

# Emotional stress and orthodontic tooth movement: effects on apical root resorption, tooth movement, and dental tissue expression of interleukin-1 alpha and calcitonin gene-related peptide immunoreactive nerve fibres in rats

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**SUMMARY** The aim of the study was to investigate the effect of emotional stress on apical root resorption (ARR) and tooth displacement during orthodontic tooth movement in rats. A further area of interest was to evaluate if the expression of interleukin-1 alpha (IL-1 $\alpha$ ) as well as the density and distribution of peptidergic nerve fibres immunoreactive to calcitonin gene-related peptide (CGRP) in the periodontal ligament (PDL) are associated with possible stress-induced changes in root resorption and tooth movement.

A total of 52 male Wistar rats, aged 6 weeks, were divided in three experimental and one control group ( $n=4$ ). Group 1 had orthodontic tooth movement and received foot shocks (OTMS;  $n=16$ ), group 2 had orthodontic tooth movement but received no foot shocks (OTMNS;  $n=16$ ), and group 3 had no orthodontic tooth movement and received foot shocks (NOTMS;  $n=16$ ). Each group was further divided into four subgroups ( $n=4$ ), corresponding to the period of the experiment, i.e. 3, 7, 13, and 21 days. At the end of each experimental period, the blood samples were taken, the animals were sacrificed, and the jaws excised, demineralized, and processed for immunocytochemistry. One-way analysis of variance was used to detect inter-group differences for all investigated variables. CGRP immunopositive nerve fibres were evaluated qualitatively.

All the experimental groups demonstrated higher corticosterone levels than the control group, suggesting a stress-induced experience by orthodontic treatment *per se*. The OTMS group had the least amount of cellular cementum throughout the experimental periods and showed significant reduction in tooth displacement, especially at 3 and 7 days. No obvious changes were observed in the dental tissue expression of IL-1 $\alpha$  and CGRP immunoreactive nerve fibres between the stressed and non-stressed orthodontically treated groups.

## Introduction

The aetiology and predictability of root resorption have been a matter of extensive research and a number of review articles summarize the knowledge on this topic (Brezniak and Wasserstein, 1993, 2002; Killiany, 1999; Hartsfield *et al.*, 2004; Segal *et al.*, 2004; Krishnan, 2005). Taking into account that the risk of apical root resorption (ARR) with fixed appliances is significantly greater than with removable appliances (Linge and Linge, 1983), numerous studies have attempted to identify the mechanotherapy that would reduce, or even eliminate root resorption. Different types of appliances, treatment modalities, and force regimens have been associated with different amounts of resorption and the results are often conflicting (Linge and Linge, 1983; Beck and Harris, 1994; Janson *et al.*, 2000; Mavragani *et al.*, 2000; Sameshima and Sinclair, 2001; Maltha *et al.*, 2004). A meta-analysis of the treatment-related factors of ARR revealed a strong correlation between root resorption and total apical displacement and treatment duration (Segal *et al.*, 2004). Age, gender, malocclusion type, and root shape

have also been positively or negatively associated with root resorption (Brezniak and Wasserstein, 1993, 2002).

The systemic condition of the patient has received less attention in the literature. A few studies have shown an increased risk of root resorption in patients with chronic asthma and/or allergies (McNab *et al.*, 1999; Owman-Moll and Kuroi, 2000). Endocrine disturbances and immune responses have also been mentioned as possible contributing factors (Goultschin *et al.*, 1982; Ng *et al.*, 1990). So far, emotional stress has not been addressed as a potential factor, although it is quite clear that stress situations are closely related to and affect both the endocrine and immune systems. Stressful events cause fear and anxiety which are regarded as risk factors for a variety of diseases, including periodontal (Peruzzo *et al.*, 2007). Hence, the aim of this study was to investigate, primarily, the effect of emotional stress on root resorption and tooth displacement during experimental orthodontic tooth movement in rats. A further area of interest was to evaluate if the expression of interleukin-1 alpha (IL-1 $\alpha$ ) at protein level as well as the density and distribution

of peptidergic nerve fibres immunoreactive to calcitonin gene-related peptide (CGRP) in the periodontal ligament (PDL) are associated with possible stress-induced changes in root resorption and tooth movement.

