Calcif Tissue Int
DOI 10.1007/s00223-014-9899-7

ORIGINAL RESEARCH

Glucocorticoid-Induced Osteoporosis in Growing Rats

Sien Lin · Jianping Huang · Liang Zheng · Yanzhi Liu · Guihua Liu · Nan Li · Kuixing Wang · Liyi Zou · Tie Wu · Ling Qin · Liao Cui · Gang Li

Received: 21 May 2014 / Accepted: 17 July 2014

Abstract This study evaluated whether growing rats were appropriate animal models of glucocorticoid-induced osteoporosis. The 3-month-old male rats were treated with either vehicle or prednisone acetate at 1.5, 3.0, and 6.0 mg/kg/day by oral gavage, respectively. All rats were injected with tetracycline and calcein before sacrificed for the purpose of double in vivo labeling. Biochemistry, histomorphometry, mechanical test, densitometry, micro-CT, histology, and component analysis were performed. We found that prednisone treatments dose dependently decreased body weight, serum biomarkers, biomechanical markers, bone formation, and bone resorption parameters in both tibial and femoral trabecular bone without trabecular bone loss. We also found that significant bone loss happened in femoral cortical bone in the glucocorticoid-treated rats. The results suggested that prednisone not only inhibited bone formation, but also inhibited bone resorption which resulted in poor bone strength but with no cancellous bone loss in growing rats. These data also suggested that the effects of glucocorticoid on bone metabolism were different between cortical bone and trabecular bone, and different between tibia and femur. Growing rats may be a glucocorticoid-induced osteoporosis animal model when evaluated the effects of drugs upon juvenile patients exposed to GC for a long time.

Keywords Osteoporosis · Glucocorticoid · Animal model · Rats

Introduction

Synthetic glucocorticoids (GCs) are widely used for the treatment of autoimmune diseases, rheumatism, gastrointestinal diseases, tumors, and organ transplantation in clinical practice for decades. Although the therapeutic effects of GC have been fully confirmed, it inevitably produced many side effects by long-term use. Glucocorticoid-induced osteoporosis (GIO) is one of the serious side effects which have become the most common secondary osteoporosis in adults. Early data reported that 30 % of patients with long-term (over 6 months) use of GC

Sien Lin and Jianping Huang contributed equally to this study.

S. Lin · L. Zheng · Y. Liu · L. Zou · T. Wu · L. Cui (✉)
Department of Pharmacology, Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical College, Zhanjiang, Guangdong 524023, China
e-mail: cuiliao@163.com

S. Lin · L. Qin · G. Li
Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China

J. Huang
Department of Stomatology, Guangdong Medical College, Zhanjiang, Guangdong, China

G. Liu
Department of Orthopaedics, Huizhou Municipal Central Hospital, Huizhou, Guangdong, China

N. Li · K. Wang · G. Li
Program of Stem Cell and Regeneration, School of Biomedical Science, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China

N. Li · K. Wang · G. Li (✉)
Li Ka Shing Institute of Health Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China
e-mail: gangli@cuhk.edu.hk

Published online: 03 August 2014

acquired osteoporosis [1]. Due to a rapid, dose-dependent bone loss, adult patients with GIO are usually suffering from back pain, height lost, humpback, and even fracture. GC-induced fractures may cause disability, bringing a heavy economic burden to families and society.

Decreased bone mineral density (BMD) was also documented in various GC-treated pediatric diseases, including asthma, rheumatoid arthritis, systemic lupus erythematosus, and organ transplantation [2]. A population-based study demonstrated that fracture risk was increased in the children who received over four courses of glucocorticoids [2]. GIO was related to the decreased osteogenesis and increased osteoblast apoptosis which reduced bone formation [3]. Since children have high bone growth rate, we believed that the growing skeleton could be highly vulnerable to adverse effects of glucocorticoid on bone formation.

Appropriate animal models are essential for research and development of new drugs. Sheep, rabbit, and minipig have been developed as large animal model of GIO in previous studies [4–6]. However, small animal models should be considered because of lower cost and easier handling. Rat is the most commonly used model for osteoporosis, based on which, histomorphometry, biomechanics, and imaging methodologies have been well established. Recently, our team has found that adult rats at the age of 6 months were appropriate animal models of GIO models [7]. We found significant decrease in BMD, bone strength, trabecular bone mass, and poor architecture in GC-treated rats. However, there is still no report on the changes in bone metabolism and bone architecture of growing rats when treated with GC. Furthermore, whether growing rats will be good candidates for GIO models for juvenile patients is not well validated. It is believed that endogenous GC is essential for the proliferation and differentiation of osteoblast in skeleton development, while long-term use of external GC inhibits bone formation. We hypothesize that the dosages of external GC may be a key factor in the development of GIO models. The current study was designed to evaluate the effects of three different doses of GC on bone of growing rats using methods of biochemistry, cytophotometry, mechanical testing, densitometry, micro-CT, histology, and components analysis. In this study, we used prednisone (Pre) which is a widely used glucocorticoid and can be orally administrated.

Materials and Methods

Animals and Treatments

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 by National Laboratory Animal Monitoring Institute of China. All experimental protocols were approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical College, Zhanjiang, China. Permit Number: SYXK (GUANGDONG) 2008–0007. The 3-month-old Sprague–Dawley male rats were acclimated to local vivarium conditions (temperature 24–26 °C, humidity 67 %) and allowed free access to water and diets containing 1.11 % calcium and 0.74 % phosphorus. All rats received subcutaneous injection of tetracycline (20 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) on days 14 and 13, and calcein (10 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) on days 4 and 3 before sacrifice.

This study was performed on 36 rats which were divided into four groups with 9 rats per group. The rats were orally administered either distilled water as vehicle control (CON), or prednisone acetate (Guangdong Xianju Pharmacy, Guangdong, China) at doses of 1.5 mg/kg/day (Pre1.5), 3.0 mg/kg/day (Pre3.0), and 6.0 mg/kg/day (Pre6.0), respectively, for 90 days. The volume for the oral gavage was 5 ml/kg/day.

Sample Collection and Applications

Rats were weighted every week. At the endpoint, the rats were sacrificed by cardiac puncture under anesthesia using 1 mg/kg sodium pentobarbitone. The blood serum was collected for biochemical assays. The left proximal tibial metaphysis (PTM), left tibial shaft (TS), and left distal femoral metaphysis (DFM) were subjected to undecalcified section for bone histomorphometry. The right PTM was subjected to decalcified section for fat tissue measurement in bone marrow. The right femurs and the 5th lumber vertebrae (LV5) were performed both for bone densitometry and biomechanics measurement. The 6th lumber vertebrae (LV6) were performed micro-CT scanning. After that, the desiccant right femur was used for examining the chemical component of calcium (Ca), phosphorus (P), and hydroxyproline (Hyp).

Serum Markers Assays

Blood was collected in specimens 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×g for 10 min and stored at −80 °C for biochemical markers assays. Tartrate-resistant acid phosphatase-5b (TRACP-5b, Biomedical technologies, Stoughton, MA, USA), osteocalcin (Immunodiagnostic System, Tyne and Wear, UK), and insulin-like growth factor-1 (IGF-1, Boster bioengineering, Wuhan, Hubei, China) in serum were

measured with commercial ELISA kit using ELX800 Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) according to the protocols attached.

