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ORIGINAL ARTICLE

Curcumin alleviates glucocorticoid-induced osteoporosis by protecting osteoblasts from apoptosis *in vivo* and *in vitro*

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SUMMARY

Curcumin, an active component of the rhizomes of *Curcumin longa L.*, possesses broad anti-inflammation and anti-cancer properties. Curcumin was previously reported to be capable of protecting ovariectomized rats against osteoporosis. However, the effect of curcumin on glucocorticoid-induced osteoporosis (GIO) is not yet clear. The present study investigated the effects of curcumin on dexamethasone (Dex)-induced osteoporosis *in vivo* and Dex-induced osteoblast apoptosis *in vivo* and *in vitro*. The GIO rat model was induced by subcutaneous injection of Dex for 60 days and verified to be successful as evidenced by the significantly decreased bone mineral density (BMD) determined using dual X-ray absorptiometry. Subsequently, curcumin administration (100 mg/kg) for 60 days obviously increased BMD and bone-alkaline phosphatase, decreased carboxy-terminal collagen cross links, enhanced bone mechanical strength, and improved trabecular microstructure, thereby alleviating Dex-induced osteoporosis. Mechanically, curcumin remarkably reversed Dex-induced femoral osteoblast apoptosis *in vivo*. In cultured primary osteoblasts, pretreatment with curcumin concentration-dependently decreased the number of Dex-induced apoptotic osteoblasts by down-regulating the ratio of Bax/Bcl-2 as well as the levels of cleaved caspase-3 and cleaved poly ADP-ribose polymerase (PARP). Moreover, curcumin pretreatment activated extracellular signal regulated kinase (ERK) signalling in Dex-induced osteoblasts by up-regulating the expression level of p-ERK1/2. Taken together, our study demonstrated that curcumin could ameliorate GIO by protecting osteoblasts from apoptosis, which was possibly related to the activation of the ERK pathway. The results suggest that curcumin may be a promising drug for prevention and treatment of GIO.

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INTRODUCTION

Glucocorticoids (GCs) have been used widely in the therapies of various diseases such as asthma, rheumatoid arthritis, organ transplantation, cancer and autoimmune disorders due to their anti-inflammatory and immunomodulatory effects.[1] However, the long-term usage of GCs has the potential to cause a progressive decrease in bone mineral density (BMD) and bone strength, thereby resulting in osteoporosis, and even fracture.[1] It is estimated that glucocorticoid-induced osteoporosis (GIO) occurs in 30–50% of patients receiving long-term GC administration, especially in postmenopausal women.[2,3] Bone remodelling is a perfect functional balance process between osteoclast-mediated resorption of primary bone and osteoblast-induced formation of new bone. Increasing evidence has shown that apoptosis of osteoblasts and osteocytes as well as prolongation of osteoclast lifespan are the principle pathogenesis of GIO.[4] Hence, reducing the lifespan of osteoclasts, or preventing osteoblast apoptosis, or both, would be a promising therapeutic strategy for the treatment of GIO.

Curcumin is an active ingredient extracted from the rhizomes of the turmeric plant (*Curcumin longa L.*) and possesses broad pharmacological activities such as anti-inflammation, anti-oxidation, anti-cancer, neuro-protection and anti-tuberculosis.[5,6] Previous studies have demonstrated that curcumin could protect rats against ovariectomy-induced osteoporosis by enhancing BMD, increasing mechanical strength, and preventing bone loss.[7,8] It is reported that treatment with curcumin could improve bone turnover and enhance BMD in the APP/PSI transgenic mice.[9] Additionally, curcumin was shown to drastically suppress osteoclastic bone resorption by stimulating osteoclast apoptosis and inhibiting osteoclast differentiation.[10–14] However, to the best of the authors' knowledge, it is not clear whether curcumin has therapeutic effects on GIO and GC-induced osteoblast apoptosis.

The present study demonstrates that curcumin has remarkably osteoprotective and anti-apoptotic effects in dexamethasone (Dex)-induced osteoporosis model rats. To further elucidate the molecular mechanisms, the effects of curcumin on Dex-induced apoptosis in primary cultures of rat osteoblasts we also investigated and the apoptosis-related signalling pathway involved was explored.

RESULTS

Curcumin alleviates Dex-induced osteoporosis in GIO model rats

To develop the GIO rat model, rats were administrated with Dex (0.1 mg/kg) once daily for 60 days. The results of dual X-ray absorptiometry (DXA) showed that the BMD of rats was significantly reduced at the end of the Dex exposure, indicating a successful GIO model (Fig. 1a, $P < 0.01$). Of note, although the rats in model group did not continue to receive Dex throughout the following 60 days of the curcumin treatment, the declined BMD did not undergo spontaneous remission. As predicted, administration with curcumin (100 mg/kg) obviously elevated the BMD compared with Dex model rats (Fig. 1a, $P < 0.05$).

