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Original Paper

Biomechanical analysis of combined treatment of high calcium and bisphosphonate in tibia of steroid-treated growing-phase rats

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Childhood systemic diseases are commonly treated with steroids. Consequently, steroid-induced osteoporosis is often observed as a side effect of steroid therapy. However, osteoporosis of tibia resulting from steroid therapy has not been reported yet. Herein we constructed a steroid-induced osteoporosis in tibia of the growing phase rats to examine internal structural changes of the bone and tried to find out the effect of bisphosphonates as a new and early treatment method. Biomechanical analysis was performed using two-dimensional microdensitometry and three-dimensional pQCT method. In addition, the following evaluations were carried out: noninvasive bone strength measurements in steroid-induced osteoporotic rat tibiae; comparing the effectiveness of single high-calcium diet *versus* combined treatment of high calcium and bisphosphonate for osteoporosis; and quantitative measurement of four elements (Ca, P, Mg, Zn) in bone matrix. Our data suggested that a combined treatment of high calcium and bisphosphonate was an effective new method to improve and treat steroid-induced osteoporosis in childhood.

Key words: Bisphosphonate, pQCT, Rat

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INTRODUCTION

Steroids are used to treat various diseases, including systemic lupus erythematosus (SLE)[1)], juvenile rheumatoid arthritis[2)], nephrotic syndrome[3,4)], serious bronchial asthma[5)], idiopathic thrombocytopenic purpura[6)], brain tumors[7)], and lymphangioma[8)] during childhood. However, steroid-induced osteoporosis (secondary osteoporosis) is one of the most common childhood metabolic bone diseases in childhood owing to the side effect of long-term steroid treatment.

Childhood is an important stage of development in which bone mass gradually increases through bone resorption and formation to reach the peak bone mass. Therefore, the application of steroids during this period inhibits bone growth and may leave permanent damage[3,9)]. During childhood, the bone is in a state of high turnover and bone density progressively increases, showing maximal increment during puberty. Since enhancement of the absolute value of peak bone mass is considered crucial for prevention of fractures and osteoporosis in late adulthood, maintenance of normal bone metabolism during childhood to reach as much as higher peak bone mass is an essential target.

Recent studies showed the effectiveness of bisphosphonate treatment in steroid-induced osteoporosis[10-14)]. However, there is no report on the effectiveness of bisphosphonate treatment for osteoporosis of tibial diaphysis at the growing stage. In the present study, we sought to assess the effectiveness of bisphosphonate treatment combined with high calcium supply in steroid-induced osteoporosis, especially considering the active metabolic turnover of bone during childhood. To this end, the effects of the proposed treatment were biomechanically analyzed in the tibial diaphyses of growing-phase rats after introducing a steroid-induced osteoporosis (secondary osteoporosis) condition. In addition, the quantitative analysis of four elements — Ca, P, Mg, Zn — was also performed. These trace elements are essential to bone growth and turnover during the growing phase.

MATERIALS AND METHODS

Rat diet was prepared in our laboratory according to a previous method described by Gunjima *et al.*[15)]. Rats were fed a standard diet (Oriental Combination A diet, Oriental Yeast Co. Ltd., Tokyo, Japan) as well as a high-calcium diet which consisted of 74% Oriental Conbination A diet and 26% UNICAL calcium (UNICAL®, Unical Calcium Food Co. Ltd., Tokyo, Japan). Table 1 shows the compositions of the experimental diets.

On the steroid used in the present study, it was prednisolone sodium succinate (water-soluble Prednine®, Shionogi Co. Ltd., Osaka, Japan), *i.e.*,

monosodium 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 21-succinate. As for the bisphosphonate, it was etidronate disodium (Didronel®, Sumitomo Pharmaceuticals, Osaka, Japan), *i.e.*, disodium dihydrogen (1-hydroxyethylidene) bisphosphonate.

