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Original Article

Stigmasterol, a Major Component of *Cornus Officinalis*, Ameliorates Osteoporosis in Diabetes Mellitus Effects by Increasing Bone Mineral Density

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Abstract

Objectives: This study investigated the therapeutic effects of stigmasterol (STG), derived from *Cornus officinalis*, on osteoporosis in rats with type 2 diabetes mellitus (T2DM). **Methods**: Twenty-four Male Sprague-Dawley rats (6 weeks old) were used to establish a T2DM model and were divided into four groups: normal diet (ND), high-fat diet (HFD), low-dose STG (STG-L, 100 mg/kg), and high-dose STG (STG-H, 200 mg/kg). The rats received daily gavage treatments for four weeks. Therapeutic effects were assessed by examining femoral bone structure, serum bone formation markers (P1NP, osteocalcin, and osteoprotegerin), bone resorption indices (CTX-1 and RANKL), and osteogenic protein expression (Runx2, osteopontin, and COL1A1). **Results**: STG significantly reduced fasting blood glucose levels and improved insulin resistance in T2DM rats. It enhanced trabecular bone microstructure, with the STG-H group demonstrating superior effects. Compared to the HFD group, STG increased bone mineral density, bone volume fraction (BV/TV), and trabecular thickness, while reducing bone surface-to-volume ratio (BS/BV) and trabecular separation. STG also elevated serum levels of P1NP, osteocalcin, and osteoprotegerin, while reducing CTX-1 and RANKL. Western blot analysis revealed increased expression of Runx2, osteopontin, and COL1A1 in femoral tissues. **Conclusions**: STG appears to alleviate osteoporosis in diabetes by improving bone microstructure, promoting bone formation, and reducing bone resorption, indicating its potential as a therapeutic option for managing osteoporosis.

Keywords: Bone Formation, Bone Resorption, Diabetes, Osteoporosis, Stigmasterol

Introduction

Diabetes mellitus (DM) is a common chronic metabolic disease encountered in clinical practice. As the disease progresses, DM can lead to dysfunction in multiple organs and tissues, significantly impacting patients' quality of life[1]. DM and osteoporosis may develop in a bidirectional cause-and-effect relationship or as a result of a shared underlying cause, such as glucocorticoid-induced DM and osteoporosis[2,3]. Increasing evidence indicates that DM is an independent risk factor for osteoporosis, with the incidence of osteoporosis in patients with DM being 4–5 times higher than in non-diabetic individuals[4,5].

Osteoporosis is characterized by an abnormal

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microstructure resulting from net bone loss and/or disruption in the delicate balance between bone formation and resorption. While the pathogenesis of osteoporosis is multifactorial, it remains incompletely understood. Chronic hyperglycemia severely impairs lipid, carbohydrate, and protein metabolism[6]. Dysregulated lipid metabolism increases levels of very low-density lipoproteins and total cholesterol, which accumulate in the subendothelium of vessels, leading to atherosclerosis, vascular stenosis, and subsequent microvascular and macrovascular complications. These vascular changes impair the blood supply to bone, contributing to abnormalities such as microfractures.

Hyperglycemia-induced oxidative stress is a key factor in osteoporosis in DM[7]. Oxidative stress promotes the release of reactive oxygen species, resulting in mitochondrial dysfunction and cell death in bone tissue[8,9]. Chronic hyperglycemia also stimulates the formation of advanced glycation end products (AGEs), which disrupt bone mass formation by promoting non-enzymatic glycation of type I collagen cross-links[10,11]. Furthermore, AGEs interact with their receptors (RAGE), inhibiting osteoblast proliferation through pathways such as Wnt, phosphoinositide 3-kinase (PI3K), and extracellular signal-regulated kinase (ERK). Additionally, DM alters the total protein content of key enzymes involved in bone formation[12,13].

