ORIGINAL RESEARCH

Protective Role of β-Sitosterol in Glucocorticoid-Induced Osteoporosis in Rats Via the RANKL/OPG Pathway

Tao Wang, MD; Songjun Li, MD; Cunguo Yi, MD; Xiuhui Wang, MD; Xianzhang Han, MD

ABSTRACT

Introduction • Osteoporosis affects approximately 10% of the population worldwide. β-sitosterol (BSS), a major phytosterol in plants, has been claimed for centuries to have numerous medical benefits, including bone strengthening. This study aimed to find the benefit of BSS in treating osteoporosis according to traditional methods and to investigate the protective effect of BSS on glucocorticoid-induced osteoporosis in rats.

Design • Wistar rats were randomly assigned to one of four groups: the control group, the dexamethasone (DEX) group and one of two BSS-treated osteoporosis groups (100 and 200 mg/kg). Blood samples and femur bones were collected for histopathology, immunohistochemistry, biochemical and mRNA expression analysis.

Results • The results indicated that BSS (100 and 200 mg/kg) increased bone length, bone weight and bone mineral density (BMD) and suppressed DEX-induced reduction in body weight, dose-dependently. Mechanistically, BSS (100 and 200 mg/kg) treatment alleviated the increase of bone resorption markers and the decline of osteogenic markers, which might be partially mediated by regulation of nuclear factor kappa-β ligand/osteoprotegerin (RANKL/OPG) and RunX2 pathways. The immunohistochemical inducible nitric oxide synthase (iNOS) results of the rats' distal femur were negative in all groups. However, except in the DEX group, the endothelial nitric oxide synthase (eNOS) color reaction in osteoblasts was strongly positive in the other 3 groups. These results suggest that BSS showed promising effects in protection against glucocorticoid-induced osteoporosis by protecting osteoblasts and suppressing osteoclastogenesis. (*Altern Ther Health Med.* 2022;28(7):18-25).

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INTRODUCTION

Glucocorticoids (GCs) are commonly used as immunosuppressive and anti-inflammatory drugs. GCs are frequently used to treat many inflammatory, allergic and autoimmune disorders. GCs can actively decrease the severity of these disorders, but their massive and long-term use is always associated with multiple complications, including osteoporosis.[1] The mechanisms involved in glucocorticoid-induced osteoporosis (GIO) are multiple. GCs suppress the formation of bone-forming cells and osteoblasts and induce their apoptosis. GCs also inhibit the activity and differentiation of osteoblast-derived cells. In addition, an important factor for the development of osteoclasts, which are an expression of nuclear factor kappa-β ligand (RANKL), is induced by GCs. On the other hand, osteoprotegerin (OPG) are downregulated by GCs, which are the decoy receptors of RANKL. The resulting imbalance between RANKL and OPG ultimately results in a state of increased bone resorption.[2] In addition, GCs induce catabolism of the bone proteins, osteocalcin and osteopontin, resulting in a decreased bone anabolic state. It was also reported that GCs could prolong the lifespan of the mature osteoclasts.

The problems in the conventional treatment of osteoporosis have contributed to the use of natural sources as an alternative to synthetic medications, which are used both as a medical formulation and a dietary substitute. Several traditional medicines formulated from various herbs have

been used since time immemorial to treat different diseases.[3,4] To understand the scientific basis of the effectiveness of herbal medication, many studies are underway, as current research is lacking that shows their effectiveness scientifically. The use of available herbal medicinal products is also being promoted by the World Health Organization (WHO) Expert Committee for the Treatment of Osteoporosis.

β-sitosterol (BSS) is a major phytosterol extracted from corn, soy, rice bran, wheat germ, peanuts, pumpkin seeds and plant oils cited in ancient medicinal history for its use in the treatment of rickets and other bone disorders. Several studies have indicated that BSS inhibits cancer cell proliferation and exhibits antihypercholesterolemic, anti-inflammatory, anti-angiogenic, antidiabetic and immune-modulating properties. For this reason, we aimed to explore the protective mechanism of BSS in glucocorticoid-induced osteoporosis in rats.

