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Original Article

BSNXD modulates mesenchymal stem cell differentiation into osteoblasts in a postmenopausal osteoporotic mouse model

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Abstract: Mesenchymal stem cells (MSCs) are a type of stem cell that has multidirectional differentiation abilities. Under certain inducing factors, MSCs can differentiate into osteoblasts and adipocytes. Adipocytes and osteoblasts are derived from MSCs, and decreased osteoblastogenesis and increased adipocytes may be a primary cause of postmenopausal osteoporosis (PMO). The present study aimed to elucidate whether BuShen NingXin Decoction (BSNXD), a traditional Chinese medicinal compound, regulates MSC differentiation into both osteoblasts and adipocytes. The effects of BSNXD on bone morphometry were measured using micro-CT and its effects on the proportion of immune cells in the spleen were measured using flow cytometry (FCM). BSNXD-mediated regulation of MSC differentiation into osteoblasts and adipocytes was verified *in vitro* using ALP and Oil Red O staining. In addition, osteoblastogenesis-related genes and adipocyte transcription factors were measured using real-time PCR. We found that BSNXD increased bone volume, bone mineral density, and bone trabecular number, but decreased bone trabecular spacing. BSNXD treatment also increased regulatory T cell (Treg) function *in vivo*. *In vitro*, BSNXD serum increased ALP activity as well as *collagen type I*, *osteocalcin*, *Runx2*, and *osterix* mRNA expression. Moreover, BSNXD decreased adipocyte numbers and *PPARγ* mRNA expression, whereas in Tregs, BSNXD enhanced ALP activity. In conclusion, BSNXD promotes the differentiation of MSCs into osteoblasts and inhibits differentiation into adipocytes. BSNXD enhanced expression of osteoblastogenesis-related genes and decreased adipocyte transcription factor expression. We propose that BSNXD may be effective for the prevention of PMO.

Keywords: Traditional Chinese medicine, osteoblast, mesenchymal stem cell, adipocyte, postmenopausal osteoporosis, regulatory T cell

Introduction

Postmenopausal osteoporosis (PMO) is a common disease in postmenopausal women that seriously impacts their health and quality of life. The pathophysiology of postmenopausal osteoporosis is likely due to estrogen deficiency, and treatment is primarily hormone replacement therapy [1-4]. However, there are disadvantages to hormone replacement therapy, including increased cancer risk. Therefore, it is important to explore new treatment modalities to reduce the risk of complications.

Mesenchymal stem cell (MSC) therapy has many benefits, in particular, MSCs are readily available from the patients themselves [5-8]. MSCs can differentiate into a variety of cells, including osteoblasts and adipocytes. Osteoblasts are key bone forming cells, and increased numbers of adipocytes could be a primary cause of postmenopausal osteoporosis. Thus, if a therapy can influence MSC differentiation by promoting osteoblast formation and inhibiting adipocyte production, it will likely be a good candidate for the treatment of PMO.

The bones and the immune system interact [9, 10], and a discipline termed osteoimmunology was developed [11-16]. Regulatory T cells (Tregs) are important immune cells and play an important role regulating immune function. The interaction between Tregs and bone metabolism has been examined, but the influence of Tregs on osteoblast differentiation of MSCs has not yet been reported.

Table 1. Primer sequences for RT-qPCR

Gene	Sequence (5'-3')	Amplicon size
Collagen1	Fw TGACTGGAAGAGCGGAGAGTA	117 b
	Rw GACGGCTGAGTAGGGAACAC	
Osteocalcin	Fw TGCCTGGCTGGAGATTCTG	190 bp
	Rw GCTGCTGTGACATCCATACTT	
Osterix	Fw GCTCGTAGATTTCTATCCTC	114 bp
	Rw CTTAGTGACTGCCTAACAGA	
PPARγ	Fw GGAATTAGATGACAGTGACTTGGC	186 bp
	Rw ATCTTCTGGAGCACCTTGGC	
Runx2	Fw GACAGTCCCAACTTCCTGTG	149 bp
	Rw GCGGAGTAGTTCTCATCATTC	
β-actin	Fw CCTCTATGCCAACACAGT	155 bp
	Rw AGCCACCAATCCACACAG	

Traditional Chinese medicine plays an important role in the treatment of diseases in China [17-20]. BuShen NingXin Decoction (BSNXD), a traditional Chinese medicine compound, is used clinically to treat the symptoms of postmenopausal women, including postmenopausal osteoporosis. In the present study, we sought to identify whether BSNXD regulated MSC differentiation into osteoblasts and adipocytes.

Materials and methods

Mice and reagents

C57BL/6 mice (aged 6-8 weeks) were provided by the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). Housing and handling in accordance with the guidelines of the Chinese Council for Animal Care. Sham mice were used as control treatment whereas ovariectomized mice were used as PMO model mice. Mice were then treated with different drugs, such as estrogen, BSNXD, or saline.