Experimental animals

Eight-week-old male Wistar rats (n=50) weighing approximately 258 g (Seac Yoshitomi Co. Ltd., Fukuoka, Japan) were randomly divided into five groups of 10 rats each. The animals were housed in individual cages and maintained at 22±1°C in a 12-hour light-dark cycle. Food and water were supplied *ad libitum*.

As shown in Table 2, the rats were classified into five groups. In the 6-week control group (code: 6Co), rats were fed a standard diet with tap water for six weeks. In the 6-week steroid group (code: 6St), rats were fed a standard diet with tap water and were administered water-soluble Prednine® (30 mg/kg/2 days) orally for six weeks.

In the 9-week control group (code: 9Co), rats were fed a standard diet with tap water for nine weeks. In the 9-week steroid and high-calcium diet group (code: 6St・3Hc), rats were fed a standard diet with tap water and given water-soluble Prednine® (30 mg/kg/2 days) orally for six weeks, followed by a high-calcium diet with tap water for three weeks. In the 9-week steroid, high calcium, and bisphosphonate group (code: 6St・3HcBp), rats were fed a standard diet with tap water and were administered water-soluble Prednine® (30 mg/kg/2 days) orally for six weeks, followed by high-calcium diet with tap water and subcutaneous injection of bisphosphonate (5 mg/kg/day) into the dorsal skin for three weeks.

At the end of six weeks (groups 6Co and 6St) or nine weeks (groups 9Co, 6St・3Hc, and 6St・3HcBp), the animals were sacrificed under deep anesthesia by intra-abdominal injection of thiamylal sodium (Isozol®, Mitsubishi Pharmaceutical Co. Ltd., Osaka, Japan) combined with diethyl ether. The tibiae were removed and fixed in 10% neutral formalin solution. All the experimental procedures were conducted with due consideration for ethical care and handling of experimental animals, based on the Rules for Animal Experimentation of Kyushu Dental College.

Table 1 Compositions of experimental diets (%)

Ingredients	Standard diet	High-calcium diet
β-Corn starch	38.00	28.12
Vitamin-free casein	25.00	18.50
α-Potato starch	10.00	7.40
Cellulose powder	8.00	5.92
Soy bean oil	6.00	4.44
Mineral mixture[1)]	6.00	4.44
Granulated sugar	5.00	3.70
Vitamin mixture	2.00	1.48
UNICAL[2)]	−−	26.00
	100.00	100.00

Published compositions:

[1)](g/100g): NaCl, 4.66; KI, 0.01; KH_2PO_4, 25.72; NaH_2PO_4, 9.35; $MgSO_4$, 7.17; $CaHPO_4$, 14.56; Fe-citrate, 3.18; $MnSO_4$・4-5H_2O, 0.12; $CuSO_4$・5H_2O, 0.03; $ZnCO_3$, 0.11; Ca-lactate, 35.09

[2)](mg/100g): P, 16.5; Fe, 1.41; Ca, 20.4×10^3; Na, 158; K, 2.4; Cu, 0.06; Zn, 0.09; Mn, 0.34; S, 0.03

Table 2 Classifications of rats by diet and treatment

	Code	Diet and treatment
6-week group	6Co	Standard diet with tap water for 6 weeks (control)
	6St	Standard diet with tap water and steroid for 6 weeks
9-week group	9Co	Standard diet with tap water for 9 weeks (control)
	6St・3Hc	Standard diet with tap water and steroid for 6 weeks followed by high-calcium diet with tap water for 3 weeks
	6St・3HcBp	Standard diet with tap water and steroid for 6 weeks followed by high-calcium diet with tap water and bisphonate for 3 weeks

Microdensitometry (MD)

After fixation in 10% neutral formalin solution, the tibiae were exposed 60 seconds to X-ray under the conditions of 35 kVp tube voltage and 5 mA tube current. Soft X-ray photographs were taken with a focus-film distance of 70 cm (EMS-2, Softex Co. Ltd., Tokyo, Japan). Under these circumstances, with a view to evaluating the bone density objectively on the soft X-ray film, an aluminum step wedge (25 mm in length, 0.01−1.0 mm in thickness) was bonded to the soft X-ray film (Fuji Softex Film FG) before exposure. Optical observation (MD method) was performed using a densitometer (PDS-15, Konica Co. Ltd., Tokyo, Japan). Measurement of the bone density was done by scanning the center of the tibial diaphyses with a light beam of 10×500 μm slit width at a speed of 0.1 mm/sec. Optical density of the bone was converted to the thickness of aluminum by the density pattern of the standard aluminum wedge, and expressed as aluminum equivalent (mmAl).

