<Original Article>

Relationship Between the Dose of Clodronate and Serum Level of Alkaline Phosphatase, Calcium, and Phosphate During Orthodontic Tooth Movement

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• Abstract

Objective : To evaluate the relationship between the dose of Clodronate and serum level of alkaline phosphatase (ALP), calcium (Ca), and phosphate (PO4) during orthodontic tooth movement

MaterialS and MethodS: A total of 18 sex-matched Wistar rats (weight=180~230g, mean age=8 weeks) were allocated into the 2.5mM Clodronate (2.5C) group, 10mM Clodronate (10C) group, or control group (n=6 for each group). After the application of a nickel-titanium closed coil spring (force of 60g) between the upper central incisors and first molars (UFM), 2.5C, 10C, or saline was injected every third day into the subperiosteum of the alveolar bone adjacent to UFM for the experimental and control groups. The animals were sacrificed 17 days later. Trunk blood was quickly collected into a heparinized tube and centrifuged at 2,000 rpm for 20 min. The plasma was used for the biochemical assays of the serum level of ALP, Ca, and PO4. Kruskall-Wallis test and Mann-Whitney test with Bonferroni correction were performed for the statistical analyses.

Results : Dose-dependent increase in the level of ALP ($P<0.01$) and decrease in the level of Ca ($P<0.001$) were observed among the control, 2.5C, and 10C groups. Although there was no significant difference in PO4 between the 2.5C and 10C groups, the 10C group showed a significantly higher level of PO4 than the control group ($P<0.01$).

Conclusion : Since Clodronate induced significant dose-dependent change in the serum level of ALP, Ca, and PO4 during orthodontic tooth movement, orthodontists should consider these biochemical markers not only as a diagnostic tool for bone turnover rate but also as a monitoring tool for orthodontic tooth movement.

• Key word : Clodronate, Orthodontic tooth movement, Alkaline phosphatase, Calcium, Phosphate

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INTRODUCTION

Bisphosphonates (BPs) are widely used in the treatment of bone disorders such as osteoporosis, Paget's disease, and bone tumors[1] because they wield direct toxic effects on the osteoclasts[2,3] and enable the indirect inhibition of osteoclast-stimulating factors released from osteoblasts[4,5]. BPs also affect and regulate the functions of the different cellular components of bone such as osteocytes and macrophages[6].

Biochemical markers of bone turnover such as alkaline phosphatase (ALP), calcium (Ca), and phosphate (PO_4) can be used to monitor the response to anti-osteoporotic therapy[7~12].

Alkaline phosphatase (ALP) is a membrane-bound metalloenzyme and a marker of osteoblastic activity[13-17] . With regard to the effects of BPs on ALP activity, Igarashi, et al[18] reported that BPs at concentrations of 2.5 ㎛ or higher caused an increase in ALP activity followed by the inhibition of PGE2 production by osteoblasts. Klein, et al 19 stressed that a 1.0㎛ concentration of Alendronate as one of BPs was optimal in increasing ALP expression without inducing any change in the cell counts in bone marrow cell suspensions after 11 days of culturing.

The level of serum Ca can be increased by the parathyroid hormone (PTH) or PTH-like substances secreted by tumor cells[20-22]. In addition, hypocalcemia itself stimulates PTH secretion from the parathyroid gland[23-25]. In the clodronate treatment of osteoblastic metastases, Francini, et al[26] found that the serum levels and urine excretion of Ca decreased significantly, whereas the serum ALP increased progressively until the end of treatment.

Nowadays, orthodontists increasingly see adult patients who suffer from osteoporosis and use BPs regularly. Most studies on the effect of BPs on the rate of orthodontic tooth movement reported a dose-dependent decrease in the rate of orthodontic tooth movement following either topical or systemic administration of BPs[27-30]. Since a complete cessation of orthodontic tooth movement was reported as a side effect of Zoledronate (one of BPs) treatment[31], clinicians should consider this aspect for orthodontic patents who are under medication of BP therapy.

To the authors' knowledge, however, the relationship between the dose of Clodronate and biochemical markers such as alkaline phosphatase (ALP), calcium (Ca), and phosphate (PO4) during orthodontic tooth movement in animals has not been proven by other studies. Therefore, this study sought to evaluate the relationship between the dose of Clodronate and serum level of ALP, Ca, and PO4 during orthodontic tooth movements.

MATERIALS AND METHODS

The samples were 18 Wistar rats (approximate weight=180-230g, mean age=8 weeks) that were sex-matched and allocated into the 2.5mM Clodronate group (n=6, three males and three females), the 10mM Clodronate group (n=6, three males and three females), or the control group (n=6, three males and three females) to avoid age- and gender-related bias. This study was conducted based on an approved protocol, and animal care was delivered according to the guidelines established by the Institutional Animal Care and Use Committee of XXXXX National University.