### Materials and methods

The study was authorized by the Norwegian Animal Research Authority and conducted in accordance with the animal welfare act.

#### Animals

The material comprised 52 six-week-old male rats (mol:WIST Han), with a mean body weight of  $243 \pm 22.7$  g. The animals were housed in polycarbonate cages with standard 12-hour light–dark cycles, temperature 21°C, and fed a standard pellet diet with tap water *ad libitum*. The animals were acclimatized for approximately 5 days before the start of the experiment.

The experimental animals ( $n=48$ ) were divided into three groups as follows: group 1 had orthodontic tooth movement and received foot shocks (OTMS;  $n=16$ ), group 2 had orthodontic tooth movement but received no foot shocks (OTMNS;  $n=16$ ), and group 3 had no orthodontic tooth movement and received foot shocks (NOTMS;  $n=16$ ). Each of the groups was further divided into four subgroups ( $n=4$ ), corresponding to the number of days the experiment lasted, i.e. 3, 7, 13, and 21 days. Four animals served as an untreated control group.

#### Experimental procedures

The operations were carried out under general anaesthesia, using a subcutaneous injection of fentanyl–fluanison—midazolam, 0.2 ml/100 g body weight. As previously described (Vandevska-Radunovic *et al.*, 1997). An orthodontic appliance consisting of a coil spring ligated to the first right maxillary molar and connected to a band cemented on the incisors was inserted in the animals undergoing experimental tooth movement. The coil exerted a mesial force to the molar of approximately 0.5 N. The animals subjected to stress received 0 to 10 foot shocks daily in a specially designed apparatus (Overmier and Murison, 1991). The shocks were given with random inter-shock intervals to avoid learning effects, each shock being at 1 mA intensity and of 1 second duration. All animals were weighed before the start of the experiment and before sacrifice. Blood samples were taken under general anaesthesia from the jugular vein at 3, 7, 13, and 21 days after the initiation of the experiment. Samples were centrifuged, and the plasma was drawn off and frozen for later fluorometric analysis of corticosterone. The animals were then sacrificed, the jaws excised, and fixed in 4 per cent paraformaldehyde for 24 hours and then demineralized in 10 per cent ethylenediaminetetraacetic acid (EDTA) plus

7.5 per cent polyvinylpyrrolidone for 4 weeks. After demineralization, the jaws were sectioned sagittally at 20 µm in a freezing microtome.

#### Histological and immunohistochemical procedures

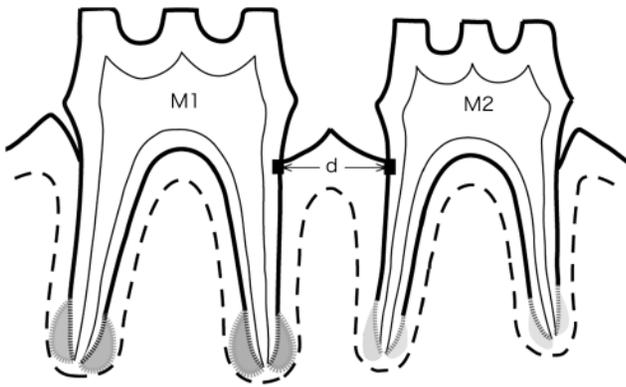
Alternate serial sections from the central part of experimental and control first maxillary molars were used for further analysis. Sections intended for evaluation of root resorption and tooth displacement were stained with haematoxylin and eosin on gelatin-coated slides. However, immunoreaction was performed on free-floating sections in tissue culture wells, 12 alternate sections for each antibody. These sections were incubated for 72 hours at 4°C in anti-rat IL-1 $\alpha$  (1:400 dilution; Endogen, Cambridge, Massachusetts, USA) and CGRP (1:6000 dilution; Diagnostika, Falkenberg, Sweden) polyclonal antibodies raised in rabbits. Antigen–antibody complexes were detected by the avidin–biotin–peroxidase (ABC) method, using a commercially available kit (Vectastain ABC kit; Vector Laboratories, Burlingame, California, USA), and visualized by 3,3'-diaminobenzidine (Sigma, St Louis, Missouri, USA) in the presence of 0.2 per cent  $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  to enhance immunostaining. Finally, the sections were mounted on gelatine-coated slides and counterstained with methylene blue/azure II in 1 per cent sodium borate and distilled water. They were then dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Eukitt (Kindler, Freiburg, Germany). The specificity of the immune reaction was tested by omitting the primary antibody. In these sections, no immunospecific staining was observed.

