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ORIGINAL ARTICLE

Diosgenin prevents bone loss on retinoic acid-induced osteoporosis in rats

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Abstract
Objective To observe the preventive and therapeutic effects of diosgenin on retinoic acid-induced osteoporosis in rats.
Methods A total 50 Sprague-Dawley rats were randomly divided into 5 groups: control group, model group (osteoporosis rats), low (10 mg kg^{-1}), middle (30 mg kg^{-1}), and high-dose diosgenin (90 mg kg^{-1})-treated groups. The osteoporosis rats model was induced by retinoic acid. The BMD and physical parameters of femoral including length, wet weight, and dry weight in each group were measured. The hematoxylin–eosin staining was used for bone histomorphology analysis. Besides, the bone calcium (Ca) and phosphorus (P) contents were measured. In order to detect the biochemical index in different treatment groups, the serum tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase (ALP), estradiol, and osteocalcin were compared among different groups.
Results The osteoporosis rat model was successfully induced by retinoic acid. Compared with the model group, the lessening of femoral length and weight and the loss of BMD were significantly improved in diosgenin groups. Both contents of Ca and P were much more increased when induced by retinoic acid ($p < 0.05$). The estradiol and osteocalcin levels in the middle and high-dose treatment groups were significantly higher than that of the model group, while the ALP and TRAP levels were much lower than the model group ($p < 0.05$).
Conclusion Diosgenin can prevent the loss of bone in experimental rats. The mechanism may be that it improves the level of estrogenic hormone of estradiol and inhibits the high bone turnover.

Keywords Diosgenin · Osteoporosis · Retinoic acid · Bone loss · Rat

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Introduction

Osteoporosis is a bone disease that leads to an increased risk of fracture with reduced bone mineral density (BMD), deteriorated bone microarchitecture, and alters amounts and types of proteins in bone [1, 2]. The purpose of osteoporosis treatment is to prevent the bone fractures by decreasing bone loss or, preferably, by enhancing bone density and strength [3, 4]. Early detection and treatment of osteoporosis can sufficiently decrease the risk of future bone diseases, while it is difficult to cure the osteoporosis by rebuilding the bone. In addition, there is no available treatment to cure osteoporosis completely. Therefore, early prevention of osteoporosis is as important as treatment [5].

Natural compounds in plants, such as polyphenols and flavonoids, have proved to be preventive efficacy to bone loss in osteoporosis rats [6–9]. Diosgenin, a steroid sapogenin (Fig. 1), extracting from *Dioscorea* wild yam tubers, such as the Kokoro [10], has been shown to inhibit proliferation, suppress inflammation, and induce apoptosis in tumor cells [11–13]. The aglycone (sugar-free), diosgenin is used for the commercial synthesis of steroid products, such as pregnenolone, cortisone, progesterone, etc. Previous studies have shown that diosgenin could be used to prevent and treat osteoporosis [14, 15]. All these studies indicate the safety and efficacy of diosgenin using as a

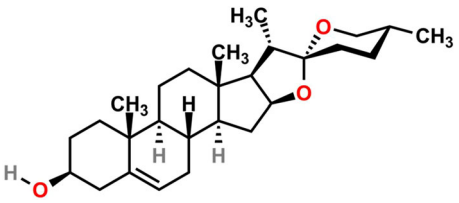

Fig. 1 Chemical structure of the steroid diosgenin

certain alternative treatment modality for osteoporosis, and it is available for diosgenin being used therapeutically for postmenopausal women who attempt to reduce osteoporotic progression. However, the molecular mechanism of diosgenin activity in bone-derived cells remains largely unknown.

The model of retinoic acid-induced osteoporosis in rats is used in several studies to evaluate the influence of substances on bone loss in human, for its easy operation, high successful rate, short time consumption, and type symptoms of osteoporosis [16–19]. Early studies observed that large dose of vitamin A was toxic to the skeletal system of rats [20, 21]. Further studies also showed that retinoic acid causes constant decrease of BMD in a short period of 1–3 weeks [20]. All these findings demonstrated that short-term effects of retinoic acid could act as an appropriate revulsive of osteoporosis [22].

In the present study, we investigated the influence and mechanisms of diosgenin activity in preventing and treating osteoporosis rat model induced by retinoic acid. Our study also provides further information to the possible therapeutic use of diosgenin on the treatment of bone-related diseases.

