Promotion of Osteogenesis by Sweroside via BMP-2 Related Signaling in Postmenopausal Osteoporosis

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Abstract

Phlomis umbrosa has been traditionally used for bone diseases such as bone fracture and rheumatism in traditional Korea Medicine. Sweroside (SOS), which is one of the active compounds of *P. umbrosa*, has been known to promote osteoblast differentiation. In this study, ameliorative effects of SOS on osteoporosis and potential target pathway were investigated. Ovariectomized mice were administered 3 doses of SOS for 4 weeks after inducing osteoporosis. Bone mineral content (BMC) and bone mineral density (BMD) were analyzed by dual energy X-ray absorptiometry. SaOS-2 osteoblasts were differentiated to clarify the promoting effects of SOS on osteoblast differentiation and bone formation. Osteoblastic bone-forming markers were evaluated by RT-PCR in lumbar vertebrae (LV) and mineralized SaOS-2 cells. Treatment of SOS increased BMC and BMD levels. SOS markedly attenuated the bone marrow adipocytes in the central bone cavity of the femoral shaft. SOS increased the formation of bone matrix in SaOS-2 cells. BMP-2 and RUNX2 in LV and SaOS-2 cells were up-regulated by treating with SOS. BMP-2/RUNX2-activated ALP, OPN and BSP-1 expressions were increased by SOS. In conclusion, SOS induced the formation of mineralized bone matrix by regulating BMP-2/RUNX2-mediated osteoblastic molecules. Therefore, SOS could be a therapeutic active compound of treatment for osteoporosis by producing the new bone matrix.

1. Introduction

Osteoporosis is characterized by low bone mass and expansion of medullary cavity, resulting in bone fracture of wrist, vertebrae and hip by even minimal bump [1]. Etiology of osteoporosis is associated with several external factors such as genetics, smoking, inflammation, environmental toxins and hormone [2]. Especially, deficiency of hormone such as estrogen, one of those factors, is a main inducer in 40% post-menopausal women of aged over 50 since estrogen is reported to reduce osteoclast formation as well as enhance osteoclast apoptosis [3]. Bone homeostasis is known to be regulated by osteoblasts and osteoclasts simultaneously [4]. After bone breakdown by the process of osteoclasts-mediated bone resorption, osteoblasts derived from mesenchymal stem cells cover the outer bone surface and release the organic matrix, enzyme, growth factors and hormones to form new mature bone [5]. In the process of the reorganization and mineralization of osteoblasts, various cellular mechanisms are responsible for bone synthesis including bone morphogenetic protein 2 (BMP-2), notch signaling, Wnt pathway and transforming growth factor-β (TGF-β) signaling [6]. Those osteoblastic molecular mechanism thus is likely to be a therapeutic target in bone loss.

Several medications are used as treatments of osteoporosis such as calcium, vitamin D, estrogen and selective estrogen receptor modulators (SERMs)[7]. Generally, patients suffered from osteoporosis receive calcium and vitamin D as supplements, which couldn't completely treat osteoporosis, only delay its progression [8,9]. Hormone replacement therapies (HRT) are used for postmenopausal woman to reduce bone loss [10], but evidences have constantly suggested that HRT increases the risks of thrombosis and breast cancer [11]. Alternative use of selective estrogen receptor modulators such as raloxifene contributes

to decrease the risks of HRT with maintaining effect of bone mineral density (BMD) and bone strength [12]. Despite of this optimistic effect, there are remains of side effect issues for hot flushes and leg cramps [13]. Previous studies regarding on bone-related treatments for osteoporosis are focused on down-regulations of osteoclast rather than up-regulations of osteoblast [14,15]. To reduce osteoclastic activity, bisphosphonates such as alendronate, risedronate, ibandronate and zoledronate, which have efficacy of skeletal anti-resorption in human clinical studies, are developed, but serious issues including gastrointestinal disorder, muscle pain and osteonecrosis of jaw have been raised by its long-term use [16]. First-developed osteogenic agent, sodium fluoride, is found to increase the number of osteoblast and deposition of bone matrix [17,18]. However, new drug development using sodium fluoride is consequentially failed because of abnormal and unmineralized matrix [19]. These issues demonstrated that bone anabolic therapy should contribute to matrix mineralization as well as osteoblast number, finally leading to increases of bone mass and strength.

Phlomis umbrosa has been traditionally used to treat the bone disease in Korean Medicine. Sweroside (SOS), belongs to cyclopentanopyran, is one of the active compounds of *P. umbrosa*, also found from *Nymphoides indica*, *Lonicera japonica*, and *Cornus offcinalis*[20–22]. In addition, SOS is used as a marker of *P. umbrosa* in the Korean Pharmacopoeia. SOS is known to have liver protective, wound healing, anti-nociceptive, anti-inflammatory and anti-allergic effects [23]. Previous reports showed that SOS promotes osteogenic differentiation of bone marrow mesenchymal stem cells [24]. Other study has demonstrated that SOS induces proliferation and differentiation and inhibits apoptosis in human osteosarcoma MG-63 cells and rat osteoblasts [21]. Those previous studies demonstrated the potentials of SOS on osteoblast differentiation, however, there is a lack of evidence for specific target pathway to form bone mineralized matrix. This study revealed the ameliorative effects of SOS on bone mineralization in osteoporosis and its underlying mechanism, providing a useful therapeutic target for osteoporosis.

2. Results

Assessment of BMC and BMD on whole body, femora and LV in OVX-induced osteoporotic mice

BMC and BMD were measured using DXA to determine value of bone minerals on experimental mice in total body and 2 specific regions including femora and L4-L6 vertebrae. In terms of total body BMC and BMD, OVX-induced osteoporotic mice showed significant reduction compared to sham-operated mice. The body BMC values in OVX group (0.8001 ± 0.0250 g) was 0.156 g lower than sham group (0.956 ± 0.0250 g), while E2 (0.882 ± 0.0817 g) and SOS 0.01, 0.1 and 1 μM groups (0.887 ± 0.0871 g, 0.888 ± 0.0880 g and 0.889 ± 0.0891 g, respectively) were significantly higher than OVX group (Fig. 1A). The value of body BMD in sham group (0.030 ± 0.0006 g/cm^2) was 0.006 g/cm^2 higher than OVX group (0.025 ± 0.0008 g/cm^2). Administration of E2 increased the body BMD level by 0.031 ± 0.0005 g/cm^2. SOS at all concentrations up-regulated the body BMD levels to 0.029 ± 0.0045 g/cm^2, 0.030 ± 0.0005 g/cm^2 and 0.029 ± 0.0014 g/cm^2, respectively, compared to OVX group (Fig. 1A).