Cornus officinalis, a plant of the Cornaceae family, produces mature fruit known as *Corni Fructus*, which is a well-known Chinese herbal medicine, sometimes referred to as "jujube peel" after pitting[14]. The active compound in *Cornus officinalis*, stigmasterol (STG), can bind to bone cell membranes, directly influencing the osteoprotegerin (OPG)/RANKL ratio in osteoblasts. STG also exhibits estrogenic activity, promoting osteogenesis and inhibiting bone resorption by inducing ovarian granulosa cells to synthesize estradiol and binding to estrogen receptors on bone cell membranes[15]. Beta-sitosterol, another bioactive compound, has demonstrated various therapeutic effects, including antipyretic, anticancer, and immunomodulatory properties, making it a potential treatment for various diseases[16,17]. Additionally, the anti-inflammatory properties of STG are well-documented[18]. For instance, Chen et al. demonstrated STG's therapeutic effects on osteoarthritis in a rabbit model[19]. However, limited research exists on the effects of STG against osteoporosis induced by diabetes mellitus.

To explore the anti-osteoporosis effects in DM of STG, different doses were tested in streptozotocin-induced diabetic mice to evaluate its therapeutic effects on osteoporosis in T2DM models, aiming to provide robust scientific evidence for its efficacy.

Materials and Methods

Experimental animals

A total of 24 six-week-old specific pathogen-free male Sprague-Dawley rats (body weight: 80 ± 5 g) were obtained from the Affiliated Hospital of Youjiang Medical University for Nationalities. The rats were acclimatized for 14 days before the formal experiment.

T2DM rat model and group administration

The rats were randomly divided into two groups: the normal diet (ND) group, which consisted of six rats, and the T2DM model group, which included the remaining 18 rats. The rats in the T2DM group were fed a high-fat diet for 16 weeks, after which diabetes was induced by an intraperitoneal injection of streptozotocin (30 mg/kg, Sigma) at week 16. The rats in the ND group received an equal volume of physiological saline (Affinity). Blood glucose and insulin levels were assessed by fasting blood glucose (FBG) and the homeostasis model assessment of insulin resistance (HOMA-IR). Rats with blood glucose levels exceeding 16.7 mmol/L for three consecutive days and maintaining levels above 16.7 mmol/L for 2–4 weeks were considered to have developed DM.

After successfully establishing the model, the T2DM rats were randomly divided into three groups: the high-fat diet (HFD) group, the low-dose STG (STG-L) group (Sigma), and the high-dose STG (STG-H) group, with six rats in each group. The ND and HFD groups received physiological saline intragastrically. Rats in the STG-L group were treated with STG (100 mg/kg) intragastrically, while rats in the STG-H group were treated with STG (200 mg/kg) intragastrically. All treatment groups were administered once daily for 4 consecutive weeks.

Hematoxylin and eosin staining

To assess femoral tissue damage, trabecular bone from the rat femur was isolated. A portion of the tissue was fixed in 4% paraformaldehyde for 24 hours. The tissues were then decalcified with ethylenediaminetetraacetic acid (EDTA) solution for 15 days and prepared into 4 μm paraffin sections. Hematoxylin and eosin staining was performed on the femoral sections following the method described by Liu et al. (20) to evaluate pathological changes in the femoral tissue.

Computer tomography examination of bone tissue morphology

The femurs of the rats were wrapped in saline-soaked gauze and placed vertically in sample cups. The femurs were then analyzed using a micro-computed tomography (CT) scanner (Scanco Medical, Switzerland) to assess trabecular bone microstructure. The following indicators were measured: trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), bone volume/total volume ratio (BV/TV), trabecular bone number, and cartilage quantity.