Bone Histomorphometry

The left proximal tibial metaphysis (PTM), left tibial shaft (TS), and left distal femoral metaphysis (DFM) were removed and bone marrow cavities were exposed using IsoMet® precision bone saw (Buehler, Lake Bluff, IL, USA), then were fixed in 10 % buffered formalin for 24 h, followed by gradient alcohol dehydration, xylene defatting, and undecalcification embedded in methyl methacrylate. The frontal PTM and DFM tissue were cut into 9- and 5-μm thick sections with the RM2155 hard tissue microtome (Leica, Wetzlar, Germany), respectively. The unstained 9-μm sections were used for dynamic histomorphometric analysis. The 5-μm sections were stained with Von Kossa, and trichrome masson goldner for static histomorphometric measurements. TS were cut using IsoMet® precision bone saw, and sections were mounted on plastic slides, grounded, and polished for histomorphometry. A semi-automatic digitizing image analysis system (OsteoMetrics, Atlanta, GA, USA) was used for quantitative bone histomorphometric measurements.

The measurement region of PTM or DFM was cancellous bone between 1 and 4 mm distal or proximal to the growth plate-epiphyseal junction. The left distal femoral growth plate was also processed for cartilaginous longitudinal growth rate analyses. The quantitative analysis was performed on each sample, with one section each. The histomorphometric measurements were done on the cancellous (Cn) and cortical (Ct) bone, respectively. The abbreviations of the bone histomorphometric parameters used were recommended by the ASBMR Histomorphometric Nomenclature Committee [8]. All measured thicknesses (except cortical thickness) were multiplied by π/4. Structural parameters were tissue volume (TV), bone volume (BV), marrow volume (Ma.V), bone surface (BS), cortical thickness (Ct.Th), marrow diameter (Ma.Dm), periosteal surface (Ps.S), and endocortical surface (Ec.S). The parameters of microarchitecture (trabecular thickness [Tb.Th], number [Tb.N], and separation [Tb.Sp]) were derived from area and perimeter measurements. Dynamic measurement parameters were inter-label width in the growth plate (G-Int.Wi), single-labeled surface (sLS), double-labeled surface (dLS), and label thickness (L.Th). Bone formation or resorption was assessed with measurements of osteoblast surfaces per bone surface (Ob.S/BS) or osteoclast surface per bone surface (Oc.S/BS). The mineral apposition rate (MAR) and the ratio of mineralizing surface to bone surface (MS/BS, calculated as double plus half of single-labeled surfaces), bone formation rate per unit of bone surface (BFR/BS), and bone formation rate per unit of bone volume (BFR/BV) were analyzed on unstained sections under ultraviolet light. All histomorphometric parameters were in accordance with the published studies [7, 9].

Bone Mineral Density (BMD) Determination

The right femurs and LV5 of rats were wrapped with saline-saturated gauze to maintain their moisture and stored at −20 °C. After thawed at room temperature, the bones were moisturized by soaking them in saline solution with the residual muscle removed. The whole femoral BMD was scanned with Prodigy Dual-Energy X-ray Absorptiometry scanner (GE Healthcare, Little Chalfont, UK) to measure the bone mineral content (BMC, g/cm^2) and bone area (BA, cm^2). The BMD was calculated as BMC/BA.

Biomechanical Test

After measuring BMD, the right femurs and LV5 were used to determine the bone mechanical properties through three-point bending or compression test using Material Testing System (MTS Systems, Eden Prairie, MN, USA), respectively. The bones were tested with a 1 mm indenter, at a speed of 0.01 mm/s with a 15 mm span for femur. Force and deflection were automatically recorded. The output parameters included elastic load (the force required to cause bone specimens to deform, N), maximum load (the maximum force the bone can resist, N), fracture load (the force required to cause bone fracture, N), and the maximum deflection (maximum degree of the bone displacement, mm). Bone diameter (B.Dm) and marrow diameter (Ma.Dm) of the fractured ends of femurs were also measured. The stiffness coefficient (load–displacement curve slope, N/mm), Young's modulus, and area moment of inertia were also calculated based on the output parameters.

Micro-computed Tomography (micro-CT) Scanning

The specimens (LV6, with 5 samples per group) were scanned using a desktop eXplore Locus SP preclinical specimen micro-CT (GE Healthcare, Madison, WI, USA) at an isotropic spatial resolution of 14 μm and a peak voltage of 80 kV. Four projections were performed for each scan angle in each slice. These projections had an exposure time of 2960 ms each and were averaged in order to improve the signal-to-noise ratio. MicroView ABA 2.1.2 software (GE healthcare, Madison, WI, USA) was used to analyse the multiplanar images of LV5 and to calculate the parameters of architecture for each specimen. A cylinder region of interest (ROI) ($\pi \times 0.6$ mm $\times 0.6$ mm $\times 4$ mm) was selected at the centre of the LV6 and separated from the cortical bone. Then, local

adaptive threshold algorithms were used during the evaluation of all specimens. The selected microstructural parameters included the bone structure model index (SMI), BV/TV and connective density (Conn. D). A calibration phantom, including air, saline, and a mineral standard material (air = 1.22832, water = 3.4134, bone = 4730), allowed calibration and conversion of X-ray attenuation such that the mineral content was proportional to greyscale values in Hounsfield Units (HUs).

Marrow Fat Cells Measurement

The right proximal tibial bone marrow cavity was exposed and decalcified at room temperature in 10 % buffered EDTA for 5 weeks. After decalcification, the samples were dehydrated and paraffin embedded. 5 μm thick sections were prepared then followed with hematoxylin and eosin stain (H&E) as previous described [10]. Five fields under a magnification of 100× were randomly selected for evaluation; the mean value of all fields measured for each animal was taken for statistical analysis. Percentage area of bone marrow fat (fat cells area divided by tissue area in the examined regions) was evaluated with imaging process software, Image-Pro-Plus 6.0 (Media Cybernetics Inc., Bethesda, MD, USA).

Sample Digestion and Component Analysis

After subjected to biomechanical test, the femurs were digested and subjected to component analysis as previous report [11]. Briefly, samples were weighed before and after placing at 80 °C oven for 72 h. Then the desiccant bones were digested with 6 mol/l hydrochloric acid in 10-ml ampoule bottles at 108 °C oven for 24 h. Calcium and phosphorus in bone samples were determined by Inductively Coupled Plasma emission spectrometer (ICP-IRIS/AP, Thermo Jarrell Ash, Franklin, MA, USA). Instrument operating conditions applied for calcium (Ca) and phosphorus (P) determined were showed as below: gas (plasma) 24 l/min, gas flow (auxiliary) 0.5 l/min, RF power 1150 W, sample aspiration rate 1.48 ml/min, pressure 30 psi, standard Ca 10 μg/ml (Sigma-Aldrich, St. Louis, MO, USA) and P 50 μg/ml (Sigma-Aldrich, St. Louis, MO, USA). Hydroxyproline (Hyp) in bone digestive solution was determined by colorimetric assays according to manufacturer's instructions (Nanjing Jiancheng Biological Bioengineering, Nanjing, Jiangsu, China).

Statistical Analysis

Data were presented as mean ± SD, and analyzed using SPSS12.0 software for Windows (SPSS, Chicago, IL, US). The statistical differences among groups were evaluated using variance (ANOVA) with Fisher's PLSD test. Probabilities (P) less than 0.05 were considered significant.

Results

Body Weight and Serum Markers

At the endpoint, mean body weights of the rats were decreased by 11.4 % ($P < 0.01$), 14.7 % ($P < 0.01$), or 19.2 % ($P < 0.01$) after treated with prednisone at 1.5, 3.0, or 6.0 mg/kg/day (Fig. 1), respectively. The linear relationship between mean body weights and the dosages of prednisone ($R^2 = 0.99$) showed a dose-dependent manner.

