Polygonum multiflorm alleviates glucocorticoid-induced osteoporosis and Wnt signaling pathway

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Abstract. It is known that long-term excessive administration of glucocorticoid (GC) results in osteoporosis. The present study aimed to evaluate the protective effects of *Polygonum multiflorm* (PM) on the bone tissue of rats with GC-induced osteoporosis (GIO). A total of 90 6-month-old female Sprague Dawley rats (weight range, 190-210 g) were randomly divided into nine groups: Control (normal saline); prednisone (GC; 6 $mg \cdot kg^{-1} \cdot d^{-1}$; Model); GC plus PMR30 (the 30% ethanol eluent fraction of PM) (H) (400 $mg \cdot kg^{-1} \cdot d^{-1}$); GC plus PMR30 (M) (200 $mg \cdot kg^{-1} \cdot d^{-1}$); GC plus PMR30 (L) (100 $mg \cdot kg^{-1} \cdot d^{-1}$); GC plus PMRF (fat-soluble fraction of PM) (H) (400 $mg \cdot kg^{-1} \cdot d^{-1}$); GC plus PMRF (M) (200 $mg \cdot kg^{-1} \cdot d^{-1}$); GC plus PMRF (L) (100 $mg \cdot kg^{-1} \cdot d^{-1}$); GC plus calcitriol (CAL; 0.045 $\mu g \cdot kg^{-1} \cdot d^{-1}$; positive). Rats were administered intragastrically with prednisone and/or the aforementioned extracts for 120 days, and weighed once/week. The serum was collected for detection of biochemical markers. The left tibia was used for bone histomorphometry analysis. The right tibia was prepared for hematoxylin and eosin staining. The left femur was used to analyze the protein expression of dickkopf-1 (DKK1), WNT inhibitory factor 1 (WIF1) and secreted frizzled related protein 4 using western blotting. Long-term excessive treatment of prednisone inhibited the bone formation rate accompanied with a decrease in bone mass, growth plate, body weight, and the level of bone-specific alkaline phosphatase and hydroxyl-terminal propeptide of type I procollagen in the serum. Furthermore, a simultaneously increase in the level of tartrate resistant acid phosphatase-5b and cross-linked carboxy-terminal telopeptide of type I collagen in the serum, in addition to DKK1, and WIF1 protein expression, was observed. PMR30 (M and L) and PMRF (H) groups were able to reduce the negative effects of GC on the bones. PMR30 (M and L) and PMRF (H) dose demonstrated a protective effect of PM on bone tissue in GIO rats. The mechanism underlying the preventive effect of PM for the treatment of GIO may be associated with direct upregulation of the canonical Wnt/β-catenin signaling pathway.

Introduction

Glucocorticoids (GCs) are anti-inflammatory agents used in the treatment of various diseases, such as asthma, rheumatoid arthritis, and systemic lupus erythematosus (1-4). Although GCs have been prescribed for many years, their potential side effects (growth retardation, osteopenia, adrenal insufficiency, etc.), can prevent their long-term use (5,6). Significantly, GC-induced osteoporosis (GIO) is thought as the most severe one of these side effects because of the increased fracture risk (7-9). GIO resulting from osteopenia has been described as the most predictable and debilitating complication of long-term GCs therapy (5,6). Therefore, the development of medications that prevent GIO is of clinical significance.

Polygonum multiflorum Thunb. (PM, He-Shou-Wu) is a kind of traditional Chinese medicine (10). PM and its extracts can be used to improve the health of blood and blood vessels, blacken hair, strengthen bones, neurosis and other diseases commonly associated with aging (11-16). Based on previous evidence in our team, we found that PM and its extracts exert beneficial effects in the prevention and treatment of osteoporosis, which have already been applied for China patents (ZL 00101246.0) (17). Furthermore, we have investigated the effects of main components [(emodin and 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (TSG)] of PM *in vitro*. The results showed that emodin and TSG can

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promote Bone Marrow Mesenchymal Stem Cells (MSCs) to differentiate into osteoblasts. Moreover, emodin can inhibit MSCs differentiate into adipocytes (18-21). The underlying mechanism of TSG may be related to regulation of Wnt signaling pathway (22). However, the exact signaling mechanism by which PM rescued impaired bone formation induced by GC has not yet been investigated.

The Wnt/β-catenin signaling is an important pathway that is required by the growth, development and maintenance of skeletal tissue (9,23). Wnt signals are extracellularly regulated by several secreted antagonists including secreted frizzled-related protein (sFRP), Wnt inhibitory factor-1 (WIF1), and dickkopf-1 (DKK1) (24). Previous evidence showed that GCs can promote the expression of DKK1 in cultured human osteoblasts (25). It is known that the cell fate of pre-osteoblasts is mainly determined by the Wnt/β-catenin signal pathway (26). Therefore, the molecules which induce the activation of Wnt/β-catenin signaling are beneficial for the treatment of osteoporosis. PM has been demonstrated to exert a simulatory effect on Wnt/β-catenin signaling pathway *in vivo* and *in vitro* (23,27). Whether the extracts of PM can increase the bone mass or not in the GIO model characteristic of decreased bone formation? If the extracts could prevent GIO, and what's the mechanism of PM on bone metabolism? Considering the above questions, this study aims to observe the effect and the mechanism of PM underlying bone loss in GIO rats.

Materials and methods

Preparation of PM extract. The dried roots of *PM* were purchased in Yulin Xiang Sheng Chinese Herbal Medicine Co., Ltd. (Henan, China), and were authenticated by Professor Yuyu Liu. A voucher specimen was deposited at the herbarium of Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical University (Guangdong, China). Air-dried roots of PM (56.0 kg) was extracted by 75% ethanol at 50~60˚C, followed by rinsing with cyclohexane. The organic solvent of PMRF was acquired by evaporation under a vacuum at 55˚C. The PMRF dissolved in water was absorbed by macroporous resin D-101, and then eluted with H_2O, 10, 20, 30, 40, 50, 60, 70, 80 and 90% ethanol successively, and PMR30 was prepared by the collection and concentration of 30% ethanol elution (28).