MATERIAL AND METHODS

Chemicals

Dexamethasone disodium phosphate (DEX) and BSS were purchased from Sigma-Aldrich Chemicals (Burlington, Massachusetts, USA). All other chemicals used for assay procedures were purchased locally and of analytical grade.

Animals

Male Wistar rats weighing 180 g ± 20 g were used in the study. The animals were housed in standard conditions (24 ± 2° C; relative humidity, 50 to 70%; 12-hour light/dark cycle). The animals were provided with a standard pellet diet and water ad libitum. The Institutional Animal Ethics Committee of Shanghai University, China, approved the animal experiments.

Study Design

Induction of Osteoporosis and Treatments. The Wistar rats were randomly assigned to one of four groups (n = 8): control, model, DEX+BSS 100 and DEX+BSS 200. The rats in the control group were given an equal volume of saline solution intramuscularly. For 5 weeks, 7 mg/kg of DEX was intramuscularly injected once a week in all groups of rats except the control group. The DEX+BSS 100 and DEX+BSS 200 groups of rats were intragastrically administered 100 or 200 mg/kg BSS once a day, respectively, starting from the second week of the osteoporosis induction and continued for 3 weeks. Blood samples were collected from the rat orbital route at the end of the fifth week, and then all the rats were euthanized. Soft tissues of the femur were carefully removed, and the wet weight and length of the left femur bone were measured; for further analysis, the bones of the right femur and tibia were stored at -20° C.[10\]

Hematoxylin and Eosin (H&E) Staining. One-third of the distal parts of the femur were taken and the attached surrounding tissues were cleanly removed. Then, the bone samples were fixed in a 4% formaldehyde solution, embedded, sectioned and stained according to the method for preparing undecalcified bone sections.

Bone Mineral Density (BMD). Dual-energy x-ray absorptiometry was used to analyze BMD; results were reported as g/cm^2.

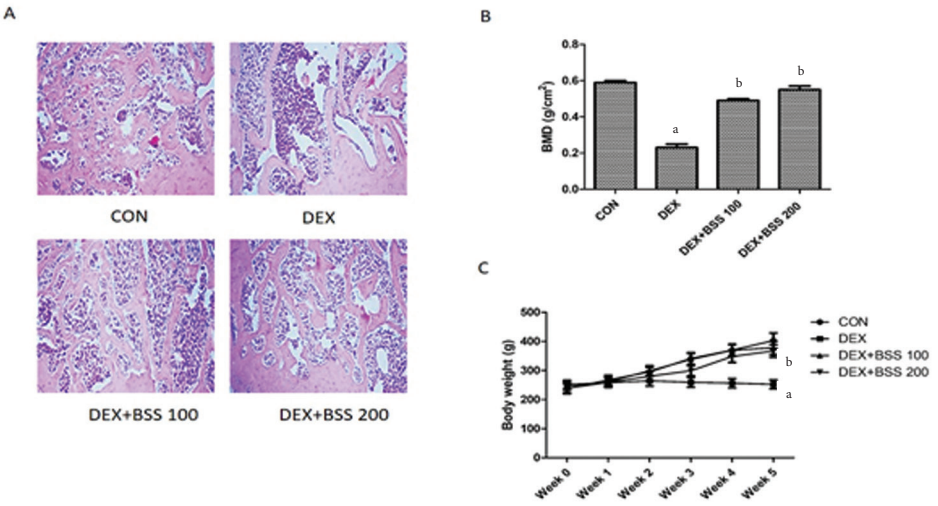
Immunohistochemical Staining. To confirm the alterations in inducible nitrogen oxide (NO) synthase and endothelial NO synthase (eNO) within the bone, immunohistochemical staining of the proximal tibia was performed. After being placed in a 10% formaldehyde solution for fixation, one-third of the proximal tibia parts were dehydrated step-by-step, immersed in 3:7 butyl methacrylate and methyl methacrylate for 24 hr (negative vacuum pressure: 8.67 kPa) and embedded in semi-polymerized methyl methacrylate. Each specimen was cut with a YD-1508R paraffin slicing machine into several undecalcified 3-μm-thick sections. Per the kit instructions, an immunohistochemical streptavidin-peroxidase method was used to detect the expression of iNOS and eNOS.

