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ORIGINAL ARTICLE

Influence of glucocorticoid on bone in 3-, 6-, and 12-month-old rats as determined by bone mass and histomorphometry

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Abstract The influence of glucocorticoid (GC) on bone in rats at different ages was investigated in order to provide insight into human glucocorticoid induced osteoporosis (GCOP). Three-, 6-, and 12-month-old female Wistar rats were divided into four groups: Zero-time control (ZT), vehicle (Cont), prednisolone (PSL) 2 mg/kg (P–L), PSL 20 mg/kg (P–H). PSL was subcutaneously administered every day for 4 weeks. Bone mineral density (BMD) at the proximal metaphysis and diaphysis of the tibia was measured by peripheral quantitative computed tomography. Histomorphometry of the tibia was performed for 3- and 6-month-old rats. GC increased trabecular and cortical BMD at the metaphysis in all 3-month-old rats with time. Trabecular BMD at the metaphysis in the P–L and P–H groups was significantly higher than in the control group. Histomorphometric parameters for both bone formation and resorption were also increased by GC treatment. In the 6-month-old rats, the metaphyseal trabecular BMD did not significantly change in any group, but the diaphyseal trabecular BMD significantly increased in the control group with time. The trabecular BMD of the metaphysis and diaphysis was significantly lower in the P–L and P–H groups than in the control group at week 4. Histomorphometric parameters for bone formation and resorption were both reduced by GC treatment. The BMD remained unchanged in all 12-month-old rats. Six-month-old rats treated with 20 mg/kg GC are suitable models for GC-induced osteoporosis with dominant cancellous bone decrease and reduced bone turnover. The pathology induced by 20 mg/kg prednisolone in 6-month-old female rats seems to be most similar to glucocorticoid-induced osteoporosis in humans.

Keywords Glucocorticoid · Osteoporosis · Rat · Histomorphometry

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Introduction

Glucocorticoid is widely used for the treatment of anaphylactic shock and collagen disease as well as renal, respiratory, and neural diseases due to its potent anti-inflammatory and immunosuppressive actions. However, it also exhibits various adverse effects such as induction of diabetes, peptic ulcers, and osteoporosis, as well as increased susceptibility to infection. Glucocorticoid-induced osteoporosis is particularly serious because it increases the frequency of fractures and seriously impairs patients' activities of daily living. The osteoporotic effect is thought to occur through simultaneous inhibition of bone formation and enhancement of bone resorption, with a resulting net reduction of bone mass [1]. However, contradictory findings have been reported after experimental glucocorticoid administration to rats; bone mineral density (BMD) was increased in some reports [2] but decreased in others [3]. Similarly, an increase [4] and decrease [5, 6] in bone volume on histological investigation have been reported. These contradictory findings may have resulted from variations in background factors such as the age of the animals and the dose of glucocorticoid. The age of rats

used in previous reports ranged from 3 weeks [2] to 7 months [7], the glucocorticoid dosing frequency varied from once a week [7] to daily [2], and the duration of administration varied from 1 week [2] to 20 weeks [8]. Therefore, no reproducible experimental model has been established that is applicable to the analysis of glucocorticoid-induced osteoporosis and drug efficacy.

In this study, to prepare an experimental model of glucocorticoid-induced osteoporosis, we investigated the influence of glucocorticoid on bone in rats at different ages using bone mass measurement, bone histomorphometry, and bone turnover markers.

Materials and methods

Animals

Three-, 6-, and 12-month-old female Wistar rats (Shimizu Laboratory Supply Co., Kyoto, Japan) were used. This experiment was carried out in accordance with the Guidelines for Animal Experimentation of the Faculty of Medicine, Tottori University. The animals were given chow (CE-2, Clea Japan, Inc., Tokyo; 1.18 g/100 g Ca, 250 IU/100 g vitamin D3) and tap water ad libitum, and maintained at 24°C under a 12 h light cycle (7:00 a.m. to 7:00 p.m.).

After 7 days of acclimation, rats were divided into the following four groups by mean body weight standardization: (1) killed at the start as a zero-time control (ZT, $n = 8$); (2) vehicle administration control (Cont, $n = 10$); (3) low dose administration group (P–L, $n = 10$); and (4) high dose administration group (P–H, $n = 10$).

Glucocorticoid administration

Prednisolone sodium succinate (PSL) (Shionogi, Osaka, Japan) was subcutaneously injected into the back at each dose every day for 4 weeks. Rats received 2 mg/kg of PSL with saline in the P–L group, 20 mg/kg of PSL with saline in the P–H group, or the vehicle alone (Cont) every day.

Measurement of bone mineral density (BMD)

Proximal tibia

Volumetric bone mineral density (vBMD; expressed in milligram per cubic centimeter) was measured by peripheral quantitative computed tomography (pQCT: model XCT-960 scanner; Norland-Stratec, Pforzheim, Germany) of the proximal metaphysis (2 mm distal to the growth cartilage) and diaphysis (5 mm distal to the growth cartilage) of the left tibia every 2 weeks for 4 weeks. Under intraperitoneal anesthesia with ketamine HCl (Ketalar; Sankyo, Tokyo, Japan) plus xylazine (Celactal; Bayer, Leverkusen, Germany) (2:1) at 1 mg/kg, the scan beam was attached to the proximal metaphysis of the left tibia perpendicularly to the bone axis, and measurement was performed with a voxel size of 0.295 mm and a slice thickness of 1 mm after identifying the metaphysis and diaphysis by scout scanning. Measurement parameters were determined as follows: contmode 2 (for bone contour), peelmode 20 (for trabecular region), contmode 1 (for cortical region), and threshold 0.63 for the vBMD of cancellous bone and 0.93 for the vBMD of cortical bone [9–14]. CVs for this measurement with repositioning were 2.8% for cancellous bone and 3.3% for cortical bone [10, 12].

Whole-body mineral content

Whole-body bone mineral content (W-BMC; g) was measured using dual-energy X-ray absorptiometry (model QDR 4500A dual-energy X-ray absorptiometers; Hologic, Waltham, MA, USA) at the start of the experiment and just before sacrificing under intraperitoneal anesthesia with ketamine HCl plus xylazine (2:1) at 1 mg/kg. The rats were placed on their stomach with the shoulder, elbow, hip, knee, and ankle joints maintained at a 90° flexion with tape. The measurements were performed in the small animal mode by adjusting the line spacing to 1.5 mm, point resolution to 0.64 mm, and scan speed to 2.5 mm/s (software: Rat Whole-Body ver. 8.26a) [15].

