Int J Clin Exp Med 2016;9(9):17628-17635
www.ijcem.com /ISSN:1940-5901/IJCEM0032972

Original Article

Effects of antihypertensive drugs on bone metabolism in ovariectomized spontaneously hypertensive rats

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Received May 29, 2016; Accepted August 3, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Osteoporosis and hypertension often coexist in the elderly, especially in postmenopausal women. However, conflicting results regarding the relationship between antihypertensive agents and bone metabolism were described in previous studies. In this study, we examined the effects of antihypertensive drugs on bone metabolism in ovariectomized (OVX) spontaneously hypertensive rats. Two weeks after ovariectomy, female spontaneously hypertensive rats (11 week old) were treated daily by intragastric administration with hydrochlorothiazide, metoprolol, amlodipine, valsartan, or benazepril for 12 weeks. Serum concentrations of aminoterminal propeptide of type I collagen (P1NP), C-terminal telopeptide of type I collagen (CTX), and estradiol were measured by enzyme-linked immunosorbent assay. Serum P1NP and CTX concentrations were increased in the OVX group, which was accompanied by a decrease in trabecular bone mineral density (BMD) and deterioration of bone microarchitecture. Hydrochlorothiazide and benazepril significantly restored serum PINP and CTX concentrations and improved microarchitecture parameters, whereas amlodipine decreased serum PINP and CTX concentrations, but showed no significant effects on BMD and bone microarchitecture. Additionally, metoprolol and valsartan had no effects on these parameters. These results suggest that hydrochlorothiazide and benazepri are effective in the prevention of OVX-associated bone deterioration in spontaneously hypertensive rats.

Keywords: Antihypertensive agents, osteoporosis, spontaneously hypertensive rats, bone metabolism

Introduction

Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue. The incidence of osteoporosis increases with age, especially in postmenopausal women [1-3]. Hypertension is associated with reduced bone mineral density (BMD) and increased fracture risk in women [4-6]. Previous studies on the correlations between antihypertensive agents (e.g. thiazide diuretics, beta-blockers, calcium channel blockers, and renin-angiotensin-aldosterone system agents) and bone health yield inconsistent results [7-10].

Due to the frequent coexistence of hypertension and osteoporosis, when selecting long-term antihypertensive therapy the potential effects of antihypertensive agents on development, worsening, or improvement of osteoporosis should also be considered. To clarify the effect of commonly prescribed antihypertensive agents on bone metabolism, we employed an ovariectomized (OVX) model of estrogen deficiency in spontaneously hypertensive rats (SHR), which was the most suitable animal model that mimics postmenopausal osteoporosis with hypertension in humans. BMD and microarchitecture were evaluated by micro-CT, which now has become the "gold standard" for evaluation of bone morphology and microarchitecture in small animal models ex vivo. In addition, we tested the bone turnover markers, P1NP (a marker of bone formation) and CTX (a marker of bone resorption), to get a better understanding of how the antihypertensive agents modulate the bone metabolism of OVX SHR.

Material and methods

Animals, groups, and treatment

Forty-two female SHRs (11 week old) were purchased from Beijing Laboratory Animal Tech-

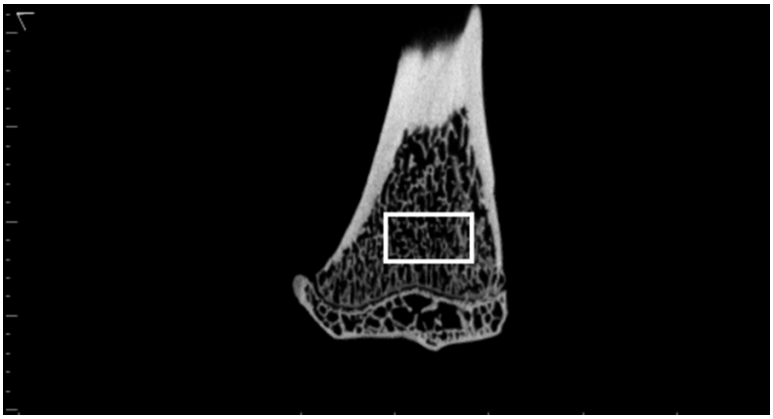

Figure 1. The region of interest (ROI) of micro-CT in right proximal tibia.