Enzyme-linked immunosorbent assay

Serum samples from the rats were thawed at room temperature and then diluted to an appropriate concentration according to the instructions provided by the enzyme-linked

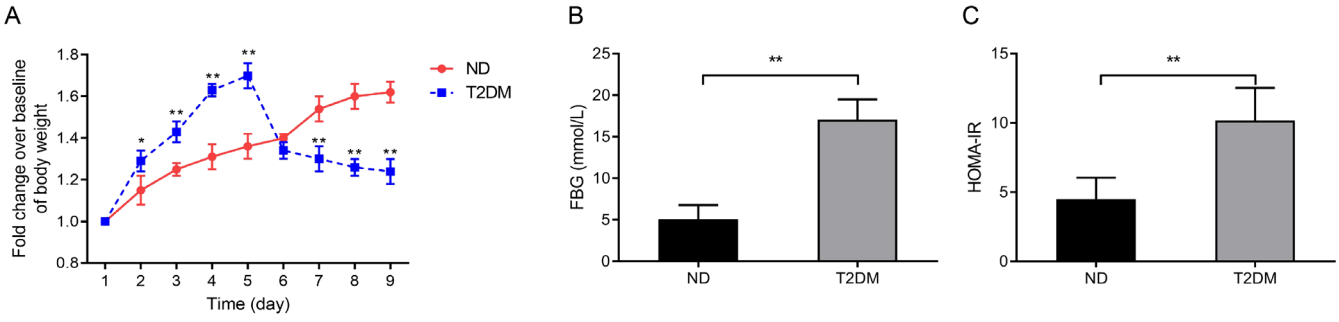

Figure 1. Changes in body weight of rats in the ND and T2DM groups during HFD and intraperitoneal STZ injection: A) Body weight of rats in the ND and T2DM groups; B) FBG levels in the ND and T2DM groups; C) HOMA-IR index in the ND and T2DM groups. ND, normal diet; T2DM, type 2 diabetes mellitus; HFD, high-fat diet; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment of insulin resistance.

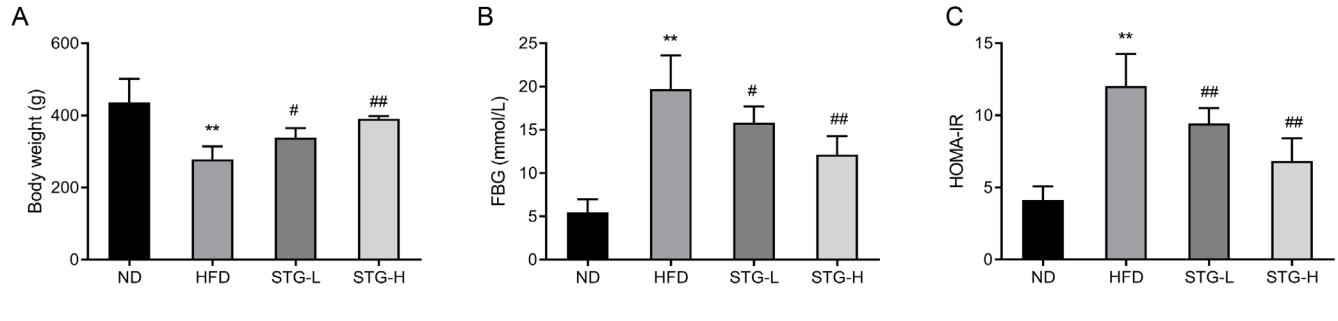

Figure 2. Effects of STG on body weight gain and hyperglycemia induced by T2DM in rats: A) Body weight, B) FBG levels, C) HOMA-IR index of rats in different treatment groups. STG, stigmasterol; T2DM, type 2 diabetes mellitus; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment of insulin resistance.

immunosorbent assay (ELISA) kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.). Reagents and samples were added to the ELISA plate, and the plate was incubated according to the standard protocol. After the reaction was complete, the optical density (OD) at 450 nm was measured using a microplate reader. Serum levels of bone formation markers, including procollagen type I N-terminal propeptide (P1NP), osteocalcin (OC), and osteoprotegerin (OPG), were calculated based on the standard curve.