To further investigate the effects of curcumin on GIO, the levels of bone-alkaline phosphatase (b-ALP) and carboxy-terminal collagen cross links (CTX) in serum were determined using enzyme-linked immunosorbent assay (ELISA) kits. As shown in Fig. 1b, the level of b-ALP in Dex-induced rats was significantly decreased compared with the control group ($P < 0.001$), whereas the CTX level was obviously increased upon Dex injections ($P < 0.001$). Importantly, such decrease of b-ALP level and increase of CTX level induced by Dex were remarkably reversed by curcumin treatment ($P < 0.05$ and $P < 0.001$, respectively). In addition, the three-point bending test was also conducted to evaluate the effect of curcumin on the ultimate load and stiffness of bone. The results presented in Fig. 1c, d clearly show that Dex exposure led to a significant reduction in ultimate load and stiffness of bone compared with the control rats ($P < 0.01$), which was obviously enhanced following curcumin treatment ($P < 0.05$). Histologically, a reduced trabecular thickness and an enhanced trabecular separation in femoral epiphysis were observed in GIO model rats (Fig. 1e). However, curcumin treatment obviously ameliorated such deleterious effects of Dex on bone trabecular microstructure. Taken together, these results indicate that curcumin could alleviate Dex-induced osteoporosis.

Curcumin inhibits Dex-induced apoptosis of osteoblasts *in vivo* and *in vitro*

Osteoblast apoptosis induced by GCs has been considered as the critical factor in pathogenesis of GIO.[15] The effect of curcumin on Dex-induced apoptosis of osteoblasts in femur tissues was determined using terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining assay. As shown in Fig. 2a, great amount of TUNEL positive osteoblasts gathered in clusters around the trabecular in Dex-induced osteoporotic rats. Nevertheless, few TUNEL positive osteoblasts were observed in the control group and curcumin treated group, suggesting that curcumin could obviously rescue osteoblasts from Dex-induced apoptosis *in vivo*.

To gain further insight into the effect of curcumin on osteoblast apoptosis, Annexin V/PI staining-based flow cytometry analysis and Hoechst staining were performed in cultured primary osteoblasts separated from rats. The results of Annexin V/PI assay showed that a large portion (50.2%) of osteoblasts underwent apoptosis following exposure to Dex for 6 h (Fig. 2b). However, pretreatment with curcumin concentration-dependently inhibited Dex-induced apoptosis of osteoblasts. Particularly, pretreatment with 2 μmol/L curcumin resulted in a significant decrease of apoptotic osteoblasts ($P < 0.001$). Meanwhile, exposure to Dex resulted in obvious appearance of apoptotic osteoblasts as evidenced by chromatin condensation and nuclear fragmentation, which was also attenuated by curcumin pretreatment in a concentration-dependent manner. These findings demonstrated that curcumin could suppress Dex-induced apoptosis of osteoblasts *in vivo* and *in vitro*.

Curcumin suppresses pro-apoptotic protein expression and activates ERK signalling pathway in Dex-induced osteoblasts

To further confirm the anti-apoptotic effect of curcumin on osteoblasts, we determined the expression of apoptosis-related proteins using Western blot analysis. As shown in Fig. 3a, the expression levels of pro-apoptotic proteins Bax, cleaved caspase-3 and cleaved poly ADP-ribose polymerase (PARP) were significantly up-regulated following Dex exposure compared with the control group, whereas the levels of pro-apoptotic protein Bcl-2 were obviously down-regulated ($P < 0.001$). However, pretreatment with 2 μmol/L curcumin remarkably inhibited such up-regulation or down-regulation of these proteins induced by Dex. These results further confirm that curcumin could protect osteoblasts from Dex-induced apoptosis.

It has been demonstrated that high dose GCs can suppress the ERK pathway, which is associated with their effects on the promotion of osteoblast apoptosis.[16] In agreement with previous reports,[17,18] the data presented here also shows that Dex significantly down-regulated the expression level of phosphorylational ERK (p-ERK) compared with the control group ($P < 0.001$). Noticeably, pretreatment with curcumin appeared to concentration-dependently elevate the p-ERK level. These findings indicated that ERK pathway in osteoblasts was inhibited by Dex exposure, but activated after curcumin treatment.

DISCUSSION

Currently, GC administration is the most common cause of secondary osteoporosis. The present study demonstrates that curcumin obviously improves Dex-induced osteoporosis in GIO model rats and protects osteoblasts from Dex-induced apoptosis *in vivo* and *in vitro*. Moreover, curcumin pretreatment is shown to activate the ERK pathway in Dex-induced osteoblasts by up-regulating p-ERK levels. To the best of the authors' knowledge, this is the first report that identifies the anti-GIO effect of curcumin and partially reveals its underlying mechanisms.

