

Original Research Article

Ginkgetin aglycone exerts anti-osteoporotic effect via regulation of NOX4/Akt/PI3K pathway

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Abstract

Purpose: To investigate the protective effect of Ginkgetin aglycone (GA) on ovariectomy-induced osteoporosis in rats, as well as the mechanism of action involved.

Methods: Adult female Wistar rats ($n = 40$) were separated into four groups: normal control, ovariectomy (OVR), 100 mg GA/kg dose, and 200 mg GA/kg dose. The rats were ovariectomized using standard procedures, except for those in normal control group. Rats in the two treatment groups received 100 or 200 mg GA/kg orally for a period of 12 weeks. Biochemical assays were performed on the urine and blood. Markers of bone formation and mediators of inflammation were assessed. Bone micro-architectural changes were examined using micro-CT scanner, while Western blotting was used to determine the expressions of NOX4, NF- κ B p65, PI3K, Akt and JNK proteins in rat femurs.

Results: Phosphorus and calcium levels in the serum varied among different groups. Levels of calcium, phosphorus and creatinine decreased ($p < 0.01$) significantly to a greater extent in the urine of GA group than in that of OVR group ($p < 0.05$). Interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF- α) and osteocalcin (OC) levels and the activity of alkaline phosphatase (ALP) decreased more in GA group than in OVR group. In GA-treated group, bone mineral density (BMD) was enhanced in a dose dependent manner than OVR group ($p < 0.05$). Treatment with GA ameliorated altered bone microarchitecture in OVR rats. Treatment of osteoporotic rats with GA led to significant and dose-dependent decrease in the expressions of JNK, NOX4, NF- κ B p65 and PI3K, and ($p < 0.05$) increase in the expression of Akt in femur tissue.

Conclusion: In conclusion, result of study proves the anti-osteoporotic activity of GA is exerted via regulation of NOX4/PI3K/Akt pathway.

Keywords: Osteoporosis, Osteocalcin, Ginkgetin aglycone, Ovariectomy, Bone mineral density, Cytokines

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INTRODUCTION

Osteoporosis leads to increased susceptibility to fractures and bone frailty due to disturbance in bone microarchitecture characterized by loss of

bone mass. Globally, the incidence of osteoporosis is put at approximately 9 million, and it has been identified as a major cause of fractures [1]. Studies have shown that estrogen deficiency in post-menopausal women is usually

triggered by inflammatory diseases [2]. BMD is markedly reduced in osteoporotic patients due to the deficiency of certain hormones, leading to impairment of cancellous metaphyseal bone [3]. Hormone replacement therapy (HRT) is the major strategy for managing menopause. However, HRT is limited by factors that predispose women to ovarian, breast or endometrial cancer [4].

Plant-derived compounds have shown potential in the management of several disorders including osteoporosis. *Ginkgo biloba*, commonly known as *ginkgo* or maidenhair tree, is the only surviving species in the division *Ginkgophyta*. This plant is indigenous to China, and it is used in treatment of disorders caused by oxidative stress [5]. *Ginkgo biloba* is reported to have high amounts of terpenoids and flavonoids, which are reputed for their potent anti-inflammatory, antioxidant and free radical scavenging effects [6].

Ginkgetin aglycone (GA), a novel *G. biloba* extract has been shown to confer protection on the kidney due to its antioxidant and anti-inflammatory activity [7]. *Ginkgetin* aglycone reduces inflammation by inhibiting the NF- κ B signaling pathway, thereby modulating apoptosis [8]. Reported study determines the antiosteoporosis property of GA in ovariectomy rats.

MATERIALS AND METHODS

Materials

Ginkgetin aglycone was obtained from Shanghai Yuanye Biotechnology Co., Ltd. (China). Osteocalcin (OC), TNF- α and IL-1 β ELISA kits were procured from Beijing Chenglin Biotechnology Co., Ltd. (China). Primary antibodies for NADPH oxidase 4 (NOX4), NF- κ B p65, protein kinase B (Akt), c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinases (PI3K), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were products of Abcam Inc. (USA). Automated biochemical analyzer was purchased from Thermo Fischer Scientific Co., Ltd. (USA). Lunar Prodigy Advance was obtained from GE Healthcare (USA). Micro-CT scanner was product of Scanco Medical (Switzerland).

Experimental rats

The adult female Wistar rats (n = 40) used in this study were obtained from Shanghai Institute of Medical Sciences, Shanghai, China. They were housed in metabolic cages under standard conditions and had standard feed and water.

