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Research Article
Salvianolic Acid B Administration Attenuate Bone Loss in Ovariectomy Induced Rat Model

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Abstract

Background and Objective: Osteoporosis is a chronic progressive skeletal disorder characterized by low Bone Mass Density (BMD) and bone quality. This study was aimed to access the anti-osteoporotic effects of salvianolic acid B (SAB-B) against ovariectomy-induced in rat model. **Materials and Methods:** Forty female (n = 40) rats were randomly chosen and divided into four groups as sham-operated control (underwent bilateral laparotomy; sham group, n=10), whereas rats underwent laparotomy followed by bilateral ovariectomy (ovariectomy model group; OVX, n= 10). Whereas, OVX underwent rats were orally supplemented with either 20 or 40 mg kg^{-1} of SAB-B (OVX+20 or 40 mg kg^{-1} SAB-B group; n= 10) for 12 weeks after 4 weeks of OVX. Statistical difference between the experimental groups were analyzed by student t-test using SPSS software at $p<0.05$. **Results:** Treatment with SAB-B (20 or 40 mg kg^{-1}), substantially increased the body weight than OVX rats. A pronounced elevation in the levels of Bone Mineral Density/Content (BMD/BMC) and body weight were observed in SAB-B treated group as compared to OVX group. Bone markers like deoxypyridinoline, alkaline phosphatase and osteocalcin as well as inflammatory markers like IL-1β, IL-6, TNF-α concentrations were significantly abolished after 12 weeks of administration with SAB-A in OVX rats. Furthermore, the bone biomechanical stability was notably improved upon supplementation with SAB-B. **Conclusion:** Both SAB-B 20 and 40 mg kg^{-1} treatment exhibit potent anti-osteoporotic activity, nevertheless SAB-B 40 showed superior anti-osteoporotic activity than SAB-B 20. Therefore, SAB-B would be recommended for treating post-menopausal osteoporosis with other standard drugs.

Key words: Osteoporosis, salvianolic acid B, ovariectomy, bone mineral density, inflammatory markers

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoporosis is chronic progressive skeletal disorder characterized by low Bone Mass Density (BMD) with altered bone micro-architecture and bone quality due to imbalanced bone remodeling[1].Osteoporosis is classified as primary (post-menopausal), secondary and idiopathic types, but primary osteoporosis is a common one, which contributes approximately 95 or 80% of all osteoporosis in female (post-menopausal) or male (aging), respectively[2]. The prevalence rate of osteoporosis is high in senior women (post-menopausal) than men, due to estrogenic interplay[3]. Every year almost 9 million people are affected globally by osteoporosis especially in China, the number is increasing enormously (due to high elderly population)with an increased incidence of osteoporotic fracture rate and thus, considered as one the major public issue[4]. The etiology of osteoporosis is multifactorial, however, lack of estrogen, inflammation (cytokines) are the crucial factors contributing to osteoporosis[5,6]. Hormone Replacement Therapy (HRT), supplementation of minerals (calcium, phosphorous) and vitamins (Vit D), calcitonin, estrogen receptor modulatory drugs, bone resorption inhibitors (bisphosphonates) like zoledronic acid, ibandronate well as bone formation stimulant like fluoride, steroids are currently recommended by FDA to treat osteoporosis[7,8]. Nonetheless, these therapies might cause several adverse effects such as gastrointestinal discomfort, endometrial or ovarian cancer, atypical fracture[9,10] and therefore, recently researchers are highly focusing on natural or complementary therapy to treat osteoporosis[11].

