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Calcitonin gingival crevicular fluid levels and pain discomfort during early orthodontic tooth movement in young patients

José Antonio Alarcón [a,*], Dolores Linde [a], Germán Barbieri [b], Patricia Solano [b], Octavio Caba [c], María Judith Rios-Lugo [b], Mariano Sanz [b], Conchita Martin [d]

[a] Department of Stomatology, Section of Orthodontics, Faculty of Odontology, University of Granada, Granada, Spain
[b] Department of Stomatology III, Section of Periodontology, Faculty of Odontology, University Complutense of Madrid, Madrid, Spain
[c] Department of Health Sciences, University of Jaén, Jaén, Spain
[d] Department of Stomatology IV, Section of Orthodontics, Faculty of Odontology, University Complutense of Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Accepted 1 October 2012

Keywords:
Calcitonin
Gingival crevicular fluid
Orthodontic movement
Dental pain
Western blot

ABSTRACT

Objectives: To investigate the previously unreported presence of calcitonin (CT) levels in gingival crevicular fluid (GCF), its variations during initial orthodontic tooth movement in both tension and compression sites, and its possible association with the experienced dental pain.

Design: Fifteen children (mean age: 12.6 years) requiring orthodontic closure of the upper midline diastema were included. We collected GCF from the compression and tension sites of the upper right central incisor (experimental) and first bicuspid (control), before and after (1 h, 24 h, 7 d, 15 d) beginning of treatment. Calcitonin levels were determined by Western blot. Pain intensity was assessed using a visual analogue scale.

Results: Calcitonin levels were higher in the compression site versus the control site at 7 d ($p = 0.014$). Intragroup comparisons showed an increment of CT between 1 h and 7 d (680.81 ± 1672.60 pg/30 s, $p = 0.010$) in the compression site. No significant changes were found in the tension and control sites. Calcitonin levels and pain intensity were negatively associated during the period from 24 h to 15 d ($r = -0.54$, $p = 0.05$).

Conclusions: CT levels in the GCF significantly increased in the compression site after the short term after application of orthodontic forces. These changes were negatively associated with the perceived patient's dental pain during the period from 24 h to 15 d.

1. Introduction

Calcitonin (CT), a 32-amino acid polypeptide hormone produced by C-cells of the thyroid gland, is involved in bone homeostasis. Calcitonin binds to a 7-membrane-spanning array G protein-coupled receptor with calcium acting as secondary messenger. This receptor is frequently located in osteoclasts, renal tubular, and neural cells, In response to a rise in blood calcium levels, CT is released and decreases these levels, mainly through the inhibition of osteoclast-mediated bone resorption,[1] the decrease of calcium tubular reabsorption,[2] and the regulation of 1,25-dihydroxyvitamin D3 production in the kidney.[3] In vitro and in vivo studies have shown

* *Corresponding author at*: Department of Stomatology, Faculty of Odontology, University of Granada, Campus Universitario de Cartuja, s/n 18071, Granada, Spain. Tel.: +34 958201480; fax: +34 958201480.
E-mail address: jalarcon@ugr.es (J.A. Alarcón).
0003–9969/$ – see front matter
http://dx.doi.org/10.1016/j.archoralbio.2012.10.002

that CT has a potent inhibitory effect on osteoclasts, where it inhibits cell motility, actin ring formation, and bone resorptive mechanisms.[4,5]

Calcitonin also has analgesic properties, especially on bone-related pain,[6–8] although its mechanism of action remains unclear. Three hypotheses have been proposed to explain this powerful analgesic effect: a direct central nervous system action involving calcitonin-binding receptors,[9,10] a peripheral mechanism inhibiting the synthesis of PGE_2 and thromboxane,[11,12] and an increase in plasma β-endorphin levels.[7,11,13]

Orthodontic tooth movement takes place by a combination of bone apposition in the tension side and bone resorption in the compression side after the application of mechanical forces on teeth.[14,15] During this biological process, many biochemical markers, either related with bone resorption (e.g. IL-1β, IL-6, IL-8, TNF-α, substance P, NTx, and β-glucouronidase) or with bone formation (e.g. osteocalcin and leptin) can be found in the gingival crevicular fluid (GCF).[16–20] Analysis of the GCF can also reveal information on the cellular response of the surrounding periodontal ligament and bone metabolic changes that occur during orthodontic treatment.

Although CT actively intervenes in bone metabolism and is secreted under conditions of increasing bone resorption,[1] to our knowledge, the CT levels in GCF during orthodontic tooth movement have not been investigated. It is, therefore, the aim of this investigation: (1) to assess whether CT can be detected in the GCF of both the tension and compression sides of teeth under orthodontic movement; (2) to measure CT levels during the early phase of orthodontic movement; and (3) to find possible associations between the CT levels in GCF and the patient's perceived intensity of pain during orthodontic tooth movement.