Rats were acclimatized to the laboratory environment for 7 days at 24 ± 3 °C, for light/dark cycles of 12 h at a humidity of 60 ± 5 %. Protocol performed on rats was approved by the Institutional Animal Care and Use Committee (no. IACUC/FAH-NU/2017/11). The study procedures were carried out according to the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) [9].

Experimental design

Rats were anesthetized by pentobarbitone (50 mg/kg, i.p.), and subjected to bilateral ovariectomy based on standard method. They were then randomly assigned to four groups of 10 rats each: normal control group, ovariectomy (OVR) group, 100 mg GA/kg bwt group and 200 mg GA/kg group. The rats were ovariectomized using standard procedure, except for those in normal control group.

Rats in the two treatment groups received 100 or 200 mg GA/kg bwt orally for a period of 12 weeks. Urine samples were collected from the rats. Serum was obtained from the blood by centrifuging it for 10 min 2000 rpm. The rats were subsequently sacrificed and their femurs were isolated for analysis.

Biochemical assays

The activity of ALP and levels of creatinine, phosphorus and calcium in serum and urine were determined using an automated biochemical analyzer. The levels of OC, TNF- α and IL-1 β in the serum were observed using their respective ELISA kits.

Determination of bone mineral density

Lunar Prodigy Advance was used for the estimation of BMC and BMD in excised femurs.

Assessment of bone microarchitecture

Micro-computed tomography was performed according to standard methods [10]. Micro-CT scanner (μ CT80 scanner) was used to determine trabecular bone microarchitecture (distal right femoral metaphysis). The ABA bone analysis software was used to estimate bone morphometric parameters at regions of interest. These parameters were trabecular separation, ratio of trabecular volume to bone total volume, structure model index, trabecular thickness, trabecular number, and BMD on the growth plate.

Western blotting

Each excised rat right tibia was trypsinized to form a cell suspension. Phosphate-buffered saline (PBS) was used to wash the cell and lysed with ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitor. BCA assay kit was used to estimate the protein concentration in the lysate. Isolated 30 μ g of protein was separated by gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 was added with gentle shaking at 37 ° C and incubated to block non-specific binding of the blot. Then, the blots were incubated with primary antibodies for Akt, PI3K, NF- κ B p65, NADPH oxidase 4 (NOX4), JNK and GAPDH, each at a dilution of 1 to 1000 for overnight at 4 °C. Thereafter, membrane was incubated with secondary antibody for at room temperature for 90 min. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Bio-rad gel imaging system. The respective protein expression levels were normalized to that of GAPDH which was used as a standard.

Statistical analysis

Results are represented as mean \pm SEM. One way ANOVA was used for analysis with Dunnett's post hoc test using 5.0 Graph Pad Prism (San Diego, CA, USA). Statistical significance level $p < 0.05$ considered as significance.

RESULTS

GA ameliorates the levels of some biochemical parameters in blood and urine

As shown in Figure 1, Phosphorus and calcium level was not altered in the serum among the groups. Calcium, phosphorus and creatinine level were enhanced in the urine of OVR group than in normal control group, but were significantly and dose-dependently reduced ($p < 0.01$) after treatment with GA.

Effect of GA on inflammatory mediators

Figure 3 shows IL-1 β and TNF- α levels were enhanced more in the serum of OVR group than normal control group, but were significantly and dose-dependently reduced after treatment with GA.

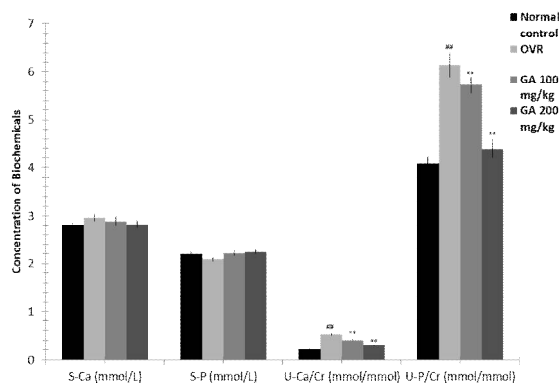


Figure 1: Effect of GA on levels of some biochemical parameters in the urine and serum of rats ## $p < 0.01$ than normal control group ** $p < 0.01$ than OVR group

GA ameliorates ALP activity and OC level

Activity of ALP and level of OC were enhanced in the serum of OVR group than in normal control group, but were significantly ($p < 0.01$) and dose-dependently reduced after treatment with GA (Figure 2).