#### Evaluation and statistical procedure

Both tooth displacement and cellular cementum volume were measured on 12 central sections per molar in a Cue-3 Image Analyzer (Galai Production, Migdal HaeMek, Israel). Cellular cementum volume was measured for both the mesial and the distal roots. The values from each root were pooled and then all sections summarized to obtain the relative volume for each molar. The amount of tooth displacement was measured between the first and second maxillary molars by drawing a line perpendicular to the long axis of the distal root of the first molar and connecting the most distant crown points just above the junctional epithelium (Figure 1). All values were summarized and divided by the number of sections in order to obtain the mean distance. Cells immunopositive to IL-1 $\alpha$  were counted in a light microscope ( $\times 25$  objective  $\times 10$  ocular; Labophot-2, Nikon, Tokyo, Japan) throughout the PDL of the most mesial and distal roots of all investigated molars, below the alveolar bone crest.

#### Statistical analysis

Means and standard deviations were calculated for corticosterone levels, tooth displacement, cellular cementum volume, and number of cells immunopositive to IL-1 $\alpha$  in all



**Figure 1** Measurement of the distance (*d*) between the first (M1) and the second (M2) maxillary molars. The grey periapical area on M1 denotes the cellular cementum.

investigated animals. One-way analysis of variance (ANOVA) with a least significant *post hoc* test was used to detect statistically significant inter-group differences. CGRP immunopositive nerve fibre morphology and distribution was assessed qualitatively, while nerve fibre density was graded as equal to (0) and increased (+) compared with the controls.

## Results

There was a mean weight loss of approximately 3–5 per cent in the 3 day experimental period in the groups with orthodontic appliances. However, there was an overall gain in weight of approximately 10–15 per cent at the end of the experimental periods in all groups.

### Corticosterone levels

Corticosterone levels and their distribution throughout the experimental periods showed individual variations both in the control and experimental groups (Figure 2A). In the control group, corticosterone values were pooled and their mean value was used for statistical analysis. All experimental groups showed a statistically significant ( $P < 0.01$ ) increase in corticosterone levels at day 3 compared with the control group but not between each other. At 7 days, the increase was significant ( $P < 0.01$ ), but only for the OTMS group compared with the control group. At days 13 and 21, there were no statistically significant differences between the groups although, on average, corticosterone levels were higher in the experimental groups than in the control group. When the experimental groups were used as a dependent variable, the only statistically significant change was in the OTMS group, where corticosterone levels decreased from 7 to 13 days.

### Cellular cementum/ARR

The relative volume of cellular cementum was taken as a measure for ARR. In general, the OTMS group showed the least amount of cellular cementum, which was significantly

less ( $P \leq 0.05$ ) than the control group at 3, 7, and 13 days (Figure 2B) and significantly less ( $P \leq 0.05$ ) than the NOTMS group at 7, 13, and 21 days. The OTMNS group also showed significantly less cellular cementum ( $P \leq 0.01$ ) compared with the NOTMS and control groups but only at 7 and 13 days (Figure 3). At 21 days, the OTMNS group approached the values of the control group. No significant differences in cellular cementum were observed between the OTMS and OTMNS groups.