Animal experiments. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Guangdong Laboratory Animal Monitoring Institute, under the National Laboratory Animal Monitoring Institute of People's Republic of China (29). The experiments have been conducted according to protocols approved for Specific Pathogen-Free animal care of the Animal Center of Guangdong Medical University, and approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical University [permit no. SYXK (Guangdong) 2008-0008; Zhanjiang, China].

The Sprague Dawley (SD) female rats were acclimated to local vivarium conditions (temperature: 24-28˚C, humidity: 60%) and under specific pathogen-free conditions. Rats were allowed free access to water and diet.

Experimental protocols. Six-month-old female SD rats weighing (190-210 g, n=90) were randomly divided into ten groups by weight: basic group, control (normal saline) group, prednisone (GC, 6 $mg \cdot kg^{-1} \cdot d^{-1}$, model) group, GC plus PMR30 (H) (400 $mg \cdot kg^{-1} \cdot d^{-1}$) group, GC plus PMR30 (M) (200 $mg \cdot kg^{-1} \cdot d^{-1}$) group, GC plus PMR30 (L) (100 $mg \cdot kg^{-1} \cdot d^{-1}$) group, GC plus PMRF (H) (400 $mg \cdot kg^{-1} \cdot d^{-1}$) group, GC plus PMRF (M) (200 $mg \cdot kg^{-1} \cdot d^{-1}$) group, GC plus PMRF (L) (100 $mg \cdot kg^{-1} \cdot d^{-1}$) group, GC plus calcitriol (CAL) (0.045 $\mu g \cdot kg^{-1} \cdot d^{-1}$) (positive group). Rats were administered intragastrically with prednisone and/or the extracts mentioned above for 120 days, and weighed once per week. Rats were injected subcutaneously with calcein on the 3rd, 4, 13, and 14th day before killed for the purpose of double labeling *in vivo*, respectively (30).

Rats were sacrified by caridiac puncture under sodium pentobarbital anesthesia at the experimental endpoint. The serum was separated for testing biochemical markers. The left tibia was used for bone histomorphometry analysis. The right tibia was prepared for H&E staining. The left femur was used to test protein expression of DKK1, WIF1 and SFRP4 using western blotting assay (31).

Serum markers assay. Blood was collected in specimen tubes and kept at 25˚C for 40-50 min in a vertical position for completely clotting. And then the serum was separated by centrifuging at 1,000 x g for 10 min and stored at -80˚C for biochemical markers assays. The serum was separated for testing biochemical markers, including Bone-specific alkaline phosphatase (BAP), Hydroxyl-terminal propeptide of type I procollagen (PICP), tartrate resistant acid phosphatase-5b (TRACP-5b), Cross-linked Carboxy-terminal telopeptide of type I collagen (CTX-I), and DKK1. BAP and OCN, as serum markers of bone formation, and OPG, sRANKL, and TRAP5b, as the markers of bone resorption, were measured in rats using commercially available ELISAs (Tuokeda Bio-Tech, Guangzhou, China).

Bone histomorphometry assay. For histomorphometric analysis, the left tibia was removed, dissected, and cut. The proximal tibial metaphysis (PTM) was opened to expose the bone marrow cavity with an Isomet low-speed saw (Buehler, Lake Bluff, IL, USA) and then fixed in 70% ethanol after they had been placed in 10% formalin for 24 h, dehydrated in increasing concentrations of ethanol, defatted in xylene, and then embedded without decalcification in methyl methacrylate (32). Frontal sections of the PTM were cut at thicknesses of 5 and 8 μm. The region of interest in the PTM was located between 1 and 4 mm distal to the growth plate-epiphyseal junction, not including cortical bone. A semiautomatic digitizing image analysis system (OsteoMetrics, Inc., Decatur, GA, USA) was used for static and dynamic histomorphometry measurements. For static histomorphometry measurements with Masson-Goldner trichrome staining (5-μm sections), the total tissue area, trabecular area, trabecular perimeter, and osteoclast number (Oc.N) were measured and used to calculate the percentage of trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), number of osteoclasts (N.Oc), percent osteoclast surface perimeter (Oc.S.Pm), and percent osteoblast surface perimeter (Ob.S.Pm). For dynamic analyses (8-μm sections),

Table I. Serum biochemical indices of bone marker in rats ($\bar{x} \pm s$).

Group	PICP (μg/l)	BAP (μg/l)	CTX-I (nmol/l)	TRACP-5b (pg/ml)	DKK1 (μg/l)
CON	10.59±4.17	13.64±3.00	36.88±9.18	1600±467	9.70±3.11
Pre	9.10±2.58	8.06±2.47[b]	45.88±10.37	2148±368[a]	12.76±2.96
CAL	11.47±3.14	12.80±2.46[d]	37.40±18.51	1758±379	8.62±1.55[d]
PMR30(H)	9.94±3.13	12.65±2.61[d]	29.84±6.43[d]	1959±360	11.45±2.62
PMR30(M)	13.58±2.50[d]	10.45±1.56	31.46±8.84[d]	1960±482	9.15±1.68[d]
PMR30(L)	12.50±2.14	13.25±1.93[d]	41.77±13.59	1726±306	9.03±3.07[d]
PMRF(H)	12.99±2.72[d]	13.01±2.31[d]	30.03±8.42[d]	1649±378[c]	10.97±3.16
PMRF(M)	12.01±3.60	12.00±3.06[d]	37.28±11.64	2325±463	11.31±4.07
PMRF(L)	11.12±4.77	10.50±3.17	29.91±5.96[d]	1820±583	10.17±2.08

[a]P<0.05, [b]P<0.01 vs. CON; [c]P<0.05, [d]P<0.01 vs. Pred; [e]P<0.05, [f]P<0.01 vs. CAL.

single-labeled perimeter, double-labeled perimeter, interlabeled width, percent of labeled perimeter (%L.Pm), mineral apposition rate (MAR), and bone formation rate per unit of bone surface (BFR/BS), bone formation rate per unit of bone volume (BFR/BV), and bone formation rate per unit of bone tissue area (BFR/TV) were measured and calculated. For the tibial shaft (40-μm sections), the cortical area (Ct.Ar), percent Ct.Ar (%Ct.Ar), percent marrow area, percent periosteal labeled perimeter (%P-L.Pm), periosteal MAR, periosteal BFR per unit of bone surface (P-BFR/BS), percent endocortical labeled perimeter, endocortical MAR, and endocortical BFR per unit of bone surface (E-BFR/BS) were calculated from the measured parameters (33).