Effect of GA on BMC and BMD

There was no alternation in BMC among the group. The BMD of OVR group was reduced ($p < 0.01$) significantly than normal control group (Figure 4.). Treatment with GA significantly ($p < 0.01$) and dose-dependently increased femoral BMD in osteoporotic rats.

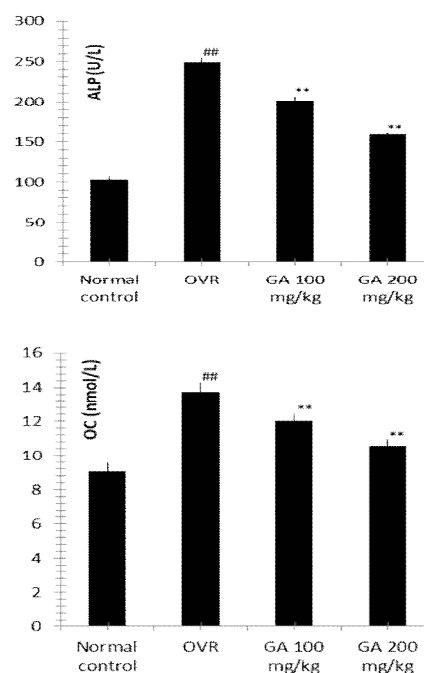


Figure 2: Effect of GA on serum ALP activity and OC level in ovariectomized rats; ## $p < 0.01$ than normal control group, ** $p < 0.01$ than OVR group

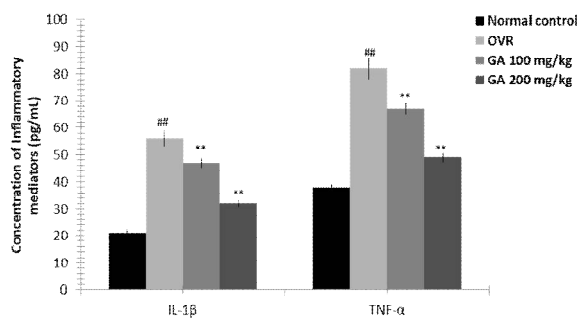


Figure 3: Effect of GA on mediators of inflammation in ovariectomized rats; ^{##} $p < 0.01$ than normal control group, ^{**} $p < 0.01$ than OVR group

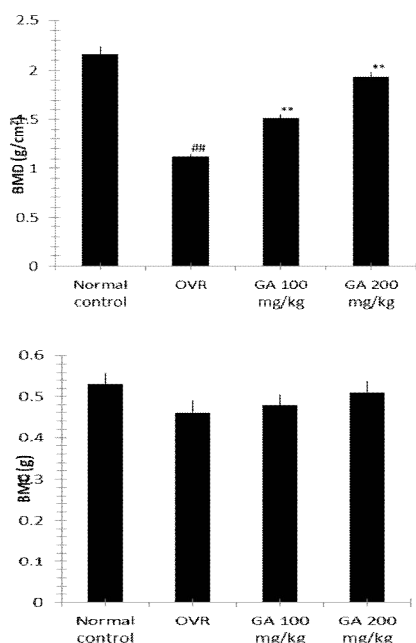


Figure 4: Effect of GA on BMC and BMD in ovariectomized rats; ^{##} $p < 0.01$ than normal control group, ^{**} $p < 0.01$ than OVR group

Effect of GA on osteoporosis-induced bone micro-architectural changes

As shown in Table 1 and Figure 5, microarchitecture of bone such as bone surface density, trabecular bone tissue volume density, trabecular number, and trabecular thickness were reduced in OVR group than in normal

control group. However, treatment with GA significantly ($p < 0.01$) reversed the osteoporosis-induced changes in bone microarchitecture.

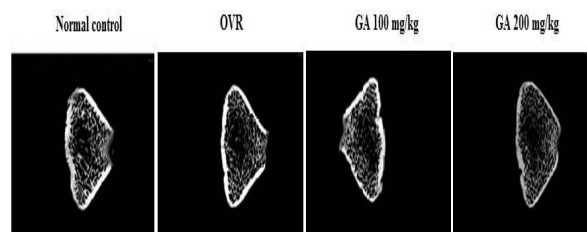


Figure 5: Effect of GA on osteoporosis-induced micro-architectural changes in ovariectomized rats

Effect of GA on expressions of NOX4/Akt/PI3K pathway proteins

Treatment of osteoporotic rats with GA led to significant and dose-dependent decreases in the expressions of JNK, NOX4, NF- κ B p65 and PI3K, and ($p < 0.01$) significant increase in the expression of Akt in femur tissue (Figure 6).

