Original Article

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DHEA promotes osteoblast differentiation by regulating the expression of osteoblast-related genes and Foxp3$^+$ regulatory T cells

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Summary **Several studies have reported that dehydroepiandrosterone (DHEA) promotes osteoblast proliferation and inhibits osteoblast apoptosis and that DHEA inhibits osteoclast maturation. However, whether DHEA regulates osteoblast differentiation remains unclear. The present study first examined the effect of DHEA on bone morphology *in vivo*. DHEA was found to increase bone volume (BV), bone mineral density (BMD), and the number of trabeculae in bone (Th.N) and it was found to decrease trabecular spacing in bone (Th.sp) in ovariectomized (OVX) mice. Next, the effect of DHEA on osteoblast differentiation was examined *in vitro* and osteoblastogenesis-related marker genes, such as *Runx2*, *Osterix*, *Collagen1*, and *Osteocalcin*, were also detected. DHEA increased osteoblast production in mesenchymal stem cells (MSCs) cultured in osteoblastogenic medium, and DHEA increased the expression of Runx2 and osterix, thereby increasing the expression of osteocalcin and collagen1. Immune cells and bone interact, so changes in immune cells were detected *in vivo*. DHEA increased the number of Foxp3$^+$ regulatory T cells (Tregs) in the spleen but it did not affect CTLA-4 or IL-10. When MSCs were treated with DHEA in the presence of Tregs, alkaline phosphatase (ALP) activity increased. Osteoblasts and adipocytes are both generated by MSCs. If osteoblast differentiation increases, adipocyte differentiation will decrease, and the reverse also holds true. DHEA was found to increase the number of adipocytes in osteoblastogenic medium but it had no effect on the number of adipocytes and expression of PPARγ mRNA in adipogenic medium. This finding suggests that osteoblasts may be involved in adipocyte production. In conclusion, the current results suggest that DHEA can improve postmenopausal osteoporosis (PMO) by up-regulating osteoblast differentiation *via* the up-regulation of the expression of osteoblastogenesis-related genes and *via* an increase in Foxp3$^+$ Tregs.**

Keywords: DHEA, postmenopausal osteoporosis, MSCs, osteoblast, adipocyte, regulatory T cells

1. Introduction

The level of dehydroepiandrosterone (DHEA) in postmenopausal women is lower than that in fertile women, which suggests the potential clinical benefit of DHEA as treatment for postmenopausal osteoporosis (PMO) (*1*). Treatment of postmenopausal osteoporosis is mainly hormone replacement therapy, but this therapy is often accompanied by adverse reactions, limiting its use (*2,3*). The pressing task is to identify a new way to treat PMO that can prevent osteoporosis without causing adverse reactions.

Mesenchymal stem cells (MSCs) are able to differentiate into multiple types of cells. Under certain conditions, MSCs can be induced to differentiate into osteoblasts, adipocytes, cartilage cells, and other types of cells (*4,5*). Inducing MSCs to differentiate into osteoblasts and inhibiting MSCs from differentiating into adipocytes is crucial to preventing PMO.

DHEA begins to increase when the adrenal cortex

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begins to function and it gradually decreases after puberty. Along with other endocrine hormones, DHEA in the blood decreases with the onset of menopause. DHEA can prevent diabetes, cancer, and heart disease and it can enhance immune function, suggesting that DHEA can prolong survival and improve the quality of life.

DHEA has a positive effect on bone metabolism (*6-8*). DHEA can promote the growth and proliferation of osteoblasts, and mitogen-activated protein kinase signaling pathways are required for expression of osteoblast-specific genes. DHEA can promote a shift in the Th1/Th2 ratio towards Th2, thus improving immunity in a model of PMO.

Immune cells and bone interact (*9-11*). Regulatory T cells (Tregs) are important immunosuppressive cells that regulate the functioning of osteoblasts and osteoclasts (*12,13*). Whether DHEA affects immune cells and then affects bone metabolism is unclear. Whether DHEA affects the differentiation of MSCs into osteoblasts or adipocytes and the means by which it does so are still unknown. The present study will explore the mechanisms by which DHEA affects the differentiation of MSCs.