Western blotting assay. Left femurs were stored at -80˚C before they were used. The whole proteins were extracted by the method previously described with a Total Protein Extraction kit (Applygen Technologies, Inc., Beijing, China) (33). Sixty micrograms of total protein extracts was separated on sodium dodecyl sulfate-polyacrylamide gels and transferred to poly (vinylene difluoride) membranes. The membranes were blocked with 5% skimmed milk for 2 h at room temperature, and were incubated overnight at 4˚C with rabbit anti-DKK1 monoclonal antibody (ab109416), WIF1 antibody (ab155101), and sFRP4 (ab154167) (all from Abcam, Cambridge, MA, USA) at a dilution of 1:300. This was followed by incubation with the corresponding secondary antibodies and goat anti-rabbit IgG antibodies (Beyotime Institute of Biotechnology, Haimen, China). Protein expression was visualized using a BeoECL Plus instrument (Beyotime Institute of Biotechnology). GADPH rabbit mAb (CST, USA) was used to normalize the sample loading. The images of bands were quantified with Image-Pro Plus 6.0.

Statistical analysis. Data were described as the means ± standard deviation (mean ± SD) and analyzed statistically with SPSS, version 13.0. One-way ANOVA was used to detect the differences in changes between the groups of various treatments after establishing if the data were normally distributed and equivalency of variances. P-value (the probabilities) <0.05 were considered statistically significant.

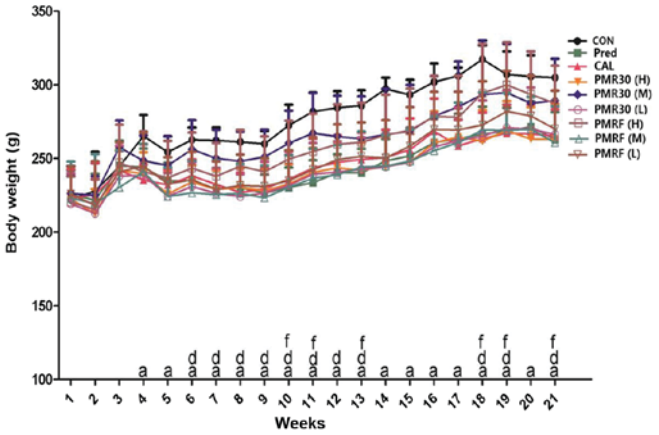

Figure 1. Body weight (g) changes during the experimental period. Body weight measurements from vehicle-treated controls (CON), prednisone 6 mg.kg^{-1}.d^{-1} (Pre), calcitriol 0.045 μg.kg^{-1}.d^{-1} (CAL), PMR30 (H) 400 mg.kg^{-1}.d^{-1}, PMR30 (M) 200 mg.kg^{-1}.d^{-1}, PMR30 (L) 100 mg.kg^{-1}.d^{-1}, PMRF (H) 400 mg.kg^{-1}.d^{-1}, PMRF (M) 200 mg.kg^{-1}.d^{-1}, and PMRF (L) 100 mg.kg^{-1}.d^{-1} treated rats. [a]P<0.05 vs CON, [d]P<0.05 PMR30 (M) vs. Pre, [f]P<0.05 PMRF (H) vs. Pre.

Results

Effects of PM on body weight. The alterations of body weight of rats were no statistically significant difference between each group during the initial stage of experiment, while the weight of rats in prednisone group decreased significantly from the fourth week compared with the CON group (P<0.05) (Fig. 1). Reversely, the changes of body weight of rats in PMR30 (M) and PMRF (H) groups increased compared with the prednisone group (P<0.05). These results indicated that PMR30 (M) and PMRF (H) groups can improve the slow growth of GIO rats.

Effects of PM on biomarkers of bone turnover. We confirmed that GC resulted in the decrease of biomarkers of the bone formation, including serum bone specific alkaline phosphatase (BAP) and serum Hydroxyl-terminal propeptide of type I procollagen (PICP) (Fig. 2 and Table I). Contrarily, GC stimulated the increase of the biomarkers related to bone resorption including serum Tartrate-resistant acid phosphatase-5b (TRAP-5b), DKK1 and C-terminal telopeptides of I collagen (CTX-1) level of bone

Table II. Histomorphometric static parameters of proximal tibial of rats ($\bar{x} \pm s$).

Group	%Tb.Ar (%)	Tb.Th (μm)	Tb.N (no./mm)	Tb.Sp (μm)
CON	29.20±5.19	52.86±7.60	5.58±0.90	131.90±32.59
Pre	18.81±6.60[b]	38.21±11.44	4.91±0.81	172.31±49.13
CAL	25.82±5.94[c]	50.64±6.07	5.10±1.02	153.21±45.76
PMR30(H)	20.52±6.25	88.84±116.84[c,e]	4.33±2.15	409.06±597.48
PMR30(M)	23.25±61.7	46.80±8.21	4.93±0.82	162.48±49.65
PMR30(L)	22.66±4.64	48.76±5.29	4.64±0.84	173.24±43.53
PMRF(H)	26.76±7.35[c]	52.84±4.65	5.01±0.98	153.45±43.48
PMRF(M)	23.62±5.63	47.09±9.69	5.01±0.53	154.57±26.18
PMRF(L)	19.83±3.89[f,g]	47.03±3.75	4.23±0.86	197.42±46.87

[a]P<0.05, [b]P<0.01 vs. CON; [c]P<0.05, [d]P<0.01 vs. Pred; [e]P<0.05, [f]P<0.01 vs. CAL; [g]P<0.05, [h]P<0.01 vs. PMRF(H).