Application of the nickel-titanium closed coil spring (Figure 1)

After the animals were placed under general anesthesia with Zylazine hydrochloride (40 mg/kg, Rompun®, Bayer Korea, Seoul, Korea) and Zolazepam (5 mg/kg, Virbac, Carros, France), the initial body weights and distances between the right and left upper central incisor tip and mesiobuccal cusp tip of the first molars were measured with a digital caliper

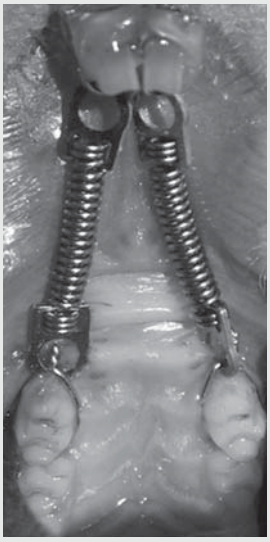

Figure 1. Application of orthodontic nickel-titanium closed coil spring.

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(Mitutoyo 700-126, Kawasaki, Japan). The grooves were prepared at the cervical line of the upper incisors using a low-speed hand piece (Vmax, Nakanishi, Inc., Tochigi, Japan) to prevent the ligatures from being dislodged.

Since several studies[32-34] have shown that 40~60g of force stimulates the substantial tooth movement of molars in rats, a nickel-titanium closed coil spring (0.10x0.30-inch, force of 60g, Ormco®, Orange, CA, USA) was connected with a 0.010-inch steel ligature wire between the right upper first molar and central incisor. After the ligatures were tied, composite resin (Transbond XT Light Cure Adhesive Paste, 3M Unitek, Monrovia, CA, USA) was cured over the ligature wire to prevent slippage. The lower incisors were reduced to prevent the breakage of the orthodontic appliance.

Injection of Clodronate

Volumetrically equivalent dosages of 2.5mM or 10mM Clodronate solution (50 ㎕; dichloromethylene diphosphonic acid, Sigma Co., St. Louis, MO, USA) were injected into the subperiosteum of the alveolar bone adjacent to the upper first molars every third day for the experimental groups using a 31-gauge ULTRA-FINE insulin syringe (BD Co., NJ, USA) during the experimental period. An equal volume of saline was injected into the same area for the control group.

Preparation of samples

Animals were sacrificed 17 days after spring placement and immediately after brief anesthesia in a carbon dioxide chamber. Trunk blood was quickly collected into a heparinized tube and centrifuged at 2,000 rpm for 20 min. The plasma was transferred into new tubes, frozen in liquid nitrogen, and stored at -80°C pending use for the assay.

Alkaline Phosphatase Activity Assay

ALP activity was measured by the time-dependent formation of p-nitrophenolate from paranitrophenyl phosphate (pNPP) (i.e., increased absorption of light at 410 nm) in alkaline solution using the ALP kit (HBI Co., Ltd., Seoul, Korea). Reactions were initiated by the addition of 30 ㎕ of plasma sample to a total reaction volume of 200 ㎕ (in clear-bottom 96-well plate) containing assay buffer (pH 10.5), 5 mM Mg acetate, and 10 mM pNPP. Optical density in 410 nm was measured initially (t=0) and after 4 min (t=4 min) on a microtiter plate reader (Powerwave X340, Bio-Tek instruments, VT, USA). ALP activity was calculated as units per liter, where 1U was defined by the conversion of 1 ㎛ol of substrate to product per minute at room temperature.

Calcium activity assay

Calcium content (HBI Co., Ltd., Seoul, Korea) was used for the calcium analysis according to the reaction of the chelate o-Cresolphthalein Complexon (OCPC), which appears red. Diluted standards and plasma samples were prepared by putting 5 ㎕ into a clear-bottom 96-well plate. The working reagent was mixed by putting 200 ㎕ into the plate. Optical density in 612 nm was measured 3 min after incubation at room temperature on a microtiter plate reader (Powerwave X340, Bio-Tek instruments, VT, USA). By measuring this biochemical optical absorption, calcium concentration can be calculated.

Phosphate activity assay

Phosphate content (HBI Co., Ltd., Seoul, Korea) was used for the phosphate analysis by utilizing the malachite green dye and molybdate, which forms a stable, colorized complex specifically with inorganic phosphate. The standard (0.28 mg/dl), distilled water, and plasma samples were prepared by putting 50 ㎕ into a clear-bottom 96-well plate. The reagent was mixed by putting 100 ㎕ into the plate. Optical density in 620 nm was measured 30 min after incubation at room temperature on a microtiter plate reader (Powerwave X340, Bio-Tek instruments, VT, USA). By measuring this biochemical optical absorption, phosphate concentration can be calculated.