The femur is the most vulnerable to fracture, and approximately 40% of European women with atypical femur fractures have been treated with GCs.[19,20] Hence, in our GIO model, the femur was chosen to evaluate the effect of curcumin on Dex-induced osteoporosis. BMD, an important index reflecting the status of bone metabolism, is widely used to assess the changes of bone mass and predict the risk of fracture. This study found that curcumin remarkably reversed Dex-induced lower BMD, which is consistent with the previous reports that curcumin could enhance BMD in ovariectomized rats and in APP/PS1 transgenic mice.[9,21] Additionally, Kaji *et al.* and Van Staa *et al.* have both demonstrated that several factors other than BMD are also considered to affect fracture.[22,23] In particular, the levels of b-ALP

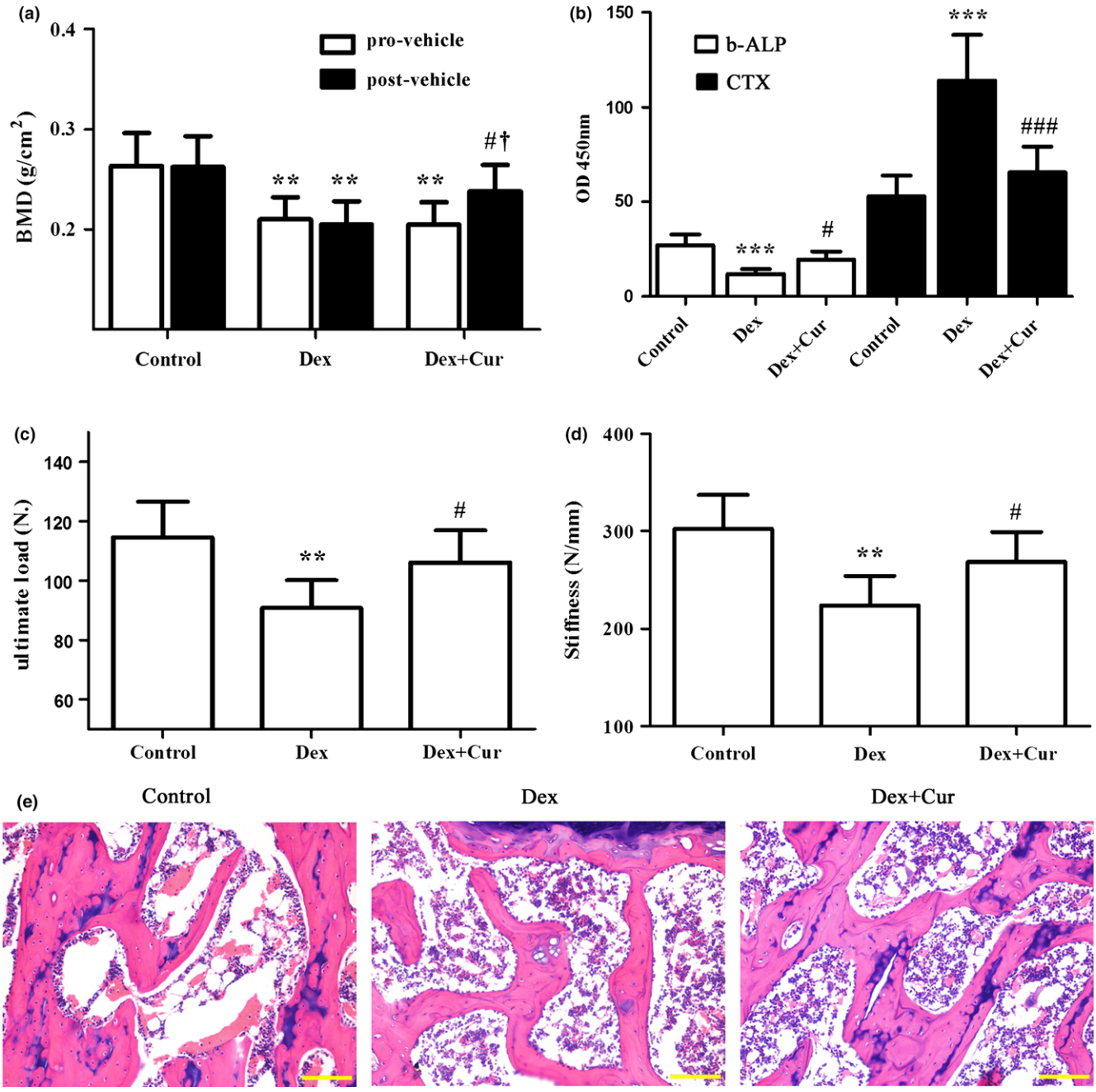

Fig. 1 The effects of curcumin on Dex-induced osteoporosis in glucocorticoid-induced osteoporosis (GIO) model rats. Rats were subcutaneously injected with 0.1 mg/kg per day Dex (Dex) or equivalent normal saline (Control) for 60 days. Then, half of Dex-treated rats were orally administrated with 100 mg/kg per day curcumin for another 60 days (Dex + Cur), and others were given an equal volume of 0.5% sodium carboxymethylcellulose (CMC-Na) for 60 days. (a) The BMD values were determined by DXA at the end of Dex administration (pro-vehicle) and at the end of curcumin treatment (post-vehicle), respectively. (b) The levels of b-ALP and CTX in serum were detected by ELISA kits. (c) The ultimate load and (d) stiffness of the femur were determined by the three-point bending test. (e) Representative sections of femur from control rats, Dex model rats and Dex in combination with curcumin treated rats were performed with H&E staining for histological assessment. The reticular-connected red areas indicate trabecular bone. Original magnification was 200 ×. Data are presented as mean ± standard deviation (SD; $n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ *vs* the Dex model group; $^{\dagger}P < 0.05$ *vs* the pro-vehicle group of Dex + Cur.

and CTX have been used widely as sensitive markers for bone formation and bone resorption, respectively.[24] Our study demonstrated that curcumin not only elevated b-ALP level but also declined CTX level in Dex-induced rats, implying the dual role of curcumin as a bone formation promoter and bone resorption suppressor. In addition, Dex administration led to significant reduction of bone mechanical strength and damage of trabecular bone in our GIO model rats, which was also observed at the early stage of GIO in Lane's study.[25] Curcumin was shown to reverse Dex-induced decrease of bone mechanical strength and