Peripheral quantitative computed tomography (pQCT)

The tibial diaphyses were scanned at a distance of 15.5 mm from the proximal growth plate by pQCT (XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany) with 0.12×0.12 mm pixel and 0.46 mm slice thickness (Fig. 1). Cortical bone mineral density (CtBMD, mg/cm^3), cortical bone mineral content (CtBMC, mg/mm), and cortical bone cross-sectional area (CtCSA, mm^2) were analyzed using pQCT software, Rev. 6.00. Cortical bone area was defined as the section that corresponded to a pixel area over a threshold of 690 mg/cm^3. Cortical bone thickness (CtThc, mm), as well as the circumferences of cortical bone periosteum (Peri, mm) and endosteum (Endo, mm), were calculated from the cross-sectional total bone area and cross-sectional cortical bone area.

Stress-strain index (SSI) was also analyzed by pQCT as a noninvasive index of whole bone strength using a threshold value of 464 mg/cm^3. SSI was calculated using the following equation:

$$SSI = CtBD \times Z/NCtBD$$

where CtBD is cortical bone mineral density in mg/cm^3; Z is section modulus in mm^3, and NCtBD is normal value of cortical bone density at 1,200 mg/cm^3 with reference to the x and polar axes (xSSI, pSSI). xSSI and pSSI were used as determinants of bending and torsional strengths respectively.

Quantitative measurements of calcium, phosphorus, magnesium, and zinc

Quantitative measurements of bone mineral contents were performed as described in our previous study by Yahara *et al.*[16]. Briefly, tibial diaphyses were washed in pure water after fixation in neutral buffer formalin. This was followed by rinsing in ethyl alcohol and drying at 60°C for one hour, and then weighed (0.06−0.12 mg). After this step, the samples were placed in a beaker (Pyrex heat-resistant glass), where 5 ml of hydrochloric acid and 3 ml of nitric acid (both of analytical grade, Wako Pure Chemical Industries Co. Ltd., Osaka, Japan) were added. Content was heated in a sand bath on a hot plate for one hour to dissociate the content. Ultrapure water was added to the resulting solution to make a sample solution with a total volume of 50 ml. This solution was diluted 10 times with ultrapure water for analysis, whereby Ca, P, Mg, and Zn were quantitatively analyzed using an inductively coupled plasma atomic emission spectrometer (ICPS-8100, Shimadzu Co. Ltd., Kyoto, Japan) and determined according to the standard calibration