In terms of femoral bone mineral level, the femoral BMC were 0.006 g reduced in OVX group (0.040 ± 0.0010 g) compared to sham group (0.046 ± 0.0005 g). Injection of E2 increased the 0.004 g BMC level (0.044 ± 0.0007 g). Administration of SOS with 0.01, 0.1 and 1 μM improved the BMC levels (0.0417 ± 0.0011 g, 0.043 ± 0.0004 g and 0.044 ± 0.0008 g), respectively (Fig. 1B). The levels of femoral BMD in sham group was 0.119 ± 0.0022 g/cm^2, while that in OVX group was 0.107 ± 0.0024 g/cm^2. E2 group showed recovery of femoral BMD level (0.123 ± 0.0025 g/cm^2). In addition, SOS treatment at all concentrations increased the BMD levels (0.111 ± 0.0024 g/cm^2, 0.122 ± 0.0019 g/cm^2 and 0.121 ± 0.0027 g/cm^2), respectively (Fig. 1B).

Additionally, OVX group exhibited a significant reduction of BMC level in L4-L6 vertebrae (0.025 ± 0.0019 g) compared to sham group (0.038 ± 0.0024 g). Injection of E2 significantly increased the BMC level of LV region (0.038 ± 0.0038 g). Administration of SOS at all concentrations markedly improved the value of BMC by 0.031 ± 0.0014 g, 0.033 ± 0.0017g and 0.0292 ± 0.0004 g, respectively (Fig. 1C). The BMD level of LV in sham group was 0.076 ± 0.0009 g/cm^2 and that in OVX group was 0.055 ± 0.0008 g/cm^2. E2 treatment significantly increased by 49.11% the value of BMD in LV (0.076 ± 0.0057 g/cm^2). The BMD levels in L4-L6 vertebrae in SOS-treated groups were 0.058 ± 0.0016 g/cm^2, 0.067 ± 0.0009 g/cm^2 and 0.067 ± 0.0007 g/cm^2, showing 6.17%, 21.05% and 22.45% recoveries, respectively, compared to OVX group (Fig. 1C).

Assessment of histomorphometrical changes in OVX-induced mice

H&E staining revealed well-formed bone marrow with a little bone marrow adiposity in normal femoral bone. In OVX-induced osteoporotic mice, bone marrow adipose tissues were markedly increased at the medullary cavity of femoral shaft. Treatment with 1 μM SOS apparently improved the increase of bone marrow fat in OVX mice (Fig. 2).

Analysis of BMP-2 expression by RT-PCR in OVX-induced mice

BMP-2 attributes to regulation of osteogenic differentiation and bone formation. As illustrated in Fig. 3A, expression mRNA level of BMP-2 was sharply declined in OVX group by 41.65% compared with sham group. On the contrary, E2 treatment slightly increased BMP-2 mRNA level compared to OVX group. mRNA levels of BMP-2 in SOS-treated groups, especially SOS SOS 1 μM group were elevated by 70.68% compared to OVX group (Fig. 3A).

Assessment of RUNX2 expression by RT-PCR in OVX-induced osteoporotic mice

Expression mRNA level of RUNX2, promoted by BMP-2, was decreased in OVX group by 61.15 ± 15.49% compared to sham group. On the other hand, E2 treatment showed a significant increase about 62% on expression mRNA level of RUNX2 compared with OVX group. Also, mRNA level of RUNX2 was recovered from SOS 0.01, 1 μM treatment by 56.70 ± 16.07% and 108.78 ± 11.23% compared to OVX group (Fig. 3B).

Assessment of bone specific matrix genes by RT-PCR in OVX-induced osteoporotic mice

Bone specific matrix genes, ALP, OPN and BSP-1, were known as turnover and maturation of osteo-relative cells [25]. Expression mRNA levels of ALP and OPN were promoted by injection of E2 (68.98 ± 6.31% and 49.7 ± 36.69%) contrary to OVX group. Compared with OVX group, particularly SOS 1 μM treatment improved mRNA levels of ALP and OPN (112.03 ± 9.11% and 71.51 ± 4.53%), approaching approximate levels of sham group. In comparison with OVX group, gene expression mRNA levels of BSP-1 were increased in equal levels with sham group in E2 (72.26 ± 19.00%) and SOS-treated group (95.88 ± 14.8%, 98.62 ± 4.63% and 105.78 ± 5.22%, respectively) (Fig. 3C).

Assessment of osteoblastogenesis in AA + β-GP-induced SaOS-2 cells

SaOS-2 cells were differentiated with AA and β-GP and stained with ARS to identify extracellular calcium deposition [26]. By contrast with non-treated group, AA + β-GP-induced group were dramatically increased in calcium deposition by 10.5 folds. In addition, SOS co-treated with AA + β-GP group were highly differentiated by 1.44%, 2.57% and 4.11%, respectively in dose-dependent manner compared to single treated with AA + β-GP group (Fig. 4A, 4B). The effect of SOS on SaOS-2 cells viability were determined by MTT assay. After treatment with concentrations of SOS 1, 10, 100 nM for 24h, there was no significant difference in all concentrations of SOS on SaOS-2 cells (Fig. 4C).

Assessment of BMP-2 and RUNX2 expression by RT-PCR in AA + β-GP-induced SaOS-2 cells

To demonstrate the effects of SOS on BMP-2 and RUNX2, which are facilitate osteoblastogenesis through differentiation of osteoblast cells, mRNA expression levels were analyzed by RT-PCR. As showed in Fig. 5A, expression mRNA level of BMP-2 was increased by 56.11% in differentiated with AA + β-GP group compared with non-differentiated group. SOS co-treated with AA + β-GP groups were considerably elevated by 1.6 folds, 1.2 folds and 2 folds, respectively compared to AA + β-GP group. Also, expression mRNA level of RUNX2 was increased by 79.73% in AA + β-GP-treated group compared with non-treated group. Especially, SOS 100 nM treatment was markedly increased mRNA level of RUNX2 about 117% compared to AA + β-GP group (Fig. 5B).