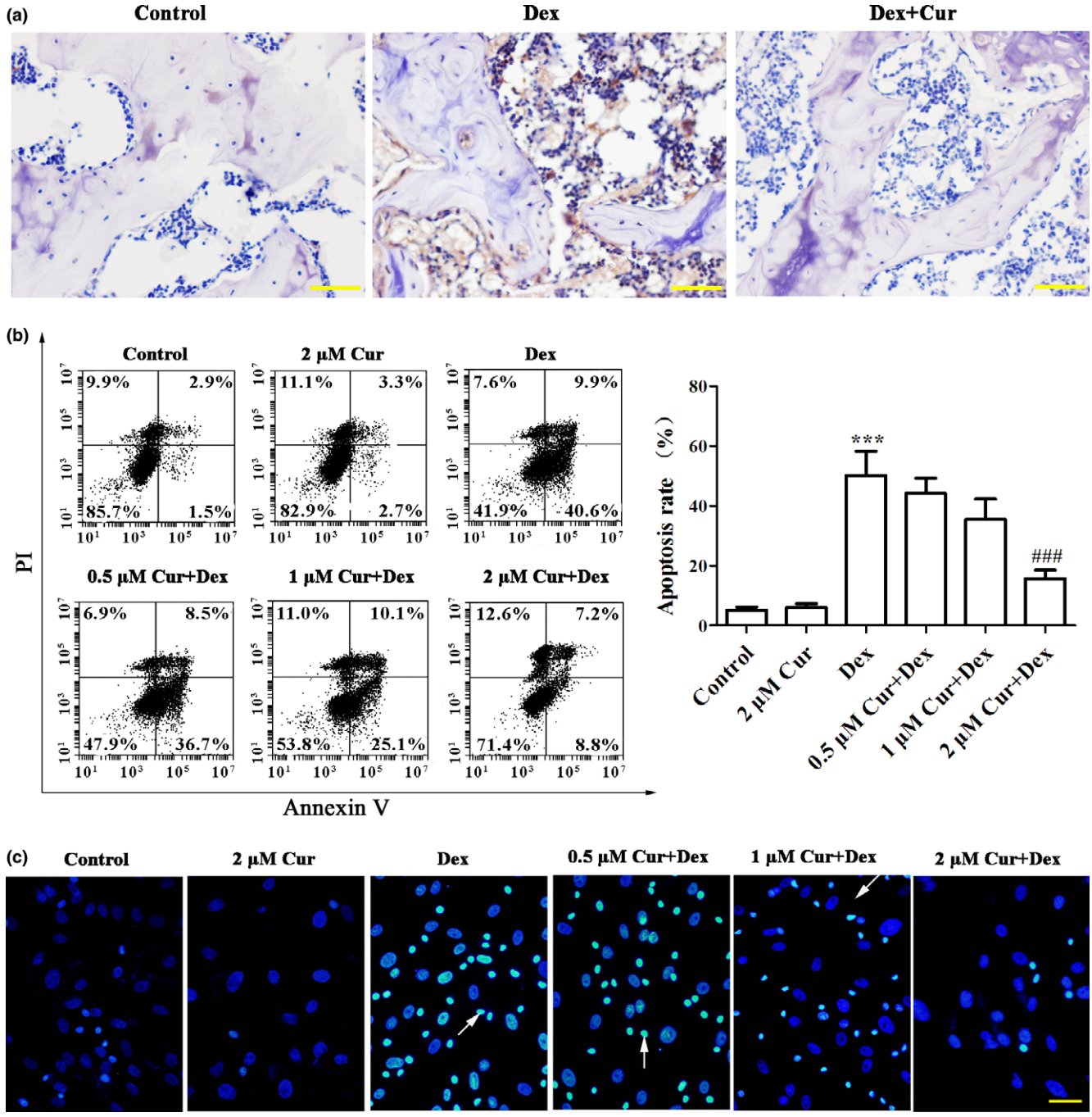

Fig. 2 The effects of curcumin on Dex-induced apoptosis of osteoblasts *in vivo* and *in vitro*. (a) Representative sections of femur from control rats, Dex model rats and Dex in combination with curcumin treated rats were performed with terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining for assessment of osteoblast apoptosis. Blue cells indicate TUNEL-negative osteoblasts; brown cells denote TUNEL positive osteoblasts. Original magnification 400 × . (b) Osteoblasts were pre-treated with or without various concentrations of curcumin (0.5, 1 and 2 μmol/L) for 2 h, followed by exposure to 1 μmol/L Dex for 6 h. The apoptotic osteoblasts were examined by Annexin V-FITC/PI staining using flow cytometry. Cells are characterized as necrosis (upper left quadrant), late apoptosis (upper right quadrant), healthy (lower left quadrant), and early apoptosis (lower right quadrant). Total apoptotic ratio was calculated as a bar chart on the right. (c) The effect of curcumin on Dex-induced osteoblast apoptosis was evaluated by Hoechst staining using laser scanning confocal microscope. Original magnification 400 ×. Arrows indicate apoptotic osteoblasts. The experiments were repeated three times, and the representative images are shown. Data are presented as mean ± standard deviation (SD; $n = 3$), $^{***}P < 0.001$ *vs* control; $^{\#\#\#}P < 0.001$ *vs* Dex.

damage of trabecular bone. Taken together, these findings indicate that curcumin could alleviate Dex-induced osteoporosis in GIO rats.

Existing evidence suggests that osteoblast apoptosis, which leads to declined bone formation and increased bone fragility, plays a critical role in GC-induced bone loss or osteoporo-