2. Materials and Methods

2.1. *Mice and reagents*

C57BL/6 mice (6-8 weeks of age) were provided by the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China) and were housed and handled in accordance with the guidelines of the Chinese Council for Animal Care. Sham-operated mice were used as controls, and ovariectomized (OVX) mice were used as a model of PMO. Mice were divided into 3 groups and then treated with different drugs, *i.e.* estrogen, DHEA, or saline. MEM, estrogen, DHEA, PBS and saline, and ALP solution/alizarin red solution were purchased from Sigma-Aldrich Co (Saint Louis, MO, USA). Flow cytometry antibodies CD4-FITC, CD25-APC, CTLA-4-PE, IL-10-PE, Foxp3-PE were purchased from eBioscience (San Diego, CA, USA).

2.2. *Experimental protocols in vivo*

Animals were anesthetized with 10% chloral hydrate. Mice in the sham-operated group underwent surgery but were not OVX. Mice in the treatment groups underwent a bilateral ovariectomy and were divided into 3 groups (OVX, OVX + DHEA, and OVX + E2). One week later, the sham-operated group was treated with saline (containing 0.1% ethanol), and OVX mice group in the treatment groups were given saline, DHEA (5 mg/kg per day orally), or estrogen (100 μg/kg per day orally) (*6*). The four groups received equal quantities of fluids at the same time during treatment. All mice were sacrificed after 3 months of treatment. Blood was collected from the heart and serum was stored at -80°C for use in cell culture, the spleen was collected for analysis of Tregs, and femurs were stored for micro-CT analysis (*14*).

2.3. *Flow cytometry (FCM)*

The spleen was harvested from mice in every group and mechanically disrupted in 10 mL of PBS. The cell suspension was then filtered through nylon mesh with a pore size of 110 μm and treated with NH4Cl/Tris buffer to remove RBC. The cells were then washed three times and transferred into tubes (100 μL per tube) for immunolabeling. The cells were fixed, permeabilized, and stained for Foxp3, IL-10, CTLA-4 using PE-labeled antibodies after cells were labeled with CD4 (FITC) and CD25 (APC). The cells were then washed twice and resuspended in PBS for FCM with a flow cytometer (Becton Dickinson, Palo Alto, CA, USA). PE-conjugated isotypes were used as controls. Statistical analysis was done using isotype-matched controls.

2.4. *MSC culture*

MSCs were cultured as previously described (*15*). Mice were anesthetized with 10% chloral hydrate and immersed in 75% ethanol for 10 min. Under aseptic conditions, the femur was removed and rinsed three times with PBS. The epiphyseal end of the femur was removed, revealing the marrow cavity. Bone marrow was removed, placed in L-DMEM with penicillin and streptomycin, and repeatedly sieved to obtain a single cell suspension. The suspension was centrifuged at 1,000 r/min for 5 min and the supernatant was discarded. The concentrated cells were placed in culture bottles at a concentration of 1×10^9 L^{-1} cells. Cells were cultured at 37°C in a 5% CO_2 environment with saturated humidity.

Medium was supplemented after 48 h. Medium was completely replaced with fresh medium every 3 d. Cells covering the bottom of the culture bottle formed a 70-80% confluent monolayer. After digestion with 0.25% trypsin, cells were subcultured at a ratio of 1:2.

2.5. *MSC-derived osteoblasts*

MSCs were seeded as previously reported (*16*) in order to induce differentiation into osteoblasts. Twenty-four hours after seeding, the growth medium was replaced with osteoblastogenic medium (OM) and 10% FCS was replaced with solvent alone (control), 10^{-7} M DHEA, or 10^{-9} M E2. Medium was changed every 2-3 days. After 14-21 days of osteogenic differentiation, cells were fixed with 70% ethanol for 1 h, washed three times with demineralized water, and then stained with an ALP

solution/alizarin red solution for 10 min. Finally, cells were washed three times with PBS.

