Original Article

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DHEA prevents bone loss by suppressing the expansion of CD4$^+$ T cells and TNFa production in the OVX-mouse model for postmenopausal osteoporosis

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Summary **Recent studies have suggested that dehydroepiandrosterone (DHEA) might serve as a form of immunomodulatory therapy for postmenopausal osteoporosis (PMO). The current study investigated the effects of DHEA administration on ovariectomy (OVX)-induced bone loss and its corresponding immunological changes. Adult OVX mice were treated with DHEA or 17-β-estradiol (E2) for 12 weeks, with or without the aromatase inhibitor letrozole. DHEA improved bone mass after OVX and displayed action like that of E2 with regard to decreasing osteoclast-related parameters. DHEA also suppressed an OVX-induced increase in CD4$^+$ T cell subsets and TNF-α production. However, DHEA elevated serum E2 levels to a lesser extent than E2. Although letrozole decreased serum E2 levels in OVX mice treated with DHEA, it did not alter DHEA's effects on corresponding immunological changes due to OVX. In conclusion, DHEA may prevent bone loss by suppressing the OVX-induced expansion of CD4$^+$ T cells and TNF-α production in mice, independent of E2.**

Keywords: Dehydroepiandrosterone, postmenopausal osteoporosis, CD4$^+$ T cells, TNF-α

1. Introduction

Several epidemiologic trials have revealed a strong inverse correlation between levels of sex steroid hormones, such as dehydroepiandrosterone (DHEA) and estrogen, and the occurrence of several well-known geriatric syndromes, such as osteoporosis and cardiovascular disease (*1,2*). DHEA and its sulfate ester, DHEA sulfate (DHEAS), are the most abundant circulating hormones produced by the adrenal gland, and levels of these hormones decrease with age, sometimes decreasing to 10-20% of the levels found in young individuals (*3*). This age-related decrease has been called the "adrenopause" phenomenon. The decrease in DHEA in the elderly is more marked than the decrease in other hormones.

DHEA and DHEAS are both pre-hormones (*4*). However, only DHEA can be converted into more potent androgens and estrogens in peripheral tissues, while DHEAS is maintained as a circulating stock (*3*). In premenopausal women, 50-75% of estrogens, and the majority of androgens, are produced from DHEA, while almost all androgens and estrogens are synthesized from it during postmenopause (*5*). Postmenopausal osteoporosis (PMO) is a well-known estrogen deficiency-induced geriatric syndrome and is also a worldwide health problem. It manifests as a progressive systemic skeletal disorder characterized by reduced bone mass that leads to increased bone fragility and fracture risk. DHEA levels may be associated with bone marker levels and DHEA treatment might increase

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bone mass (*6,7*). An *in vitro* analysis of primary human osteoblasts indicated that aromatase converts DHEA into estrogen (*8*). Thus, the role of DHEA in regulating bone metabolism in PMO has garnered increasing attention.

Many studies have suggested a beneficial effect of DHEA administration on preventing trabecular bone mineral density (BMD) loss in women with an estrogen deficiency (*8,9*). The current authors have conducted a number of previous studies on the effect of DHEA on bone. DHEA improved bone mass in ovariectomized (OVX) mice and appeared to have greater potential clinical value for the prophylactic and therapeutic treatment of PMO than estrogen (E2) (*10*); DHEA improved murine osteoblast growth *via* the mitogen-activated protein kinase (MAPK) signaling pathway, independent of either androgen receptors or estrogen receptors (*11*); DHEA protected OVX rabbits from atherosclerosis by alleviating inflammatory injury in endothelial cells (*12*); and DHEA promoted osteoblast differentiation by regulating the expression of osteoblast-related genes and $Foxp3^+$ regulatory T cells in mice (*13*). However, what is still unclear is how DHEA affects other immune cells that may play a role in bone metabolism and whether DHEA regulates the immune cells only through its hormonal end products or as a result of its direct action through certain receptors.

Recent studies have reported an association between pathogenetic changes due to PMO and disturbances in the endocrine-immune network. Some researchers have contended that PMO can be classified as an inflammatory condition (*14*). Skeletal system homeostasis is greatly influenced by endocrine as well as immune factors (*15*). After menopause, diminished ovarian function has a deleterious effect on bone metabolism. Estrogen has been also known to regulate the functions of the immune system and T cells. Increased proliferation of activated T cells, and their longer lifespan, in OVX mice results in an expansion of the T cell pool in bone marrow (*16*). Bone loss has been induced in nude mice by OVX and it has been restored by transferring wild-type T cells into nude mice (*17*), and depletion of T cells by treatment with anti-CD4/CD8 antibodies protects mice from bone loss due to OVX (*18*). These findings indicate that T cells may play an essential role in the development of PMO. Immunophenotypical analyses of peripheral blood lymphocytes in osteoporotic patients suggested an increase in several subsets of T lymphocytes, including $CD4^+$ T cells (*19*). OVX may also up-regulate TNF-α production, which is the most potent stimulator of osteoclastogenesis when estrogen is depleted (*20*). Moreover, $CD4^+$ T cells secrete TNF-α and receptor activator for nuclear factor-κ B ligand (RANKL), which is an osteoclastogenic mediator (*21*). Therefore, enhancing the endocrine-immune network appears to positively modulate bone metabolism in PMO.

Various positive immunologic actions of DHEA have been noted in various human diseases and animal models (*22-24*). These findings, coupled with the fact that DHEA promotes additional factors that modulate BMD in postmenopausal women, emphasizes the need for more information concerning the potential beneficial impact of DHEA on the interplay between the immune and skeletal systems. The current study investigated the effects of DHEA administration, with or without an aromatase inhibitor, on bone morphometry, E2 levels, $CD4^+$ T cell expansion, and TNF-α production in OVX mice in order to determine the potential value of DHEA as a therapy for PMO and its mechanism of action.