nology Company (Beijing, China) and housed in the cages (two rats/cage) under automatically controlled conditions of temperature (23 ± 1°C), humidity (50 ± 10%), and a 12:12-h light/dark cycle. They were given a standard rat chow and water ad libitum. All rats were acclimatized for 1 week. After the rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g body weight), bilateral OVX or sham operation was performed. The experiment protocol was approved by the Animal Experiment Ethics Committee of authors' institute (Ref. no. 2014121001).

SHRs were randomly assigned to seven groups (n = 6 for each group): Group 1 (sham), sham-operated + water; Group 2 (control), OVX + water; Group 3 (hydrochlorothiazide), OVX + hydrochlorothiazide (10 mg/kg body weight), Group 4 (metoprolol), OVX + metoprolol (50 mg/kg body weight), Group 5 (amlodipine)' OVX + amlodipine (5 mg/kg body weight), Group 6 (valsartan), OVX + valsartan (30 mg/kg body weight), and Group 7 (benazepril), OVX + benazepril (10 mg/kg body weight).

All drugs were dissolved in drinking water, and the water or drugs were given once daily by intragastric administration from day 14 after operation for 12 weeks. Body weight of the rats were recorded weekly for adjustment of the dosage of antihypertensive agents. Hydrochlorothiazide was purchased from Chen Xin Pharmaceutical Company, metoprolol succinate from AstraZeneca Pharmaceutical Company, amlodipine from Pfizer Pharmaceutical Company, valsartan and benazepril from Beijing Novartis Pharmaceutical Company.

Blood pressure

Systolic blood pressure (BP) was measured by tail-cuff method using a noninvasive BP measurement system (Medlab-u/8C502 biological signal collection and processing system, China) in conscious rats before operation and 12 weeks after treatment. The measurement was performed according to the manufacturer's instructions. Three repeated measurements were performed at each time point for each SHR and their average was used for data analysis.

Sampling

At 12 weeks after treatment, all SHRs were sacrificed by drawing whole blood from postcava under anesthetized with 10% chloral hydrate (0.3 ml/100 g body weight). Following euthanasia, the right tibia was excised and fixed in 10 % formaldehyde (w/v) for measurement of BMD and bone microarchitecture by micro-CT. Serum samples were collected and stored at -80°C until use.

Evaluation of bone-related markers

Serum concentrations of calcium, phosphorus and alkaline phosphatase were measured using an automatic biochemical analyzer (Beckman Coulter, Beckman, CA, USA). Serum concentrations of P1NP, CTX, and estradiol were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All samples were assayed in duplicate.

Evaluation of BMD and trabecular bone microarchitecture

The right proximal tibia was scanned with micro-CT (ZKKS-MCT-Sharp). The region of interest (ROI) was selected under the tibial growth plate (**Figure 1**). The ROI was drawn semi-automatically at each two-dimensional section of slices of interest. The BMD and microarchitecture of ROI were automatically