DISCUSSION

Osteoporosis is common among post-menopausal women. In rats, ovariectomy-induced osteoporosis results in marked reductions in bone strength and mass [11]. In the last few decades, compounds of plant origin have shown great potential in the management of bone disorders. This reports the protective effect of GA on ovariectomy-induced osteoporosis in rats. Several biochemical parameters are involved in bone remodeling, including ALP and OC which are markers of bone formation [12]. Levels of calcium, phosphorus and creatinine were enhanced more in the urine of OVR group than in normal control group, but were significantly and dose-dependently reduced after treatment with GA. Serum activity of ALP and level of OC were also reduced in GA group than in OVR group.

Table 1: Effect of GA on osteoporosis-induced bone micro-architectural changes

Group	Bone surface density (mm ⁻¹)	Trabecular bone tissue volume density (%)	Trabecular number (mm ⁻¹)	Trabecular thickness (mm)
Normal control	3.92 ± 0.42	37.21 ± 2.97	1.42 ± 0.04	0.28 ± 0.04
OVR	2.36 ± 0.28 ^{##}	22.49 ± 1.42 ^{##}	0.83 ± 0.03 ^{##}	0.25 ± 0.02 [#]
100 mg GA/kg bwt	3.17 ± 0.29 ^{**}	32.84 ± 2.16 ^{**}	1.27 ± 0.02 ^{**}	0.26 ± 0.01 [*]
200 mg GA/kg bwt	3.72 ± 0.32 ^{**}	36.42 ± 2.46 ^{**}	1.58 ± 0.03 ^{**}	0.28 ± 0.03 [*]

^{##} $P < 0.01$ than normal control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ than OVR group

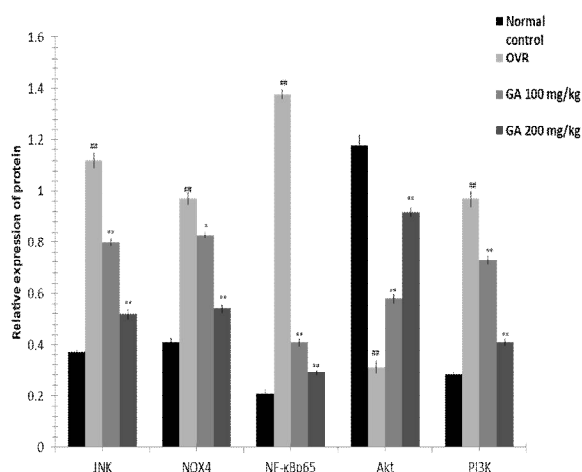


Figure 6: Relative expressions of NOX4, NF-κB p65, PI3K, Akt and JNK in the femur of osteoporotic rats; ^{##} $p < 0.01$ than normal control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ than OVR group

Treatment with GA ameliorates the altered level of biochemical parameters in the serum and urine of ovariectomized rats. BMD is the most important parameter for estimating the degree of osteoporosis and predicting the risk of fracture. In post-menopausal women, decreases in estrogen levels result in permanent loss of trabecular bone [13]. BMD was enhanced in GA treated osteoporosis rats.

Inflammatory cytokines regulate bone resorption, and osteoporosis has been shown to occur due to enhanced level of inflammatory cytokines [14]. Level of IL-1 β and TNF- α were reduced in GA group than OVR group. It is likely that GA reduces the levels of inflammatory mediators in the serum of osteoporotic rats. NOX4 regulates the function of osteoblasts, while bone formation is promoted via suppression of NOX4 expression [15]. Similarly, NF-κB p65 and JNK proteins regulates the osteoblast function [16]. Treatment of osteoporotic rats with GA may significantly reverse the altered expressions of NOX4, JNK and NF-κB p65 proteins in femoral tissue. It is an established fact that bone formation is also regulated via PI3K/Akt pathway. Treatment of osteoporotic rats with GA may significantly alter PI3K/Akt signaling pathway in femoral tissue.

CONCLUSION

The findings of this study show that the anti-osteoporotic effect of GA is exerted via regulation of NOX4/PI3K/Akt pathway. Thus, GA has potentials for clinical use in the management of osteoporosis.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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