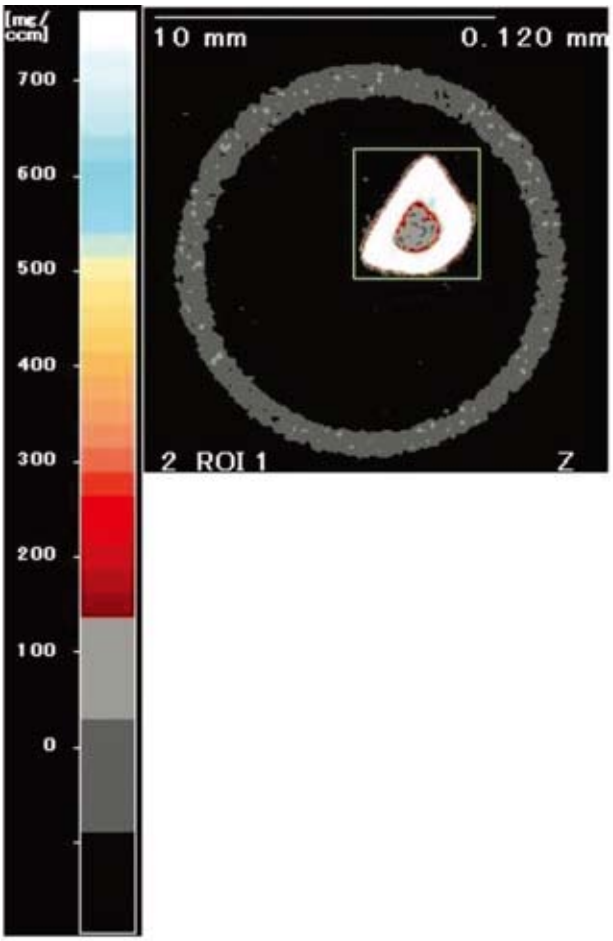

Fig. 1 A representative image of tibial diaphyseal bone scanned at a distance of 15.5 mm from the proximal growth plate.

curves of these four minerals (Ca: 0, 8, 50 ppm; P: 0, 8, 40 ppm; Mg: 0, 0.4, 2 ppm; Zn: 0, 0.02, 0.1 ppm). Ratios of these four elements in the samples were expressed in percentage.

Statistical analysis

Statistical analyses were performed using t-test and one-way analysis of variance (ANOVA) for data with a significant difference in *post hoc* test. Data were expressed as mean±standard deviation (SD).

RESULTS

MD

No significant difference was detected between groups 6Co and 6St. A significant difference was observed between groups 6St･3HcBp and 6St･3Hc ($p<0.01$) (Table 3).

pQCT

pQCT analysis demonstrated that CtBMD and Peri were significantly lower in group 6St than in group 6Co ($p<0.01$ and $p<0.05$, respectively). On the other hand, CtBMC and CtCSA were significantly higher in group 6St than in group 6Co ($p<0.05$ and $p<0.05$, respectively). With the 9-week groups (9Co, 6St･3Hc, and 6St･3HcBp), all bone parameters were lower in group 6St･3Hc than in control group 9Co. CtBMD, CtBMC, CtCSA, and Peri were significantly lower in group 6St･3Hc than in group 9Co ($p<0.05$, $p<0.05$, $p<0.01$, and $p<0.01$, respectively). On the other hand, most of the cortical bone parameters, CtBMD, CtBMC, CtCSA, Peri, and Endo were significantly higher in group 6St･3HcBp than in group 6St･3Hc

Table 3 Densities of tibial diaphyses in rats based on equivalent thickness of aluminum

	6-week group		9-week group		
	6Co	6St	9Co	6St・3Hc	6St・3HcBp
Density (mmAl)	1.10±0.07	1.16±0.08	1.12±0.03	1.13±0.04[a]	1.23±0.09[b]

Data are shown as mean ± SD. a: $p<0.01$ *vs.* 9Co b: $p<0.01$ *vs.* 6St･3Hc

Table 4 pQCT measurement results of tibial diaphyses in rats

	6-week group		9-week group		
	6Co	6St	9Co	6St・3Hc	6St・3HcBp
Cortical bone mineral density (CtBMD, 10^3mg/cm^3)	1.28±0.01	1.26±0.01[x]	1.28±0.02	1.26±0.01[b]	1.28±0.02[d]
Cortical bone mineral content (CtBMC, mg/mm)	5.54±0.36	5.93±0.25[y]	6.72±0.22	5.46±0.91[b]	6.46±0.50[d]
Cortical bone cross-sectional area (CtCSA, mm^2)	4.32±0.30	4.70±0.18[y]	5.26±0.10	4.30±0.63[a]	5.20±0.43[c]
Cortical Thickness (CtThc, mm)	0.68±0.03	0.65±0.03	0.68±0.04	0.60±0.07	0.65±0.06
Periosteum (Peri, mm)	8.97±0.18	8.66±0.24[y]	9.86±0.25	9.02±0.42[a]	9.76±0.31[c]
Endosteum (Endo, mm)	3.97±1.17	4.56±0.82	5.57±0.48	5.04±0.51	5.68±0.13[c]
Stress Strain Index with reference to the x-axis (xSSI)	2.48±0.12	2.17±0.26[y]	3.28±0.17	2.29±0.55[a]	3.04±0.29[c]
Stress Strain Index with reference to the polar-axis (pSSI)	5.81±0.90	4.33±0.50[y]	6.65±0.62	4.95±0.43[a]	4.90±0.44[a]