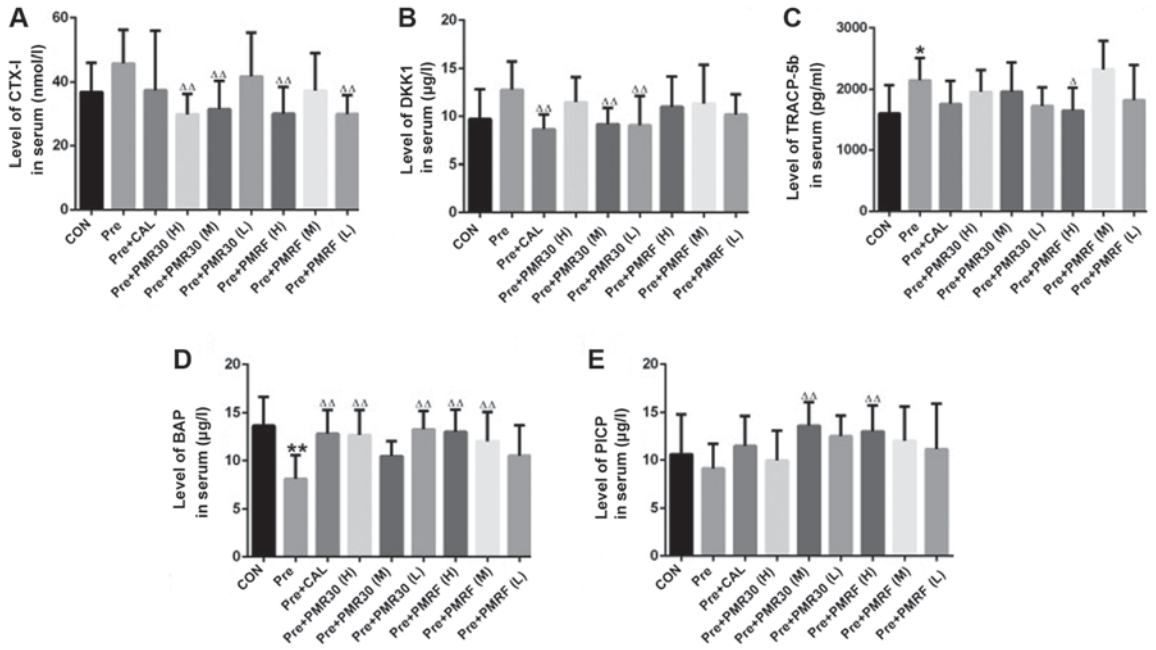

Figure 2. Endpoint levels of serum biochemical markers (A) CTX-I, (B) DKK1, (C) TRACP-5b, (D) BAP, (E) PICP in the rats treated with vehicle (CON), prednisone (Pre), calcitriol (CAL), and various PMR (30, F) dose levels. *P<0.05, **P<0.01 vs. CON; ΔP<0.05, ΔΔP<0.01 vs. Pred; #P<0.05, ##P<0.01 vs. CAL.

tissue (Fig. 2 and Table I). Encouragingly, PMR30 (M, L) and PMRF (H) groups showed a capacity to reverse the deleterious impacts of bone turnover elicited by GC, as effectively as CAL.

Effects of PM on the parameters of the two-dimensional histomorphometry of the proximal tibial metaphysis. The changes of the two-dimensional histomorphometry of the proximal tibial metaphysis: Compared with the control group, %Tb.Ar, percent labeled perimeter (%L.Pm), mineral apposition (MAR), BFR/TV and %Ob.S.Pm were decreased (P<0.05), while percentage of osteoclast surface perimeter (%Oc.S.Pm) and number of osteoclast per millimeter (Oc.N) were increased (P<0.05) in prednisone group. Compared with the prednisone group, %Tb.Ar and BFR/TV were increased (P<0.05); and %Oc.S.Pm was decreased (P<0.05) in CAL group. Compared with the prednisone group, %L.Pm and new bone formation rate per unit of bone surface (BFR/BS) were increased in PMR30 (H) group (P<0.05). Compared with the prednisone group, number of osteoclast per millimeter (Oc.N) was decreased (P<0.05) in PMR30 (H, M, L) groups. Compared with the prednisone group, %Tb.Ar and BFR/TV were increased (P<0.05) in PMRF (H) group. Compared with the prednisone group, percentage of osteoclast surface perimeter (%Oc.S.Pm) was decreased (P<0.05) in PMRF (H, M, L) groups (Tables II-IV and Fig. 3). These results indicated that PMR30 (M, L) and PMRF (H) groups can improve the bone loss and decreased activity of osteoclast in GIO rats.

Effects of PM on the parameters of two-dimensional histomorphometry of the tibial shaft. The changes of the two-dimensional histomorphometry of the tibial shaft: Compared with the control group, Ct.Ar, %Ct.Ar and P-MAR were decreased (P<0.05) while %Ma.Ar and E-BFR/BS were increased (P<0.05) in prednisone group. Compared with the

Table III. Histomorphometric dynamic parameters of proximal tibial of rats ($\bar{x}\pm s$).