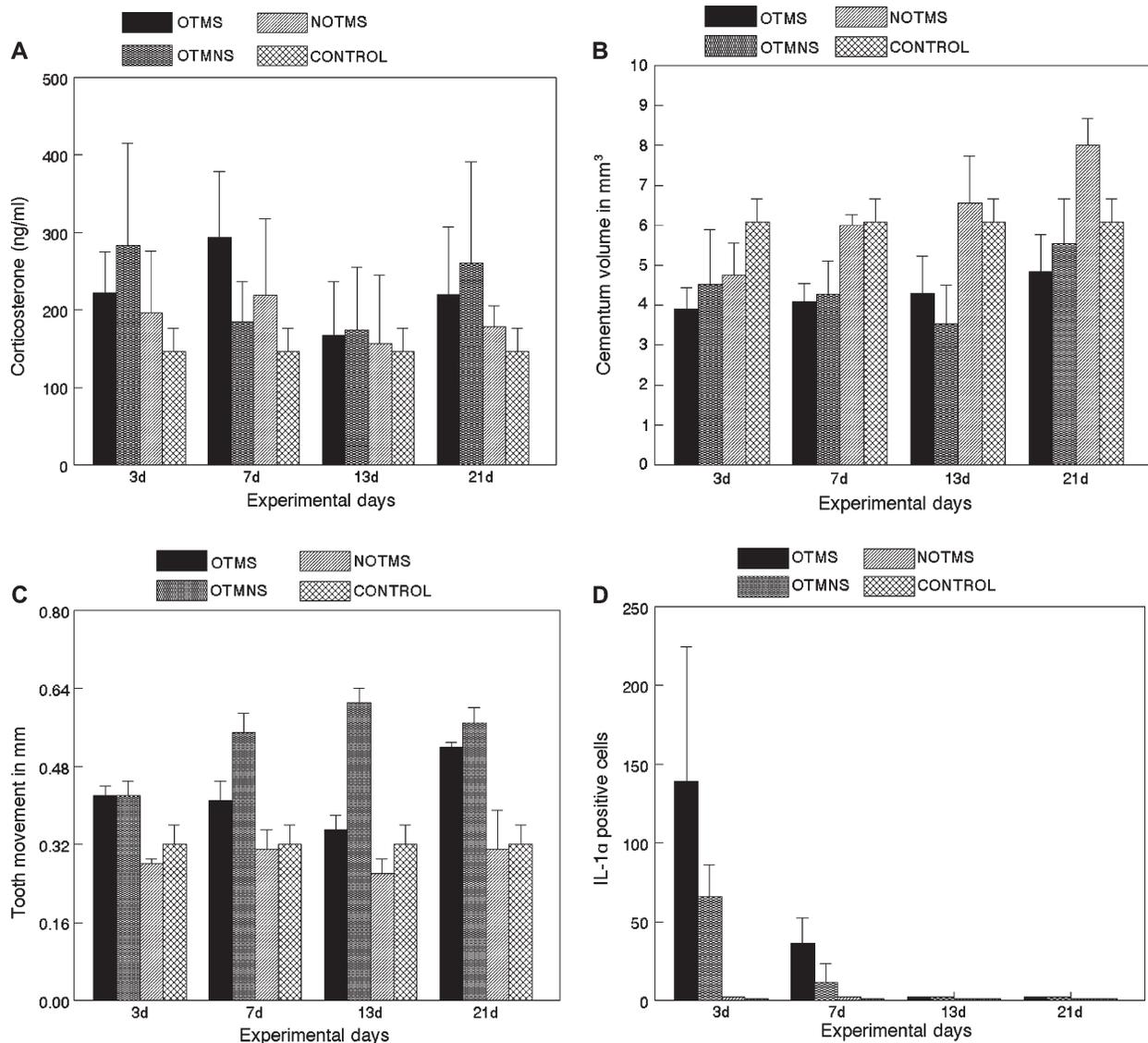
### Tooth movement

At 3 days, the first maxillary right molar in both the OTMS and OTMNS groups showed significantly greater mesial displacement than the molars in the NOTMS and control groups ( $P < 0.05$ ; Figure 2C). Mesial tooth movement of the right first maxillary molar in the OTMNS group gradually increased through days 7 and 13, thus gaining statistical significance when compared with the OTMS group ( $P < 0.01$ ; Figure 2C) and maintaining significance when compared with the control and NOTMS groups ( $P < 0.01$ ). At 21 days, mesial tooth movement of the maxillary first molar in the OTMNS group was still significantly greater than in the NOTMS and control groups ( $P < 0.05$ ) but not when compared with the OTMS group. The right first maxillary molar in the OTMS group demonstrated greater mesial movement than previously; this was on the border of being statistically significant but only when compared with the NOTMS group ( $P = 0.051$ ).