For all quantitative studies, duplicate aliquots of each plasma sample were measured; the average value was used for analyses. All the assays were done with Olympus, AU 400 (Olympus, Tokyo, Japan).

Statistical Analysis

Kruskall-Wallis test and Mann-Whitney test with Bonferroni correction were conducted.

Statistical analyses were performed with the STATVIEW 4.11 J (Abacus, Berkeley, CA, USA) program.

RESULTS

Comparison of the amount of tooth movement (mm) over time (Figure 2)

Statistically significant differences were noted between the control and 2.5mM Clodronate groups and between the

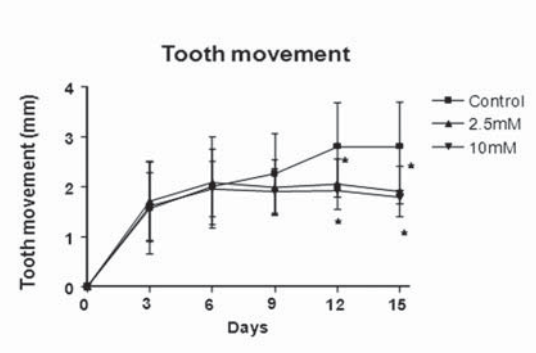

Figure 2. Comparison of the amount of tooth movement (mm) over time.

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control and 10mM Clodronate groups on the 12th and 15th days (P<0.05), respectively.

Comparison of ALP among the Control, 2.5mM Clodronate, and 10mM Clodronate groups (Table I)

There was a significant increase in the serum level of ALP in the 2.5mM Clodronate (P<.0167) and 10mM Clodronate groups (P<.0003) on day 17th compared to the control group. Moreover, the 10mM Clodronate group showed dose-dependent increase in the serum level of ALP compared to the 2.5mM Clodronate group (P<.0033).

Comparison of Ca among the Control, 2.5mM Clodronate, and 10mM Clodronate groups (Table II)

The serum level of Ca on day 17th significantly decreased in the 2.5mM Clodronate group (P<.0033) and in the 10mM Clodronate group (P<.0003) compared to the control group. Moreover, the 10mM Clodronate group showed dose-dependent decrease in the serum level of Ca compared to the 2.5mM Clodronate group (P<.0003).

Comparison of PO4 among the Control, 2.5mM Clodronate, and 10mM Clodronate groups (Table III)

The 10mM Clodronate group showed significantly high levels of PO_4 on day 17th compared to the control group (P<.0167). There was no significant difference in the serum level of PO4 between the Control and 2.5mM Clodronate groups and between the 2.5mM Clodronate and 10mM Clodronate groups on day 17th.

Table I. Comparison of ALP among the Control, 2.5mM Clodronate, and 10mM Clodronate groups

17 days after orthodontic force application	Mean	SD	p-value †	Multiple comparison ††	
				2.5mM Clodronate	10mM Clodronate
Control (N=6)	938.00	188.70	0.002 **	0.0155 ʃ	⟨0.0001 ʃʃʃ
2.5mM Clodronate (N=6)	1134.83	77.89		-	0.0028 ʃʃ
10mM Clodronate (N=6)	1389.70	88.10		0.0028 ʃʃ	-

† Kruskall-Wallis test was done. ** means P ⟨ 0.01.
†† Mann-Whitney U-test with Bonferroni correction was done. ʃ means p←0.0167, ʃ ʃ, p←0.0033, and ʃ ʃ ʃ, p ⟨ 0.0003. The data refers to Control group ⟨ 2.5mM Clodronate ⟨ 10mM Clodronate.
SD means standard deviation.

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Table II. Comparison of Ca among the Control, 2.5mM Clodronate, and 10mM Clodronate groups

17 days after orthodontic force application	Mean	SD	p-value †	Multiple comparison ††	
				2.5mM Clodronate	10mM Clodronate
Control (N=6)	12.32	0.26	0.0001***	0.0003 ʃʃ	⟨0.0001 ʃʃʃ
2.5mM Clodronate (N=6)	11.48	0.31		-	⟨0.0001 ʃʃʃ
10mM Clodronate (N=6)	10.22	0.31		⟨0.0001 ʃʃʃ	-

† Kruskall-Wallis test was done. *** means P ⟨ 0.001.
†† Mann-Whitney U-test with Bonferroni correction was done. ʃ ʃ means p ⟨ 0.0033, and ʃ ʃ ʃ, p ⟨ 0.0003. The data refers to Control group ⟨ 2.5mM Clodronate ⟨ 10mM Clodronate.
SD means standard deviation.