Bone histomorphometry

Bone histomorphometry was performed in 3- and 6-month-old rats. Bone labeling by intraperitoneal injection of calcein at 10 mg/kg was performed twice: at 6 days and 1 day before sacrificing (schedule of 1-4-1-1). Four weeks after the initial sensitization, rats were anesthetized by intraperitoneal injection of ketamine HCl plus xylazine (2:1) at 1 mg/kg, blood was collected by cardiac puncture, and the animals were sacrificed. The left femur and left tibia were excised for analysis and fixed in 70% alcohol.

The tibia was embedded in methylmethacrylate resin without decalcification and sectioned in the frontal plane at a thickness of 5 μm with a Jung Model K microtome (Reichert-Jung, Nussloch, Germany).

For histomorphometric analysis, the following items were measured in the secondary spongiosa extending 1.3–3.9 mm distally from the proximal growth cartilage of the tibia: bone volume/tissue volume (BV/TV) (expressed as a percentage) [16], osteoblast surface/bone surface (Ob.S/BS) (expressed as a percentage), osteoid surface/bone

surface (OS/BS) (expressed as a percentage), trabecular number (Tb·N) (N/mm), trabecular separation (Tb.Sp) (mcm), eroded surface/bone surface (ES/BS) (expressed as a percentage), osteoclast number/bone surface (N.Oc/BS) (per millimeter), mineralizing surface/bone surface (MS/BS) (expressed as a percentage), single-label surface/bone surface (sLS/BS) (expressed as a percentage), double-label surface/bone surface (dLS/BS) (expressed as a percentage), mineral apposition rate (MAR) (micrometer/day), and bone formation rate/bone surface (BFR/BS) (square millimeter per square millimeter per year).

Bone histomorphometric parameters were measured using a semiautomatic digitizer connected to a personal computer (System Supply, Nagano, Japan).

Bone length and diameter of the left femur

The bone length of the left femur of rats was measured using a digimatic caliper (Mitsutoyo Co., Kawasaki, Japan) after sacrificing. Femoral length (from the femoral head to condyle) and the central transverse diameter at the middle of the femur were measured.

Biochemical markers

Bone markers were measured in 6-month-old rats. Serum and urine were isolated by centrifugation and stored at −70°C. Serum calcium (Ca, mg/dl), inorganic phosphate (phosphate, mg/dl), and alkaline phosphatase (ALP, IU/l) were measured by the *O*-Cresolphthalein complexone (OCPC) method, molybdic acid extraction procedure, and the *p*-nitrophenyl phosphate (PNP) rate assay, respectively. Osteocalcin (OC, ng/ml) was measured using a specific radioimmunoassay based on rat osteocalcin (Biomedical Technologies, Inc., Stoughton, MA, USA). Growth hormone (GH, ng/ml) and parathyroid hormone (PTH: intact PTH (1–84) and N-terminal PTH, pg/ml) were measured using the solid-phase method of the immunoradiometric assay. Urine type I collagen *N*-telopeptide (NTX, nM BCE/mM creatinine) was measured with ELISA (Ostex International, Seattle, WA, USA).

Statistical analysis

Fisher's protected least significant difference procedure was performed after a repeated measures analysis of variance (ANOVA) for comparison of body weight, bone histomorphometry, histological evaluation, vBMD, w-BMC, bone marker, and bone length among groups. Diachronic change was analyzed by comparing middle and final values with initial values using paired *t*-tests. During the experiment period, one rat in the Cont group, two rats in the P–L group, and one rat in the P–H group died. These rats died after anesthesia for BMD measurement and were excluded from analysis.

Statistical analysis was performed using Stat View software (Version 5.0; SAS Institute Inc., Cary, NC, USA) and $P < 0.05$ was considered significant.

Results

Body weight

Body weight increased with time in all 3-month-old rat groups ($P < 0.01$) (Table 1). Body weight at week 4 was significantly lower in the P–H group than in the Cont group ($P < 0.05$).

In 6-month-old rats, body weight was significantly increased at week 4 when compared to week 0 in the Cont group ($P < 0.01$). In the P–H group, body weight was significantly decreased at week 2 when compared to week 0 ($P < 0.01$). No significant difference was found among treatment groups at any time point.

In 12-month-old rats, the body weight at week 2 was significantly lower than at week 0 ($P < 0.01$) in the Cont group, but no significant difference was found at week 4. In the P–L and P–H groups, body weight was significantly decreased at weeks 2 and 4 when compared to week 0 ($P < 0.01$). Comparison among the groups revealed that body weight at week 2 was significantly lower in the P–H group than in the Cont group, and body weight at week 4

Table 1 Body weight

	Cont	P–L	P–H
3-month-old			
0 week	221.9 ± 5.6	218.4 ± 8.4	214.7 ± 7.0
2 weeks	239.7 ± 7.2*	239.4 ± 10.7*	231.3 ± 12.9*
4 weeks	242.9 ± 7.4*[a]	241.9 ± 11.3*	226.3 ± 11.6*[a]
6-month-old			
0 week	264.2 ± 8.7	268.4 ± 13.4	271.2 ± 14.6
2 weeks	266.8 ± 9.7	264.0 ± 16.9	264.4 ± 17.1*
4 weeks	273.3 ± 7.8*	265.7 ± 14.7	265.3 ± 21.1
12-month-old			
0 week	328.4 ± 24.0	317.0 ± 19.6	305.3 ± 22.2
2 weeks	321.1 ± 26.3*[b]	301.2 ± 21.2*	292.7 ± 15.6*[b]
4 weeks	320.6 ± 28.1[cd]	294.1 ± 21.1*[c]	281.6 ± 19.6*[d]

Values represent the mean ± SD (g)

[a,b] $P < 0.05$

[a] $P < 0.05$ (Cont vs. P–H)

[b] $P < 0.05$ (Cont vs. P–H)

[c,d] $P < 0.05$ (Cont vs. P–L, P–H)

* Versus 0 weeks $P < 0.05$

was significantly lower in the P–L and P–H groups than in the Cont group ($P < 0.05$).