Prednisone treatment at the highest dose level significantly decreased the serum osteocalcin, TRACP-5b, and IGF-1 level (Fig. 2). Despite a reduction in the osteocalcin level in the rats treated with prednisone at 3.0 mg/kg/day, the TRACP-5b and IGF-1 level was not changed at this lower prednisone dose group (Fig. 2).

Cancellous Bone Histomorphometry

For the tibial cancellous bone, histomorphometric data showed that there was no significant difference in the static parameters (BV/TV, Tb.Th, Tb.N, and Tb.Sp) between the rats treated with vehicle and prednisone at all three dosages (Fig. 3; Table 1). However, the dynamic parameters (MAR, BFR/BS, and BFR/BV) of trabecular bone significantly decreased at the two higher prednisone dose levels compared with controls (Fig. 3; Table 2). Furthermore, parameters of bone cells (Ob.S/BS and Oc.S/BS) significantly decreased in the rats treated with prednisone at all three dose levels compared with that in the vehicle-treated ones (Fig. 3; Table 1).

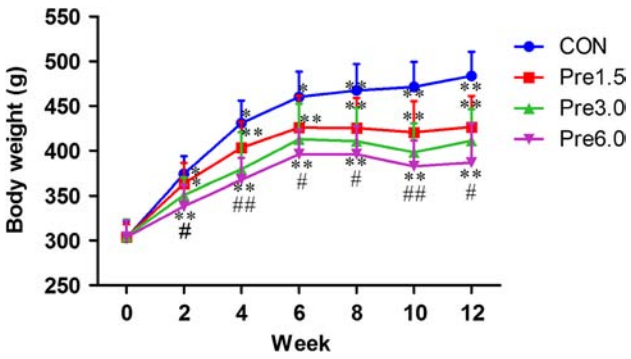

Fig. 1 Body weight (g) changes during the experimental period. Body weight measurements from vehicle-treated controls (CON), prednisone 1.5 mg/kg/day (Pre1.5), prednisone 3.0 mg/kg/day (Pre3.0), and prednisone 6.0 mg/kg/day (Pre6.0) treated rats. $^{*}P < 0.05$, $^{**}P < 0.01$ versus CON; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Pre1.5

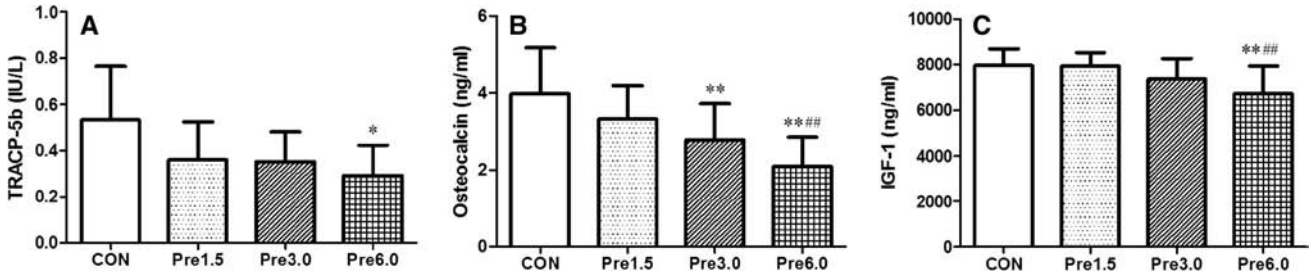

Fig. 2 Endpoint levels of serum biochemical markers (**a** TRACP-5b, **b** Osteocalcin, **c** IGF-1) in the rats treated with vehicle (CON) and various prednisone (Pre) dose levels. $^{*}P < 0.05$, $^{**}P < 0.01$ versus CON; $^{\#\#}P < 0.01$ versus Pre1.5

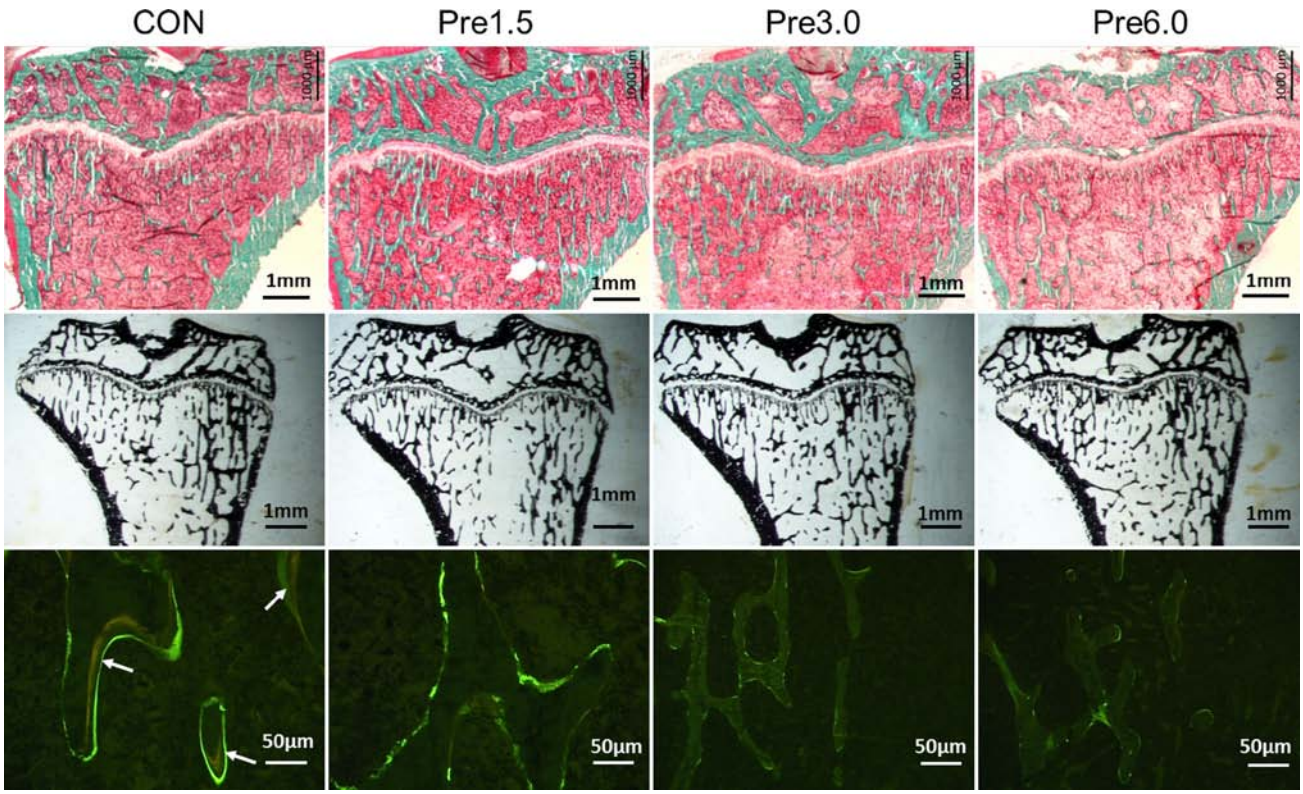

Fig. 3 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on the proximal tibial metaphyses (PTM) bone structure and mineral bone formation. Arrows point to the tetracycline and calcein labeling. Quantitative measurements of histomorphometric parameters of DFM are showed in Tables 1 and 2. (*Upper panel* Goldner's Trichrome stain, *middle panel* Von Kossa stain, *lower panel* fluorescence images of undecalcified sections)

Table 1 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on proximal tibial metaphysis cancellous bone structure histomorphometry, osteoblast, and osteoclast surface