2. Materials and Methods

2.1. *Reagents and chemicals*

Mouse lymphocytes from bone marrow and the spleen were cultured in complete RPMI-1640 medium (Wisent Inc., St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS), penicillin (500 U/mL), and streptomycin (500 mg/mL). Saline, DHEA, 17-β-estradiol, letrozole, calcein, hematoxylin and eosin, and a tartrate-resistant acid phosphatase (TRAP) staining kit and a von Kossa staining kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). A toluidine blue staining solution was purchased from Leagene, Inc. (Beijing, China). An estradiol (E2) enzyme immunoassay (EIA) kit was purchased from BioCheck Inc. (Burlingame, CA, USA). A TNF-α enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN, USA). FITC-conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4, and PE-CY5-conjugated anti-mouse CD8a antibodies were purchased from BD Biosciences (Mississauga, ON, CA). Magnetic beads coated with anti-mouse CD4 antibody were supplied by Miltenyi Biotech (Auburn, CA, USA). Trizol was purchased from GIBCO-BRL, Invitrogen Corp. (Carlsbad, CA, USA). A Revert AidTM H Minus first strand cDNA synthesis kit was obtained from Fermentas (St. Leon-Rot, Germany). A Light Cycler 480 SYBER Green I Master was supplied by Roche Diagnostics Pvt., Ltd. (Mumbai, India).

2.2. *Creation of an animal model of PMO and in vivo experiments*

Animal experiments were performed in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication number 85-23, revised 1985). The animals used were 64 female BALB/c mice, 8 weeks old, with a body mass of between 20 and 30 g, that were purchased from the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China).

In the first *in vivo* experiment, 24 mice were randomly divided into four groups. The sham group (5 mice) underwent surgery without an ovariectomy.

One week after surgery, the mice were treated with a vehicle (gum acacia in distilled water) daily. A bilateral oophorectomy was performed on 19 mice. During the experimental period, 4 mice died during the administration of anesthesia, but there were no deaths due to other causes. The mice that died were excluded from analysis. One week after OVX, the mice were then randomly divided into 3 groups (OVX, OVX + DHEA, and OVX + E2; $n = 5$ per group).

The OVX control group was intragastrically administered saline ($n = 5$), the OVX + DHEA group was intragastrically administered 5 mg/kg per day of DHEA ($n = 5$) (*10*), and the OVX + E2 group was intragastrically administered estrogen (17-β-estradiol, E2) (100 μg/kg per day, $n = 5$) (*10,11,21*) daily. Twelve weeks after treatment, all mice were weighed, sacrificed after the final treatment, and blood and tissue samples were harvested for further investigation. A successful ovariectomy was confirmed in all OVX animals by observing the lack of ovarian tissue and atrophied uterine horns.

An additional *in vivo* experiment was performed to determine whether DHEA acted *via* an estrogen derivative or metabolite. A bilateral oophorectomy was performed on 40 mice; 20 were then treated with saline while the other 20 were treated with DHEA. OVX mice treated with saline or OVX mice treated with DHEA were then divided into the following 4 treatment groups: a group receiving the carrier solvent, and 3 groups receiving a 0.04, 0.2, or 2-μg/d injection of the aromatase inhibitor letrozole for 3 months ($n = 5$, all groups). Letrozole was dissolved in 0.1 mL of 0.3% hydroxyl propyl cellulose and given as a subcutaneous injection. Letrozole doses were selected as previously described (*25*).

2.3. *Analysis of bone mineral density*

To determine bone mineral density, the left femur and lumbar vertebrae were isolated after dissection. Dual energy X-ray absorptiometry (DEXA) was performed using an animal PIXImus densitometer (Lunar; GE Copr.). A consistent region of interest (the distal 4 mm of the femur) was selected to maintain uniformity during the analysis of samples.

2.4. *Bone histomorphometric analysis*

All bone histomorphometric analyses were performed in accordance with a previously described protocol (*26*). Briefly, the left tibia was isolated from each mouse; later, the proximal end was trimmed off and fixed in PBS-buffered 3.7% formaldehyde for 18 hours at 4°C. After incubation in 70% ethanol for 24 h, the undecalcified left proximal tibia was dehydrated in ascending alcohol concentrations, cleared in xylene, and embedded in methyl methacrylate; later, the tibia was sectioned (5 μm). These sections were stained with toluidine blue and the von Kossa procedure as indicated in a standard protocol (*27*).

The parameters for static and dynamic histomorphometry were quantified using undecalcified proximal tibia sections (5 μm). To evaluate the number of osteoclasts, the decalcified proximal tibia was embedded in paraffin. Serial sections were prepared from paraffin blocks (6 μm thickness) and sections were stained for TRAP activity. The bone volume (BV), osteoblast surface (Ob. S), osteoclast number (Oc. N), bone surface (BS), bone volume/tissue volume (%) (BV/TV), osteoblast surface/bone surface (%) (Ob. S/BS), osteoid surface/bone surface (%) (OS/BS), osteoclast surface/bone surface (%) (Oc. S/BS), osteoclast number/bone perimeter (1/mm) (Oc. N/BP), and eroded surface/bone surface (%) (ES/BS) were measured using the Osteo-Measure Histomorphometry System (Osteometrics, Atlanta, GA, USA) according to standardized protocols. All of these parameters were in accordance with the histomorphometric nomenclature and definitions of the American Society of Bone Mineral Research.

2.5. *Calcein labeling*

To label the sites of active bone formation in mice, double labeling was used, with calcein as a marker (*28*). First, 2.5 mg/mL of calcein was prepared in a 2% solution of sodium bicarbonate. The mice were weighed and injected twice, intraperitoneally, with calcein at a dose of 10 mg/kg body weight at 3-day intervals (*29*). The mice were sacrificed 2 days after the second injection.