Fetal bovine serum (FBS) and phenol red-free minimum essential media (MEM) were purchased from Gibco (Grand Island, NY, USA). Adipogenic induction media, osteogenic induction media, α-minimum essential media, 17-β-estradiol (E2), the alkaline phosphatase (ALP) staining kit, and collagenase type 2 were purchased from Sigma-Aldrich Co. The following monoclonal antibodies against mouse lymphocytes were used: fluorescein isothyocyanate (FITC)-conjugated anti-CD4, anti-IL-10-PE, anti-Foxp3-PE, anti-CD25-APC, anti-cytotoxic T lymphocyte antigen-4 (anti-CTLA4)-PE. Antibodies and their corresponding isotype controls were purchased from eBioscience (San Diego, CA, USA).

BuShen NingXin Decoction (BSNXD)

Based on traditional Chinese medicinal theory and our clinical experience, BSNXD is composed of eight crude herbs: dried Rehmannia root (15 g), common Anemarrhena rhizome (15 g), Chinese Corktree bark (9 g), Barbary wolfberry fruit (15 g), Chinese dodder seed (15 g), short-horned Epimedium herb (12 g), Spina date seed (9 g), and Oriental water plantain rhizome (12 g).

Drug-derived serum preparation

Mice were divided into three groups: control, BSNXD, and 17-β-estradiol (E2). Groups were treated with saline, BSNXD, or estrogen, respectively. The groups received the same volumes of fluid at the same time for 7 days. One hour after the last BSNXD lavage, serum was acquired from the heart. It was inactivated at 56°C for 30 min, filtered through at 0.2 mm filter, and stored at -80°C until use.

In vivo experimental protocols

Animals were anesthetized with 10% chloral hydrate. The sham group underwent the surgical procedure but without the ovariectomy. The ovariectomy group (OVX) had bilateral incisions and was divided into three groups (OVX, OVX+BSNXD, and OVX+E2). After 1 week, the sham group was treated with saline (containing 0.1% ethanol), and the OVX groups were treated with saline, BSNXD (5 ml mixed row herbs orally per day), or 17-β-estradiol (100 mg/kg/day orally), respectively. The four groups received equal volumes of fluid during treatment. All mice were sacrificed 3 months after treatment. Blood was collected from the hearts and serum was stored at -80°C until use in cell culture. Spleens were used for regulatory T cells analyses, and femurs were stored for micro-CT analyses.

Flow cytometry (FCM) analyses

Harvested spleens were mechanically grinded from the stroma in 10 ml PBS. Cell suspensions