### IL-1 $\alpha$ immunopositive cells and CGRP immunopositive nerve fibres

The number of IL-1 $\alpha$  immunopositive cells was highest in the OTMS group both at 3 and at 7 days (Figure 2D). However, this was not statistically significant when compared with the OTMNS group. The majority of the immunopositive cells were observed in the distal PDL of the distal root corresponding to the tension area and in the gingival papillae mesial and distal to the maxillary first molars (Figure 3). At 13 and 21 days, almost no IL-1 $\alpha$ -positive cells were seen in the PDL below the alveolar crest of the investigated molars. The control and NOTMS groups showed almost no IL-1 $\alpha$ -positive cells in the PDL, but individual positive cells could be observed in the gingival papillae. Large individual differences were present in the experimental groups undergoing orthodontic tooth movement.

The CGRP immunopositive fibres were short, mainly located in the periapical area and close to the alveolar bone, although individual fibres extended towards the cellular cementum. No obvious differences in the number and distribution of immunopositive nerve fibres were observed among the various groups at 3 and 21 days. An increase in nerve fibre density was noted in the apical and cervical half of the PDL of the OTMNS and OTMS groups at 7 days when compared with the NOTMS and control groups (Table 1). At



**Figure 2** Means and standard deviations of (A) corticosterone levels (ng/ml), (B) cellular cementum volume (mm<sup>3</sup>) taken as a measure for apical root resorption of the first maxillary molar, (C) mesial tooth displacement (mm) of the first maxillary molar, and (D) interleukin-1 alpha immunopositive cells in the periodontal ligament of the first maxillary molar in orthodontically treated and stressed (OTMS), orthodontically treated non-stressed (OTMNS), non-treated stressed (NOTMS), and control groups at 3, 7, 13, and 21 days.

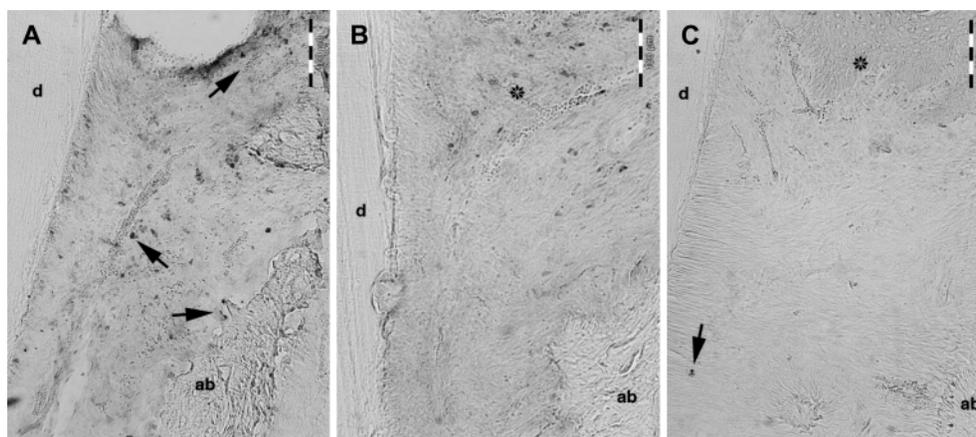
13 days, these changes were obvious only in the apical area. No apparent differences in nerve fibre distribution and density could be seen between the experimentally moved teeth of the OTMS and OTMNS groups.

## Discussion

Emotional or psychological stress has been recognized as a risk factor in the aetiology and pathogenesis of a number of diseases. Chronic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease are some of the conditions that can be initiated or exacerbated by stressful events (Straub *et al.*, 2005; Mawdsley and Rampton, 2006; Kemeny and Schedlowski,

2007). Moreover, periodontal disease, bruxism, orofacial pain, and even relapse tendency after orthodontic treatment have been shown to have positive relationship with stress (Fried, 1976; Rosales *et al.*, 2002; Peruzzo *et al.*, 2007). The results from the present study provide evidence that emotional stress is also associated with orthodontic tooth movement. Animals subjected to stress and experimental orthodontic treatment demonstrated reduced amounts of tooth movement when compared with controls and non-stressed orthodontically treated animals. They also showed the greatest amount of root resorption throughout the experimental period.