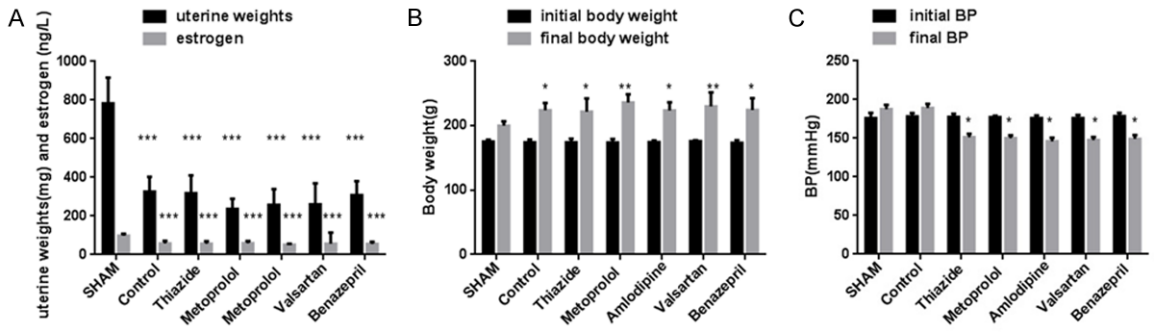

Figure 2. The comparison of uterine weights (mg), estrogen (ng/L), body weight (g), and BP (mmHg) of SHR in each group. A. Uterine wet weight and E2; B. Body weight; C. Blood pressuer. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with sham group; $^{\#}P<0.05$, compared with the control group. Values are expressed as means ± S.E.M. (n = 6).

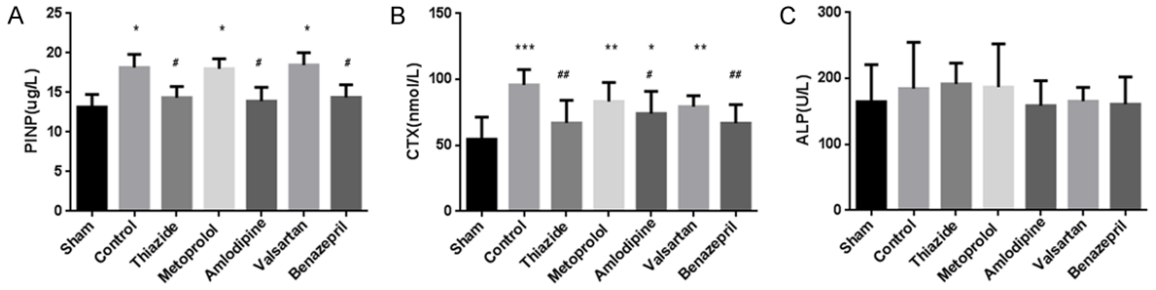

Figure 3. The comparison of PINP (ug/L), CTX (nmol/L), and ALP of SHR in each group. A. PINP; B. CTX; C. ALP. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with sham group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the control group. Values are expressed as means ± S.E.M. (n = 6).

evaluated using the analysis software of med-Project with direct three-dimensional morphometry. The microarchitectural parameters for trabecular bone were obtained, including total volume (TV), bone volume (BV), bone volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp) and the structure model index (SMI). Description and nomenclatures followed guidelines for the assessment of bone microstructure with micro-CT analysis [11].

Statistical analysis

The data were interpreted as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and LSD post hoc test were used for multiple comparisons between groups. Correlations between parameters were presented as pearson correlation coefficient (r). All statistical analyses were two-sided and a *p* value of less than 0.05 was regarded as statistically significant. SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Results

General observations

To clarify the effect of commonly used antihypertensive agents on bone metabolism, we used an OVX model of estrogen deficiency in a hypertension model, SHR. At 14 weeks after bilateral OVX, the uterine wet weight and serum E2 concentration decreased, while body weight increased significantly compared with those in sham-operated group. No statistical differences in uterine wet weight, serum E2 concentration and body weight were found among all drug -treated groups (**Figure 2**). Correlation analysis showed that body weight were negatively correlated with E2 level and uterine wet weight (r = -0.544, r = -0.407, respectively). The systolic BP of all groups was comparable at baseline. Whereas, at the end of the treatment, the BP of all drug -treated groups were significantly lower than that of sham and OVX-control groups. No statistical differences in BP were found among all drug-treated groups (**Figure 2**).