Group	%L.Pm (%)	MAR (μm/d)	BFR/BS (%/year)	BFR/BV (%/year)	BFR/TV (%/year)
CON	18.26±4.76	1.17±0.09	7.70±1.77	243.61±48.03	70.28±13.99
Pre	12.13±4.01	1.12±0.21	4.87±1.39	224.78±59.36	39.98±13.83[a]
CAL	20.70±6.76	1.22±0.18	9.43±4.08	314.71±140.23	78.41±31.23[d]
PMR30(H)	40.17±57.19[d,e]	1.01±0.27	15.05±21.64[d]	257.30±95.17	51.51±22.82[e]
PMR30(M)	11.67±2.13	1.16±0.32	5.03±2.01	179.41±67.01[f]	41.62±18.92[e]
PMR30(L)	19.05±8.04	1.03±0.14	7.12±2.69	241.55±83.75	55.31±23.95[e]
PMRF(H)	19.24±5.74	1.08±0.20	7.79±3.10	245.23±93.87	65.81±29.02[c]
PMRF(M)	16.49±5.40	0.99±0.24	6.12±2.76	216.27±85.37[e]	50.16±20.68[e]
PMRF(L)	18.31±3.59	1.14±0.16	7.73±2.27	276.57±90.36	54.91±20.09[e]

[a]P<0.05, [b]P<0.01 vs. CON; [c]P<0.05, [d]P<0.01 vs. Pred; [e]P<0.05, [f]P<0.01 vs. CAL.

Table IV. Oc and Ob parameters of proximal tibial of rats ($\bar{x}\pm s$).

Group	Oc.N (no./mm)	%Oc.S. Pm (%)	%Ob.S. Pm (%)
CON	0.10±0.06	0.31±0.14	0.20±0.12
Pre	0.20±0.09[a]	0.87±0.54[b]	0.09±0.06
CAL	0.12±0.06	0.19±0.11[d]	0.08±0.04
PMR30(H)	0.19±0.22	0.39±0.49[c]	0.16±0.28[e]
PMR30(M)	0.08±0.04[d,g]	0.19±0.11[d]	0.08±0.04
PMR30(L)	0.10±0.02[c,g]	0.18±0.05[c]	0.10±0.06
PMRF(H)	0.08±0.03[d]	0.12±0.06[c]	0.23±0.14
PMRF(M)	0.06±0.05[d,h]	0.13±0.14	0.13±0.06
PMRF(L)	0.07±0.03[d]	0.12±0.06[c]	0.14±0.10

[a]P<0.05, [b]P<0.01 vs. CON; [c]P<0.05, [d]P<0.01 vs. Pred; [e]P<0.05, [f]P<0.01 vs. CAL; [g]P<0.05, [h]P<0.01 vs. PMR30(H).

Table V. Histomorphometric static parameters of tibial shaft in rats ($\bar{x}\pm s$).

Group	Ct.Ar (mm^2)	%Ct.Ar (%)	%Ma.Ar (%)
CON	4.59±0.34	78.22±1.77	21.77±1.77
Pre	4.05±0.50[a]	75.32±2.72[a]	24.68±2.72[a]
CAL	4.07±0.62	75.37±5.58	24.63±5.58
PMR30(H)	4.17±0.39	77.02±3.56	22.98±3.56
PMR30(M)	4.21±0.44	76.43±4.76	23.57±4.76
PMR30(L)	4.28±0.39	75.61±5.99	24.39±5.99
PMRF(H)	4.40±0.32	76.38±2.18	23.62±2.18
PMRF(M)	4.49±0.33	75.46±2.46	24.54±2.46
PMRF(L)	4.29±0.47	76.85±2.67	23.15±2.67

[a]P<0.05, [b]P<0.01 vs. CON; [c]P<0.05, [d]P<0.01 vs. Pred; [e]P<0.05, [f]P<0.01 vs. CAL.

prednisone group, %Ct.Ar and %P-L.Pm were showed a trend toward increased (P>0.05) while %Ma.Ar and E-BFR/BS were showed a trend toward decreased (P>0.05) in CAL, PMR30 and PMRF groups (Fig. 4 and Tables V and VI). These results indicated that PMR30 and PMRF groups can improve the thickness of cortical bone in GIO rats.

Effects of PM on the growing epiphyseal plate of proximal tibial. The results of growth plate, compared with the control group, the growth plate was become thinner in prednisone group. Compared with the prednisone group, the growth plate was become more thicken in CAL, PMRF (M, L) and PMR30 (M, L) groups (Fig. 4). It is indicated that *PM* may be stimulate growth hormone secretion of rat.

Effects of PM on marrow fat tissue deposistion of the proximal tibial metaphysis. Compared with the control group, the number of adipocytes was become more and dense in prednisone group. Compared with the prednisone group, the number of adipocytes were became less in CAL, PMR30 (M, L) and PMRF (H, M) groups (Fig. 5). These results indicated that PMR30 (M, L) and PMRF (H, M) groups can decrease the number of adipocytes in GIO rats. It is prompted that *PM* can inhibit the differentiation of bone marrow stromal cells into adipocytes.

Effects of PM on the Wnt signaling pathway. Compared with control group, the expression of DKK1 and WIF1 were increased in prednisone group (P<0.05). Compared with prednisone group, the expression of DKK1 were decreased (P<0.05) in CAL, PMR30 (M, L) and PMRF (H) groups. Compared with prednisone group, the expression of WIF1 was decreased (P<0.05) significantly in PMR30 (M) and PMRF (F) groups (Fig. 6). These results indicated that PMR30 (M) and PMRF (F) groups can increase the expression of DKK1 and WIF1 aim to regulate the Wnt signaling pathway in GIO rats.