Both salvianolic acid A (SAB-A) and salvianolic acid B (SAB-B) are the hydrophilic phenolic compounds of herbal plant *Salvia miltiorrhiza* (Red sage) also known as danshen in Chinese. Danshen (roots) is one of the renowned Traditional Chinese Medicine (TCM) used as hemorrheologic drug (increase blood flow) and thus prescribed for treating various blood related ailments (remove blood stasis and menstrual cycle diseases) as well as to protect heart, liver, bones and brain[12,13]. Out of these two salvianolic acids, SAB-B is of great interest owing to its various pharmacological properties like cardioprotective, neuroprotective, gastroprotective, renoprotective and hepatoprotective[14-16]. Previous studies have shown that *Salvia miltiorrhiza* exhibit anti-osteoporotic activity, owing to various phenolic compounds (by enhancing estrogenic property) in the various model[13,17]. Moreover, few researchers also demonstrated that salvianolic acid B from danshen would promote osteogenesis or bone loss in both cell line and animal model[2,18,19]. However, till date, no animal studies have conducted with salvianolic acid B against ovariectomy-induced osteoporosis. Therefore, the current study was intended to explore the anti-osteoporotic effects of SAB-B by evaluating the Bone Mineral Density/Content (BMD/BMC), bone markers, inflammatory markers and bone biomechanical stability.

MATERIALS AND METHODS

Chemicals: Salvianolic acid B, sodium pentobarbital, physiological saline were purchased from Sigma-Aldrich (MO, USA). All the other chemicals used for this study were of analytical grade.

Experimental animals: Forty female healthy Sprague Dawley (SD) rats weighing 240-260 g (adult) were purchased from a local experimental animal vendor (Gansu, China). Experimental rats were kept in a steel cage with full access to water and food with 12/12 h dark/light cycle at 22-24°C (65% humidity). The experimental (surgical) protocols were conducted by following the guidelines laid by NIH for handling and caring experimental animals. The current animal study was approved by Lanzhou University Second Hospital ethical committee board members (LU-12-10/112). This animal study was conducted in the animal house of Lanzhou University Second Hospital during March-July, 2017.

Grouping and OVX induction: After 2 weeks of adaptation period, rats were divided into four groups with 10 in each. All the rats were fasted overnight and anesthetized with 50 mg of sodium pentobarbital through intraperitoneal injection. Sham-operated control rats (n = 10) underwent only bilateral laparotomy without any treatment. Whereas, remaining rats (n = 30) also underwent bilateral laparotomy and followed by bilateral ovariectomy (OVX) via midline dorsal incision. OVX rats (n = 10) were supplemented with water (no treatment). After 4 weeks of OVX induction, the remaining rats (n = 20) were orally supplemented with either 20 or 40 mg kg^{-1} of SAB-B by dissolving with water (OVX+20 or 40 SAB-B rats) for 12 weeks (post-treatment).

Sample preparation: For collecting 12 h urine sample, all the rats were housed in a special cage (individually) and their urine sample was collected before sacrifice (after 16 weeks). The collected urine samples were filtered and acidified with 2 mL of hydrochloric acid (HCL; 1 mol L^{-1}) and stored at -20°C for biochemical analysis. All the rats were fasted overnight and euthanized (after the collection of a urine sample) with 40 mg of diethyl ether via intraperitoneal injection and the blood samples were collected by cardiac puncture (aorta). The

collected blood samples are centrifuged at 3000 rpm for 15 min at 4°C to separate serum sample and stored at -20°C for biochemical analysis. Later, the femurs were dissected from each rat and covered in saline-soaked cotton gauze stored at -20°C for biomechanical analysis. The body weight was checked every 4 weeks and before sacrifice (16th week) using OHAUS Ranger 7000 Compact Scale from Fisher Scientific (Hampton, NH, USA).

Biochemical analysis

Bone markers: The levels of serum calcium (S-Ca), serum phosphorus (S-P), serum alkaline phosphatase (S-ALP) and urinary creatinine (U-Cr) were measured using commercial kit bought from Immunodiagnostic Systems (Boldons, UK). In addition, serum osteocalcin (S-OC) was determined by an OC ELISA kit purchased from Biovalue Biomedical Engg. Co., Ltd., (Shanghai, China). Furthermore, urinary deoxypyridinoline (DPD) (U-DPD) concentration was evaluated using commercial ELISA kit (METRA DPA) bought from Quidel Corp., (CA, USA) and expressed as the ratio of DPD to Cr (U-DPD/Cr).