Bone mineral measurement

Proximal tibia

Three-month-old rats Metaphyseal trabecular vBMD significantly increased with time in all groups ($P < 0.01$) and was significantly higher in both glucocorticoid treatment groups than in the Cont group at weeks 2 and 4 ($P < 0.01$ vs. P–H group at week 2, $P < 0.05$ vs. P–L group at week 2 and P–H group at week 4) (Fig. 1). Cortical vBMD significantly increased with time in all groups ($P < 0.01$) but showed no significant difference across groups.

Diaphyseal trabecular and cortical BMDs significantly increased with time in all groups ($P < 0.01$) but showed no significant difference across groups.

Six-month-old rats Metaphyseal trabecular vBMD did not significantly change in any group (Fig. 2). Trabecular vBMD at week 4 was significantly lower in both glucocortioid treatment groups than in the Cont group ($P < 0.05$). No significant change over time or difference across groups was noted in the cortical vBMD.

Diaphyseal trabecular vBMD at week 4 was significantly higher than at week 0 in the Cont group ($P < 0.01$). In both glucocorticoid treatment groups, no time-course changes were observed for the trabecular vBMD. Comparison of the groups revealed that trabecular vBMD was significantly lower in both glucocorticoid treatment groups than in the Cont group at week 4 ($P < 0.05$). No significant change over time or difference across groups was noted in cortical vBMD.

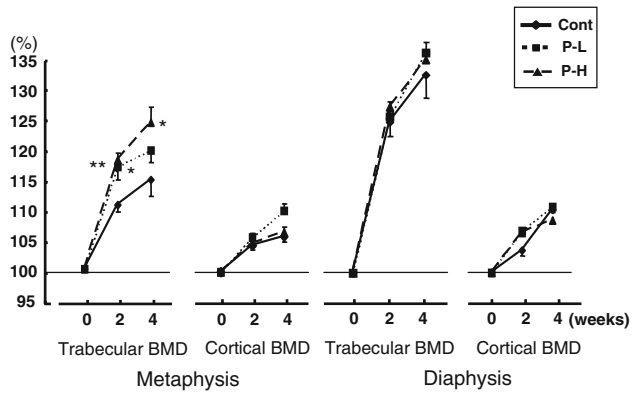

Fig. 1 Bone mineral density (BMD) changes in 3-month-old rats at 4 weeks. At up to 2 weeks of prednisolone treatment, there was a significant increase in the trabecular BMD of the metaphysis and diaphysis in all groups. Data represent the mean ± SEM. $*P < 0.05$; $**P < 0.01$ (vs. Cont)

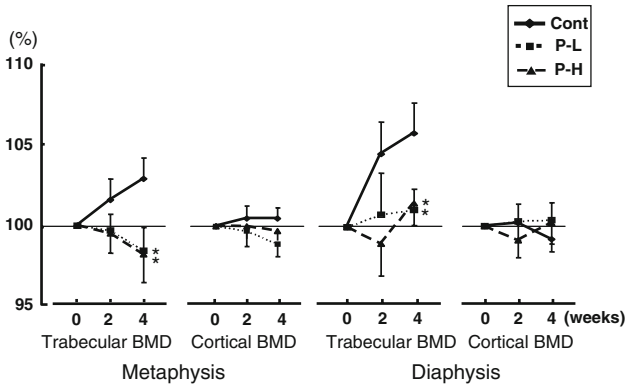

Fig. 2 Bone mineral density changes in 6-month-old rats at 4 weeks. After 4 weeks of prednisolone treatment, there was a significant decrease in the trabecular BMD of the metaphysis and diaphysis. Data represent the mean ± SEM. $*P < 0.05$ (vs. Cont)

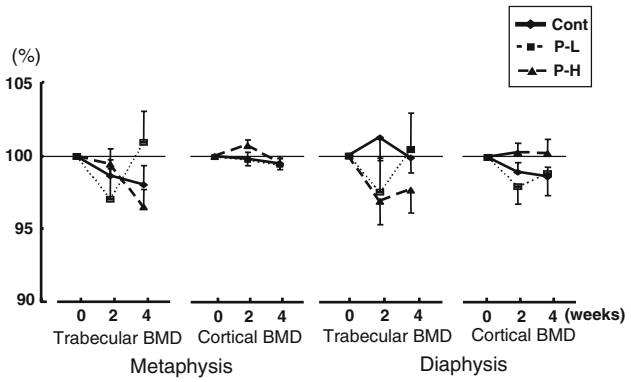

Fig. 3 Bone mineral density changes in 12-month-old rats at 4 weeks. There were no significant differences between groups. Data represent the mean ± SEM

Twelve-month-old rats No significant changes over time or differences across groups were found for the trabecular or cortical vBMD in the metaphysis or diaphysis (Fig. 3).

Whole-body BMD

In 3-month-old rats, the whole-body BMD was 6.9 ± 0.2, 6.8 ± 0.3, and 6.6 ± 0.2 g at week 0 in the Cont, P–L, and P–H groups, respectively, and 8.0 ± 0.3, 8.0 ± 0.3, and 7.6 ± 0.3 g at week 4, respectively. The rates of change were 116.7 ± 1.8, 118.9 ± 1.8, and 116.4 ± 1.5%, respectively, showing no significant difference among groups. The whole-body BMD significantly increased in all groups over time ($P < 0.01$).

In 6-month-old rats, the whole-body BMD was 9.1 ± 0.3, 8.8 ± 0.5, and 9.1 ± 0.3 g at week 0 in the Cont, P–L, and P–H groups, respectively, and 9.4 ± 0.5, 9.1 ± 0.5, and 9.2 ± 0.4 g at week 4, respectively. The rates of change were 102.3 ± 1.6, 102.9 ± 0.7, and 101.9 ± 0.8%, respectively, showing no significant difference among

groups. The whole-body BMD only significantly increased in the P–L group over time ($P < 0.05$).