Measurement of Bone Tissue Oxidative Stress. Per the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute) of the commercially available kit, plasma glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) activities were calculated and the malondialdehyde (MDA) content was measured.

Analysis of Serum IGF and Transforming Growth Factor β. According to the manufacturer's instructions, the serum levels of IGF and transforming growth factor beta (TGF-β) were assayed with the rat ELISA kit (Nanjing Jiancheng Bioengineering Institute). Then, alkaline phosphatase (ALP) activity was measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute). Briefly, serum samples were incubated with the test solutions at 37° C for 1 hour. Finally, absorbance was assayed using a microplate reader (BioTek Epoch, Winooski, Vermont, USA) at 520 nm (ALP). Osteocalcin (OCN) and C-terminal telopeptide of type I collagen (CTX) serum levels were determined using a commercial kit (USCN Life Science, China) according to the manufacturer's protocol. Briefly, serum samples were mixed with the antibody solutions (OCN and CTX) at 37° C for 1 hour. After adding streptavidin-HRP, the solution was incubated at 37° C for 30 min. Finally, absorbance was assayed using a microplate reader at 450 nm.

Reverse Transcription Polymerase Chain Reaction. Per the manufacturer's protocol of Trizol reagent (Takara, China), an assay of mRNA expression of RANKL, OCN, OPG and runt-related transcription factor 2 (RunX2) was performed via reverse transcription polymerase chain reaction (RT-PCR) of the right femur tissue. First, total RNA isolation was done from the tissues of the frozen femur. Then, the First Strand cDNA Synthesis kit was used to synthesize cDNA from total RNA, and the SYBR Green qPCR Master Mix kit was used to perform RT-PCR. The quantitative PCR (qPCR) process was carried out twice; GAPDH acted as internal control and the RT-PCR amplification reaction conditions included primer sequences of 40 cycles of 95° C for 10 seconds, 60° C for 30 seconds and 72° C for 15 seconds. The 2–DDCT method was used for the calculation of relative mRNA expression.

Figure 1. H&E staining of the rats' femurs (×100) (A) Effects of BSS on the changes in BMD (B) and body weight (C) in DEX-induced osteoporosis in rats. Data are reported as mean ± SEM (n = 8/group).



[a]$P < .01$ vs the CON group
[b]$P < .001$ vs the DEX group (ANOVA)

Abbreviations: BBS, β-sitosterol; BMD, bone mineral density; CON, control; DEX, dexamethasone disodium phosphate; H&E, hematoxylin and eosin; SEM, standard error of mean.

Statistical analysis

Data are reported as mean ± SEM. Between-group differences were compared using one-way ANOVA followed by Tukey's multiple comparison test for post hoc analysis using GraphPad Prism software (San Diego, California, USA). $P < .05$ was considered statistically significant.

RESULTS

Effects of BSS on the Morphology of Rats with DEX-induced Osteoporosis

The pathological examination of the slices of one-third of the distal parts of the rat femurs under a light microscope demonstrated that: the number of trabeculae decreased, they were sparse and ruptured, most of them could not connect with the mesh, the bone marrow cavity was enlarged, the trabecular structure had larger blank regions and a large number of fat cells could be found in the model group compared with the control group. Furthermore, the femoral trabeculae were wider and thicker with a significant increase in their number; ruptured trabeculae and fat cells were rarely found and the trabeculae were smooth in the BSS (100 and 200 mg/kg) groups compared with the DEX group. These findings were similar to those seen in the control group (Figure 1A).