Calcein double labeling was verified with fluorescence microscope measurements to determine the mineral apposition rate (MAR) and bone formation rate (BFR), which were evaluated on two nonconsecutive sections for each animal. The double calcein-green labels were measured on bone trabeculae using fluorescence microscopy (Olympus BX-60) with an excitation wavelength of 485 nm and emission wavelength of 510 nm. The mineralizing surface/BS (%) (MS/BS), MAR (μm/day), and BFR ($\mu m^3/\mu m^2$ per year) were measured using the Osteo-Measure Histomorphometry System (Osteometrics, Atlanta, GA, USA) according to standardized protocols. All of these parameters were in accordance with the histomorphometric nomenclature and definitions of the American Society of Bone Mineral Research.

2.6. *Flow cytometry*

After autopsy, bones and spleens were collected and placed in PBS. Bone marrow was flushed out and labeled with fluorescent antibodies for analysis of $CD4^+$ T cells. In brief, total lymphocytes were isolated from bone marrow with Hisep LSM 1084 (Himedia) by

means of the density (1.084 ± 0.0010 g/mL) gradient centrifugation technique.

Cells from bone marrow or the spleen were labelled with anti-CD3, CD4, and CD8a antibodies, FITC-conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4, and PE-CY5 conjugated anti-mouse CD8a antibodies to assess the percentage of $CD4^+$ in $CD3^+$ cells. The specificity of immunostaining was ascertained based on the background fluorescence of cells incubated with Ig isotype controls. Fluorescence data from at least 10,000 cells were collected from each sample. Immunostaining was done according to the manufacturer's instructions. FACS Caliber and FACS Arya (BD Biosciences Mississauga, ON, CA) were used to quantify the percentage of $CD4^+$ in $CD3^+$ cells in all groups.

2.7. *Isolation of $CD4^+$ T cells from the spleen or bone marrow*

Single cell suspensions were obtained from the spleen or bone marrow and incubated with magnetic beads coated with anti-mouse CD4 antibody. $CD4^+$ T cells were isolated according to the manufacturer's instructions. These purified cells were then collected in Trizol for real-time PCR.

2.8. *Quantitative real-time PCR*

Total RNA was extracted from isolated $CD4^+$ T cells using Trizol. cDNA was synthesized from 1 μg of total RNA with the Revert AidTM H Minus first strand cDNA synthesis kit. SYBR green chemistry was used for quantitative determination of TNF-α and GAPDH mRNAs according to an optimized protocol. The design of the sense and antisense oligonucleotide primers was based on published cDNA sequences using the Universal Probe Library (Roche Diagnostics, USA). The resulting cDNA was used in a PCR reaction using gene-specific primers for TNF-α (5'-TCTTCTCATTCCTGCTTGTGG-3' and 5'-GGTCTGGGCCATAGAACTGA-3') and GAPDH (5'- AGCTTGTCATCAACGGGAAG -3' and 5'- TTTGATGTTAGTGGGGTCTCG -3'). For real-time PCR, the cDNA was amplified with Light Cycler 480 (Roche Diagnostics Pvt., Ltd.). Double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the Light Cycler 480 SYBER Green I Master to allow for quantitative detection of PCR products in a 20-μL reaction volume. The temperature protocol for the reaction was 95°C for 5 min, 40 cycles of denaturation at 94°C for 2 minutes, annealing and extension at 62°C for 30 seconds, and extension at 72°C for 30 seconds. GAPDH was used to control for differences in RNA isolation, RNA degradation, and the efficiency of reverse transcription.

2.9. *Measurement of serum E2 and TNF-α levels*

At the end of the experiment, mice were anesthetized and sacrificed. A blood sample was quickly obtained by cardiac puncture. Blood volumes up to 1 mL have frequently been obtained from mice *via* this method. Serum samples were prepared by centrifugation. The serum samples were inactivated at 56°C for 30 minutes and filtered with a 0.2 -μm filter and then stored at -20°C for determination of the levels of E2 and TNF-α. Furthermore, the E2 level in serum samples was measured using an E2 ELISA kit according to the manufacturer's protocol. Serum TNF-α was also measured in all groups using an ELISA kit according to the manufacturer's instructions.

2.10. *Statistical analysis*

All values are expressed as the mean ± SD. Data were analyzed with aid of the software SPSS, and variance was evaluated using one-way ANOVA. Differences were accepted as significant at $p < 0.05$.

3. Results

3.1. *Both DHEA and E2 administration increased BMD in OVX mice*

As shown in Figure 1A, a significant decrease in femur and vertebra BMD was noted in OVX mice compared to sham-treated mice ($p < 0.01$, Figure 1). The OVX animals that were administered DHEA or E2 had a significantly higher femur and vertebra BMD compared to the OVX group ($p < 0.01$, Figure 1). Femur and vertebra BMD did not differ significantly in the OVX + DHEA group and the OVX + E2 group ($p > 0.05$, Figure 1).

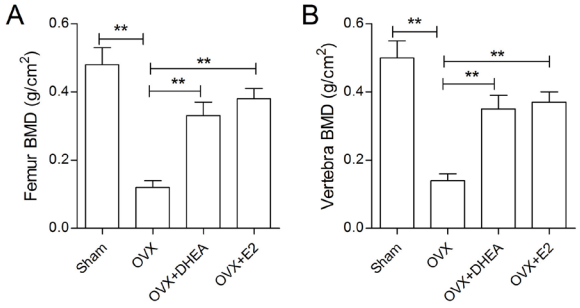

Figure 1. DHEA treatment improved BMD in OVX mice. **(A)** The sham group ($n = 5$) underwent surgery without an ovariectomy (OVX). OVX mice were treated with saline including 0.1% ethanol daily, 5 mg/kg per day of DHEA, or 100 μg/kg per day of E2 ($n = 5$). All treatments were administered to mice intragastrically. **(A)** Analysis of the BMD of the left femur in different groups. **(B)** Analysis of the BMD of the lumbar vertebrae. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$.