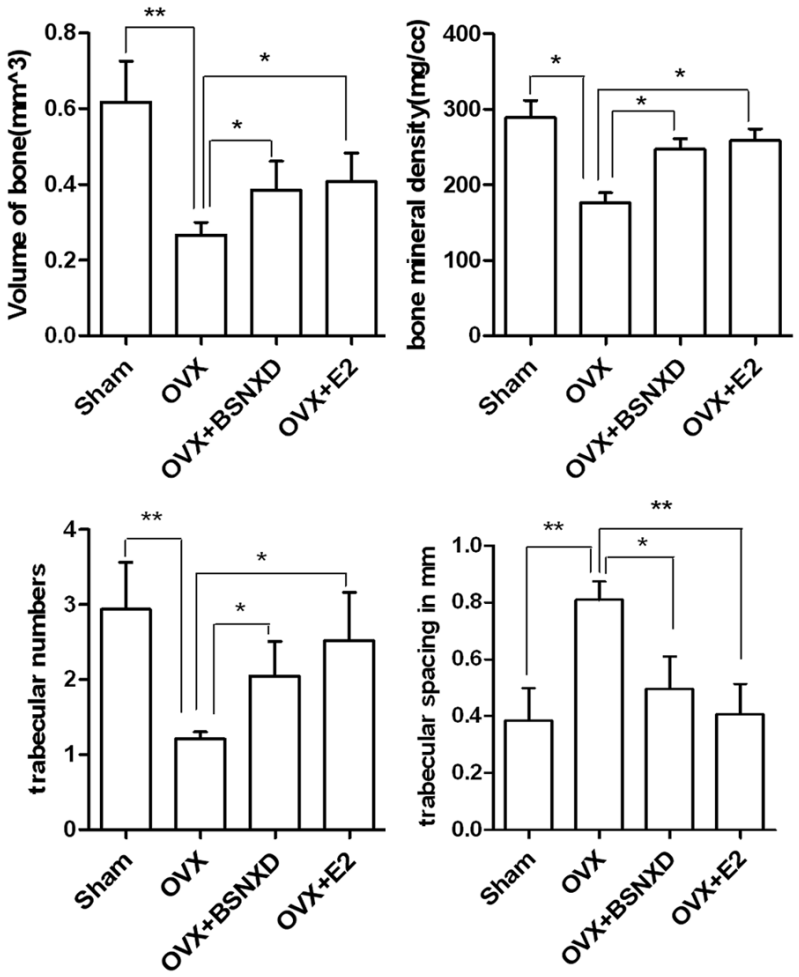

Figure 1. Effects of BSNXD on bone morphology. Sham mice underwent a mock operation and received saline. Ovariectomized mice underwent bilateral oophorectomy and were randomly divided into three groups: OVX (treated with saline), OVX+BSNXD (treated daily with 5 ml mixed row herbs [BSNXD] per kg body weight), and the OVX+E2 (treated daily with 5 ml E2 per kg body weight). Femur samples were harvested after treatment for 12 weeks. Micro-CT for bone morphology was performed in femurs. Original magnification (200×), Bone volume, Bone mineral density, trabecular numbers, and trabecular pacing were measured. Data are expressed as the mean ± S.E.M. (n = 6). *$P < 0.05$, **$P < 0.01$ compared with the OVX group.

were filtered through 110-μm nylon mesh and treated with NH_4Cl/Tris buffer to remove red blood cells. Thereafter, cells were washed three times and distributed for immunolabeling (100 μl per tube). Cells were fixed, permeabilized, and stained with Foxp3, IL-10, and CTLA-4 using PE-labeled antibodies after being labeled with CD4 (FITC) and CD25 (APC). Next, cells were washed twice and resuspended in PBS for FCM analyses using a flow cytometer (FACS Calibur, BD). PE-conjugated isotypes were used as controls. Statistical analyses were conducted using isotype-matched controls.

MSC culture

Mice were anesthetized using 10% chloral hydrate and immersed in 75% ethanol for 10 min. Under aseptic conditions, the femur was isolated and cleaned three times in PBS. The epiphyseal end of the femur was removed, revealing the marrow cavity. Using L-DMEM media with penicillin and streptomycin, bone marrow was repeatedly beat to get single cell suspensions. Suspensions were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The cell pellet was transferred to culture bottles at a concentration of 1×10^9 L-1 cells and maintained at 37°C with 5% CO_2 saturated humidity.

After 48 hours, the media were replaced with fresh media. Subsequently, the media were changed every 3 days. Cells covered the culture bottle bottom fused into a single, 70%~80% confluent monolayer. After digestion with 0.25% trypsin, cells were replated at a 1:2 ratio.

MSC-derived osteoblasts

For osteogenic induction of MSCs, MSC were seeded as previously described [21]. Twenty-four hours after seeding, growth media were replaced with osteogenic induction media (OIM) and 10% FCS with solvent control, 10% BSNXD, or 10^{-9} M E2. Media were changed every 2-3 days. After 14 to 21 days of osteogenic differentiation, cells were fixed for 1 hour with 70% ethanol, washed three times with demineralized water, and stained for 10 min with an ALP solution/Alizarin red solution (Sigma). Finally, cells were washed three times with PBS.

To quantify ALP activity, ALP precipitates were solubilized. Briefly, stained samples were incu-

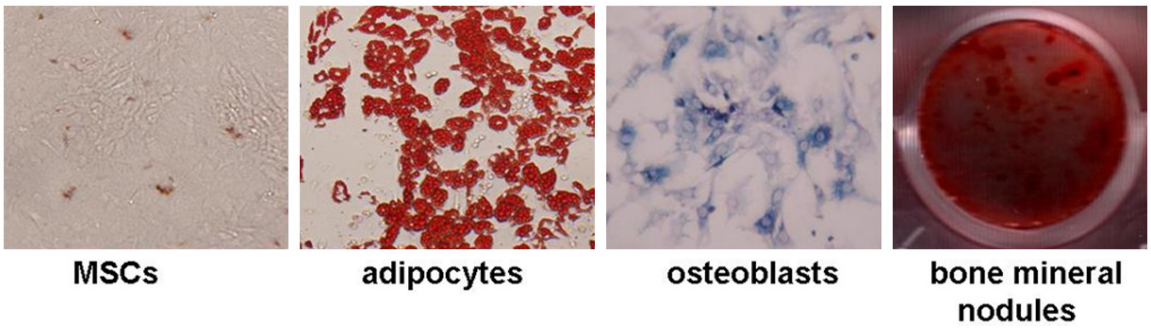

Figure 2. MSCs differentiate into osteoblasts and adipocytes. Primary MSCs were cultured in both osteoblast induction conditions and adipocyte induction conditions for 10 days. ALP staining was used to evaluate osteoblast production, Alizarin red was used to stain bone mineral nodules, and Oil Red O staining was used to assess adipocyte production. Original magnification, 400×.