Data are shown as mean ± SD. x: $p<0.01$, y: $p<0.05$ *vs.* 6Co. a: $p<0.01$, b: $p<0.05$ *vs.* 9Co. c: $p<0.01$, d: $p<0.05$ *vs.* 6St･3Hc

Table 5 Ca, P, Mg, and Zn mineral contents of tibial diaphyses in rats

	6-week group		9-week group		
	6Co	6St	9Co	6St・3Hc	6St･3HcBp
Ca	27.00±2.00	25.00±1.00[y]	26.00±1.00	24.00±1.00[a]	26.00±1.00[b]
P	14.00±1.00	13.00±2.00[y]	12.00±1.00	12.01±1.00	13.00±1.00[ac]
Mg	0.50±0.04	0.45±0.05[x]	0.47±0.00	0.42±0.04[a]	0.47±0.03[b]
Zn	0.04±0.00	0.04±0.00[x]	0.05±0.00	0.05±0.01	0.10±0.04[ab]

Data are shown as mean ± SD. x: $p<0.01$, y: $p<0.05$ *vs.* 6Co a: $p<0.01$ *vs.* 9Co b: $p<0.01$, c: $p<0.05$ *vs.* 6St･3Hc

($p<0.05$, $p<0.05$, $p<0.01$, $p<0.01$, and $p<0.01$, respectively) (Table 4).

After six weeks, both xSSI and pSSI were markedly lower in group 6St than in group 6Co ($p<0.05$ and $p<0.05$, respectively). Similarly, after nine weeks, xSSI and pSSI were significantly lower in groups 6St•3Hc and 6St•3HcBp than in group 9Co. On the other hand, xSSI of group 6St•3HcBp was significantly higher than that of group 6St•3Hc ($p<0.01$) (Table 4).

Quantitative measurements of Ca, P, Mg and Zn

Amounts of Ca, P, Mg, and Zn were significantly lower in group 6St than in group 6Co ($p<0.05$, $p<0.05$, $p<0.01$, and $p<0.01$, respectively). On the other hand, all the amounts of Ca, P, Mg, and Zn were higher in group 6St•3HcBp than in group 6St•3Hc ($p<0.01$, $p<0.05$, $p<0.01$, and $p<0.01$, respectively) (Table 5).

DISCUSSION

Osteoporosis is now considered as a patho-physiological condition of calcium deficit during aging depending on the acquired nutritional life. This condition is specifically characterized by a decrease in bone density without abnormalities of metabolic functions of the body. Therefore, prevention and treatment of osteoporosis are now well described. Recently, a new treatment method is proposed for osteoporosis due to calcium deficit during childhood[15-18]. On the other hand, it is now clear that the use of steroids for systemic diseases during childhood causes steroid-induced osteoporosis (secondary osteoporosis) as a side effect. However, prevention and treatment of this situation is not established yet.

Although osteoporosis due to steroids occurs early due to the high metabolic turnover in childhood, its quick detection and early treatment are also possible. By means of a steroid-induced secondary osteoporosis animal model in the present study, we clearly showed the efficiency of a combined treatment of high-calcium diet with bisphosphonate in the tibial diaphyses of growing-phase rats. The main component of steroids, glucocorticoid, inhibits bone matrix formation in childhood, which then results in a decrease in bone density[19]. On the other hand, etidronate used as a bisphosphonate reverses the decrease of bone density by inhibiting bone resorption[20,21]. Furthermore, since a high calcium intake raises calcium absorption in the intestine, we considered the combined treatment of high calcium and bisphosphonate for the treatment of secondary osteoporosis in childhood.