Inflammatory markers: The concentration of various serum inflammatory markers (cytokines) like Tumour Necrosis Factor alpha (TNF-α), Interleukin one beta (IL-1β) and Interleukin six (IL-6) were quantified by commercial ELISA kit supplied by Thermo Fisher Scientific Inc., (MA, USA).

Bone mineral density/content: Both Bone Mineral Density (BMD) and bone mineral content (BMC) of the femur were estimated by Dual-Energy X-ray Absorptiometry (DEXA) using Lunar Prodigy DPXA -IQ-7040 instrument from GE Healthcare (WI, USA). The values are calculated using an inbuilt software-Encore from GE Healthcare (WI, USA).

Bone bio-mechanical stability: Femur bio-mechanical stability/strength was determined by 3-bending test. In brief, the freeze-dried femur was thawed and removed from cotton gauze and its length and midshaft diameter were measured (by caliper) and finally tested using MTS-858 MiniBionix instrument from MTS Systems Corp., (MN, USA) based on supplier's protocol. The femur diaphysis stability was evaluated at a speed of 2 mm min^{-1} and load-deformation cure was calculated as specified by Zhang *et al.*[20]. Based on the load-deformation cure the values of the energy absorption, maximum load, stiffness and maximum stress were calculated using special software provided by MTS Systems Corp., (MN, USA).

Statistical analysis: The average values were expressed as the mean±standard deviation (n = 10). The significant difference between experimental groups was determined using one-way analysis of variance (ANOVA) with Duncan's multiple range test (for multi-comparison) by special software (SPSS, version 21) from IBM (NY, USA). The p-value<0.05 was deemed as statistically significant.

RESULTS

Body weight change: Figure 1 shows the efficacy of SAB-B on body weight of experimental rats. A pronounced increase ($p<0.01$) in the body weight was observed in every week of OVX-induced rats than sham-operated control rats. Especially on the 16th week, the body weight was peaked in OVX group. However, supplementation with both doses of SAB-B (20; $p<0.05$ or 40; $p<0.01$) could considerably abolish those weight gain in OVX-induced rats.

Inflammatory markers: The efficacy of SAB-B on various inflammatory markers of experimental rats is shown in Fig. 2. The mean values of various inflammatory markers like TNF-α, IL-1β and IL-6 were exponentially elevated in OVX underwent rats than those of sham control rats. As compared with OVX-induced rats, the average values of various inflammatory markers were significantly diminished upon administration with SAB-B 20 or 40 mg kg^{-1}.

Bone markers: Efficacy of SAB-B in serum (S) and urinary (U) bone markers of experimental rats is epitomized in Table 1. The levels of serum Ca and P were significantly decreased

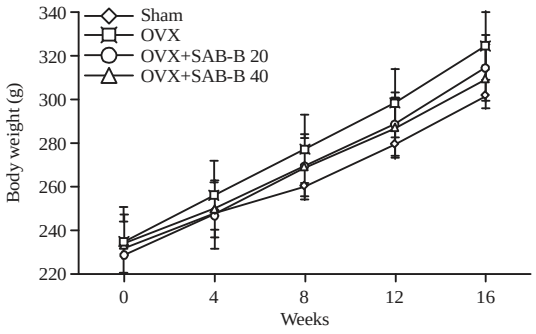

Fig. 1: Efficacy of SAB-B on body weight of experimental rats. OVX: Ovariectomy, SAB-B: Salvianolic acid B

Average values were expressed as the mean±standard deviation (n = 10). Statistical comparison (p-value; *p<0.05, #p<0.01): Where "a" represents the comparison between OVX-induced group and sham-operated control group, while "b" represents the comparison between SAB-B 20 or 40 and OVX-induced group

Table 1: Efficacy of SAB-B on serum (S) and urinary (U) bone markers of experimental rats