Assessment of bone specific matrix genes by RT-PCR in AA + β-GP-induced SaOS-2 cells

To evaluate the effects of SOS on ALP, OPN and BSP-1, which are key factors of bone turnover and maturation were measured by RT-PCR. Expression mRNA levels of ALP, OPN and BSP-1 were increased by 52.05%, 56.78% and 81.1%, respectively in differentiation of SaOS-2 cells treated with AA + β-GP. The three of bone specific genes, SOS treatment prominently improved expression mRNA levels of ALP, OPN and BSP-1 in dose-dependent manner. In particular, SOS 100 nM has remarkable effects on mRNA levels of ALP, OPN and BSP-1 that were increased by 7.7 folds, 4.3 folds and 3.8 folds, respectively (Fig. 5C).

3. Discussion

The integrity and mass of bone is maintained by a physiological bone remodeling process supervised by bone-resorbing osteoclasts and bone-forming osteoblasts [27]. Bone formation by osteoblastic cells contributes to bone remodeling, fracture healing and embryonic developments [28]. The impaired osteoblastic bone formation, caused by hormones, growth factors and cellular mechanisms, results in decreases of newly formed trabecular bone [29]. Hence, abnormal and unmineralized bone due to osteoblast inactivation in osteoporosis exhibits loss of bone minerals and the collapse of bone microstructure [30]. Especially, low BMD level is a main characteristic of osteoporosis. -2.5 or lower T-score on BMD is a standard for diagnosis of osteoporosis in human [31]. Definition of osteoporosis are based on results from values of BMD on total body and specific regions such as femur, lumbar spine, wrist and hip [32]. Additionally, recent reports have revealed that increase of bone marrow fat is found in osteoporotic patients. The bone cavity consists of 85% bone marrow and the remaining trabecular bones [33]. As development of bone loss in osteopenia or osteoporosis, bone marrow adipocyte is known to reduce the bone quantity and quality by suppressing osteoblastogenesis [34]. In this study, BMC and BMD levels of whole body, femur and LV were significantly decreased compared to normal bones. SOS administration markedly increased the levels of BMD as well as BMC on whole body, femur and LV. And treatment with 0.1 and 1 mM of SOS markedly attenuated the adipose tissues of bone marrow in medullary cavity compared to OVX group. These results demonstrate that SOS ameliorated osteoporosis. In addition, the cell mineralization stained by Alizarin Red S solution was dose-dependently increased in SOS-treated groups. These data suggested that SOS accelerated the differentiation of osteoblasts for further mineralization.

In previous study, *P. umbrosa* possessed the osteogenic effects by increasing RUNX2 expression [22]. Because SOS is a marker for quality control of *P. umbrosa* in the Korean Pharmacopoeia, we confirmed whether SOS is an effective compound of *P. umbrosa* for treating osteoporosis to investigate the underlying mechanisms of osteoblasts-induced bone formation. RUNX2 is known to contribute to differentiation and maturation of osteoblasts from mesenchymal stem cells at early stage [35]. Although BMP-2, notch signaling, Wnt pathway and transforming growth factor-β (TGF-β) are involved in osteoblast differentiation, we assumed SOS could influence BMP-2/RUNX2 signaling pathway in osteoporosis based on the previous report that *P. umbrosa* up-regulated the expressions of BMP-2 in bones [36]. BMP-2 has been reported to promote the differentiation and maturation of osteoblasts from mesenchymal progenitor cells following the initiation of transcription of RUNX2. And this response activates the ALP, OPN and BSP-1 [37], which act to enhance the osteoblastic mineralization [35]. Bone anabolic molecules, ALP, OPN and BSP-1, promote bone matrix apposition at late stage of bone mineralization [38]. ALP, an earliest enzyme of osteoblast differentiation and mineralization, is present in 50% of mineralized matrix and 50% of blood [39]. ALP levels were diminished in either bloodstream or bone of patients suffered from osteoporosis. OPN is composed non-collagenous bone matrix as a regulator of bone remodeling and bone strengthen. During the process of osteoblast differentiation and osteogenesis, BSP-1 leads to assemble calcium in mineralized matrix [35]. In current study, BMP-2 mRNA expression was decreased in OVX mice and that was reversed by SOS treatment with increase of RUNX2. Likewise, SOS

treatment also increased BMP-2 and RUNX2 mRNA levels in differentiated SaOS-2 cells. The molecular mechanism of SOS on BMP-2/RUNX2 signaling pathway was verified by additional experiments, that SOS significantly increased the activation of Smad-4 and Smad-5 (Fig. S3), intracellular transducers of BMP-2 signal to participate in bone metabolism [40]. The mRNA level of ALP in OVX mice was recovered by SOS treatment to normal level. Also, the ALP mRNA expressions in differentiated SaOS-2 cells were raised by SOS treatment. Following that, *in vivo* and *in vitro* experiments showed that SOS treatment up-regulated the mRNA levels of OPN and BSP-1 in both bone tissues and differentiated SaOS-2 cells. Especially, administration with 1 μM of SOS showed increases of bone-forming molecules including RUNX2, ALP, OPN and BSP-1 as much as E2. However, the expressions of those osteogenic biomarkers in SOS-treated groups were not increased much higher than the sham group. In addition, there is no sign of toxicity throughout the whole experiment and the body weights were observed on a certain level or steadily increase. Based on the evidences, SOS is believed to be used as an alternative for osteoporosis as a derived from natural compound without side effects, while other drugs for osteoporosis has been reported to induce adverse effects such as breast cancer risk of E2.

In the previous literatures, SOS as a one of the cyclopentanopyran regulated the ALP activity, type collagen expression and osteocalcin secretion with its inhibitory effects of apoptosis of osteoblasts. Recently, there is report that sweroside induced the osteogenic differentiation by mTORC1 signaling pathway with up-regulation of BUNX2, osterix and OCN (Ref. J Cell Biochem. 2019;120(9):16025–16036). In this study, SOS increased the mineralization of bone matrix and up-regulated the mRNA levels of BMP-2 and its series transcriptional factors including RUNX2, ALP, OPN and BSP-1. From those evidences, we assumed that osteogenic effects of SOS could be associated with the RUNX2 and ALP mRNA expressions and BMP-2/RUNX2 signaling pathway would be one of its underlying mechanisms.