ALP precipitates were solubilized to quantify ALP activity. Briefly, stained samples were incubated with 800 mL acetic acid (10%) for 30 min, the supernatant was transferred into a 1.5 mL tube and boiled for 10 min at 85°C, and then the supernatant was placed on ice for 5 min. After centrifugation (15,000/g, 15 min), 500 μL of the supernatant was transferred into another 1.5-mL tube and mixed with 200 μL of 10% ammonium hydroxide. Samples were transferred into a 96-well microtiter plate and optical density was measured at 405 nm using a standard ELISA reader. *P* was calculated with a student's *t*-test ($n = 3$) to detect statistically relevant differences.

2.6. *MSC-derived adipocytes*

Adipogenic culture of MSCs as reported (*17*) was performed to induce differentiation into adipocytes. Briefly, MSCs in the second or third passage were induced to form adipocytes in an adipogenic medium (a-MEM and 10% FBS) for up to 12 days as determined by peak adipogenesis. At that point, the medium was changed to an adipocyte-sustaining medium consisting of high-glucose DMEM with 10 μg/mL insulin and 10% FBS to promote adipocyte maturity. Cultures were analyzed prior to adipogenesis on day 0 and at specific points in time over a period of 25 days.

2.7. *Oil Red O staining*

Cells were fixed with 4% paraformaldehyde for 20 min at 4°C. Cells were rinsed and washed and then stained with Oil Red O solution for 15 min to stain lipid droplets/vacuoles. Cells in random fields were manually counted and the number in 5 high power fields was averaged.

2.8. *Real-time PCR*

RNA treated with DNase was isolated from MSC-derived osteoblasts and adipocytes, enriched adipofibroblasts, and lipid-laden adipocyte cultures at specific time-points using an RNeasy Mini Kit in accordance with the manufacturer's instructions (Qiagen). Cells were lysed on plates in buffer containing GITC (Buffer RLT). Reverse Transcription was performed immediately after RNA isolation using a Transcriptor First Strand cDNA synthesis kit and oligo-dT primers (Roche, Branchburg, NJ). Real-time PCR was performed using SybrGreen and Taqman technology. Briefly, 10 mL of SybrGreen Master Mix (Applied Biosystems, Darmstadt, Germany) was mixed with 1 mL (10 pg) of forward primer, 1 mL (10 pg) of reverse primer, 6.8 mL of water, and 1.2 mL (60 ng) of template. Levels of expression of the beta-actin gene were used to normalize mRNA expression for real-time PCR. Reactions were performed using the following conditions: 95°C for 10 min and cycles of 95°C for 15 sec, 55-60°C for 30 sec, and 72°C for 30 sec for 40 cycles. The primers used for each gene were as follows: Runx2: Forward GACAGTCCCAACTTCCTGTG, Reverse GCGGAGTAGTTCTCATCATTC; Osterix: Forward GCTCGTAGATTTCTATCCTC, Reverse CTTAGTGACTGCCTAACAGA; Collagen1: Forward TGACTGGAAGAGCGGAGAGTA, Reverse GACGGCTGAGTAGGGAACAC; Osteocalcin: Forward TGCCTGGCTGGAGATTCTG, Reverse GCTGCTGTGACATCCATACTT; PPARγ: Forward GGAATTAGATGACAGTGACTTGGC, Reverse ATCTTCTGGAGCACCTTGGC; β-actin: Forward CCTCTATGCCAACACAGT, Reverse AGCCACCAATCCACACAG.

2.9. *Statistical analysis*

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

3. Results

3.1. *The effects of DHEA on bone morphology*

Bone volume (BV), bone mineral density (BMD), and the number of trabecules in bone (Tb.N) were lower in the OVX group than in the sham-operated group ($p < 0.05$). Trabecular spacing (Tb.sp) was wider in the OVX group than in the sham-operated group ($p < 0.05$), suggesting that a mouse model of PMO was successfully established. Compared to the OVX group that was treated with saline, the OVX + DHEA and OVX + E2 groups had a higher BV, BMD, and Tb.N and a smaller Tb.sp, suggesting that DHEA and E2 improved bone morphology in mice with osteoporosis (Figure 1).