Group	BV/TV (%)	Tb.Th (μm)	Tb.N (#/mm)	Tb.Sp (μm)	Ob.S/BS (%)	Oc.S/BS (%)
CON	14.1 ± 3.2	58.1 ± 6.4	2.4 ± 0.5	369.6 ± 91.8	9.4 ± 3.2	0.67 ± 0.28
Pre1.5	17.1 ± 5.0	60.4 ± 4.4	2.8 ± 0.7	319.9 ± 112.0	4.8 ± 1.6**	0.16 ± 0.05**
Pre3.0	15.9 ± 4.8	58.8 ± 7.0	2.7 ± 0.6	334.0 ± 91.7	4.2 ± 1.0**	0.17 ± 0.07**
Pre6.0	13.9 ± 5.2	52.0 ± 8.1$^{\#,\triangle}$	2.6 ± 0.7	366.5 ± 166.4	4.1 ± 1.5**	0.23 ± 0.11**

Note Value are mean ± SD, ** $P < 0.01$ versus CON; $^{\#}$ $P < 0.05$ versus Pre1.5; $^{\triangle}$ $P < 0.05$ versus Pre3.0

Table 2 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on proximal tibial metaphysis cancellous bone dynamic parameter

Group	MS/BS (%)	MAR (μm/day)	BFR/BS (μm/day × 100)	BFR/BV (%/year)
CON	28.8 ± 8.7	1.7 ± 0.8	48.2 ± 20.6	516.6 ± 239.9
Pre1.5	23.9 ± 4.9	1.3 ± 0.4	30.7 ± 10.1*	314.9 ± 121.8*
Pre3.0	23.8 ± 3.9	0.9 ± 0.4**	22.4 ± 11.0**	233.0 ± 118.7**
Pre6.0	20.9 ± 5.2**	0.7 ± 0.5**,#	16.4 ± 12.4**,#	196.7 ± 162.1**

Note Value are mean ± SD, * $P < 0.05$, ** $P < 0.01$ versus CON; # $P < 0.05$ versus Pre1.5

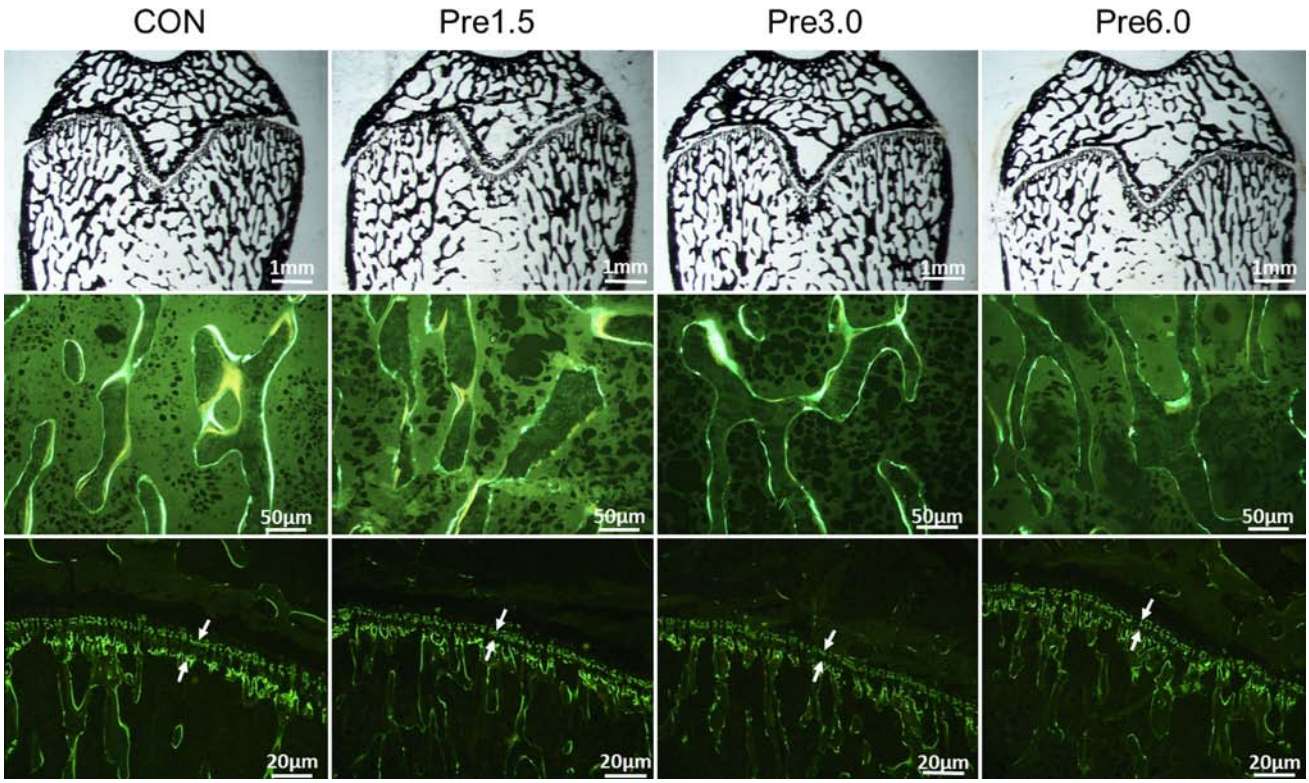

Fig. 4 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on the distal femoral metaphyses (DFM) bone structure, mineral bone formation, and cartilage growth. *Arrows point* to inter-labeling distances after double labeling with tetracycline and calcein. The inter-labeling distance in the growth plate was used to determine longitudinal growth rate (LGR). Quantitative measurements of histomorphometric parameters of DFM are showed in Tables 3 and 4. (*Upper panel* Von Kossa stain, *middle and lower panel* fluorescence images of undecalcified sections)

Table 3 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on distal femoral metaphyses cancellous bone structure

Group	BV/TV (%)	Tb.Th (μm)	Tb.N (#/mm)	Tb.Sp (μm)
CON	20.3 ± 4.3	67.0 ± 7.9	3.0 ± 0.5	271.5 ± 52.1
Pre1.5	22.4 ± 4.1	66.8 ± 7.2	3.4 ± 0.6	238.5 ± 51.2
Pre3.0	23.0 ± 3.3	58.9 ± 5.9*,#	3.9 ± 0.5*	200.8 ± 33.4*
Pre6.0	22.4 ± 6.9	62.6 ± 8.5	3.5 ± 0.7	234.0 ± 76.7

Note Value are mean ± SD, * $P < 0.05$ versus CON; # $P < 0.05$ versus Pre1.5

For the femoral trabecular bone, as showed by Fig. 4 and Table 3, prednisone treatment at all three dosages did not decrease the static parameters. As same as that of the tibial trabecular bone, prednisone treatment at the highest dose level significantly decreased the dynamic parameters (MS/BS, MAR, BFR/BS, and BFR/BV) (Fig. 4; Table 4).

