Oral Diseases (2007) 13, 544–549. doi:10.1111/j.1601-0825.2006.01331.x

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ORIGINAL ARTICLE

Effects of bisphosphonate on the mandible of rats in the growing phase with steroid-induced osteoporosis

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AIM: The objective of this study was to investigate the effects of bisphosphonate on the mandible of rats in the growing phase with steroid-induced osteoporosis, and to estimate the biomechanical response in the mandibular bone.

MATERIALS AND METHODS: Eight-week-old male Wistar rats ($n = 50$) were assigned to a 6-week control (Co-6) group, 6-week steroid (St) group, 9-week control (Co-9) group, 9-week steroid + standard diet (StSD) group, or 9-week steroid + standard diet + bisphosphonate (StSDBp) group. The mandibular bone was evaluated by two-dimensional bone density measurement (PDS-15), three-dimensional pQCT, and quantitative analysis of Ca, P, Mg, and Zn using a sequential high frequency plasma spectrometer (ICPS-8000).

RESULTS: In PDS-15 analysis, the bone density converted to aluminum equivalent was higher in StSDBp group when compared with the StSD group, and no significant difference was observed in bone density between St group and Co-6 group. In pQCT analysis, trabecular bone density and mineral content were significantly higher, while all other bone parameters were significantly lower in St group when compared with the Co-6 group. The densities of trabecular and cortical bones, mineral content and cross-sectional area of cortical bone, and non-invasive stress strain index with reference to *x* and yaxes were higher in StSDBp group than in StSD group. In quantitative analyses, Ca and P were markedly higher in StSDBp group than in StSD group, while there were no differences in Mg and Zn.

CONCLUSION: Bisphosphonate treatment increases trabecular and cortical bone parameters in the mandible of growing rats with steroid-induced osteoporosis.

Oral Diseases (2007) **13**, 544–549

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Received 18 July 2006; accepted 26 July 2006

Keywords: steroid-induced osteoporosis; growing phase mandible; bisphosphonate; rat

Introduction

Osteoporosis is a disease of reduced bone density and bone mineral content caused by calcium deficiency. During childhood, the bone is in a state of high turnover with a gradual increase of bone mass. In this stage, the bone growing along the direction of the long axis reaches the peak bone mass for life. Previous studies have shown that secondary osteoporosis occurs as a result of the adverse effect of steroid treatment for systemic diseases in childhood such as bronchial asthma, nephrotic syndrome, and juvenile rheumatoid arthritis (Wolthers and Pedersen, 1991; Lettgen *et al*, 1994; Cetin *et al*, 1998). Steroids induce osteoporosis by decreasing the absorption of calcium from the intestine, suppressing the differentiation of osteoblasts, and promoting bone resorption by osteoclasts (Patschan *et al*, 2001; Canalis and Delany, 2002; Sivagurunathan *et al*, 2005). There are few reports on using bisphosphonate as a treatment for steroid-induced osteoporosis in childhood (Cimaz, 2002; Srivastava and Alon, 2003). In the oral field, although the use of grape seed proanthocyanidins for the treatment of osteoporosis (Gunjima *et al*, 2004; Kamitani *et al*, 2004; Kojima *et al*, 2004; Ishikawa *et al*, 2005) and the relation between decreased maxillary bone mineral content and loss of teeth in osteoporosis (Krall *et al*, 1996; Gilles *et al*, 1997; Jeffcoat, 1998) have been reported, so far there is no study for the effect of bisphosphonate on the jawbone in steroid-induced osteoporosis during childhood.

In the present study, therefore, we tried to use bisphosphonate to treat the mandible of rats in growing phase weakened by steroid application. Using two-dimensional bone density measurement (PDS-15) and three-dimensional peripheral Quantitative Computed Tomography (pQCT), we analysed density, mineral content, cross-sectional area, and non-invasive strength of the trabecular and cortical bone

components, and quantitation of minerals (Ca, P, Mg and Mn).