Effects of BSS on Changes in BMD and Body Weight

DEX-induced osteoporosis was successfully established by intramuscular injection of DEX. As shown in Figure 1B, the BMD index was reduced in the DEX group, partially alleviated by dose-dependent treatment with BSS (100 and 200 mg/kg). As shown in Figure 1C, in line with the reduction in the BMD index, body weight was decreased in the DEX group. However, with dose-dependent BSS treatment (100 and 200 mg/kg), body weight was significantly increased compared with the DEX group ($P < .001$). Hence, our preliminary data implied that treatment with BSS did not decrease body weight and showed no observable toxicity.

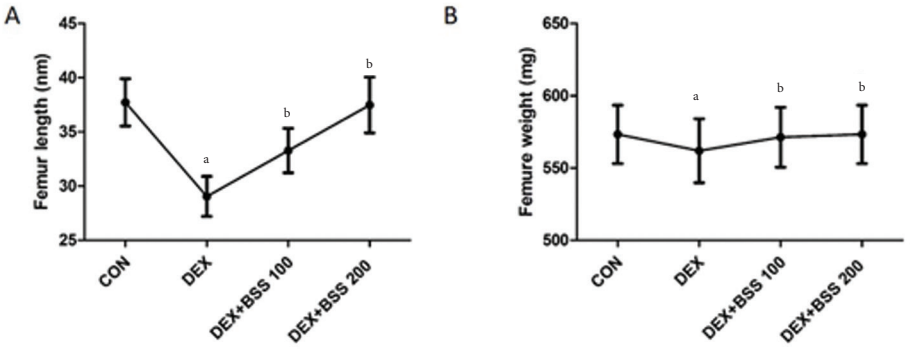
Effects of BSS on DEX-induced Bone Physical Change

As shown in Figure 2A and 2B, administration of DEX alone resulted in a decline in femur length and weight compared with the control group ($P < .001$). However, treatment with BSS (100 and 200 mg/kg) ameliorated the DEX-induced reduction of femur length and weight compared with the DEX group ($P = .05$), dose-dependently.

Immunohistochemical Observation

Immunohistochemical observation revealed that the results of iNOS were negative, eNOS-immunoreactive substances were sepia precipitates, and eNOS was expressed in the trabeculae and bone marrow cavity, mainly distributed in osteoblasts and bone marrow stromal cells surrounding the trabeculae. The color reaction of osteoblasts in the DEX group was weakly positive, the cytoplasm was light grey; only a few regions demonstrated a brown residue and the osteoblasts were dispersed with no dense particles in them. The color reaction of osteoblasts in the DEX+BSS 100 and DEX+BSS 200 groups was dose-dependently strongly positive, the cytoplasm was filled with brown precipitates and the osteoblasts were distributed uniformly, but the density was slightly lower than that in the control group. The color

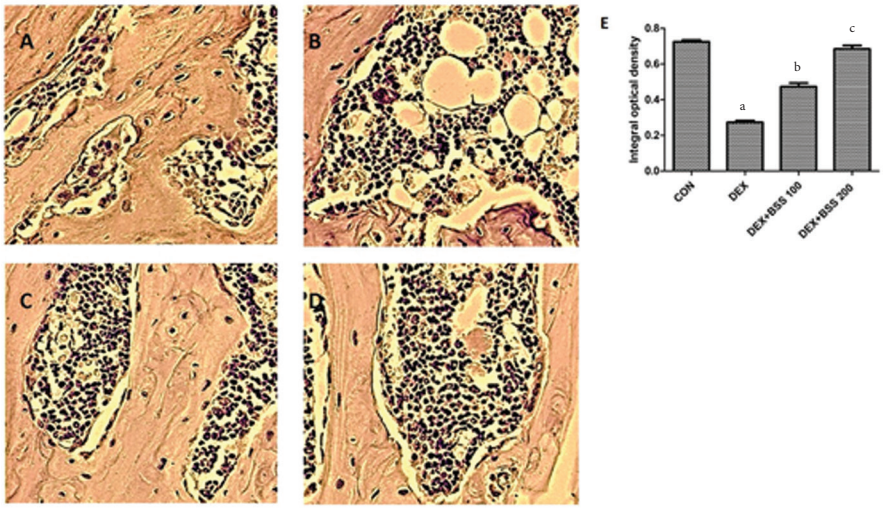
Figure 2. Effects of BSS on femur length (A), femur weight (B). Data are reported as mean ± SEM (n = 8/group).