Table 4 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on distal femoral metaphyses cancellous bone histomorphometry dynamic parameters

Group	MS/BS (%)	MAR (μm/day)	BFR/BS (μm/day × 100)	BFR/BV (%/year)	LGR (μm/day)
CON	45.2 ± 7.3	1.1 ± 0.1	52.1 ± 10.1	474.0 ± 79.5	84.4 ± 5.7
Pre1.5	37.7 ± 7.2*	0.9 ± 0.1**	35.0 ± 6.0**	322.0 ± 64.8*	70.7 ± 12.2**
Pre3.0	23.8 ± 3.9**,#	0.8 ± 0.1**,#	26.5 ± 5.1**,#	272.8 ± 43.1**	62.3 ± 11.2**
Pre6.0	20.9 ± 5.2*,△	0.9 ± 0.1**,△	35.4 ± 6.8**,△	353.1 ± 94.3**	74.6 ± 16.3**,△

Note Value are mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ versus CON; $^{\#}P < 0.05$ versus Pre1.5; $^{\triangle}P < 0.05$ versus Pre3.0

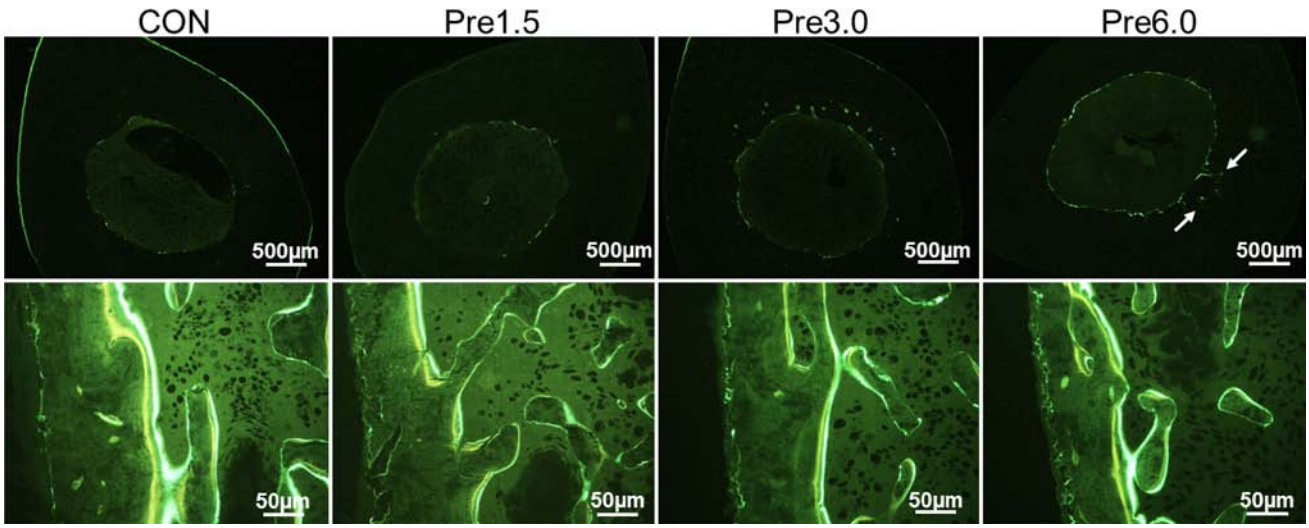

Fig. 5 Effects vehicle (CON) and various prednisone (Pre) dose treatments on the tibial shaft (TS) and distal femoral metaphyses (DFM) cortical bone structure and mineral bone formation. *Arrows point* to the porosities along the endocortical bone. Quantitative measurements of histomorphometric parameters of cortical bone are shown in Tables 5 and 6. (Fluorescence images of undecalcified sections)

Table 5 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on cortical bone of tibia shaft (TS) histomorphometry parameters

Group	Ma.V/TV (%)	Ps-MS/BS (%)	Ps-MAR (μm/day)	Ps-BFR/BS (μm/day × 100)	Ps.S (mm)	Ec.S (mm)
CON	25.5 ± 4.9	46.4 ± 22.5	0.80 ± 0.25	420.3 ± 285.9	10.8 ± 1.2	5.7 ± 1.0
Pre1.5	25.3 ± 4.6	36.8 ± 16.7	0.83 ± 0.40	344.5 ± 217.7	9.6 ± 1.1	4.9 ± 1.0
Pre3.0	25.0 ± 5.4	26.9 ± 17.7	0.48 ± 0.37*	166.0 ± 136.6*,#	9.6 ± 0.7	5.4 ± 1.6
Pre6.0	27.5 ± 6.4	31.9 ± 16.4	0.59 ± 0.13*	200.6 ± 123.3	10.0 ± 1.2	5.5 ± 1.1

Note Value are mean ± SD, $^{*}P < 0.05$ versus CON; $^{\#}P < 0.05$ versus Pre1.5

Data also showed that prednisone treatment at all three dosages decreased the femoral LGR (Fig. 4; Table 4).

Cortical Bone Histomorphometry

For the tibial cortical bone, histomorphometric data showed prednisone treatment at the highest dose level significantly decreased the periosteal MAR (Ps-MAR) (Fig. 5; Table 5). While no significant change in static parameters (Ma.V/TV, Ps.S, and Ec.S) of all the three doses of prednisone treatment was found (Fig. 5; Table 5). Furthermore, some resorptive porosities along the endocortical bone with fluorescence were found at the two higher prednisone dose levels. Moreover, prednisone treatment at all three dose levels significantly decreased the femoral cortical bone thickness (Ct.Th), endocortical MS/BS (Ec-MS/BS), Ec-MAR, and Ec-BFR/BS (Fig. 5; Table 6).

Table 6 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on cortical bone of distal femoral metaphyses histomorphometry parameters

Group	Ct.Th (μm)	Ec-MS/BS (%)	Ec-MAR (μm/day)	Ec-BFR/BS (μm/day × 100)
CON	352.0 ± 37.7	90.3 ± 5.9	2.8 ± 0.6	258.8 ± 69.8
Pre1.5	274.0 ± 36.8**	82.7 ± 5.0*	1.9 ± 0.4**	160.6 ± 39.4**
Pre3.0	290.2 ± 60.0**	85.0 ± 5.9*	1.8 ± 0.2**	157.0 ± 20.2**
Pre6.0	247.2 ± 45.3**	82.0 ± 9.0*	2.1 ± 0.3**	172.9 ± 31.2**

Note Value are mean ± SD, * $P < 0.05$, ** $P < 0.01$ versus CON

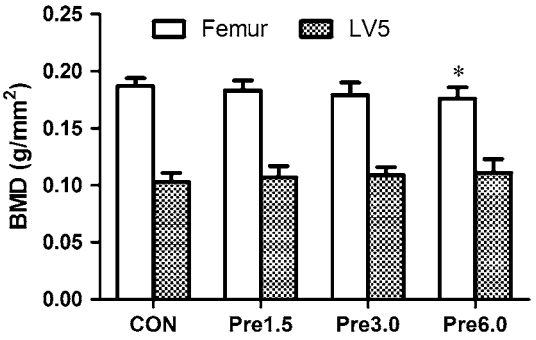

Fig. 6 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on BMD of whole femur or LV5. $^{*}P < 0.05$ versus CON

Bone Mineral Density

Prednisone treatment at the highest dose level significantly decreased the BMD of whole femur ($P < 0.01$) (Fig. 6). While prednisone treatment at all three dose levels did not decrease the BMD of whole LV5 (Fig. 6).

Bone Biomechanics

At the endpoint, prednisone treatment at the highest dose level significantly reduced elastic load ($P < 0.01$), maximum load ($P < 0.01$), stiffness ($P < 0.01$), and area moment of inertia ($P < 0.01$) of femur and significantly decreased the maximum load ($P < 0.01$) and elastic modulus ($P < 0.05$) of LV5 (Fig. 7).

Microarchitecture of Cancellous Bone

Trabecular microarchitecture of LV6 was analyzed by micro-CT, and the representative 3D images were shown in Fig. 8. No significant change of bone volume and microarchitecture (BV/TV, SMI, and Conn.D) of all the three doses of prednisone treatment was found (Fig. 8).

Marrow Fat Tissue Deposition

Histological data of decalcified tibia showed prednisone treatment at all three dose levels significantly increased the fat tissue area in the bone marrow cavity (Fig. 9).

