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Short-term etidronate treatment prevents glucocorticoid-induced bone debility of the mandible in growing rats

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Structured Abstract

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Objectives – To analyse the effects of short-term treatment with etidronate on the glucocorticoid-induced retardation of bone growth and deterioration of bone structure in the prepubertal rat mandible.

Materials and Methods – Fifty 5-week-old male rats were divided into five groups. Etidronate or vehicle treatment (5 mg/kg/day, daily, subcutaneous injection) was initiated after glucocorticoid administration (30 mg/kg/day, on alternate days, orally) for 6 weeks and was continued for 3 weeks. Then, bone growth was measured using lateral cephalometric analysis. Peripheral quantitative computed tomography was used to determine bone density, bone cross-sectional area and bone strength.

Results – Glucocorticoid-treated rats had significantly lower body weight, mandibular length, cortical bone density, bone strength and cross-sectional area in trabecular and cortical bone, but had significantly higher trabecular bone density than untreated rats. No significant difference in mandibular height was observed between the glucocorticoid-treated group and the untreated control group. Etidronate treatment improved the glucocorticoid-induced decrease in bone strength and increased density in trabecular and cortical bone above the untreated control level, but had no significant effects on the reduction in mandibular length.

Conclusion – These findings suggest that etidronate can potentially reverse the glucocorticoid-induced deterioration of internal bone structure, but has no beneficial effects on the glucocorticoid-induced retardation of bone growth in the growing rat mandible.

Key words: bisphosphonate; bone density; bone growth; glucocorticoid; growing rats

Introduction

Glucocorticoids (GCs) are widely used to treat inflammatory and autoimmune conditions such as severe asthma and systemic lupus erythematosus because of their immunosuppressive and anti-inflammatory actions (1). However, clinical studies have reported that a high-dose, long-term GC therapy leads to a decrease in bone mass and an increase in the risk of bone fracture (2, 3). In particular, juvenile bone tissues are sensitive

to GCs because the turnover rate is faster than in adult bone tissues; thus, secondary osteoporosis in children progresses quickly and growth disorders caused by GC therapy pose a major problem (4, 5). The number of children in Japan suffering from asthma has more than tripled in the last 20 years and the use of GCs for asthma patients has also increased (6). Furthermore, growth retardation was found in 35% of children with asthma who had taken GCs dairy for more than 2 years (7). Currently, children are often treated with calcium and vitamin D supplementation, but these do not provide beneficial effects over the long term (8).

In recent years, bisphosphonates have been used as powerful inhibitors of bone resorption and shown to be effective clinically for treating metabolic bone disorders, such as Paget's disease (9), tumour bone metastasis (10) and various types of osteoporosis (11–13). Bisphosphonates have been used as the first-line therapeutic agents for the treatment of GC-induced osteoporosis in adults (11–13). However, a study warned that the use of bisphosphonates in paediatric patients should be evaluated carefully and no evidence of their benefit has been presented (14); oral bone tissue bisphosphonate increased the bone mass of the mandible in 8-week-old rats, which corresponds to adolescents administered GC (15). Another study showed that the growth of the rat mandible reached its maximum at 8 weeks (16). Therefore, little is known of the response of mandibular bone structures to bisphosphonate in rats administered high-dose GC when mandibular bone growth is incomplete. This study investigated whether etidronate, a first generation bisphosphonate, reverses the negative effect of prepubertal GC administration on the mandible in young rats.

Materials and methods

Experimental animals

Fifty 5-week-old male Wistar rats weighing approximately 135 g were purchased from Seac Yoshitomi (Fukuoka, Japan). Each rat was housed individually in a small cage under a 12-h light:dark cycle at a constant temperature of 22 ± 1°C and a humidity of 50 ± 5% and had free access to a standard powder diet containing calcium 480 mg/100 g (Oriental Yeast, Tokyo, Japan), as described in Kimura *et al.* (15). All rats had been fed this diet since weaning. Distilled water was available *ad libitum*.