In this study, biomechanical analyses of trabecular and cortical bones were conducted using two-dimensional microdensitometry and three-dimensional pQCT method. In addition, the following evaluations were carried out to examine the effect of the combined treatment of high calcium and bisphosphonate in steroid-induced osteoporotic rat tibiae: noninvasive bone strength measurements and quantitative measurements of four elements (Ca, P, Mg, Zn) in bone matrix. Two-dimensional microdensitometry demonstrated significantly higher bone density in group 6St•3HcBp than in group 6St•3Hc ($p<0.01$). This could have resulted from an increase in calcium absorption in the intestine due to UNICAL® treatment[22] coupled with a suppression of bone density decrease due to bisphosphonate administration.

Three-dimensional pQCT analysis demonstrated that CtBMD and Peri were significantly lower in group 6St than in group 6Co ($p<0.01$ and $p<0.05$, respectively). This result suggested that steroids had a prominent effect on the internal structures of bone. CtBMD, CtBMC, CtCSA, and Peri were also significantly lower in group 6St•3Hc than in group 9Co ($p<0.05$, $p<0.05$, $p<0.01$, and $p<0.01$, respectively). This result suggested that a shift occurred rapidly in steroid-induced bone from a high-turnover metabolic condition of childhood to a lower metabolic turnover. In previous studies, calcium, vitamin D, or bisphosphonates were reportedly used as single agents in the treatment of steroid-induced osteoporosis[11,23,24]. Our study showed that single, early high-calcium treatment was not enough to recover the metabolic condition of bone as compared to the control group. On the other hand, all the bone measurement parameters including CtBMD, CtBMC, CtCSA, Peri, and Endo were significantly higher in high calcium+bisphosphonate treatment group (6St•3HcBp) than in high-calcium-only treatment group (6St•3Hc) ($p<0.05$, $p<0.05$, $p<0.01$, $p<0.01$, and $p<0.01$, respectively). These results clearly showed that a combined administration of high calcium and bisphosphonate was an effective therapeutic method in improving the fragile state of bone health, especially during the growing phase of childhood with high bone turnover.

On SSI index, the bone strength indices with reference to the x-axis (xSSI) and polar-axis (pSSI) were significantly lower in group 6St than in group 6Co ($p<0.05$ and $p<0.05$, respectively). This result meant that the bending and torsional strength decreased, thereby suggesting a remodeling situation of bone formation. On the other hand, xSSI of group 6St•3HcBp became significantly higher than that of group 6St•3Hc ($p<0.01$). This result indicated that the combined administration of high calcium and bisphosphonate could improve the bending strength of bone.

On the four elements in bone matrix, group

6St•3Hc failed to reach the level present in group 9Co. This result suggested that a single treatment with high calcium supply was not sufficient to recover the bone. Group 6St•3HcBp showed significantly larger amounts of Ca, P, Mg, and Zn than in group 6St•3Hc, whereby the mineral content levels reached or exceeded those of 9Co. This result indicated that bisphosphonate could have contributed significantly to the increase in mineral content.

Mg and Zn play a vital role in bone metabolism. Mg is assumed to be stored in bone as well as Ca, and that it displaces Ca in apatite crystals. It is included in the substrate follicle, and works as a catalyst when alkaline phosphatase plays the role of supplying P necessary for calcification in bone formation[25]. Zn also plays an important role in bone metabolism, such as accelerating bone formation by activating aminoacyl-tRNA synthetase and stimulating bone protein synthesis[26]. It also works as an inhibitor to bone resorption by exhibiting an inhibitory effect on osteoclast-like cell formation in mouse marrow culture[27]. On the effects of bisphosphonate on Mg and Zn, no other data were available apart from the present study. On this note, further research might be required.

The present study revealed that all the elements, Ca, P, Mg and Zn, significantly increased through a combined treatment of high calcium and bisphosphonate for osteoporosis. Therefore, results of this study indicated that such a combined treatment might be effective for the early treatment of steroid-induced osteoporosis.

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