3.2. *DHEA increases osteoblastogenesis by up-regulating the expression of osteoblastogenesis-related genes*

In osteoblastogenic medium, DHEA promoted MSCs to differentiate into osteoblasts. The ALP activity of osteoblasts was higher in groups treated with DHEA or E2 than that in the control group ($p < 0.05$) (Figure 2A). The number of bone nodules also increased in the groups treated with DHEA or E2 compared to the control group ($p < 0.05$) (Figures 2B and 2C). The expression of mRNA by osteogenesis-related genes was examined using real-time PCR in order to explore the mechanisms by which DHEA regulates MSC differentiation. Compared to the control treatment, DHEA and E2 promoted expression of *collagen I*, *osteocalcin*, *Runx2*, *and osterix*. However, there was no difference in levels of expression in the two groups (Figure 2D).

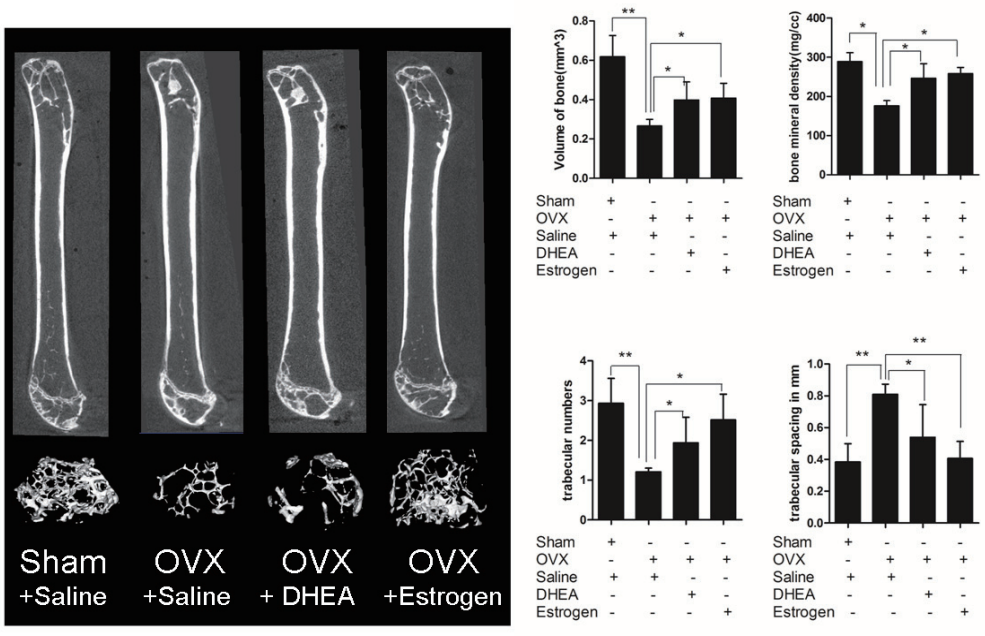

Figure 1. Effects of DHEA on bone morphology in OVX mice. Sham mice underwent mock surgery and received saline. Ovariectomized (OVX) mice underwent bilateral oophorectomy and were randomly divided into three groups: OVX (treated with saline), OVX + DHEA (treated daily with 5 mL of mixed raw herbs [BSNXD] per kg body weight), and OVX + E2 (treated daily with 5 mL of DHEA per kg body weight). Femur samples were harvested after 12 weeks of treatment. Micro-CT was performed to determine the bone morphology in femurs. Bone volume, bone mineral density, the number of trabecules in bone, and trabecular spacing were measured at the original magnification (200×). Data are presented as the mean ± SEM. ($n = 6$). * $p < 0.05$, ** $p < 0.01$ compared to the OVX group.