The rats were divided randomly into five groups of ten: control (Cont.) 6 week, GC 6 week, Cont. 9 week, GC + vehicle and GC + etidronate [disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP)]. The experimental protocol is shown in Fig. 1. The GC, prednisolone sodium succinate (Prednine; Shionogi & Co., Osaka, Japan) was administered orally at 30 mg/kg/day on alternate days for 6 weeks to establish bone debility. EHDP (Sumitomo Pharmaceutical, Osaka, Japan) was injected subcutaneously at 5.0 mg/kg/day once daily starting immediately after the 6 weeks of GC administration. The doses of prednisolone sodium succinate and EHDP were based on the results of previous studies on growing rats (15, 17, 18). The rats were weighed on alternate days for the initial 6 weeks and then daily during the final 3 weeks. At the end of the experiment, each rat was deeply anaesthetized with diethyl ether and given a lethal injection of thiamylal sodium (Isozole; Mitsubishi Pharma, Osaka, Japan) intraperitoneally. The heads were separated into left and right halves and then fixed with a 10% neutral buffered formalin solution, after which the lower jaw was extracted. The experimental protocol was approved by the committee for the Care and Use of Laboratory Animals of Kyushu Dental College.

Lateral cephalometric analysis

After extracting the lower jaw, the soft tissues around the alveolar part of the mandibular bone were carefully detached to expose the mental formation. The lingual surface of the head was mounted in contact with the

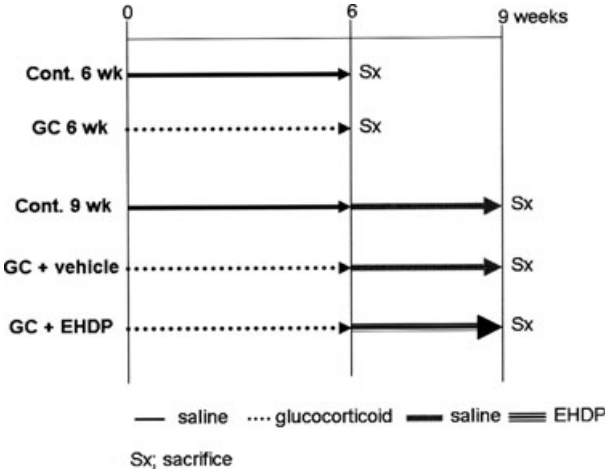

Fig. 1. Experimental protocol.

film surface, with the mental foramen set immediately under the focus. Soft radiographs were taken using a ESM-2 (Softex, Tokyo, Japan) with Fuji Softex film (Fuji Film, Tokyo, Japan) at 28 kVp and 6 mA, with a 60-s exposure and a focus-to-film distance of 60 cm. Cephalometric analysis was performed according to the method of Kiliaridis *et al*. (19). A wire of standard length (10 mm) was attached to each film and reproduced on the X-ray film. The developed radiographic images were enlarged five times and printed on photographic paper, after which the points for determination were traced and the length and height of the mandible were measured. To determine the length and height of the mandible, a line was drawn through the menton and antegonion, and a parallel line was made through the top the condyle. Then, two perpendicular lines were drawn through the anterior and posterior edges of the mandible (20) (Fig. 2). The measurements were calibrated using the image of the standard length of wire measured with callipers with an accuracy of 0.01 mm and the results were five times greater than the actual length.

Bone density and bone cross-sectional area

Peripheral quantitative computed tomography (pQCT) measurements were obtained using an XCT Research SA + series (StraTec-Medizintechnik, Pforzheim, Germany). We scanned around the centre of the first molar mesial root in the mandible at three different positions at 0.1-mm intervals, and measured the trabecular and cortical components in each with a voxel size of 0.08 mm and a height of 0.26 mm. This position contains adequate cortical and trabecular bone for observing the response to drugs.