Materials and methods

Materials

Rat diet (supplied as a powder diet by Oriental Yeast Co., Ltd, Tokyo, Japan) was blended in our laboratory and used in the experiment. The calcium content of the rat diet was 480 mg 100 g^{-1}. The composition of the diet is shown in Table 1 (Kamitani *et al*, 2004). The steroid used was prednisolone sodium succinate [monosodium 11β, 17,21-trihydroxypregna-1,4-diene-3,20 dione 21-succinate; water-soluble Prednine®] obtained from Shionogi & Co., Ltd (Osaka, Japan). The bisphosphonate used was etidronate disodium [disodium (1-hydroxyethylidene) bisphosphonate; Didronel®] obtained from Sumitomo Pharmaceuticals (Osaka, Japan).

Experimental animals

Eight-week-old male Wistar rats ($n = 50$) weighing approximately 255 g (SEAC Yoshitomi, Ltd, Fukuoka, Japan) were randomized into five groups of 10 rats each. The animals were maintained in individual cages at 22 ± 1°C and a 12-h dark–light cycle. Food and water were supplied *ad libitum*.

The 6-week control (Co-6) group was fed with a standard diet and tap water for 6 weeks. The 6-week steroid (St) group was fed with a standard diet and tap water, and steroid (30 mg kg^{-1} 2 $days^{-1}$) was administered orally for 6 weeks. The 9-week control (Co-9) group was fed with a standard diet and tap water for 9 weeks. The 9-week steroid + standard diet (StSD) group was fed with a standard diet and tap water, and steroid (30 mg kg^{-1} 2 $days^{-1}$) was administered orally for 6 weeks, followed by standard diet with tap water for 3 weeks. The 9-week steroid + standard diet + bisphosphonate (StSDBp) group was fed with a standard diet and tap water, and steroid (30 mg kg^{-1} 2 $days^{-1}$) was administered orally for 6 weeks, followed by standard diet with tap water and subcutaneous injection of bisphosphonate (5 mg kg^{-1} day^{-1}) into the dorsal skin for 3 weeks.

Table 1 Composition of experimental diet (%)

Ingredients	*Standard diet (Ca 480 mg 100 g^{-1})*
β-Corn starch	38.00
Vitamin-free casein	25.00
α-Potato starch	10.00
Cellulose powder	8.00
Soybean oil	6.00
Mineral mixture	6.00*
Granulated sugar	5.00
Vitamin mixture	2.00
Total	100.00

*Mineral mixture of standard diet (g 100 g^{-1}): NaCl, 4.66 g; KI, 0.01 g; KH_2PO_4, 25.72 g; NaH_2PO_4, 9.35 g; $MgSO_4$, 7.17 g; $CaHPO_4$, 14.56 g; Fe-citrate, 3.18 g; $MnSO_4{\cdot}4\text{-}5H_2O$, 0.12 g; $CuSO_4{\cdot}5H_2O$, 0.03 g; $ZnCO_3$, 0.11 g; Ca-lactate, 35.09 g.

At the end of 6 weeks (Co-6, and St groups) or 9 weeks (Co-9, StSD, and StSDBp groups), the rats were sacrificed under deep anesthesia with diethyl ether combined with thiamylal sodium (Isozol® from Mitsubishi Pharma Corp., Osaka, Japan). The mandible was removed, divided into right and left, and fixed in 10% neutral formalin solution. All the experimental procedures were conducted with consideration for ethical care and handling of experimental animals, based on the Rules for Animal Experimentation of Kyushu Dental College.

Body weight

Body weights of the rats were measured at a fixed time every week during the experimental period.

