

# The Effects of Antihypertensive Drugs on Bone Mineral Density in Ovariectomized Mice

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## INTRODUCTION

Osteoporosis is characterized by an increase in bone fragility, resulting in fractures caused by minor trauma or even of a spontaneous nature. The disease is classified clinically as either primary or secondary osteoporosis. Primary osteoporosis is bone loss that occurs in postmenopausal women during normal aging. Postmenopausal osteoporosis, the most common form of the disease, commences when estrogen production decreases (1). Approximately forty percent of women over 50 years of age will suffer a fracture related to postmenopausal osteoporosis during their lifetime (2).

The prevalence of hypertension also increases markedly with aging, suggesting that hypertension and osteoporosis coexist. Because antihypertensive drugs are widely used for the treatment of hypertension, it is important to understand the effects of these drugs on bone. Antihypertensive drugs impact osteoporosis directly and indirectly by affecting bone metabolism,

The effects of several antihypertensive drugs on bone mineral density (BMD) and micro-architectural changes in ovariectomized (OVX) mice were investigated. Eight-week-old female C57/BL6 mice were used for this study. Three days after ovariectomy, mice were treated intraperitoneally with nifedipine (15 mg/kg), telmisartan (5 mg/kg), enalapril (20 mg/kg), propranolol (1 mg/kg) or hydrochlorothiazide (12.5 mg/kg) for 35 consecutive days. Uterine atrophy of all mice was confirmed to evaluate estrogen deficiency state. BMD and micro-architectural analyses were performed on tibial proximal ends by micro-computed tomography (micro-CT). When OVX mice with uterine atrophy were compared with mice without atrophy, BMD decreased ( $P < 0.001$ ). There were significant differences in BMD loss between different antihypertensive drugs ( $P = 0.005$ ). Enalapril and propranolol increased BMD loss in mice with atrophied uteri compared with control mice. By contrast, thiazide increased BMD in mice with uterine atrophy compared with vehicle-treated mice ( $P = 0.048$ ). Thiazide ( $P = 0.032$ ) and telmisartan ( $P = 0.051$ ) reduced bone loss and bone fraction in mice with uterine atrophy compared with the control. Thiazide affects BMD in OVX mice positively. The reduction in bone loss by thiazide and telmisartan suggest that these drugs may benefit menopausal women with hypertension and osteoporosis.

**Key Words:** Antihypertensive Agents; Osteoporosis; Ovariectomized Mice; Thiazides; Telmisartan

strength and density (3). Meta- and epidemiological analyses of national databases illustrate an association between antihypertensive drugs and bone function (4-7). A reduced fracture risk has been reported with thiazide diuretics (5, 6), whereas conflicting results have been reported in other antihypertensive drugs (4-7).

There are few studies on the effects of antihypertensive drugs on bone function in animal models of postmenopausal osteoporosis. Ovariectomized (OVX) mice represent an animal model that mimics postmenopausal osteoporosis in humans. We used this model to investigate whether antihypertensive drugs affect bone density and cause micro-architectural changes that associate with estrogen deficiency.

## MATERIALS AND METHODS

### Animals and drug treatment

Forty eight 8-week-old female C57/BL6 mice weighing 18-20 g

were purchased from Orient Bio (Seongnam, Korea). C57/BL6 mice were anesthetized by inhalation of 2% isoflurane. Bilateral ovariectomy was performed, and mice were sacrificed 5 weeks after surgery by an overdose of anesthetics. Uterine atrophy confirmed estrogen deficiency. Three days after ovariectomy, mice were treated intraperitoneally with different antihypertensive drugs for 35 consecutive days. Mice were randomly divided into 6 groups ( $n = 8$  for each group) as follows: group 1, control (vehicle-treated); group 2, nifedipine (15 mg/kg body weight [b.w.]); group 3, telmisartan (5 mg/kg b.w.); group 4, enalapril (20 mg/kg b.w.); group 5, propranolol (1 mg/kg b.w.); and group 6, hydrochlorothiazide (12.5 mg/kg b.w.). Mice were housed in standard cages (4 mice per cage), and maintained at  $22^{\circ}\text{C} \pm 5^{\circ}\text{C}$  with constant humidity  $50\% \pm 10\%$  and a 12 hr light: 12 hr dark cycle. Animals had free access to autoclaved water and pellet diet. This experiment was conducted in accordance with institutional guidelines.