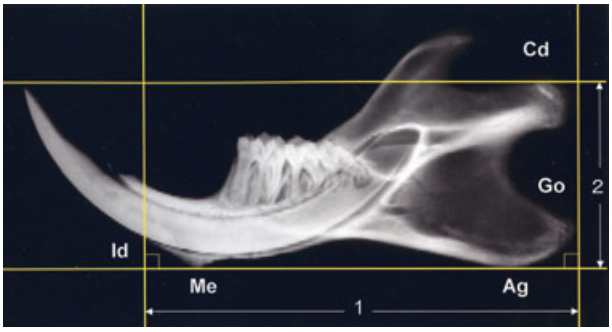

Fig. 2. To determine the length of the mandible, a tangent was drawn through the menton (Me) and antegonion (Ag), with two perpendicular lines drawn through the rear and front edges of the mandible and a parallel tangent drawn through the top of the condyle. 1, mandible length; 2, mandibular ramus height; Cd, superior-most point of the condylar head; Go, gonion; Id, infradentale (labial side).

The trabecular region was defined manually and we determined the trabecular bone density (TrBD, mg/cm^3) and trabecular bone cross-sectional area (TrCSA, mm^2). The cortical region was defined using cortical mode 1 and a threshold value of 690 mg/cm^3, and we determined the cortical bone density (CtBD, mg/cm^3) and cortical bone cross-sectional area (CtCSA, mm^2) (20–23).

Non-invasive assessment of bone strength

As a non-invasive indicator of bone strength, the strength strain index (SSI) was determined using a threshold of 690 mg/cm^3 and then assessed using the following formula: $SSI = CtBD \times Z/NCtBD$, where CtBD is the cortical bone density (mg/cm^3), Z is the section modulus (cm^3) and NCtBD is the normal physiological value for CtBD (1200 mg/cm^3) (21).

Statistical analysis

The data are presented as the mean ± SD, and the Cont. 6 week and GC 6 week groups were compared statistically using a two-sided *t*-test for unpaired samples. Multiple comparisons amongst the three 9-week treatment groups were performed using ANOVA and using Scheffé's method. The level of significance was set at 5% on a two-tailed test. The methodological procedure used for cephalometric analysis and pQCT analysis was repeated for each ten of mandibular bones, 2 weeks after the initial measurements. The error of the method was calculated using Dahlberg's formula (24).

Results

Body weight

The average body weights for each group are shown in Table 1. The final body weights in the GC administration groups were significantly lower than those in the untreated control groups ($p < 0.05$ or $p < 0.01$). Etidronate treatment did not significantly affect body weight.

Lateral cephalometric analysis

The results of the lateral cephalometric analysis are shown in Table 1. In the GC administration groups,

the mandibles were significantly shorter than in the untreated control groups ($p < 0.05$ or $p < 0.01$). Etidronate treatment did not significantly affect mandibular height. Although the mean mandibular length in the GC + EHDP group was greater than that in the GC + vehicle group, the difference was not significant. The error of the method was 0.30 mm for the length of the mandible and 0.48 mm for the mandibular ramus.

Bone density and bone cross-sectional area as measured using pQCT

The results for bone density and bone cross-sectional area are summarized in Table 2. TrCSA, CtBD and CtCSA were significantly lower in the GC 6 week group than in the Cont. 6 week group ($p < 0.05$ or $p < 0.01$), whilst TrBD was significantly higher in the GC 6 week group than in the Cont. 6 week group ($p < 0.01$). All parameters in the GC + EHDP group were significantly higher than those in the GC + vehicle group ($p < 0.05$ or $p < 0.01$). Furthermore, TrBD and CtBD were significantly higher in the GC + EHDP group than in the Cont. 9 week group ($p < 0.01$ and $p < 0.05$, respectively).

Figure 3 shows representative features in the pQCT images of the mandibular bone structure. The images of the GC 6 week and GC + vehicle groups show significant losses of trabecular and cortical bone. The degree of trabecular bone mineralization in the GC + EHDP group was significantly higher than in the GC + vehicle group. The error of the method was 1.99 mg/cm^3 for TrBD, 0.13 mm^2 for TrCSA, 1.47 mg/cm^3 for CtBD and 0.12 mm^2 for CtCSA.