3.2. *DHEA reduced the OVX-induced enhancement of osteoclast-mediated bone resorption and improved osteoblast-mediated bone formation*

As expected, OVX significantly increased bone resorption in combination with enhanced bone formation. However, increased bone resorption is superior to enhanced bone formation. Thus, an imbalance in bone transition led to the loss of bone microarchitecture in the OVX group in comparison to the sham-treated group (Figures 2A and 3A). Analysis of dynamic and static bone histomorphometric parameters (*i.e.* Ob. S/BS, OS/BS, MS/BS, MAR, and BFR) indicated an increase in bone formation in the OVX group in comparison to bone formation in the sham group ($p < 0.01$ or $p < 0.05$, Figures 2B and 3B). Twelve weeks of treatment with DHEA increased bone mass and formation (*i.e.* BV/TV, Ob. S/BS, OS/BS, MS/BS, and BFR, $p < 0.01$ for all, Figures 2B and 3B) in comparison to bone mass and formation in the OVX group, thus indicating further

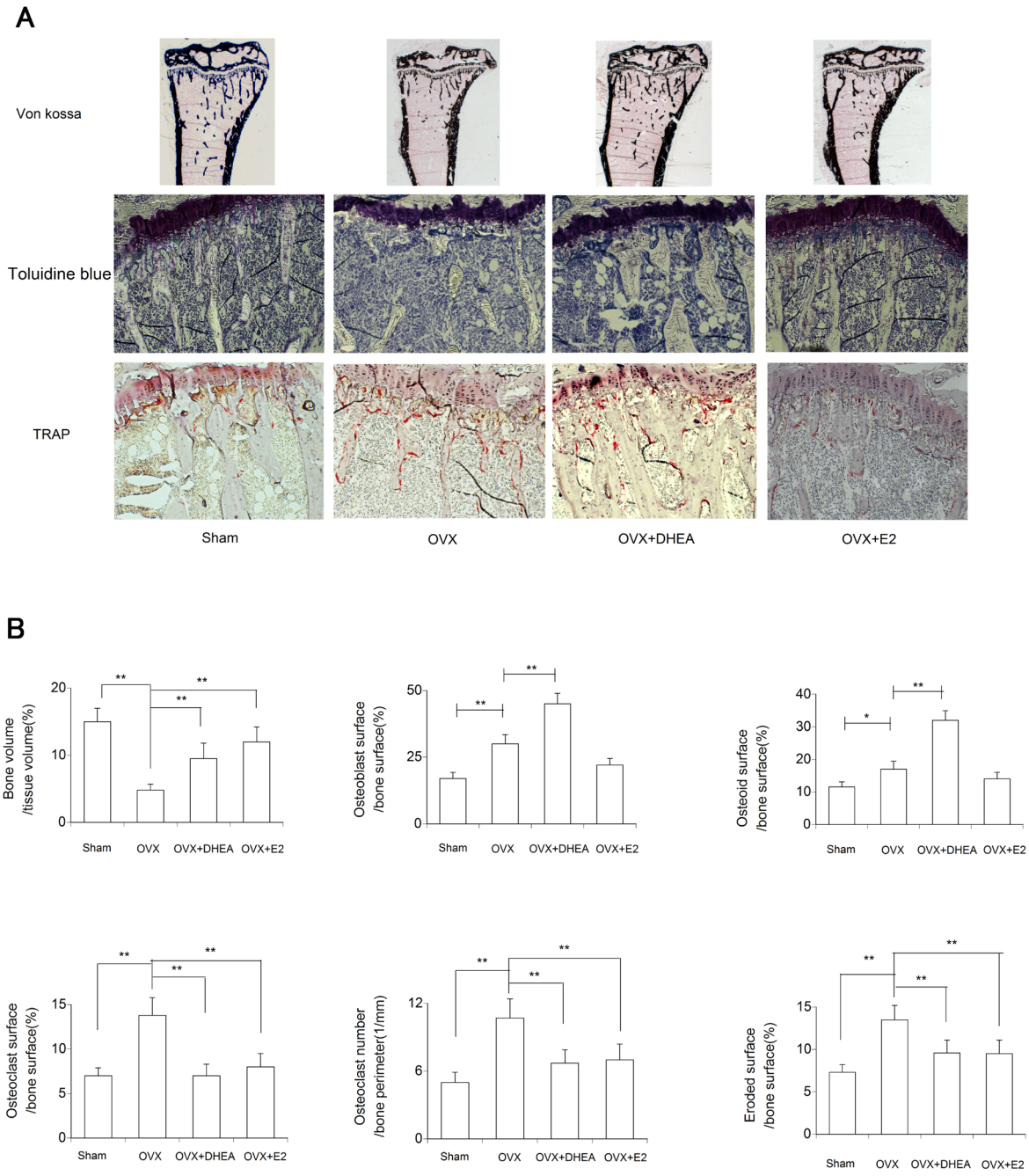

Figure 2. DHEA improved the bone phenotype of OVX mice. Static histological analysis of the proximal tibia in each group. **(A)** The first panels are von Kossa staining, the middle panels are toluidine blue staining, and the panels on the far right are TRAP staining. **(B)** The bone volume/tissue volume (%), osteoblast surface/bone surface (%), osteoid surface/bone surface (%), osteoclast surface/bone surface (%), osteoclast number/bone perimeter (1/mm), and eroded surface/bone surface (%) were measured according to standardized protocols using the Osteo-Measure Histomorphometry System. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$.

enhancement of osteoblast differentiation. However, E2 administration did not significantly affect osteoblast-related dynamic bone histomorphometric parameters in comparison to those parameters in the OVX group ($p > 0.05$, Figures 2B and 3B).