Chemical Compositions of Bone

As showed by Table 7, except for a significant decrease in level of phosphorus was observed in the lowest dose of prednisone treatment level, no significant difference in Ca or Hyp was found in all of the three prednisone levels compared with vehicle-treated controls (Table 7).

Discussion

The results indicated that prednisone significantly reduced body weight and the longitudinal bone growth in the rats treated with prednisone. The secretion of endogenous GC was regulated by hypothalamus–hypophysis–adrenal cortex system. Previous data showed that long-term steroid treatment inhibited the secretion of growth hormone (GH), and caused growth retardation in children suffering nephrotic syndrome [12]. Similar to GH, IGF-1 promotes longitudinal bone growth by augmenting chondrocyte hypertrophy [13]. A reduction of serum IGF-1 caused by long-term use of glucocorticoid may inhibit the process of endochondral ossification, then subsequently impaired the longitudinal growth [14]. Our data also showed that both serum IGF-1 level and LGR were significantly decreased in the rats treated with prednisone at the highest dose level, which was consistent with the previous results [14].

Long-term use of glucocorticoid may cause osteoporosis or osteonecrosis in human [10]. Our previous study found a significant cancellous bone loss in 6-month-old GC-treated rats which was different from the result of this study [7]. Although there was no comparable data to show the different effects of GC on bone architecture of rats at different ages, we believed it may be due to the changes in bone metabolism of rats at different ages. In cancellous bone, there was a gradual transition from the predominance of modeling activity in rapidly growing bones to the predominance of remodeling in slowly growing or nongrowing bones [15]. In physiological conditions, bone modeling predominantly occurred in the growing rats while bone remodeling happened in the adult or aged rats [15].

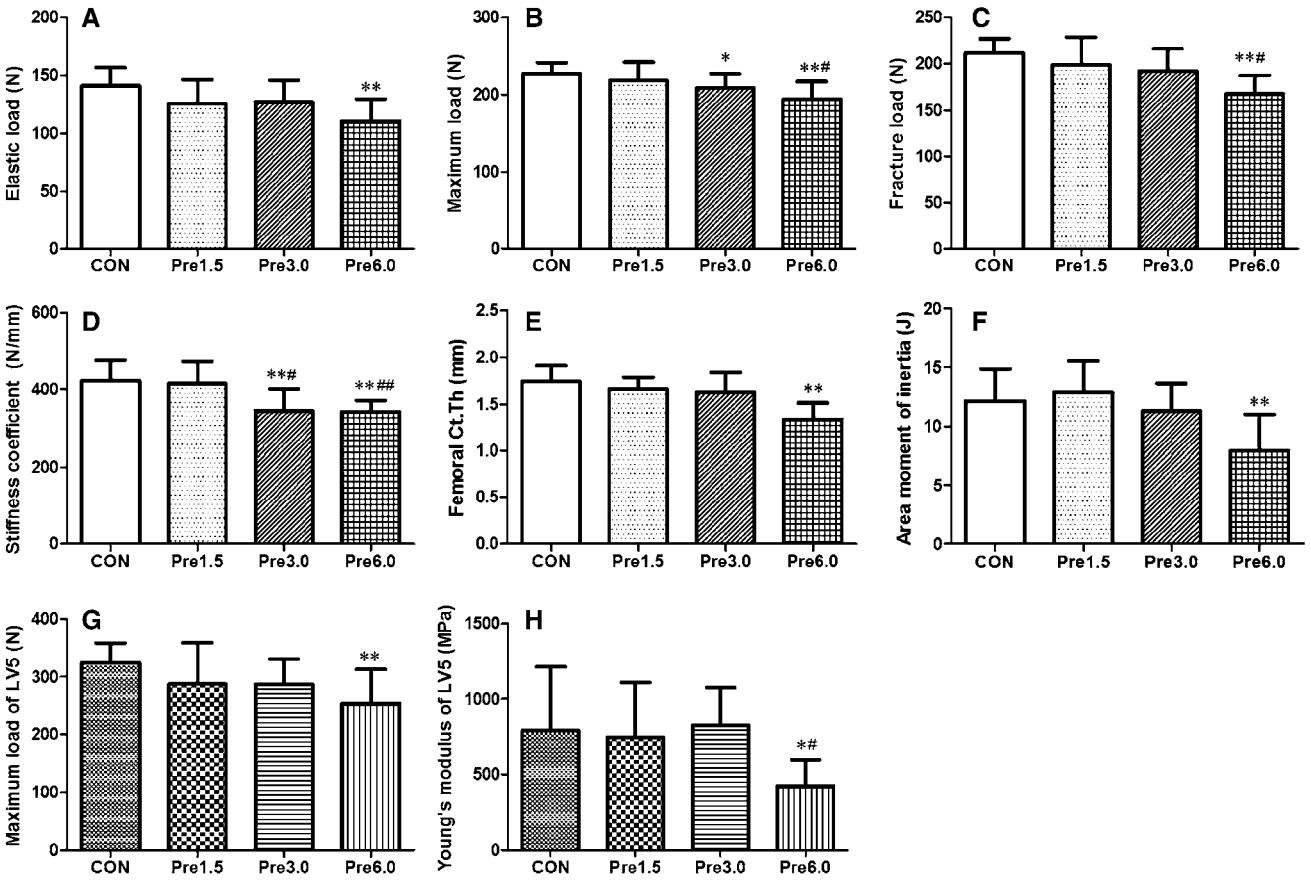

Fig. 7 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on biomechanics of femurs (**a**–**f**) and LV5 (**g**, **h**). $^{*}P < 0.05$, $^{**}P < 0.01$ versus CON; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Pre1.5

Our data indicated the effects of prednisone on cortical bone differed from trabecular bone. For the femoral cortical bone, a significant bone loss determined by BMD measurement, with a reduction in the cortical bone thickness and a markedly reduction in the biomechanical properties at the highest prednisone dose level were investigated. Some resorptive porosities in the endocortical bone were also found at the two higher prednisone dose levels. However, both histomophometric data and micro-CT data indicated there was no glucorcorticoid-induced bone loss in the trabecular bone area in this study. No significant change was found in the trabecular microarchitechture (SMI and Conn.D) as indicated by the micro-CT results, neither. Although there were significant decreases in bone formation parameters (MS/BS, MAR, BFR/BS, BFR/BV, and Ob.S/BS) in the trabecular bone area, bone resorption (Oc.S/BS) also decreased at the highest prednisone dose level. The decreased serum osteocalcin and TRACP-5b level were consistent with the histomophometric data. All these suggested that an inhibitory effect of prednisone on both bone formation and bone resorption might be responsible for the maintaining of trabecular bone volume.

Our data also showed that prednisone treatment significantly increased fat tissue in bone marrow, which implied there might be a increase in adipogenesis and decrease in osteogenesis of mesenchymal stem cells in bone marrow of the rats treated with prednisone (data not showed). Previous study found that Ob.S/BS and Oc.S/BS were decreased after GC treatment, with a reduction or maintaining of the trabecular bone volume [16]. But a reduction of cortical bone volume was always observed [16]. Previous data also demonstrated that bone loss in femoral cortical bone with no bone loss in the trabecular bone after GC treatment in adult mice [17]. It was believed that increased apoptosis of osteoblasts in trabecular bone and apoptosis of osteocytes in the metaphyseal cortical bone in the mice treated with GC [17]. Furthermore, decreased production of osteoclasts and osteoblasts may also contribute to the diminished bone formation and bone turnover caused by GC [17]. In addition, a paper recently showed that osteocyte autophagy was increased at the distal femur cortical bone region but not at trabecular region after low dose GC treatment while osteocytes apoptosis increased at the same region after high dose GC treatment for 1 month in growing mice [3].