Bone strength

The SSI for the reference x-, y- and z-axes (xSSI, ySSI and pSSI, respectively) is summarized in Table 3. All of

Table 1. **Body weight during the study period and mandibular bone size**

	Cont. 6 week	GC 6 week	Cont. 9 week	GC + vehicle	GC + EHDP
Initial BW (g)	133.40 ± 6.49	134.56 ± 2.47	130.47 ± 3.40	135.20 ± 3.77	134.30 ± 7.38
Final BW (g)	401.15 ± 23.51	318.97 ± 19.52*	462.87 ± 23.20	429.36 ± 14.95†	406.70 ± 32.20†
Mandibular length (mm)	117.88 ± 0.75	115.22 ± 1.22*	119.75 ± 1.22	116.03 ± 0.63††	117.56 ± 0.99†
Mandibular height (mm)	54.60 ± 1.15	52.84 ± 2.07	57.02 ± 2.35	54.36 ± 0.81	55.19 ± 0.57

BW, body weight; EHDP, disodium ethane-1-hydroxy-1,1-diphosphonate (etidronate); Cont. 6, Control 6 week; GC 6 week, 6 week glucocorticoid treatment; Cont. 9 week, Control 9 week; GC + vehicle: injected with vehicle after 6 week glucocorticoid treatment; GC + EHDP: injected with etidronate after 6 week glucocorticoid treatment.
Data are mean ± SD.
*Compared with the Cont. 6 week group, $p < 0.01$ (*t*-test).
†Compared with the Cont. 9 week group, $p < 0.05$; ††Compared with the Cont. 9 week group, $p < 0.01$ (ANOVA and Scheffe's test).
Note that mandibular length and height are ×5 the actual size.

Table 2. **Bone density and bone cross-sectional area of mandible**

	Cont. 6 week	GC 6 week	Cont. 9 week	GC + vehicle	GC + EHDP
Trabecular bone density (TrBD, mg/cm^3)	517.63 ± 44.99	662.43 ± 77.26**	550.00 ± 60.59	597.75 ± 70.96	675.95 ± 35.09††,‡
Trabecular cross-sectional area (TrCSA, mm^2)	2.26 ± 0.46	1.92 ± 0.89*	3.04 ± 0.38	1.87 ± 0.16††	2.48 ± 0.33††,‡‡
Cortical bone density (CtBD, mg/cm^3)	1258.83 ± 14.63	1244.75 ± 9.74**	1271.65 ± 9.62	1267.48 ± 17.00	1280.67 ± 9.64†,‡‡
Cortical bone cross-sectional area (CtCSA, mm^2)	4.88 ± 0.39	3.86 ± 0.14**	4.89 ± 0.18	4.27 ± 0.22††	4.48 ± 0.35††,‡

Cont. 6, Control 6 week; GC 6 week, 6 week glucocorticoid treatment; Cont. 9 week, Control 9 week; GC + vehicle: injected with vehicle after 6 week glucocorticoid treatment; GC + EHDP: injected with etidronate after 6 week glucocorticoid treatment; EHDP, disodium ethane-1-hydroxy-1,1-diphosphonate (etidronate).
Data are mean ± SD.
*Compared with the Cont. 6 week group, $p < 0.05$; **Compared with the Cont. 6 week group, $p < 0.01$ (*t*-test).
†Compared with the Cont. 9 week group, $p < 0.05$; ††Compared with the Cont. 9 week group, $p < 0.01$ (ANOVA and Scheffe's test).
‡Compared with the GC + vehicle group, $p < 0.05$; ‡‡Compared with the GC + vehicle group, $p < 0.01$ (ANOVA and Scheffe's test).