Taken together, SOS treatment markedly recovered the decrease of BMD level with bone marrow reconstitution of medullary cavity in osteoporotic mice. SOS increased the mRNA level of BMP-2 and its down-stream molecules including RUNX2, ALP, OPN and BSP-1 in both of OVX-induced bone tissue and mineralized osteoblasts. Regulation of BMP-2/RUNX2 signaling molecules by SOS induced the construction of bone mineralized matrix. SOS would be prospective compound derived from natural products for treating osteoporosis.

4. Methods

Animal experiment

5-week-old female ICR mice were purchased from Raon Bio Inc. (Yongin, Korea). Mice were acclimated under controlled animal facility with 22 ± 2℃ of temperature, 50 ± 5% of humidity and light/dark cycle. Thirty-five mice were undergone OVX surgery to induce post-menopausal osteoporosis. The rest 7 mice were sham-operated as a normal control group (Sham). After 12 weeks, all mice were divided 6 groups (n = 7); sham-operated mice as a normal control group (Sham), OVX mice as a negative control group (OVX), OVX mice treated with 10 μg/kg of 17β-estradiol as a positive control group (E2), OVX mice treated with

0.01, 0.1 and 1 μM of SOS ((SOS0.01), (SOS0.1) and (SOS1)). SOS was purchased from Sigma-Aldrich (Cat. No. PHL89802; MO, USA). 1 mM stock solution of SOS was prepared in dimethyl sulfoxide (Sigma-Aldrich) and dissolved in saline. 100 μL per mice of sample were intraperitoneally injected once a day for 5 days per week. During whole animal procedures, body weights were measured once a week to check the condition of mice. After 4 weeks of treatment period, all mice were sacrificed under anesthesia with avertin (Sigma-Aldrich). The blood was collected to obtain serum and uterus, tibia, femur and lumbar vertebrae (LV) was dissected right after sacrifice. All experiments were performed according to the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by Committee on Care and Use of Laboratory Animals of Kyung Hee University (KHUASP(SE)-18-071). The animal study was carried out in compliance with the ARRIVE guidelines.

Dual energy X-ray absorptiometry

The levels of bone mineral content (BMC) and bone mineral density (BMD) were analyzed by dual energy X-ray absorptiometry (DXA; Medikors, Seongnam, Korea). The value was indicated by g for BMC and g/cm^2 for BMD. Before sacrifice, alive mice were anesthetized and measured whole body BMC and BMD. In addition, extracted tibiae, femora and LV (L4-L6 vertebrae) were determined BMC and BMD in detail.

Bone Histomorphometry

The left femurs were fixed in 10% neutralized formalin for 24 h. Femurs were washed with distilled water and demineralized with 0.1 M ethylene diamine tetra acetic acid solution for 2 weeks. After decalcification, femurs were dehydrated by series concentration gradient of ethanol and xylene. Middle shafts of femur were embedded in paraffin and sagittal sections of the femoral body were cut at 10 μm thickness on gelatin-coated slides. Bone marrow adipocytes of the medullary cavity in the femoral shaft were stained with hematoxylin and eosin solution. The central area with bone marrow between compact bone (woven bone; width of bone cavity = 222.5 μm) were monitored under a ZEN-blue edition software (ZEN 2.6., Carl Zeiss Microscopy GmbH, Thornwood, NY, USA) to confirm the bone structure.

Osteoblast-like cells culture

SaOS-2 human osteoblast-like cells were obtained from Korean Cell Line Bank (Seoul, Korea). Cells were cultured Dulbecco's modified minimal essential medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin at 37℃ in a 5% CO2 of humidity atmosphere.

MTT assay

The SaOS-2 cells were seeded 96-well plates at 1×10^4 cells/well and allowed to stabilize for 24 h. Cells were treated with serum-free medium contain 3 different doses of SOS (1, 10 and 100 nM) for next 24 h. To reduce the interruption by cell differentiation and growth of SaOS-2 cells, serum-free/starvation was incubated for 24 h. Sample medium was removed and 2 mg/mL of MTT solution was added into each

well for 2 h. The supernatant was discarded and DMSO was treated to dissolve formazan crystals for 30 min. The optical density was detected at 570 nm using ELISA microplate reader (BioTek, PA, USA).

Osteoblasts differentiation analysis

SaOS-2 cells were seeded 6-well plates 0.8×10^5 cells/well. Differentiation media consists of DMEM supplemented with 10% FBS, 1% penicillin, 50 μg/mL L-ascorbic acid (AA) and 10 mM β-glycerophosphate (β-GP) was incubated into cells in presence or absence of 1, 10, 100 nM SOS to confirm the maturation and mineralization of osteoblasts [41]. Differentiation media containing SOS was changed every 3 days for all 9 days. At the end of the differentiation, the mineralized cells by differentiation media were rinsed with phosphate-buffered saline without calcium and magnesium. 10% neutralized formalin was used for fixation of cells at least 30 min. After washing 2 times, the cells were stained with 40 mM alizarin red solution (ARS) for 10 min at room temperature. All differentiated and un-differentiated cells were visualized under a microscope. Then, the extraction solution including 20% methanol and 10% acetic acid was incubated into cells for 2 h and collected to detect the value of 450 nm wavelength using a microplate reading instrument.

RT-PCR analysis

Excised LV were pulverized in liquid nitrogen and incubated in Trizol (Invitrogen Corp., Carlsbad, CA, USA) for overnight at 4°C. The bone tissues in Trizol were homogenized and extracted ribonucleic acid (RNA). Differentiated SaOS-2 cells treated with SOS were prepared according to the above experiments. The cells were treated with Trizol and harvested to extract RNA. Complementary DNA was synthesized from 2 μg RNA of LV and mineralized SaOS-2 cells at 45°C for 1 h and 95°C for 5 min using Maxime RT premix kit (Invitrogen). The levels of BMP-2 (*BMP2*), RUNX2 (*CBFA1*), ALP (*ALPL*), OPN (*SPP1*), BSP-1 (*BSPH1*) and GAPDH were amplified using Maxime PCR premix kit (Invitrogen). The expressions of each genes were detected by 1% agarose gel and normalized to GAPDH. Visualized genes were analyzed by Image J software (ver.1.8.0. National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical significance was determined by unpaired one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure. Data from three independent experiments (n = 3). In all analyses, $p < 0.05$ was taken to indicate statistical significance.