2. Materials and methods

2.1. Subjects

Fifteen young patients (8 female and 7 male; mean age: 12.6 years) presenting an upper midline diastema and requiring orthodontic treatment participated in this study. All of the patients met the following criteria: aged 10–15 years; presence of an upper midline diastema ≥ 1.5 mm; in good general and oral health; without evidence of periodontal or gingival disease, as demonstrated by presence of probing depths ≤ 3 mm, and no radiographic evidence of bone loss; and without a record of antibiotic therapy within the last 6 months, or chronic anti-inflammatory drug medication in the month preceding the study. The parents of all patients were informed on the characteristics of the study and agreed for their children to participate by signing an EC-approved informed consent form.

The sample size was estimated from data obtained in a similar clinical study evaluating the biomarkers IL-1β, substance P and PGE_2.[18] Under the assumption of a mean difference of 0.10 pg at the 20 s sample and an expected pooled standard deviation of 0.05, a sample size of 12 patients provided a power of 0.8.

2.2. Study design

Two weeks before the beginning of this prospective longitudinal case series study, each participant received prophylaxis and oral hygiene instructions. All participants indicated their willingness to adhere strictly to these instructions. Patients were instructed not to take any medications, including non-steroidal anti-inflammatory drugs, during the study period. Plaque control and motivation were performed during the whole study.

Brackets (0.022×0.028 in. Mini Master, American Orthodontics, Sheboygan, WI, USA) were placed on the buccal surface of the upper central incisors. The upper midline diastema was closed orthodontically by applying a force of 100 g with a pre-stretched elastomeric chain (Memory chain, American Orthodontics, Sheboygan, WI, USA). The elastomeric chain was activated and tied at one of the two braces using a 0.008-in. ligature wire once the 100 g force activation was achieved. The magnitude was adjusted to produce equivalent compressive stresses between subjects using an orthodontic dynamometer (YS-31D Push pull gauge. YDM CORPORATION, Saitama 355-0042, Japan). To reduce the occurrence of vertical aligning movements, no arch-wire was inserted in the bracket slot during this experiment.

The GCF was collected from the mesial (compression) and distal (tension) sites of the upper right central incisor (experimental) and from the mesial site of the right or left upper first bicuspid (control), just before (0 h, baseline) and after (1 h, 24 h, 7 d, and 15 d) from the start of the orthodontic treatment. At each appointment, the plaque index (PI),[21] modified gingival index (GI)[22] and bleeding on probing index (BOP)[23] were assessed in experimental, control, and adjacent teeth, just after GCF sampling.

Discomfort or pain was estimated by a visual analogue scale (VAS) at each moment of the study, previous to GCF sampling and assessment of periodontal indices.

2.3. GCF sampling

The GCF samples were taken with periopaper strips (Harco, Tustin, CA, USA). Selected teeth were isolated with cotton rolls, cleaned of plaque deposits, and dried gently with air. The paper strips were gently introduced subgingivally at 1 mm for 30 s. Each sample was placed in a centrifuge tube (Millipore Ultrafree-MC) and stored at $-80\ ^\circ C$ until analysis. The volume of the sample on the paper strips was measured using a calibrated Periotron 8000 (Harco). The readings from the Periotron were converted to an actual volume (microliters) by reference to the standard curve calibrated with human serum.

2.4. Calcitonin determination

The CT level in GCF from experimental and control teeth was analyzed by Western blot analysis with specific antibodies for CT. On the day of analysis, the periopaper was diluted in 100 μl of 1% Triton X-100 in 50 mM Tris–HCl (pH 7.5) (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) and centrifuged at 12,500 rpm for 5 min. Proteins were separated by 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini Protean II cell (Bio-Rad, Hercules, CA, USA)

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at 80 V for 2 h at room temperature and transferred to a nitrocellulose membrane (15 V at room temperature for 20 min). Blots were treated with blocking solution (PBS, 5% nonfat milk) for 1 h at room temperature and then reacted with a CT mouse monoclonal IgG1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution overnight at 4 °C. Membranes were washed (10 min in 5% PBS) 3 times and incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse IgG (Sigma–Aldrich, St Louis, MO, USA) diluted at 1:200.

Protein–antibody complexes were visualized by enhanced chemiluminescence (ECL, Bonus, Amersham, Little Chalfont, UK). Relative amounts of CT were determined by densitometric analysis with Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA). All of the GCF samples were run in duplicate. The levels of CT were reported as the total amount (in pg) per 30 s sample.

2.5. Evaluation of discomfort or pain

A baseline questionnaire-based interview was established to evaluate the previous experience of each patient with general and dental pain. Previous experience of general pain was evaluated with a 100-mm VAS, with two end-points, labelled ''no pain'' on the left and ''worst pain'' on the right.[24] The same scale was used to assess dental pain relative to previously experienced situations.

