

Original Research Article

Mollugin attenuates glucocorticoid-induced osteoporosis in rats via Akt/P13K pathway

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Abstract

Purpose: To investigate the protective effect of mollugin on glucocorticoid (GC)-induced osteoporosis in rats.

Methods: A total of 30 female Sprague Dawley rats (weighing 180 to 200 g) were randomly assigned to five groups of six rats each: control, GC and mollugin groups (20, 40 and 80 mg/kg, respectively). Except for the control group, osteoporosis was induced in the rats by intramuscular administration of dexamethasone at a dose of 2.5 mg/kg twice weekly for nine weeks. Bone mineral density (BMD) and serum activities of tartrate-resistant acid phosphatase (TRAP) and specific alkaline phosphatase (ALP), and levels of collagen type I fragment (CTX) and osteocalcin were estimated. The effect of mollugin alone, and in the presence of PI3K/Akt inhibitor on the proliferation of bone marrow osteoblasts was investigated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-tetrazolium bromide (MTT) assay. Western blotting was used for determination of the expressions of p-Akt, Akt and cyclin D1 protein.

Results: There were significant increases in body weights of rats in GC group, when compared with the control group. However, treatment with mollugin significantly reduced the body weights in a dose-dependent manner ($p < 0.05$). The BMD was significantly reduced in GC group, relative to the control group ($p < 0.05$). Serum activities of TRAP and ALP were significantly higher in GC group than in control group, but were significantly reduced by mollugin treatment ($p < 0.05$). Serum level of CTX was significantly increased and osteocalcin reduced in the GC group, relative to control ($p < 0.05$). Osteoblast proliferation was significantly higher in the mollugin-treated groups. The expressions of p-Akt, Akt and cyclin D1 were significantly and dose-dependently higher in mollugin-treated groups ($p < 0.05$). There were more viable osteoblasts in the mollugin-treated groups than in the untreated group. However, treatment with mollugin in the presence of PI3K/Akt inhibitor significantly reduced their viability ($p < 0.05$).

Conclusion: Mollugin has therapeutic potential for GC-induced osteoporosis via mechanism involving the PI3K/Akt pathway.

Keywords: Mollugin, Osteoporosis, Bone, PI3K/Akt inhibitor, Osteoblast

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INTRODUCTION

Osteoporosis is a metabolic disorder that results in decreases in the mass and quality of bone; and deterioration of bone architecture [1].

Glucocorticoids (GCs) are used for the treatment of several chronic disorders such as cancer, gastrointestinal and autoimmune diseases [2]. However, chronic use of glucocorticoids leads to the development of secondary osteoporosis [3].

A balance between functions of osteocytes and osteoblasts is responsible for bone remodeling. These cells are the targets of glucocorticoids which induce apoptosis in them via a redox pathway [4]. Chronic use of glucocorticoids inhibits the maturation and proliferation of osteoblasts [5]. Drugs used for the treatment of osteoporosis are very few. Studies have shown that several naturally-occurring compounds exert anti-osteoporotic effect by acting as antioxidants.

Mollugin is a naphthohydroquinone isolated from *Rubia cordifolia* (*Rubiaceae*); a herb used in traditional Chinese medicine for the treatment of several chronic disorders including osteoporosis [6,7]. Studies have shown that mollugin possesses antiviral, anti-cancer and anti-inflammatory properties [8,9]. Its neuroprotective effect is exerted through the inflammatory pathway [10]. Mollugin inhibits collagen and arachidonic acid pathways thereby preventing platelet aggregation [10]. The aim of this study was to investigate the protective effect of mollugin on glucocorticoid-induced osteoporosis in rats.