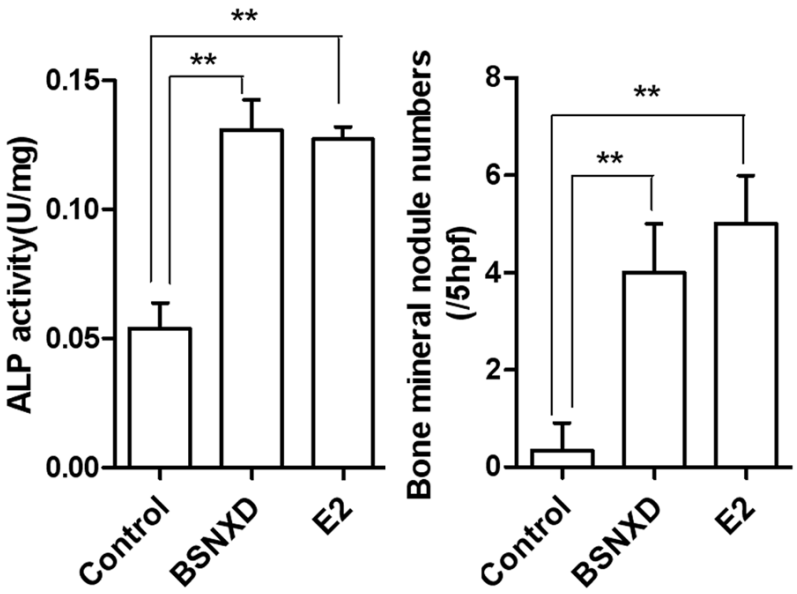

Figure 3. BSNXD increases ALP activity in osteoblasts. Primary MSCs were exposed for 48 h to control serum, 10% BSNXD-derived serum, or 10^{-9} M E2 in osteoblast induction conditions. The ALP activity of osteoblasts was determined using an ALP activity analysis kit. Data are expressed as the mean ± S.E.M. (n = 6). $^{**}P < 0.01$ compared with the control group.

bated with 800 ml acetic acid (10%) for 30 min. The supernatant was then transferred to a 1.5 ml tube and boiled for 10 min at 85°C, followed by 5 min on ice. After centrifugation (15 min at 15,000 g), supernatants (500 µl) were transferred into 1.5 ml tubes and mixed with 200 µl of 10% ammonium hydroxide. Samples were transferred to 96 well microtiter plates and the optical density was measured at 405 nm using a standard ELISA reader. *P*-values were calculated using student's t-tests to detect statistically relevant differences (n = 3 with two replicates each).

MSC-derived adipocytes

For adipogenic induction of MSCs, MSCs at the second or third passage were induced to form adipocytes using adipogenic induction media (α-MEM plus 10% FBS) for up to 12 days as determined by peak adipogenic expression. Then, media were changed to adipocyte maintenance media composed of high-glucose DMEM with 10 µg/ml insulin and 10% FBS, which promotes adipocyte maturity. Cultures were analyzed prior to adipocyte induction on day 0 and at specific time points during the 25-day time-course.

Oil Red O staining

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

Real-time PCR

Dnase-treated RNA was isolated from MSC-derived osteoblasts, adipocytes, enriched adipofibroblasts, and lipid laden adipocyte cultures at specific time points using the RNeasy Mini kit according to the manufacturer's instruc-

