



RESEARCH ARTICLE

Effects of Estradiol Alone and Estradiol combined with Trivalent Chromium on Bone Metabolism in Ovariectomized Rats

Lin-Jing Kong¹, Tien-Huan Hsu^{1,2}, Hsein-Chi Wang^{1,2}, Kuan-Sheng Chen^{1,2} and Wei-Ming Lee^{1,2*}

¹Department of Veterinary Medicine; ²Veterinary Medical Teaching Hospital, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, R.O.C. 145, Xingda Rd., Taichung city, Taiwan, R.O.C.

*Corresponding author: wmlee@dragon.nchu.edu.tw

ARTICLE HISTORY (18-245)

Received: July 18, 2018
Revised: November 29, 2018
Accepted: December 01, 2018
Published online: January 24, 2019

Key words:

Calcium
Estradiol
Osteocalcin
Parathyroid hormone
Phosphorus
Trivalent chromium

ABSTRACT

Estrogen deprivation is associated with an increased morbidity due to osteoporosis and other metabolic disorders such as diabetes and obesity. Cr³⁺ has been shown to be beneficial with respect to carbohydrate, protein, and lipid metabolism. Therefore, the aim of this study was to investigate the effects of estradiol (E2) alone and E2 combined with Cr³⁺ on bone metabolism in ovariectomized rats. Thirty-six 8-week-old female Sprague-Dawley rats were randomly divided into 6 groups after undergoing ovariectomy. Body weight and plasma concentrations of calcium, phosphorus, parathyroid hormone, and osteocalcin were measured weekly. Both femurs and both tibiae were dissected to measure the percentage of the trabecular bone (BV/TV%). The final VarBW% in the control group was significantly higher ($P < 0.05$) than those in the other groups. The BV/TV% of the tibia and that of the femur in the HE2 group were significantly higher ($P < 0.05$) than those in the other groups. However, the percentage of the trabecular bone in the proximal metaphysis of the tibia in the HE2 group was significantly decreased ($P < 0.05$) after Cr supplementation. The plasma concentration of calcium in the low-dose estradiol groups was significantly higher ($P < 0.05$) than that in the high-dose estradiol groups. The plasma concentration of phosphorus fluctuated in different groups. The plasma concentration of phosphorus in the control group was the lowest ($P < 0.01$) among all experimental groups. The calcium-phosphate product in the control group was the lowest ($P < 0.01$) among all groups. The concentration of PTH in the HE2 group was significantly higher ($P < 0.05$) than that in the control group, but there was no significant statistical difference ($P > 0.05$) among the Cr, LE2, LE2+Cr, and HE2+Cr groups with respect to the concentration of PTH.

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To Cite This Article: Kong LJ, Hsu TH, Wang HC, Chen KS and Lee WM, 2019. Effects of estradiol alone and estradiol combined with trivalent chromium on bone metabolism in ovariectomized rats. *Pak Vet J*, 39(2): 278-284. <http://dx.doi.org/10.29261/pakvetj/2019.005>

INTRODUCTION

Bone metabolism is a complicated process that involves the metabolism of calcium, phosphorus, and collagen. At the cellular level, bone formation is controlled by osteoblasts, whereas bone resorption is controlled by osteoclasts, which complete the bone remodeling cycle during the process of bone metabolism (Zhu *et al.*, 2018). Estrogens act directly on bone cells by binding on the estrogen receptors α and β that are present on osteoblasts, osteocytes, and osteoclasts (Oursler *et al.*, 1991). By inhibiting osteoblast and osteocyte apoptosis (Huang *et al.*, 2018) and by increasing the number and function of the osteoblasts, estrogens favor bone

formation. In contrast, estrogens inhibit bone resorption by suppressing osteoclast formation and activity as well as by inducing osteoclast apoptosis (Khosla *et al.*, 2012; Souza Faloni *et al.*, 2012); therefore, estrogen plays a role as bone protector. The rapid decrease in estrogen is believed to be the main cause of bone loss in postmenopausal women, and this can finally result in osteoporosis (Khosla *et al.*, 2012). The traditional treatment for postmenopausal osteoporosis is hormone replacement therapy (HRT), which comprises estrogen supplementation. Estradiol in the dosage of 2 mg/kg per person daily has been proved to be efficient in preventing postmenopausal osteoporosis in the past. However, long-term administration of estradiol at this dosage was

observed to be associated with an increased risk of mammary tumors. To avoid this adverse effect of high-dose estradiol, a lower dosage is currently recommended to maintain the estradiol level in postmenopausal women.