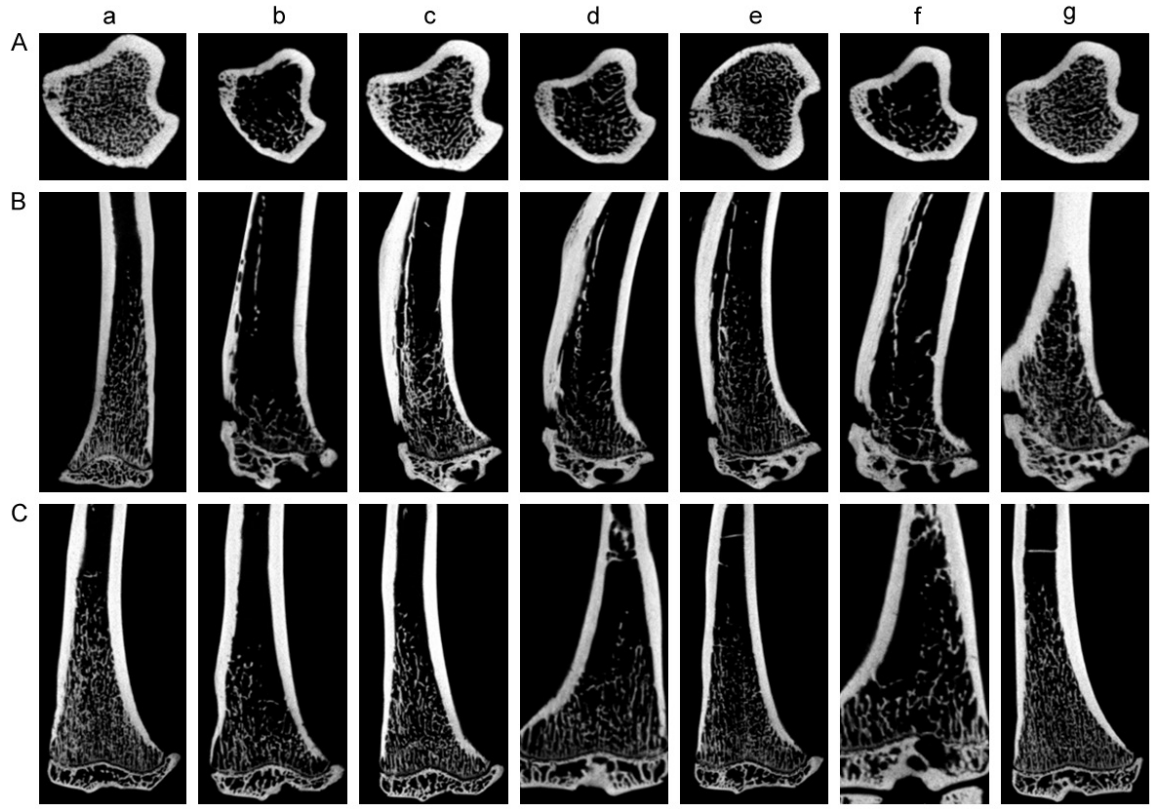

Figure 4. Representative two-dimensional micro-CT images of the right proximal tibia in SHR. a. Sham group; b. OVX-control group; c. Hydrochlorothiazide group; d. Metoprolol group; e. Amlodipine group; f. Valsartan group; g. Benazepril group. A. Transverse section of the image; B and C. Longitudinal section of the image. Trabecular bone loss was observed in the OVX- control group compared with sham group. Increased in bone mass was observed in hydrochlorothiazide and benazepril groups compared with OVX-control group.

Serum bone-related markers

At 14 weeks after bilateral OVX, the serum PINP and CTX concentrations increased significantly compared with sham group ($P<0.001$). Treatment with hydrochlorothiazide, amlodipine and benazepril attenuated OVX-induced increase in bone turnover, leading to a significant decline in serum PINP and CTX concentrations. In contrast, treatment with metoprolol and valsartan had no effect on bone turnover induced by OVX (**Figure 3**). However, no significant differences in serum PINP and CTX concentrations were found among the hydrochlorothiazide, amlodipine and benazepril groups. Similarly, no significant differences were found between metoprolol and valsartan groups. In addition, we analyzed several other serum bone-related markers. There were no significant differences in serum calcium and phosphate and alkaline phosphatase (ALP) levels before and after drug treatment (**Figure 3**).