### Reagents

Nifedipine, enalapril maleate, telmisartan, propranolol and hydrochlorothiazide were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in dimethyl sulfoxide (DMSO).

### Bone mineral density and morphometry measurements

Bone mineral density (BMD) and morphometric analyses were performed on fixed proximal tibias by micro-computed tomography (micro-CT) using a SkyScan 1172 (SkyScan, Aartselaar, Belgium). BMD ( $\text{mg}/\text{cm}^3$ ) was measured 0.3-0.8 mm distal to the growth plate of tibial proximal ends and analyzed in 77 continuous sections. Samples were fixed in 3.7% formaldehyde (w/v) for approximately 24 hr, and scanned at 141 A/70 kVp for 590 ms through a 0.5 mm-thick filter. Description and nomenclatures followed guidelines for the assessment of bone microstructure with micro-CT analysis (8). To set the trabecular bone region range consistently across samples, data from each sam-

ple were resampled with CTAn software (SkyScan) after reconstructing scanned images with SkyScan reconstruction program NRecon software. Morphometric parameters, including total volume (TV,  $\mu\text{L}$ ), bone volume (BV,  $\mu\text{L}$ ), bone volume fraction (BV/TV, %), trabecular thickness (Tb. Th,  $\mu\text{m}$ ), trabecular number (Tb. N/mm) and connectivity density (Conn D/ $\mu\text{L}$ ) were measured with CTAn software. Morphometric quantification was determined 0.3-0.8 mm distal to the growth plate of tibial proximal ends.

### Statistical analyses

Statistical analyses were performed using SPSS software (version 18.0). Data are presented as mean values with standard deviation (SD). The effects of different antihypertensive drugs on BMD were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test (post hoc analysis). Differences between two groups were analyzed by the independent t-test or Mann-Whitney test (two-tailed) as appropriate. Correlations between parameters are presented as Spearman's correlation coefficient ( $r$ ).  $P$  values of  $< 0.05$  were considered significant.

## RESULTS

To study the effects of antihypertensive drugs on BMD in postmenopausal women, we used an ovariectomy mouse model of estrogen deficiency. Five weeks after bilateral ovariectomy, BMD was measured in tibial proximal ends of OVX mice (Fig. 1A). Except for thiazide, which significantly increased BMD ( $P = 0.048$ ), there were no significant differences in BMD in drug-treated OVX mice compared with vehicle-treated OVX mice (Fig. 1B).

To investigate differences in BMD based on the severity of estrogen deficiency, OVX mice were divided into two subgroups, those with normal uteri and those with atrophied uteri, since uterine atrophy in OVX mice associates with the degree of estrogen deficiency (9). When OVX mice with normal uteri