Static histomorphometry confirmed the loss of bone microarchitecture in OVX mice in comparison to sham-treated mice (Figure 2A). Specifically, OVX significantly increased osteoclast-related parameters (*i.e.* Oc. S/BS, Oc. N/BP, and ES/BS) in comparison to sham treatment ($p < 0.01$ for all, Figure 2B). After 12 weeks of treatment, both DHEA and E2 administration significantly decreased those parameters in comparison to parameters in the OVX group ($p < 0.01$ for all, Figure 2B). Dynamic bone histomorphometry parameters related to bone resorption did not differ significantly in the OVX + DHEA group and the OVX + E2 group ($p > 0.05$ for all, Figure 2B).

In other words, E2 primarily reduced osteopenia after OVX by decreasing osteoclast-related parameters,

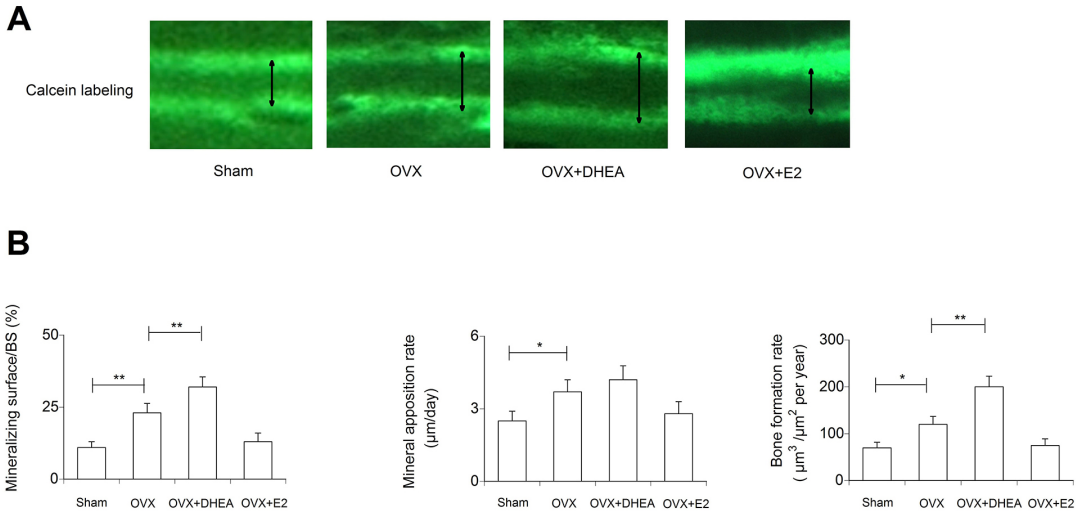

Figure 3. Bone dynamic histomorphometry of the proximal tibia. (A) Calcein double labels were measured on bone trabeculae by fluorescence microscope measurements with an excitation wavelength of 485 nm and emission wavelength of 510 nm for each group. (B) To determine the mineralizing surface/BS, mineral apposition rate and bone formation rate, calcein double labeling was analyzed with the Osteo-Measure Histomorphometry System. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$.

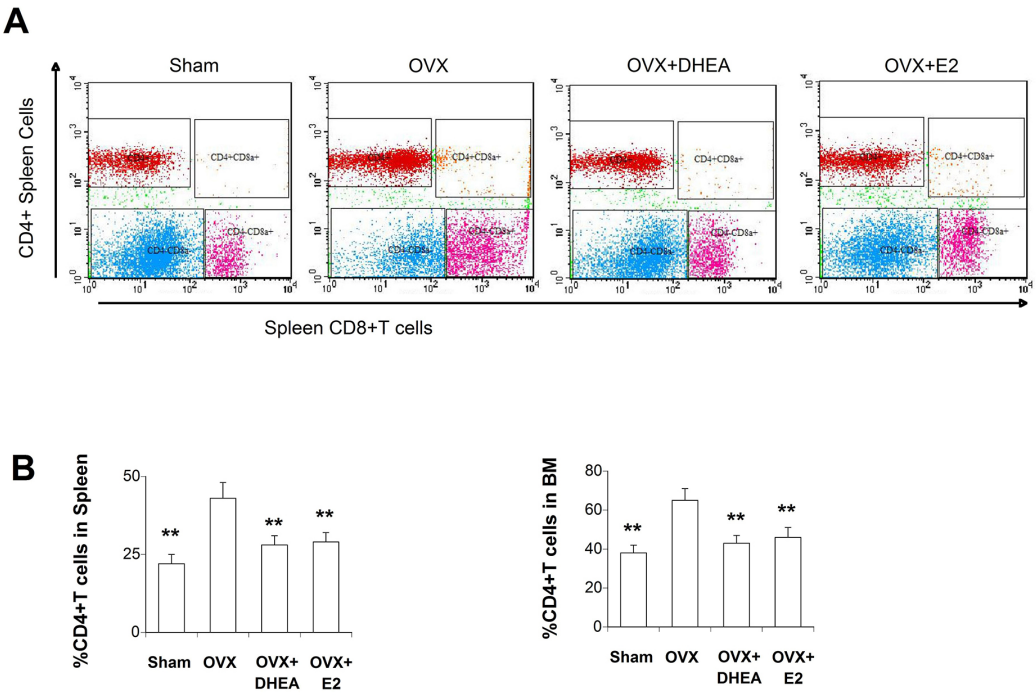

Figure 4. DHEA treatment significantly decreased OVX-induced increases in $CD4^+$ T cells subsets in bone marrow and the spleen. Mice were treated as described in Figure 1. **(A)** $CD4^+$ T cells in the spleen were quantified using flow cytometry. **(B)** Bar graphs indicate the percentage of $CD4^+$ T cells in the spleen or in bone marrow. Data are expressed as the mean ± SD; **$p < 0.01$ compared to the OVX group.

while DHEA alleviated OVX-induced bone loss by inhibiting osteoclast-mediated bone resorption and by improving osteoblast-mediated bone formation. Moreover, DHEA and E2 did not differ significantly in terms of their decreasing osteoclast-related parameters.