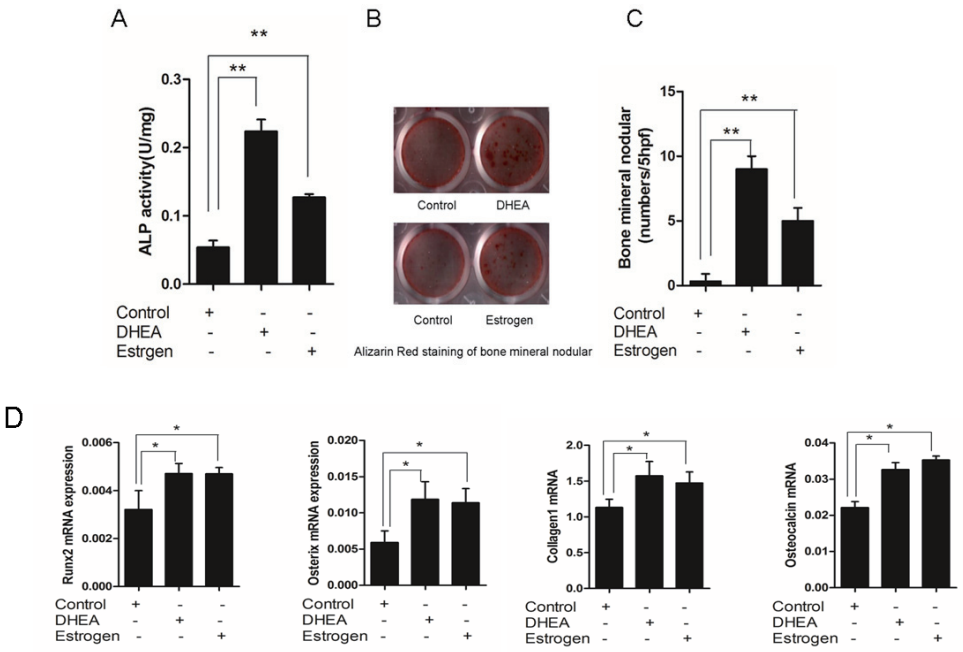

Figure 2. DHEA increased ALP activity and expression of mRNA by osteoblastogenesis-related genes. Primary MSCs were exposed to control serum, 10^{-7} M DHEA, or 10^{-9} M E2 for 48 h under osteoblastogenic conditions. The ALP activity of osteoblasts was determined with a kit to analyze ALP activity after 7 days of culturing (**2A**), alizarin red staining was performed after 21 days of culturing (**2B**), and the number of mineralized bone nodules was counted (**2C**). *Runx2*, *osterix*, *collagen I*, and *osteocalcin* mRNA were analyzed (**2D**). Data are presented as the mean ± SEM. ($n = 6$). * $p < 0.05$, ** $p < 0.01$ compared to the control group.

3.3. *DHEA increases the proportion of Foxp3$^+$ Tregs in the spleen and enhances osteoblastogenesis*

Immune cells can influence bone metabolism, so this study determined whether immune cells were affected after DHEA treatment. Changes in spleen-derived immune cells after DHEA treatment were examined first. Compared to the sham-operated group, the OVX