Glucocorticoids, such as cortisol and corticosterone, are the main hormones released after stress stimuli and their high plasma levels indicate a positive stress response. All animals



**Figure 3** Distal periodontal area of the distal root of the first maxillary molar at 3 (A), 7 (B), and 13 (C) days of an orthodontically treated and stressed [orthodontic tooth movement and received foot shocks (OTMS)] rat. (A and B) Interleukin-1 alpha immunocompetent cells (arrows) are seen in the periodontal ligament close to dentine (d) and alveolar bone (ab) and in the interdental papilla (\*). At day 13 (C), only individual immunopositive cells can be seen (arrow). Bar = 100 µm.

in the experimental groups in the present study showed higher corticosterone levels at all experimental periods when compared with the control group. The groups having orthodontic tooth movement, with and without foot shocks, demonstrated similar temporal distribution and attained high corticosterone levels 3 weeks after the placement of the orthodontic appliance. This suggests that orthodontic tooth movement *per se* elicits stress effects and that adjoining psychological stress does not have an additional effect on corticosterone plasma levels. Behavioural testing of animals subjected to experimental tooth movement has confirmed that emotional stress can be evoked after the placement of an orthodontic appliance and ahead of pain-related animal behaviour (Yozgatian *et al.*, 2008). This is most probably due to occlusal disharmony and stimulation of the periodontal receptors, which in turn affect corticosterone plasma levels and lead to stress-related behaviour (Yoshihara *et al.*, 2001). To date, there are no human investigations that evaluate the effect of orthodontic treatment on corticosterone levels.

However, behavioural responses to mechanical tooth movement associated with pain and even anxiety of the procedure have been well documented and indicate possible stress effects in humans (Giddon *et al.*, 2007).

If orthodontic treatment itself evokes stressful responses, the question that arises is if additional emotional stress has confounding effects on root resorption and tooth movement. Severe cementum resorption in mammalian teeth, with and without repair, has been reported in connection with stressful experiences including starvation, temperature extremes, injury, and fear (Myrick, 1988). That author suggested that these stimuli lead to hypersecretion of glucocorticoids and in turn evoke hypocalcaemia which results in dental tissue resorption. Experiments on hypocalcaemic rats subjected to orthodontic tooth movement showed that the occurrence and severity of root resorption was increased when compared with normal animals, and that these changes could be related to an increase in alveolar bone turnover (Engström *et al.*, 1988). The present findings are partly in agreement with

**Table 1** Evaluation of the density of the calcitonin gene-related peptide immunopositive nerve fibres in different areas of the periodontal ligand (PDL) of the experimental groups: orthodontic tooth movement and shock (OTMS), orthodontic tooth movement and no shock (OTMNS), and no orthodontic tooth movement and shock (NOTMS).

Tissue location	Experimental period (days)											
	3			7			13			21		
	OTMS	OTMNS	NOTMS	OTMS	OTMNS	NOTMS	OTMS	OTMNS	NOTMS	OTMS	OTMNS	NOTMS
Apical half of the PDL	0	0	0	+	+	0	+	+	0	0	0	0
Cervical half of the PDL	0	0	0	+	+	0	0	0	0	0	0	0

The control group is taken as having zero (0) value. 0, equal to the control group; +, increased density compared with the control group.