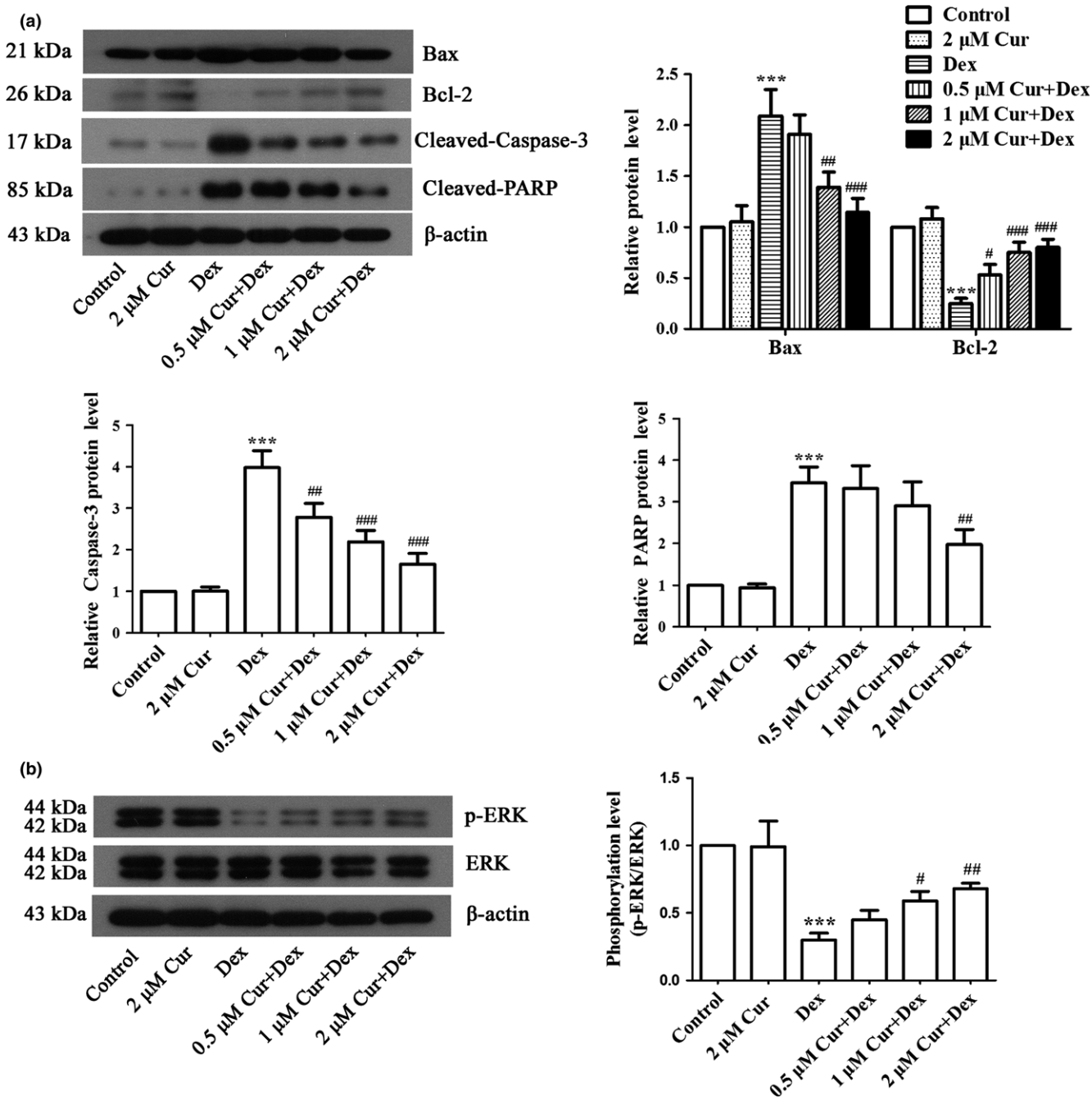

Fig. 3 The effects of curcumin on apoptosis-related proteins and the extracellular signal regulated kinase (ERK) pathway in Dex-induced osteoblasts. Osteoblasts were pre-treated with or without various concentrations of curcumin (0.5, 1 and 2 μmol/L) for 2 h, followed by exposure to 1 μmol/L Dex for 6 h. (a) The expression levels of apoptosis-related proteins including Bax, Bcl-2, cleaved caspase-3, and cleaved PARP were determined by Western blot analysis. β-actin was used as an internal reference. Representative bands are presented and the relative band intensity ratio was analyzed. (b) The expression levels of p-ERK and ERK in osteoblasts with different treatments were detected by Western blot analysis. Representative blots are showed and the densitometric ratio between p-ERK and ERK was analyzed. Data are shown as mean ± SD (n = 3); ***$P < 0.001$ *vs* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* Dex.

sis.[18,26,27] It has also been reported that the reduction of cancellous bone area, wall and trabecular width, as well as the decrease of bone formation rate, are the typical characteristics of GIO, which result from an inadequate number of osteoblasts and incomplete repair of erosion cavity during bone remodelling.[28] Thus, discovering an effective therapeutic drug that prevents GC-induced apoptosis of osteoblasts is imperative. In agreement with previous reports,[18,28] osteoblast apoptosis was obviously induced by Dex exposure *in vivo* and *in vitro* in the present study. Notably, curcumin remarkably reduced Dex-induced apoptosis of osteoblasts in rats and concentration-dependently inhibited osteoblast apoptosis mediated by Dex *in vitro*, suggesting