Discomfort or pain during the study period also was evaluated with VAS. Patients indicated their current level of spontaneous pain intensity and experienced pain while clenching with cotton rolls for the experimental and control teeth.

2.6. Statistical analysis

Longitudinal changes of GCF CT levels were expressed as percentages of baseline values (0 h). After testing for normality, repeated measures ANOVA with post hoc tests were used to compare differences between sites (tension, compression, and control) and to analyze intragroup differences between time points (1–24 h, 7 d, or 15 d; 24 h to 7 d or 15 d; and 7–15 d). Multiple linear regression analysis was performed to reveal correlations between the GCF CT level and pain intensity, and between pain intensity and previously experienced general and dental pain. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3. Results

All patients maintained good oral hygiene throughout the study. No significant changes in PI, GI, or BOP were found at any time point at any site.

Patients showed a central diastema that ranged from 1.5 to 2.4 mm (mean value: 1.86 mm, SD: 0.27 mm).

3.1. Longitudinal GCF CT changes

Table 1 shows descriptive statistics of CT levels, expressed as the total amount per 30-s sample, in the tension and compression sites of experimental teeth and in the mesial site of control teeth. A pronounced increase in percent CT levels was found from 1 h to 7 d in the compression site; these levels remained fairly stable in the tension and control sites (Fig. 1). Differences in longitudinal CT changes between sites, expressed as the percentage of the respective baseline values (0%), are shown in Table 2. A pronounced increment in the percent CT level in the compression site compared to the control site was detected at 7 d ($p = 0.014$). Intragroup comparisons also showed an increment of the percent CT levels between 1 h and 7 d (680.81 ± 1672.60 pg/30 s, $p = 0.010$) in the compression site. No significant percent differences were found between the tension and control sites over time (Table 3).

The volume of GCF samples ranged from 0.1 to 0.9 μl. Differences between sites and time points were tested. No significant differences were found, although the pooled mean values at baseline (mean = 0.4 μl; SD = 0.32 μl) were slightly lower than during the study (mean = 0.67 μl; SD = 0.48 μl).

3.2. Evaluation of discomfort or pain

A questionnaire-based interview revealed that the strongest previously experienced general pain was associated with ''spraining one's ankle'', followed by ''cutting one's finger''. The strongest previously experienced dental pain was associated with ''injection'' and ''drilling''. Substantial intersubject variation was observed for previously experienced general and dental pain.

Fig. 2 shows the evolution of mean VAS pain scores for experimental and control teeth throughout the study. In experimental teeth, spontaneous pain intensity increased from 1 h to 24 h, whereas pain intensity when clenching cotton rolls increased substantially from the application of orthodontic forces to 24 h. Pain intensity scores returned to

Table 1 – Descriptive statistics of calcitonin levels (pg per 30 s sample) at tension, compression and control sites throughout the study.

	Tension pg/30 s			Compression pg/30 s			Control pg/30 s		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Baseline	23.0	34.4	13.5	5.7	4.4	6.0	25.9	25.9	18.0
1 h	29.9	24.7	21.0	6.7	6.9	4.8	33.8	45.1	17.3
24 h	36.3	44.4	18.9	11.2	17.0	2.9	25.8	27.5	13.8
7 d	43.7	64.4	14.3	38.2	32.0	26.0	21.8	23.6	12.9
15 d	31.7	31.8	16.8	27.7	22.6	22.1	27.8	23.7	19.8

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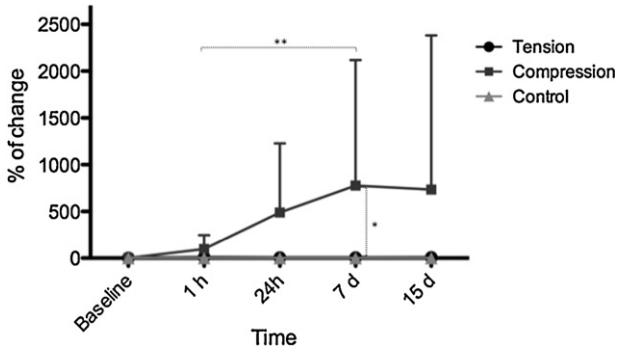

Fig. 1 – Evolution of mean calcitonin values at tension, compression, and control sites throughout the study ($^{*}p < 0.05$, $^{**}p < 0.01$).