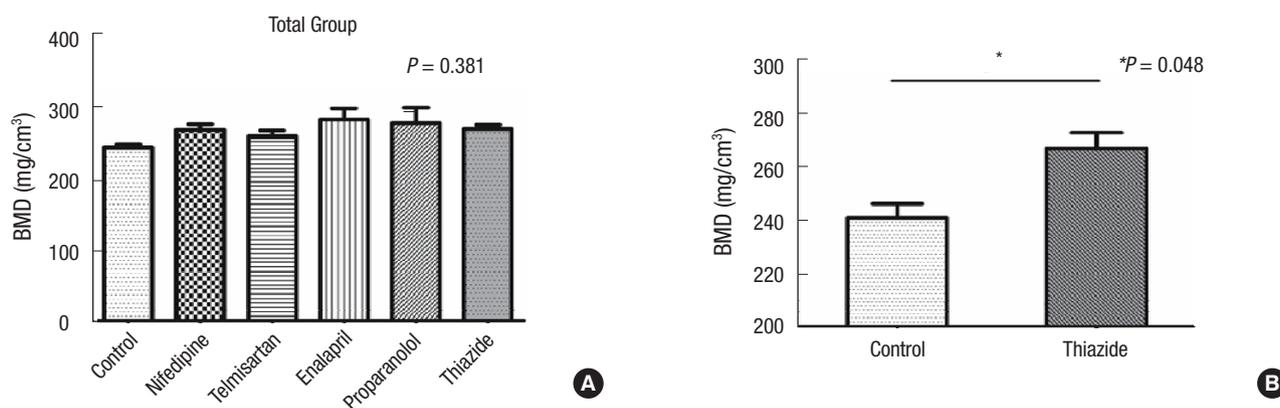
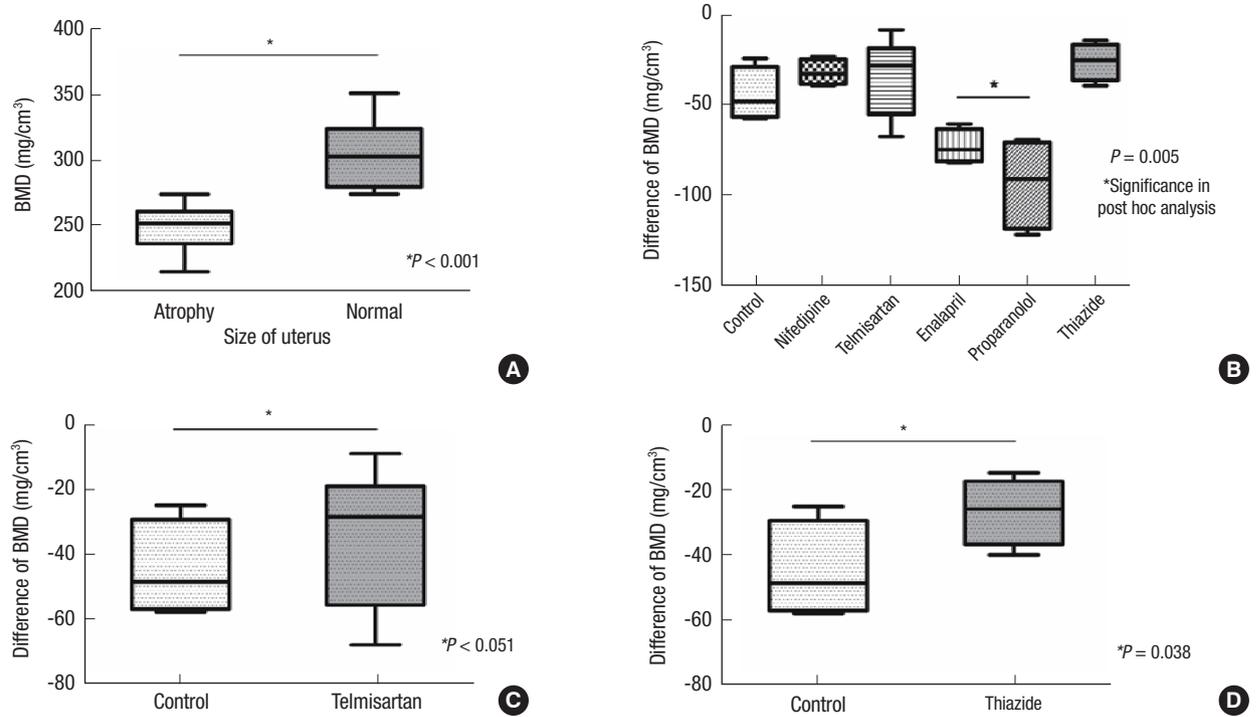