Groups	S-Ca	S-P	S-ALP	S-OC	U-DPD/Cr
Sham	2.64±0.33	2.31±0.41	124.42±15.41	13.21±2.1	57.14±6.93
OVX	2.37±0.27^{a*}	2.14±0.25^{a*}	240.02±23.87$^{a\#}$	19.45±3.22$^{a\#}$	92.62±11.02$^{a\#}$
OVX+SAB-B 20	2.48±0.38^{b*}	2.22±0.39^{b*}	196.24±18.96^{b*}	16.68±1.9^{b*}	79.24±8.32^{b*}
OVX+SAB-B 40	2.57±0.41^{b*}	2.28±0.40^{b*}	157.33±21.92$^{b\#}$	14.92±2.7$^{b\#}$	65.89±9.18$^{b\#}$

Average values were expressed as the mean±standard deviation (n = 10). Statistical comparison (p-value; *p<0.05, #p<0.01): Where "a" represents the comparison between OVX-induced group and sham-operated control group, while "b" represents the comparison between SAB-B 20 or 40 and OVX-induced group

Table 2: Efficacy of SAB-B on biochemical stress parameters (stability- bending test) in the femur diaphysis of experimental rats

Groups	Energy (N)	Maximum load (N)	Stiffness (N mm^{-1})	Maximum stress (MPa)
Sham	49.82±4.13	109.75±12.58	174.78±21.03	196.42±21.20
OVX	38.24±4.24$^{a\#}$	87.93±11.26$^{a\#}$	149.93±20.74$^{a\#}$	162.92±30.13$^{a\#}$
OVX+SAB-B 20	42.45±5.45^{b*}	99.38±13.73^{b*}	158.58±18.90^{b*}	176.82±32.57^{b*}
OVX+SAB-B 40	46.32±6.77$^{b\#}$	104.59±14.34$^{b\#}$	167.99±20.26$^{b\#}$	188.56±24.45$^{b\#}$

Average values were expressed as the mean±standard deviation (n = 10). Statistical comparison (p value; *p<0.05, #p<0.01): Where "a" represent the comparison between OVX-induced group and sham-operated control group, while "b" represent the comparison between SAB-B 20 or 40 and OVX-induced group

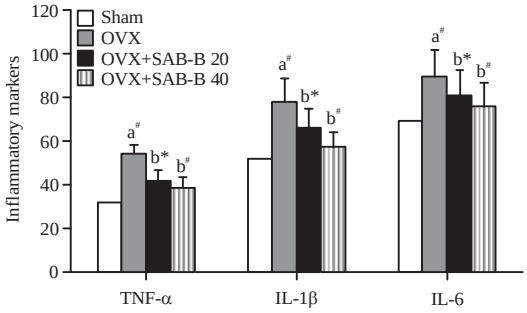

Fig. 2: Efficacy of SAB-B on various inflammatory markers of experimental rats. OVX: Ovariectomy, SAB-B: Salvianolic acid B, TNF-α: Tumour Necrosis Factor alpha, IL-1β: Interleukin one beta, IL-6: Interleukin six

Average values were expressed as the mean±standard deviation (n = 10). Statistical comparison (p-value; *p<0.05, #p<0.01): Where "a" represents the comparison between OVX-induced group and sham-operated control group, while "b" represents the comparison between SAB-B 20 or 40 and OVX-induced group

