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Inhibition on angiotensin-converting enzyme exerts beneficial effects on trabecular bone in orchidectomized mice

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ABSTRACT

Background: This study aimed to study the osteo-preservative effects of captopril, an inhibitor on angiotensin-converting enzyme (ACE), on bone mass, micro-architecture and histomorphology as well as the modulation of captopril on skeletal renin-angiotensin system (RAS) and regulators for bone metabolism in mice with bilateral orchidectomy.
Methods: The orchidectomized (ORX) mice were orally administered with vehicle or captopril at low dose (10 mg/kg) and high dose (50 mg/kg) for six weeks. The distal femoral end, the proximal tibial head and the lumbar vertebra (LV) were stained by hematoxylin and eosin, Safranin O/Fast Green and masson-trichrome. Micro-computed tomography was performed to measure bone mineral density (BMD).
Results: Treatment with captopril increased trabecular bone area at distal metaphysis of femur, proximal metaphysis of tibia and LV-4, moreover, high dose of captopril significantly elevated trabecular BMD of LV-2 and LV-5. The mRNA expressions of renin receptor, angiotensinogen, carbonic anhydrase II, matrix metalloproteinase-9, and tumor necrosis factor-alpha were significantly decreased in tibia of ORX mice following treatment with captopril. The administration with captopril enhanced the ratio of OPG/RANKL mRNA expression, the mRNA expression of transforming growth factor-beta and the protein expression of bradykinin receptor-1.
Conclusions: The inhibition on ACE by captopril exerts beneficial effects on trabecular bone of ORX mice. The therapeutic efficacy may be attributed to the regulation of captopril on local RAS and cytokines in bone.

Introduction

Osteoporosis is a disease that thins and weakens bones to the point that they become fragile and easily break. It is well-known that osteoporosis-related fractures due to low-trauma or fragility result in heavy health-related costs, substantial disability and mortality among postmenopausal women and older men [1]. Osteoporosis is now recognized as a major threat to health in aging people. Men sustain bone loss of approximately 0.5–1% per year from the sixth decade despite not undergoing a menopausal transition as women do [2]. Overall, the trend of age-adjusted prevalence of osteoporosis was similar between women and men [3,4]. However, the mortality and morbidity caused by osteoporotic fractures are greater in men with testosterone deficiency-induced osteoporosis than those in women with postmenopausal osteoporosis [5,6].

Bone metabolism is normally modulated by regulators and cytokines in bone micro-environment. The expression ratio of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL), both of which are secreted by osteoblasts, determines the maturation of osteoclasts. Matrix metalloproteinase (MMP)-9 and carbonic anhydrase (CA)II produced from osteoclasts are responsible for resorbing organic proteins and inorganic minerals, respectively. Bradykinin manages bone metabolism through modulating osteogenesis and osteoclastogenesis by binding to its receptor. Recently, various cytokines like tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β are found to play major roles in bone health.