Western blot

Bone homogenate was prepared from femur tissue following the method described in a previous study[21]. Total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer (Sigma), and protein concentration was measured. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Germany) using the wet transfer method. The membranes were blocked with 5% skimmed milk (Amresco, USA) at room temperature for 2 hours. Primary antibodies were added at a 1:1000 dilution and incubated overnight at 4°C on a shaker. The next day, the membranes were incubated with secondary antibodies at a 1:5000 dilution at 37°C for 45 minutes. Afterward, enhanced chemiluminescence (ECL) reagent (Sigma) was added for detection. β-actin was used as an internal reference, and the relative expression levels of runt-related transcription factor 2 (Runx2), osteopontin (OPN), and collagen type I alpha 1 chain (COL1A1) proteins (Abcam, USA) were determined by calculating the ratio of their respective gray values to that of β-actin.

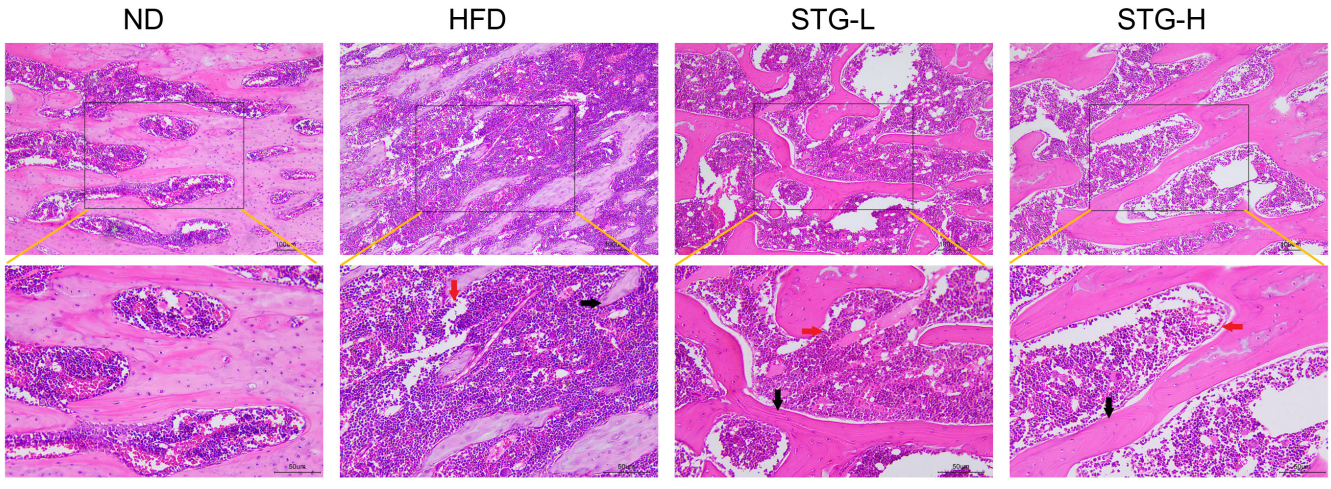

Figure 3. Hematoxylin and eosin staining was used to observe the trabecular bone structure of the femurs in the ND, HFD, STG-L, and STG-H groups. ND, normal diet; HFD, high-fat diet; STG-L, low-dose stigmasterol; STG-H, high-dose stigmasterol.

Statistical Analysis

All experimental data were statistically analyzed using SPSS 26.0 software. A t-test was used for comparisons between two groups, while one-way analysis of variance (ANOVA) was applied for comparisons among multiple groups. The results are presented as mean ± standard deviation. A p-value of < 0.05 was considered statistically significant.

Results

Establishment of the T2DM rat model

The results shown in Figure 1A-C indicate that, during the high-fat diet (HFD) period, the body weight of rats in the T2DM group was significantly higher compared to the ND group ($P < 0.05$). Following STZ injection, the T2DM group rats exhibited a decrease in body weight. In addition, FBG levels and the homeostasis model assessment of HOMA-IR index were significantly elevated in the T2DM group compared to the ND group ($P < 0.05$).

Effects of STG on high body weight and hyperglycemia in T2DM rats

The results showed that, following successful model establishment, body weight in the HFD group was significantly lower compared to the ND group ($P < 0.05$) (Figure 2A). In contrast, body weight was notably increased in the STG-L and STG-H groups compared to the HFD group ($P < 0.05$). Additionally, FBG levels and the homeostasis model assessment of HOMA-IR index were measured. FBG levels and HOMA-IR index were significantly higher in the HFD group compared to the ND group ($P < 0.05$), while both FBG levels and the HOMA-IR index were significantly lower in the STG-L and STG-H groups than in the HFD group ($P < 0.05$) (Figures 2B-C).