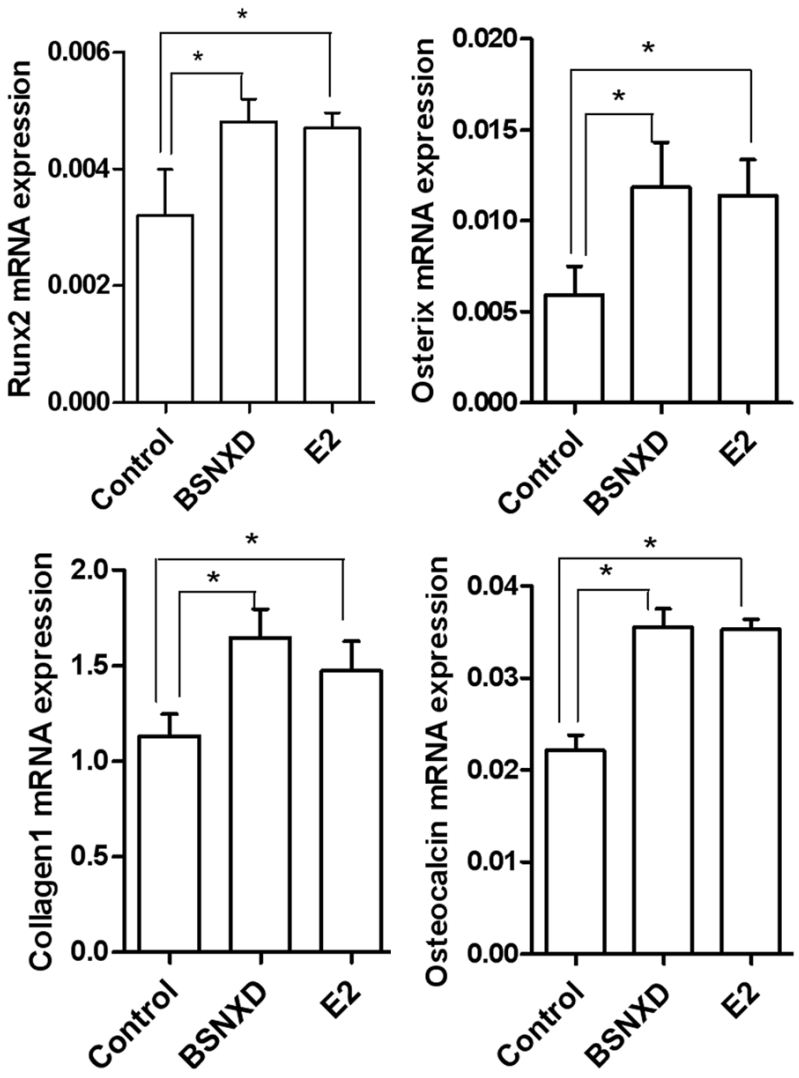

Figure 4. BSNXD regulates osteoblastogenesis-related gene expression. Primary MSCs were exposed for 48 h to control serum, 10% BSNXD-derived serum, or 10^{-9} M E2 in osteoblast induction conditions. *Runx2*, *osterix*, *collagen type I*, and *osteocalcin* mRNAs (a-d) were analyzed. Data are expressed as the mean ± S.E.M. (n = 6). *$P < 0.05$ compared with the control group.

tions (Qiagen). Cells were lysed in GITC-containing buffer (Buffer RLT). Reverse transcription was performed immediately after RNA isolation using the Transcriptor First Strand cDNA synthesis kit and oligo-dT primers (Roche, Branchburg, NJ). Real-time PCR was performed using Sybr Green and Taqman technology. Briefly, 10 ml SybrGreen Master Mix (Applied Biosystems, Darmstadt, Germany) was mixed with 1 ml (10 pg) of each primer, 6.8 ml water, and 1.2 ml (60 ng) template. mRNA expression was normalized to *b-actin* expression. Reactions were performed using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55-60°C for 30 sec, and 72°C for 30 seconds. Primer sequences are listed in **Table 1**.

Statistical analyses

All values are expressed as the mean ± standard error of the mean (S.E.M.). Data were analyzed using SPSS, and variance was evaluated using one-way analyses of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Effects of BSNXD on bone morphology

Compared with the sham group, bone volume (BV), bone mineral density (BMD), and trabecular bone number (Tb.N) in the OVX group were lower ($P < 0.05$), whereas trabecular bone spacing (Tb.sp) was wider ($P < 0.05$), suggesting the PMO model was successfully established. Compared with the OVX group, the OVX+BSNXD and OVX+E2 groups had higher BV, BMD, Tb.N, and thinner Tb.sp, suggesting that BSNXD and E2 improved bone morphology in PMO mice (**Figure 1**). There were no differences between the BSNXD and E2 group ($P > 0.05$).

MSCs can differentiate into osteoblasts and adipocytes

In the presence of osteogenic induction media, MSCs could differentiate into osteoblasts. This was verified using ALP staining for osteoblasts and Alizarin red staining for bone mineral nodules. When cultured in adipocytic induction media, MSCs could differentiate into adipocytes, as shown by Oil red O staining (**Figure 2**).