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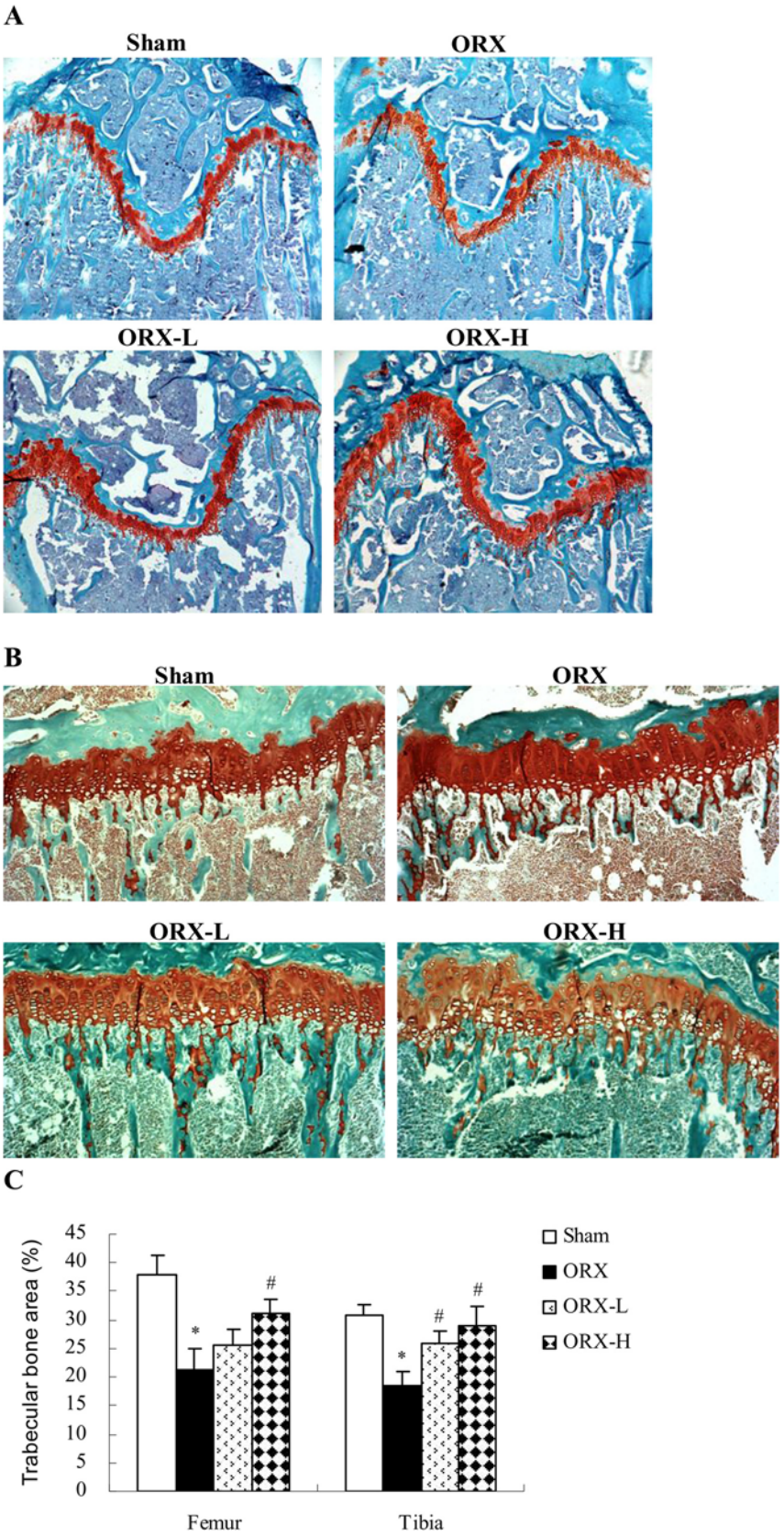

Fig. 1. Histological image measured by Safranin O/Fast Green staining. Femurs and tibias were collected from Sham mice and ORX mice orally treated with vehicle (ORX), or captopril with low dose (ORX-L, 10 mg/kg) or high dose (ORX-H, 50 mg/kg) for 6 weeks. A, distal femoral end (magnification, ×50). B, proximal tibial head (magnification, ×100). C, trabecular bone area under growth plate in femur and tibia was quantified. Values were expressed as means ± SEM, n = 8; * $p < 0.05$ *vs.* Sham group, # $p < 0.05$ *vs.* ORX group.

The renin-angiotensin system (RAS) components exist in tissues and organs, namely tissue RAS, participating in kinds of pathophysiologic processes [7,8]. Recent animal studies indicated the existence of RAS components in trabecular bone [9,10], and cell studies found the expression of angiotensin receptors in primary osteoblasts [11], suggesting there locally exist RAS components in bone micro-environment. The previous functional studies indicated that the skeletal RAS displays key biological actions on mediating bone metabolism [12–14].

Angiotensin II, key effector in RAS, is produced from angiotensin I by catalyzation of angiotensin-converting enzyme (ACE), a vital molecule in RAS. The clinical studies found that ACE inhibitor (ACEI) was capable of elevating bone mineral density (BMD) and reducing fracture risk in patients [15,16]. In line with the clinical observations of the bone-preservative properties following ACE inhibition, animal studies showed that treatment with ACEI repressed estrogen deficiency-induced decrease in bone density [17,18] and accelerated bone healing [19,20].