[a]$P < .01$ vs the CON group
[b]$P < .001$ vs the DEX group (ANOVA)

Abbreviations: BBS, β-sitosterol; CON, control; DEX, dexamethasone disodium phosphate; SEM, standard error of mean.

Figure 3. Effects of BSS on the eNOS expression in rats with DEX-induced osteoporosis (×400). (A) CON; (B) DEX; (C) DEX+BSS 100; (D) DEX+BSS 200; integral optical density values (E). Data are reported as mean ± SEM (n = 8/group).



[a]$P < .01$ vs the CON group
[b]$P < .01$
[c]$P < .001$ vs the DEX group (ANOVA)

Abbreviations: BBS, β-sitosterol; BMD, bone mineral density; CON, control; DEX, dexamethasone disodium phosphate; eNOS, endothelial nitric oxide synthase; SEM, standard error of mean.

reaction of osteoblasts in the control group was strongly positive, and the whole cytoplasm was uniformly stained with a dense brown precipitate, as seen in Figure 3 (A-D).

Statistical analysis demonstrated that the integral optical density values of femoral eNOS expression in the DEX group was significantly decreased ($P < .001$) compared with the control group. However, after administering BSS (100 and 200 mg/kg), the optical density of femoral eNOS expression in rats with DEX was significantly higher ($P < .01$ and $P < .001$, respectively) than those in the only DEX group, as illustrated in Figure 3E.

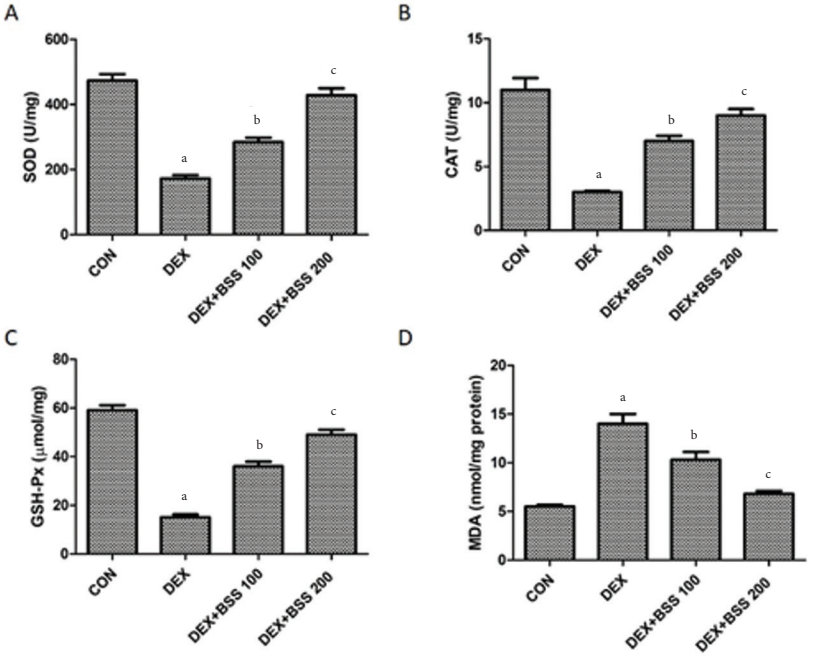
Effects of BSS on Oxidative Stress

As shown in Figure 4, in the DEX group, SOD, GSH-Px and CAT activity was lower and the content of MDA in the femur tissue was higher than in the control group ($P < .001$). However, treatment with BSS (100 and 200 mg/kg) improved ($P < .01$ and $P < .001$) the activity of SOD, GSH-Px and CAT and dose-dependently decreased the content of MDA.