Effects of STG on the trabecular bone structure of femur in T2DM rats

Hematoxylin and eosin staining (Figure 3) was performed to observe femoral tissue damage. In the ND group, the femur exhibited abundant, continuous, and dense trabecular bone, indicating normal trabecular bone structure. In contrast, the HFD group showed destruction of the trabecular bone structure, with partial loss of trabeculae, enlargement of the bone marrow cavity, and an increase in fat cells. Compared to the HFD group, the trabecular bone structure was improved in the STG-L and STG-H groups, with a reduction in fat cell number. Notably, the trabecular bone restoration was more pronounced in the STG-H group than in the STG-L group.

Effects of STG on the microstructure of femur in T2DM rats

Micro-CT scan results (Figure 4A-E) showed that, compared to the ND group, the HFD group exhibited a significant decrease in bone mineral density (BMD), BV/TV, and Tb.Th in femur tissues ($P < 0.05$), along with a notable increase in the BS/BV ratio and Tb.Sp ($P < 0.05$). In contrast, the STG-L and STG-H groups had significantly higher BMD, BV/TV, and Tb.Th, and significantly lower BS/BV and Tb.Sp compared to the HFD group ($P < 0.05$).

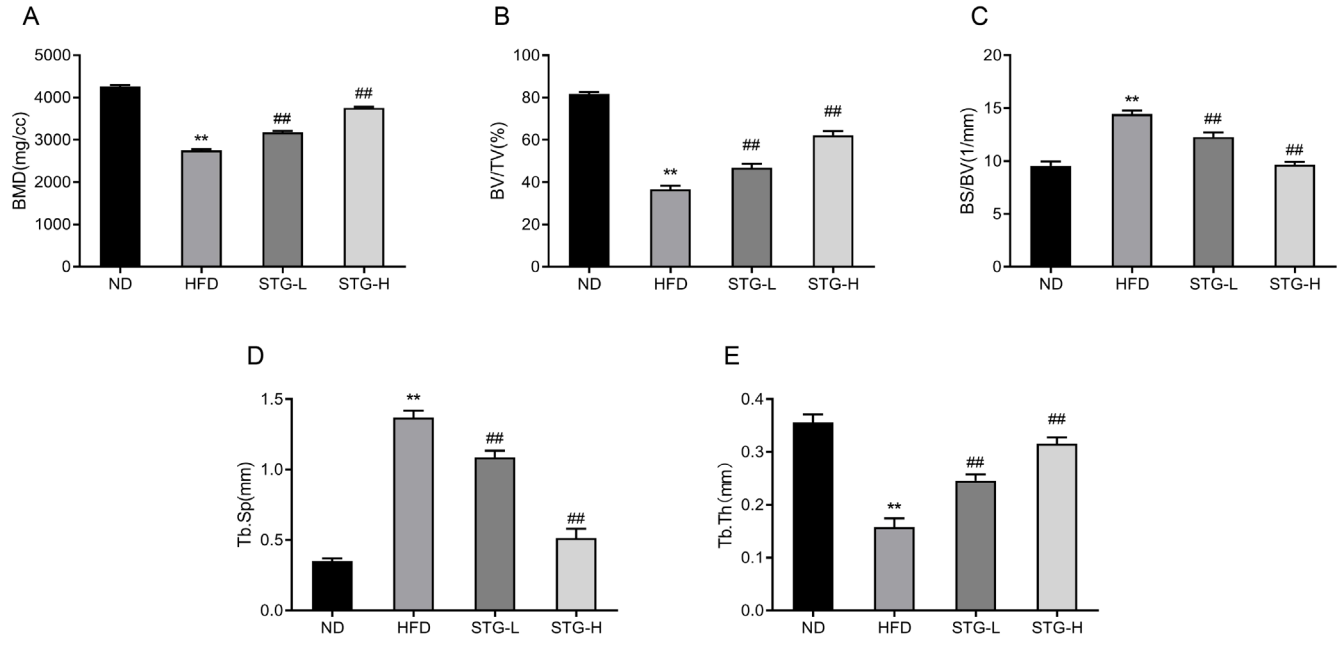

Figure 4. Effects of STG on the trabecular bone microstructure of femurs in T2DM rats. A, BMD; B, BV/TV; C, BS/BV; D, Tb.Sp; E, Tb.Th. STG, stigmasterol; T2DM, type 2 diabetes mellitus; BMD, bone mineral density; BV/TV, bone volume/total volume; BS/BV, bone surface/bone volume ratio; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness.

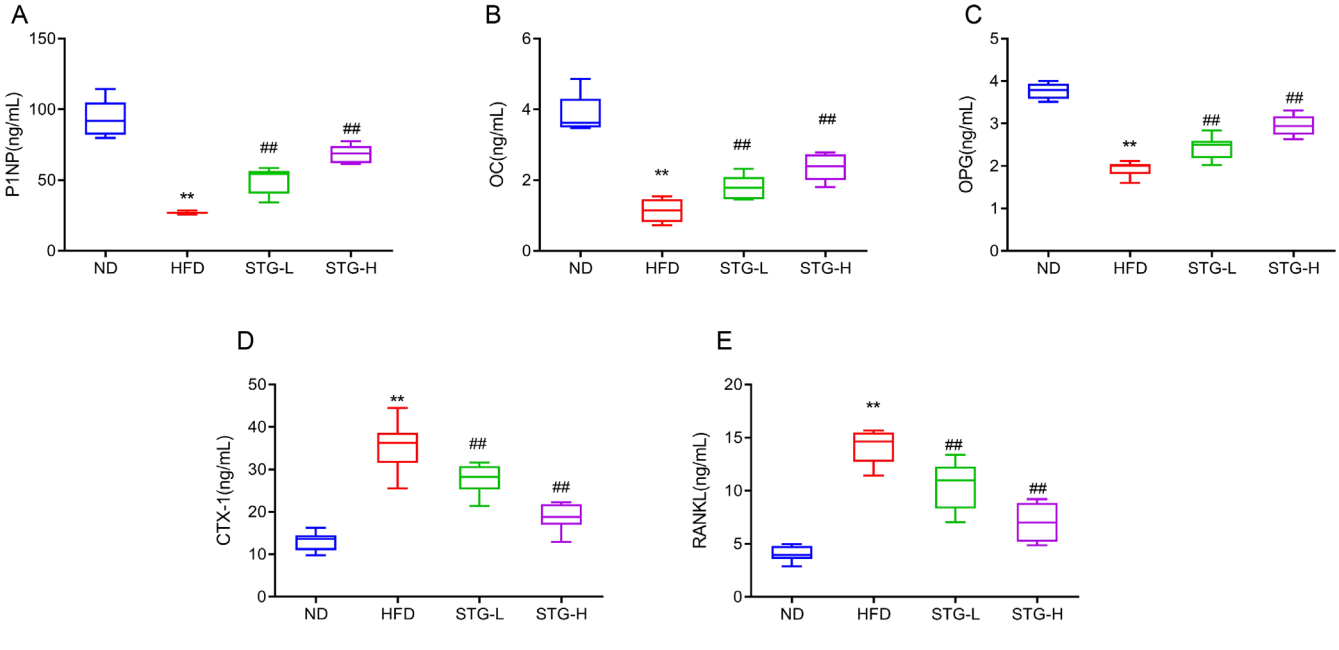

Figure 5. Effects of STG on serum bone formation and resorption markers in T2DM rats. A–E: ELISA analysis of the serum levels of PINP (A), OC (B), OPG (C), CTX-1 (D), and RANKL (E) in different treatment groups. STG, stigmasterol; T2DM, type 2 diabetes mellitus; PINP, Procollagen type I N-terminal propeptide; OC, osteocalcin; OPG, osteoprotegerin; CTX-1, C-terminal telopeptide of type I collagen; RANKL, receptor activator of nuclear factor kappa-B ligand.