EXPERIMENTAL

Materials

Dexamethasone was product of Tianjin Pharmaceutical Group Xinzheng Co., Ltd., Zhengzhou, China; ALP and TRAP kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. TRAP microplate reader (ELX-800) was purchased from Bio-Tek Instruments, Winooski, VT, USA). Osteocalcin and CTX ELISA kits were products of USCN Life Science, Wuhan, China; Dulbecco's Modified Essential Medium (DMEM) was obtained from Gibco Life Technologies, Carlsbad, CA, USA), while fetal bovine serum (FBS) was from HyClone, Logan, UT, USA. Radio-immunoprecipitation (RIPA) lysis buffer, and horseradish peroxidase-labeled goat anti-rabbit IgG and bicinchoninic acid (BCA) protein assay kits were purchased from Beyotime Institute of Biotechnology, Haimen, China. Polyvinylidene difluoride (PVDF) membranes were products of Millipore, Bedford, MA, USA, while β -actin, p-Akt, Akt and cyclin D1 were obtained from Santa Cruz Biotechnology, Inc., Dallas, TX, USA.

Animals

A total of 30 female Sprague Dawley rats weighing between 180 and 200 g were procured from Shanghai Medical College, China. They were housed under controlled conditions

(temperature: 25 ± 2 °C; humidity: 60 ± 5 %; and 12 h light/dark cycle). The study protocol was approved by Institutional Animal Care and Use Committee (IACUC) of Hunan Provincial People's Hospital, the First Affiliated Hospital of Hunan Normal University, Hunan, China, and followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use [11]. The rats were randomly assigned to five groups of six rats each: control, GC, mollugin (20 mg/kg), mollugin (40 mg/kg), and mollugin (80 mg/kg). Except for rats in the control group, osteoporosis was induced in all rats by intramuscular administration of 2.5 mg/kg bwt dexamethasone twice a week for nine weeks, and body weights of the rats were measured on weekly basis.

Sample collection

At the end of the treatment period, blood was collected from the rats through retroorbital puncture, and centrifuged at 2000 rpm for 20 min to obtain serum samples used for biochemical analyses. The rats were sacrificed by cervical dislocation and their femurs excised and used for analysis.

Determination of bone mineral density (BMD)

Dual-energy x-ray absorptiometry bone densitometry was used for the estimation of BMD. The measurement was done in triplicate and the mean calculated.

Measurement of the activities of TRAP and ALP

Activities of TRAP and ALP were determined using their respective kits according to the instructions of the manufacturers. Microplate reader was used to read absorbance at wavelengths of 530 nm (TRAP) and 520 nm (ALP).

Assessment of collagen type I fragment and osteocalcin

Enzyme-linked immunosorbent assay (ELISA) was used for the estimation of collagen type I fragment and osteocalcin according to the instructions of kits manufacturers.

Cell culture

Sprague-Dawley rats of either sex aged 2 months or less were procured from Beijing Institute of Medical Sciences, China and used for primary osteoblast cell culture. The rats were

sacrificed and their bones (tibia and femur) excised. The bone marrows were flushed with 5 ml of phosphate-buffered saline (PBS), followed by the addition of lymphocyte separation medium, and centrifuged at 2000 rpm for 20 min to obtain mononucleated cells. Fetal bovine serum-supplemented DMEM was added to the isolated cells and incubated at 37 °C in an incubator. The culture medium was replaced with fresh medium every three days and the cells were trypsinized after attaining 80 % confluency. Differentiation of osteoblasts was induced by adding 1×10^4 cells into 96-well plates filled with osteoblast differentiation medium. The positive control group (cells that were not treated with mollugin nor exposed to standard medium and so had no differentiation factors) were treated with concentrations of mollugin ranging from 0 - 10 μ M.

Determination of cell viability (MTT assay)

The viability of cells i.e. their ability to reduce tetrazolium salts was determined using MTT assay. The cells (1×10^4 cells/well) were seeded into a 96-well plate by adding DMEM and were incubated for 24 h. Mollugin (0 - 10 μ M) was added to the cells and they were further incubated for 3 days. At the end of the third day, 20 μ l of MTT solution was added to the wells, followed by incubation for another 4 h. The medium was finally replaced with dimethyl sulfoxide (DMSO) solution, agitated at 50 oscillations/min for 10 min, and absorbance of the samples were read using a microplate reader at 450 nm.