In 12-month-old rats, the whole-body BMD was 10.7 ± 0.8, 10.4 ± 0.8, and 10.0 ± 0.6 g at week 0 in the Cont, P–L, and P–H groups, respectively, and 10.8 ± 0.5, 10.2 ± 0.6, and 10.0 ± 0.6 g at week 4, respectively. The rates of change were 101.4 ± 1.8, 98.6 ± 1.7, and 99.5 ± 1.8%, respectively, showing no significant difference among groups. No significant changes were found with respect to time.

Bone length and diameter

In 3-month-old rats, bone length was significantly longer in the Cont, P–L, and P–H groups than in the ZT group (Table 2) ($P < 0.05$ vs. Cont and P–L groups, $P < 0.01$ vs. P–H group). In 6-month-old rats, bone length was significantly longer in the Cont group than in the ZT group ($P < 0.05$), and significantly shorter in the P–H group than in the Cont group ($P < 0.05$). In 12-month-old rats, no significant difference was noted among the groups.

No significant differences were noted in the central vertical or central transverse diameter among the four groups at any age.

Table 2 Bone length and diameter

	ZT	Cont	P–L	P–H
3-month-old				
Length	34.1 ± 0.6[ade]	35.1 ± 0.3[e]	35.1 ± 0.4[d]	34.8 ± 0.9[a]
Transverse diameter	3.8 ± 0.1	3.8 ± 0.07	3.8 ± 0.1	3.8 ± 0.1
Vertical diameter	3.1 ± 0.07	3.0 ± 0.08	3.1 ± 0.1	3.1 ± 0.09
n	8	10	11	11
6-month-old				
Length	36.0 ± 0.5[a]	36.6 ± 0.8[ab]	36.4 ± 0.5	36.0 ± 0.6[b]
Transverse diameter	3.9 ± 0.09	4.0 ± 0.2	4.0 ± 0.2	4.0 ± 0.2
Vertical diameter	2.9 ± 0.2	3.0 ± 0.1	3.0 ± 0.2	3.0 ± 0.2
n	8	9	10	10
12-month-old				
Length	37.0 ± 0.6	37.2 ± 0.7	37.0 ± 0.7	36.2 ± 0.7
Transverse diameter	4.0 ± 0.07	4.1 ± 0.2	4.1 ± 0.2	4.1 ± 0.2
Vertical diameter	3.1 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.1 ± 0.1
n	8	10	10	11

Values represent the mean ± SD (mm)

[a,b] $P < 0.01$ (ZT vs. Cont, P–L)

[c] $P < 0.05$ (ZT vs. P–H)

[d,e] $P < 0.05$ (ZT vs. Cont, P–H)

Histomorphometry

Three-month-old rats

BV/TV was significantly higher in the P–H group than in the Cont and ZT groups (Table 3) ($P < 0.05$ vs. ZT group, $P < 0.01$ vs. Cont group). Tb·N was significantly lower in the Cont than in the ZT and P–H groups ($P < 0.05$ vs. ZT group, $P < 0.01$ vs. P–H group), and significantly higher in the P–H group than in the Cont and P–L groups ($P < 0.05$ vs. P–L group, $P < 0.01$ vs. Cont group). Tb.Sp was significantly lower in the P–H group than in the Cont group ($P < 0.01$). OS/BS was significantly lower in the ZT and P–H groups than in the Cont group ($P < 0.05$ vs. ZT group, $P < 0.01$ vs. P–H group), and significantly lower in the P–H group than in the P–L group ($P < 0.01$). Obs/BS was significantly lower in the P–H group than in the P–L and Cont groups ($P < 0.01$). ES/BS was significantly higher in the P–L group than in the Cont group ($P < 0.05$). No significant difference was found in N.Oc/BS across groups. dLS was found in six, seven, seven, and seven animals in the ZT, Cont, P–L, and P–H groups, respectively. No significant differences were found in MS/BS or BFR/BS across groups, but MAR was significantly higher in the P–H group than in the ZT and Cont groups ($P < 0.05$) (Fig. 4).

Six-month-old rats

No significant difference was found in BV/TV among the groups (Table 4) (Fig. 5a, b). OS/BS was significantly higher in the Cont and P–L groups than in the ZT group ($P < 0.05$ vs. P–L group, $P < 0.01$ vs. Cont group). ES/BS was significantly lower in the ZT and P–H groups than in the Cont group ($P < 0.05$ vs. ZT group, $P < 0.01$ vs. P–H group), and significantly lower in the P–H group than in the P–L group ($P < 0.05$). N.Oc/BS was significantly lower in the P–H group than in the Cont and P–L groups ($P < 0.05$). dLS was found in seven, seven, seven, and one animals in the ZT, Cont, P–L, and P–H groups, respectively (Fig. 6a, b). MS/BS was significantly lower in the P–H group than in the other groups ($P < 0.05$ vs. P–L group, $P < 0.01$ vs. ZT and Cont groups). MAR was significantly lower in the P–L group than in the Cont and ZT groups ($P < 0.05$ vs. ZT group, $P < 0.01$ vs. Cont group), and BFR/BS was significantly lower in the P–L group than in the ZT group ($P < 0.05$) (Fig. 7). dLS was found in only one animal in the P–H group, and no significant differences were found in MAR or BFR/BS.

Bone turnover markers

Calcium levels were significantly lower in the P–L group than in the Cont group ($P < 0.05$). ALP levels were