While, in a contrary to the beneficial effects of ACEI on bone health, the recent emerging evidences indicated that the use of ACEI might potentially accelerate bone loss [21–23]. Therefore, we are keen to know whether the inhibition on ACE could preserve bone tissue of mice with testosterone deficiency-induced osteoporosis. This study investigated the osteo-preservative effects of captopril on bone density, histology and micro-structure of trabecular bone as well as the modulation of captopril on skeletal RAS and regulators for bone metabolism in mice with bilateral orchidectomy.

Material and methods

Animal study design

Thirty-two three-month-old male ICR mice (Slac Laboratory Animal, Shanghai, China) received commercial diet and distilled water *ad libitum* during experimental period. The acclimatized mice underwent either bilateral laparotomy (Sham, n = 8) or bilateral orchidectomy (n = 24). After recovery for 1 week, the orchidectomized (ORX) mice were randomly divided into three groups: ORX mice with vehicle treatment (n = 8), ORX mice with orally administration of captopril with low dose (ORX-L, 10 mg/kg, n = 8) or high dose (ORX-H, 50 mg/kg, n = 8) by intragastric gavage. The dosage for captopril used in this study was referred as described previously [24,25]. Six weeks after drug administration, tibias, femurs and lumbar vertebras were immediately collected for a variety of analyses. The animal experiment complied with the ARRIVE guidelines, and the study protocol was approved by the Animal Ethics Committee.

Histological staining

The femurs, tibias, and lumbar vertebras were fixed in 4% formaldehyde in PBS (pH 7.2), decalcified in EDTA (0.5 M, pH 8.0), and embedded in paraffin. The bone sections with 3 μm were cut on a microtome. Safranin O (Sigma-Aldrich)/Fast Green staining was performed on femurs and tibias. Additionally, both the hematoxylin and eosin staining and the masson-trichrome staining were performed on lumbar vertebra (LV)-4. The images for all stained slides were captured under microscope (Leica DM-2500). Trabecular bone area (%) and thickness were measured using an OsteoMeasure system (OsteoMetrics Inc., Decatur, GA, USA) as described previously [26] and the whole process was performed in a blind manner.

Micro-computed tomography (Micro-CT) scanning

The LV-2 and LV-5 without decalcification were scanned with micro vivaCT 40 system (Scanco Medical, Bassersdorf, Switzerland). The detecting parameters were set as previously described [27]. Trabecular bone was determined by a fixed threshold. After images were captured (110 μA), 100 slices were established as the volume of interest. Trabecular BMD was obtained.

Reverse transcription-polymerase chain reaction

Tibias were crushed under liquid nitrogen condition and total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA). Agarose gel electrophoresis was performed to verify RNA integrity. Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA) was used to synthesize cDNAs by reverse transcription reactions with 3 μg of total RNA. DNA Engine (ABI) was applied for regular PCR with cDNAs as the template. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as a reference control to measure the amount of PCR products. The primer sequence utilized in this study was referred as previously described [27].

Western blotting

The femur was homogenized and extracted in Laemmli buffer (Boston Bioproducts, Worcester, MA, USA), followed by 5 min boiling and centrifugation to obtain the supernatant. Protein concentration was determined by Bio-Rad Protein Assay kit. 30 μg protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, which was blocked with 5% (w/v) nonfat dry milk in TBST containing 0.1% (w/v) Tween 20, and incubated with one of the following primary antibodies (Santa Cruz Biotechnology, USA) at 4 °C overnight: mouse anti-renin monoclonal antibody, goat anti-bradykinin receptor polyclonal antibody. After washes with TBST, the membranes were incubated with secondary antibody with dilution of 1:10,000. The protein bands were captured by Odyssey Infrared Imaging System (LI-COR, USA), quantified (Odyssey Application Software version 3.0) and normalized to β-actin signal using mouse monoclonal anti-β-actin antibody (Sigma, USA).

Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). The analysis for statistical difference was carried out with PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison tests which were applied to compare the values between groups and the difference with p value of less than 0.05 was considered statistically significant.