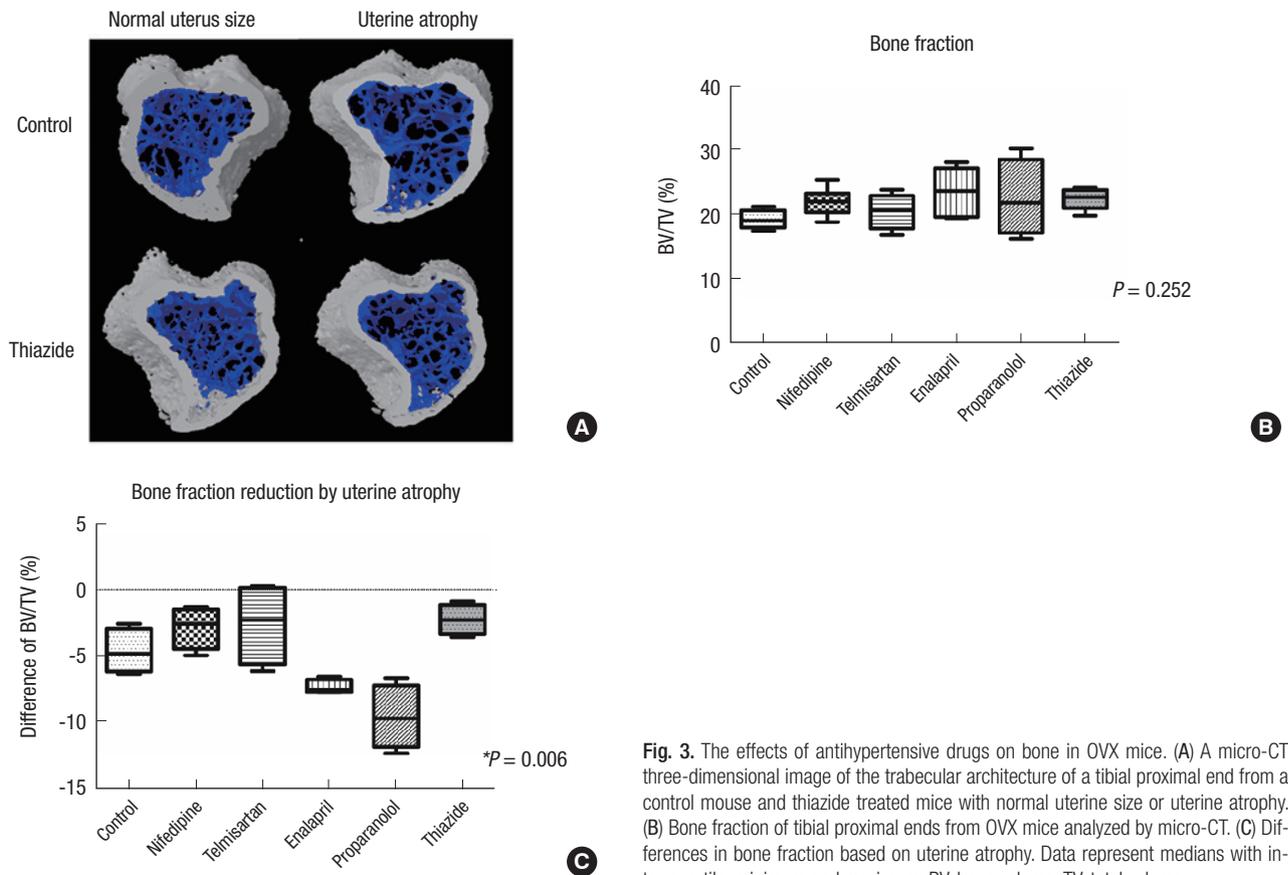