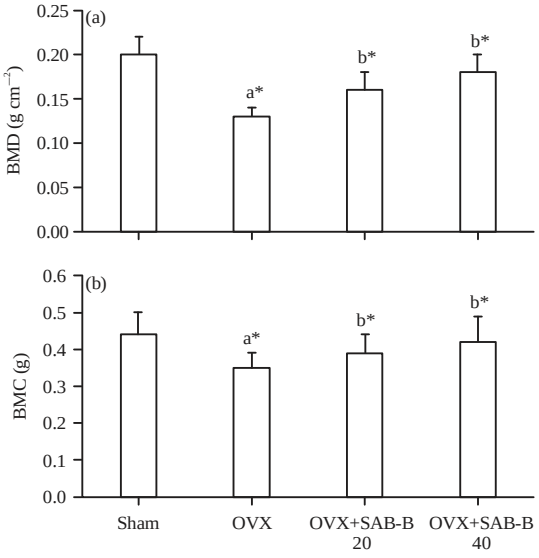

Fig. 3(a-b): Efficacy of SAB-B on (a) Bone Mineral Density (BMD) and (b) Bone mineral content (BMC) of experimental rats. OVX: Ovariectomy, SAB-B: Salvianolic acid B

Average values were expressed as the mean±standard deviation (n = 10). Statistical comparison (p value; *p<0.05, #p<0.01): Where "a" represents the comparison between OVX-induced group and sham-operated control group, while "b" represents the comparison between SAB-B 20 or 40 and OVX-induced group

(p<0.05) with a concomitant increase (p<0.01) in serum ALP, OC and Urinary DPD were noted in OVX rats, as compared with sham rats. Treatment with SAB-B for 12 weeks significantly improved (p<0.05) the serum Ca and P with considerable decline (p<0.01) in serum ALP, OC and urinary DPD on equivalence with OVX group.

Bone mineral density/content: Figure 3 represents the efficacy of SAB-B on Bone Mineral Density (BMD; a) and bone mineral content (BMC; b) of experimental rats. The femoral BMD and BMC of OVX-induced rats were significantly decreased as compared with sham group. Whereas, those lowered femoral BMD and BMC values are markedly reversed back to normal, on supplementation with different doses of SAB-B (20/40) than OVX rats.

Bone bio-mechanical stability: Efficacy of SAB-B on biochemical stress parameters (stability-bending test) in the femur diaphysis of experimental rats is shown in Table 2. OVX protocol results is notable lowered in various biochemical stress parameters like maximum load, stiffness, maximum stress and energy on the femur diaphysis in comparison with control group. Both SAB-B 20 and 40 consumptions rat's results in markable increase in those

biochemical stress parameters like maximum load, stiffness, maximum stress and energy of the femur diaphysis. Both SAB-B dosages (20 and 40) showed the potent anti-osteogenic property. Nevertheless, SAB-B 40 mg kg^{-1} dose showed highest anti-osteogenic property than SAB-B 20.

DISCUSSION

The result of the current animal study demonstrates that treatment with SAB-B could significantly improve BMD/BMC and bone bio-mechanical stability with decreased bone markers and inflammatory markers. The body weight change was assessed to confirm the removal of ovaries (lack of estrogenic property) in experimental rats and its regulation by SAB-B. The body weight was considerably increased in OVX-induced rats due to lack of ovary (estrogen). Treatment with SAB-B significantly reduced the body weight in OVX-induced rats due to the estrogenic property of SAB-B. Ample amount of studies has indicated that salvianolic acid B would act as an estrogen analog and thus exert estrogenic activity[17,21].

One of the major pathophysiological consequences of osteoporosis is inflammation. The inflammatory response and oxidative stress are the vicious events which are triggered by the altered bone remodeling process owing to modulation in the estrogen-related signaling pathway[22,23]. The levels of inflammatory markers like TNF-α, IL-1β and IL-6, were remarkably increased in OVX-induced rats attributing to excessive oxidative stress and subsequent inflammatory response[24]. Supplementation with SAB-B considerably diminished the average values of inflammatory markers like TNF-α, IL-1β and IL-6. Present study results are in corroboration with the results of Yang *et al.*[25]. Previous animal studies conducted by Chen *et al.*[15], also demonstrated that treatment with salvianolic acid B could considerably abolish the levels of various inflammatory markers like TNF-α, IL-1β and IL-6 in anischemic rat model.