Results

Safranin O/Fast Green staining on trabecular bone at femur and tibia

The affection of captopril on trabecular bone was evaluated by Safranin O/Fast Green staining at the distal end of femur (Fig. 1A) and the proximal metaphysis of tibia (Fig. 1B). The loss of bone mass and the deterioration of network connection were clearly shown at spongiosa zones at both femur (Fig. 1C, $p < 0.05$) and tibia ($p < 0.05$) in orchidectomized (ORX) group as compared to those in Sham group. Treatment with low dose of captopril (ORX-L) increased the trabecular bone area at proximal tibial metaphysis ($p < 0.05$), and treatment with high dose of captopril (ORX-H) significantly elevated ($p < 0.05$) the area of trabecular bone at both femur and tibia in ORX mice.

Histological staining on trabecular bone at lumbar vertebra

Great amount of trabecular bone exists at spine bone, thus, hematoxylin and eosin (HE) staining (Fig. 2A) and masson-trichrome staining (Fig. 2B) were performed on lumbar vertebra (LV)-4. Similarly as observed at femur and tibia, the orchidectomy induced the loss of bone mass and the impairment of cancellous bone at LV-4 as demonstrated by the decrease of trabecular bone thickness (Fig. 2C, $p < 0.05$) and trabecular bone area (Fig. 2D, $p < 0.01$). Captopril with low dose ($p < 0.05$) and high dose ($p < 0.01$) dramatically increased the trabecular bone area of LV-4 in ORX mice. Captopril dose-dependently enhanced the thickness of trabecular bone even though there was no statistical difference between captopril-treated groups and ORX group.

Bone mass of lumbar vertebra

The measurement by micro-computed tomography (Fig. 3) showed that the orchidectomy alone induced significant decrease in BMD at both LV-2 ($p < 0.01$) and LV-5 ($p < 0.05$). The treatment with high dose of captopril dramatically ($p < 0.05$) increased BMD at both LV-2 and LV-5, which was well consistent with the histological result at lumbar vertebra.

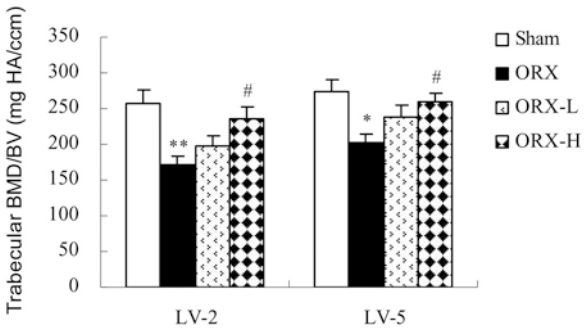

Fig. 3. Trabecular bone mineral density (BMD) at lumbar vertebra (LV)-2 and LV-5 determined by micro-computed tomography in Sham mice and ORX mice orally treated with vehicle (ORX), or captopril with low dose (ORX-L, 10 mg/kg) or high dose (ORX-H, 50 mg/kg) for 6 weeks. Values were expressed as means ± SEM, n = 8: * $p < 0.05$, ** $p < 0.01$ *vs.* Sham group, # $p < 0.05$, *vs.* ORX group.

mRNA expression of bone regulators

To clarify that tissue RAS was involved in the action of captopril on bone, the mRNA expressions of RAS components in tibia, such as angiotensinogen (AGT) and renin receptor (Renin-R), were

