 0.05, ** p < 0.02 and *** p < 0.01, compared to dexamethasone cells

Effect of methylbenzoxime on Dickkopf-1 and sclerostin expressions and GSK-3 β phosphorylation in primary osteoblasts

Dexamethasone administration to the rats up-regulated the expressions of Dickkopf-1 and sclerostin (SOST, Figure 4). However, methylbenzoxime treatment of the dexamethasone-exposed rats at doses of 5 and 10 mg/kg blocked the upregulations of Dickkopf-1 and SOST mRNA expressions (Figure 4). Dexamethasone administration led to inhibition of the phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) (Figure 4). On the other hand, treatment of the dexamethasone-administered rats with methylbenzoxime at doses of 5 and 10 mg/kg prevented the inhibition of GSK phosphorylation (Figure 4).

Effect of methylbenzoxime on serum osteocalcin and CTX

Assay of serum samples from the dexamethasone-treated rats showed a marked decrease in

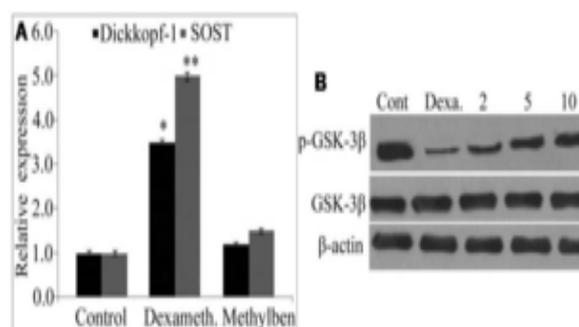


Figure 4: Effect of methylbenzoxime on Dickkopf-1 and SOST expressions and GSK-3 β phosphorylation. (A) SOST and Dickkopf-1 mRNA expressions, and (B) glycogen synthase kinase-3 β (GSK-3 β) activation in primary osteoblasts were determined after 24 h of exposure to dexamethasone and 48 h of treatment with methylbenzoxime. The results are presented as mean \pm SD of triplicate experiments; * p < 0.05, ** p < 0.02 and *** p < 0.01, compared to dexamethasone-treated cells

the level of osteocalcin and increase in CTX level (Figure 5). On the other hand, the expression levels of osteocalcin and CTX in the dexamethasone administered rats were similar to those of the control group on treatment with methylbenzoxime at a dose of 10 mg/kg. The level of osteocalcin was slightly lower, and that of CTX slightly higher in the dexamethasone-administered rats on treatment with methylbenzoxime at a dose of 5 mg/kg (Figure 5).

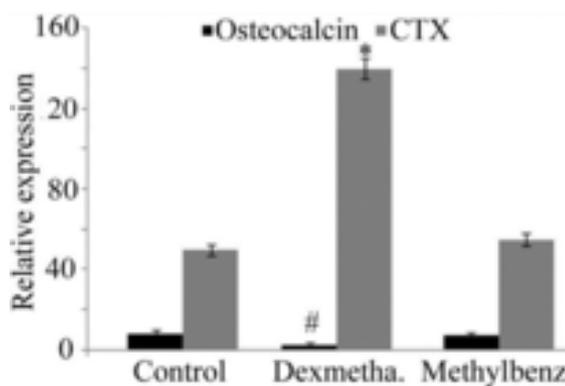


Figure 5: Effect of methylbenzoxime on serum levels of osteocalcin and collagen-type I fragments (CTX). Following dexamethasone administration, the rats were treated with methylbenzoxime at doses of 2, 5 and 10 mg/kg. The expressions of osteocalcin and CTX in the rat serum were assayed. Data are presented as mean \pm SD; * p < 0.05, ** p < 0.02 and *** p < 0.01, compared to the dexamethasone group

DISCUSSION

The present study demonstrates the therapeutic effect of methylbenzoxime on dexamethasone-

mediated osteoporosis in primary osteoblasts and rat model. Methylbenzoxime exhibited this effect through reversal of dexamethasone-induced changes in the expressions of osteocalcin and collagen-type I fragments, as well as activation of Wnt/ β -catenin signalling pathway.

