Effects of Glucocorticoids on Diurnal Variations in Experimental Tooth Movement

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Abstract: Diurnal variations in bone remodeling, which are regulated by the central circadian clock in the suprachiasmatic nuclei, have previously been identified. Glucocorticoids induce a circadian rhythm in osteoclasts, causing a circadian rhythm in bone resorption. It has been reported that the extent of experimental tooth movement (ETM) also exhibits diurnal variations, but this has not been thoroughly investigated. The aim of this study was to examine the role of glucocorticoids in the diurnal variations in the extent of ETM. Male C57BL/6J mice were divided into three groups: the whole-day group (WDG); ETM was performed during both the light and dark periods, the light period group (LPG); ETM was performed during the light period (7:00–19:00), and the dark period group (DPG); ETM was performed during the dark period (19:00–7:00). Orthodontic force was applied for a total of 48 hr in all groups. A piece of an orthodontic elastic band was inserted between the right upper first and second molars (M1 and M2) according to the Waldo method. During the study period, the distance between M1 and M2 increased by 185.1±7.0 μm in the WDG, 186.0±6.6 μm in the LPG, and 152.7±9.9 μm in the DPG. The amount of ETM-induced tooth movement was significantly larger in the WDG and LPG than in the DPG. The osteoclast surface/bone surface (compressed side) and osteoclast number/bone surface (compressed side) ratios were also significantly larger in the WDG and LPG than in the DPG. Consistent with the results of osteoclast parameters, the immune reactivity of receptor activator of nuclear factor-κB ligand (RANKL) was higher in the WDG and LPG than in the DPG. Adrenalectomy eliminated the differences in osteoclast parameters, RANKL immune reactivity and the extent of ETM between the LPG and DPG. These differences were restored by the daily administration of the synthetic glucocorticoid dexamethasone to adrenalectomized mice. These results suggest that circulating glucocorticoids contribute to the diurnal variations in the osteoclast parameters which result in the diurnal variation of the tooth movement, and the diurnal variation of RANKL immune reactivity may contribute to it.

Key words: Diurnal variation, Glucocorticoids, Tooth movement, Osteoclast, Adrenalectomy

Introduction

Autonomic nervous system activity, metabolism, and hormonal secretion display circadian rhythms, which contribute to the maintenance of homeostasis in physiological functions1–9. Circadian rhythms are derived from the suprachiasmatic nucleus, which is located in the hypothalamus and controls the peripheral clock of each tissue6,7. The central clock is controlled by light stimulation, and central time information is thought to be transmitted to peripheral tissues by the sympathetic nervous system and glucocorticoids9. We and others have shown that glucocorticoids contribute to the transmission of central time information to bone tissue6,10.

Glucocorticoids, which are steroid hormones produced in the adrenal cortex, raise the blood glucose level in the liver by promoting gluconeogenesis11. Mineralocorticoids, which induce the reabsorption of sodium ions, promote potassium ion excretion, and regulate electrolyte and water levels in the body, are secreted from the adrenal cortex12. Fujihara et al.9 reported that there are diurnal variations in the expression levels of osteoclast-related genes. Adrenalectomized (ADX) mice lose such variations, but they can be restored by administering dexamethasone (DEX) to ADX mice. These results indicate that glucocorticoids influence the circadian rhythms of bone metabolism by regulating the diurnal variations in osteoclast activity.

Orthodontic force stimulates local bone remodeling, which results in tooth movement13. In studies in which an open coil was placed between the upper first molars, Igarashi et al.14 and Miyoshi et al.15 showed that to induce lateral expansion of the maxilla, applying orthodontic force during the daytime was more effective than applying it at night in rats. Their results indicated that diurnal variations are also seen in the extent of experimental tooth movement (ETM), but it is not clear whether glucocorticoids are involved in this.

The receptor activator of nuclear factor-κB ligand (RANKL) regulates osteoclast differentiation and activity, which is upregulated by ETM in periodontal ligament16–18. We have previously reported that the mRNA expression of RANKL shows circadian rhythms in bone tissues and ETM based on the Waldo method is controlled by the sympathetic nervous system8,19,20. In the Waldo method, teeth are moved by inserting an elastic band between the first and second molars. In the present study, we investigated whether the extent of ETM induced by the Waldo method exhibits diurnal variations, and we also elucidated the effects of
adrenalectomy on such tooth movement and the effects of glucocorticoid administration on such tooth movement after ADX.