Fig. 1. The effects of antihypertensive drugs on BMD in OVX mice. (A) Comparison of BMD by multiple comparison. (B) Comparison of BMD in thiazide-treated mice vs control mice. Data represent means  $\pm$  SE. Each group consisted of  $n = 8$ .



**Fig. 2.** Comparison of BMD based on uterine atrophy. (A) BMD in mice with uterine atrophy (n=28) vs mice without uterine atrophy (n=16). (B) Differences in BMD based on uterine atrophy. BMD difference is the mean BMD of mice with atrophied uteri minus the mean BMD of mice with normal uteri. BMD in mice treated with telmisartan (C) or thiazide (D) vs vehicle-treated mice. Data represent medians with inter-quartile, minimum and maximum.



**Fig. 3.** The effects of antihypertensive drugs on bone in OVX mice. (A) A micro-CT three-dimensional image of the trabecular architecture of a tibial proximal end from a control mouse and thiazide treated mice with normal uterus size or uterine atrophy. (B) Bone fraction of tibial proximal ends from OVX mice analyzed by micro-CT. (C) Differences in bone fraction based on uterine atrophy. Data represent medians with inter-quartile, minimum and maximum. BV, bone volume; TV, total volume.

**Table 1.** The relationship between BMD and morphometric parameters obtained by micro-CT

| Group                 |                                 | TV ( $\mu\text{L}$ ) | BV ( $\mu\text{L}$ ) | BV/TV (%)          | Tb. N (/mm)        | Tb. Th ( $\mu\text{m}$ ) |
|-----------------------|---------------------------------|----------------------|----------------------|--------------------|--------------------|--------------------------|
| Total                 | BMD ( $\text{mg}/\text{cm}^3$ ) | -0.227               | 0.767 <sup>†</sup>   | 0.971 <sup>†</sup> | 0.938 <sup>†</sup> | 0.534 <sup>†</sup>       |
| Subgroup <sup>‡</sup> |                                 |                      |                      |                    |                    |                          |
| Normal                | BMD ( $\text{mg}/\text{cm}^3$ ) | -0.028               | 0.629*               | 0.958 <sup>†</sup> | 0.951 <sup>†</sup> | 0.545                    |
| Atrophy               | BMD ( $\text{mg}/\text{cm}^3$ ) | 0.258                | 0.793 <sup>†</sup>   | 0.920 <sup>†</sup> | 0.821 <sup>†</sup> | 0.503*                   |

\* $P < 0.05$ ; <sup>†</sup> $P < 0.01$  (ANOVA); <sup>‡</sup>Mice were grouped based on uterus size. BMD, bone mineral density; TV, total volume; BV, bone volume; Tb. N, trabecular number; Tb. Th, trabecular thickness.

were compared with mice with atrophied uteri, BMD decreased significantly ( $P < 0.001$ , Fig. 2A). The mean BMD loss in the atrophied uterine group was  $-21 \pm 7 \text{ mg}/\text{cm}^3$ .

The effects of antihypertensive drugs on BMD loss induced by severe estrogen deficiency were assessed by calculating the BMD difference, which is the mean BMD of mice with atrophied uteri minus the mean BMD of mice with normal uteri. This difference reflected bone loss affected by severe estrogen deficiency. BMD loss in each group was as follows: control,  $-45 \pm 15 \text{ mg}/\text{cm}^3$ ; nifedipine,  $-34 \pm 6 \text{ mg}/\text{cm}^3$ ; telmisartan,  $-32 \pm 21 \text{ mg}/\text{cm}^3$ ; enalapril,  $-73 \pm 7 \text{ mg}/\text{cm}^3$ ; propranolol,  $-93 \pm 25 \text{ mg}/\text{cm}^3$ ; and thiazide,  $-26 \pm 10 \text{ mg}/\text{cm}^3$ . When BMD was compared across all groups, there was a significant difference in multiple comparison ( $P = 0.005$ , Fig. 2B). By post hoc analysis, enalapril and propranolol increased BMD loss in mice with atrophied uteri compared with control mice. By two group analysis (i.e., vehicle-treated versus antihypertensive drug-treated), telmisartan affected bone loss moderately (Fig. 2C); however, thiazide significantly reduced bone loss by severe estrogen deficiency ( $P = 0.038$ , Fig. 2D).

Bone volume (BV) and trabecular thickness decreased in mice with uterine atrophy by micro-CT; however, BV and trabecular thickness increased in thiazide-treated mice with uterine atrophy compared with control mice (Fig. 3A). In OVX mice, BMD correlated with BV, bone fraction, trabecular number and trabecular thickness (Table 1). While there was no correlation between BMD and trabecular thickness in mice with normal uteri, an association was evident in mice with atrophied uteri ( $r = 0.503$ ,  $P = 0.012$ ). BMD associated most significantly with bone fraction ( $r = 0.971$ ,  $P < 0.001$ ).

Following multiple comparison of micro-CT parameters, only BV showed a significant difference between groups ( $P = 0.044$ ). No significant differences in bone fraction were observed ( $P = 0.252$ , Fig. 3B); however, bone fraction decreased in mice with atrophied uteri treated with nifedipine, telmisartan and thiazide (Fig. 3C). Enalapril and propranolol increased bone fraction in mice with atrophied uteri compared with normal mice ( $P = 0.006$ ).