Osteoporosis is characterized by bone deterioration leading to the development of irregularities as a result of loss of osteoblasts [10]. Studies have revealed marked degradation in the femur bone of experimental animals treated with glucocorticoids [11]. Bone mineral content is also decreased in animals by glucocorticoid treatment [12,13]. In the current study, results showed that dexamethasone administration to rats led to a marked decrease in mineral content of the femur. However, treatment of rats with methylbenzoxime prevented dexamethasone-induced reduction in bone mineral content. Trabecular bone constitutes a major portion of proximal femur, and it is highly sensitive to glucocorticoids [14]. In the present study, dexamethasone administration to rats caused marked degradation in femur which was evident in thinning down of trabeculae and formation of lacunae. However, methylbenzoxime treatment prevented dexamethasone-induced thinning of trabecular bone and development of cavities in femurs. Collagen type I fragments are associated with disturbance in equilibrium between bone formation and bone resorption [15]. Bone formation is indicated by the presence of osteocalcin, while bone resorption is marked by serum collagen type I fragments [16]. In the present study, dexamethasone administration to rats down-regulated osteocalcin and up-regulated serum collagen type I fragments in rat serum. This disturbance in equilibrium between bone formation and resorption causes osteoporosis. However, treatment of the rats with methylbenzoxime prevented dexamethasone-mediated disturbance in equilibrium between bone formation and bone resorption.

The activity of alkaline phosphatase in osteoblasts was reduced markedly on exposure to dexamethasone. The dexamethasone-induced decrease in alkaline phosphatase activity was reversed with methylbenzoxime treatment of the osteoporotic. In addition, dexamethasone exposure led to marked down-regulations in the expressions of Runx2, osterix, osteocalcin, Col1A1 and osteonectin mRNA. In adults, bone formation is regulated by the expressions of Runx2 and osterix [17]. The expressions of genes responsible for matrix proteins i.e. Col1A1,

osteoponin and osteocalcin are regulated by Runx2 [18].

The results from the present study showed that methylbenzoxime prevented dexamethasone-induced decreases in mRNA expressions of Runx2, osterix, osteocalcin, Col1A1 and osteonectin. Osteoblasts express OPG and RANKL cytokines which play important roles in the process of bone remodelling. The results from the present study revealed that dexamethasone exposure decreased OPG expression and upregulated RANKL. However, methylbenzoxime blocked dexamethasone-mediated decrease in OPG expression in osteoblasts, and increased RANKL expression. These findings suggest that methylbenzoxime inhibits bone resorption, and so blocks the development of osteoporosis.

It has been reported that Wnt/ β -catenin pathway is associated with various cellular developmental processes in animals [19]. Optimum functioning of LRP5 is very important because alteration in its level leads to the disorders [20]. In this study, dexamethasone exposure provoked decreases in the levels of Wnt, β -catenin and LRP5, but these decreases were reversed by methylbenzoxime. During the process of bone formation, SOST and Dickkopf-1 play vital roles in the regulation of Wnt signalling pathway [20]. The association of SOST with LRP6 blocks the interaction between LRP6 and Wnt [20]. Wnt signalling pathway is inhibited by expression of Dickkopf (Dkk-1) through its combination with LRP5/6 [21].

In the present study, dexamethasone exposure upregulated SOST expression, but this inhibition was relieved by treatment with methylbenzoxime. Moreover, dexamethasone enhanced the expression of Dickkopf (Dkk-1) mRNA in osteoblasts, but this was reversed by methylbenzoxime treatment. Activation of GSK-3 β causes translocation of β -catenin to the nucleus where it regulates target gene transcription [22]. It has been observed that GSK-3 β activation prevents degradation of bones [23]. In the present study, dexamethasone exposure inhibited activation of GSK-3 β and down-regulated β -catenin expression, but these effects were blocked by treatment with methylbenzoxime.

CONCLUSION

The findings of the present study show that methylbenzoxime prevents dexamethasone-induced osteoporosis both *in vitro* and in rats. This effect is exerted via a mechanism involving up-regulation of Wnt/ β -catenin pathway. Thus,

methylbenzoxime may be an effective therapeutic agent for the management of osteoporosis.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

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. Mingcong Ding, Pandeng Wei, Xuchang Hu, Tongqun Yang and Mingxuan Yang performed the experimental work. Qian Zhang carried out the literature study and compiled the data. Lin Wan designed the study and wrote the paper. All the authors approved the paper for publication.

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