**Materials and Methods**

**Animals**

Male C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were raised in our animal facility and kept under a 12-hr light/12-hr dark cycle, with lights on at 7:00 and off at 19:00. All animals were supplied with food and water ad libitum. The experimental protocols were reviewed and approved by the animal experiment committee of the School of Dentistry, Aichi Gakuin University (AGUD-415-1,2,3 and AGUD-458-1,2).

**Experimental tooth movement**

Seven- or 8-week-old mice were randomized by weight and divided into the following three groups: the whole-day group (WDG), in which orthodontic force was applied continuously for 48 hr; the light period group (LPG), in which orthodontic force was applied throughout the light period (7:00–19:00) for 4 days; and the dark period group (DPG), in which orthodontic force was applied throughout the dark period (19:00–7:00) for 4 days. Then, the mice were anesthetized using an anesthetic mixture of 0.375 mg/kg medetomidine (Medetomin, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 2 mg/kg midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan), and 2.5 mg/kg butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) (MMB), and a piece of elastic band (No. 404-136, Unitek Co., Ltd., Saint Paul, USA) was inserted between the upper right first molar (M1) and second molar (M2) according to the Waldo method. After the piece of rubber band had been inserted, atipamezole (Mepatia, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) was injected immediately to help the mice recover from the anesthesia. The mice were sacrificed via carbon dioxide inhalation at the end of the study period. The body weights of the mice were assessed every day during the ETM (FY-3000; A and D Co., Ltd, Tokyo, Japan) (Table 1-3).

**Adrenalectomy**

Using the MMB anesthetic mixture, a dorsal incision was made, and the bilateral adrenal glands were removed from 5-week-old mice. The ADX mice were supplied with food ad libitum and 0.9% NaCl to prevent hyponatremia and kept under a 12-hr light/12-hr dark cycle, with lights on at 7:00 and off at 19:00, for at least two weeks, before being subjected to ETM.

**Three-dimensional micro-computed tomography (3D-μCT) analysis**

The amount of tooth movement induced by the ETM was measured using 3D-μCT (an R-mCT μCT scanner; Rigaku Co., Ltd, Tokyo, Japan) and the TRI/3D-Bon software (Ratoc Systems, Inc., Tokyo, Japan). The maxilla was scanned using an X-ray current of 90 keV, a tube voltage of 88 µA, and an isotropic voxel size of 10-µm. In order to measure the amount of tooth movement induced by the ETM, objects were adjusted using 3D-μCT (an R-mCT μCT scanner; Rigaku Co., Ltd, Tokyo, Japan), and the TRI/3D-Bon software (Ratoc Systems, Inc., Tokyo, Japan). The amount of tooth movement induced by the ETM was measured using an anesthetic mixture of 0.375 mg/kg medetomidine (Medetomin, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 2 mg/kg midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan), and 2.5 mg/kg butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) (MMB), and a piece of elastic band (No. 404-136, Unitek Co., Ltd., Saint Paul, USA) was inserted between the upper right first molar (M1) and second molar (M2) according to the Waldo method.

**Bone histomorphometry**

The right maxilla was dissected out and fixed in 4% paraformaldehyde (EDTA) for two weeks for decalcification. Five-µm-thick occlusal sections were obtained from the region located between 50 µm and 125 µm above the furcation. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP). The number of TRAP-positive multinucleated cells was counted to determine the number of osteoclasts on the bone surface (compressed side) (the Oc.N/BS ratio) and the relative size of the osteoclast surface area compared with the bone surface area (compressed side) (the Oc.S/BS ratio), as reported previously. Osteoclasts were stained with hematoxylin and eosin (HE), and the number of osteoblasts on the bone surface (tensioned side) (Ob.N/BS) was counted. For immunohistochemistry, antigen inactivation treatment was carried out in a hot bath at 90°C for 40 min, and tissue sections were incubated with the primary antibody; i.e., anti-receptor activator of nuclear factor-κB ligand (RANKL) mouse monoclonal antibody (ab45039, Abcam Plc., Cambridge, UK) (diluted 1:100), at 4°C overnight, before being incubated with the secondary antibody; i.e., simple stain mouse MAXPO (414322, Nichirei Co., Tokyo, Japan), at room temperature for 10 min. For negative control, mouse IgG1 antibody (X0931, Agilent, Santa Clara, USA) (diluted 1:100), at 4°C over night. The number of RANKL positive cells were counted and expressed that as a percentage of the total number of cells present on the compressed side.

**Statistical analysis**

Data are shown as mean±SEM values (For body weight, data are shown as mean±SDM). Statistical analyses were conducted with the Student’s t-test or ANOVA using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, USA). Post-hoc analyses, which were performed using Tukey’s multiple comparisons test, were conducted on parameters that exhibited interactive effects, as described previously. P-values of <0.05 or <0.01 were considered to be statistically significant.

