Chinese Journal of Physiology 57(5): 231-237, 2014
DOI: 10.4077/CJP.2014.BAC242

Effect of Intermittent Administration of hPTH(1-34) on Cortical Bone Geometry in Rats Treated with High-Dose Glucocorticoids

Jun Iwamoto[1], Azusa Seki[2], and Yoshihiro Sato[3]

[1]*Institute for Integrated Sports Medicine, Keio University School of Medicine, Tokyo, Japan*
[2]*Hamri Co., Ltd., Ibaraki, Japan*
and
[3]*Department of Neurology, Mitate Hospital, Fukuoka, Japan*

Abstract

High-dose glucocorticoids reduce cortical bone gain in rats. The aim of the present study was to examine the effect of the intermittent administration of human parathyroid hormone (1-34) (hPTH[1-34]) on cortical bone in rats treated with high-dose prednisolone (PSL). Twenty-five female Sprague-Dawley rats (6 weeks old) were randomized into the following three groups: a vehicle administration (control) group, a PSL (10 mg/kg s.c., 5 times a week) administration group, and a PSL + hPTH(1-34) (30 μg/kg s.c., 3 times a week) administration group. After 8 weeks of treatment, the bone mineral density (BMD) of the femoral diaphysis was determined using peripheral quantitative computed tomography, and a static bone histomorphometric analysis was performed on the tibial diaphysis. PSL administration induced a decrease in the BMD of the femoral diaphysis, compared with the control group, as well as decreases in the total tissue area, cortical area, percent cortical area, and periosteal perimeter and increases in the marrow area, percent marrow area, and endocortical perimeter of the tibial diaphysis, compared with the control group. The intermittent administration of hPTH(1-34) to PSL-treated rats attenuated PSL-related changes in the BMD of the femoral diaphysis and the percent cortical area, marrow area, percent marrow area, and endocortical perimeter of the tibial diaphysis. The findings of the present study suggest that the intermittent administration of hPTH(1-34) improves cortical BMD, acts on the endocortical bone surface, and improves cortical bone geometry, in rats treated with high-dose PSL.

Key Words: bone histomorphometry, cortical bone, glucocorticoid, hPTH(1-34)

Introduction

Glucocorticoids are potent anti-inflammatory agents that are frequently used for the treatment of inflammatory diseases such as arthritis, pulmonary diseases, and skin diseases. High-dose or long-term treatment with glucocorticoids is known to have several adverse side effects including osteoporosis (14). Epidemiological studies have revealed a high incidence of fractures at the spine (a skeletal site rich in trabecular bone) and hip (a skeletal site rich in both trabecular and cortical bone) (27).

Bisphosphonates (alendronate and risedronate) are the front-line choices for the prevention of fractures in glucocorticoid-treated patients, with teriparatide and zoledronic acid as possible second-line options (3). Alendronate and risedronate have been confirmed to reduce the incidence of vertebral fractures in glucocorticoid-treated patients (12). Teriparatide is reported to be more effective than alendronate for increasing bone mineral density (BMD) and for reducing the incidence of vertebral fractures in patients with glucocorticoid-induced osteoporosis (22, 23). Zoledronic acid has been reported to be non-inferior,

Corresponding author: Dr. Jun Iwamoto, Institute for Integrated Sports Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel: +81-3-3353-1211, Fax: +81-3-3352-9467, E-mail: jiwamoto@a8.keio.jp
Received: September 2, 2013; Revised: December 22, 2013; Accepted: January 8, 2014.
 ISSN : 0304-4920. http://www.cps.org.tw

possibly more effective, and more acceptable to patients than risedronate for the prevention and treatment of BMD loss caused by glucocorticoids (21). Although bisphosphonates and teriparatide are considered to be good candidates for the treatment of glucocorticoid-induced osteoporosis, a strategy for preventing nonvertebral and hip fractures remains to be established.

From this point of view, a preclinical study to test the effect of anti-fracture agents on cortical bone in animal models of severe glucocorticoid-induced osteoporosis in terms of the substantial deterioration in cortical bone strength is important. Our previous bone histomorphometry studies showed that the administration of prednisolone (PSL) to rats decreased the percent cortical bone area, increased the percent marrow area, and increased the cortical porosity (7, 10, 11). Thus, PSL-treated rats can be utilized as a model for cortical osteopenia induced by glucocorticoid treatment.