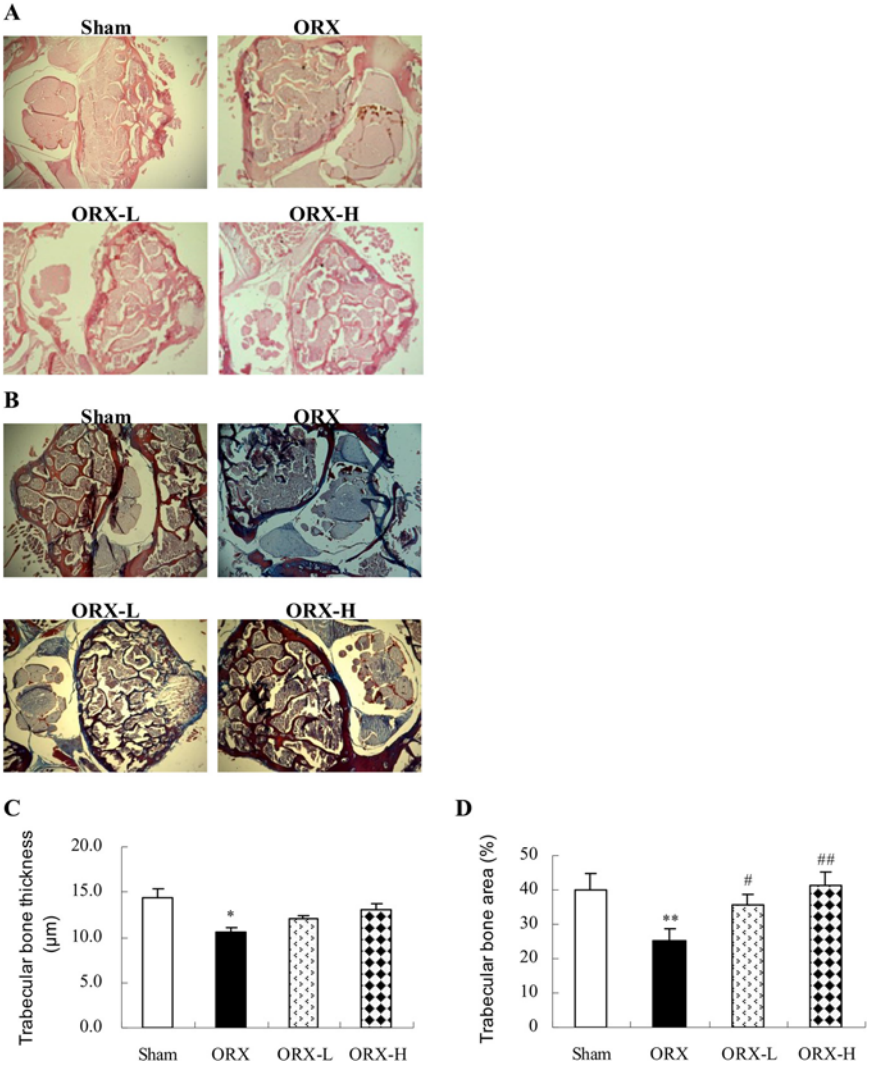

Fig. 2. Histological staining by hematoxylin and eosin (HE) (A, magnification, ×50) and masson-trichrome (B, magnification, ×50) at lumbar vertebra (LV)-4 in Sham mice and ORX mice orally treated with vehicle (ORX), or captopril with low dose (ORX-L, 10 mg/kg) or high dose (ORX-H, 50 mg/kg) for 6 weeks. The thickness (C) and area (D) of trabecular bone were quantified. Values were expressed as means ± SEM, n = 8; * $p < 0.05$, ** $p < 0.01$ *vs.* Sham group, # $p < 0.05$, ## $p < 0.01$ *vs.* ORX group.

determined (Fig. 4). The expressions of Renin-R (Fig. 4B, $p < 0.05$) and AGT (Fig. 4B, $p < 0.001$) were significantly up-regulated in tibia of vehicle-treated ORX mice. Gene expression of Renin-R was down-regulated by captopril with both doses ($p < 0.05$) and mRNA expression of AGT was markedly reduced only in ORX-H group ($p < 0.001$) when compared to those in ORX group.

The expression ratio of osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) plays vital role in formation and maturation of osteoclasts, thus, the expression of OPG and RANKL and the ratio of OPG/RANKL were determined (Fig. 4). The mRNA expression of OPG was lower in ORX group than that in Sham group (Fig. 4C, $p < 0.05$), and captopril significantly increased OPG mRNA expression as compared to that of ORX group ($p < 0.05$). The statistical difference for RANKL mRNA expression was not found among groups. While, the expression ratio of OPG/RANKL was decreased in ORX group ($p < 0.01$) and this ratio in ORX mice after treatment with low and high dose of captopril was recovered to the level in Sham group ($p < 0.01$).

The mRNA expressions of markers for bone resorption were measured in tibia for further evaluating the potential effect of captopril on bone resorptive activity (Fig. 4). The orchidectomy significantly increased (Fig. 4D, $p < 0.05$) the mRNA expression of CAII. The captopril treatments with low dose and high dose dramatically reversed this change ($p < 0.05$). The captopril treatment at high dose significantly down-regulated the mRNA expression of MMP-9 as compared to that in vehicle-treated ORX group ($p < 0.05$). Thus, this study indicated the potential regulation of captopril on osteoclastic activity.