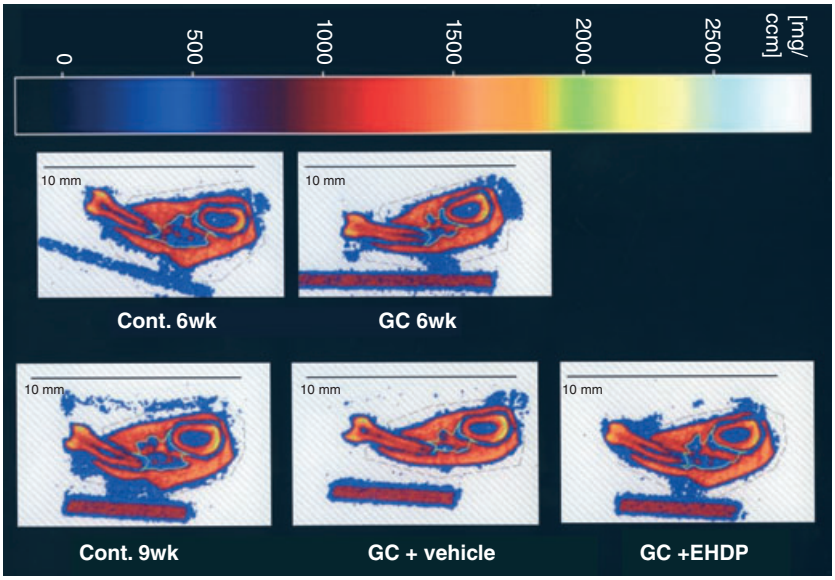

Fig. 3. Peripheral quantitative computed tomography of Wistar rat mandibles. Representative images of the Cont. 6 week, GC 6 week, Cont. 9 week, GC + vehicle and GC + EHDP groups are shown. The trabecular bone regions are enclosed by green lines. The trabecular and cortical bone regions in the GC 6 week group clearly decreased compared to the Cont. 6 week group. EHDP treatment increased the trabecular bone area and the degree of trabecular bone mineralization, as depicted in orange. In addition, the cortical bone area increased compared to the GC + vehicle group. Cont. 6, Control 6 week; GC 6 week, 6 week glucocorticoid treatment; Cont. 9 week, Control 9 week; GC + vehicle: injected with vehicle after 6 week glucocorticoid treatment; GC + EHDP: injected with etidronate after 6 week glucocorticoid treatment; EHDP, disodium ethane-1-hydroxy-1,1-diphosphonate.

Table 3. **Strength strain index of mandible**

	Cont. 6 week	GC 6 week	Cont. 9 week	GC + vehicle	GC + EHDP
xSSI	2.18 ± 0.28	1.70 ± 0.95*	2.56 ± 0.32	2.14 ± 0.20**	2.38 ± 0.38***
ySSI	4.30 ± 0.62	2.78 ± 0.19*	4.28 ± 0.22	3.16 ± 0.25**	3.33 ± 0.39**
pSSI	5.32 ± 0.78	3.56 ± 0.21*	5.42 ± 0.36	4.08 ± 0.29**	4.45 ± 0.53**,***

EHDP, disodium ethane-1-hydroxy-1,1-diphosphonate (etidronate); xSSI, strength strain index to the reference axis *x*; ySSI, strength strain index to the reference axis *y*; pSSI, strength strain index to the reference axis *z*; Cont. 6, Control 6 week; GC 6 week, 6 week glucocorticoid treatment; Cont. 9 week, Control 9 week; GC + vehicle: injected with vehicle after 6 week glucocorticoid treatment; GC + EHDP: injected with etidronate after 6 week glucocorticoid treatment.

Data are mean ± SD.

*Compared with the Cont. 6 week group, $p < 0.01$ (*t*-test); **Compared with the Cont. 9 week group, $p < 0.01$; ***Compared with the GC + vehicle group, $p < 0.05$ (ANOVA and Scheffe's test).

the SSI parameters were significantly lower in the GC 6 week group than in the Cont. 6 week group ($p < 0.01$), whereas the values for xSSI and pSSI were significantly greater in the GC + EHDP group than in the GC + vehicle group ($p < 0.05$). Furthermore, xSSI in the GC + EHDP group reached the level of the Cont. 9 week group.