Human parathyroid hormone (1-34) (hPTH[1-34], teriparatide acetate), which is used as an intermittent (56.5 μg, once weekly) injection regimen in clinical settings, has been generated by a Japanese pharmaceutical company (Asahi Kasei Pharma Co., Ltd., Tokyo, Japan) (16). Teriparatide acetate is different from teriparatide produced by genetic recombination, which is used as a daily (20 μg) injection regimen (17). A few preclinical studies have shown that the intermittent (3 times a week) injection of hPTH(1-34) (teriparatide acetate) prevented the development of ovariectomy-induced osteopenia in rats in a dose-dependent manner (1.5-6.0 μg/kg) and increased trabecular bone mass of the proximal tibial metaphysis in a dose-dependent manner (1.2 and 12 μg/kg) as a result of the increase in bone formation without the induction of cortical bone loss of the tibial diaphysis in intact rats (6, 26). Another study showed that a low-dose intermittent (3 times a week) injection of hPTH(1-34) (0.375 μg/kg) stimulated bone formation and increased trabecular bone mass but did not decrease either cortical thickness or cortical porosity in the lumbar vertebrae in intact male beagles (30). However, the effect of the intermittent administration of hPTH(1-34) on the cortical bone in animal models of glucocorticoid-induced osteoporosis remains uncertain. The aim of the present study was to examine the effect of the intermittent administration of hPTH(1-34) on cortical bone in rats treated with high-dose PSL.

Materials and Methods

Handling of Animals

Thirty female Sprague-Dawley rats (5 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animals were fed a standard pellet diet containing 1.25% calcium and 0.9% phosphorus (CRF-1; Oriental Yeast, Co., Ltd., Tokyo, Japan). The rats were housed in the local animal room at a temperature of 24°C, a humidity of 50%, and a 12-h on/off cycle for lighting. Free access to water and the pellet diet were allowed. After 1 week of adaptation to this environment, the rats (6 weeks old) were sorted into strata according to body weight and were then randomized using the stratified weight method into the following three groups of 10 animals each: a vehicle administration (control) group, a PSL administration group, and a PSL + hPTH(1-34) administration group. PSL (KMK, 100 mg/10 ml; Kawasaki Mitaka Pharmaceutical Co. Ltd., Tokyo, Japan) was subcutaneously administered at a dose of 10 mg/kg body weight five times a week. hPTH(1-34) (teriparatide acetate, 6.003 mg/vial; Asahi Kasei Pharma Co., Ltd., Tokyo, Japan) was dissolved in 0.1% BSA (Nakalai Tesque, Inc., Kyoto, Japan) and then was administered subcutaneously at a dose of 30 μg/kg body weight three times a week. The dose of hPTH(1-34) was higher than those (1.5-20 μg/kg) previously used in ovariectomized rats (6, 29). In the control group, 0.1% BSA (Nakalai Tesque, Inc., Kyoto, Japan) was used as a vehicle. The weight of the rats was monitored weekly, and the duration of treatment was 8 weeks. Two rats in the PSL group and three rats in the PSL + hPTH(1-34) group were omitted because they died during the experimental period. This experiment was performed at the laboratory of Hamri Co., Ltd. (Ibaraki, Japan), which has been approved by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Hamri Co., Ltd. (Ibaraki, Japan).

Preparation of Specimens

At 8 weeks after the start of the experiment, the animals were sacrificed by exsanguination after being anesthetized *via* the inhalation of 2-3% isoflurane (Mylan Inc., Tokyo, Japan) using the Table Top Laboratory Animal Anesthesia System (V1 Type; VetEquip, Inc., Pleasanton, CA, USA). The bilateral femora and the right tibia were harvested from each animal. The length of the left femur was measured using a dial caliper, and the weight was measured using an electronic balance (A&D Company, Tokyo, Japan). Then, the femur was preserved in saline, stored in a freezer (-60°C), and processed for the biomechanical testing. The right femur and tibia were preserved in 70% ethanol and were processed for peripheral quantitative tomography and bone histomorphometric