Declarations

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; L.Y.C., M.H.K., J.M.H. and W.M.Y. contributed to analysis design, L.Y.C. analyzed data, L.Y.C, M.H.K and WMY drafted the manuscript, and WMY provided supervision of study. All authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no competing interests.

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Supplemental Information

Supplementary Figure S1. Effect of sweroside on osteoblastic markers in lumbar vertebrae mice. Full-length gel of osteoblast differentiation initiation marker was analyzed by RT-PCR.

Supplementary Figure S2. Effect of sweroside on osteoblastic markers in differentiated SaOS-2 cells. Full-length gel of osteoblast differentiation initiation marker was analyzed by RT-PCR.

Supplementary Figure S3. Effect of sweroside on mediators (smad) of BMP-2/RUNX2 signaling in lumbar vertebrae. Significance was determined by one-way ANOVA with Tukey's multiple comparisons test. Results are presented as mean ± S.E.M $^{\#\#}$ and $^{\#\#\#}$ indicates the mean differs significantly between sham group and OVX group ($p < 0.01$ and $p < 0.001$, respectively). ** and *** indicates the mean differs significantly between SOS-treated or E2 group and OVX group ($p < 0.01$ and $p < 0.001$, respectively). Data from three independent experiments (n = 3). The band density was quantified using ImageJ software (ver.1.8.0). OVX, ovariectomized group; E2, 17β-estradiol group.

Supplementary Figure S4. Effect of sweroside on mediators (smad) of BMP-2/RUNX2 signaling in lumbar vertebrae. Full-length gel of mediators (smad) of BMP-2/RUNX2 signaling was analyzed by RT-PCR.

Figures

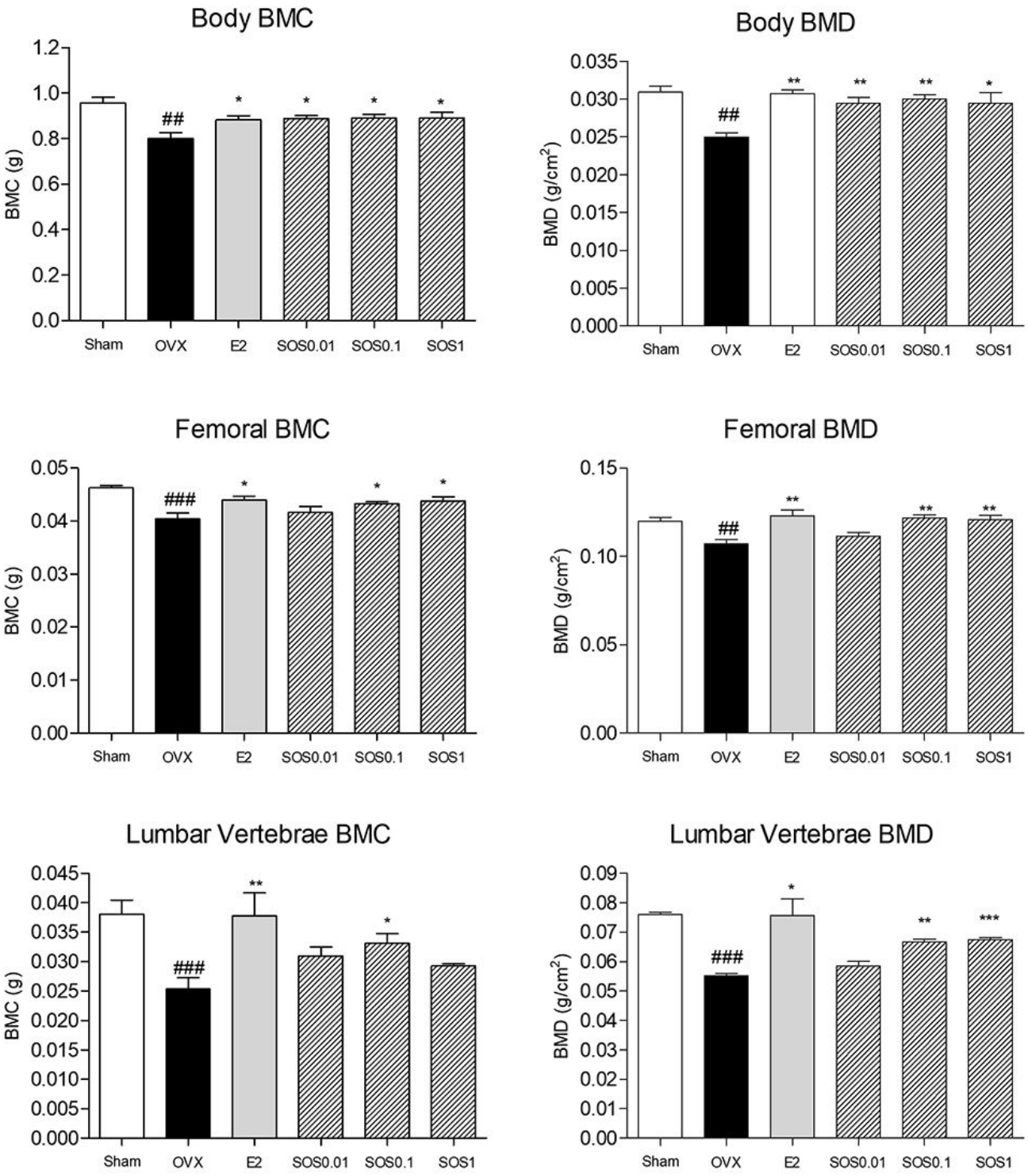

Figure 1

Effect of sweroside on BMC and BMD in ovariectomized mice. Bone mineral content and bone mineral density of (A) body (n = 7), (B) both sides of femora (n = 14) and (C) lumbar vertebrae (n = 7) were analyzed by DXA. To confirm the efficacy of SOS, osteoporotic mice were treated with SOS for 4 weeks. SOS was treated as 0.01, 0.1 and 1 μM. Significance was determined by one-way ANOVA with Tukey's multiple comparisons test. Results are presented as mean ± S.E.M ## indicates the mean differs

significantly between sham group and OVX group ($p < 0.01$). *, ** and indicates that the mean differs significantly between SOS-treated or E2 group and OVX group ($p < 0.05$ and $p < 0.01$, respectively). OVX, ovariectomized group; E2, 17β-estradiol group; LV, lumbar vertebrae; BMC, bone mineral content; BMD, bone mineral density.