Two-dimensional bone density measurement (bone mineral content)

Soft radiographs were taken on the mandibles fixed in 10% neutral formalin. The mandible samples together with an aluminum step wedge (25 mm in length, 0.01–1.0 mm in thickness) were fixed by adhesive tape to the film (Fuji Softex film FG; Fuji Photo Film, Tokyo, Japan). Soft radiographs were taken under the conditions of 35 kvp, 5 mA, 60 s, and a focus-film distance of 70 cm. The radiograph was scanned using a Sakura microphotometer (PDS-15; Konica Inc., Tokyo, Japan). The alveolar bone anterior to the first molar was scanned at five positions vertical to the mandibular plane at a speed of 0.1 mm s^{-1} using a light beam with a slit size of 10 × 500 μm, and the scanning pattern was depicted. The optical density of the bone was converted to the thickness of aluminum by the density pattern of the standard aluminum wedge, and expressed as equivalent aluminum thickness (mmAl).

Density, mineral content, and cross-sectional area of the bone

Biomechanical analysis was conducted according to the methods reported by our study group (Kamitani *et al*, 2004) using three-dimensional peripheral quantitative computed tomography (pQCT) (XCT Research SA model; Stratec-Medizintechnik GmbH, Pfozheim, Germany). To display both the scout view and tomographic image on the monitor, the bone sample was positioned centrally between the scanner unit and the detector using a supportive device. The mandibular bone was scanned around the center of the mesial root of the first molar, at three different positions each 0.1 mm apart (Figure 1). Three slices containing the trabecular bone and cortical bone at a slice thickness of 0.26 mm were measured at a voxel size of 0.08 mm. The trabecular bone region was determined manually, and the trabecular bone density (TrBD, mg cm^{-3}), mineral content (TrBMC, mg mm^{-1}), and cross-sectional area (TrCSA, mm^2) were measured. The cortical bone region was determined using contour mode 2, cortical mode 1, and peel mode 2 with a threshold of 690 mg cm^{-3}, and cortical bone density (CtBD, mg cm^{-3}), mineral content (CtBMC, mg mm^{-1}), and cross-sectional area (CtCSA, mm^2) were measured.

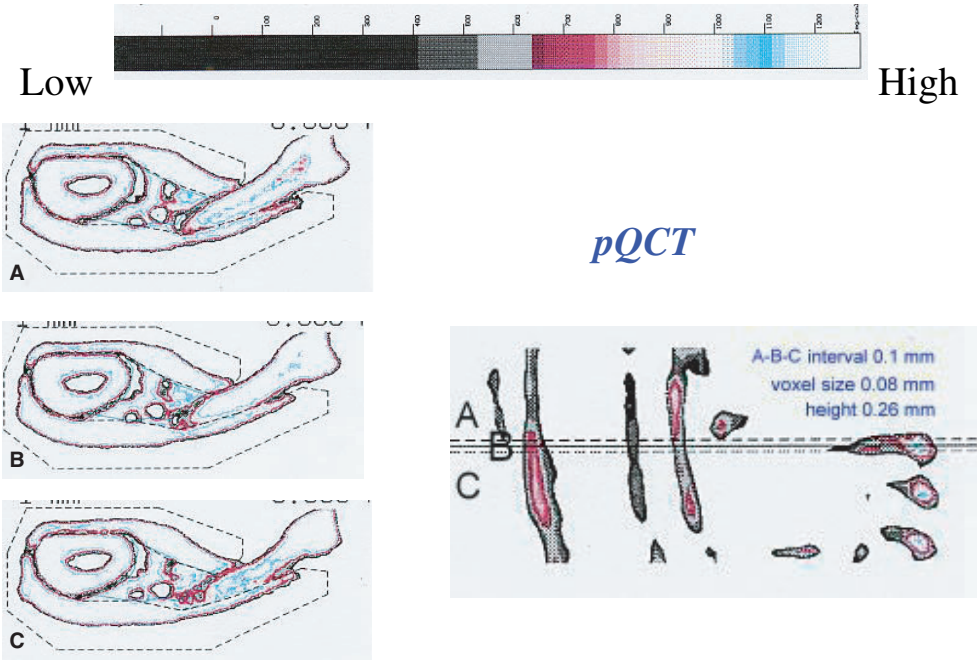

Figure 1 Mandibular bone of Wistar rat. The center of the mesial root of the first molar is depicted in three different positions (A, B, and C) at intervals of 0.1 mm (precisely). This figure is a representative image of peripheral quantitative computed tomography (left)