Table 1. Body weight, femoral length, and femoral weight

	Control	PSL	PSL + hPTH(1-34)
Initial Body Weight (g)	89.4 ± 4.9	89.5 ± 5.7	90.2 ± 5.0
Final Body Weight (g)	159.0 ± 8.7	67.6 ± 5.1*	67.6 ± 6.9*
Femoral Length (mm)	29.7 ± 0.5	24.5 ± 0.4*	24.6 ± 0.6*
Femoral Weight (g)	0.61 ± 0.04	0.40 ± 0.02*	0.40 ± 0.02*

Data are expressed as the mean ± SD.
An ANOVA with Fisher's PLSD test was used to compare data among the three groups.
*: significant *vs*. Control. PSL: prednisolone, hPTH: human parathyroid hormone.

analysis, respectively.

Peripheral Quantitative Tomography of the Femoral Diaphysis

The diaphysis of the femur was scanned using peripheral quantitative computed tomography (pQCT; XCT-Research SA+; Stratec Medizintechnik GmbH, Pforzheim, Germany) in 70% ethanol/saline. The bones were placed horizontally in a polypropylene tube and scanned at a voxel size of 0.12 mm. The scan line was adjusted using the scout view. According to the length of the femur, the sites 12 mm (control group) and 9 mm (PSL group and PSL + hPTH[1-34] group) proximal to the distal growth plate were scanned. For the analyses of bone mineral content (BMC), BMD, area, and thickness, a threshold of 690 mg/cm^3 in contour mode 1 was used to separate the bone tissue from the marrow. For analyses of the x-axis, y-axis, and polar strain stress index (SSI), a threshold of 464 mg/cm^3 in contour mode 1 was used.

Histomorphometry of the Tibial Diaphysis

The tibia was cut into two parts (proximal metaphysis and diaphysis plus distal metaphysis) using a Diamond Band Saw (EXAKT BS 3000; Norderstedt, Germany), and the diaphysis plus distal metaphysis part was stained according to the method of Villanueva (28). After dehydration with ethanol and acetone, the bone tissue was embedded in methyl methacrylate (Wako Pure Chemical, Osaka, Japan). Cross-sections of the tibial diaphysis were obtained 4 mm proximal to the tibiofibular junction at a thickness of 20-30 μm using a microgrinder (Exakt KG 4000; Norderstedt, Germany). Then, the specimens were observed under a fluorescence microscope (Zeiss Axiplan 2; Jena, Germany) coupled with a video camera (CCD Color Camera CS 5270 I; Tokyo Electronic Industry Co., Ltd., Tokyo, Japan). A bone morphometry software program (Winroof Version 3.5; Mitani Corporation, Fukui, Japan) was used for the static histomorphometric analysis.

The parameters measured were total tissue area, marrow area, periosteal perimeter, and endocortical perimeter, in accordance with the method described by Chen *et al*. (2) Two large cavities with and without vessels, which were independent of osteocytes and lacunae (7-9), were observed in the intracortical area of each specimen to measure the cavity area. These data were then used to calculate the cortical area (total tissue area – marrow area), percent cortical area (cortical area/total tissue area × 100), percent marrow area (marrow area/total tissue area × 100), and percent cavity area (cavity area/cortical area × 100).

Biomechanical Testing of the Femoral Diaphysis

The mechanical strength of the femoral diaphysis was evaluated using a three-point bending test. A load (100 kgf) was applied to the bone midway between two supports placed 18 mm apart. The femur was positioned so that the loading point was at the center of the femoral diaphysis, and bending occurred at the medial-lateral axis. Each specimen was submerged for about 1 h before testing to enable temperature equilibration. The load-displacement curves were recorded at a crosshead speed of 5 mm/min using a material-testing machine (MZ-500S; Maruto Instrument, Tokyo, Japan). The parameters analyzed were the bone stiffness, work to failure, and ultimate force.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Comparisons among the groups were performed using an analysis of variance (ANOVA) using Fisher's protected least significant difference (PLSD) test. All statistical analyses were done using the Stat View J-5.0 program on a Windows computer, and $P < 0.05$ was regarded as significant.