that curcumin may alleviate GIO via protecting osteoblasts against Dex-induced apoptosis. Additionally, osteoclasts, the predominant cells involved in bone resorption, are the other key players in GIO.[4] More noteworthy is that curcumin has been shown to dramatically inhibit osteoclastic bone resorption by stimulating osteoclast apoptosis.[10,11] Accordingly, these combined results further confirmed that curcumin may play a dual role in the treatment of GIO, one as bone formation enhancer via promoting osteoblast survival, the other as bone resorption inhibitor via stimulating osteoclast apoptosis.

Bcl-2 family proteins have been reported to play an important role in GC-induced apoptosis of osteoblasts.[29–31] Bcl-2 acts as an anti-apoptotic protein, while Bax is a pro-apoptotic protein, thus the ratio of Bax/Bcl-2 determines the fate of many cells.[32] The results of this study are consistent with previous studies, and demonstrate that Dex exposure leads to a remarkable up-regulation of Bax and down-regulation of Bcl-2 during osteoblast apoptosis. Nevertheless, pretreatment with curcumin significantly prevents such up-regulated Bax and down-regulated Bcl-2 induced by Dex, suggesting that Bax/Bcl-2 complex is involved in the anti-GIO effects mediated by curcumin. Moreover, in response to apoptotic stimuli, an imbalance between Bax and Bcl-2 can result in the release of cytochrome c, in turn triggering the caspase cascade.[33] Caspase-3 is a major effector protease and its activation leads to the cleavage of PARP, ultimately cells undergo apoptosis.[34] Here, curcumin pretreatment obviously suppressed Dex-induced activation of caspase-3 and PARP in osteoblasts. Taken together, these findings further identified the anti-apoptotic effect of curcumin on Dex-induced osteoblasts.