Postmenopausal women also have a high morbidity due to metabolic disorders such as diabetes and obesity in addition to osteoporosis. In the past, it was believed that obesity acts as a protective factor against osteoporosis because of its positive loading effect on bone mineral density (BMD). However, some recent studies have suggested that high levels of fat mass can be a risk factor for osteoporosis and fragility fractures (Greco *et al.*, 2010; Kim, *et al.*, 2010; Compston *et al.*, 2014); this effect may be mediated by the loading forces or calciotropic hormones (Cummings *et al.*, 1985; Reid *et al.*, 1993). The serum levels of trivalent chromium in obese humans are lower than those in non-obese humans, which is similar to the condition in diabetes (Sundararaman *et al.*, 2012). In addition, supplementation of chromium can increase serum levels of DHEA-sulfate, which plays a physiological role in maintaining bone mineral density (Chen *et al.*, 2015). Consequently, it can be speculated that trivalent chromium partially participates in bone metabolism. However, the effects of supplementation with Cr³⁺ alone or Cr³⁺ combined with HRT on bone metabolism remain to be investigated. Therefore, this study was aimed to investigate the effects of E2 alone and E2 combined with trivalent chromium on bone metabolism in ovariosterectomized rats.

MATERIALS AND METHODS

Animals: Thirty-six 8-week-old female Sprague-Dawley (SD) rats weighing 250-300 g were obtained from Biolasco Taiwan Co., Ltd. Two rats were housed in each cage and maintained under controlled conditions (temperature, 22-24°C; humidity, 55-60%; light hours, 12; dark hours, 12). Food and water were administered *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (IACUC NO. 104-033).

Experimental design: After 7 days of acclimation to the housing conditions, all animals underwent ovariectomy and were randomized into six groups (n=6 per group). Trivalent chromium was available as powder capsule (0.15 g per capsule) (Maxluck Biotech Corp., Taipei) containing chromium trichloride (100 µg), whey protein, and lactoferrin. Chromium chloride (CrCl₃·6H₂O) is composed of 19.5% Cr. The dosage was based on that used in a previous study by Sahin *et al.* (2011). Trivalent chromium (80 µg/kg) was administered. The trivalent chromium powder was mixed with deionized distilled water (DDW). The concentration of the CrCl₃ solution was 20 µg/ml, and the rats were force-fed a dose of 4 ml/kg (equal to 80 µg/kg of CrCl₃).

Estradiol was administered in the form of estradiol valerate (2 mg per pill) (Synmosa Biopharma Corp., Taipei). The lower dosage of estradiol was based on the dosage used in a previous study (Zhao *et al.*, 2011) in which a daily administration of 0.025 mg/kg of E2 to ovariectomized rats produced a BMD that was similar to that in sham-operated rats. The higher dosage of estradiol

was modified from the standard E2 dosage in the traditional HRT (2 mg/day per person) for postmenopausal osteoporosis in humans (Gambacciani and Levancini, 2014).

After 7 days of recuperation, the animals were force-fed orally daily the following treatments: control group – deionized distilled water (4 ml/kg); Cr group – trivalent chromium (80 µg/kg); LE2 group – estradiol (0.025 mg/kg); LE2+Cr group – estradiol (0.025 mg/kg) and trivalent chromium (80 µg/kg); HE2 group – estradiol (0.5 mg/kg); and HE2+Cr group – estradiol (0.5 mg/kg) and trivalent chromium (80 µg/kg).

All animals were killed 15 weeks after the operation. Blood samples were collected by heart punctures. Both femurs and both tibias were collected and fixed in 4% neutral-buffered formalin.

Histology and histomorphometric evaluations: After the fixation in 4% neutral-buffered formalin for 24 hours, the samples of the femurs and the tibias were decalcified using Morse's solution (20% methanoic acid and 10% sodium citrate) at room temperature for 22 hours. Then, bone samples were dehydrated in graded ethanol and embedded in paraffin. The bone samples were then cut sagittally into 2-µm-thick sections using a Leica RM2245 Semi-Automated Rotary Microtome (Leica SpA, Milan, Italy) followed by a hematoxylin and eosin staining. Images were obtained using an Olympus BX51 microscope (OLYMPUS corp., Tokyo, Japan) equipped with a Canon DS126201 camera (Canon inc., Japan). Histomorphometric analysis was performed using Image-Pro Plus, a two-dimensional image analysis software.