Results

Body Weight, Femoral Length, and Femoral Weight

Table 1 shows that the initial body weight did not differ significantly among the three groups. PSL

Table 2. pQCT Analysis of the femoral diaphysis

	Control	PSL	PSL + hPTH(1-34)
BMC (mg/mm)	4.61 ± 0.16	2.75 ± 0.15*	2.89 ± 0.15*
BMD (mg/cm^3)	1263 ± 7	1135 ± 5*	1151 ± 13*, #
Area (mm^2)	3.65 ± 0.13	2.42 ± 0.13*	2.51 ± 0.12*
Thickness (mm)	0.53 ± 0.02	0.34 ± 0.02*	0.36 ± 0.02*
Stress Strain Index			
X-axis	1.71 ± 0.10	1.22 ± 0.11*	1.22 ± 0.13*
Y-axis	1.91 ± 0.10	1.19 ± 0.10*	1.24 ± 0.07*
Polar	3.14 ± 0.16	2.09 ± 0.16*	2.22 ± 0.13*

Data are expressed as the mean ± SD.
An ANOVA with Fisher's PLSD test was used to compare data among the three groups.
*: significant *vs*. Control. #: significant *vs*. PSL. pQCT: peripheral quantitative computed tomography, PSL: prednisolone, hPTH: human parathyroid hormone, BMC: bone mineral content, BMD: bone mineral density.

Table 3. Bone histomorphometric analysis of the tibial diaphysis

	Control	PSL	PSL + hPTH(1-34)
Total Tissue Area (mm^2)	3.33 ± 0.14	2.89 ± 0.13*	2.82 ± 0.12*
Cortical Area (mm^2)	2.38 ± 0.10	1.57 ± 0.07*	1.59 ± 0.07*
Marrow Area (mm^2)	0.95 ± 0.06	1.32 ± 0.09*	1.23 ± 0.08*, #
Cavity Area (mm^2)	0.015 ± 0.008	0.025 ± 0.008*	0.022 ± 0.007
Periosteal Perimeter (mm)	6.93 ± 0.17	6.42 ± 0.17*	6.28 ± 0.14*
Endocortical perimeter (mm)	3.58 ± 0.10	4.28 ± 0.17*	4.13 ± 0.12*, #
Percent Cortical Area (%)	71.4 ± 1.1	54.5 ± 1.7*	56.4 ± 1.7*, #
Percent Marrow Area (%)	28.6 ± 1.1	45.5 ± 1.7*	43.6 ± 1.7*, #
Percent Cavity Area (%)	0.63 ± 0.33	1.60 ± 0.47*	1.36 ± 0.41*

Data are expressed as the mean ± SD.
An ANOVA with Fisher's PLSD test was used to compare data among the three groups.
*: significant *vs*. Control, #: significant *vs*. PSL: prednisolone, hPTH: human parathyroid hormone.

administration induced a decrease in the final body weight, but hPTH(1-34) administration to PSL-treated rats did not significantly influence the final body weight. PSL administration induced decreases in the femoral length and weight, but hPTH(1-34) administration to PSL-treated rats did not significantly influence the femoral length and weight.

pQCT Analysis of the Femoral Diaphysis

Table 2 shows that PSL administration induced decreases in the BMC, BMD, area, thickness, x-axis, y-axis, and polar SSI, but hPTH(1-34) administration to PSL-treated rats did not significantly influence any of these parameters except the BMD.

Bone Histomorphometric Analysis of the Tibial Diaphysis

Table 3 shows that PSL administration induced decreases in the total tissue area, cortical area, periosteal perimeter, and percent cortical area and increases in the marrow area, cavity area, endocortical perimeter, percent marrow area, and percent cavity area. hPTH(1-34) administration to PSL-treated rats attenuated increases in the marrow area, endocortical perimeter, and percent marrow area and a decrease in the percent cortical area.

Biomechanical Testing of the Femoral Diaphysis

Table 4 shows that PSL administration induced decreases in stiffness, work to failure, and ultimate force, but hPTH(1-34) administration to PSL-treated rats did not significantly influence any of these parameters.