3.3. *Enhanced TNF-α-production of $CD4^+$ T cells in OVX mice is restored by treatment with either DHEA or E2*

To determine whether DHEA treatment affected the endocrine-immune network in PMO, the effects of DHEA on the $CD4^+$ T cells and its production of TNF-α were analyzed. OVX mice treated for 12 weeks had a greater number of $CD4^+$ T cells in bone marrow or the spleen in comparison to the sham group. Treatment of OVX mice with either DHEA or E2 resulted in a significant reduction in the OVX-induced expansion of $CD4^+$ T cells in bone marrow or the spleen in comparison that in the OVX group ($p < 0.01$, Figure 4). However, the OVX-induced expansion of $CD4^+$ T cells did not differ significantly in the OVX + DHEA group and OVX + E2 group ($p > 0.05$, Figure 4).

E2 deficiency is known to increase levels of circulating TNF-α (*17,30*). The current findings indicated that OVX led to a significant increase in levels of circulating TNF-α ($p < 0.01$, Figure 5A) and levels of TNF-α mRNA in bone marrow $CD4^+$ T cells ($p < 0.01$, Figure 5B) in comparison to levels in the sham group. However, treatment with DHEA or E2 led to a significant reduction in TNF-α production ($p < 0.01$, Figure 5). Results indicated that TNF-α production did not differ significantly in the OVX + DHEA group and the OVX + E2 group ($p > 0.05$, Figure 5).

3.4. *DHEA led to a slight elevation of serum E2 levels that was eliminated by the aromatase inhibitor letrozole*

The findings above indicated that both DHEA and E2 can prevent bone loss and suppress the OVX-induced expansion of $CD4^+$ T cells and TNF-α production. Given that DHEA is the pre-hormone for E2, the serum E2 level was measured to determine if DHEA improved bone mass *via* its end metabolites.

As expected, the serum level of estrogen decreased significantly in the OVX group in comparison to that in the sham-treated group ($P < 0.01$, Figure 6A). An increase in the E2 level was noted in both OVX + DHEA mice and OVX + E2 mice in comparison to OVX mice ($p < 0.05$, $p < 0.01$, respectively, Figure 6A). However, DHEA had less of an effect on the elevation of serum estrogen levels ($p < 0.01$, Figure 6A) than E2 did. Serum estrogen levels in the OVX + DHEA group were lower than those in the sham-treated group ($p < 0.05$, Figure 6A) while serum estrogen levels in the OVX + E2 group were higher than those in the sham-treated group ($p < 0.05$, Figure 6A). The *in vivo* experiment (DHEA plus letrozole) indicated that a DHEA-induced increase in E2 was suppressed by injection of the aromatase inhibitor letrozole (0.04, 0.2, 2 μg/d) ($p < 0.05$, $p < 0.01$, Figure 6B). However, letrozole did not affect E2 levels in OVX mice ($p > 0.05$, Figure 6B). The findings above indicate that conversion of DHEA into estrogen might be one way in which DHEA can be used to prevent PMO.

3.5. *The DHEA-mediated suppression of the OVX-induced expansion of $CD4^+$ T cells and TNF-α production was not eliminated by the aromatase inhibitor letrozole*

Although DHEA and E2 have almost the same action with regard to decreasing osteoclast-related parameters and modulating TNF-α-producing $CD4^+$ T cells, DHEA only moderately elevated the serum estrogen level. Thus, DHEA was hypothesized to inhibit TNF-α-producing $CD4^+$ T cells in OVX mice *via* its conversion into metabolites, such as E2. To confirm this hypothesis, the effects of DHEA plus letrozole on $CD4^+$ T cells and

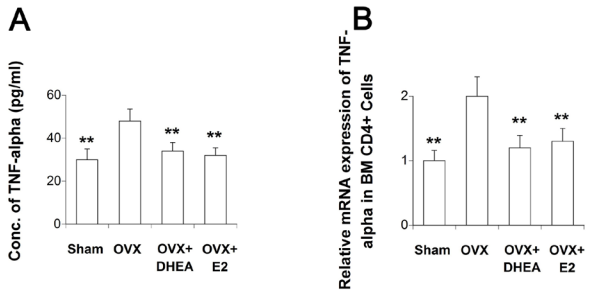

Figure 5. DHEA treatment significantly decreased OVX-induced increases in TNF-α production. (A) Circulating levels of TNF-α were measured in different groups using ELISA. **(B)** Levels of TNF-α mRNA in bone marrow $CD4^+$ T cells were measured in different groups using qPCR. Data are expressed as the mean ± SD; $^{**}p < 0.01$ compared to the OVX group.

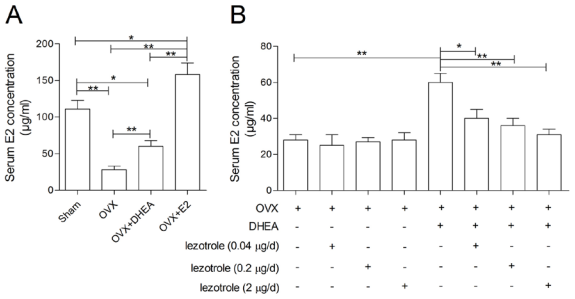

Figure 6. DHEA led to a slight elevation of serum E2 levels and this effect was eliminated by the aromatase inhibitor letrozole in OVX mice. (A) E2 levels in different group of mice treated as described in Figure 1. **(B)** OVX mice were intragastrically administered saline or DHEA ($n = 20$), and then both OVX mice treated with saline ($n = 20$) and OVX mice treated with DHEA ($n = 20$) were divided into thew following 4 treatment groups: a group receiving the carrier solvent, and 3 groups receiving a 0.04, 0.2, or 2-μg/d letrozole injection for 3 months ($n = 5$ for each group). After the experiment, mice were sacrificed and serum was collected to measure E2 levels. E2 levels in sera were measured with ELISA. Data are expressed as the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