Non-invasive bone strength measurement
Bone strength was measured in non-invasive stress strain index (SSI) by the pQCT method using contour mode 2, cortical mode 1, and peel mode 2, at a threshold of 464 mg cm^{-3}. SSI was calculated by the equation: $SSI = CtBD \cdot Z/NCtBD$ (where CtBD is the cortical bone density in mg cm^{-3}; Z is the section modulus in mm^3, and NCtBD is the normal value of cortical bone density of 1200 mg cm^{-3}). The SSI with reference to the *x*, *y*, and *z* axes were determined.

Quantitative measurements of calcium, phosphorus, magnesium, and zinc
From each of the mandibular alveolar bone, a cortical bone fragment measuring 5 mm in length was used for quantitative mineral measurements. Each sample was measured after washing in ethyl alcohol and drying at 60°C for 1 h. Then the sample was placed in a beaker (Pyrex heat-resistant glass), 5 ml of hydrochloric acid, and 3 ml of nitric acid (both analytical grade; Wako Pure Chemical Industries Ltd, Osaka, Japan) were added and heated in a hot plate for 1 h to dissociate the content. The resulting mixture was dissolved in 50 ml of distilled water. The solution was diluted 10 times and used as the analytical sample. Standard solutions (calcium 0, 8 and 40 ppm; phosphorus 0, 8 and 40 ppm; magnesium 0, 0.4 and 2 ppm; and zinc 0, 0.02 and 0.1 ppm) were measured to obtain calibration curves. The samples were measured using a sequential high frequency plasma spectrometer (Shimadzu Corp., Kyoto, Japan), and the ratios of calcium (Ca), phosphorus (P), magnesium (Mg), and zinc (Zn) in the samples were expressed in percentage.

Statistical analysis
The data are expressed as mean ± standard deviation (s.d.). Statistical analyses were performed using *t*-test and one-way analysis of variance for data showing a significant level in *post hoc* test. $P < 0.05$ was considered significant.

Results

Body weight
The initial and final body weights (mean ± s.d.) of rat in Co-6, St, Co-9, StSD, and StSDBp groups are given in Table 2. The final body weight was significantly lower in St group than in Co-6 group ($P < 0.01$), and lower in StSD and StSDBp groups when compared with the Co-9 group ($P < 0.05$, $P < 0.01$, respectively) (Table 2).

Two-dimensional bone density (bone mineral) measurements
The results of 2-D bone density based on the alveolar bone mineral content measured as aluminum equivalent (mmAl) are given in Table 3. The bone density was markedly higher in StSDBp group than in StSD group ($P < 0.05$). Although the bone density of St group showed a tendency to be lower than that of Co-6 group, there was no significant difference between them.

Density, mineral content, and cross-sectional area of the bone
A markedly higher trabecular bone density (TrBD) and mineral content (TrBMC) were observed in St group than in Co-6 group ($P < 0.01$ and $P < 0.05$, respectively), whereas all other bone parameters were

Table 2 Body weights (g) during the study period

	Co-6	*St*	*Co-9*	*StSD*	*StSDBp*
Initial BW	251.35 ± 8.13	257.89 ± 4.15	254.20 ± 11.36	260.16 ± 6.07	260.42 ± 14.95
Final BW	470.75 ± 45.32	388.16 ± 26.20[a]	528.83 ± 14.39	457.20 ± 43.38[c]	426.76 ± 36.96[b]

Data are shown as mean ± s.d. *vs* Co-6: [a]$P < 0.01$; *vs* Co-9: [b]$P < 0.01$, [c]$P < 0.05$.
BW, body weight; Co-6, 6-week control group; St, 6-week steroid group; Co-9, 9-week control group; StSD, 9-week steroid + standard diet group; StSDBp, 9-week steroid + standard diet + bisphosphonate group.