Histomorphometric evaluations of the femoral and tibial sagittal sections were performed in a relatively constant area (10±1 mm²) of the trabecular bone that was selected between the medial borders of cortex and a line parallel to the cartilaginous plate (broken line in Fig. 1). The percentage of the trabecular bone (BV/TV%), the ratio between the trabecular bone area (BV) and the total area (TV) in the proximal metaphysis of the tibia and the distal metaphysis of the femur, was determined.

Determination of the plasma concentrations of calcium and inorganic phosphorus: The plasma levels of total calcium and inorganic phosphorus were determined by colorimetry using commercial test kits (Roche Diagnostic, Milan, Italy). The Roche Hitachi 717 Chemistry Analyzer (Roche Diagnostic) was used.

Determination of the plasma concentrations of parathyroid hormone (PTH) and osteocalcin (OC): The plasma levels of parathyroid hormone (PTH) and osteocalcin were measured using Milliplex MAP Rat Bone Panel 2 Magnetic Bead Kit (RBN2MAG-31K; Millipore Corp., Billerica, MA, USA); the Magpix immunobead assay technology with xPONENT® software (Luminex Corp., Austin, TX, USA) was used.

Statistical analysis: Statistical analysis was performed using IBM SPSS software (version 21.0, SPSS Inc., Chicago, IL). The relationship between groups and the outcome was tested using the Tukey's test. A p value that was less than 0.05 indicated statistical significance.

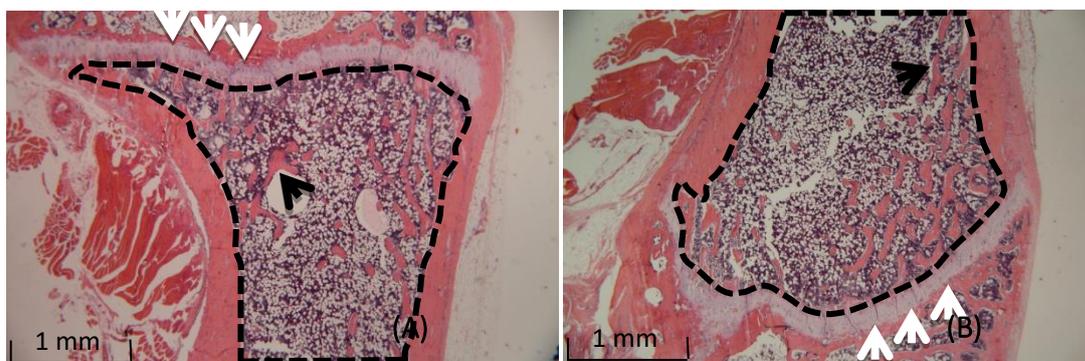


Fig. 1: Histological sections (2- μ m thick) taken from the control group showing the regions in which the histomorphometrical analyzes were performed: (A) sagittal section of the proximal metaphysis of the tibia; (B) sagittal section of the distal metaphysis of the femur. The broken lines indicate the areas in which evaluations were performed. Trabecular bone is indicated by black arrows. Cartilaginous plate is indicated by white arrows.

RESULTS

The body weight increased in all experimental groups during the experiment. The final VarBW% in the control group was significantly higher ($P < 0.05$) than the final VarBW% in the other experimental groups. There was no significant difference among the Cr, LE2, LE2+Cr, HE2, and HE2+Cr groups with respect to the final VarBW% (Fig. 2). The percentage of the trabecular bone in the proximal metaphysis of the tibia (Fig. 3A) and the femur (Fig. 3B) was increased in the Cr group, and the increase tended to be higher after estradiol supplementation. The BV/TV% of the tibia and that of the femur in the HE2 group were significantly higher ($P < 0.05$) than those in the other groups. However, the percentage of the trabecular bone in the proximal metaphysis of the tibia in the HE2 group was significantly decreased ($P < 0.05$) after Cr supplementation (Fig. 4A), although the percentage of the trabecular bone in the proximal metaphysis of the femur was not significantly decreased (Fig. 4B).