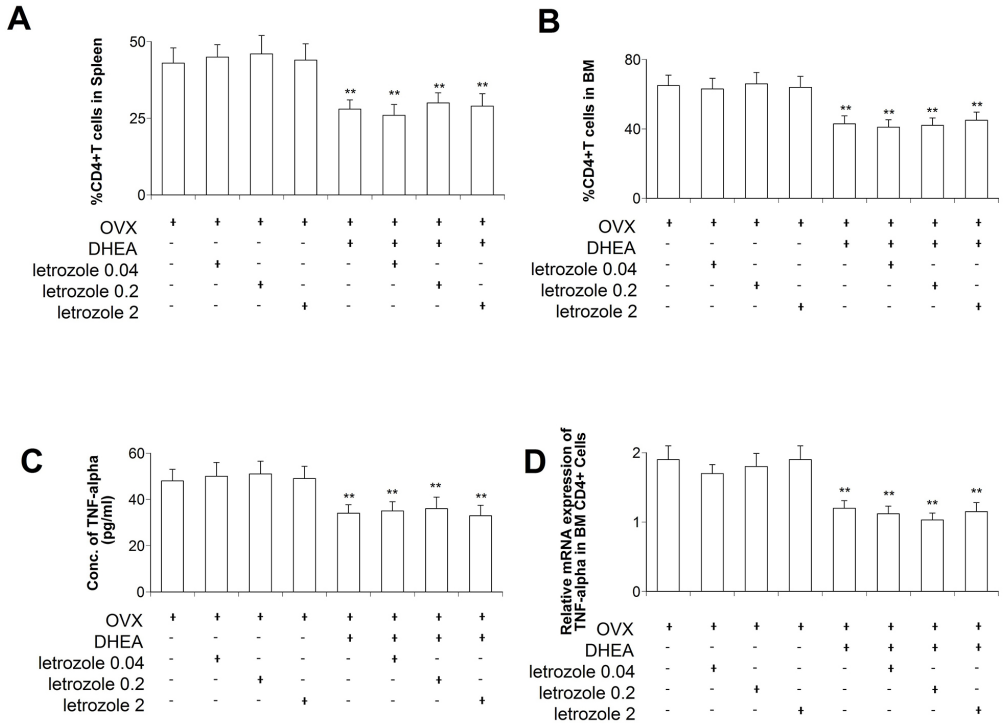

Figure 7. The aromatase inhibitor letrozole did not affect suppression of OVX-induced expansion of CD4$^+$ T cells and TNF-α production by DHEA. OVX mice were intragastrically administered saline or DHEA (n = 20), and then both OVX mice treated with saline (n = 20) and OVX mice treated with DHEA (n = 20) were divided into the following 4 treatment groups: a group receiving the carrier solvent, and 3 groups receiving a 0.04, 0.2, or 2-μg/d letrozole injection for 3 months (n = 5 for each group). **(A)** CD4$^+$ T cells in the spleen and **(B)** CD4$^+$ T cells in bone marrow were quantified using flow cytometry. **(C)** Circulating levels of TNF-α were measured in different groups using ELISA. **(D)** Levels of TNF-α mRNA in bone marrow CD4$^+$ T cells were measured in different groups using qPCR. Data are expressed as the mean ± SD; **p < 0.01 compared to the OVX group.

TNF-α production were analyzed in OVX mice.

Compared to the OVX group, each group of mice treated with DHEA had fewer CD4$^+$ T cells in bone marrow or the spleen (p < 0.01, Figures 7A and 7B). However, in both OVX mice and mice treated with DHEA, treatment with serial concentrations of letrozole did not result in significant changes in the OVX-induced expansion of CD4$^+$ T cells in either bone marrow or the spleen (p > 0.05, Figures. 7A and 7B). Furthermore, both DHEA administered alone and DHEA plus letrozole administration led to a significant increase in levels of circulating TNF-α (p < 0.01, Figure 7C) and levels of TNF-α mRNA in bone marrow CD4$^+$ T cells (p < 0.01, Figure 7D) in comparison to levels in the OVX group. In addition, treatment with letrozole did not affect the production of TNF-α in OVX mice treated with DHEA (p > 0.05, Figures 7C and 7D). However, when OVX mice treated with E2 were subsequently treated with letrozole, both the increases in levels of circulating TNF-α and levels of TNF-α mRNA in bone marrow CD4$^+$ T cells were inhibited (data not shown). Thus, suppression of the OVX-induced expansion of CD4$^+$ T cells and TNF-α production by DHEA was not *via* its conversion into E2.

4. Discussion

DHEA has been known to function as an inert precursor of sex steroids such as E2 and testosterone (*4*). However, the identification of DHEA receptors in the liver, kidneys, and testes of rats suggests that DHEA has specific physiologic actions of its own (*4,31-33*). As far as existing studies are concerned, DHEA may prevent PMO *via* an indirect or direct pathway. In the indirect pathway, DHEA may be converted to estrogen locally or systematically (*4,8*). In the direct pathway, DHEA may regulate the function of osteoblasts and osteoclasts *via* its own specific physiologic actions (*10,11,34,35*). The current results revealed the following: (*i*) DHEA treatment improved the bone phenotype of OVX mice *via* its action on osteoclast-related parameters (Figures 1-3), it reduced the OVX-induced enhancement of osteoclast-mediated bone resorption, and it improved osteoblast-mediated bone formation (Figures 2 and 3) while E2 only reduced the OVX-induced increase in osteoclast parameters (Figures 2 and 3). (*ii*) DHEA restored the enhanced TNF-α-production of CD4$^+$ T cells in OVX mice (Figures 4 and 5). (*iii*) DHEA elevated the serum E2 level, but the effect was moderate and was eliminated by the aromatase inhibitor letrozole (Figure 6). (*iv*) Although letrozole suppressed a DHEA-induced elevation of E2 levels, it did not affect the suppression of the OVX-induced expansion of CD4$^+$ T cells and TNF-α production by DHEA (Figure 7).