Table 3 Histomorphometric analysis of 3-month-old rats

Parameter	ZT	Cont	P–L	P–H
BV/TV (%)	24.9 ± 3.7[a]	22.7 ± 5.1[b]	28.0 ± 6.3	33.1 ± 7.7[ab]
Tb·N (N/mm)	2.5 ± 0.2[c]	1.9 ± 0.3[cd]	2.1 ± 0.3[e]	2.5 ± 0.2[de]
Tb.Sp (μm)	305.8 ± 38.9	415.3 ± 99.6[f]	356.4 ± 78.3	263.7 ± 56.0[f]
OS/BS (%)	15.1 ± 7.1[g]	19.5 ± 2.6[gh]	17.1 ± 5.6[i]	11.2 ± 3.0[hi]
Obs/BS (%)	15.2 ± 7.3	15.2 ± 2.6[j]	16.8 ± 5.9[k]	10.5 ± 2.7[jk]
ES/BS (%)	3.7 ± 1.1	4.6 ± 1.4[l]	8.1 ± 5.6[l]	7.5 ± 5.7
N.Oc/BS (N/mm)	0.28 ± 0.11	0.34 ± 0.12	0.38 ± 0.090	0.34 ± 0.15
sLS/BS (%)	13.5 ± 4.9[m]	5.5 ± 4.0[mn]	13.3 ± 5.3[n]	9.3 ± 4.5
dLS/BS (%)	3.2 ± 4.5	2.4 ± 2.8	3.6 ± 2.1	3.5 ± 4.5
MS/BS (%)	9.7 ± 6.3	5.2 ± 3.5	9.2 ± 3.6	7.0 ± 5.7
MAR (μm/day)	2.0 ± 0.5[o]	1.9 ± 0.4[p]	2.4 ± 0.5	3.2 ± 1.2[op]
BFR/BS (mm^2/mm^2/year)	0.11 ± 0.059	0.045 ± 0.030	0.092 ± 0.046	0.082 ± 0.065

Values represent the mean ± SD

[a] $P < 0.05$ (ZT vs. P–H)

[b] $P < 0.01$ (Cont vs. P–H)

[c] $P < 0.05$ (ZT vs. Cont)

[d] $P < 0.01$ (Cont vs. P–H)

[e] $P < 0.05$ (P–L vs. P–H)

[f] $P < 0.01$ (Cont vs. P–H)

[g] $P < 0.05$ (ZT vs. Cont)

[h] $P < 0.01$ (Cont vs. P–H)

[i] $P < 0.05$ (P–L vs. P–H)

[j,k] $P < 0.01$ (P–H vs. Cont, P–L)

[l] $P < 0.05$ (Cont vs. P–L)

[m,n] $P < 0.01$ (Cont vs. ZT, P–L)

[o,p] $P < 0.05$ (P–H vs. ZT, Cont) R

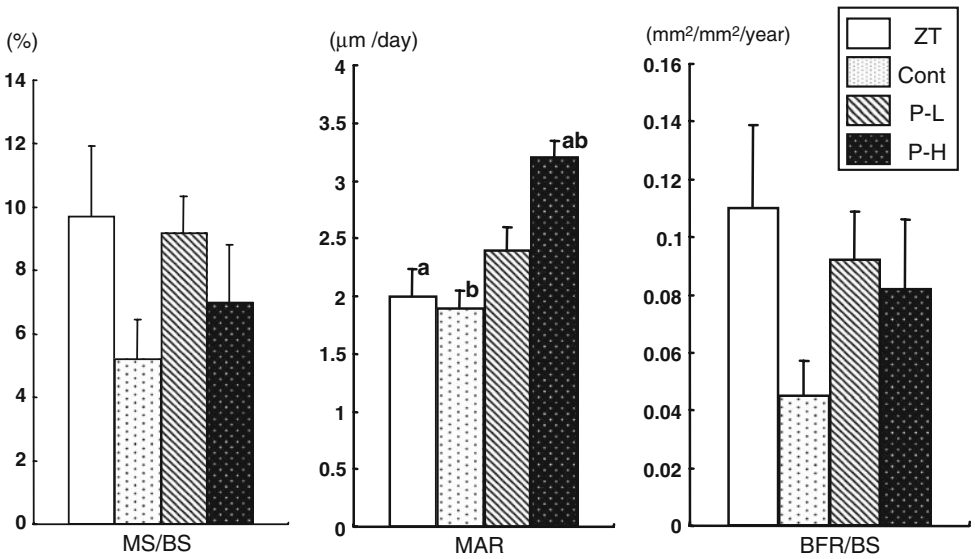

Fig. 4 Bone formation parameters determined by histomorphometry at the proximal tibial metaphysis in 3-month-old rats. Data represent the mean ± SEM. *a* $P < 0.05$ (vs. ZT), *b* $P < 0.05$ (vs. Cont) for MAR

significantly higher in the P–L and P–H groups than in the Cont group ($P < 0.05$). Osteocalcin levels were significantly lower in the P–H group than in the Cont group ($P < 0.05$) (Table 5). No significant differences were found in PTH, GH or urine NTX among the groups.

Table 4 Histomorphometric analysis of 6-month-old rats

Parameter	ZT	Cont	P–L	P–H
BV/TV (%)	27.7 ± 6.1	24.6 ± 3.3	26.6 ± 3.1	25.5 ± 3.0
Tb·N (N/mm)	2.1 ± 0.4	2.4 ± 0.5	2.5 ± 0.5	2.2 ± 0.3
Tb.Sp (μm)	364.7 ± 118.2	321.8 ± 66.8	307.9 ± 72.9	342.1 ± 56.7
OS/BS (%)	2.0 ± 2.3[ab]	5.3 ± 2.4[a]	4.7 ± 2.4[b]	3.4 ± 2.3
Obs/BS (%)	3.9 ± 3.5	5.8 ± 2.4	5.1 ± 2.7	3.8 ± 2.3
ES/BS (%)	8.1 ± 4.6[c]	14.5 ± 4.5[cd]	13.3 ± 4.9[e]	7.8 ± 3.5[de]
N.Oc/BS (N/mm)	0.19 ± 0.11	0.26 ± 0.12[f]	0.25 ± 0.072[g]	0.13 ± 0.083[fg]
sLS/BS (%)	6.2 ± 3.0	8.6 ± 5.5[h]	8.9 ± 3.6[i]	3.4 ± 1.9[hi]
dLS/BS (%)	7.7 ± 6.2[jkl]	3.7 ± 3.2[j]	3.6 ± 2.8[k]	0.068 ± 0.22[l]
MS/BS (%)	13.4 ± 13.4[m]	22.0 ± 17.6[n]	9.6 ± 4.7[o]	2.1 ± 1.1[mno]
MAR (μm/day)	2.3 ± 0.6[p]	2.8 ± 0.6[q]	1.6 ± 0.7[pq]	1.63[s]
BFR/BS (mm^2/mm^2/year)	0.11 ± 0.070[r]	0.10 ± 0.064	0.044 ± 0.023[r]	0.019[s]