In the non-Cr groups, there was a statistically significant strong negative correlation ($r = -0.639$, $P < 0.05$) between the BV/TV% of the proximal tibial metaphysis and the VarBW and a statistically significant moderate negative correlation ($r = -0.531$, $P < 0.05$) between the BV/TV% of distal femoral metaphysis and the VarBW% (Fig. 5).

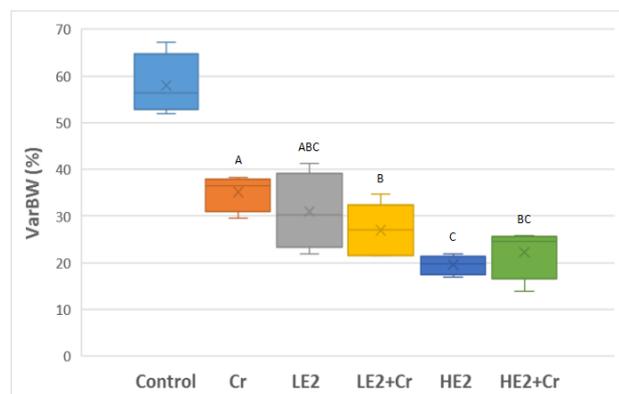


Fig. 2: Box plot of the final VarBW (%) in the six groups after 14 weeks of different treatments. The plot displays the median (line), mean (cross), interquartile range (box), and extreme values (whiskers) for each group. The same alphabet means that there were no significant differences among the groups.

The plasma concentration of calcium in all experimental groups remained in a relatively steady state; the lowest mean was 9.17 mg/dL in the HE2 group, and the highest mean was 10.12 mg/dL in the LE2+Cr group. A significant difference ($P < 0.05$) with respect to the plasma concentration of calcium was observed between the low-dose estradiol groups and high-dose estradiol groups.

The plasma concentration of calcium in the low-dose estradiol groups was significantly higher ($P < 0.05$) than that in the high-dose estradiol groups (Fig. 6A). The plasma concentration of phosphorus fluctuated in different groups (Fig. 6B). The plasma concentration of phosphorus in the control group was the lowest ($P < 0.01$) among all experimental groups.

The calcium-phosphate product in the control group was the lowest ($P < 0.01$) among all groups (Fig. 6C). The level of PTH in the control group was 32.91 pg/mL, which was significantly lower ($P < 0.05$) than the levels in other groups. The concentration of PTH in the HE2 group was 442.86 pg/mL, which is significantly higher ($P < 0.05$) than that in the control group, but there was no significant statistical difference ($P > 0.05$) among the Cr, LE2, LE2+Cr, and HE2+Cr groups with respect to the concentration of PTH (Fig. 7A). There was no significant difference among the control, Cr, LE2, LE2+Cr, HE2 and HE2+Cr groups with respect to the concentration of PTH (Fig. 7B).

DISCUSSION

In the non-Cr groups, there was a negative relationship between the VarBW% and the dosage of estradiol. This is because estrogen plays an essential role in regulating the metabolism and energy balance and this includes the regulation of growth hormone (Fernández-Pérez *et al.*, 2014), total cholesterol, glucose tolerance, and insulin response (Nelson *et al.*, 1990). The significant difference between the control group and the E2 groups showed that even a low-dose supplement of estradiol can restore the balance in energy metabolism and control weight.

With respect to BV/TV%, there was a positive correlation between estradiol dosage and BV/TV% in both the proximal metaphysis of the tibia and the distal metaphysis of the femur in the non-Cr groups; this confirmed that the estrogenic effect on the bone is dose-dependent. This effect is because estrogens act directly on bone cells by binding on the estrogen receptors α and β