Many studies have suggested a beneficial effect of

DHEA administration on the prevention of trabecular BMD loss in postmenopausal women (*8,9*). DHEA levels in blood fall following OVX in rats and DHEA replacement counters the skeletal effects of OVX (*36*). These results coincide with the current findings. The current results indicated that DHEA treatment improves the bone phenotype of OVX mice by limiting osteoclast-mediated bone resorption and by improving osteoblast-mediated bone formation (Figures 1-3). However, E2 primarily increases bone mass after OVX by decreasing osteoclast-related parameters (Figures 1-3). A previous study by the current authors also noted DHEA's preferential stimulatory effect on bone, thus supporting DHEA's potential clinical value over estrogen in the prophylactic and therapeutic treatment of PMO (*10*). Furthermore, DHEA and E2 did not differ significantly in terms of the extent to which they decreased osteoclast-related parameters (Figure 2).

Researchers have begun to recognize the fact that the skeletal and immune systems mutually regulate one another to a much greater degree than previously believed, and "osteoimmunology", an interdisciplinary research principle, plays many roles in the cross-talk between the bone and immune systems (*37*). PMO is known to be associated with a variety of endocrine and immune alterations (*38*). Researchers have contended that positive effects on immune status can be achieved by altering the hormonal milieu (*39*). Such cross-talk between the endocrine and immune systems might be exploited to treat PMO. Various positive immunologic actions of DHEA have been noted in various human diseases and animal models, *e.g*. it influences the systemic concentrations of inflammatory cytokines, it promotes the proliferation of T-lymphocytes, and it alters subsets of T-lymphocytes, thus improving immune functions (*40-44*). The current findings indicated that OVX caused an expansion of the $CD4^+$ T cell pool in bone marrow and the spleen, which may occur as a result of increased proliferation of $CD4^+$ T cells and cells with a longer lifespan (Figure 5). DHEA and E2 reduced the proportion of the OVX-induced increase in bone marrow and spleen $CD4^+$ T cells (Figure 4). The current findings also indicated that OVX led to a significant increase in levels of circulating TNF-α and levels of TNF-α mRNA of bone marrow $CD4^+$ T cells in OVX mice in comparison to levels in sham-treated mice (Figure 5). This finding coincides with previous results (*30*). Additionally, treatment with DHEA or E2 inhibited OVX induced production of TNF-α (Figure 5). Together, the current findings suggest that both DHEA and E2 limit expansion of the $CD4^+$ T cell pool and the systemic and local (*via* bone marrow T cells) increase in TNF-α caused by a deficiency of E2.

RANKL is an osteoclastogenic mediator that is mainly expressed in bone by cells of mesenchymal origin (*37*). However, T cells can also be an important source of RANKL under certain conditions, such as PMO (*37*). $CD4^+$ T cells have the potential to both positively and negatively impact osteoclastogenesis by secreting pro-osteoclastogenic cytokines like RANKL and by secreting cytokines to induce anti-osteoclastogenic cytokine OPG expression (*45,46*). RANKL/RANK/OPG represents the primary triad in the cross-talk between osteoblasts and osteoclasts that regulates osteoclastogenesis. Upregulating TNF-α directly, or by augmenting RANKL secretion, may lead to enhanced osteoclastogenesis (*47*).

DHEA is a pre-hormone for estrogen. *In vitro* analysis indicated that primary human osteoblasts exhibit aromatase activity that converts DHEA into estrogen (*8*). The current study also indicated that DHEA elevated the serum E2 level in OVX mice (Figure 6A), and this phenomenon was eliminated by the aromatase inhibitor letrozole (Figure 6B). However, the current results indicated that DHEA suppresses OVX-induced expansion of $CD4^+$ T cells and TNF-α-production but not *via* the conversion of DHEA into E2. Moreover, DHEA has specific physiologic actions of its own. A previous study by the current authors indicated that DHEA inhibited osteoclastogenesis *via* an estrogen receptor α-dependent pathway (*48*). Furthermore, another study by the current authors suggested that DHEA improved murine osteoblast growth *via* the MAPK signaling pathway, independent of either androgen receptors or estrogen receptors (*11*). Although DHEA and E2 did not differ significantly in terms of their suppression of OVX-induced expansion of TNF-α-producing $CD4^+$ T cells and their decreasing osteoclast-related parameters, serum estrogen levels in the OVX + DHEA group were only slightly elevated. Those levels were lower than physiological levels in the sham-treated group and they were markedly lower than levels in the OVX + E2 group. Moreover, the aromatase inhibitor letrozole suppressed a DHEA-induced elevation of E2 levels in OVX mice but it did not affect the suppression of the OVX-induced expansion of $CD4^+$ T cells and TNF-α production by DHEA (Figure 7). This suggested that suppression of the OVX-induced expansion of $CD4^+$ T cells and TNF-α production by DHEA is not *via* its conversion into a metabolite. Thus, the conversion of DHEA into E2 might be one way in which DHEA prevents OVX-induced bone loss, but not the only way. Another mechanism might account for DHEA's ability to modulate immunity and improve the bone phenotype after OVX. Further work should be done to establish the causal relationship between changes in T-cells and bone-preserving effects of DHEA in OVX mice.

In conclusion, immune function is disrupted in OVX mice. DHEA reversed detrimental immunological changes due to OVX and it provided protection against bone loss. Moreover, these effects were not eliminated by the aromatase inhibitor letrozole. Based on the current findings, DHEA may prevent bone loss by suppressing the OVX-induced expansion of $CD4^+$ T

cells and TNF-α production instead of its conversion into estrogen, its end metabolite.

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