Curcumin has been documented to possess multiple pharmaceutical activities and mediate a diverse range of molecular mechanisms. A number of studies have shown that curcumin can inhibit cell proliferation and induce apoptosis in a variety of cancer cells through multiple signalling pathways such as Wnt, the nuclear factor (NF)-κB, Notch, and mitogen-activated protein kinase (MAPK) signalling pathways.[35–37] In contrast, several reports indicate that curcumin can protect cells from apoptosis in various disease models including hepatic injury, testicular injury, and Alzheimer's disease.[38–40] Besides, it has been reported that curcumin can produce antidepressant effects by activating ERK signalling pathway in hippocampus.[41] ERK signalling activation is essential for cell survival, during which ERK1/2 is phosphorylated and translocated to the nucleus to activate multiple downstream substrates. Existing evidence has shown that Dex-induced apoptosis of osteoblasts is associated with the decrease in phosphorylation levels of ERK1/2.[17,18,42] The present results show that exposure to Dex significantly down-regulated the levels of p-ERK1/2 in osteoblasts, which was prevented by curcumin pretreatment. On the basis of these findings, it seems likely that the ERK signalling activation may be involved in the anti-apoptotic effects of curcumin. However, the detailed mechanisms of osteoblast apoptosis mediated by the ERK pathway need further exploration.

In conclusion, the present study clearly demonstrates that curcumin alleviates Dex-induced osteoporosis in rats. Mechanically, curcumin protects osteoblasts against Dex-induced apoptosis *in vivo* and *in vitro* through inhibiting the expression of pro-apoptotic proteins as well as activating the ERK pathway. The data presented in this study provide evidence to support the osteoprotective effects of curcumin on GIO, and suggest that curcumin may serve as an effective therapeutic strategy for the treatment of GIO.

MATERIALS AND METHODS

Animal model and drug administration

All animal care and experimental procedures were approved by the Animal Care Ethics and Use Committee of China Medical University, and performed in accordance with the guidelines of this Committee. Eighteen female Sprague-Dawley rats (5 MC) were purchased from Vital River Laboratory Animal Technology (Beijing, China). Curcumin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.5% sodium carboxymethylcellulose (CMC-Na).

The GIO rat model was induced by Dex as previously described.[24] All rats were subcutaneously injected 0.1 mg/kg per day Dex (Melonepharma, Dalian, China; $n = 12$) or equivalent normal saline (Control group, $n = 6$) for 60 days. Then, all animals underwent BMD detection to validate the presence of GIO. Subsequently, half of the Dex-treated rats were orally administrated with 100 mg/kg per day curcumin for another 60 days (Dex + Cur group, $n = 6$). Meanwhile, normal control rats and other Dex-treated rats (Dex group, $n = 6$) were given equal volume of 0.5% CMC-Na by gavage for 60 days. At the end of curcumin treatment, BMD values were determined again.

Bone mineral density measurements

Bone mineral density was measured by DXA using a PIXImus II densitometer (GE Medical Systems, Lunar Division, Madison, WI, USA). During BMD measurements, rats were kept in an anaesthetized state with sodium pentobarbital (50 mg/kg, i.p.). All rats were placed at the same position and measured by the same technician. The measurement was limited to the proximal femur area of the rats.

Biochemical analysis

Following BMD measurements, peripheral blood was collected to assess b-ALP and CTX levels using Rat b-ALP ELISA Kit and Rat CTX ELISA Kit (Becton-Dickinson, Franklin Lakes, NJ, USA) respectively according to the manufacturer's instructions. The absorbance was read at 450 nm in a microplate reader (BIOTEK, Winooski, VT, USA).

Mechanical testing

Rats were killed after collecting blood, and each right femur was removed from the rats. The three-point bending test was carried out to determine bone mechanical strength as previously reported.[43] Each femur was positioned on the two lower supports in the anvil of a Universal Testing Machine (Instron 4202; Instron, Canton, MA, USA). Load was applied to the middle of the femur with a crosshead speed of 1.5 mm/min for all tests. The values of load versus displacement were automatically recorded and the mechanical parameters were calculated by the Instron software (Instron series IX Automated Materials Tester, version 8.04.00; Instron, Canton, MA, USA).