In addition, the mRNA expressions of two cytokines, TNF-α and transforming TGF-β, were determined in tibia (Fig. 4). The mice

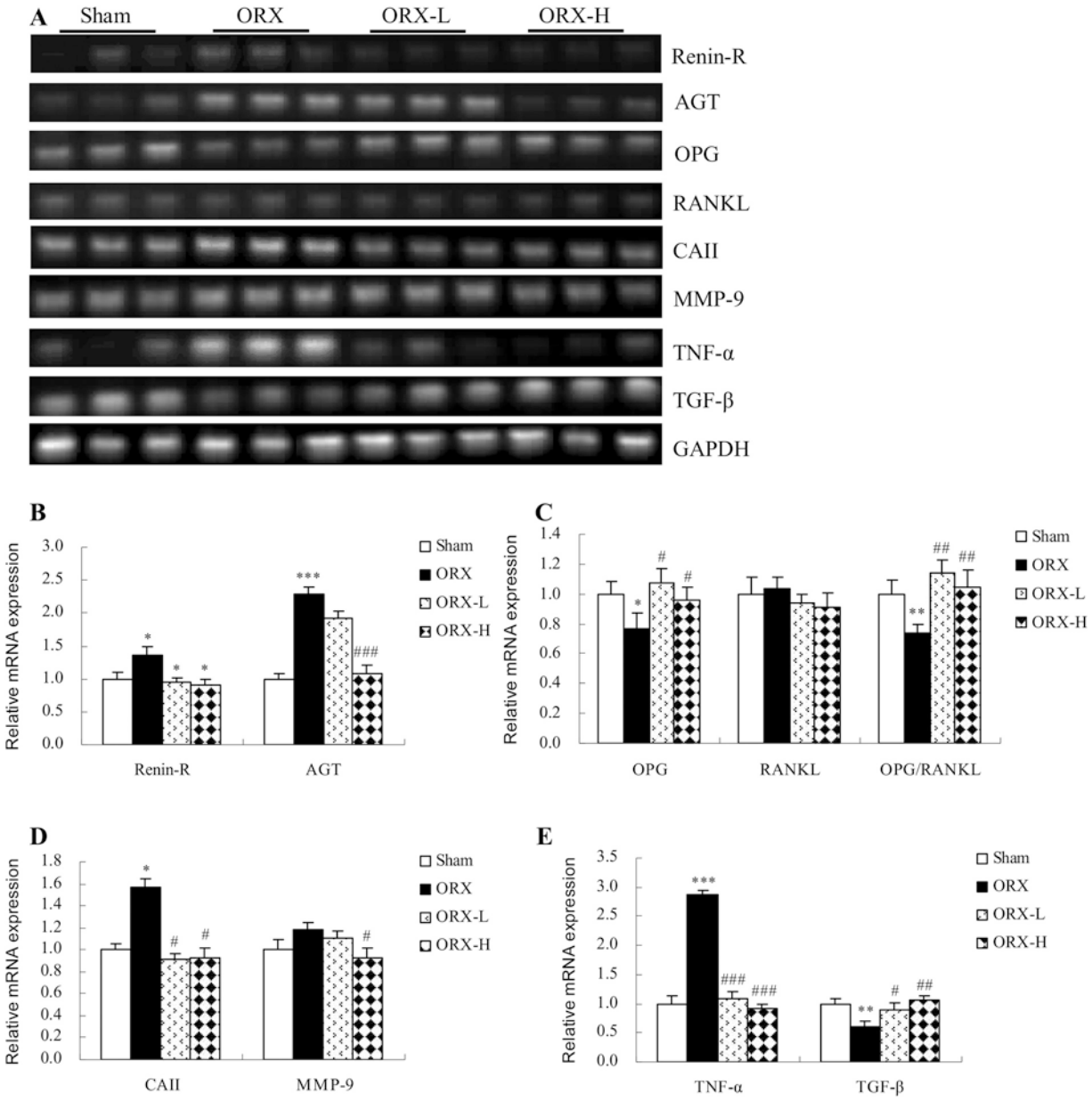

Fig. 4. mRNA expressions of bone regulators in tibia of Sham mice and ORX mice orally treated with vehicle (ORX), or captopril with low dose (ORX-L, 10 mg/kg) or high dose (ORX-H, 50 mg/kg) for 6 weeks. A, PCR bands. B, RAS components. C, OPG/RANKL. D, regulators for bone resorption. E, cytokines for bone metabolism. B-E, the densitometric quantification for RT-PCR. Renin-R, renin receptor; AGT, angiotensinogen; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; CAII, carbonic anhydrase II; MMP-9, matrix metalloproteinase-9; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta. Values were expressed as means ± SEM, n = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Sham group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* ORX group.