Discussion

It is widely known that GC treatment induces osteoporosis. In our previous study, GC exerts a series of deleterious actions

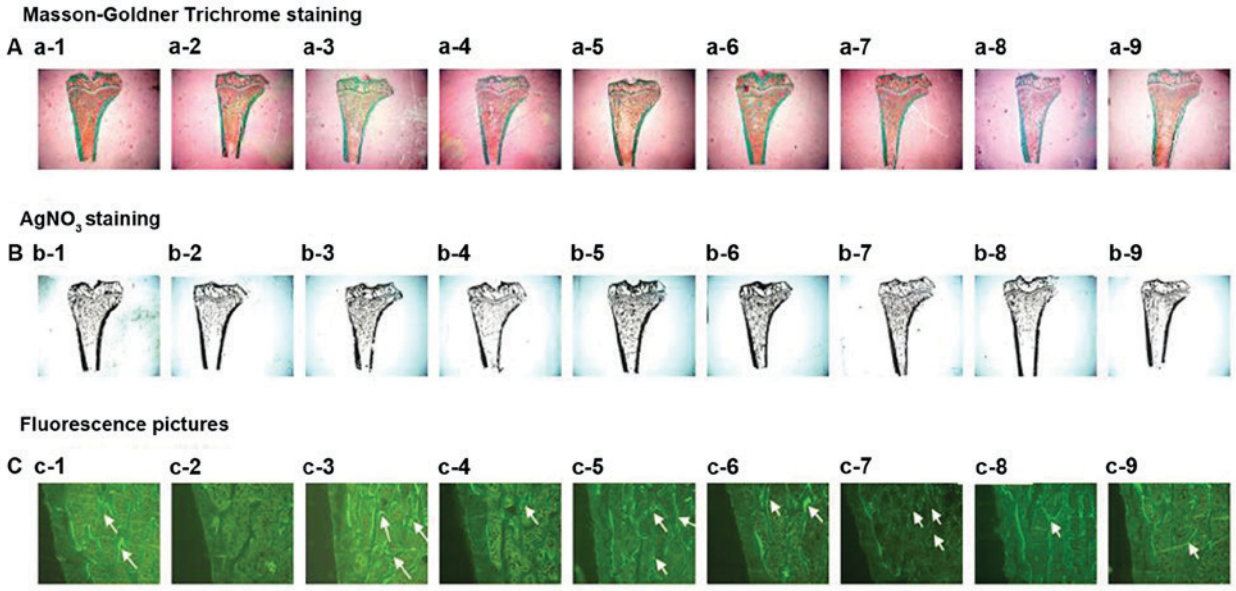

Figure 3. Effects of vehicle (CON), prednisone (Pre), calcitriol (CAL), and various PMR (30, F) dose treatments on the proximal tibial metaphysis (PTM) bone structure and mineral bone formation. Arrows point to the tetracycline and calcein labeling. Quantitative measurements of histomorphometric parameters of PTM are showed in Tables II and III. (A) Goldner's Trichrome stain, (B) $AgNO_3$ stain,(C) fluorescence images of undecalcified sections a-1, b-1, c-1:CON; a-2, b-2, c-2:Pre (6 $mg.^{-1}.d^{-1}$); a-3, b-3, c-3:Pre+CAL (0.045 $\mu g.kg^{-1}.d^{-1}$); a-4, b-4, c-4:Pre+PMR30 (H) (400 $mg.kg^{-1}.d^{-1}$); a-5, b-5, c-5:Pre+PMR30 (M) (200 $mg.kg^{-1}.d^{-1}$); a-6, b-6, c-6:Pre+PMR30 (L) (100 $mg.kg^{-1}.d^{-1}$); a-7, b-7, c-7:Pre+PMRF (H) (400 $mg.kg^{-1}.d^{-1}$); a-8, b-8.c-8:Pre+PMRF (M) (200 $mg.kg^{-1}.d^{-1}$); a-9, b-9, c-9: Pre+PMRF (L) (100 $mg.kg^{-1}.d^{-1}$).

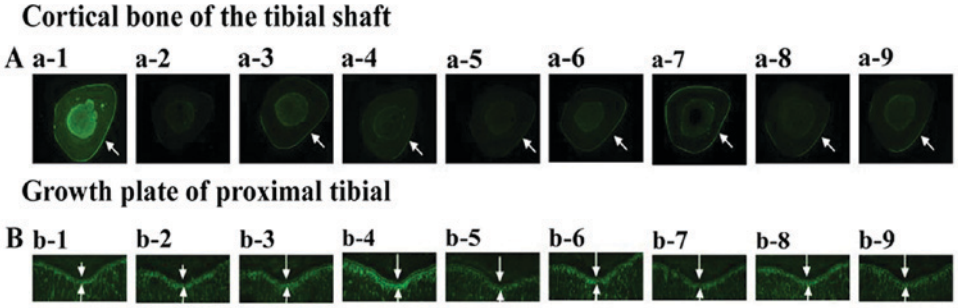

Figure 4. Effects of vehicle (CON) and various prednisone (Pre) dose treatments on cortical bone of the tibial shaft and cartilage growth. Arrows point to interlabeling distances after double labeling with tetracycline and calcein. Quantitative measurements of histomorphometric parameters of tibial shaft are shown in Tables V and VI. (A) Fluorescence images of the tibial shaftmiddle and (B) fluorescence images of the growth plate

on bone tissue in both male and female rats. Furthermore, previous evidence demonstrated that extracts of PM exhibits a pretective effect on ovariectomized rats (20). In the present study, extracts of PM (PMR30 and PMRF) were exhibits a pretective effect on bone loss in GIO rats, owing to restoration of bone micro-architecture and the serum levels of biomarkers related to bone formation. In addition, *Polygonum Multiflorm* alleviates GIO may be through regulation of Wnt signaling pathway to protect the bone.

Physiologically, the secretion of endogenous GC was regulated by hypothalamus-hypophysis-adrenal cortex system (30), thus inhibited the secretion of growth hormone (GH) (34). However, excessive exposure to excessive exogenous clinically GC could induce rapidly bone loss resulting to the increase of fracture risk. In the present study, we found that long-term excessive administration of prednisone caused a decrease of bone formation parameters (MAR and BFR/TV) in the trabecular bone area and a decrease of growth of longitudinal bone, and an increase of bone resorption (Oc.S/BS) (Figs. 1 and 4). It has generally been thought that GIO results from impaired bone formation as well as exaggerated bone resorption. Possible pathological mechanisms of GIO are listed in the following: (1) impairing osteoblast or osteoclast function directly, and (2) secondary hyperparathyroidism, due to the increased renal excretion and decreased intestinal absorption of calcium (35).