Effects of *Cuscuta chinensis* Extract (CCE) on Bone Metabolism

As shown in Figure 5 A-E, the levels of IGF, TGF-b, RANKL (OCN) and alkaline phosphatase (AP) activity in

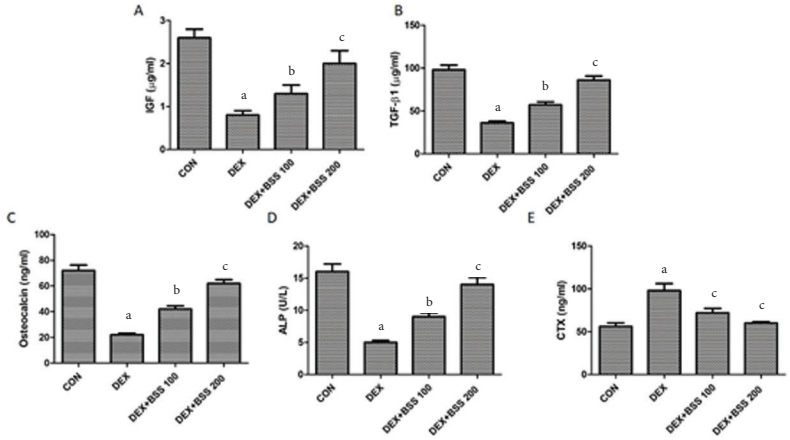
Figure 4. Effects of BSS on SOD activity (A), CAT activity (B), GSHPx activity (C), and MDA level (D) in femur tissue of DEX-induced osteoporotic rats. Data are reported as mean ± SEM (n = 8/group).



[a]$P < .01$ vs the CON group
[b]$P < .01$
[c]$P < .001$ vs the DEX group (ANOVA)

Abbreviations: BBS, β-sitosterol; BMD, bone mineral density; CAT, catalase; CON, control; DEX, dexamethasone disodium phosphate; GSHPx, glutathione peroxidase; MDA, malondialdehyde; SEM, standard error of mean; SOD, superoxide dismutase.

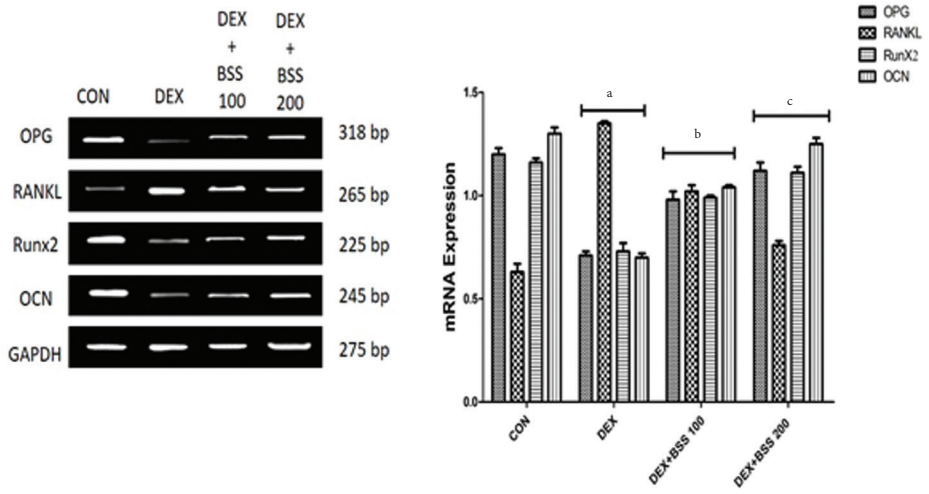
Figure 5. Effects of BSS on bone turnover markers in DEX-induced osteoporotic rats. Serum IGF (A) serum TGF-β (B) serum levels of OCN. (C) ALP activity in serum. (D) Serum levels of CTX (E). Data are reported as mean ± SEM (n = 8/group).