The bone mass or content are inversely proportional to various serum and urinary bone turn over markers like ALP, OS, Ca, P and DPD[11,26]. Especially, ALP and DPD are considered as the best biochemical markers for osteoblastic activity[27].The levels of serum Ca, P, ALP, OC and Urinary DPD were considerably altered in OVX rats, as compared with sham rats. These considerable alterations are may be due to excessive oxidative stress, inflammation which contribute to increased bone modulation[22]. Twelve weeks of treatment with SAB-B significantly altered those bone markers and thus maintain normal bone rigidity. Salvianolic acid B (estrogenic property) has been reported to stimulate bone marrow cells to differentiate into osteoblast and thereby, alter the bone remodeling process and mimic the effect of osteoblast inducer[19]. Moreover, salvianolic acids B can exhibit potent antioxidant and anti-inflammatory activities[10] and thus, protect the bone from excessive resorption.

Bone strength, bone mineral density/content or mass are the crucial factors that maintain the bone integrity or quality. Therefore, the Bone Mineral Density/Content (BMD/BMC) were evaluated by DEXA instrument. The left femoral BMD and BMC levels of OVX-induced rats were concomitantly reduced than sham-operated rats due to loss of estrogenic property (ovariectomy). The levels of femoral BMD and BMC were significantly escalated upon oral administration of SAB-B (due to estrogenic property) for 12 weeks. Studies have indicated that treatment with *Salvia miltiorrhiza* extract rich in salvianolic acid for 8 weeks could significantly improve the levels of both BMD and BMC[28].

Monitoring of bone stability or rigidity is essential for diagnosis or treatment of osteoporosis and hence 3 bending tests were done to evaluate the bone strengthening or anti-osteoporotic activity. OVX leads to considerable reduction in various biochemical stress parameters like maximum load, stiffness, maximum stress and energy of the femur diaphysis. Consumption of SAB-B (20 and 40) results in significant increase in those biochemical stress parameters like maximum load, stiffness, maximum stress and energy of the femur diaphysis. As indicated previously SAB-B could significantly increase the bone mass/content (BMD/BMC) and thus increase bone strength (bone micro-architecture) attributing to its osteogenic property. Salvianolic acid B (different doses), also completely reversed the bone loss condition (osteopenia) by enhancing osteoblast cell formation and thus maintain optimal bone mass and thus increase bone strength or rigidity[19]. Moreover, Xu *et al.*[18], concluded that supplementation with salvianolic acid B could enhance the expression of osteopontin, runt-related transcription factor-2 (Runx2) via ERK signaling pathway and thereby promote the osteogenesis (mineralization) in human mesenchymal stem cell model. Therefore, it is concluded that SAB-B can promote osteogenesis and thus modulate bone remodeling process in OVX induced rats and eventually results in improve bone mass or density.

CONCLUSION AND FUTURE RECOMMENDATIONS

The results of study inferred that the SAB-B (20 and 40 mg kg^{-1}) displayed potent anti-osteoporotic activity by significantly attenuating the body weight gain, inflammatory markers, bone turn over markers with improved levels of

BMD/BMC as well as various biochemical stress or biomechanical stability parameters in ovariectomized rats (OVX). The anti-osteoporotic activity of SAB-B 40 mg kg^{-1} is much better than SAB-B 20 mg kg^{-1} due to higher estrogenic property and thus, efficiently modulated the bone remodeling process in OVX induced rats. Further studies are required to confirm the exact mechanism for the anti-osteoporotic property in relationship with signaling pathways.

SIGNIFICANCE STATEMENT

This study demonstrated that salvianolic acid B possess anti-osteoporotic potential by altering various various biochemical (bone and inflammatory markers) as well as improving bone mass density and bone stability (rigidity) in post-menopause. Therefore, SAB-B with potent anti-osteoporotic activity would pave a path for discovering a novel natural anti-osteoporotic regimen, by combining SAB-B with other standard synthetic anti-osteoporotic agents. Therefore, this study contribute in developing a novel anti-osteoporotic drug.

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