Values represent the mean ± SD

[a] $P < 0.01$ (ZT vs. Cont)

[b] $P < 0.05$ (ZT vs. P–L)

[c] $P < 0.05$ (ZT vs. Cont)

[d] $P < 0.05$ (Cont vs. P–H)

[e] $P < 0.05$ (P–L vs. P–H)

[f,g] $P < 0.05$ (P–H vs. Cont, P–L)

[h,i] $P < 0.01$ (P–H vs. Cont, P–L)

[j,k] $P < 0.05$ (ZT vs. Cont, P–L)

[l] $P < 0.05$ (ZT vs. P–H)

[m,n] $P < 0.01$ (P–H vs. ZT, Cont)

[o] $P < 0.05$ (P–L vs. P–H)

[p] $P < 0.05$ (ZT vs. P–L)

[q] $P < 0.01$ (Cont vs. P–L)

[r] $P < 0.05$ (ZT vs. P–L)

[s] Only one rat showed double labeled surface

Discussion

It is widely known that glucocorticoid treatment reduces bone mass and induces osteoporosis. In adults, oral prednisolone administration for 6 months reduced the lumbar bone mass by 5–15% [19] and subsequently increased the risk of fractures [1]. Various direct developmental mechanisms have been proposed including reduction of osteoblast function, enhancement of osteoclast function, and reduction of intestinal calcium absorption and calcium reabsorption from the convoluted tubule. Indirect action through hypogonadism has also been proposed. However, the actual mechanism has not been definitively established even though the pathophysiology has been investigated at the cellular, tissue, and organ levels.

Investigations at the cellular level reported that glucocorticoid promoted osteoblast apoptosis [18] and differentiation from precursor cells [19] as well as stimulating osteoclast-like cell formation [20], suggesting that glucocorticoid exhibits both catabolic and anabolic effects on bone, and that its actions vary depending on the individual condition, dose, and dosing frequency.

Tissue-level investigations reported that methylprednisolone reduced the bone volume by promoting bone resorption and inhibiting bone formation in 11-week-old rats [21], and that prednisolone inhibited both bone formation and resorption with subsequent decreases in bone volume in 8-week-old rats [22], whereas 12-week administration of prednisolone did not significantly affect lumbar vertebral trabecular bone formation, resorption, or volume in 28-week-old rats [23], showing that the findings were not consistent.

Investigations at the organ level using the DXA method reported that methylprednisolone increased the tibial metaphyseal BMD in 5-week-old rats [24] and that prednisolone decreased tibial BMD in 8-week-old rats [3] and the lumbar vertebral BMD in 32-week-old rats [25], showing that glucocorticoid-induced changes in BMD were

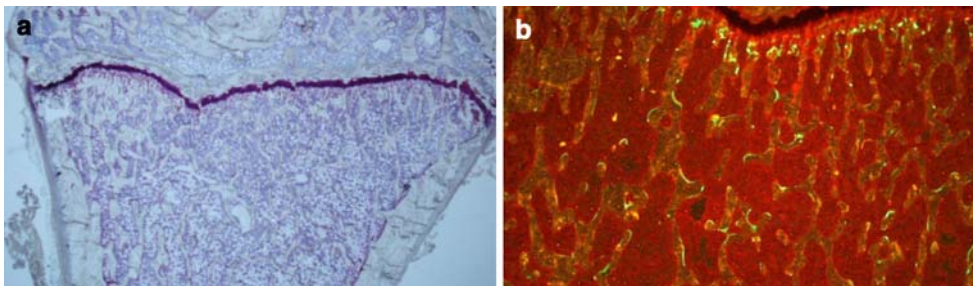

Fig. 5 **a** Photomicrograph of proximal metaphysis of the tibia from Cont of 6-month-old. (Villanueva bone stain ×40). **b** Fluorescent photomicrographs of the proximal metaphysis of the tibia from Cont of 6-month-old (Villanueva bone stain ×80)

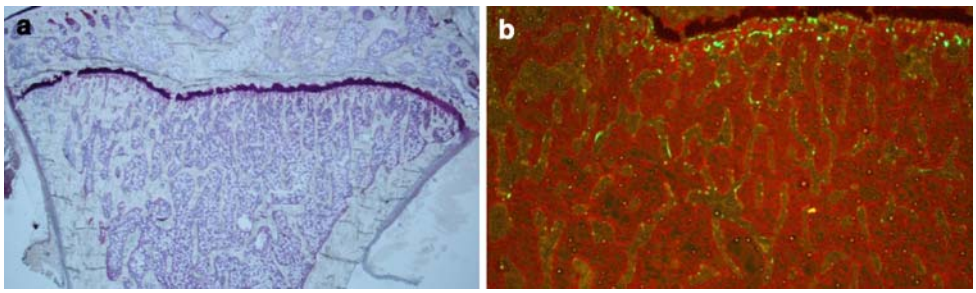

Fig. 6 **a** Photomicrograph of proximal metaphysis of the tibia from P–H of 6-month-old. (Villanueva bone stain ×40). **b** Fluorescent photomicrographs of the proximal metaphysis of the tibia from P–H group of 6-month-old. (Villanueva bone stain ×80) The labeled surface was decreased compared with Cont