In this study, PMR30 (M, L) and PMRF (H) groups were found to be effective at attenuating GIO *in vivo*, as evidenced through its restoration of %Tb.Ar, Tb.Th, %L.Pm, BFR/BS, and BFR/TV (Tables II-IV and Fig. 3). This indicates that the compressive strength and mechanical properties of the bone tissue and the activity of bone formation were enhanced. Furthermore, it would promote bone formation and increase bone turnover rate. This is benefit to replace bone matrix between old and new, and self-repair the bone micro-structure, so that fighting brittle fracture by long-term use of prednisone.

Table VI. Histomorphometric dynamic parameters of tibial shaft in rats ($\bar{x}\pm s$).

Group	%P-L. Pm (%)	P-MAR (μm/d)	P-BFR/BS (%/year)	%E-L. Pm (%)	E-MAR (μm/d)	E-BFR/BS (%/year)
CON	27.40±6.73	0.89±0.23	24.66±10.51	14.62±9.11	0.41±0.19	5.38±3.98
Pre	29.18±7.72	0.65±0.05[b]	19.27±5.62	20.35±8.95	0.59±0.23	11.70±6.63[a]
CAL	34.49±6.81	0.91±0.13[d]	28.72±8.48[c]	16.35±6.83	0.52±0.09	7.96±3.30
PMR30(H)	30.67±13.05	0.78±0.31	21.21±7.80	19.94±4.77	0.72±0.25[e]	11.38±5.87
PMR30(M)	33.97±7.33	0.47±0.27	16.81±13.47[e]	19.08±5.00	0.31±0.24[c,e]	6.68±6.04
PMR30(L)	26.64±11.30	0.83±0.23	19.25±6.68[e]	14.85±4.91	0.45±0.19	6.83±5.04
PMRF(H)	38.56±6.11[c]	0.65±0.17	23.33±7.90	18.90±5.22	0.42±0.22	8.38±5.32
PMRF(M)	29.02±10.05	0.73±0.11	21.35±8.49	17.37±4.59	0.40±0.12	6.42±3.02
PMRF(L)	27.16±4.26	0.49±0.16	14.52±7.39[f]	18.26±8.16	0.51±0.16	8.58±3.34

[a]P<0.05, [b]P<0.01 vs. CON; [c]P<0.05, [d]P<0.01 vs. Pred; [e]P<0.05, [f]P<0.01 vs. CAL.

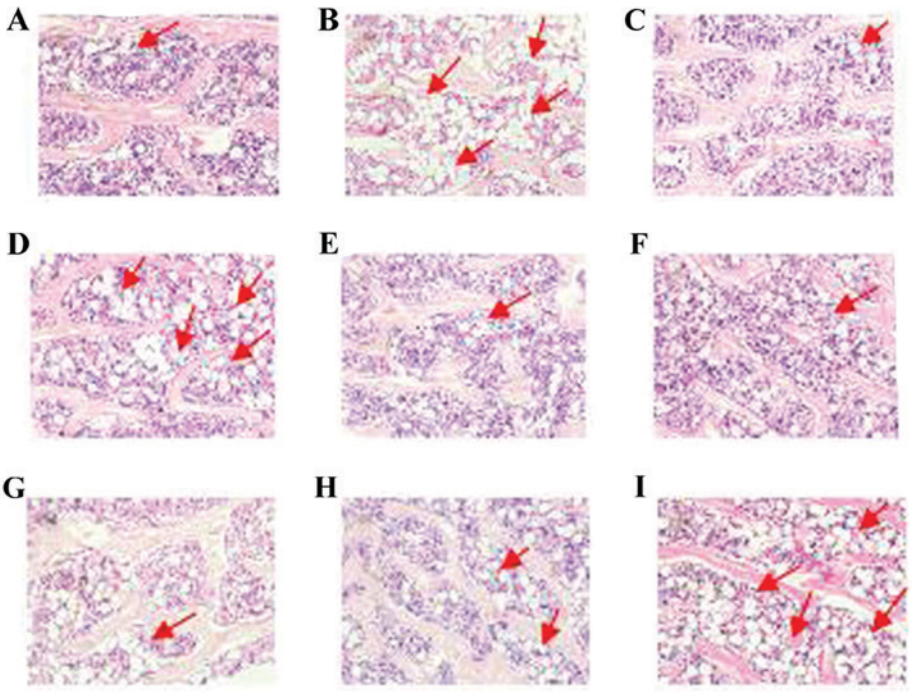

Figure 5. Effects of vehicle-treated controls (CON), prednisone 6 mg.kg^{-1}.d^{-1} (Pred), calcitriol 0.045 μg.kg^{-1}.d^{-1} (CAL), and variuos PMR (30, F) dose treatments on adipocyte distribution in bone marrow of PTM. (A) CON; (B) Pre (6 mg.$^{-1}$kg^{-1}.d^{-1}); (C) Pre+CAL (0.045 μg.kg^{-1}.d^{-1}); (D) Pre+PMR30 (H) (400 mg.kg^{-1}.d^{-1}); (E) Pre+PMR30 (M) (200 mg.kg^{-1}.d^{-1}); (F) Pre+PMR30 (L) (100 mg.kg^{-1}.d^{-1}); (G) Pre+PMRF (H) (400 mg.kg^{-1}.d^{-1}); (H) Pre+PMRF (M) (200 mg.kg^{-1}.d^{-1}); (I) Pre+PMRF (L) (100 mg.kg^{-1}.d^{-1}).

PMR30 and PMRF can increase the parameters of the outer membrane and decrease the inner membrane of tibial shaft in different degree (Tables V-VII and Fig. 4). This shows that the PM can promote the periosteal bone formation of the outer membrane and inhibit endosteal bone resorption of the inner membrane, decrease bone turnover, and increase the bone mass and thickness of cortical bone, against long-term use of GC-induced %Ma.Ar increase, then make cortical become thinner, thereby increasing the quality and bone biomechanical properties to avoid fracture. Of these results we also found the ratio of bone conversion are different proximal tibia and tibia shaft. The conversion rate of proximal tibia was faster than tibia shaft, this may related to blood vessel is rich in proximal tibial (37).