Materials and methods

Animal grouping

A total of 50 female Sprague-Dawley rats (National Grade A experimental animal) aged 90 days old weighing between 190 and 260 g were offered by the Center of Experimental Animals, China Pharmaceutical University (Nanjing, China). The animal care and animal experimentations were processed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research and Council, 1996).

Forty of the rats were treated with 70 mg/kg retinoic acid suspension (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) once daily for 14 days. The rat osteoporosis model induced by retinoic acid was examined by the cortex, size, and beam of bone. Thirty of the these rats were randomly allocated to low (10 mg/kg), middle (30 mg/kg), and high (90 mg/kg) dose-treated groups with administration of three doses of diosgenin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), respectively, by oral gavage for another 14 days. The doses of diosgenin are similar to 1 time, 2 times, and 4 times dose of steroidal saponins in clinical prescription for treatment of patients with myocardial ischemia or coronary heart disease (1.44 g/60 kg weight/-day) [23]. Others feed with 0.5 % 70 mg/kg sodium carboxymethylcellulose (CMC-Na, Longhe food ingredient co., LTD, Nanjing, China) were defined as model group.

Additionally, another 10 healthy rats were supplemented as the healthy control group and treated with 0.5 % 70 mg/kg CMC-Na once daily for 28 days. The dosing was adjusted according to the daily weight conditions. All rats were raised under consistent conditions during the study.

Bone physical parameters

Bilateral femur bones were got from the killed rats for histomorphology analysis. The right one was used to analyze the length and wet weight. For dry weight determination, the femur bone was first dehydrated and then carbonized by burning into ashes at 800 °C for 8 h. The left femur bone was cut at the mid-diaphysis to test BMD using dual energy X-ray bone densitometer (Hologic, USA).

Bone histomorphometry

The femoral bones of the selected rats from each group were used to analyze the bone histomorphometry by hematoxylin–eosin (HE) staining. Briefly, the bone samples were fixed in 4 % formalin for 24 h followed by 2-week decalcification at 4 °C by 10 % ethylene diamine tetraacetic acid (EDTA) solution. After that, bone samples were trimmed and embedded in paraffin. They were cut into 5-μm-thick sections and stained with HE for light microscope examination.

Bone mineral detection

The levels of Ca and P (mmol/g) were determined by inorganic calcium and phosphorus assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) when some ashes were dissolved in 6 mol/L HCl (Norman Biotechnology Co. Ltd., Nanjing, China).

Biochemical indexes of serum ALP, TRAP, estradiol, and osteocalcin

The serum samples were obtained from the rats 24 h after the last dose of the study drugs given. The levels of ALP and TRAP were measured with reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. The colorless *para*-nitrophenyl phosphate (pNPP) is converted to

yellow encounter with ALP and TRAP at the base condition and acid condition, respectively. Thus, the enzymatic activities of ALP and TRAP could be evaluated by colorimetric analysis of pNPP. The absorbance of serum samples was measured at 400–415 nm under the base and acid conditions, respectively, to detect their enzymatic activity. The levels of estradiol (ng/mL) and osteocalcin (pg/mL) were detected by radioimmunoassay [24] with ^{125}I-labeled tracer (Aladdin reagent Co., Shanghai, China).

Statistical analysis

Statistical comparison analysis was performed by ANOVA. Results were expressed as mean ± SE with significance defined as $p < 0.05$.

Results

Effect of diosgenin on bone physical parameters

As shown in Table 1, the BMD, length, wet weight, and dry weight of femur bones in model group were much less than that in the control group ($p < 0.05$), indicating a successful osteoporosis model was constructed. Compared with the model group, different dose of diosgenin-treated groups showed significant anti-osteoporosis effect ($p < 0.05$), especially the rats treated with high dose of diosgenin.

Effect of diosgenin on bone histomorphology

In order to detect the effect of diosgenin on bone histomorphology, the histomorphometry was performed by HE staining (Fig. 2). Compared with the control group, rats in model group showed sparse and thin trabeculae, as well as loss of connectivity. However, the trabeculae were relatively wider and reticulate structure was more obvious after treated with different concentrations of diosgenin.