[a]$P < .01$ vs the CON group
[b]$P < .01$
[c]$P < .001$ vs the DEX group (ANOVA)

Abbreviations: ALP, alkaline phosphatase; BBS, β-sitosterol; CAT, catalase; CON, control; CTX, C-terminal telopeptide of type 1 collagen; DEX, dexamethasone disodium phosphate; IGF, insulin-like growth factor; MDA, malondialdehyde; OCN, osteocalcin; SEM, standard error of mean; SOD, superoxide dismutase; TGF-β, transforming growth factor beta.

Figure 6. Effects of BSS on mRNA expression of OPG, RANKL, RunX2, and OCN in DEX-induced osteoporotic rats. Data are reported as mean ± SEM (n = 8/group).



[a]$P < .01$ vs the CON group
[b]$P < .01$
[c]$P < .001$ vs the DEX group (ANOVA)

Abbreviations: BBS, β-sitosterol; CON, control; DEX, dexamethasone disodium phosphate; OCN, osteocalcin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-β ligand; SEM, standard error of mean.

serum were decreased, and the CTX content was increased in the DEX group compared with the control group ($P < .001$). However, with BSS treatment (100 and 200 mg/kg), these changes were reversed, dose-dependently.

Effects of BSS on DEX-induced Changes in mRNA Expression

As shown in Figure 6, mRNA expression of osteoprotegerin (OPG) , RunX2, and OCN were decreased, and the mRNA expression of RANKL was increased in the DEX group compared with the control group ($P < .001$). However, dose-dependently, BSS (100 and 200 mg/kg) treatment markedly upregulated OPG, RunX2, OCN, and down-regulated RANKL mRNA expression.

DISCUSSION

Glucocorticoids are used extensively in the treatment of autoimmune and rheumatic disease and several acute medical conditions, such as traumatic damage of the spinal cord. Overuse of glucocorticoids is associated with various adverse events in the vascular system, ultimately resulting in a reduction in the blood supply to the femoral head, and they have also been reported to be involved in the suppression of mesenchymal stem cell osteogenic differentiation. Inhibitory action of glucocorticoids can be linked to the induction of downregulation of RunX2, which can be considered significant in the development of osteoblasts. In addition, oxidative stress is evoked by glucocorticoids and involved in the acceleration of osteoblast apoptosis, leading to the suppression of osteoblast proliferation.

Consequently, because of their antioxidant effects, natural products have been suggested to produce osteoclast differentiation and suppression in the resorption of bone.[18,19] DEX, a popular glucocorticoid used in hospitals, is involved in exhibiting harmful effects on bone structure, turnover and minerals. Therefore, Wistar rats were intramuscularly injected with DEX once a week for 5 weeks in our study to induce osteoporosis and develop *in vivo* osteoporosis models in rats.

Osteoporosis can be characterized as a reduction in the weight and strength of the bones, which ultimately results in a high risk for fracture. Loss of BMD and a decrease in body weight are also observed during glucocorticoid therapy; these are still considered the main markers for osteoporosis.[20] In line with previous studies, our findings indicate that DEX is involved in the BMD decrease, and body weight gain is suppressed. Treatment with BSS showed prevention of osteoporosis via alleviation of a DEX-induced decline in body weight gain and BMD. Regulation of osteoblast function is cooperatively achieved by TGF-β and IGF. TGF-β is promotes type I collagen synthesis and regeneration of bone is also improved, and β-catenin's transcriptional activity, location and stability are regulated by IGF. This study shows that BSS is involved in the raised TGF-β and IGF levels in rats with DEX-induced osteoporosis.[21] The balance between osteoblast-regulated bone formation and osteoclast regulated bone resorption is called bone homeostasis; the primary cause for the pathogenesis of osteoporosis is believed to be the destruction of this equilibrium. Moreover, glucocorticoids contribute to osteoporosis, including bone resorption and

bone formation; the indicators of bone formation, ALP and OCN, are measured in this study. OCN is an osteoblast activity's late index and is said to be the bone's richest non-collagenous protein, and in the early stages of osteogenic differentiation, ALP is considered an essential index participating in calcification of the skeleton by enhancing the contents of local inorganic phosphates.[22,23]

We observed that BSS was involved in preventing suppression of the markers involving DEX-induced bone formation, which is in line with previous studies. CTX is another important marker of bone resorption. Similar to previous studies, it was found that DEX reduced CTX's serum content, and BSS treatment mitigated the DEX-induced increase in the markers of bone resorption.