Interestingly, we found that longitudinal growth plate of proximal tibial were increased in PMR30 (H, M, L) and PMRF (H) groups (Fig. 4), compared with prednisone group. This may be related to PM inducing the growth hormone release. Lo has been reported that an emodin derivatives isolated from *PM*, this emodin derivative, tentatively named emoghrelin, was demonstrated to stimulate growth hormone secretion of rat primary anterior pituitary cells, presumably via the same molecular mechanism of GHSR activation (38). In the present study, the content of combined anthraquinone

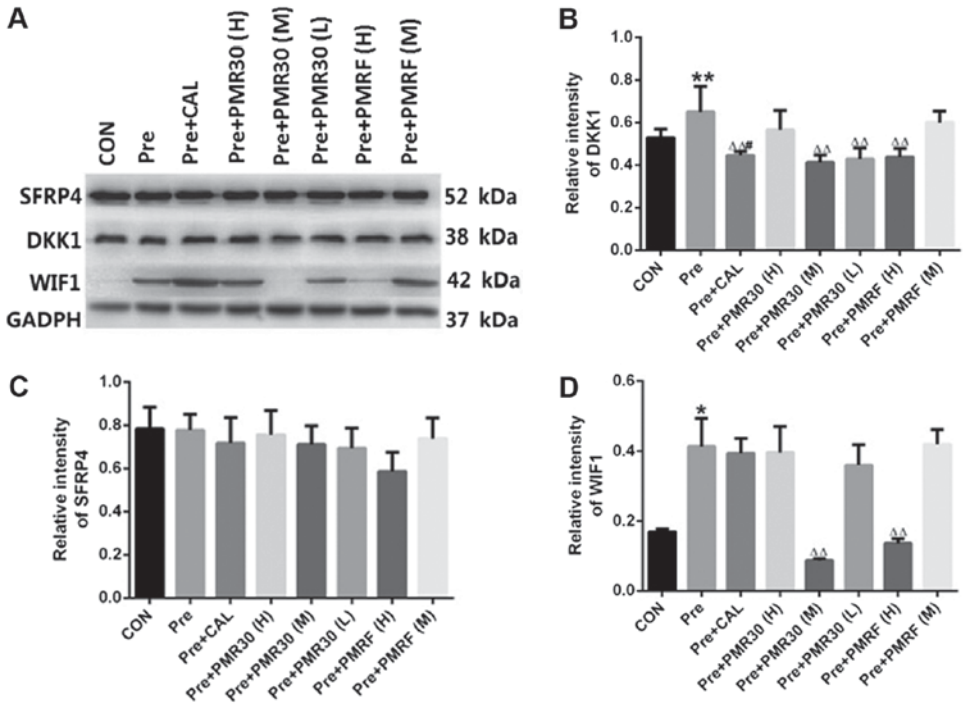

Figure 6. Effects of the extracts of PM on target protein of wnt signaling pathways in GIO rats. (A) The scanned image on X-rays was after exposure of western blot analysis. (B) The DKK1 protein of wnt signaling pathways in GIO rats. (C) The SFRP4 protein of wnt signaling pathways in GIO rats. (D) The WIF1 protein of wnt signaling pathways in GIO rats. *P<0.05, **P<0.01 vs. CON; $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 vs. Pred; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CAL.

(CAQ) in the sample of PMR30 was over than PMRF, that the longitudinal growth plate of proximal tibial were increased in PMR30 (H, M, L) over than PMRF (H) groups. The deficiency is not to determine the longitudinal growth rate (LGR).

The disruption of the bone formation-resorption balance plays a key role in osteoporosis (39). Serum BAP is known as a marker of bone formation, and TRACP-5b is known as a marker of bone resorption (35). In the present study, we found that PM recovered the serum BAP and PICP levels and significantly reversed the prednisone-induced decrease. We have also found that the serum of DKK1 and TRACP-5b level were increased in GC group which were consistent with the histomophometric data (Tables I-VI). TRACP-5b mainly exists in bone tissue, which is mainly derived from osteoclasts, and presents the activity of osteoclasts and its function of bone resorption (36). The level of TRACP-5b was increased in prednisone group, suggesting that long-term use of GCs increased osteoclast activity. Our study data indicated that PM can promote bone formation and inhibit bone resorption.

Futhermore, it has been reported that high level of DKK1 which suppresses the Wnt signal of bone formation in osteoblasts, resulted from activation of transcription through GRE in the DKK1 gene promoter. In the present study, DKK1 was increased expression in prednisone group (Fig. 6). The Wnt/β-catenin signaling pathway was also found to be re-activated by PM, which may be related to the bone-protective effects of PM. These results indicate that PM exerts protective effects against GIO. In previous studies, GC-treated animals also exhibited decreased BMD and bone mineral content (BMC) (27). In addition, in the previous experiments, we verified that TSG, as a major constituent in *PM* Thunb, showed anti-osteoporosis activity *in vitro* and *in vivo* (22). These data suggest that the bone-protective effects of PM are mediated through the regulation of Wnt signaling pathway. Wnt binds with specific cell-surface receptors Frizzled and LRP5/6, thereby leading to binding with Axin, which in turn mediates the proteolysis of β-catenin. DKK1 is also known to inhibit Wnt signaling by binding to LRP5/6 (41). DKK1 are receptor inhibitors which play a key role in the regulation of the Wnt signaling pathway in bone formation (42). As expected, treatment with PM recovered the activity of this signaling pathway. These results suggest that the Wnt/β-catenin signaling pathway is involved in the bone-protective effects of PM against prednisone-induced osteoporosis.

In conclusion, we demonstrated that PM can attenuate GIO and the mechanism of the preventive effect on GIO may be linked to direct up-regulation of canonical Wnt/β-catenin pathway.

Acknowledgements

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