BMD and trabecular bone microarchitectural parameters

As shown in **Figure 4**, trabecular bone loss was observed in two-dimensional micro-CT images of the right proximal tibia in the OVX group compared with sham group. Increased in bone mass was observed in hydrochlorothiazide and benazepril treated groups compared with the OVX group.

After OVX, the BMD was significantly decreased, compared with sham group ($P<0.001$). Treatment with hydrochlorothiazide and benazepril increased BMD significantly when compared with OVX-control group ($P = 0.001$). However, no statistical differences in BMD were found among the metoprolol, amlodipine, valsartan, and OVX groups (**Figure 5A**). On the other hand, after OVX, the BV/TV ($P = 0.007$) and Tb. N ($P = 0.001$) was significantly decreased, while Tb. Sp ($P = 0.000$) and SMI ($P =$

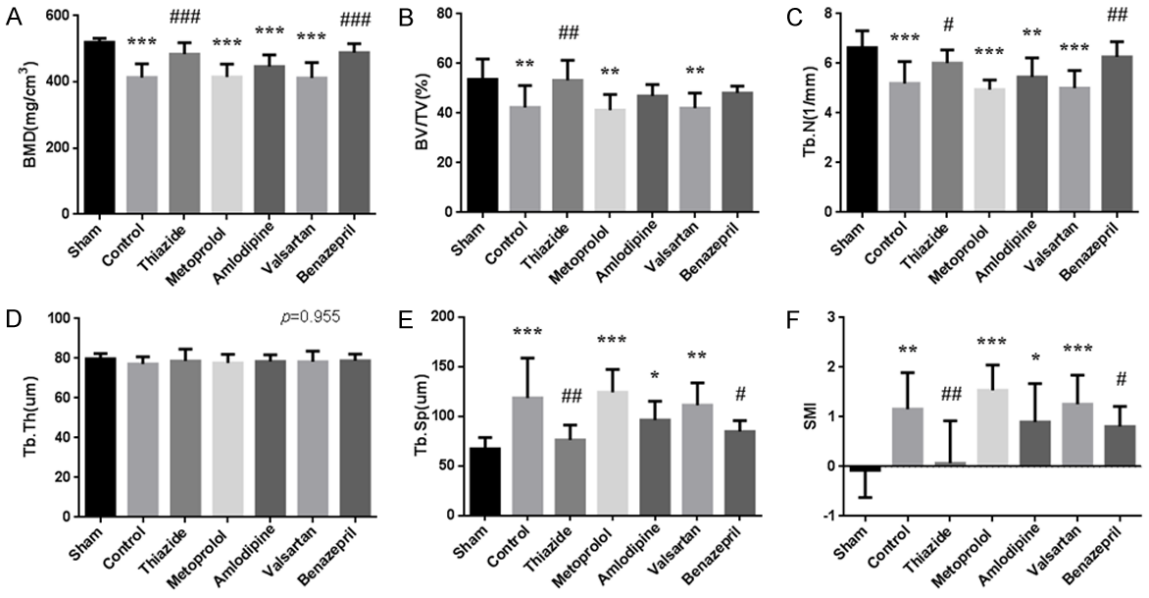

Figure 5. Effect of antihypertensive agents on BMD and trabecular bone microarchitecture of proximal tibia in SHR. A. BMD; B. BV/TV; C. Tb. N; D. Tb. Th; E. Tb. Sp; F. SMI. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with sham group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the control group. Values are expressed as means ± S.E.M. (n = 6).