Discussion

The pharmacological actions of GCs on bone metabolism have not been fully elucidated. An *in vitro* examination found that GCs suppress osteoblastogenesis in bone marrow and promote the apoptosis of osteoblasts and osteocytes, leading to decreased bone formation (25) and increased bone resorption by extending the life span of osteoclasts (26). In our study, 30 mg/kg of prednisolone were administered for 6 weeks to cause growth retardation, bone loss and bone debility in growing rats. Previous studies demonstrated that oral prednisolone doses of 30 or 100 mg/kg and experimental periods of 6 or 8 weeks inhibited bone formation in rats. We chose the minimum dose of prednisolone and shortest period of administration based on these studies (15, 17).

Several studies have reported that GC treatment in growing animals reduces body weight and longitudinal bone growth (27, 28). In our study, GCs resulted in a reduction in body weight and mandibular length. Therefore, we believe that the retardation in sagittal mandibular growth resulted from generalized growth retardation owing to GC administration. However, GC had no significant effect on the mandibular ramus height, although the mean mandibular height was smaller in the GC 6 week group than in the Cont. 6 week group. These findings were consistent with the findings of Davidovitch (29), who demonstrated that although excess GC had more suppressive effects on tibial growth, it had little effect on growth of the mandibular condyle, which may result because the cartilages in these two sites have different embryonic origins and different modes of growth. In addition, a recent study also found that 6 weeks of GC administration significantly decreased mandibular length and height (membranous ossification), while condylar length and height (endochondral ossification) did not change in young rats (27). Growth hormone and insulin-like growth factor (IGF-I) play major roles in condylar growth regulation (30). But, several studies have demonstrated that functional factors such as masticatory function, rather than hormonal factors which play a major role in mandibular condylar growth regulation in growing rats (31, 32). Therefore, we believe that these observations resulted from the synergistic effect of functional factors and extensive effects of hormonal factors on the ossification of condylar cartilage that exceeded the suppressive effect of GC.

The mandible grows by not only via endochondral ossification such as in the mandibular condyle, but also via membranous ossification in the mandibular body and mandibular angle. The mandibular body contains adequate trabecular and cortical bone. Many animal studies have measured the bone in the mandibular first molar region and the results are highly reproducible (20–23). However, little is known of the relationship between mandible growth and bone density following GC and bisphosphonate administration in rats. Therefore, in the pQCT analysis, we chose this position to clarify the difference in the response to these drugs on trabecular and cortical bone in the rat mandible. Despite using pQCT, the trabecular bone in the mandibular condyle and mandibular angle cannot be measured completely in rats. However, these positions are growth centres, so additional studies are needed. Furthermore, for analysing bone strength, we used SSI which is determined by the non-invasive mechanical analysis of CtBD and modulus sections. Ferretti *et al.* (33) demonstrated that SSI values and a three-point bending test had a high correlation for the long bones in animals, and SSI could be used as an indicator of bone strength. In the pQCT analysis, GC administration resulted in decreases in TrCSA, CtBD, CtCSA, xSSI, ySSI and pSSI, but a significant increase in TrBD. In humans (2, 3) and animals (17, 34), high-dose GC generally causes a decrease in bone mineral density. By contrast, several histomorphometric animal studies have reported that GCs increase the trabecular bone mass of the tibial metaphysis, which might be because of altered endochondral ossification (35, 36). Our findings regarding the areas of mandibular membranous ossification are consistent with these previous studies. Dempster *et al.* (37) reported that GC treatment increased osteoclast apoptosis in rats. Furthermore, we used pQCT, which measures the degree of bone mineralization rather than bone mineral density. Therefore, we postulate that GC inhibited bone formation and bone resorption, resulting in an increase in trabecular bone mineralization. Consequently, high-dose GC administration reduced trabecular and cortical bone mass and bone strength and the suppressive effect of GC was greater in cortical bone than in trabecular bone.