after orchidectomy showed the increased expression of TNF-α (Fig. 4E, $p < 0.001$) and the decreased expression of TGF-β ($p < 0.01$). Treatment with captopril reduced the TNF-α expression by 62% ($p < 0.001$) and enhanced the TGF-β expression by 46% ($p < 0.05$) as comparison with those in ORX group.

Expression of renin and bradykinin receptor-1 proteins in femur

Orchidectomy significantly induced the down-regulation of bradykinin receptor-1 (B1R) protein (Fig. 5B, $p < 0.001$), but did not alter the protein expression of renin in femur. Treatment with low dose and high dose of captopril in ORX mice markedly increased the expression of B1R ($p < 0.001$). Of noted, the protein expression of renin was higher ($p < 0.05$) in ORX-H group than that in vehicle-treated ORX group.

Discussion

Angiotensin-converting enzyme inhibitors (ACEIs) are classically applied for anti-hypertension treatment [28], and are currently in wide use for the treatment of diabetic complications [29,30]. Given the key role of tissue renin-angiotensin system (RAS) in maintaining bone health, it is vital to study the effect of ACEIs on bone injury induced by testosterone deficiency. In the present study, we investigated the therapeutic effect of captopril, one typical ACEI, on trabecular bone histology and bone mass at long bone and spine bone as well as studied the modulation of captopril on regulators for bone metabolism in orchidectomized (ORX) mice.

This study demonstrated that the treatment with captopril effectively attenuated the orchidectomy-induced pathological alterations of micro-structure of trabecular bone at lumbar vertebra (LV)-4, distal metaphysis of femur and proximal metaphysis of tibia as observed by histological staining, moreover, bone mineral density (BMD) at both LV-2 and LV-5 was significantly enhanced in ORX mice in response to captopril treatment for 6 weeks. These results revealed the preventive effects of inhibiting angiotensin-converting enzyme (ACE) on decrease of bone mineral density and impairment of trabecular bone structure at axial and appendicular bones.

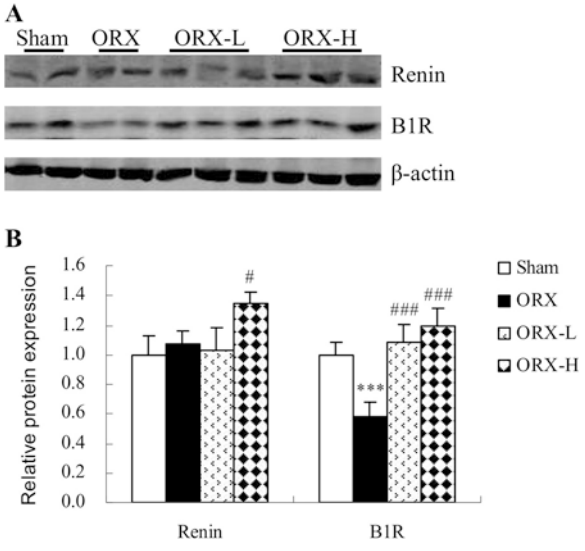

Fig. 5. Protein expressions of renin and bradykinin receptor-1 (B1R) in femur of Sham mice and ORX mice orally treated with vehicle (ORX), or captopril with low dose (ORX-L, 10 mg/kg) or high dose (ORX-H, 50 mg/kg) for 6 weeks. A, western blot bands. B, the densitometric quantification for western blotting. Values were expressed as means ± SEM, n = 8. *** $p < 0.001$ *vs.* Sham group, # $p < 0.05$, ### $p < 0.001$, *vs.* ORX group.