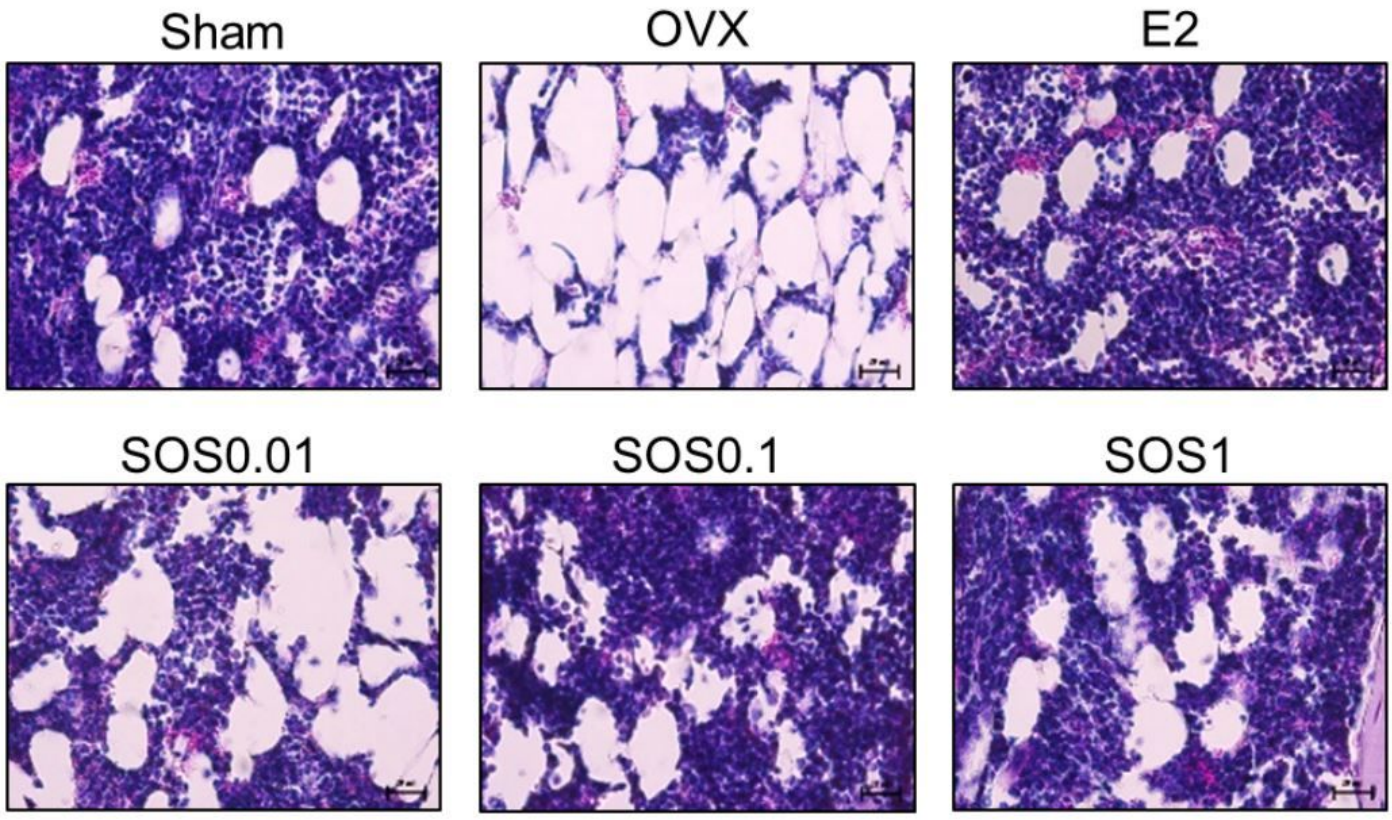

Figure 2

Effect of sweroside on histomorphometrical changes in ovariectomized mice (n = 7, each group). Evaluation of histomorphometrical changes using hematoxylin and eosin staining (magnification 400×, 20 μm). SOS was treated as 0.01, 0.1 and 1 μM. Experiments were performed in all osteoporotic mice with similar results. The images were captured by ZEN-blue edition software (ZEN 2.6., Carl Zeiss Microscopy GmbH, Thornwood, NY, USA). The representative images show the average of all experimental mice. OVX, ovariectomized group; E2, 17β-estradiol group; SOS, Sweroside.