Effect of diosgenin on bone calcium (Ca) and phosphorus (P)

Both bone Ca and P levels in model group and diosgenin-treated groups were significantly increased compared with that in control group ($p < 0.05$). As shown in Fig. 3, there was a significant difference in bone Ca content

Table 1 Bone BMD and physical parameters in different treatment groups

Group	BMD (g/cm^2)	Length (mm)	Wet weight (mg)	Dry weight (mg)
Control	0.198 ± 0.005	38.00 ± 0.13	0.79 ± 0.08	0.64 ± 0.08
Model	0.173 ± 0.007*	34.35 ± 0.08*	0.60 ± 0.10*	0.50 ± 0.04*
Low	0.182 ± 0.003*,#	35.21 ± 0.04*,#	0.65 ± 0.03*	0.54 ± 0.07*
Middle	0.187 ± 0.006*,#	36.05 ± 0.15*,#	0.69 ± 0.08*,#	0.58 ± 0.05#
High	0.192 ± 0.018#	36.97 ± 0.08*,#	0.73 ± 0.10#	0.59 ± 0.06#

* $p < 0.05$ compared with control group

# $p < 0.05$ compared with model group

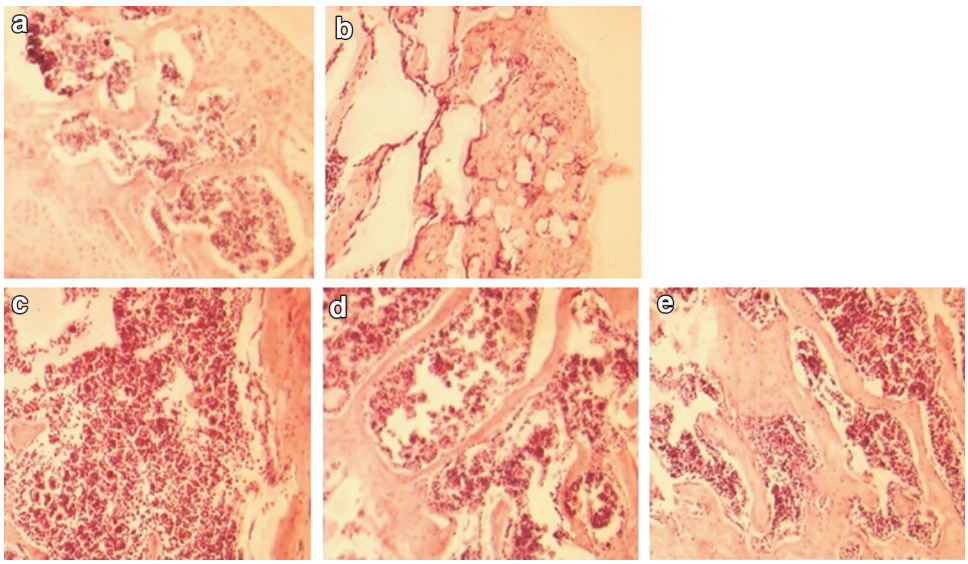

Fig. 2 Pathomorphology observation of femur in rat (HE, ×100). **a** Control group, **b** model group, **c** low-dose diosgenin group of 10 mg/kg, **d** middle-dose diosgenin group of 30 mg/kg, **e** high-dose diosgenin group of 90 mg/kg

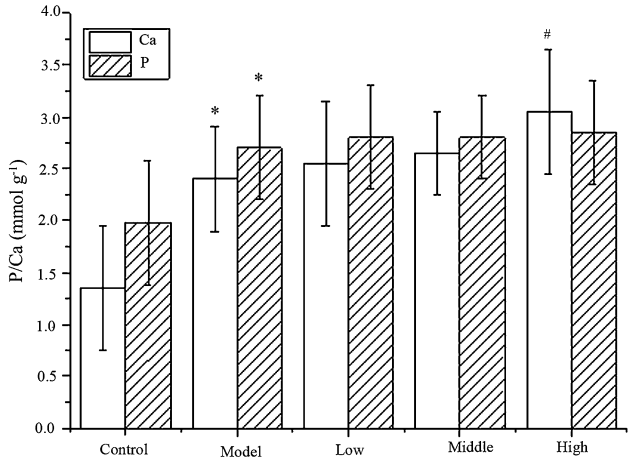

Fig. 3 Effect of diosgenin on bone Ca and P. *Significant difference of bone Ca and P level compared with model group, #significant difference in bone Ca content between high-dose group and model group ($p < 0.05$)