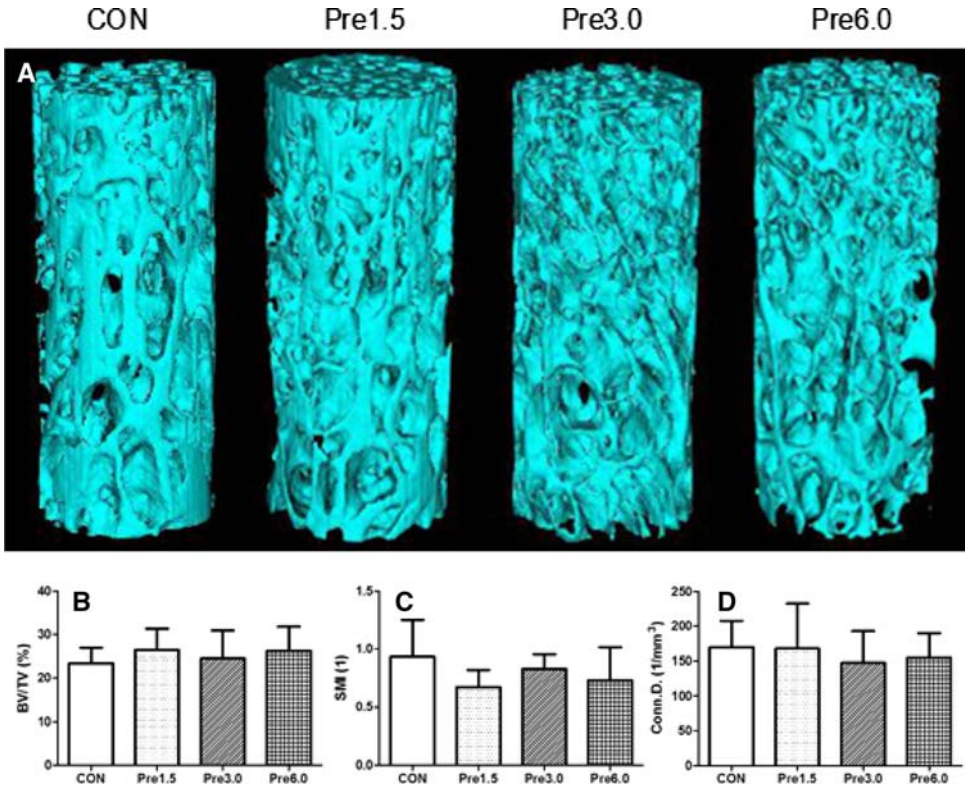

Fig. 8 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on the trabecular microarchitecture of LV6. The representative 3D images (**a**) and the parameters (**b** BV/TV, **c** SMI, **d** Conn.D) were presented, showing no statistical difference

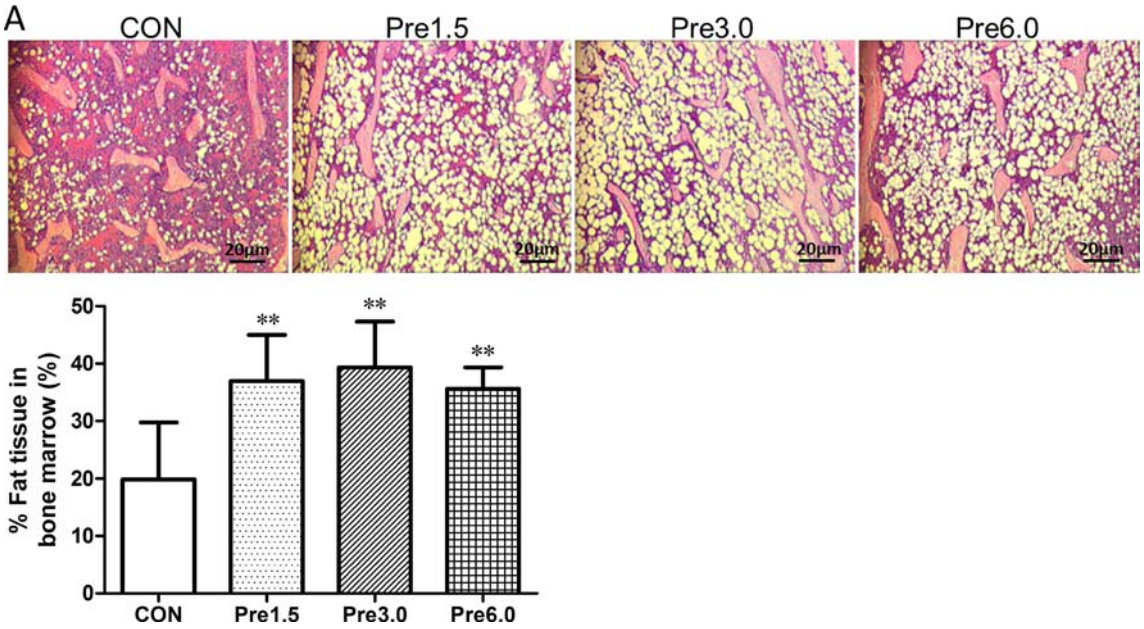

Fig. 9 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on adipocyte distribution in bone marrow of PTM. $^{**}P < 0.01$ versus CON

Therefore, we suggested that the effect of GC on bone metabolism is different between cortical bone and trabecular bone. Indeed, recent data indicated that GC at pharmacological dosage may have divergent effects on cortical and trabecular bone [18, 19]. Despite there was no direct evidence in our study to explain the different function of bone cells between cortical bone and trabecular bone, recent studies revealing a large cohort of functionally distinct glucocorticoid receptor subtypes that control specific sets of genes and/or differentially regulate common sets

Table 7 Effects of vehicle (CON) and various prednisone (Pre) dose treatments on the chemical compositions of femur

Group	Ca (mg/g)	P (mg/g)	Hyp (mg/g)
CON	25.13 ± 1.61	13.71 ± 0.01	3.12 ± 0.24
Pre1.5	25.94 ± 2.67	12.44 ± 1.29*	3.14 ± 0.22
Pre3.0	27.34 ± 1.67	13.59 ± 0.79	3.29 ± 0.25
Pre6.0	26.45 ± 1.21	13.41 ± 0.60	3.29 ± 0.29

Note Value are mean ± SD, $*P < 0.05$ versus CON

provided enormous potential for signaling diversity, further contributed to the tissue- and cell-specific effects of glucocorticoids [20]. It also revealed that, not only there were some differences in bone architecture between cortical bone and trabecular bone after GC treatment, but there were also some differences between endosteal bone surface and periosteal bone surface [21].

The ability of bone to provide mechanical support and resist fracture depends on both bone mass and material properties of bone, both of which must be actively maintained to respond to changing force condition [22]. Given the clinical interest in material properties of bone, it refers to the influence of factors that affect fracture but are not accounted for by bone mass [23]. Interestingly, GC usage is associated with fragility fractures, which occur often with higher BMD levels than those of postmenopausal women with osteoporotic fractures [24, 25]. Bone mass and the chemical compositions of bone cannot be recognized as predictors of material properties of bone in this study, which suggested that material properties may be deteriorated by exogenous GC without bone mass changed. Low bone formation and resorption in the prednisone-treated rats in this study may partially contribute to the higher fracture risk. It was reported that the amount of bone turnover in the skeleton has been identified as a predictor of fracture risk independent of areal BMD and was increasingly cited as an explanation for discrepancies between areal BMD and fracture risk [26]. It was also speculated that the increased bone fragility observed with GC treatment partially results from localized changes within the bone around the osteocyte [27]. In GC-treated mice, bone matrix mineralization and material properties are most profoundly affected in the perilacunar regions, suggesting an important role for osteocytes in bone metabolism [28]. In our future study, it is valuable to explore the relationship between osteocytes and GC and the underlying mechanism.