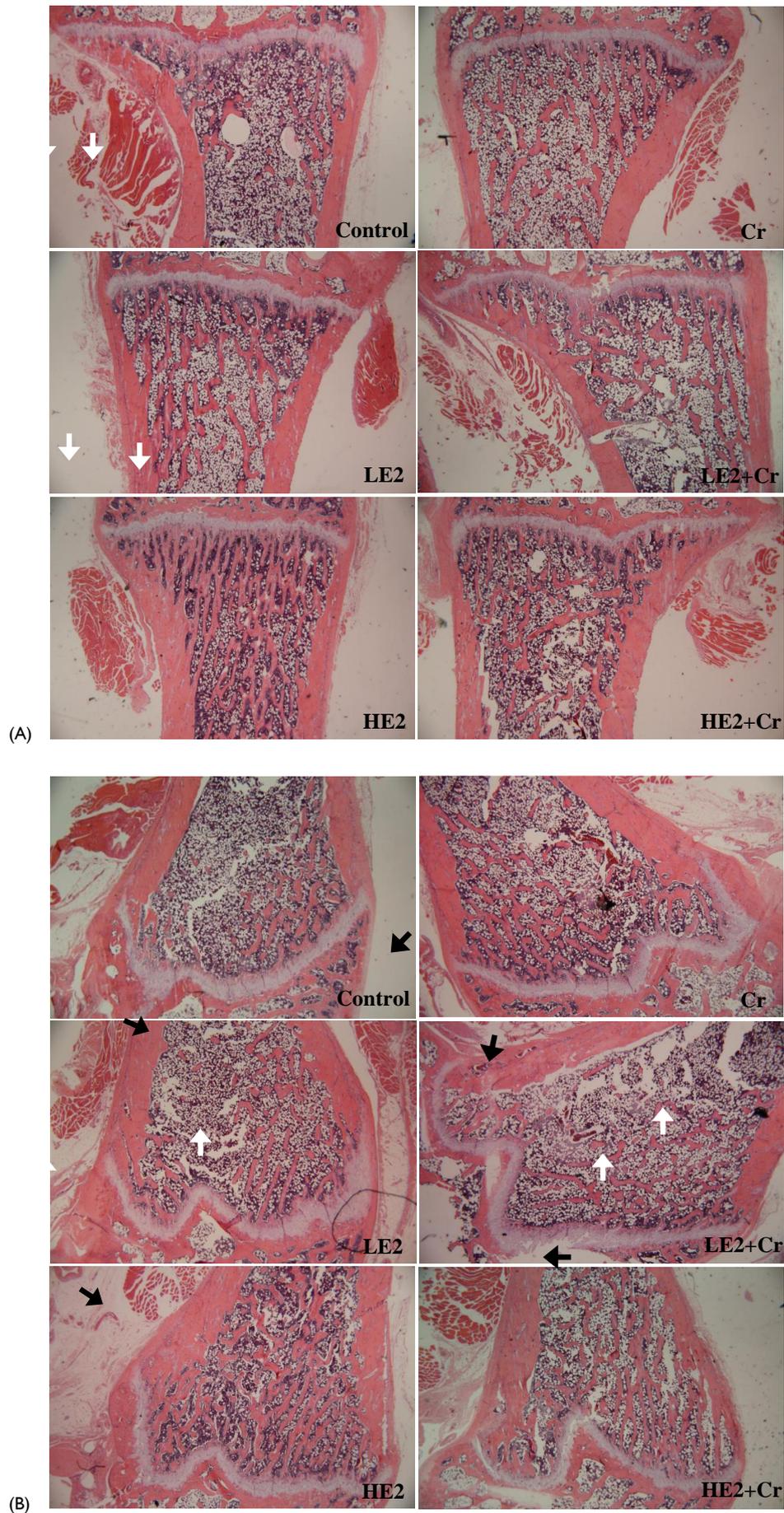


Fig. 3: Histological sections showing the trabecular bone structure in the six groups after 14 weeks of different treatments. (A) Sagittal sections of the proximal metaphysis of the tibia; (B) sagittal sections of the distal metaphysis of the femur. Bony tissue is indicated by the black arrows. The cartilaginous plate is indicated by the white arrows.