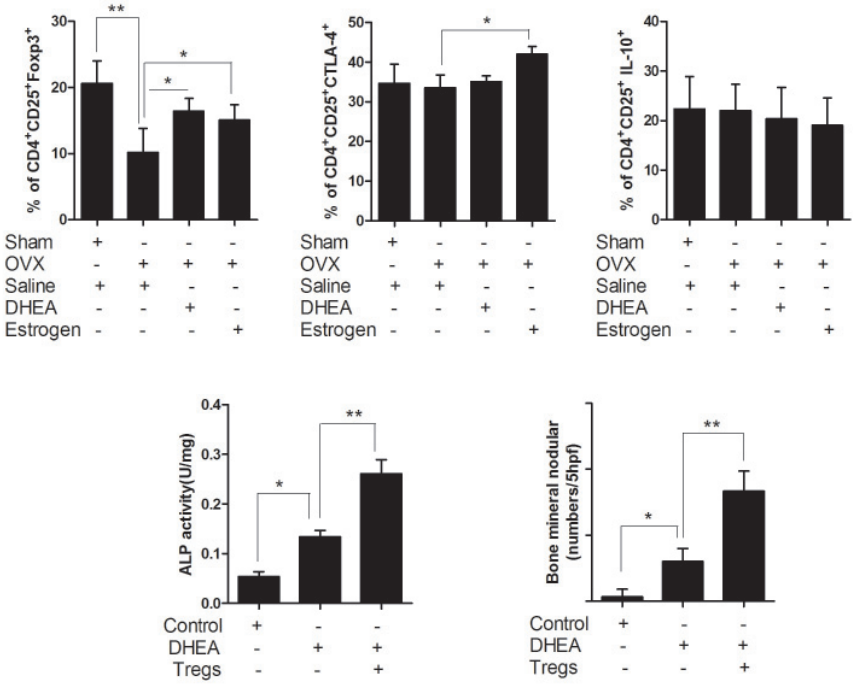

Figure 3. DHEA increased the number of Foxp3$^+$ Tregs, and Tregs enhanced the effects of DHEA on osteoblast differentiation. Sham mice underwent mock surgery and received saline. OVX mice underwent bilateral oophorectomy and were randomly divided into three groups: OVX (treated with saline), OVX + DHEA (treated daily with 5 mL of E2 per kg body weight), and OVX + E2 (treated daily with 5 mL of E2 per kg body weight). Spleen samples were harvested after 12 weeks. Flow cytometry was performed to ascertain immune cells in the spleen. Data are presented as the mean ± SEM. (n = 6). * $p < 0.05$, ** $p < 0.01$ compared to the OVX group. Primary MSCs were exposed to control serum, 10^{-7} M DHEA, or 10^{-9} M E2 for 48 h under osteoblastogenic conditions in the presence or absence of Tregs. ALP activity of osteoblasts was determined using a kit to analyze ALP activity. The number of bone nodules was assessed using alizarin red staining. Data are presented as the mean ± SEM. (n = 6). * $p < 0.05$, ** $p < 0.01$.

group had fewer Foxp3-positive Tregs. DHEA treatment increased the percentage of Foxp3$^+$ Tregs ($p < 0.05$) while E2 did not. Conversely, E2 treatment increased the percentage of CTLA-4$^+$ Tregs but DHEA did not significantly influence the number of Tregs (Figure 3). MSCs were cultured in the presence or absence of Tregs in order to determine the effects of Tregs on DHEA-mediated differentiation of MSCs. In the presence of Tregs, ALP activity was higher and bone nodular production increased, suggesting that Tregs can enhance the effect of DHEA on differentiation of MSCs (Figure 3).

3.4. *DHEA does not directly affect adipocyte differentiation*

MSCs can differentiate into osteoblasts and adipocytes. Increased numbers of adipocytes indicate a significant risk of PMO developing. MSCs cultured in osteoblastogenic medium and treated with DHEA had increased numbers of adipocytes. However, MSCs cultured in adipogenic medium and treated with DHEA or E2 did not produce different numbers of adipocytes. The transcription factor PPARγ regulates adipogenesis. Expression of this gene was assessed using real-time PCR, and the levels of expression in the group treated with DHEA did not differ from those in the control group and the group treated with E2 (Figure 4).

4. Discussion

Mature OVX mice were previously used to model postmenopausal bone loss (*18*). Analysis of bone morphology has revealed that OVX mice have significantly reduced bone mass, bone density, and fewer trabecules in bone as well as significantly wider trabecular spacing compared to sham-operated mice (*19*). These findings were evident in the present study, suggesting that a model of PMO was successfully established.

After treatment with DHEA, the OVX mice had significantly increased bone mass, bone density, and more trabecules in bone as well as significantly smaller trabecular spacing, suggesting that DHEA significantly improves bone morphology. An improvement in bone morphology can increase the mechanical strength and integrity of bone and affect bone growth, suggesting that DHEA has clinical significance in the prevention and treatment of PMO.