Table 1. Correlation analysis of BMD with indicated variables

Variables	*r*	*P*
BV/TV	0.768	0.000
Tb. N	0.899	0.000
serum E2	0.341	0.027
Tb. Sp	-0.877	0.000
Tb. Th	-0.382	0.013
SMI	-0.810	0.000
PINP	-0.381	0.013
CTX	-0.567	0.000
Baseline BP	-0.432	0.004
Terminal BP	0.056	0.726
	0.172	0.275

0.002) was increased, relative to the sham group (**Figure 5B-F**). Treatment with hydrochlorothiazide increased BV/TV ($P = 0.009$) and Tb. N ($P = 0.039$) and reduced Tb. Sp ($P = 0.002$) and SMI ($P = 0.006$), compared with the OVX group. Treatment with benazepril increased Tb. N ($P = 0.009$) and decreased Tb. Sp ($P = 0.013$), compared with the OVX group.

Correlation analysis

Correlation analysis showed that BMD were positively correlated with BV/TV, Tb. N, E2 level ($r = 0.768$, 0.899, and 0.341, respectively) and negatively correlated with Tb. Sp, Tb. Th, body weight, serum PINP and CTX ($r = -0.877$, -0.377, -0.381, -0.567, and -0.432) (**Table 1**).

Discussion

The main findings of this study were that treatment with hydrochlorothiazide and benazepril attenuated OVX-induced increase in serum PINP and CTX, decrease in BMD, deterioration in trabecular bone microarchitecture, suggesting their potential in the prevention of bone deterioration associated with OVX in SHR. Hydrochlorothiazide, a thiazide diuretics, is one of the most commonly prescribed antihypertensive agents worldwide. Thiazides act at the distal convoluted tubule to increase calcium absorption, reduce urinary calcium excretion. In addition, a recent study has shown that thiazides can stimulate duodenal Ca^{2+} absorption as well as osteoblast differentiation and bone Ca^{2+} storage by inhibiting thiazide-sensitive Na^{+}-Cl^{-} cotransporter (NCC) gene, therefore potentially prevent osteoporosis [12].

As expected, our findings showed that hydrochlorothiazide treatment reduced bone turnover markers (serum PINP and CTX), increased BMD and improved bone microarchitectural in

OVX-SHR. Our results were consistent with prior animal and epidemiologic studies, where thiazides were found to elevate BMD in OVX mice [13], and to reduce bone turnover markers in older adults and postmenopausal women [14]. Furthermore, a Cochrane review, including twenty-one observational studies with nearly 400,000 participants, reported reduction in risk of hip fracture by 24% among long-term thiazide users compared with nonusers [15].

Metoprolol, a selective beta1-adrenoceptor antagonist (β_1-blocker), is one of the most widely used drugs in the management of hypertension, ischemic heart disease and heart failure. Beta-adrenergic receptors have been subdivided into three types: beta-1 (heart and vessels), beta-2 (pulmonary tissues), and beta-3 (adipose tissue). In recent years, both in vitro and in vivo experimental studies have been demonstrated that human osteoblastic as well as osteoclastic cells express adrenergic receptors [16]. Increased sympathetic nervous activity causes bone loss via an increase in bone resorption and a decrease in bone formation [17]. Such findings indicate that β-blockers (BB) may be effective toward osteoporosis attributed to increased sympathetic nervous activity. However, our data demonstrated that there were no significant difference in BMD, trabecular bone microarchitecture parameters and bone turnover markers between the metoprolol-treated and control groups. Our finding was consistent with a previous study, which reported that the cardioselective β-blockers do not modify bone mass or the structural bone parameters in males with acute myocardial infarction [18]. Furthermore, a recent retrospective nationwide claim study showed that BB users was associated with increased risk of fracture compared with non-users [19]. However, some epidemiology studys and meta-analysis reported that BB use was associated with higher BMD and a lower fracture risk [20], and that the association between BB use and reduced fracture risk were independent of BMD, and was mainly found in beta-1 selective BB, not in the nonselective agents, compared to other classes of antihypertensive agents [21]. The reason for the conflicting results regarding the effects of BB on bone health has not been fully understood. One possible explanation was that β_1- and β_2-adrenergic signals exert opposite effects on bone, with β_1 exerting a predominantly anabolic stimulus in response to mechanical stimulation and during growth, whereas β_2-adrenergic receptor signaling mainly regulates bone resorption during aging [22]. This view is supported by the findings that β_2 adrenoceptor agonists have a negative effect on bone geometry, mass, microarchitecture and bone mechanical properties [23]. Another possible explanation was that the fracture-lowering effect of BBs could be mediated systemically or indirectly in humans, unlike the direct effects on the β_2 receptors of osteoblasts or osteoclasts in animal or experimental studies.