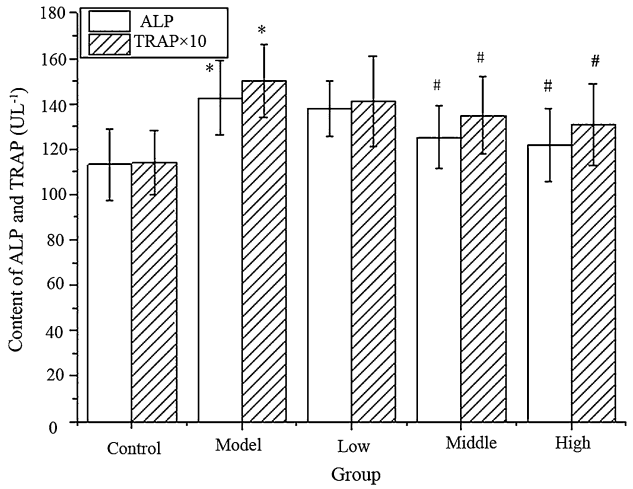

Fig. 4 Effect of diosgenin on serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). *$p < 0.05$ compared with control group; #$p < 0.05$ compared with model group ($p < 0.05$)

between high-dose-treated group and model-treated group ($p < 0.05$).

Effect of diosgenin on ALP and TRAP

As shown in Fig. 4, enzymatic activities of ALP and TRAP in serum of model group were much more than those of control group ($p < 0.05$). However, ALP and TRAP activities in serum of middle and high-dose-treated groups were evidently less than those of model group ($p < 0.05$).

Effect of diosgenin on estradiol and osteocalcin

Figure 5 shows estradiol and osteocalcin contents in model group were evidently lower than those of control group

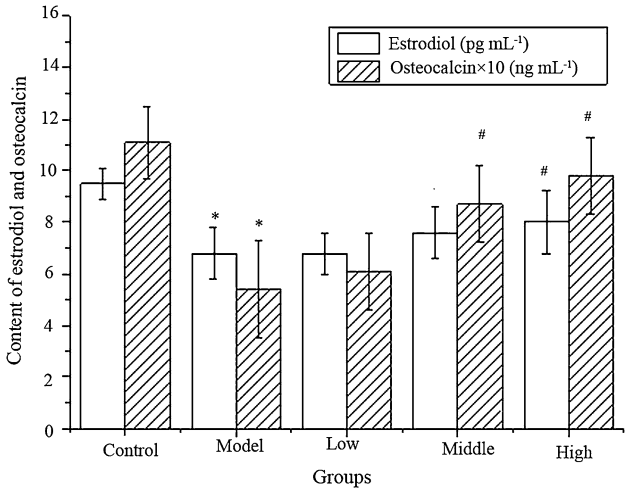

Fig. 5 Effect of diosgenin on serum estradiol and osteocalcin. *$p < 0.05$ compared with control group; #$p < 0.05$ compared with model group

($p < 0.05$). Both estradiol and osteocalcin contents increased in a dose-dependent manner. In addition, estradiol content in high-dose-treated group and osteocalcin content in middle and high groups were much more than model group ($p < 0.05$), indicating diosgenin could reduce estradiol and osteocalcin loss induced by retinoic acid.

Discussion

In the present research, we investigated whether diosgenin intake inhibits osteoporosis in retinoic acid-induced rats by regulating bone metabolism toward negative balance. The retinoic acid-induced rats showed a decreased femur length and estradiol and osteocalcin level, and increased level of ALP and TRAP, indicating a successful model. Indeed, significant promotion of bone formation and inhibition of bone absorption were observed in diosgenin-treated groups, indicating the improvement of bone metabolism and bone mineralization. All these changes closely imitate the human bone metabolism.

The histopathological studies have already revealed the sparse and disrupted bone in osteoporosis rats, and this disruption may lead to the decline of bone strength [25]. The experimental results showed the reduced weight of femoral shaft, shortened width of bone, and decreased bone mineral substances, and collagen in rats model group administered retinoic acid for 14 days, implying successfully induced osteoporosis-like changes. All the indexes have obvious improvement in middle, high dose of diosgenin-treated groups compared with model group. These changes support with our hypothesis that there is a protective effect of diosgenin against osteoporosis induced by retinoic acid in rats.

Nutritional factors also contribute to the development of osteoporosis. Contents of Ca and P in model group were higher than in control, and the reason could be explained by the slow lost process of these mineral substances. Previous study has also proved that the phases of bone resorption were much longer than bone formation when estrogen level declined caused by ovariectomization [26]. Contents of Ca and P were much higher in diosgenin-treated group than in model group, especially the bone Ca content between high-dose group (90 mg/kg) and model group. These changes may be due to the botanical composition (e.g., flavonoids, phenolics) in diosgenin, which could exhibit strong bone protective properties in rat models of osteoporosis [27].