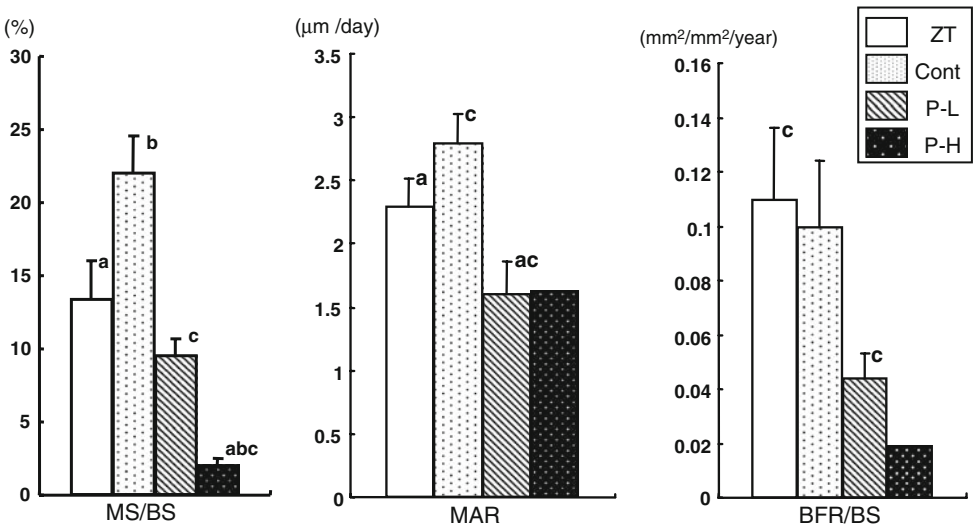

Fig. 7 Bone formation parameters determined by histomorphometry at the proximal tibial metaphysis in 6-month-old rats. Data represent the mean ± SEM. *a*, *b* $P < 0.01$ (vs. ZT, Cont), *c* $P < 0.05$ (vs. P–L) for MS/BS. *a* $P < 0.01$ (vs. Cont), *c* $P < 0.05$ (vs. ZT) for MAR *c* $P < 0.05$ (vs. ZT) for BFR/BS

not consistent due to variations in the age and measurement site of experimental animals. Separate measurement of cortical and trabecular BMD using pQCT has recently been reported. Ferretti et al. administered dexamethasone at a high dose to 30-day-old rats and found that femoral cortical BMD was decreased [26]. Nansei et al. [2] reported that 20 mg/kg methylprednisolone increased the tibial cortical BMD in 7-day-old rats. These studies were performed in rats in the developmental period, but there has been no report of pQCT measurements in mature rats.

The existing literature shows that there is no consistent understanding of the influence of glucocorticoid. Thus, we measured whole-body bone mass by the DXA method and tibial trabecular and cortical bone masses by pQCT at the organ level, performed morphometry of the tibia at the tissue level, and measured bone turnover markers in 3-, 6-, and 12-month-old female rats of the specified strain, for comprehensive analysis. It has been reported that the length, weight, and bone mineral density of the femur increase at a fast rate from 1–3 months of age, after which the rate of increase becomes gradual. Changes in femoral bone mineral density are minimal after 6 months and by 12 months, all bone measured parameters had plateaued. Based on these findings we chose 3-, 6-, and 12-month-old female rats for this experiment [27]. In 3-month-old rats, trabecular BMD was increased at the organ level and both bone formation and bone resorption were enhanced at the tissue level. In 6-month-old rats, trabecular BMD was

Table 5 Serum and urine biochemical markers in 6-month-old rats

Parameter	Cont	P–L	P–H
Calcium (mg/dl)	10.2 ± 0.8[a]	9.5 ± 0.3[a]	10.2 ± 1.1
Phosphate (mg/dl)	8.5 ± 1.7	7.5 ± 1.1	6.3 ± 1.2
Alkaline phosphatase (IU/l)	302.8 ± 102.8[bc]	349.3 ± 63.1[b]	358.8 ± 77.6[c]
Osteocalcin (ng/ml)	12.4 ± 3.2[d]	11.4 ± 1.8	7.85 ± 2.1[d]
Parathyroid hormone (pg/ml)	106.4 ± 56.4	104.2 ± 53.0	72.0 ± 51.1
Growth hormone (ng/ml)	18.4 ± 28.2	7.4 ± 1.4	13.9 ± 12.0
Urine NTX (nM BCE/mM creatinine)	50.2 ± 26.2	50.9 ± 35.8	42.2 ± 22.1

Values represent the mean ± SD

[a] $P < 0.05$ (Cont vs. P–L) for Calcium

[b,c] $P < 0.05$ (P–H vs. Cont, P–L)

[d] $P < 0.05$ (Cont vs. P–H)

decreased at the organ level, bone formation and resorption were inhibited at the tissue level, and bone formation was also inhibited with respect to expression of bone turnover markers. In 12-month-old rats, no change was found in the BMD.

It has been reported that in human glucocorticoid-induced osteoporosis, the inhibition of bone formation and promotion of bone resorption mainly reduced trabecular bone mass, but no changes were observed in the blood Ca level or in ALP expression, whereas osteocalcin expression was decreased, and NTX was increased [28]. This was similar to the findings in 6-month-old rats in our study, although in our study bone formation and resorption were inhibited in rats. However, the inhibition of bone resorption in human glucocorticoid-induced osteoporosis has recently been reported [29]. Reduction of cortical bone mass by glucocorticoid has also been reported in humans [30], but we did not observe such changes in 6-month-old rats.

Several drawbacks may have limited this study. First, remodeling of cortical bone does not occur in rats because of the absence of the Haversian canal in rat cortical bone [27], suggesting that the influence of glucocorticoid is different from that in humans. However, trabecular bone is remodeled in both rats and humans. Thus, we focused on trabecular bone for BMD measurement and histomorphometry. Second, the growth cartilage does not close in rats, and growth continues. To exclude growth-associated changes in bone mass, the sites of bone mass measurement and histomorphometry were set 1.3–3.9 mm diaphyseal to the growth cartilage, where the influence of growth is absent. However, we did not performed pair-feeding and it is not clear whether changing in body weight influenced bone mass or not. Third, the duration of glucocorticoid administration was limited to 4 weeks. Since glucocorticoid is administered over a long period in clinical cases, investigations at the tissue and organ levels after experimental administration for long time may be necessary. Fourth, further experiments at the cell and gene levels are needed to elucidate the molecular mechanism of glucocorticoid osteoporosis. Finally, mechanical investigation was not performed but is important for understanding the high prevalence of fractures in glucocorticoid-induced osteoporosis.

In conclusion, glucocorticoid increased the metaphyseal trabecular bone mass at the organ level, and enhanced both bone formation and resorption at the tissue level in 3-month-old rats. In 6-month-old rats, the trabecular bone mass was decreased in the metaphysis and diaphysis at the organ level, and both bone formation and resorption were inhibited at the tissue level. In 12-month-old rats, no changes were noted at the organ level. The pathology induced by administration of 20 mg/kg prednisolone in 6-month-old female rats seems to be most similar to glucocorticoid-induced osteoporosis in humans.