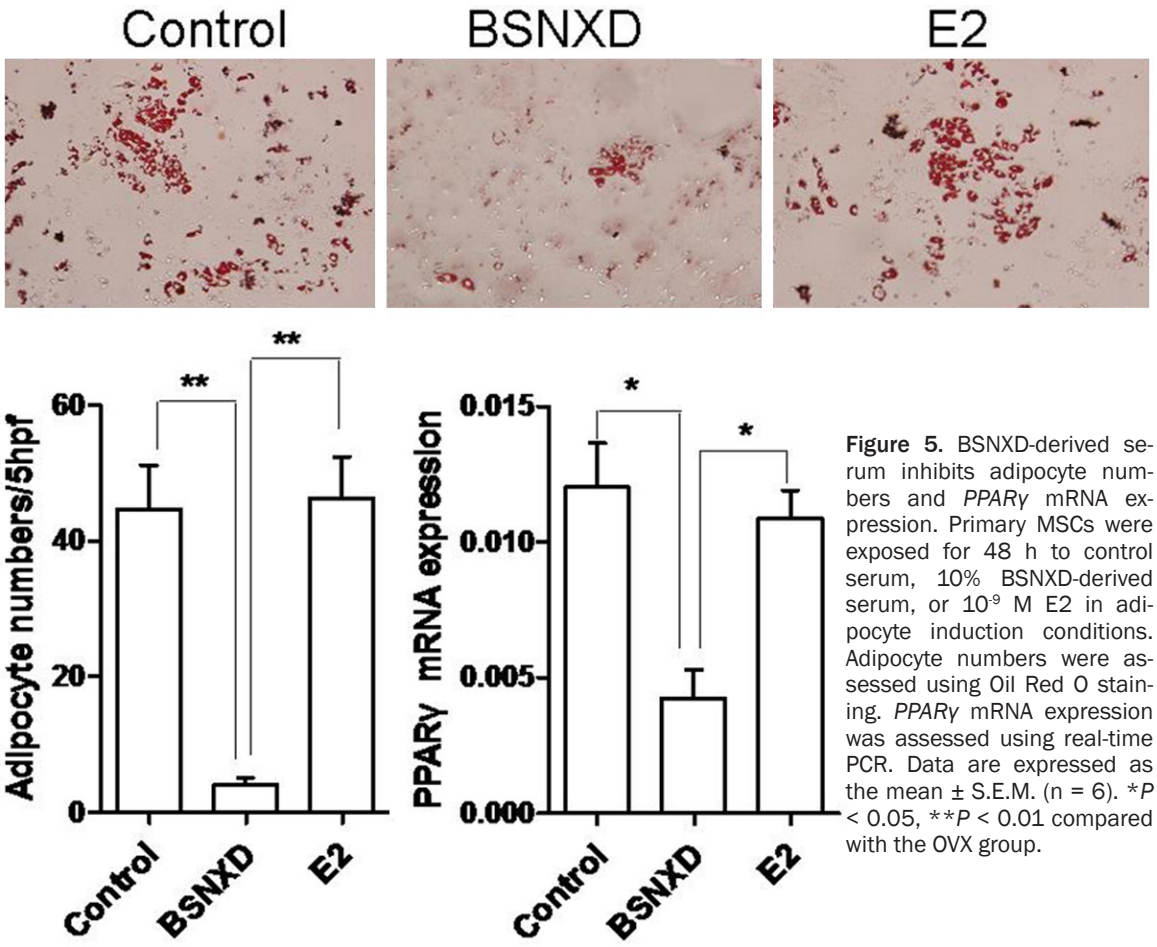

Figure 5. BSNXD-derived serum inhibits adipocyte numbers and *PPARγ* mRNA expression. Primary MSCs were exposed for 48 h to control serum, 10% BSNXD-derived serum, or 10^{-9} M E2 in adipocyte induction conditions. Adipocyte numbers were assessed using Oil Red O staining. *PPARγ* mRNA expression was assessed using real-time PCR. Data are expressed as the mean ± S.E.M. (n = 6). **P* < 0.05, ***P* < 0.01 compared with the OVX group.

BSNXD-derived serum increases ALP activity and bone nodular numbers

In osteogenic induction conditions, BSNXD-derived serum affected MSC differentiation into osteoblasts. We found that the ALP activity of osteoblasts in the BSNXD and E2 groups was higher than that of the serum control group. Bone nodular numbers were also increased in the BSNXD and E2 groups compared to the serum control group (**Figure 3**).

BSNXD-derived serum upregulates osteogenesis-related gene expression

In order to explore the regulation mechanism of BSNXD on MSC differentiation, we examined mRNA expression of osteogenesis-related genes using real-time PCR. Compared to the serum control treatment, BSNXD-derived serum and E2 promoted *collagen type I*, *osteocalcin*, *Runx2*, and *osterix* expression. However, there was no difference between these two groups (**Figure 4**).

BSNXD-derived serum suppresses adipocyte differentiation by inhibiting PPARγ expression

Our results show that MSCs can differentiate into osteoblasts and adipocytes. Increased adipocyte numbers indicate a significant risk of PMO occurrence. We cultured MSCs under adipocytic induction conditions and found no differences between the E2 and serum control group. However, there was less adipocyte production in the BSNXD group compared to the serum control group (**Figure 6**). We examined expression of the adipocytic transcriptional gene PPARγ using real-time PCR. Expression was suppressed in the BSNXD group compared to the serum control and E2 groups (**Figure 5**).