**Results**

**The amount of ETM induced via the Waldo method exhibited diurnal variations**

To investigate the diurnal variations in the amount of ETM induced using the Waldo method, mice were kept under a 12-hr light/12-hr dark cycle for at least two weeks before tooth movement was induced and then were divided into three groups (the WDG, LPG, and DPG). In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days (force was applied for a total of 48 hr). In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days (force was applied for a total of 48 hr). The amount of tooth movement achieved was measured using 3D-μCT (Fig. 1a). The mean increase in the distance between M1 and M2 induced by the ETM (hereafter referred to as the tooth movement distance) was 185.1±7.0 µm in the WDG, 186.0±6.6 µm in the LPG, and 152.7±9.9 µm in the DPG. In the DPG, the mean tooth movement distance was 17.5% lower than that seen in the WDG and 17.9% lower than that seen in the LPG (Fig. 1b). There was no significant difference in the mean tooth movement distance between the WDG and LPG.

To examine the role played by osteoclasts in the diurnal variations in the tooth movement distance, we measured the Oc.S/BS and Oc.N/BS ratios as osteoclast parameters. The mean Oc.S/BS ratio was 10.8±0.9% in the WDG, 16.0±1.2% in the LPG, and 4.2±1.6% in the DPG (Fig. 1c). In the DPG, the mean Oc.S/BS ratio was 61.2% lower than that seen in the WD and 73.7% lower than that seen in the LP (Fig. 1b). The mean Oc.N/BS ratio was 3.2±0.4% in the WDG, 4.4±0.5% in the LPG, and 1.2±0.5% in the DPG. In the DPG, the mean Oc.N/BS ratio was 62.5% lower than that seen in the WD and 72.7% lower than that seen in the LGP (Fig. 1c). The mean Oc.N/BS ratio was 3.0±0.5% in the WDG, 4.9±1.0% in the LPG, and 5.3±0.6% in the DPG.
There were no significant differences in the mean Ob.N/BS between any of the three groups (Fig. 1d). The immunohistological examinations showed that there were $14.4\pm0.7/\text{mm}^2$, $14.9\pm0.8/\text{mm}^2$, and $7.7\pm0.7/\text{mm}^2$ RANKL-positive cells in the WDG, LPG, and DPG, respectively (Fig. 1e). In the DPG, the number of RANKL-positive cells was 46.5% lower than that seen in the WDG and 48.3% lower than that seen in the LPG (Fig. 1e).

The ETM led to reductions in body weight in the LPG and DPG. The mean body weight of the mice in the WPG was higher than those of the mice in the LPG and DPG (Table 1). There was no significant difference in body weight between the LPG and DPG (Table 1).

Figure 1. Diurnal variations were detected in the tooth movement distance achieved using the Waldo method. (a) 3D-μCT images of the right upper molars. Scale bars: 500 μm. (b) Mean tooth movement distance. The WDG and LPG exhibited greater tooth movement than the DPG. (c) Representative images of the staining of tartrate-resistant acid phosphatase (TRAP) in osteoclasts. Scale bars: 50 μm. The osteoclast (stained red) surface/bone surface (Oc.S/BS) (%) and osteoclast number/bone surface (Oc.N/BS) (number/mm) ratios were analyzed. Both ratios were lower in the DPG than in the WPG and LPG. (d) Representative images of the hematoxylin-eosin (HE) staining of osteoblasts. The osteoblast number/bone surface (Ob.N/BS) (number/mm) ratio was analyzed. There were no significant differences in the Ob.N/BS ratio between the groups. Scale bars: 50 μm. (e) Immunohistochemistry of receptor activator of nuclear factor-κB ligand (RANKL). The ratio of RANKL-positive cells (%) was lower in the DPG than in the WPG and LPG. Arrow indicates RANKL-positive cells. IgG: negative control. Scale bars: 50 μm. In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days. In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days. Six, six, and seven animals were used in the WDG, LPG, and DPG, respectively. The error bar indicates the mean±SEM. * statistically significant difference vs. the WDG at $p<0.05$ and $p<0.01$, respectively, according to ANOVA; † statistically significant difference vs. the LPG at $p<0.05$ and $p<0.01$, respectively, according to ANOVA; T: teeth, B: bone.
The diurnal variations in the tooth movement distance disappeared in the adrenalectomized (ADX) mice. To elucidate the effects of endogenous glucocorticoids on the diurnal variations in the tooth movement distance, mice were subjected to adrenalectomy before being kept under a 12-hr light/12-hr dark cycle for two weeks, and then ETM was conducted. The mean tooth movement distance was measured using 3D-μCT (Fig. 2a). The mean tooth movement distance was 141.0±3.6 μm in the WDG, 149.4±4.6 μm in the LPG, and 158.7±6.2 μm in the DPG. There were no significant differences in the mean tooth movement distance between the three groups (Fig. 2b). The mean Oc.S/BS (%) and Oc.N/BS (number/mm) ratios were analyzed. There were no significant differences between the groups. Scale bars: 50 μm. (d) Representative images of the staining of tartrate-resistant acid phosphatase (TRAP) in osteoclasts. The Oc.S/BS (%) and Oc.N/BS (number/mm) ratios were analyzed. There were no significant differences between the groups. Scale bars: 50 μm. (e) Immunohistochemistry of RANKL. There were no significant differences between the groups. Arrow indicates RANKL-positive cells. IgG: negative control. Scale bars: 50 μm. In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days. In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days. Six, seven, and seven ADX animals were used in the WDG, LPG, and DPG, respectively. The error bar indicates mean±SEM. T: teeth, B: bone.
Sae Kusafuka et al.: Glucocorticoids Regulate the Diurnal Variations in Tooth Movement