Western blotting

The cells treated with mollugin (0 – 10 μ M) were washed with PBS and ice-cold radio-immunoprecipitation assay buffer (RIPA) was used to lyse them. Determination of protein content was done using BCA protein assay kit. Protein separation was achieved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the bands transferred to PVDF membranes. Membranes with target proteins were incubated with primary antibodies (p-Akt, Akt, cyclin D1 and β -actin), washed and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies. The densities of the bands were estimated using Chemi-doc XRS imaging system.

Statistical analysis

Data are expressed as mean \pm SEM. The statistical analysis was performed using

Graphpad Prism (6.1). Groups were compared using Dunnett's post-hoc test. Values of $p < 0.05$, were considered statistically significant.

RESULTS

Effect of mollugin on body weight and BMD

The body weights of rats increased significantly in GC group, relative to the control. However, they were significantly reduced in a dose-dependent manner after treatment with mollugin ($p < 0.05$). Measurement of BMD was done before and after treatment. It was significantly reduced in GC group when compared to control group, but the reduction was significantly reversed with mollugin treatment ($p < 0.05$), as shown in Figure 1.

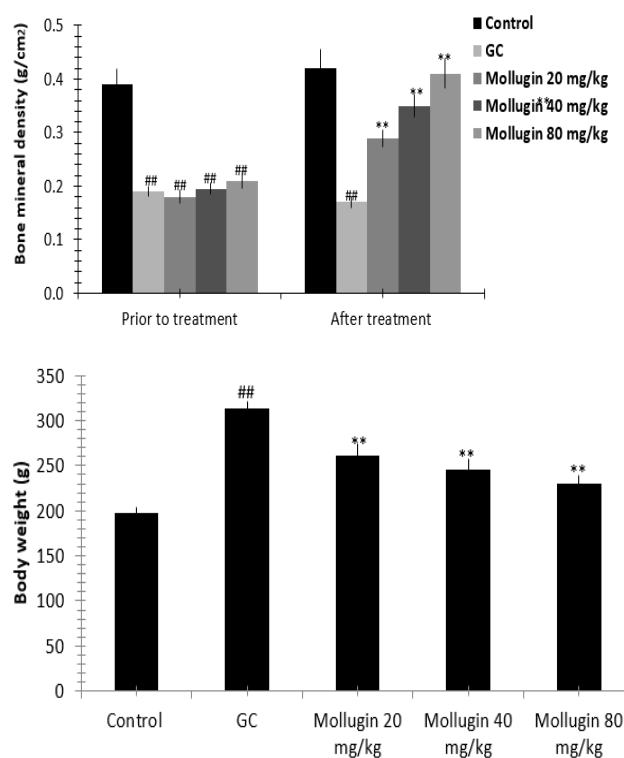


Figure 1: Effect of mollugin on body weight and BMD in GC-induced osteoporotic rats. [#] $p < 0.01$, compared to control group; * $p < 0.05$, ** $p < 0.01$, compared to GC group

Effect of mollugin on the activities of TRAP and ALP

Serum activities of bone-specific TRAP and ALP were significantly higher in GC group than in control group, but was significantly reduced with mollugin treatment ($p < 0.05$), as shown in Figure 2.

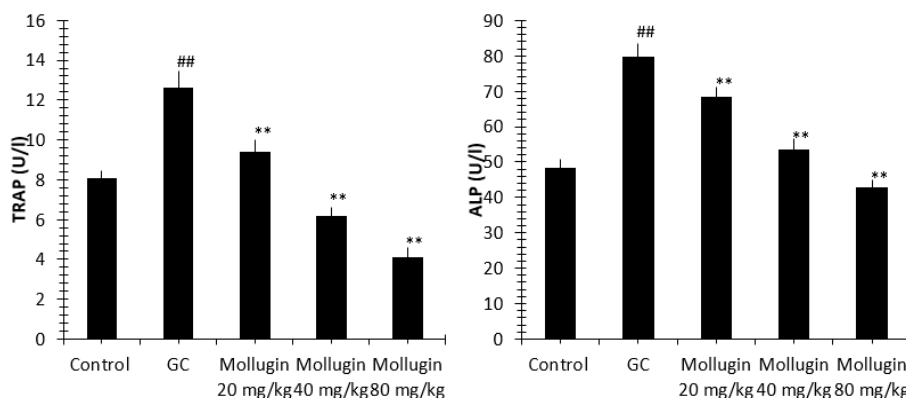


Figure 2: Effect of mollugin on serum activities of TRAP and ALP in GC-induced osteoporotic rats. ^{##}*p* < 0.01, compared to control group; **p* < 0.05, ***p* < 0.01, compared to GC group