The effects of BSNXD on spleen immune cells

To study the interaction between immune cells and BSNXD, we first analyzed changes in the spleen-derived immune cells after BSNXD treatment. Compared to the sham group, Foxp3-positive Tregs were reduced in the OVX

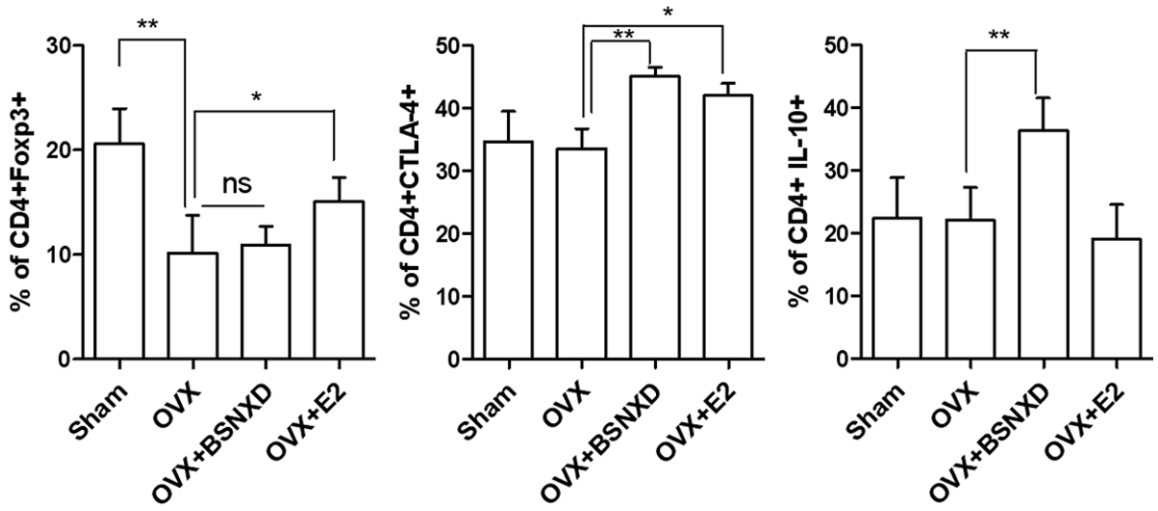

Figure 6. BSNXD affected the percentage of Foxp3$^+$, CTLA-4$^+$, and IL-10$^+$ in Tregs. Sham mice underwent a mock operation and received saline. Ovariectomized mice underwent bilateral oophorectomy and were randomly divided into three groups: OVX (treated with saline), OVX+BSNXD (treated daily with 5 ml mixed row herbs [BSNXD] per kg body weight), and OVX+E2 (treated daily with 5 ml E2 per kg body weight). Spleen samples were harvested after 12 weeks. FCM for immune cells was performed in spleens. Data are expressed as the mean ± S.E.M. (n = 6). *P < 0.05, **P < 0.01 compared with the OVX group.

group. Compared with the OVX group, BSNXD treatment did not change the percentage of Foxp3$^+$ Tregs, whereas E2 increased the percentage ($P < 0.05$). Conversely, BSNXD treatment increased the percentage of CTLA-4$^+$ and IL-10$^+$ cells but E2 did not significantly influence their numbers (**Figure 6**).

Tregs enhance BSNXD-derived serum effect on osteoblastogenesis

In order to determine the effects of Tregs on BSNXD-mediated MSC regulation, we cultured MSCs in the presence or absence of Tregs. In the presence of Tregs, ALP activity was higher and bone nodular production increased (**Figure 7**).

Discussion

Postmenopausal osteoporosis (PMO) is a common bone metabolic disease in postmenopausal women. Its primary characteristics are bone loss and bone microstructural changes [22]. The bone microstructure includes bone volume, bone mineral density, bone trabecular number, and trabecular bone spacing. This study selected ovariectomized female mice to simulate PMO and used different drugs to examine their effects on bone microstructure. Postmenopausal women lack estrogen, therefore, we used estrogen as a positive control and physiological saline lavage treatment in the sham group. Three months after the treatment, bone volume, bone density, and the bone trabecular numbers of ovariectomized mice were significantly decreased with concomitantly wider trabecular spacing. These data suggest the PMO model was successfully established [23-25]. In ovariectomized mice with BSNXD and E2 lavage treatment, bone mass, bone density, and bone trabecular numbers were significantly increased, whereas trabecular spacing was decreased, suggesting these compounds can significantly improve the bone tissue microstructure. There was no significant difference between estrogen and BSNXD treatment, suggesting both are effective for the prevention and treatment of PMO [26].

Osteoblasts and adipocytes are derived from MSCs. As osteoblast differentiation increases, adipocyte differentiation decreases. It has been proposed that increased adipocytes in the bone are a primary cause of PMO [27-30]. Osteoblasts are the most important cells in bone formation and function in PMO development. Thus, designing drugs that promote osteoblast generation is a novel strategy for the prevention and treatment of PMO.