Runx2 and osterix are transcription factors associated with osteoblastogenesis while collagen1 and osteocalcin are genes expressed in osteoblasts; all four of these genes are markers of different stages of bone

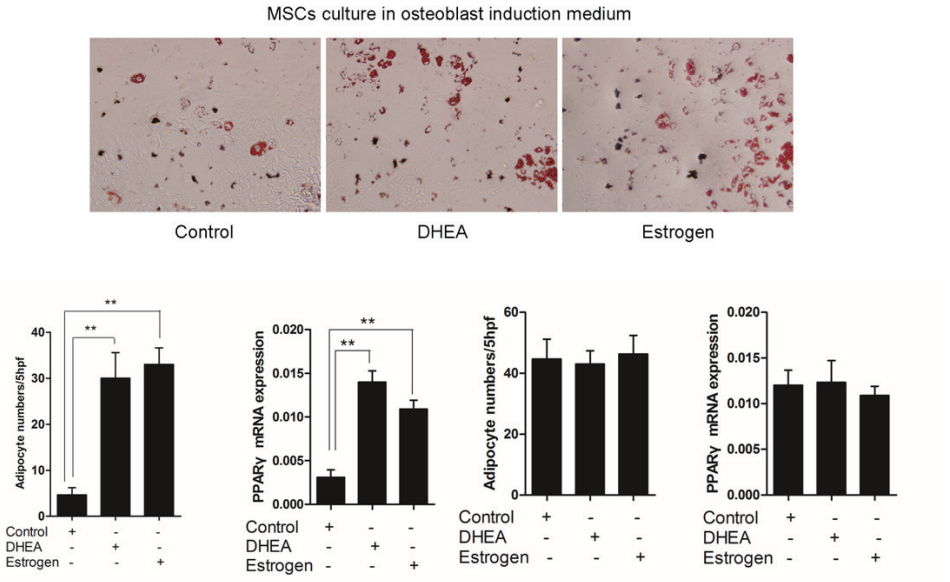

Figure 4. DHEA affected adipocyte differentiation *via* osteoblast production. Primary MSCs were exposed to control serum, 10^{-7} M DHEA, or 10^{-9} M E2 for 48 h under adipogenic conditions. The number of adipocytes was assessed using oil red O staining. Expression of *PPARγ* mRNA was assessed using real-time PCR. Data are presented as the mean ± SEM. ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the OVX group.

development (*20-22*). PPARγ is a transcriptional factor that regulates adipogenesis (*23*). Although DHEA increases the number of adipocytes in osteoblastogenic medium, it produces no changes in the number of adipocytes in adipogenic medium (*24-30*).

Tregs affect bone metabolism, and this action is directly regulated by Foxp3 and CTLA-4 or it is indirectly regulated by TGF-β and IL-10 (*31-36*). DHEA can markedly increase the proportion of $Foxp3^{+}$ Tregs and encourage osteoblast differentiation to prevent PMO. DHEA does not affect the ratio of CTLA 4^{+} or IL-10^{+} Tregs, suggesting that DHEA plays a role on osteoblast differentiation *via* Foxp3.

In a previous study, the current authors found that DHEA significantly promoted proliferation and inhibited apoptosis of osteoblast *via* mitogen-activated protein kinase signaling pathways independent of either androgen receptors or estrogen receptors, suggesting that it may play a direct role *via* a DHEA-specific receptor instead of *via* conversion to androgens or estrogens (*6*).

Results of *in vivo* and *ex vivo* experiments revealed that DHEA can directly promote differentiation of MSCs into osteoblasts. DHEA increased the expression of Runx2, Collagen1, Osterix, and Osteocalcin mRNA, increasing the ALP activity of osteoblasts and the number of mineralized bone nodules. DHEA increases the number of $Foxp3^{+}$ Tregs, increasing osteoblast differentiation. Therefore, DHEA is likely to be an ideal solution to prevent and treat PMO.

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