Amlodipine, a dihydropyridine calcium channel blockers, ranks among the most frequently prescribed drugs for hypertension. Its principal action is to inhibit calcium entry through voltage-gated transmembrane L-type channels. This produces a decrease in the intracellular calcium concentration and subsequent relaxation of smooth muscle cells in the peripheral and coronary vessels [24]. Osteoblasts have been found to express voltage-dependent calcium channels [25]. Activation of L-type calcium channels is required for gap junction-mediated intercellular calcium signaling in osteoblastic cells [26]. This fact suggests that amlodipine could influence bone metabolism via the calcium channels of osteoblasts. In the present study, we demonstrated that amlodipine treatment had no significant effects on BMD and trabecular bone microarchitecture compared with control group. Our results are consistent with a previous study where cilnidipine (L-/N-type CCB), but not amlodipine, ameliorated osteoporosis in ovariectomized hypertensive rats through inhibition of the N-type calcium channel [27]. In addition, amlodipine had no impact on bone healing [28]. In contrast, amlodipine increased the bone density dose-dependently in ovariectomy-induced osteopenic rats [29]. A clinical study found that amlodipine increased vitamin D levels significantly in patients with a newly diagnosed hypertension on a 12-week treatment duration compared to valsartan [30]. Moreover, a nationwide case-control study found that treatment with calcium channel blockers is associated with a reduced fracture risk [31]. These findings suggest that amlodipine have beneficial effects on the prevention of osteoporosis.

Valsartan, one of the angiotensin II type 1 receptor (AT1R) blockers (ARBs), and benazepril, one of the angiotensin converting enzyme

inhibitors (ACEI), belong to renin-angiotensin system (RAS) inhibitors. It has been shown that angiotensin II indirectly promoted the differentiation and activation of osteoclasts via upregulation of receptor activator of NF-kappaB ligand (RANKL) in osteoblasts [32]. Some clinical studies reported that patients treated with an ACE inhibitor showed an increased BMD and more importantly reduced fracture risks [33]. Our findings were very similar to prior work showing that it was ACEIs (benazepril), but not ARBs (valsartan), have positive effects on bone metabolism, as assessed by decreasing PINP and CTX, increasing BMD and improving microarchitecture parameters. In addition, a prospective cohort study of 5995 older American men showed that use of ACEI but not ARB may marginally increase bone loss in older men [34]. The reasons for the different effects on bone of ARB and ACEI might partly be due to the differences in action mechanisms.

The present study also has several limitations. First, the number of SHR was limited, the dose-response relationships between antihypertensive drugs and bone metabolism were not examined. Second, the molecular mechanisms of antihypertensive drugs affect bone metabolism were not examined deeply.

In summary, our results show that hydrochlorothiazide and benazepri treatment have positive effects on the prevention of bone deterioration associated with OVX in SHR. These findings suggest that thiazides and ACEIs have potential antiosteoporotic effects on hypertensive postmenopausal osteoporosis. Long-term prospective randomized studies are needed to further assess the effects of antihypertensive agents on bone metabolism in postmenopausal women.

Disclosure of conflict of interest

None.

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