Table 1. Body weight (g) changes after tooth movement

|       | Before   | After   |
|-------|----------|---------|
| WDG   | 20.9±0.7 | 20.3±0.7|
| LPG   | 21.8±0.4 | 18.9±0.7**|
| DPG   | 20.8±0.7 | 18.2±1.0**|

In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days. In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days. Data are shown as mean±SDM values. *p<0.01 vs. before the experimental tooth movement, according to the Student’s t-test; ** statistically significant difference vs. the WDG at p<0.05 and p<0.01, respectively, according to ANOVA

The injection of DEX restored the diurnal variations in the tooth movement distance in the ADX mice

The adrenal glands secrete glucocorticoids, mineral corticoids, and

Figure 3. The injection of DEX restored the diurnal variations in the tooth movement distance induced in ADX mice. (a) 3D-μCT images of the right upper molars. Scale bars: 500 μm. (b) Mean tooth movement distance. Greater tooth movement was seen in the LPG than in the DPG. (c) Representative images of the staining of TRAP in osteoclasts. Both osteoclast ratios were lower in the DPG than in the LPG. Scale bars: 50 μm. (d) Representative images of the HE staining of osteoblasts. Scale bars: 50 μm. The Oc.N/BS (number/mm) ratio was analyzed. There were no significant differences between the groups. (e) Immunohistochemistry of RANKL. The ratio of RANKL-positive cells was lower in the DPG than in the LPG. Arrow indicates RANKL-positive cells. IgG: negative control. Scale bars: 50 μm. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days. In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days. Eight and nine animals were used in the LPG and DPG, respectively. The error bar indicates the mean±SEM. *p<0.05 and p<0.01, respectively, according to the Student’s t-test; T: teeth, B: bone.
In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days (force was applied for a total of 48 hr). In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days (force was applied for a total of 48 hr). Data are shown as mean±SDM values. None of the parameters analyzed with ANOVA exhibited significant differences. *p<0.01 vs. before the tooth movement, according to the Student’s t-test.

In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days (force was applied for a total of 48 hr). In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days (force was applied for a total of 48 hr). Data are shown as mean±SDM values. None of the parameters analyzed with ANOVA exhibited significant differences. *p<0.01 vs. before the tooth movement, according to the Student’s t-test.

Table 3. Body weight (g) changes after tooth movement in dexamethasone (DEX)-injected adrenalectomy (ADX) mice

|       | Before | After |
|-------|--------|-------|
| LPG   | 23.6±1.8 | 20.6±2.0 * |
| DPG   | 24.0±1.4 | 20.5±1.6 * |

In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days (force was applied for a total of 48 hr). In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days (force was applied for a total of 48 hr). Data are shown as mean±SDM values. None of the parameters analyzed with ANOVA exhibited significant differences. *p<0.01 vs. before the tooth movement, according to the Student’s t-test.

Table 2. Body weight (g) changes after the tooth movement in ADX mice

|       | Before | After |
|-------|--------|-------|
| WDG   | 23.9±1.3 | 22.4±1.7 |
| LPG   | 23.9±1.1 | 21.6±1.1 * |
| DPG   | 22.1±2.0 | 21.4±1.8 |

In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days (force was applied for a total of 48 hr). In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days (force was applied for a total of 48 hr). Data are shown as mean±SDM values. None of the parameters analyzed with ANOVA exhibited significant differences. *p<0.01 vs. before the tooth movement, according to the Student’s t-test.