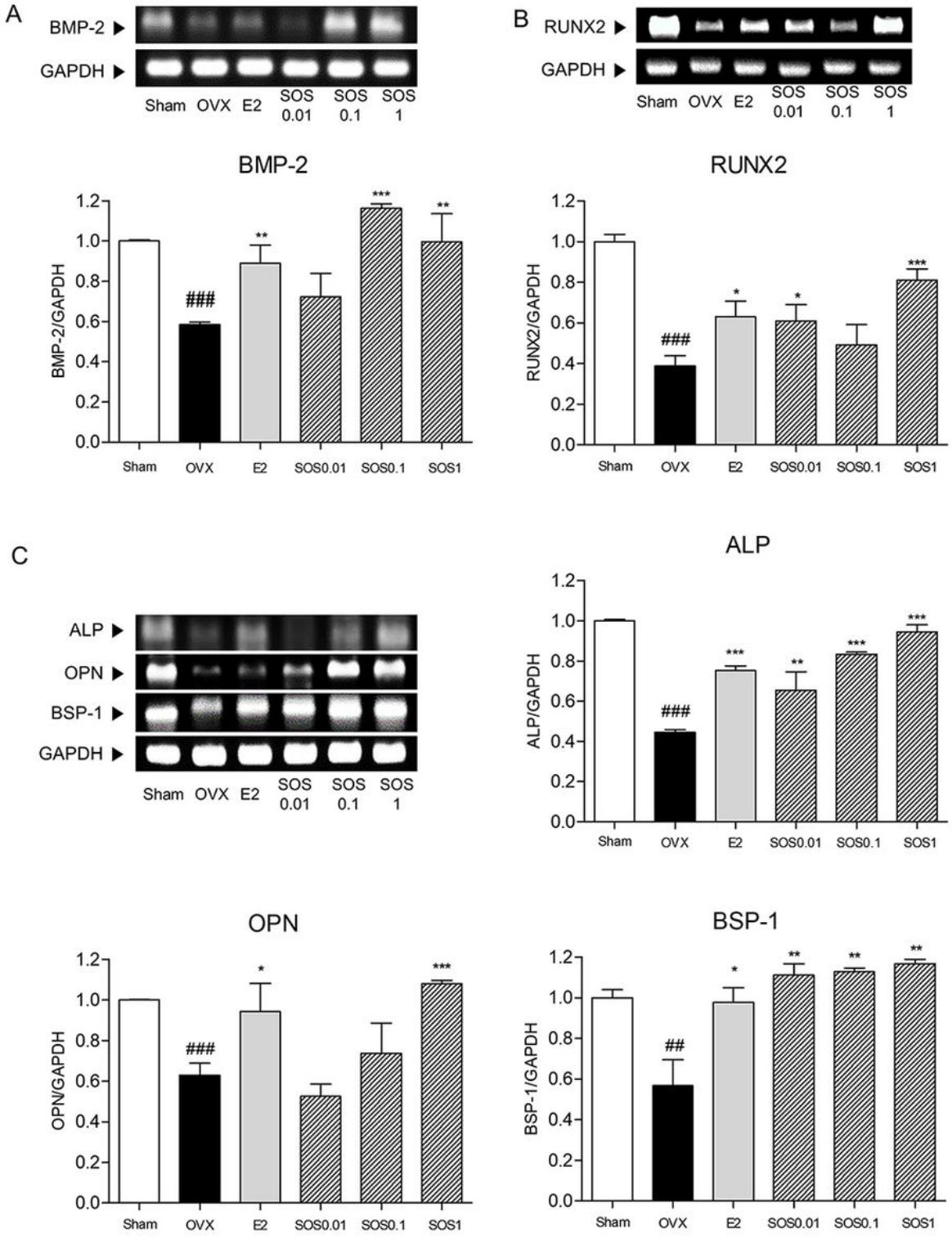

Figure 3

Effect of sweroside on osteoblastic markers in lumbar vertebrae mice (n = 7, each group). Osteoblast differentiation initiation marker was analyzed by RT-PCR. SOS was treated as 0.01, 0.1 and 1 μM. (A) mRNA expression of osteoblast differentiation initiation marker for BMP-2, (B) mRNA expression of transcription factor of osteoblast differentiation for RUNX2, (C) mRNA expression of osteoblast-mediated bone formation markers for ALP, OPN and BSP-1 expression in lumbar vertebrae. Full-length gels are

presented in Supplementary Fig. S1. Significance was determined by one-way ANOVA with Tukey's multiple comparisons test. Results are presented as mean ± S.E.M #, ## and ### indicates the mean differs significantly between sham group and OVX group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). *, ** and *** indicates that the mean differs significantly between SOS-treated or E2 group and OVX group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Data from three independent experiments (n = 3). The band density was quantified using ImageJ software (ver.1.8.0). OVX, ovariectomized group; E2, 17β estradiol group; BMP-2, bone morphogenetic protein 2; RUNX2, Runt-related transcription factor 2; ALP, alkaline phosphatase; OPN, osteopontin; BSP-1, bone sialoprotein 1.