In conclusion, after 3 months of GC treatment, despite no bone loss and no microarchitechture change in the trabecular bone, decreased longitudinal growth and cortical bone mass accompanied with deteriorated mechanical competence, low bone formation, and low bone resorption were found in the growing rats, which were similar to that of the juvenile patients exposed to GC for a long time. Trabecular bone loss which usually found in GIO patients was not found in the current models. Growing rats may be used as a glucocorticoid-induced osteoporosis animal model when evaluated the effects of drugs upon juvenile patients.

Acknowledgments This project was funded by the National Science Foundation of China (No: 30973574), Science & Technology Innovation Fund of Guangdong Medical College (No: STIF201104), the Science and Technology Fund of Zhan Jiang (No: 2012C3102015), and the Youth Fund of Guangdong Medical College (No: 201001 & 201022). We wish to thank Dr. David William Green at the University of Hong Kong and Dr. Yuk Wai Lee at the Chinese University of Hong Kong for their contribution on English language editing and grammar correcting of the manuscript.

Conflict of interest Sien Lin, Jianping Huang, Liang Zheng, Yanzhi Liu, Guihua Liu, Nan Li, Kuixing Wang, Liyi Zou, Tie Wu, Ling Qin, Liao Cui, and Gang Li declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All animal experiments were approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical College, Zhanjiang, China. Permit Number: SYXK (GUANGDONG) 2008–0007.

References

1. Gudbjornsson B, Juliusson UI, Gudjonsson FV (2002) Prevalence of long term steroid treatment and the frequency of decision making to prevent steroid induced osteoporosis in daily clinical practice. Ann Rheum Dis 61:32–36
2. Leonard MB (2007) Glucocorticoid-induced osteoporosis in children: impact of the underlying disease. Pediatrics 119:S166–S174
3. Jia J, Yao W, Guan M, Dai W, Dai W, Shahnazari M, Kar R, Bonewald L, Jiang JX, Lane NE (2011) Glucocorticoid dose determines osteocyte cell fate. FASEB J 25:3366–3376
4. Ding M, Cheng L, Bollen P, Schwarz P, Overgaard S (2010) Glucocorticoid induced osteopenia in cancellous bone of sheep: validation of large animal model for spine fusion and biomaterial research. Spine 35:363–370
5. Baofeng L, Zhi Y, Bei C, Guolin M, Qingshui Y, Jian L (2010) Characterization of a rabbit osteoporosis model induced by ovariectomy and glucocorticoid. Acta Orthop 81:396–401
6. Bauss F, Russell RG (2004) Ibandronate in osteoporosis: preclinical data and rationale for intermittent dosing. Osteoporos Int 15:423–433
7. Cui L, Li T, Liu Y, Li P, Xu B, Huang L, Chen Y, Liu Y, Tian X, Jee WS, Wu T (2012) Salvianolic acid B prevents bone loss in prednisone-treated rats through stimulation of osteogenesis and bone marrow angiogenesis. PLoS ONE 7:e34647
8. Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM (2013) Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 28:2–17
9. Lin SE, Huang JP, Wu LZ, Wu T, Cui L (2013) Prevention of osteopenia and dyslipidemia in rats after ovariectomy with combined aspirin and low-dose diethylstilbestrol. Biomed Environ Sci 26:249–257

10. Sheng H, Zhang G, Wang YX, Yeung DK, Griffith JF, Leung KS, Qin L (2009) Functional perfusion MRI predicts later occurrence of steroid-associated osteonecrosis: an experimental study in rabbits. J Orthop Res 27:742–747
11. Mahanti HS, Barnes RM (1983) Determination of major, minor and trace elements in bone by inductively-coupled plasma emission spectrometry. Analytica Chimica Acta 151:409–417
12. Dong F, Ren J (2003) Insulin-like growth factors (IGFs) and IGF-binding proteins in nephrotic syndrome children on glucocorticoid. Pharmacol Res 48:319–323
13. Wang J, Zhou J, Bondy CA (1999) Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. FASEB J 13:1985–1990
14. Siebler T, Robson H, Shalet SM, Williams GR (2001) Glucocorticoids, thyroid hormone and growth hormone interactions: implications for the growth plate. Horm Res 56:7–12
15. Erben Reinhold G (1996) Trabecular and endocortical bone surfaces in the rat: modeling or remodeling? Anat Rec 246:39–46
16. Takahashi M, Ushijima K, Hayashi Y, Maekawa T, Ando H, Tsuruoka S, Fujimura A (2010) Dosing-time dependent effect of dexamethasone on bone density in rats. Life Sci 86:24–29
17. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC (1998) Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Investig 102:274–282
18. Leonard MB, Feldman HI, Shults J, Zemel BS, Foster BJ, Stallings VA (2004) Long-term, high-dose glucocorticoids and bone mineral content in childhood glucocorticoidsensitive nephrotic syndrome. N Engl J Med 351:868–875
19. Wetzsteon RJ, Shults J, Zemel BS, Gupta PU, Herskovitz RM, Burnham JM, Howard KM, Leonard MB (2009) Divergent effects of glucocorticoids on cortical and trabecular compartment BMD in childhood nephrotic syndrome. J Bone Miner Res 24:503–513
20. Oakley RH, Cidlowski JA (2011) Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. J Biol Chem 286:3177–3184
21. Henneicke H, Herrmann M, Kalak R, Zemel BS, Foster BJ, Stallings VA (2011) Corticosterone selectively targets endocortical surfaces by an osteoblast-dependent mechanism. Bone 49:733–742
22. Gounarides JS, Korach-Andre M, Killary K, Argentieri G, Turner O, Laurent D (2008) Effect of dexamethasone on glucose tolerance and fat metabolism in a diet-induced obesity mouse model. Endocrinology 149:758–766
23. Watts NB (2002) Bone quality: getting closer to a definition. J Bone Miner Res 17:1148–1150
24. VanStaa TP, Laan RF, Barton IP, Cohen S, Reid DM, Cooper C (2003) Bone density threshold and other predictors of vertebral fracture in patients receiving oral glucocorticoid therapy. Arthritis Rheum 48:3224–3229
25. Pluijm SM, Koes B, de Laet C, Van Schoor NM, Kuchuk NO, Rivadeneira F, Mackenbach JP, Lips P, Pols HA, Steyerberg EW (2009) A simple risk score for the assessment of absolute fracture risk in general practice based on two longitudinal studies. J Bone Miner Res 24:768–774
26. Hernandez CJ (2008) How can bone turnover modify bone strength independent of bone mass? Bone 42:1014–1020
27. Lane NE, Yao W, Balooch M, Nalla RK, Balooch G, Habelitz S, Kinney JH, Bonewald LF (2006) Glucocorticoid-treated mice have localized changes in trabecular bone material properties and osteocyte lacunar size that are not observed in placebo-treated or estrogen-deficient mice. J Bone Miner Res 21:466–476
28. Buttgereit F, Zhou H, Kalak R, Gaber T, Spies CM, Huscher D, Straub RH, Modzelewski J, Dunstan CR, Seibel MJ (2009) Transgenic disruption of glucocorticoid signaling in mature osteoblasts and osteocytes attenuates K/BxN mouse serum induced arthritis in vivo. Arthritis Rheum 60:1998–2007