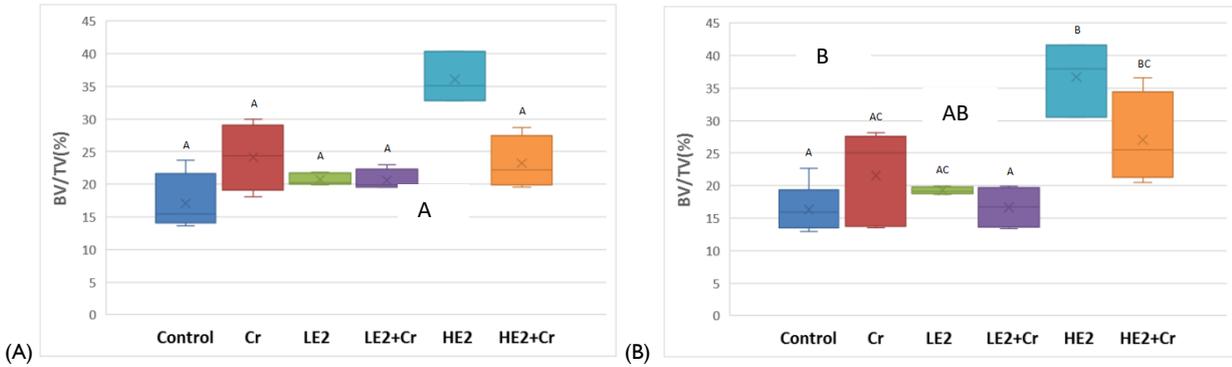


Fig. 4: Box plot of BV/TV% in the (A) proximal metaphysis of the tibia and (B) distal metaphysis of the femur in the six groups after 14 weeks of different treatments. The plot displays the median (line), mean (cross), interquartile range (box), and extreme values (whiskers) for each group. The same alphabet means there were no significant differences among the groups.

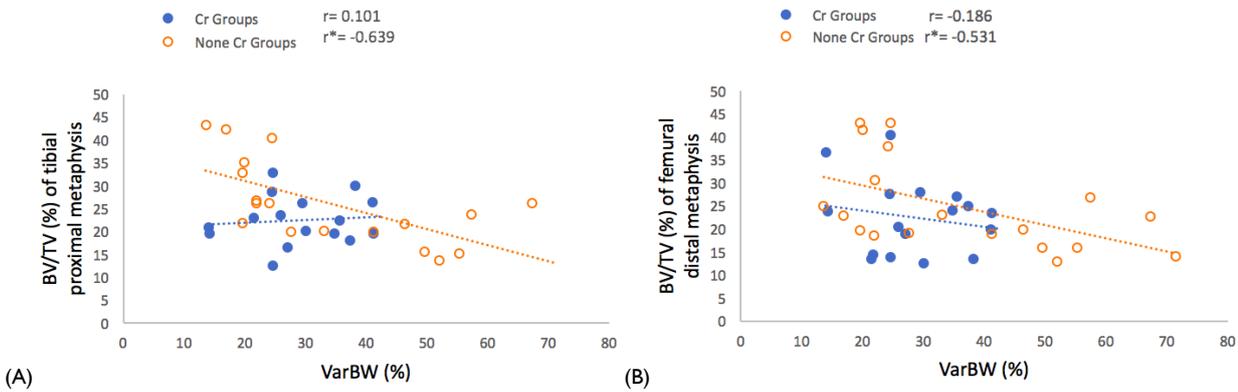


Fig. 5: Scatter plots of BV/TV% in proximal tibial metaphysis (A) and distal femoral metaphysis (B) against VarBW in all groups. The data were divided into the Cr groups (represented by solid blue points) and non-Cr groups (represented by orange hollow points). The dotted lines through the data and r values (the Pearson product-moment correlation coefficient) represent the respective regressions across Cr groups and non-Cr groups (n = 15-19 per site). *, P<0.05; **, P<0.01; ***, P<0.001.

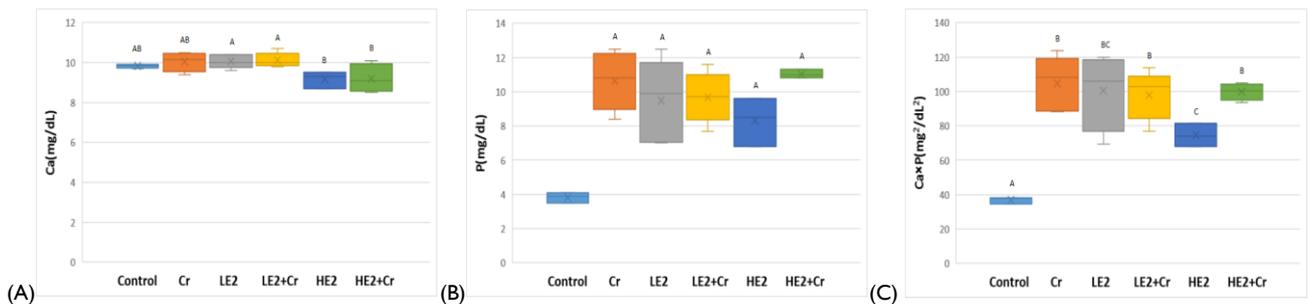


Fig. 6: Box plot of calcium levels (A), phosphorus levels (B), and product of calcium and phosphorus (C) in the six experimental animal groups after 14 weeks of different treatments. The plot displays the median (line), mean (cross), interquartile range (box), and extreme values (whiskers) for each group. The same alphabet means that there were no significant differences among the groups.