Table 3 Density of alveolar bone based on equivalent thickness of aluminium in Wistar rats

	Co-6	*St*	*Co-9*	*StSD*	*StSDBp*
Density (mmAl)	1.105 ± 0.021	1.104 ± 0.025	1.145 ± 0.043	1.118 ± 0.017	1.143 ± 0.034*

Data are shown as mean ± s.d. *vs* StSD: *$P < 0.05$.
Co-6, 6-week control group; St, 6-week steroid group; Co-9, 9-week control group; StSD, 9-week steroid + standard diet group; StSDBp, 9-week steroid + standard diet + bisphosphonate group.

markedly lower in St group when compared with the Co-6 group ($P < 0.01$) (Table 4). Similarly all the bone parameters except TrBD were significantly lower ($P < 0.01$) in StSD group when compared with the Co-9 group (Table 4). Furthermore, the TrBD in StSDBp group was markedly higher than that in StSD group ($P < 0.01$), and was even higher than that in Co-9 group ($P < 0.05$). On the other hand, all the cortical bone parameters including the density (CtBD), mineral content (CtBMC), and cross-sectional area (CtCSA) of the cortical bone were also markedly higher in StSDBp group than in StSD group ($P < 0.01$).

Non-invasive bone strength measurement
The stress strain indices with reference to the *x*-axis (xSSI), *y*-axis (ySSI), and *z*-axis (pSSI) are shown in Table 4. All the SSI parameters were markedly lower in St group than in Co-6 group ($P < 0.01$), and were also significantly lower in StSD group when compared with the Co-9 group ($P < 0.01$). On the other hand, the xSSI was markedly higher in StSDBp group than in StSD group ($P < 0.05$).

Quantitative measurements of Ca, P, Mg, and Zn
Table 5 shows the calcium (Ca), phosphorus (P), magnesium (Mg), and zinc (Zn) contents expressed in percentage. In St group, the calcium content was significantly lower ($P < 0.05$), but the magnesium content was significantly higher ($P < 0.01$) when compared with the Co-6 group. In the StSDBp group, calcium and phosphorus contents were markedly higher ($P < 0.01$) when compared with the StSD group, while there were no differences in magnesium and zinc contents between the two groups.

Discussion

Childhood is a stage of active growth and nutrition with exercise and lifestyle in daily life being especially important in this stage. In the present study, while the body weight was reduced in rats in the St group with bone weakened by 6 weeks of steroid administration when compared with the rats in Co-6 group, 3 weeks of bisphosphonate treatment normalized the body weight of the steroid-treated rats.

Two-dimensional alveolar bone density (PDS-15) was significantly higher in the StSDBp group than in the StSD group ($P < 0.05$), and no significant difference was observed in St group and Co-6 group. However, it is not possible to know in detail with these results alone how the trabecular bone and cortical bone are affected. We therefore introduced the three-dimensional pQCT

Table 4 Bone density, bone mineral content, cross-sectional area, and Stress Strain Index of the mandible in Wistar rats