Discussion

The present study found that high-dose PSL administration to rats reduced cortical bone gain, BMC, and BMD and enlarged the marrow cavity and that the

Table 4. Biomechanical testing of the femoral diaphysis

	Control	PSL	PSL + hPTH(1-34)
Stiffness (N/m)	226.8 ± 21.8	82.0 ± 11.4*	91.0 ± 14.9*
Work to Failure (N · m)	39.0 ± 8.0	28.7 ± 5.5*	27.2 ± 4.0*
Ultimate Force (N)	80.7 ± 3.9	41.9 ± 2.8*	42.3 ± 2.8*

Data are expressed as the mean ± SD.
An ANOVA with Fisher's PLSD test was used to compare data among the three groups.
*: significant *vs*. Control. PSL: prednisolone, hPTH: human parathyroid hormone.

intermittent administration of hPTH(1-34) to rats treated with high-dose PSL attenuated the cortical BMD loss, the decrease in marrow area, or the increase in percent cortical area. These findings suggest that the intermittent administration of hPTH(1-34) improves cortical BMD, acts on the endocortical bone surface, and affects cortical bone geometry in rats treated with high-dose PSL.

We did not show the results of the pQCT analysis for the distal femoral metaphysis together with those of the bone histomorphometric analysis for the proximal tibial metaphysis because of the existence of artifacts from the metaphyseal trabecular bone. High-dose PSL increased the trabecular BMD of the distal femoral metaphysis and the trabecular bone mass of the proximal tibial metaphysis as a result of the suppressed longitudinal growth rate and the decreased bone length. It has been argued that rats are a poor model of glucocorticoid-induced osteoporosis because glucocorticoids inhibit longitudinal bone growth as well as bone resorption by osteoclasts in the trabecular bone, resulting in a protective effect on the skeleton in mature rats (25). The results regarding metaphyseal trabecular bone support this suggestion.

However, PSL administration induced decreases in the BMC and BMD of the femoral diaphysis, as well as decreases in the total tissue area, cortical area, percent cortical area, and periosteal perimeter and increases in the marrow area, percent marrow area, endocortical perimeter, cavity area, and percent cavity area of the tibial diaphysis. These results of bone histomorphometry are consistent with those of previous studies showing a decreased percent cortical bone area and an increased percent marrow area as a result of the decreased periosteal bone formation and the increased endocortical bone erosion and increased cortical porosity in rats treated with glucocorticoids (5, 7, 10, 11, 25). High-dose PSL might have decreased the periosteal bone apposition and increased the bone erosion on the endocortical and intracortical cavity surfaces. Namely, high-dose PSL reduced the cortical bone gain, enlarged the marrow cavity, and increased the cortical porosity.

We also tried to evaluate bone formation and resorption. The single and double labeling surfaces, mineral apposition rate, and bone formation rate were evaluated after calcein double labeling (4 days interval). The osteoblast surface, osteoclast surface, and number of osteoclasts were also evaluated. However, osteoblasts, osteoclasts, and double labeling were hardly found in rats belonging to the PSL group and the PSL + hPTH(1-34) group, and single labeling was not found in more than half of the rats in these two groups because of the extremely high-dose of PSL usage and the subsequent severe suppression of osteoblast activity after 8 weeks of PSL treatment. Thus, the effects of hPTH(1-34) on bone formation and resorption in cortical bone during the early phase of PSL treatment were considered important.

The intermittent administration of hPTH(1-34) to rats treated with high-dose PSL attenuated the enlargement of the marrow cavity, but not the decrease in the percent cavity area and the increase in percent cortical area. These findings suggest that the intermittent administration of hPTH(1-34) acts on the endocortical bone surface without influencing the cortical porosity and improves the cortical bone geometry in rats treated with high-dose PSL. However, hPTH(1-34) did not suppress a reduction in periosteal bone apposition.