ALP, a biomarker of bone information, releasing from human osteoblast cells, can reflect the activity of osteoblast [28]. TRAP mainly expressed by osteoclast, having a pivotal role in many biological processes including bone mineralization, skeletal development, etc., can indicate the amount and activity of osteoclast [29]. As bone metabolic markers, the serum ALP and TRAP level associated with bone formation was increased in osteoporosis and other bone metabolic disorders [30]. Similar changes happened in our study, the increased activity levels of ALP and TRAP under osteoporosis conditions were significantly decreased by diosgenin-mediated suppression, indicating an antagonistic effect of diosgenin on bone absorption and bone loss, and an enhancement of bone absorption. Our study also showed a compensated bone formation in diosgenin-treated group.

As we know, osteoporosis is a direct result of a disorder balance between bone formation and its resorption. The imbalance of the two processes is acted as basic regulatory mechanism contributing to bone construction and reconstruction [31]. In addition, osteocalcin, secreted by osteoblasts, plays an important role in bone regeneration progress [31]. Upon evolution of the different osteocalcin levels between the control and treatment groups, a significant decrease was observed in model group; however, osteocalcin in diosgenin-treated groups was gradually increased with dose up, indicating diosgenin could induce ossification, producing much bone matrix, and leading to the normal balance of bone metabolism. From a mechanistic point of view, the diosgenin treatment can either raise the amount of osteoblasts, or activate the activity of the existed cells [32]. In fact, recent published data have already proved that consumption diet with rich olive oil was associated with the increase in serum osteocalcin levels [33, 34]. It seems that the diosgenin-mediated increase of osteocalcin is likely to be a key factor associated with the inhibition of bone loss.

Estradiol is an estrogenic hormone with two hydroxyl groups in molecular structure. The absence of estradiol is a well-known and probably the most important cause of osteoporosis in premature and menopause female [35]. Our results indicate that the protective effect of estradiol on diosgenin-induced bone loss occurs, and estradiol content in diosgenin-treated groups was increased in different degrees. Ferretti et al. [36] suggested that the estradiol can prevent osteoblast apoptosis by suppression of estrogen receptor β (ERβ). Our results suggest the possible mechanism that diosgenin improves the level of estradiol and inhibits the high bone turnover, while the inhibition principle was still completely blunted. The role of diosgenin influencing estradiol expression needs further study.

Recently, the possibility that naturally phytochemicals from edible materials may reduce the risk of bone diseases has gained considerable interest. Zhang et al. [25] demonstrated that higher dose of ethanol extract of *Lepidium meyenii* was effective in preventing bone loss. Rao et al. [37] reported that lycopene in tomato inhibited osteoclastic mineral resorption by decreasing tartrate-resistant acid phosphatase formation. Citrus fruits are rich in micronutrients, limonoids, and polyphenolic compounds, which were important in improving the BMD and bone biomechanical properties and decreasing the bone resorption [38]. A recent report showed that an extract of *Prunus mume* affected the proliferation and differentiation of pre-osteoblastic MC3T3-E1 cells [39]. Almost all these researches based on the mechanisms that antioxidant vitamins may exert favorable effects on BMD and osteoporotic risk by scavenging free radicals and thereby reducing oxidative stress [40]. The present study also proved that diosgenin has certain prevention and cure function for osteoporosis rats, however, by promoting bone formation, inhibiting bone absorption, and regulating bone metabolism. Although Shishodia and Aggarwal [41] suggest that the diosgenin suppresses osteoclastogenesis through restrain of NF-κB-regulated gene expression and reinforce of cytokines-induced apoptosis, the detailed mechanisms of inhibiting effect of diosgenin are still unknown; therefore, further studies are needed to identify the bioactive components and the mechanisms of the action.

Conclusions

According to the experimental results mentioned above, we can make the following conclusions. First, diosgenin possesses a potential inhibitory effect against osteoporosis via promoting bone formation, inhibiting bone absorption, and regulating bone metabolism toward negative balance. Second, diosgenin has certain prevention and cure function for osteoporosis in rats induced by retinoic acid. Our study provides a theoretical base for the further development of diosgenin.

Conflict of interest None.

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