Effect of mollugin on the levels of CTX and osteocalcin

Serum level of CTX was significantly increased while serum osteocalcin was reduced in the GC group, when compared with the control group (*p* < 0.05). However, treatment with mollugin significantly reversed the altered levels of these biomarkers (Figure 3).

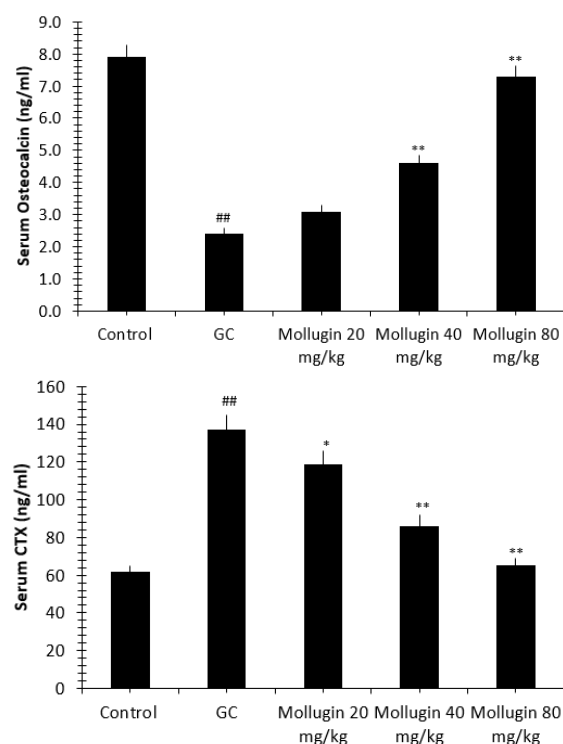


Figure 3: Effect of mollugin on serum levels of CTX and osteocalcin in osteoporotic rats; ^{##}*p* < 0.01, compared to control group; **p* < 0.05, ***p* < 0.01, compared to GC group

Effect of mollugin on the proliferation of osteoblasts

The proliferation of osteoblasts was significantly

higher in mollugin-treated groups than in the untreated group (*p* < 0.05, Figure 4).

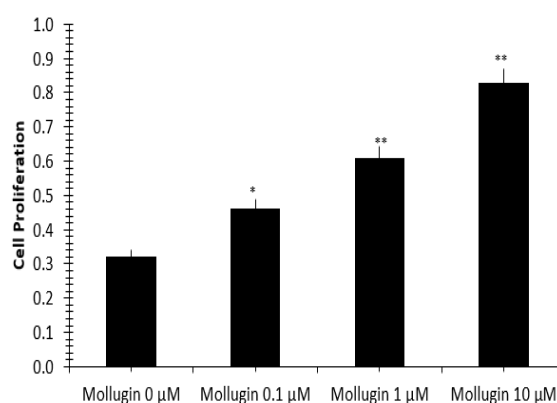


Figure 4: Effect of different concentrations of mollugin on the proliferation of osteoblasts; ^{##}*p* < 0.01, compared to control group, **p* < 0.05, ***p* < 0.01, compared to untreated group

Effect of mollugin on expressions of p-Akt, Akt and cyclin D1

The expressions of p-Akt, Akt and cyclin D1 were significantly and dose-dependently higher in mollugin-treated groups than in the untreated group (*p* < 0.05, Figure 5).