The intermittent administration of hPTH(1-34) reportedly restores the cortical BMC and the cortical bone area of the lumbar spine in ovariectomized rats (30 μg/kg, 3 times a week) (1) and stimulates bone formation in trabecular and cortical bone, leading to positive effects on the mass and structure of the bone in the lumbar spine or femur of ovariectomized rats (10 and 90 μg/kg, once a week) (20). Regarding rodent models of decreased mechanical loading caused by sciatic neurectomy or tail-suspension, however, the administration of hPTH(1-34) was found to prevent trabecular bone loss of the proximal tibial metaphysis (80 μg/kg, daily or 40 μg/kg, 5 times a week), but not to restore cortical bone loss of the tibial diaphysis (15, 24). The present study showed that the intermittent administration of hPTH(1-34) did not suppress a decrease in periosteal bone formation and a subsequent decline in the total tissue area and cortical area. hPTH(1-34) may be more responsive to trabecular and endocortical

bone than to periosteal bone in rodent models with decreased bone formation arising from glucocorticoid treatment or decreased mechanical loading. Probably because of the severe suppression of osteoblast activity on the periosteal surface by high-dose PSL administration, hPTH(1-34) might not have been able to counteract the decrease in bone formation on the periosteal surface significantly and thereby did not significantly improve the total tissue area and cortical area. However, hPTH(1-34) was speculated to improve the imbalance of bone formation and resorption and to suppress bone erosion on the endocortical surface during an 8-week experiment period, based on the results of a phase 3 clinical trial showing that the intermittent (once weekly) injection of hPTH(1-34) (teriparatide acetate) increased the serum osteocalcin level and decreased the urinary cross-lined N-terminal telopeptides of type I collagen (NTX) level in patients with osteoporosis (16).

Bone strength indices including stiffness, work to failure, and ultimate force as well as the x-axis, y-axis, and polar SSI of the femoral diaphysis deteriorated with PSL administration, but hPTH(1-34) was unable to improve theses indices in PSL-treated rats, probably because of non-significant alterations in periosteal bone formation, the percent cavity area, and the total tissue area and cortical area. Bone strength depends on BMD and bone quality, including both structural and material factors (19). In the present study, the effect of hPTH(1-34) was quite small. Substantial anabolic action, and subsequent increases in bone size and the BMC and BMD might have been needed to improve the strength of cortical bone.

The strength of the present study is that the effect of the intermittent administration of hPTH(1-34) on cortical bone geometry was demonstrated for the first time in rats treated with high-dose PSL. Conversely, the study has several limitations. First, we were unable to evaluate bone formation and resorption, as discussed above. Thus, the mechanism responsible for the changes in the cortical bone structure remains to be confirmed. Second, we did not evaluate the serum bone turnover markers. Some clinical studies have demonstrated that osteocalcin is a sensitive marker of the glucocorticoid-induced suppression of osteoblast activity (4, 13, 18). Thus, the effect of hPTH(1-34) on serum bone turnover markers, including osteocalcin, may be important for translating the results of our preclinical study into clinical practice. Thus, further time course studies are needed to explain the effect of hPTH(1-34) on cortical bone structure and bone metabolism.

In conclusion, the present study was conducted to examine the effect of the intermittent administration of hPTH(1-34) on cortical bone in rats treated with high-dose PSL. The results of the present study suggest that the intermittent administration of hPTH(1-34) improves cortical BMD, acts on the endocortical bone surface, and improves cortical bone geometry in rats treated with high-dose PSL.

Disclosures

This research was supported by Asahi Kasei Pharma, Co. Ltd., Tokyo, Japan. This paper was reviewed by scientists (Toyonobu Uzawa and Ryoko Takao) of Asahi Kasei Pharma, Co. Ltd., Tokyo, Japan.

Acknowledgments

We thank Drs. Kiichi Nonaka and Kaori Shindo (ELK Corporation, Tokyo, Japan) for pQCT analysis, Dr. Toshimi Masaki (Mitani Institute for bone histomorphometry, Tokyo, Japan) for bone histomorphometric analysis, and Dr. Tsuyoshi Ishii (Maruto Instrument Co., Ltd., Tokyo, Japan) for biomechanical testing.