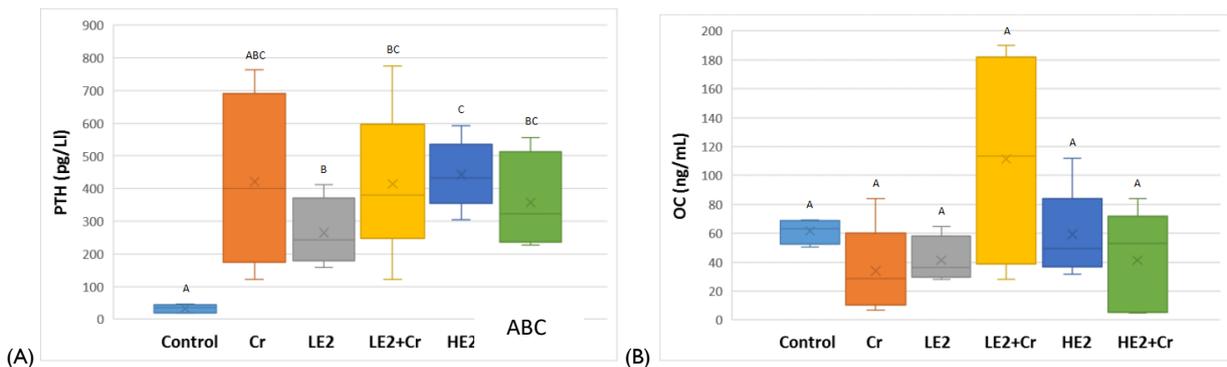


Fig. 7: Box plot of the concentrations of PTH (A) and OC (B) in the six groups after 14 weeks of different treatments. The plot displays the median (line), mean (cross), interquartile range (box), and extreme values (whiskers) for each group. The same alphabet means that there were no significant differences among the groups.

that are present on osteoblasts, osteocytes, and osteoclasts (Oursler *et al.*, 1991). By inhibiting osteoblast and osteocyte apoptosis (Huang *et al.*, 2018) and by increasing the number and function of the osteoblasts, estrogens favor bone formation. In contrast, estrogens inhibit bone resorption by suppressing osteoclast formation and activity as well as by inducing osteoclast apoptosis (Khosla *et al.*, 2012; Souza Faroni *et al.*, 2012). Although there was no significant difference of BV/TV% between the control group and the LE2 group, the bone-protective effect of administration of a high-dose (500 µg/kg/day) of estradiol was obvious. This observation was also consistent with effect observed with the use of the recommended dosage of estradiol (2 mg of oral estradiol per day per person) to prevent postmenopausal bone loss in postmenopausal women (Gambacciani and Levancini 2014).

The results of the current study showed that the plasma concentration of PTH was also positively correlated with the dosage of estradiol in the non-Cr groups, without significant difference between each two groups. It is well known that the synthesis and release of PTH are mainly regulated by ionized calcium and sometimes by calcitriol and phosphate (Tryfonidou *et al.*, 2010). However, estrogen can inhibit PTH-stimulated osteoclast-like cell formation; this effect is probably mediated through blockage of the cAMP-dependent protein kinase pathway (Kaji *et al.*, 1996). Thus, the more the estradiol suppresses the PTH function, the higher the PTH concentration is in the circulation. The higher levels of PTH may modify the bone remodeling process and the calcium metabolism. As discussed before, a high-dose of E2 causes BV/TV% to increase in a process that is dominated by bone formation. As a result, plasma concentration of calcium originating from bone resorption can be reduced; this is what was confirmed by the relatively low plasma calcium concentration in the estradiol group. The calcium concentration in this case lowered the set-point and triggered the secretion of PTH; thus, higher levels of PTH were observed in the estradiol groups. This effect may be explained by the fact that the catabolic effect of a higher concentration of PTH was inferior to the anabolic effect of estradiol; therefore, the BMD was increased after estradiol administration. In contrast, the plasma concentration of calcium in the LE2 group, although the difference was not statistically significant, was slightly higher than that in the HE2 group. This was probably due to the negative feedback of PTH on calcium concentration; it is possible that the relatively low calcium concentration first stimulated the production of PTH. However, the level of PTH was only able to enhance the reabsorption of calcium in kidney, but it was not enough to induce a catabolic action on the bone (Tryfonidou *et al.*, 2010). In comparison to the control group, other groups, including the Cr group, had significantly increased levels of plasma phosphorus. This indicates that Cr had the same effect on phosphorus level as estradiol did. However, the exact mechanism of the interaction between CR and estradiol should be studied further. The plasma calcium-phosphate product, which is used to predict the likelihood of bone formation or resorption. It was believed in the past that a high level of calcium-phosphate product promotes the deposition of