	Co-6	*St*	*Co-9*	*StSD*	*StSDBp*
Trabecular bone density (TrBD, mg cm^{-3})	549.77 ± 69.93	724.02 ± 31.48[x]	591.90 ± 83.93	533.20 ± 126.75	654.61 ± 62.66[bc]
Trabecular bone mineral content (TrBMC, mg mm^{-1})	1.50 ± 0.16	1.71 ± 0.28[y]	1.58 ± 0.36	1.21 ± 0.35[a]	1.40 ± 0.30
Trabecular bone cross-sectional area (TrCSA, mm^2)	2.75 ± 0.13	1.88 ± 0.26[x]	2.70 ± 0.62	1.87 ± 0.49[a]	1.90 ± 0.15[a]
Cortical bone density (CtBD, mg cm^{-3})	1294.20 ± 5.44	1271.50 ± 12.55[x]	1297.53 ± 16.42	1276.44 ± 13.50[a]	1291.79 ± 10.48[c]
Cortical bone mineral content (CtBMC, mg mm^{-1})	6.71 ± 0.88	5.79 ± 0.57[x]	7.55 ± 0.53	5.44 ± 1.11[a]	6.50 ± 0.55[ac]
Cortical bone cross-sectional area (CtCSA, mm^2)	5.27 ± 0.61	4.46 ± 0.39[x]	5.82 ± 0.41	4.59 ± 0.29[a]	4.97 ± 0.31[ac]
Stress strain index to the reference axis *x* (xSSI)	2.80 ± 0.25	2.20 ± 0.25[x]	3.39 ± 0.38	2.50 ± 0.42[a]	2.80 ± 0.35[ad]
Stress strain index to the reference axis *y* (ySSI)	4.51 ± 0.74	3.35 ± 0.32[x]	5.31 ± 0.54	3.88 ± 0.30[a]	3.90 ± 0.34[ac]
Stress strain index to the reference axis *z* (pSSI)	5.81 ± 0.90	4.33 ± 0.50[x]	6.65 ± 0.62	4.95 ± 0.43[a]	4.90 ± 0.44[a]

Data are shown as mean ± s.d. *vs* Co-6: [x]$P < 0.01$, [y]$P < 0.05$ *vs* Co-9: [a]$P < 0.01$, [b]$P < 0.05$ *vs* StSD: [c]$P < 0.01$,[d]$P < 0.05$
Co-6, 6-week control group; St, 6-week steroid group; Co-9, 9-week control group; StSD, 9-week steroid + standard diet group; StSDBp, 9-week steroid + standard diet + bisphosphonate group.

Table 5 Ca, P, Mg, and Zn mineral contents of the mandible in Wistar rats (%)

	Co-6	*St*	*Co-9*	*StSD*	*StSDBp*
Calcium (Ca)	26.699 ± 0.929	25.899 ± 0.653^{y}	26.000 ± 0.200	25.500 ± 0.928	26.799 ± 1.150^{bc}
Phosphate (P)	13.600 ± 0.512	13.300 ± 0.200	13.800 ± 0.066	13.700 ± 0.0333	14.200 ± 0.339^{ac}
Magnesium (Mg)	0.368 ± 0.024	0.423 ± 0.009^{x}	0.454 ± 0.004	0.446 ± 0.021	0.443 ± 0.053
Zinc (Zn)	0.024 ± 0.002	0.024 ± 0.000	0.022 ± 0.001	0.021 ± 0.009	0.022 ± 0.002

Data are shown as mean ± s.d. *vs* Co-6: $^{x}P < 0.01$, $^{y}P < 0.05$; *vs* Co-9: $^{a}P < 0.01$, $^{b}P < 0.05$; *vs* StSD: $^{c}P < 0.01$.
Co-6, 6-week control group; St, 6-week steroid group; Co-9, 9-week control group; StSD, 9-week steroid + standard diet group; StSDBp, 9-week steroid + standard diet + bisphosphonate group.

method (Ferretti, 2000). All the bone parameters were significantly different between the St group and the Co-6 group; St group had markedly lower trabecular bone cross-sectional area, cortical bone density, cortical bone mineral content, and cortical bone cross-sectional area when compared with the Co-6 group ($P < 0.01$). On the other hand, while previous studies have reported a reduction in bone density by steroid administration (Wimalawansa and Simmons, 1998; Hara *et al*, 2002), our three-dimensional pQCT analysis demonstrated significantly higher trabecular bone density and trabecular bone mineral content in the St group than in the Co-6 group. These increases may be due to the high bone turnover in the growing phase, in addition to a sensitive response to steroid treatment by trabecular bone that is known to have high turnover activity, and we suspect that this may be a transient phenomenon.