Bisphosphonates not only inhibit osteoclast function and induce apoptosis in osteoclasts (38), but also prevent apoptosis in osteoblasts (39). In addition, bisphosphonates promote the differentiation of rat osteoblasts (40). Etidronate can be incorporated into non-hydrolysable analogues of adenosine triphosphate metabolically, and the intracellular accumulation of these metabolites is likely to inhibit osteoclast function (41).

In this study, we investigated whether treatment with etidronate can reverse the insufficient bone growth and bone mass in the mandible in young rats following GC administration. Etidronate treatment did not improve the GC-induced reduction in body weight and sagittal growth of the mandible, although the mean mandibular length was greater in the EHDP group than in the vehicle group. Therefore, we believe that EHDP does not protect against the GC-induced growth retardation of bone throughout the body, including the mandible

or mandibular growth may be almost completed at 11 weeks old of age when EHDP therapy was initiated, or both.

The pQCT analysis showed that etidronate treatment increased the TrCSA, CtBD and CtCSA in GCs-treated rats compared with the vehicle treatment group, suggesting that etidronate inhibited the GC-induced loss of trabecular and cortical bone mass. Furthermore, the TrBD and CtBD with etidronate treatment were significantly higher than in the untreated controls. Iwamoto *et al.* (42) reported that etidronate treatment markedly decreased bone turnover of the lumbar vertebral body in orchidectomized rats. Therefore, we believe that etidronate reduced bone resorption and lengthened the bone formation period by slowing the bone turnover rate, resulting in an increase in the degree of mineralization of trabecular and cortical bone to above normal.

In addition, the etidronate-treated group had significantly higher xSSI and pSSI values compared with the vehicle group, and the xSSI reached the untreated control level. Kimura *et al.* (15) reported that 3-weeks of etidronate treatment had a beneficial effect on the GC-induced decrease in bone strength, but did not fully restore the young adult rat mandible. These findings suggest that the effect of etidronate treatment on the GC-induced bone debility decreased in an age-dependent manner in growing rats.

Thus, 6 weeks of GC administration resulted in growth retardation (mandibular length), bone loss and bone debility of the mandible in pre-pubertal rats. A recent animal study revealed GC-induced root resorption during orthodontic tooth movement (43). Furthermore, in our results, 3 weeks of vehicle treatment did not allow recovery of the GC-induced decrease in bone strength of the mandible, suggesting that the commencement of orthodontic and surgical treatments immediately after the cessation of GC administration should be avoided.

Short-term etidronate therapy can potentially reverse the GC-induced bone debility and loss of mass, although it did not have significant effect on the growth of the mandible in growing rats, suggesting that these problems should be solved by orthodontic or surgical treatment. However, Adachi *et al.* (44) reported that bisphosphonates inhibit orthodontic tooth movements by inhibiting the activity of and decreasing the number of osteoclasts in rats. Furthermore, recent studies have reported cases of osteonecrosis of the mandible associated with bisphosphonate, although we did not see any osteonecrosis in our study. Most cases of jaw osteonecrosis occurred in patients receiving therapy with nitrogen-containing bisphosphonates, such as pamidronate and zoledronic acid (45). Very few studies have reported on jaw osteonecrosis associated with non-nitrogen-containing bisphosphonates such as etidronate, indicating that etidronate has a comparatively low risk of osteonecrosis of the jaw compared with nitrogen-containing bisphosphonates. However, when we consider the safety of young patients, orthodontic or surgical treatment should be avoided during bisphosphonate therapy and commenced after etidronate therapy is over.

Clinical relevance

High-dose GC administration induced growth retardation, bone loss and bone debility in the growing rat mandible. Therefore, orthodontic and surgical treatments involving excess invasion of oral bone tissues should be avoided in young patients receiving GC therapy. Etidronate treatment for 3 weeks inhibited the GC-induced decrease in bone mass and improved the bone debility in growing rats. Systemic treatment with short-term etidronate thus reversed the GC-induced osteoporosis on oral osseous tissues in young patients. However, the growth disorders induced by GC administration were not improved therefore, patients who received GC administration may be recommended to commence orthodontic or surgical treatment after etidronate therapy is over.

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