those results which show that stressed animals with orthodontic treatment (OTMS) had significantly less cellular cementum throughout the experimental periods when compared with the control and stressed animals without orthodontic treatment (NOTMS). However, the differences between the two orthodontic treatment groups were small at 3, 7, and 13 days, but increased at 21 days, at which time the stressed animals displayed the least amount of cellular cementum or the greatest amount of root resorption. These data can be interpreted as a delay in the reparative processes in the stress group, which should have taken place 3 weeks after the onset of orthodontic tooth movement. It may also be that the measuring of the apical cellular cementum underscored the amount of root resorption and that group differences would have been greater if lateral resorption lacunae had been included. This has been confirmed in a group of animals treated with corticosteroids (Verna *et al.*, 2006). The relative volume of cellular cementum, and not lateral root resorption, was taken as an indirect measure of root resorption mainly because of its clinical relevance. Root shortening and not lateral root surface resorption is considered to be detrimental to orthodontically treated teeth. On the other hand, precise delineation of resorptive lacunae in the cellular cementum area, particularly in rats, is difficult. This problem was avoided by measuring the relative volume of cellular cementum as a quantitative indicator of resorbed cementum.

A clear linear increase in tooth movement was noted in the non-stressed orthodontically treated group. The displacement was significantly greater than that observed in the stressed and orthodontically treated group at 7 and 13 days. These periods correspond to the pro-inflammatory processes triggered by force application and encompass increased vascular permeability, cellular infiltration, and secretion of cytokines and neuropeptides (Vandevska-Radunovic, 1999; Krishnan and Davidovitch, 2006). Stress-induced elevation of glucocorticoids can transiently suppress cytokine production and decrease leukocyte proliferation and mobilization (Mercado *et al.*, 2002; Dhabhar, 2003). Knowing that these mediators serve central roles in the process of tooth movement, the decrease in tooth displacement in the stressed group was not unexpected. However, longer experimental periods might have given different results, as chronic administration of corticosteroids has been shown to increase tooth movement in orthodontically treated rats (Kalia *et al.*, 2004).

IL-1 $\alpha$  is a highly inflammatory multifunctional cytokine localized, among others, in mononuclear cells, osteoclasts, osteoblasts, and fibroblasts (Lossdörfer *et al.*, 2002; Kamolmatyakul *et al.*, 2004). Increased production has been reported during inflammatory infectious and autoimmune diseases, while corticosteroids are shown to suppress its production (Dinarelo, 1996). Plasma corticosterone levels did not seem to have such an effect in the early experimental periods in the current study. Similar responses were observed in the density changes of nerve fibres immunoreactive to

CGRP, where the stressed and non-stressed animals with orthodontic treatment showed almost no differences. The local inflammatory processes induced by orthodontic force seemed to override the possible suppressive effects of systemic corticosterone and caused a transient increased IL-1 $\alpha$  expression and CGRP fibre density in the dental tissues. On the other hand, evidence exists that the cytokine suppressive effect of glucocorticoids is observed at supraphysiological ranges and *in vitro*, while experiments with basal or stress-related doses enhance cytokine production *in situ* (Wilckens and De Rijk, 1997). Furthermore, stress hormones, e.g. corticosterone and catecholamines, can sometimes have a different if not opposing effect, and different concentrations and combinations of these hormones may lead to different responses (Dhabhar, 2003). Glucocorticoids may also induce different systemic and local tissue-specific effects for the same cytokines (Gibb *et al.*, 2008). These data coupled with the large individual differences can explain the observed effect of joint emotional stress and orthodontic treatment on local dental tissue expression of IL-1 $\alpha$  and CGRP immunoreactive nerve fibres. In contrast, their confounding effect on cellular cementum resorption and tooth displacement was more obvious, although individual differences were also present.

Finally, it is important to note that the investigation was undertaken in accordance with the three 'R's of animal testing, i.e. reduction, refinement, and replacement. In view of the reduction principle, the number of animals in each subgroup was minimal and yet suitable for statistical analysis. The variables investigated were assumed to be normally distributed and therefore a one-way ANOVA was used. Obtaining significant results in these circumstances clearly shows that there are inter-group differences. It is possible that the differences could have been greater if the number of animals was greater, but this would have undermined the basic principles of animal experimentation.

## Conclusions

Orthodontic tooth movement *per se* can evoke emotional stress. Additional emotional stress leads to increased cellular cementum resorption and particularly to decreased tooth movement in the early experimental periods but has no confounding effect on the local dental tissue expression of IL-1 $\alpha$  and CGRP immunoreactive nerve fibres.

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