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Table III. Comparison of PO4 among the Control, 2.5mM Clodronate, and 10mM Clodronate groups

17 days after orthodontic force application	Mean	SD	p-value †	Multiple comparison ††	
				2.5mM Clodronate	10mM Clodronate
Control (N=6)	17.50	0.14	0.0034**	0.3664	0.0039 ∫
2.5mM Clodronate (N=6)	19.10	0.10		-	0.0365
10mM Clodronate (N=6)	25.22	4.40		0.0365	-

† Kruskall-Wallis test was done. ** means P < 0.01.

†† Mann-Whitney U-test with Bonferroni correction was done. ∫ means p < 0.0167. The data refers to (Control group, 2.5mM Clodronate) < (2.5mM Clodronate,10mM Clodronate).

SD means standard deviation.

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Discussion

Comparison of ALP among the Control, 2.5mM Clodronate, and 10mM Clodronate groups

Although Perinetti, et al[35] stressed that ALP activity should be investigated as a diagnostic tool for monitoring orthodontic tooth movement in clinical practice, different results on the ALP activity in the periodontium of teeth undergoing orthodontic tooth movement have been reported. Lilja, et al[36] and Engström, et al[37] observed that high ALP activity occurred in the tension sites, whereas enzymatic activity decreased in pressure sites in rats. In in vitro studies on human periodontal ligament cells, however, tensional stress decreased ALP activity[38]. Klein-Nulend, et al[39] and Ozawa, et al[40] observed both increases and decreases in ALP activity in animal periodontal ligament cells depending on whether compressive forces were intermittent or continuous.

King, et al[41] and Keeling, et al[42] showed that the remodeling process might be complex; in the early phases of bone remodeling, a resorption activity (3~5days) was followed by its reversal (5~7days), and then by a late phase of bone deposition (7~14days) in both tension and pressure sites of the alveolar wall. In the early phase of tooth movement, bone resorption is greater than bone deposition; in a later phase, however, resorption and deposition can become synchronous. This may be due to the high acid phosphatase activity observed in the early period of tooth movement; high levels of ALP activity have been noted after 7 days when bone deposition begins[42].

This study showed that the serum level of ALP on day 17 showed significant dose-dependent increase in the control group, 2.5mM Clodronate group, and 10mM Clodronate group (Kruskall-Wallis test, P<0.01; Mann-Whitney U-test with Bonferroni correction, C<2.5C<10C, Table I). This finding was consistent with that of Igarashi et al[18], who demonstrated that three types of BPs significantly increased ALP activity in the clonal osteoblast-like cell line MC3T3-E1 at 2.5 ㎛ or higher concentration. According to Klein, et al[19], however, structurally different BPs wielded opposing effects on ALP activity and stromal cell proliferation and cell-mediated mineralization in marrow osteoprogenitors. Although those studies[18,19] analyzed the levels of bone marker such as ALP on cells with BPs, none of them were done in vivo.

Comparison of Ca among the Control, 2.5mM Clodronate, and 10mM Clodronate groups

The findings of the serum level of Ca on day 17th showing significant dose-dependent decrease in the control group, 2.5mM Clodronate group, and 10mM Clodronate group (Kruskall-Wallis test, P<0.001; Mann-Whitney U-test with Bonferroni correction, 10C<2.5C<C, Table II) may be attributable to the fact that the inhibitory effect of Clodronate on bone resorption is dose-dependent[27-30].

Comparison of PO_4 among the Control, 2.5mM Clodronate, and 10mM Clodronate groups

This study showed that the serum level of PO_4 on day 17 significantly increased in the 10 mM Clodronate group compared to the control group (Kruskall-Wallis test, P<0.01; Mann-Whitney U-test with Bonferroni correction, (C,2.5C)<(2.5C,10C); Table III). This finding suggests that a high dose (10mM) of Clodronate significantly affected PO4 production during orthodontic tooth movement compared to the control group with orthodontic tooth movement alone.

Although the serum levels of biochemical markers of bone turnover are known to be positively correlated with the

histomorphometric parameters of bone formation[43-45], further studies are needed to confirm the osteoblast activity with the level of bone Gla-protein (BGP); the additional measurement of the hydroxyproline excretion level will also be helpful in getting a complete biochemical analysis for the assessment of the patients.

Conclusion

Since Clodronate induced significant dose-dependent change in the serum levels of ALP, Ca, and PO4 during orthodontic tooth movement, orthodontists should consider these biochemical markers not only as a diagnostic tool for bone turnover rate but also as a monitoring tool for orthodontic tooth movement.

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