In a previous study that examined the effect of steroid formulations in rats, a decrease in bone density was observed in the tibial diaphysis whereas an increase in bone density was found in the metaphysis (Wang *et al*, 2002). As glucocorticoids suppress both bone formation and bone resorption, local differences in the balance between bone formation and bone resorption may also account for the difference in bone density depending on the location of the bone. In the StSDBp group, the trabecular bone density (TrBD), cortical bone density (CtBD), cortical bone mineral content (CtBMC), and cortical bone cross-sectional area (CtCSA) were markedly higher when compared with the StSD group ($P < 0.01$), and the stress strain index with reference to the *x*-axis (xSSI) was also elevated when compared with the StSD group ($P < 0.05$). In particular, the trabecular bone density was even higher than that of the control group (Co-9 group).

Disodium (1-hydroxyethylidene) bisphosphonate possesses an alkyl base in this chemical structure and this compound belongs to etidronate (HEBP), a first generation bisphosphonate. The effects of HEBP on the bone, especially the effect on the mandible of steroid-induced osteoporosis rat models, have not been reported. In the present study, we investigated the effect of a HEBP agent on the fragile state of rat mandibular bone induced by steroid administration. Etidronate (HEBP) has a strong suppressive effect on bone resorption, and is effective in increasing bone mineral content and preventing fracture (Adachi *et al*, 2000; Kushida *et al*, 2004). Intermittent therapy with this agent actively promotes calcification and increases bone growth and bone content, and is being used clinically for the treatment of osteoporosis (Watts *et al*, 1990; Adachi *et al*, 1997). The present study showed that the bone formation process was more active than the bone resorption process in the mandibular bone, indicating that etidronate treatment promotes bone formation and subsequently increases bone strength.

Regarding the four elements that affect bone metabolism and turnover, both clinical (Ali and Siktberg, 2001) and experimental studies (Risco *et al*, 1995; Creedon and Cashman, 2001; Maki *et al*, 2002) have provided evidence that Ca, P, Mg, and Zn play important roles in bone metabolism. In the present study, bone Ca content was significantly reduced ($P < 0.05$) in the St group than in the Co-6 group. In a previous report, a considerable decrease in Ca content was found in rat femur as a result of steroid administration (Simeckova *et al*, 1985). This phenomenon is thought to be due to decreased intestinal absorption of calcium induced by steroids (Canalis and Giustina, 2001). As a result of bisphosphonate administration, Ca and P were significantly higher ($P < 0.01$) in the StSDBp group when compared with the StSD group, and these elements were significantly elevated even compared with the control (Co-9 group) ($P < 0.05$ and $P < 0.01$, respectively). These findings probably reflect an increase in bone mass through bone turnover. Zinc is related to Ca and P from the nutritional point of view, but no difference was observed in this study. Although Mg was significantly higher in the St group when compared with the Co-6 group ($P < 0.01$), the relationship with the disease state is unknown.

In the present study, steroid-treated rats that were given bisphosphonate (StSDBp group) showed significantly lower values in all bone parameters except trabecular bone density when compared with the control rats. However, when these rats were compared with steroid-treated rats that were fed with a standard diet (StSD group), trabecular bone density and all cortical bone parameters were markedly increased ($P < 0.01$), although there were no significant differences in trabecular bone mineral content and trabecular bone cross-sectional area. In addition, bone strength was significantly increased in rats treated with bisphosphonate (StSDBp group) when compared with those fed a standard diet (StSD group). These findings indicate that bisphosphonate treatment is effective in improving the

fragile state of the rat mandible in growing phase with steroid-induced osteoporosis. However, although not found in the present study, recent reports have also indicated an association between long-term treatment with bisphosphonate and intraoral bone necrosis (Merigo *et al*, 2005; Pires *et al*, 2005). Further studies are required to examine bisphosphonate therapy.

Acknowledgements

The authors would like to express their gratitude to Professor Emeritus Mitsutaka Kimura, Kyushu Dental College and Professor Yoshio Kozono, Division of Biomaterials, Department of Oral Functional Reconstruction, Kyushu Dental College and Dr. Kiichi Nonaka from the Research Laboratory of Elk Corporation for their helpful advice, and to Dr. Toru Takagi, PhD.

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