In the WDG, force was applied continuously for 48 hr. In the LPG, force was only applied during the light period (7:00–19:00) for 4 days (force was applied for a total of 48 hr). In the DPG, force was only applied during the dark period (19:00–7:00) for 4 days (force was applied for a total of 48 hr). Data are shown as mean±SDM values. None of the parameters analyzed with ANOVA exhibited significant differences. *p<0.01 vs. before the tooth movement, according to the Student’s t-test.

**Discussion**

The present study revealed that: 1) There were diurnal variations in the tooth movement distance, 2) The ablation of the adrenal glands abrogated the diurnal variations in the tooth movement distance, 3) The administration of DEX restored the diurnal variations in the tooth movement distance and 4) The changes of osteoclast parameters and RANKL immune reactivity were consistent with the tooth movement distance. These results indicate that glucocorticoids play a role in the mediation of the signaling responsible for the diurnal variations in the osteoclast activity resulting in the diurnal variation of the tooth movement, and the diurnal variation of RANKL immune reactivity may contribute to it.

In this study, we showed for the first time that diurnal variations exist in the tooth movement distance achieved using the Waldo method. The amount of tooth movement achieved was much larger in the LPG than in the DPG, which is consistent with the findings of previous studies. As mice are nocturnal animals, these results suggest that performing ETM during the rest period is more effective than performing it in the active period. Although tooth movement-induced weight loss was observed, there were no significant differences in body weight between the LPG and DPG, suggesting that the observed differences in tooth movement were not due to the effects of weight loss. In addition, the mean body weight of the mice after the ETM was lower in the LPG and DPG than in the WDG, which might have been due to the differences in the number of total doses of anesthetic that were administered and the length of the experimental period. Histological examinations also showed that osteoclast parameters, such as the Oc.S/BS and Oc.N/BS ratios, exhibited larger values in the light period than in the dark period, which suggests that tooth movement occurred due to increased osteoclast activity and/or osteoclastogenesis. Bone resorption and calcium release display diurnal variations and are most active when animals are resting. It is considered that the diurnal variations in such osteoclast activity and/or osteoclastogenesis contribute to the diurnal variations in the tooth movement distance.

Miyoshi et al. reported that intermittent force moves teeth more effectively than sustained force. In our study, no significant differences in the tooth movement distance between the WDG and the LPG. However, there was significant difference in Oc.S/BS between the WDG and the LPG. This indicates that intermittent force induce Oc.S/BS more effectively than sustained force. The partial discrepancies between Miyoshi’s and our experimental results might have been due to differences in the animal species and orthodontic devices used, but further investigation of these issues is needed. They also reported that on day 7, greater osteogenesis was seen in the LPG than in the DPG, as was found for bone resorption. In our experiments, there were no such differences in osteoblast parameters, but this could be explained by the short experimental period (4 days). These results suggest that ETM using the Waldo method might not have any effect on 4 days tooth movement-induced bone formation changes.

Fujihara et al. reported that glucocorticoids mediate signals from the central circadian clock to peripheral bone tissue. As glucocorticoids are secreted from the adrenal gland, we investigated whether adrenalectomy affected the diurnal variations in the tooth movement distance induced by ETM. As a result, it was found that adrenalectomy eliminated the differences in osteoclast parameters, RANKL immune reactivity and the diurnal variations in the tooth movement distance between the LPG and DPG. As adrenalectomy causes the loss of glucocorticoids, mineral corticoids, and sex steroids, we examined whether the daily administration of a synthetic DEX affected the diurnal variations in the tooth movement distance in ADX mice. The administration of DEX restored the differences in osteoclasts parameters, RANKL immune reactivity and diurnal variations in the tooth movement distance came between the LPG and DPG in the ADX mice. These findings suggest that glucocorticoids transmit central circadian rhythms to peripheral osteoclasts, which results in the diurnal variations in the tooth movement distance induced by ETM. In addition, our data indicates that glucocorticoids also transmit central circadian rhythms to RANKL produced cells such as osteoblasts and osteocytes.

In summary, these results suggest that circulating glucocorticoids contribute to the diurnal variations in the osteoclast parameters which result in the diurnal variation of the tooth movement, and the diurnal variation of RANKL immune reactivity may contribute to it. In this study, it was clarified that an efficient orthodontic tooth movement method that takes diurnal rhythms into consideration and the distur-
bance of circadian rhythms due to stress and irregular lifestyles could affect orthodontic tooth movement.

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Conflict of Interest
The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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