Hematoxylin-eosin staining assay

Femur tissues were dehydrated through a gradient ethanol of 70% for 2 h, 80% overnight, 90% for 2 h and 100% for 1 h (100% is repeated twice). Then, these tissues were post-fixed in dimethylbenzene for 30 min and kept in dimethylbenzene-paraffin for 2 h at 60°C. After embedding in paraffin, the sections (5 μm) were cut, placed onto slides, and dried in a 60°C chamber for 24 h. Following dewaxing with ethanol, the sections were soaked in sequence with hematoxylin (Solarbio, Beijing, China), water, and 1% hydrochloric ethanol for 5 min, 5 min, and 3 s, respectively, at room temperature. After staining with eosin for 3 min, the slices were dehydrated through a gradient ethanol of 75% for 2 min, 85% for 2 min, 95% for 2 min, and 100% for 5 min (100% is repeated twice), and subsequently permeabilized twice with xylene for 10 min. Finally, the slices were mounted with resin and observed under a microscope (DP73; Olympus, Tokyo, Japan).

TUNEL assay

Sections were permeabilized in 0.1% Triton X-100 for 8 min. After washing with phosphate buffered saline (PBS) three times, sections were blocked with 3% H_2O_2 at room temperate for 10 min. TUNEL reaction was performed using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) strictly according to the manufacturer's instructions. Thereafter, sections were visualized by diaminobenzidine (DAB, Solarbio, Beijing, China) and stained with hematoxylin for 3 minutes. Finally, the apoptotic cells were observed under a microscope and photographed.

Osteoblasts isolation and culture

Primary osteoblasts were isolated from rats as described previously.[44] Calvaria were removed from neonatal rats, the fibrous tissues around the bone were gently scraped, and the sutures were removed. Then, calvaria were digested by 0.25% trypsin for 30 min at 37°C. After centrifugation, cells were resuspended and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and streptomycin/penicillin (100 U/mL) at 37°C in a humidified atmosphere containing 5% CO_2. When reaching 70% confluences, cells were digested for subsequent experiments.

For the experiment *in vitro*, osteoblasts were pre-treated with various concentrations of curcumin (0.5, 1 and 2 μmol/L) for 2 h. Then, cells were exposed to 1 μmol/L Dex for 6 h for further experiments. The untreated-cells, 2 μmol/L curcumin-treated-cells and 1 μmol/L Dex-treated-cells were tested in parallel as control.

Hoechst staining assay

Osteoblasts were inoculated onto coverslips in 12-well plates at a density of 5×10^4 cells/well. When reaching 80% confluences, cells were exposed to corresponding drugs for indicated time. According to the protocol of the Hoechst Staining Kit (Beyotime, Haimen, China), the coverslips were fixed for 20 min and washed twice with PBS. Then, the cells were incubated in Hoechst 33258 solution (0.5 mL) at room temperature for 5 min. After washing with PBS, the coverslips were mounted inversely onto slides with an anti-fluorescein quencher (Solarbio) and observed under a laser scanning confocal microscope (FV1000S-SIM/IX81; Olympus, Tokyo, Japan).

Flow cytometric analysis of osteoblast apoptosis

Cells were seeded in culture flasks at a density of 1×10^5 per flask for 24 hours, and exposed to different concentrations of drugs for indicated time. Cell apoptosis was measured by Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's directions. Briefly, the collected cells were resuspended in 500 μL binding buffer containing 5 μL Annexin V-FITC and 5 μL PI, and incubated for 15 min in a dark place. Apoptotic osteoblasts were detected immediately by flow cytometry (C6, Becton Dickinson, Franklin Lakes, NJ, USA) and the apoptotic ratio was analyzed by CELLQUEST software.

Western blot

Total proteins were extracted by lysing the cells with NP-40 lysate (Beyotime) containing 1% phenylmethanesulfonyl fluoride (PMSF). The concentration of total proteins was measured by a Bicinchoninic acid (BCA) Protein Assay Kit (Beyotime). Equal amounts of proteins from each sample were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were probed with primary antibodies against Bax (1 : 400), Bcl-2 (1 : 400; Boster, Wuhan, China), cleaved PARP (1 : 1000), cleaved caspase-3 (1 : 500; Abcam, Cambridge, MA, USA), or p-ERK (1 : 500), ERK (1 : 500; Bioss, Beijing, China) at 4°C overnight. Then, the blots were washed with Tris buffered saline with Tween 20 (TBST) three times and incubated with secondary goat anti-rabbit IgG-HRP antibody (1 : 5000, Beyotime, Jiangsu, China) at 37°C for 45 min. The interest bands were visualized by enhanced chemiluminescence (ECL) solution (Qihai Biotec, Shanghai, China) and the band intensity was analyzed by GEL-PRO-ANALYZER software (Bethesda, MD, USA). β-actin was served as an internal control.

Statistical analysis

Statistical analysis was performed with GRAPHPAD PRISM 5.0 software. All values are presented as mean $\pm$ standard deviation (SD), and differences between groups were analyzed with one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

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