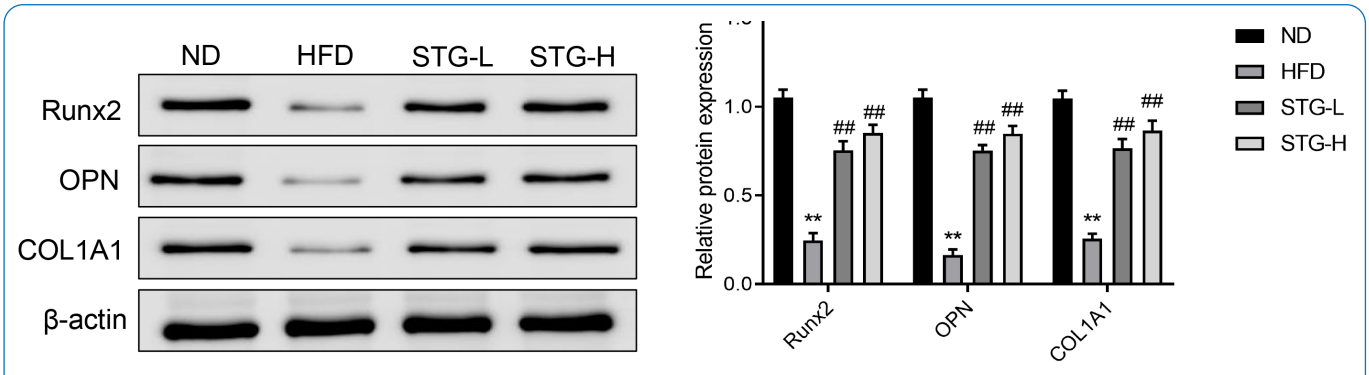

Figure 6. Effects of STG on osteogenic marker protein levels in the femurs of T2DM rats. Western blot analysis was used to detect the protein expression of Runx2, OPN, and COL1A1 in femur tissues from different treatment groups. STG, stigmasterol; T2DM, type 2 diabetes mellitus; Runx2, runt-related transcription factor 2; OPN, osteopontin; COL1A1, collagen type I alpha 1 chain.

Effects of STG on serum bone formation and resorption markers in T2DM rats

The ELISA results (Figure 5A–E) showed that serum bone formation markers, including P1NP, osteocalcin (OC), and osteoprotegerin (OPG), were significantly lower in the HFD group compared to the ND group ($P < 0.05$). In contrast, the levels of bone resorption markers, such as C-terminal telopeptide of type I collagen (CTX-1) and receptor activator of nuclear factor kappa-B ligand (RANKL), were significantly higher in the HFD group compared to the ND group ($P < 0.05$). Compared to the HFD group, the STG-L and STG-H groups exhibited a significant increase in P1NP, OC, and OPG levels, along with a notable reduction in CTX-1 and RANKL levels ($P < 0.05$).

Effects of STG on osteogenic marker protein levels in the femurs of T2DM rats

The western blot results (Figure 6) showed that, compared to the ND group, the protein levels of Runx2, osteopontin (OPN), and collagen type I alpha 1 chain (COL1A1) were significantly reduced in the femur tissues of the HFD group ($P < 0.05$). In contrast, the STG-L and STG-H groups exhibited a significant increase in the protein levels of Runx2, OPN, and COL1A1 compared to the HFD group ($P < 0.05$).