Previous studies have shown that one other cause of osteoporosis is oxidative stress.[24] Cumulative evidence shows elevated reactive oxygen species (ROS) behaviour and a decline in antioxidants often occurs during osteoporosis. An increase in oxidative stress was identified with a rise in MDA levels in DEX-induced osteoporosis, which is also in line with the previous study. We found a decline in antioxidant enzyme activity along with these modifications, and concurrent BSS therapy showed improvement of oxidative stress in the tissue of the femur.

Glucocorticoid hormones mainly acted on the bone osteoblasts, and NO played a mediating role in a series of reactions induced by these hormones. NO carries biological information, serving as a second messenger and neurotransmitter, mediating and regulating various pathological and physiological reactions. NOS is classified into 3 subtypes: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), with different genetic codes. It was confirmed that eNOS and iNOS exist primarily in bone tissue, and osteoblasts and osteoclasts secrete NO in autocrine and paracrine manners and involved in the functional regulation of osteoblasts and osteoclasts. Under normal physiological conditions, eNOS generates a low concentration of NO (pmol). Some clinical studies on eNOS and osteoporosis prove that eNOS plays a key role in regulating osteoblast activity and bone formation. In this study, the immunohistochemical results of eNOS in osteoblasts in the DEX group were weakly positive, whereas the reaction in the control group was strongly positive, indicating that the decreased expression of eNOS could play an important role in the pathogenesis of osteoporosis. After administering BSS (100 and 200 mg/kg), the color reaction of eNOS in osteoblasts was strongly positive, indicating that BSS (100 and 200 mg/kg) could stimulate the osteoblasts to increase eNOS-dependent NO levels and thus promote bone formation.

RANKL and OPG are considered the 2 major osteoblast-expressed cytokines that play crucial roles in bone metabolism, where resorption of bone is facilitated by RANKL combining with its osteoclast receptors. By combining with RANKL, OPG prohibits it from combining with RANK, thereby developing a suppression in bone resorption. We observed an upregulated RANKL expression and a down-regulated OPG expression to be evoked by DEX, which is in line with previous studies.[25]

Furthermore, role of BSS in suppressing DEX-evoked bone resorption was indicated in our findings. RunX2 is a critical osteoblast differentiation and bone structure modulator. A decline in expressing the main transcription factor resulted in a decrease in OCN expression, an essential bone extracellular matrix protein. Previous studies have also reported glucocorticoid-evoked down-regulation of OCN and RunX2 expression.[12] In accordance, our findings showed that the levels of OCN and RunX2 expression in DEX-induced osteoporotic rats were lower in femur tissue. These findings speak to concurrent therapy with BSS-replenished expression of OCN and RunX2 in femoral tissue as the possible mechanism of BSS osteoporosis prevention.

CONCLUSION

In conclusion, the results of our study revealed that by preventing bone resorption and enhancing bone formation, BSS relieved osteoporosis evoked by DEX. Partly by modulating OPG/RANKL and RunX2 signals, BSS demonstrated a significant antioxidant effect on the progression of osteoporosis. In addition, they can improve pathological changes in the microstructure and stimulate the expression of eNOS in osteoblasts. Bone protection in osteoporotic rats might be mediated by eNOS-dependent NO. Thus, to avoid and treat DEX-evoked osteoporosis, BSS may be considered a potential candidate drug. More studies addressing its use in bone disorders are expected to offer further insight into its anti-osteoporosis effects.

CONFLICT OF INTEREST

None.

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AUTHOR CONTRIBUTIONS

Tao Wang, MD; Songjun Li, MD; Cunguo Yi, MD; contributed equally to the article.

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