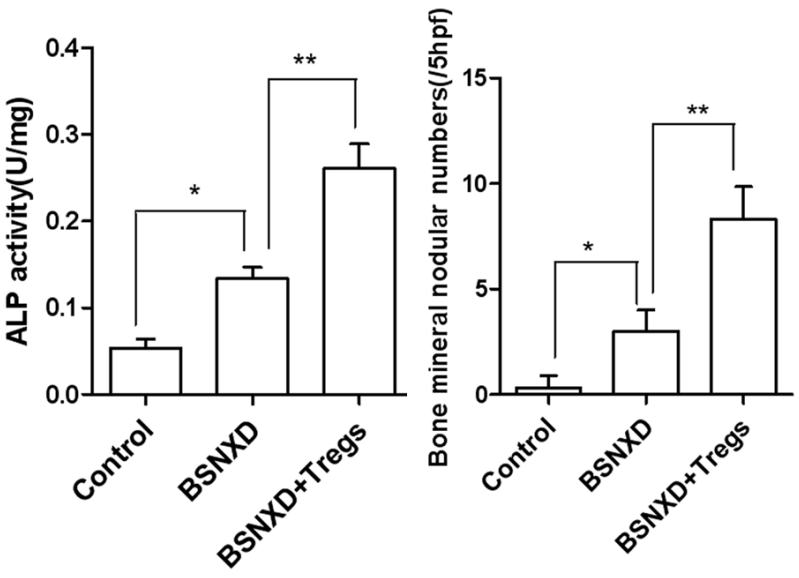

Figure 7. Treg cells enhance the effects of BSNXD-derived serum on ALP activity and the number of bone mineral nodules. Primary MSCs were exposed for 48 h to control serum, 10% BSNXD-derived serum, or 10^{-9} M E2 in osteoblast induction conditions in the presence or absence of Tregs. ALP activity of osteoblasts was determined using an ALP activity analysis kit. The number of bone nodules was assessed using Alizarin red staining. Data are expressed as the mean ± S.E.M. (n = 6). $*P < 0.05$, $**P < 0.01$.

We also found that BSNXD-derived serum increased osteoblast ALP activity, promoted the formation of osteoblasts, and accelerated osteoblast differentiation of MSCs. These results are consistent with the animal experiments that found that BSNXD increased bone volume, bone density, and bone trabecular numbers. Compared with the E2 group, there were no obvious differences in promoting osteoblast generation.

The generated osteoblasts include three different stages: the growth stage, the mature stage, and the mineralization stage. Different stages have different marker genes. For example, the growth and mature stage osteoblasts primarily express *collagen type* I, whereas mineralization stage osteoblasts express *osteocalcin* [31-34]. Osteoblast formation also requires transcription factors such as *Runx2* and *osterix* [35, 36]. We evaluated the expression of the osteoblastogenesis-related genes *collagen type I*, *osteocalcin*, *Runx2*, and *osterix* using real-time PCR. Compared with the control serum group, BSNXD-derived serum treatment and E2 treatment increased *collagen type I*, *osteocalcin*, *Runx2*, and *osterix* mRNA expression. These data illustrate that BSNXD and E2 participate in all stages of osteoblast development and accelerate osteoblast differentiation of MSCs.

Here, we evaluated the effect of BSNXD on MSCs' differentiation into adipocytes. Compared with the control group, the number of adipocytes in the BSNXD-derived serum group was reduced. The E2 treatment group did not significantly differ, compared with the control group. Thus, BSNXD-derived serum can inhibit MSC differentiation into adipocytes. PPARγ is a key transcription factor that modulates adipocyte generation. Real-time PCR results showed that *PPARγ* mRNA expression is lower in the BSNXD-derived serum group compared to the control and E2 groups. These results are also consistent with cellular staining results.

We cultured MSCs in the presence or absence of Tregs. In the presence of Tregs, there was higher ALP activity and more bone nodule production, suggesting that Tregs promoted MSC differentiation into osteoblasts [37, 38]. In order to define the role of Tregs, we also evaluated the effect of Tregs after treatment with BSNXD and E2. We found that $Foxp3^+$ Tregs did not increase with BSNXD treatment, whereas CTLA-4^+ and IL-10^+ Tregs did increase. These data suggest that BSNXD plays a role through both direct and indirect contact. In the presence of E2, there was a significant increase in $Foxp3^+$ and CTLA-4^+ Tregs, suggesting E2 functions through direct contact.

In summary, BSNXD improved bone volume and bone microstructure in an ovariectomized mouse model of PMO. It also promoted MSC differentiation into osteoblasts and inhibited differentiation into adipocytes. Tregs enhanced the effect of BSNXD on osteoblastogenesis. Based on our data, we propose that BSNXD is superior to estrogen in promoting MSC differentiation into osteoblasts, therefore, it presents

potential therapeutic use for the prevention of PMO.

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Disclosure of conflict of interest

None.

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