The beneficial effect of captopril on bone of ORX mice shown in this study was well consistent with previous animal studies, which demonstrated that the intervention of Tsukuba hypertensive mice with ACEI enalapril attenuated osteoporosis [11] as well as the ovariectomized (OVX) rats treated with captopril showed the increased area of trabecular bone at LV-4 and the improved mechanical properties at LV-5 [31]. Additionally, the patients after treatment with ACEIs had an increase in BMD and a reduced risk in fracture [32]. Thus, the preclinical and clinical studies fully suggested the potential of ACEI in exerting osteoprotective efficacy.

Of noted, the recent studies demonstrated that captopril repaired cortical morphometric features [18] and improved cortical bone thickness [33] in OVX rats, while, the potential effect of captopril on cortical bone in ORX animals, such as the middle-shaft of tibia and femur, require further investigation.

It is evident that the activation of skeletal RAS would result in bone injuries [9,11]. This study revealed that orchidectomy alone increased the mRNA expressions of renin receptor (Renin-R) and angiotensinogen (AGT) in bone, and the similar experimental results were found in tibia of rats [34] and mice [14] with diabetes. Upon to the treatment with captopril, the abnormal expression of AGT and Renin-R in ORX mice was almost reduced to the level of Sham group. Thus, this study indicated the biological role of tissue RAS in development of testosterone deficiency-induced osteoporosis and suggested that the skeletal RAS may a candidate target in drug discovery for anti-osteoporosis of elderly males.

Our results clearly showed that the expressions of genes involved in bone resorption (CAII, MMP-9) were significantly induced and the ratio of OPG/RANKL expression (a marker for osteoclastogenesis) was decreased in tibia of ORX mice, a typical feature of bone turnover associated with testosterone deficiency. Treatment of ORX mice with captopril significantly suppressed these changes of CAII, MMP-9 and OPG/RANKL. Similar results were reported by others that captopril increased the ratio of OPG/RANKL in bone [18] and serum [33] of OVX rats. Both MMP-9 and CAII, produced from osteoclasts, are enzymes to dissolve organic component and inorganic substance of bone, respectively [27]. Thus, our results clearly indicated that captopril exerted the suppression on bone resorption by which captopril protected against bone deteriorations induced by testosterone deficiency in male mice.

Besides the local RAS in bone tissue, bradykinin, the major active peptide in the kallikrein-kinin system (KKS), is able to regulate bone metabolism through acting on bradykinin receptor-1 (B1R) [35]. The expression of bradykinin receptor was decreased in osteoblasts with exposure to high glucose level and in bone of OVX mice [26,36]. This study demonstrated that captopril effectively reversed orchidectomy-induced down-regulation of B1R protein expression in mice, indicating that bradykinin receptor was involved in management of captopril on bone metabolism in ORX mice.

TNF-α, one of inflammatory markers, could activate osteoclasts, consequently stimulating bone resorption and resulting in osteoporosis. *In vivo* studies showed that the administration of animals with RAS inhibitors could reduce TNF-α expression in tissue [37,38], similarly, in this study captopril dramatically decreased mRNA expression of TNF-α in bone of ORX mice. In addition, TGF-β is an important modulator for bone formation and repair. The present study showed the down-regulation of TGF-β in bone of ORX mice, which was in consistent with early study [39]. The increase in TGF-β expression in response to captopril treatment was found in ORX mice in this study, and the similar action of captopril on TGF-β was also reported in human lung fibroblasts [40]. Our study indicated that the regulation of captopril on TNF-α and TGF-β might be involved in its protective effects on bone tissue of ORX mice.

Although treatment with captopril attenuated bone injury in ORX mice, we found that captopril at high dose in this study induced the elevation of renin protein expression in bone. The compensatory increase in renin expression is a common problem for ACEIs due to the disruption of the feedback inhibitory loop in renin production [41]. The increase in RAS activity might ultimately limit the therapeutic effectiveness of ACE inhibition. Thus, the clinical practice currently suggests the application of RAS blockers with aliskiren, one renin inhibitor which could suppress the first and rate-limiting step within RAS [42]. While, whether the combination of ACEI with aliskiren could synergistically ameliorate bone impairments induced by testosterone deficiency need to be further clarified.

Taken together, this study clearly showed the beneficial effect of captopril on trabecular bone in orchidectomized mice with testosterone deficiency, and the underlying mechanism may, at least partially, be attributed to the regulation of captopril on skeletal RAS and local cytokines.

Conflict of interest

The authors declare no conflict of interest.

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