References

1. Arita, S., Ikeda, S., Sakai, A., Okimoto, N., Akahoshi, S., Nagashima, M., Nishida, A., Ito, M. and Nakamura, T. Human parathyroid hormone (1-34) increases mass and structure of the cortical shell, with resultant increase in lumbar bone strength, in ovariectomized rats. *J. Bone Miner. Metab.* 22: 530-540, 2004.
2. Chen, M.M., Yeh, J.K., Aloia, J.F., Tierney, J.M. and Sprintz, S. Effect of treadmill exercise on tibial cortical bone in aged female rats: a histomorphometry and dual energy X-ray absorptiometry study. *Bone* 15: 313-319, 1994.
3. Compston, J. Management of glucocorticoid-induced osteoporosis. *Nat. Rev. Rheumatol.* 6: 82-88, 2010.
4. Ekenstam, E., Stålenheim, G. and Hällgren, R. The acute effect of high dose corticosteroid treatment on serum osteocalcin. *Metabolism* 37: 141-144, 1988.
5. Hara, K., Kobayashi, M. and Akiyama, Y. Vitamin K_2 (menatetrenone) inhibits bone loss induced by prednisolone partly through enhancement of bone formation in rats. *Bone* 31: 575-581, 2002.
6. Hori, M., Uzawa, T., Morita, K., Noda, T., Takahashi, H. and Inoue, J. Effect of human parathyroid hormone (PTH(1-34)) on experimental osteopenia of rats induced by ovariectomy. *Bone Miner.* 3: 193-199, 1988.
7. Iwamoto, J., Matsumoto, H., Takeda, T., Sato, Y., Liu, X. and Yeh, J.K. Effects of vitamin K_2 and risedronate on bone formation and resorption, osteocyte lacunar system, and porosity in the cortical bone of glucocorticoid-treated rats. *Calcif. Tissue Int.* 83: 121-128, 2008.
8. Iwamoto, J., Matsumoto, H., Takeda, T., Sato, Y. and Yeh, J.K. Effects of vitamin K_2 on cortical and cancellous bone mass, cortical osteocyte and lacunar system, and porosity in sciatic neurectomized rats. *Calcif. Tissue Int.* 87: 254-262, 2010.
9. Iwamoto, J., Seki, A., Sato, Y. and Matsumoto, H. Vitamin K_2 Improves renal function and increases femoral bone strength in rats with renal insufficiency. *Calcif. Tissue Int.* 90: 50-59, 2012.
10. Iwamoto, J., Seki, A., Takeda, T., Yamada, H., Sato, Y. and Yeh, J.K. Effects of combined administration of alfacalcidol and risedronate on cancellous and cortical bone mass of the tibia in glu-