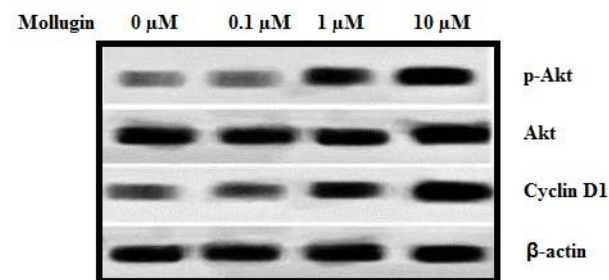


Figure 5: Expressions of p-Akt, Akt and cyclin D1 proteins in mollugin-treated groups

Effect of mollugin on the viability of osteoblasts

There were more viable osteoblasts in mollugin-treated groups than in the untreated group. However, treatment with mollugin in the presence of PI3K/Akt inhibitor significantly reduced their viability (Figure 6).

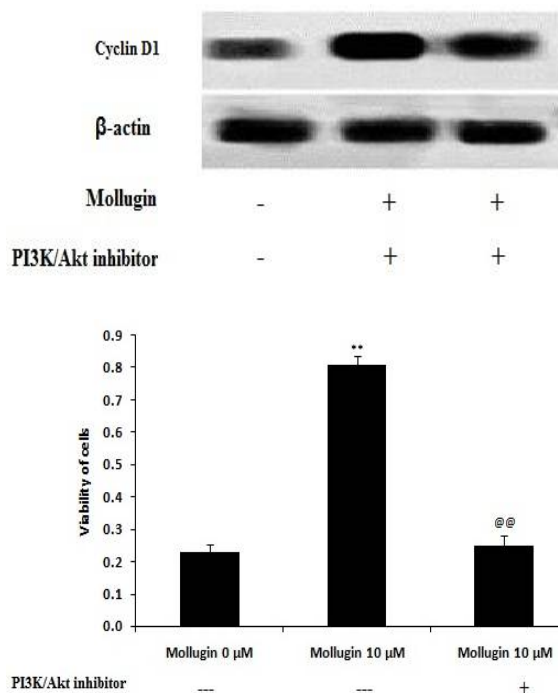


Figure 6: Effect of mollugin on viability of osteoblasts in the presence of PI3K/Akt inhibitor. ** $p < 0.01$, compared to untreated group; @@ $p < 0.05$, compared to 10 μM mollugin-treated group

DISCUSSION

Osteoporosis is characterized by deterioration of bone microarchitecture and reduction in bone mass due to decrease in the BMD. Thus, drugs that prevent reduction of BMD are used for its treatment [12,13]. In the present study, body weights of rats increased significantly in GC group, when compared to control. However, body weight increases were significantly reduced in a dose-dependent manner after treatment with mollugin, and the treatment significantly reversed the decrease in BMD caused by osteoporosis.

In osteoporosis, the balance between bone formation and resorption is altered; while CTX is the marker for bone resorption, osteocalcin is the marker for bone formation [14]. The altered serum levels of CTX and osteocalcin were significantly reversed by treatment with mollugin. The activities of TRAP (marker for bone resorption) and ALP in osteoblasts are significantly altered in osteoporosis [15]. In this

study, treatment with mollugin significantly reversed the altered activities of TRAP and ALP in osteoporotic rats. Studies have shown that drugs used in the treatment of osteoporosis usually induce the proliferation of osteoblasts [16].

In the present study, proliferation of osteoblasts was significantly higher in mollugin-treated groups than in the untreated group. Some authors have speculated that PI3K/Akt pathway plays an important role in the proliferation and differentiation of osteoblasts [17]. The results obtained in the present study showed that the proliferation of osteoblasts was significantly reduced in mollugin and PI3K/Akt inhibitor-treated group than in mollugin alone-treated group.

CONCLUSION

Mollugin has therapeutic potential for the treatment of GC-induced osteoporosis, and its anti-osteoporotic effect occurs via PI3K/Akt pathway.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

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. Zhen Jia designed the project, supervised the experiments and wrote the manuscript, Ge Yan, Yusi Wang and Pan He analysed the data and provided comments.

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