calcium phosphate, whereas a low level inhibits mineralization and even result in bone absorption. The apparently low calcium-phosphate product in the control group, compared with product in the estradiol- and Cr-treated groups, suggests that estradiol and Cr performed the role of bone protectors.

OC, which is secreted by mature osteoblasts, is recognized as a marker for later bone formation (Hu *et al.*, 2005). Increased levels of osteocalcin can be detected in diseases with accelerated bone turnover, particularly those associated with rapid bone loss (Rahnama, 2015). There was no significant difference among the control, Cr, LE2, LE2+Cr, HE2, HE2+Cr groups with respect to the concentration of OC. This may be because the rats had already passed the period of rapid bone turnover and were undergoing the same degree of bone formation when they were killed.

VarBW% was significantly higher in the control group. Cr and estradiol can decrease the levels of VarBW%. The difference in the VarBW% among the non-Cr groups was reduced with the supplementation of Cr³⁺, which implies chromium may modify estradiol's function. In contrast, supplementation with Cr³⁺ alone inhibited weight gain, an effect similar to that of low-dose E2. These seemingly contradictory results can be explained by the similar mechanisms of action of estradiol and chromium in regulating energy metabolism; the two probably affect the same pathway. E2 can enhance pancreatic β-cell function and stimulate insulin synthesis by acting on estrogen receptors (Tiano, *et al.*, 2015), and Cr³⁺ can regulate carbohydrate metabolism by increasing insulin sensitivity (Al-Qatati *et al.*, 2012). Both of them affect lipid metabolism and can prevent weight gain (Kuryl and Debski, 2008).

Besides modifying energy metabolism, chromium also modifies estradiol's dose-related effect on bone mineral density. With the exception of the combination of chromium and high-dose estradiol, which had bone-protective effect on the distal metaphysis of the femur, the treatments did not maintain the trabecular bone density in either the proximal metaphysis of the tibia or the distal metaphysis of the femur. Administration of chromium alone resulted in a higher BV/TV% compared with treatment administered in the control group; however, this difference was not statistically significant. This suggests that a supplement of chromium can be helpful in preventing bone loss in individuals with estrogen deficiency. However, the supplementation of chromium sulfate in male rats has deleterious effects on the skeleton and decreases the bone density (Bieńko *et al.*, 2017).

The concentrations of PTH in the HE2 and HE2+Cr groups were significantly higher than those in the other groups. Supplementation of chromium may not modify the effect of estradiol on PTH. However, Cr supplementation alone can increase the level of PTH, although the increase is not statistically significant. This suggests that trivalent chromium can modify the metabolism, but not the regulation, of estradiol. This effect may be mediated by insulin, an inhibitor of PTH function (Iida-Klein and Hahn, 1991), the level of which was increased by E2 and decreased by chromium (Al-Qatati, *et al.*, 2012; Tiano, *et al.*, 2015).

Conclusions: Based on the results of this study, we conclude that the effect of Cr³⁺ on BMD was similar to that of low-dose estradiol. The effect of estradiol on BMD was dose dependent and can be modified by Cr³⁺. Thus, we suggested that Cr³⁺ can be used in estrogen-deprived animals to increase BMD.

Authors contribution: LJ and WM conceived and designed the study. TH, HC, and KS executed the experiment and analyzed the sera and tissue samples. LJ analyzed the data.

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