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References

1. Reid IR. Glucocorticoid osteoporosis-mechanisms and management. Eur J Endocrinol. 1997;137:209–17.
2. McHugh NA, Vercesi HM, Egan RW, Hey JA. In vivo rat assay: bone remodeling and steroid effects on juvenile bone by pQCT quantification in 7 days. Am J Physiol Endocrinol Metab. 2003;284:E70–E5.
3. Lindgren JU, Merchant CR, De Luca HF. Effect of 1, 25-dihydroxyvitamin D3 on osteopenia induced by prednisolone in adult rats. Calcify Tissue Int. 1982;34:253–7.
4. King CS, Weir EC, Gundberg CW, Fox J, Insogna KL. Effects of continuous glucocorticoid infusion on bone metabolism in the rat. Calcif Tissue Int. 1996;59:184–91.

5. Unoki E. Effect of human PTH on steroid-induced osteopenia: a histomorphometric study of decalcified and undercalcified trabecular bone sections in rat. Nippon Seikeigeka Gakkai Zasshi (J Jpn Orthop Assoc). 1995;69:1064–75 (in Japanese).
6. Hulley PA, Conradie MM, Langeveldt CR, Hough FS. Glucocorticoid-induced osteoporosis in the rat is prevented by the tyroisin phosphatase inhibitor, sodium orthovanadate. Bone. 2002;31(1):220–9.
7. Wimalawansa SJ, Chapa MT, Yallampalli C, Zhang R, Simmons DJ. Prevention of corticosteroid-induced bone loss with nitric oxide donor nitroglycerin in male rats. Bone. 1997;21:275–80.
8. Uzawa T, Hori M, Noda T, Takahashi H, Inoue J. Effect of prednisolone on bone metabolism in rats. In: Watanabe M, Yanagisawa M (eds) Kotsukeitaikeisoku (Quantitative analysis method of bone). Niigata, Nishimurashoten; 1986. p. 242–9 (in Japanese).
9. Yamane I, Hagino H, Okano T, Enokida M, Yamasaki D, Teshima R. Effect of minodronic acid (ONO-5920) on bone mineral density and arthritis in adult rats with collagen-induced arthritis. Arthritis Rheum. 2003;48:1732–41.
10. Gasser JA. Assessing bone quantity by pQCT. Bone. 1995;17:145S–54S.
11. Sato M. Comparative X-ray densitometry of bones from ovariectomized rats. Bone. 1995;17:157S–62S.
12. Fukata S, Hagino H, Okano T, Yamane I, Kameyama Y, Teshima R. Effect of intermittent administration of human parathyroid hormone on bone mineral density and arthritis in rats with collagen-induced arthritis. Arthritis Rheum. 2004;50:4060–9.
13. Enokida M, Yamasaki D, Okano T, Hagino H, Morio Y, Teshima R. Bone mass changes of tibial and vertebral bones in young and adult rats with collagen- induced arthritis. Bone. 2001;28:87–93.
14. Yamasaki D, Enokida M, Okano T, Hagino H, Teshima R. Effects of ovariectomy and estrogen replacement therapy on arthritis and bone mineral density in rats with collagen-induced arthritis. Bone. 2001;28:634–40.
15. Hagino H, Kuraoka M, Kameyama Y, Okano T, Teshima R. Effect of a selective agonist for prostaglandin E receptor subtype EP4 (ONO-4819) on the cortical bone response to mechanical loading. Bone. 2005;36:444–53.
16. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. J Bone Miner Res. 1987;2:595–610.
17. Lukert BP, Raisz LG. Glucocorticoid-induced osteoporosis; pathogenesis and management. Ann Intern Med. 1990;112:352–64.
18. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest. 1998;102:274–82.
19. Tenenbaum HC, Heersche JN. Dexamethasone stimulates osteogenesis in chick periosteum in vitro. Endocrinology. 1985;117:2211–7.
20. Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Chihara K. Dexamethasone stimulates osteoclast-like cell formation by directly acting on Hemo poietic blast cells and enhances osteoclast-like cell formation stimulated by parathyroid hormone and prostaglandin E2. J Bone Miner Res. 1997;12:734–41.
21. Nakamuta H, Nitta T, Hoshino T, Koida M. Glucocorticoid-induced osteopenia in rats: histomorphometrical and microarchitectural characterization and calcitonin effect. Biol Pharm Bull. 1996;19:217–9.
22. Lindgren JU, Merchant CR, DeLuca HF. Effect of 1, 25-dihydroxyvitamin D3 on osteopenia induced by prednisolone in adult rats. Calcif Tissue Int. 1982;34:253–7.
23. Narita Y. Effect of intermittent administration of human PTH on experimental osteopenia in adult rat: a histomorphometric study of both trabecular and cortical bone of the vertebrae. Nippon Seikeigeka Gakkai Zasshi (J Jpn Orthop Assoc). 1995;69:1027–36. (in Japanese).
24. Wang Y, Ohtsuka M, Shao P, Sakamoto S, Shinoda H. Effects of methylprednisolone on bone formation and resorption in rats. Jpn J Pharmacol. 2002;90:236–46.
25. Wimalawansa SJ, Simmons DJ. Prevention of corticosteroid-induced bone loss with alendronate. Proc Soc Exp Biol Med. 1998;217:162–7.
26. Ferretti JL, Gaffuri O, Capozza R, Cointry G, Bozzini CE, Olovera M, et al. Dexamethasone effects on mechanical, geometric and densitometric properties of rat femur diaphyses as described by peripheral quantitative computerized tomography and bending tests. Bone. 1995;16:119–24.
27. Dike N Kalu. The ovariectomized rat model of postmenopausal bone loss. Bone and Mineral 1991; 15:175–92.
28. Godschalk MF, Downs RW. Effect of short-term glucocorticoids on serum osteocalcin in healthy young men. J Bone Miner Res. 1988;3:113–5.
29. Ton FN, Gunawardene SC, Lee H, Neer RM. Effects of low-dose prednisolone on bone metabolism. J Bone Miner Res. 2005;20:464–70.
30. Canalis E. Mechanisms of glucocorticoid action in bone: implications to glucocorticoid-induced osteoporosis. J Clin Endocrinol Metab. 1996;81:3441–7.