cocorticoid-treated young rats. *Chinese J. Physiol.* 51: 121-128, 2008.
11. Iwamoto, J., Seki, A., Takeda, T., Yamada, H., Sato, Y. and Yeh, J.K. Effects of alfacalcidol on cancellous and cortical bone mass in rats treated with glucocorticoid: a bone histomorphometry study. *J. Nutr. Sci. Vitaminol. (Tokyo)*. 53: 191-197, 2007.
12. Iwamoto, J., Takeda, T. and Sato, Y. Effects of antifracture drugs in postmenopausal, male and glucocorticoid-induced osteoporosis-usefulness of alendronate and risedronate. *Expert Opin. Pharmacother.* 8: 2743-2756, 2007.
13. Jan, J.S. and Wu, W.F. Acute effect of glucocorticoid treatment on serum osteocalcin levels in asthmatic children. *J. Microbiol. Immunol. Infect.* 33: 25-28, 2000.
14. Longui, C.A. Glucocorticoid therapy: minimizing side effects. *J. Pediatr. (Rio J)*. 83 (5 Suppl): S163-S171, 2007.
15. Moriyama, I., Iwamoto, J., Takeda, T. and Toyama, Y. Comparative effects of intermittent administration of human parathyroid hormone (1-34) on cancellous and cortical bone loss in tail-suspended and sciatic neurectomized young rats. *J. Orthop. Sci.* 7: 379-385, 2002.
16. Nakamura, T., Sugimoto, T., Nakano, T., Kishimoto, H., Ito, M., Fukunaga, M., Hagino, H., Sone, T., Yoshikawa, H., Nishizawa, Y., Fujita, T. and Shiraki, M. Randomized teriparatide [human parathyroid hormone (PTH) 1-34] once-weekly efficacy research (TOWER) trial for examining the reduction in new vertebral fractures in subjects with primary osteoporosis and high fracture risk. *J. Clin. Endocrinol. Metab.* 97: 3097-3106, 2012.
17. Neer, R.M., Arnaud, C.D., Zanchetta, J.R., Prince, R., Gaich, G.A., Reginster, J.Y., Hodsman, A.B., Eriksen, E.F., Ish-Shalom, S., Genant, H.K., Wang, O. and Mitlak, B.H. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N. Engl. J. Med.* 344: 1434-1441, 2001.
18. Nielsen, H.K., Thomsen, K., Eriksen, E.F., Charles, P., Storm, T. and Mosekilde, L. The effects of high-dose glucocorticoid administration on serum bone gamma carboxyglutamic acid-containing protein, serum alkaline phosphatase and vitamin D metabolites in normal subjects. *Bone Miner.* 4: 105-113, 1988.
19. NIH Consensus Development Panel. Osteoporosis prevention, diagnosis, and therapy. *J. Am. Med. Assoc.* 285: 785-795, 2001.
20. Okimoto, N., Tsurukami, H., Okazaki, Y., Nishida, S., Sakai, A., Ohnishi, H., Hori, M., Yasukawa, K. and Nakamura, T. Effects of a weekly injection of human parathyroid hormone (1-34) and withdrawal on bone mass, strength, and turnover in mature ovariectomized rats. *Bone* 22: 523-531, 1998.
21. Reid, D.M., Devogelaer, J.P., Saag, K., Roux, C., Lau, C.S., Reginster, J.Y., Papanastasiou, P., Ferreira, A., Hartl, F., Fashola, T., Mesenbrink, P. and Sambrook, P.N. Zoledronic acid and risedronate in the prevention and treatment of glucocorticoid-induced osteoporosis (HORIZON): a multicentre, double-blind, double-dummy, randomised controlled trial. *Lancet* 373: 1253-1263, 2009.
22. Saag, K.G., Shane, E., Boonen, S., Marín, F., Donley, D.W., Taylor, K.A., Dalsky, G.P. and Marcus, R. Teriparatide or alendronate in glucocorticoid-induced oosteoporosis. *N. Engl. J. Med.* 357: 2028-2039, 2007.
23. Saag, K.G., Zanchetta, J.R., Devogelaer, J.P., Adler, R.A., Eastell, R., See, K., Krege, J.H., Krohn, K. and Warner, M.R. Effects of teriparatide versus alendronate for treating glucocorticoid-induced osteoporosis: thirty-six-month results of a randomized, double-blind, controlled trial. *Arthritis Rheum.* 60: 3346-3355, 2009.
24. Sakai, A., Sakata, T., Ikeda, S., Uchida, S., Okazaki, R., Norimura, T., Hori, M. and Nakamura, T. Intermittent administration of human parathyroid hormone(1-34) prevents immobilization-related bone loss by regulating bone marrow capacity for bone cells in ddY mice. *J. Bone Miner. Res.* 14: 1691-1699, 1999.
25. Shen, V., Birchman, R., Liang, X.G., Wu, D.D., Lindsay, R. and Dempster, D.W. Prednisolone alone, or in combination with estrogen or dietary calcium deficiency or immobilization, inhibits bone formation but does not induce bone loss in mature rats. *Bone* 21: 345-351, 1997.
26. Uzawa, T., Hori, M., Ejiri, S. and Ozawa, H. Comparison of the effects of intermittent and continuous administration of human parathyroid hormone(1-34) on rat bone. *Bone* 16: 477-484, 1995.
27. van Staa, T.P., Leufkens, H.G.M. and Cooper, C. The epidemiology of corticosteroid-induced osteoporosis: a meta-analysis. *Osteoporos. Int.* 13: 777-787, 2002.
28. Villanueva, A.R. A bone stain for osteoid seams in fresh, unembedded, mineralized bone. *Stain Technol.* 49: 1-8, 1974.
29. Washimi, Y., Ito, M., Morishima, Y., Taguma, K., Ojima, Y., Uzawa, T. and Hori, M. Effect of combined human PTH(1-34) and calcitonin treatment in ovariectomized rats. *Bone* 41: 786-793, 2007.
30. Zhang, L., Takahashi, H.E., Inoue, J., Tanizawa, T., Endo, N., Yamamoto, N. and Hori, M. Effects of intermittent administration of low dose human PTH(1-34) on cancellous and cortical bone of lumbar vertebral bodies in adult beagles. *Bone* 21: 501-506, 1997.