## DISCUSSION

In the present study, we investigated the effects of antihypertensive drugs on BMD and bone morphometry in an animal model

of postmenopausal osteoporosis. Our data showed thiazide to decrease bone loss. When data were analyzed by the severity of estrogen deficiency, thiazide and telmisartan reduced bone loss in the severely deficient estrogen group. To our knowledge, this is the first report to investigate the effects of the most commonly prescribed antihypertensive drugs on bone function in OVX mice in a single study, as suggested in the hypertension guideline (10). Because osteoporosis can coexist with hypertension, the choice of antihypertensive drugs could be influenced by their potential effect on bone and fracture risk, especially in postmenopausal women.

It is uncertain whether antihypertensive drugs affect bone directly or indirectly. Epidemiological studies report certain antihypertensive drugs such as thiazide diuretics to lower fracture risk in postmenopausal women, suggesting that antihypertensive drugs affect bone positively. For other antihypertensive drugs, the effects on bone are controversial (11). In epidemiological studies, thiazide increases BMD in postmenopausal women (12-14), and these results are consistent with our findings. Thiazide protects against age-related bone loss and osteoporotic fractures (6, 15). Cross-sectional and longitudinal observational studies show that thiazide diuretics increased BMD (12, 15); however, randomized controlled studies could not confirm the increase in BMD by thiazide (3, 14, 16). In other studies, thiazide significantly reduced all types of fractures (6, 12), suggesting that thiazide reduces sodium reabsorption and promotes calcium reabsorption. Indeed, normal calcium homeostasis can affect BMD positively (17). Thiazide also acts on bone cells directly by decreasing osteoclast differentiation (18).

The effects of beta-blocker and calcium channel blockers (CCB) on BMD in humans are controversial, with some drugs reducing fracture risk and others not affecting fracture risk (4, 13, 19-21). In OVX mice, propranolol did not reduce BMD loss in femurs (22). The results of this experiment are consistent with the earlier one. In our study, BMD was unaffected by CCB. In the case of CCB, use of non-dihydropyridine drugs was associated with a fracture risk reduction than use of dihydropyridine drugs (4). Therefore, additional studies are needed to understand the effects of CCB on bone.

Previous studies show bone to be under the influence of the rennin-angiotensin-aldosterone system, with angiotensin II affecting bone homeostasis adversely. Inhibition of angiotensin II is beneficial for bone function. Treatment of angiotensin re-

ceptor blocker (ARB), olmesartan, attenuated the ovariectomy-induced decrease in BMD (23). In a recent cohort study, ARB reduced the risk of fracture in elderly women (6). Although angiotensin converting enzyme (ACE) inhibitor use was reported with higher BMD in elderly women, captopril did not increase BMD in OVX mice (24). Enalapril also did not influence bone metabolism and BMD (25). Our data suggest that ARB plays a protective role in severely deficient estrogen mice but that BMD is unaffected by an ACE inhibitor.

Our results showed that thiazide and telmisartan reduced BMD loss in OVX mice severely deficient in estrogen. These results were consistent with a recent epidemiological study where Solomon et al. reported a reduction in fracture risk by ARB and thiazide in elderly women (6). These findings suggest that ARB and thiazide are beneficial to elderly women with osteoporosis and hypertension. Because of the increasing population of elderly people with osteoporosis, the need for preventive therapy is a public health priority. Our findings are likely to help health-care professionals prescribe antihypertensive drugs without further affecting bone function, especially in postmenopausal hypertensive women with osteoporosis.

In summary, thiazide positively affects BMD in OVX mice. Thiazide and telmisartan also reduced bone loss in severely deficient estrogen mice. Thiazide and telmisartan may be front-line therapy for hypertensive patients who are at an increased risk for osteoporosis or already have postmenopausal osteoporosis. Long-term prospective randomized studies will need to be performed to further assess the effects of antihypertensive drugs on postmenopausal osteoporosis.

## DISCLOSURE

All authors state that they have no conflicts of interest.

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