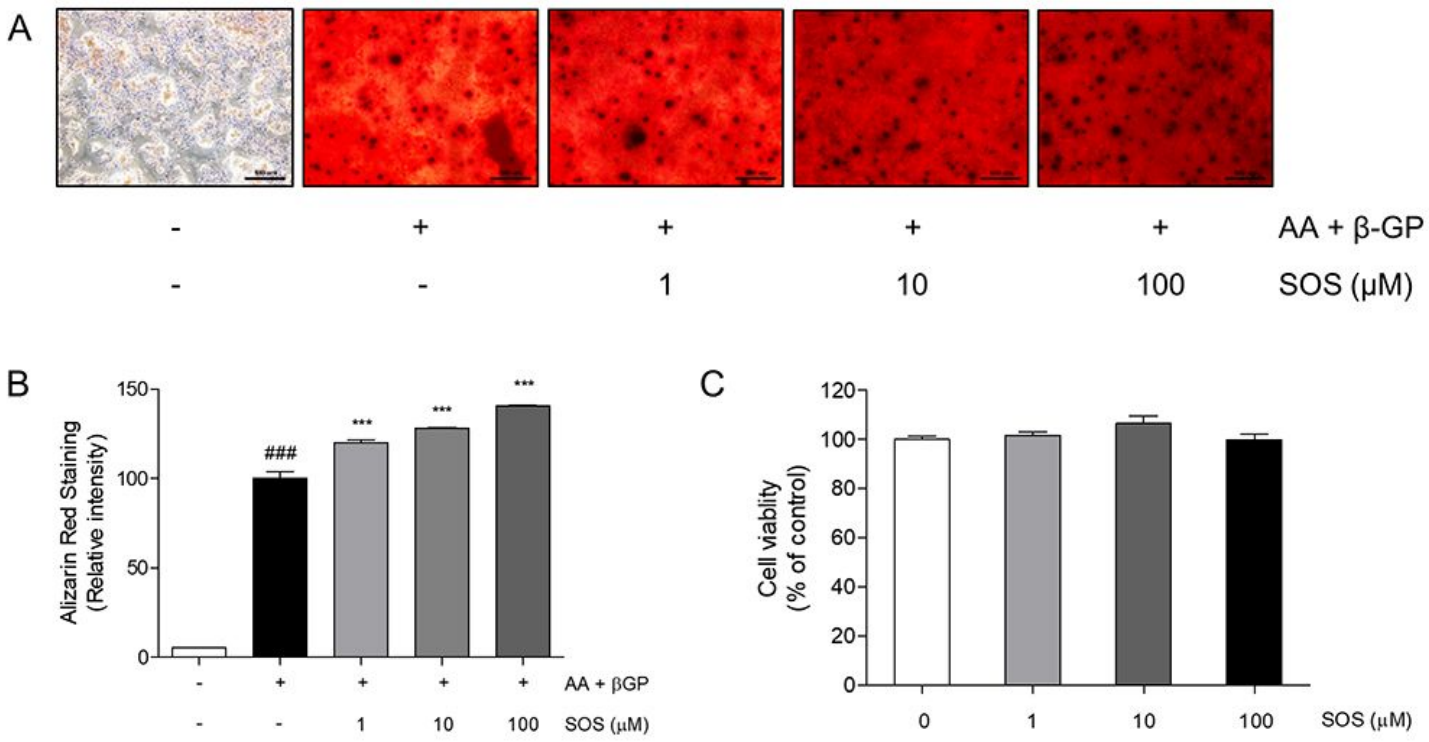

Figure 4

Effect of sweroside on mineralization with Alizarin Red staining and cell viability in SaOS-2 cells. SOS was treated as 1, 10, 100 nM. SaOS-2 cells were mineralized for 9 days with AA and β-GP. (A) Mineralized nodules stained with ARS. Magnification, 400×, 500 μm. (B) The red intensity of ARS staining. (C) SOS effects on cell viability in SaOS-2 cells. Significance was determined by one-way ANOVA with Tukey's multiple comparisons test. Results are presented as mean ± S.E.M ### indicates the mean differs significantly between sham group and OVX group ($p < 0.001$). *, ** and *** indicates that the mean differs significantly between SOS-treated or E2 group and OVX group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Data from three independent experiments (n = 3). AA, L-ascorbic acid; β-GP, β-glycerophosphate; SOS, sweroside.

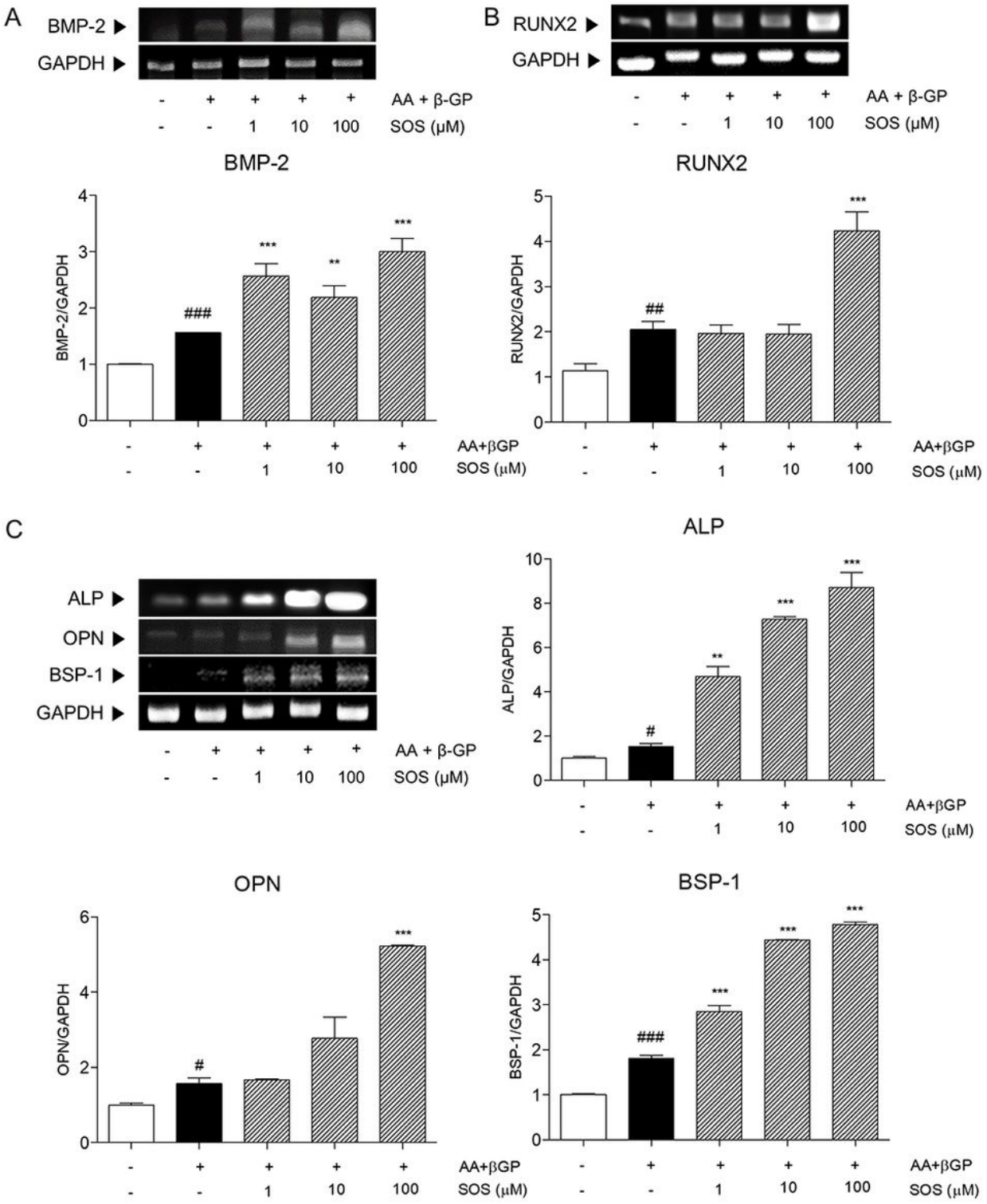

Figure 5

Effect of sweroside on osteoblastic markers in differentiated SaOS-2 cells. Osteoblast differentiation initiation markers were analyzed by RT-PCR. SOS was treated as 1, 10, 100 nM. BMP-2 (A), RUNX2 (B), ALP, OPN and BSP-1 (C) mRNA expression in mineralized SaOS-2 cells. Full-length gels are presented in Supplementary Fig. S2. Significance was determined by one-way ANOVA with Tukey's multiple comparisons test. Results are presented as mean ± S.E.M ## and ### indicates the mean differs

significantly between sham group and OVX group ($p < 0.01$ and $p < 0.001$, respectively). *, ** and *** indicates the mean differs significantly between SOS-treated or E2 group and OVX group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Data from three independent experiments (n = 3). The band density was quantified using ImageJ software (ver.1.8.0). AA, L-ascorbic acid; β-GP, β-glycerophosphate; BMP-2, bone morphogenetic protein 2; RUNX2, Runt-related transcription factor 2; ALP, alkaline phosphatase; OPN, osteopontin; BSP-1, bone sialoprotein 1.

Supplementary Files

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