Discussion

With the rising global prevalence of DM and osteoporosis, these conditions contribute to high morbidity and mortality rates among the elderly[22]. Both type 1 and type 2 DM are linked to decreased bone strength and an increased risk of fractures[23]. Osteoporosis in DM is a systemic metabolic bone disorder, characterized by reduced bone mass, altered microstructure, decreased bone strength, and increased fragility, making it a major complication affecting the skeletal system[24]. Consequently, the treatment of osteoporosis in DM has become a prominent focus in the field of systemic metabolic diseases. *Cornus officinalis* has been suggested to have potential in preventing and managing osteoporosis through various components, targets, and signaling pathways. Preliminary screening of the main components of *Cornus officinalis* identified ethyl octadecatrienoate (cis-9,12,15), STG, cornoside, and 3-deazaneplanocin A. These components have been shown to prevent osteoporosis, enhance the expression of bone differentiation factors, and regulate bone formation and mineralization by mediating endoplasmic reticulum stress, thus promoting osteogenesis and inhibiting bone resorption[25,26]. In this study, STG from *Cornus officinalis* was used as the intervention substance. The anti-osteoporosis effect in DM of STG, through improving BMD, was investigated by measuring the trabecular bone structure of the femur, serum levels of bone formation markers (P1NP, OC, and OPG), and the expression of related proteins in femur tissues.

In this study, STG significantly reduced FBG levels and the HOMA-IR index in the STG-L and STG-H groups, consistent with the findings of Zhou et al.[27]. Additionally, STG improved trabecular bone structure in these groups, with a reduction in the number of fat cells. The effect in the STG-H group was more pronounced than in the STG-L group, suggesting that STG is a potential therapeutic agent with a dose-dependent effect. The micro-CT results revealed that a HFD could damage trabecular bone structure. Compared with the HFD group, the STG-L and STG-H groups showed a significant increase in BMD, BV/TV, and Tb.Th, along with a notable decrease in BS/BV ratio and Tb.Sp. The trabecular bone structure was markedly improved in the STG-H group, indicating that STG could mitigate DM-induced trabecular bone damage, in line with related studies[28]. Furthermore, STG notably increased serum levels of P1NP, OC, and OPG,

while decreasing the levels of CTX-1 and RANKL. Previous studies have shown that *Cornus officinalis* may enhance osteogenesis-related proteins such as Runx2, OPN, OC, and OPG, potentially promoting osteogenic differentiation and alleviating the osteoporotic phenotype[29].

Promoting bone formation is crucial in the treatment of osteoporosis and serves as a guiding principle for the development of new therapeutic agents[30]. Osteoblasts play a vital role in synthesizing the bone matrix, regulating mineralization, and eventually differentiating into osteocytes or bone lining cells[31]. The growth and differentiation of osteoblasts involve continuous cell proliferation, extracellular matrix maturation, and osteonectin expression, all of which contribute to matrix mineralization and enrichment[32,33]. The transcriptional activation of osteogenic factors such as Runx2, OPN, and COL1A1 increases trabecular bone volume, mineralized matrix, and BMD, while also inducing the expression of osteoblast markers like COL1A1 and OC[34,35]. These findings suggest that STG may enhance the expression of osteoblast markers, thereby promoting bone formation.

Conclusion

In conclusion, STG promotes bone remodeling by alleviating trabecular bone damage in DM-induced osteoporotic rats. Its potential mechanisms include improving trabecular bone microstructure, upregulating bone formation markers such as P1NP, OC, and OPG, and enhancing related proteins in femur tissue to promote bone formation. Additionally, STG downregulates CTX-1 and RANKL to inhibit bone resorption. This study established a rat model of osteoporosis associated with DM using a HFD, suggesting that STG may have potential effects in mitigating this condition by enhancing BMD. However, further research is needed to fully elucidate the regulatory mechanisms of STG in bone metabolism in osteoporosis.

Ethics approval

This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities (approval number: 20230210O1).

Authors' contributions

Study concept and design: HW; Analysis and interpretation of data: HW, ZM, HX, XY, and CY; Drafting of the manuscript: HW, ZM, HX, and HH; Critical revision of the manuscript for important intellectual content: XY, CY; Statistical analysis: HW, ZM, HX, and HH; Study supervision: all authors. All authors made significant contributions to the work, whether in the conception, study design, execution, data acquisition, analysis, interpretation, or all of these areas. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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