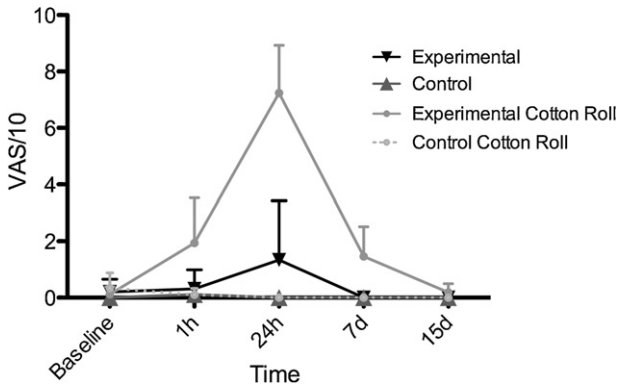

Fig. 2 – Evolution of mean VAS scores and standard error for each of the tested teeth.

initial values after 7 and 15 d, respectively. In contrast, VAS pain scores for control teeth did not change over time. No correlation between pain intensity during the study and previously experienced general and dental pain was found.

A negative association was found between the percent CT level increment and VAS scores of pain intensity changes during clenching with a cotton roll in the experimental tooth over the 24 h to 15 d period ($r = -0.54$, $p = 0.05$).

4. Discussion

This study aimed to investigate the previously unreported presence of CT levels in GCF, its variations during initial orthodontic tooth movement in both tension and compression sites, and its possible association with the experienced dental pain. This prospective case series has clearly shown that CT levels in the GCF can be measured using western blot biochemical analysis. Significant increases in percent CT levels were found in the compression site from 1 h to 7 d after application of orthodontic forces. These changes in CT levels were negatively associated with the perceived dental pain change during clenching with a cotton roll in the experimental tooth during the period from 24 h to 15 d. In contrast, no significant changes in CT levels were found in the tension and control sites.

The CT levels were reported as the total amount (pg) per 30-s sample, which is consistent with previous similar investigations that have used total amounts rather than concentrations of biomarkers, due to the inherent problem of the accurate determination of GCF volume.[25,26] Nevertheless, we cannot rule out the possibility that the increased percent CT levels found could be influenced by a potential increase in GCF volume associated with orthodontic treatment.[27,28] However, in our study no differences in GCF were found between sites and time points.

Although CT values in the compression group were lower at baseline relative to the other sites, the fact of expressing the

Table 2 – Differences between sites for calcitonin levels expressed as percentage of the baseline values at tension, compression and control sites.

	Tension-compression			Tension-control			Compression-control		
	Mean diff.	SD	*p* value	Mean diff.	SD	*p* Value	Mean diff.	SD	*p* value
1 h	−87.98	213.97	0.643	7.35	18.68	0.160	95.33	209.82	0.770
24 h	−484.80	1032.43	0.492	3.67	7.46	0.059	488.50	1031.29	0.770
7 d	−772.40	1876.70	0.131	4.26	9.92	0.123	776.70	1874.87	0.014*
15 d	−727.50	2305.60	0.770	4.98	12.02	0.432	732.50	2301.16	0.160

* $p < 0.05$.

Table 3 – Intragroup differences for levels expressed as percentage of the baseline values.

	Tension			Compression			Control		
	Mean	SD	*p* value	Mean	SD	*p* value	Mean	SD	*p* value
1–24 h	4.08	15.79	0.322	−392.74	828.93	0.547	0.401	0.99	0.160
1 h–7 d	3.652	8.9	0.492	−680.81	1672.6	0.010**	5.89	2.78	0.375
1 h–15 d	1.87	8.75	0.557	−637.7	2129.69	0.131	−0.505	1.89	1
24 h–7 d	−0.428	9.25	1	−288.07	1081.17	0.193	0.158	0.47	0.275
24 h–15 d	−2.21	9.28	0.846	−244.96	1756.6	0.160	−0.906	2.52	0.846
7–15 d	−1.782	3.41	0.129	43.11	782.79	0.695	−1.06	2.7	0.426

** $p = 0.01$.

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results as the % of change relative to these basal values overcomes these differences, which could be due to the biological variability associated to GCF determination.

In this study, we attempted to assess the reported anti-resorptive and analgesic activities of CT[1,6,7] during orthodontic teeth movement through the analysis of GCF CT levels. The observed rapid increase in percent CT levels at the compression site of the experimental teeth was probably due to changes in the bone calcium homeostasis induced by the orthodontic forces. In other words, the increment in calcium levels at the compression site during active bone resorption may have elicited the immediate increase in CT release.

Reported biological activities of CT include lowering serum calcium concentrations in pathological states of increasing bone resorption, mainly by inhibition of osteoclast activity.[1,4,5] It is, therefore conceivable that a short-term stress exerted on the periodontium by the orthodontic forces induced an increase in the GCF CT levels, which intervened in the bone remodelling during the early stages of orthodontic tooth movement. GCF CT levels may thus be considered a biomarker of bone remodelling during early orthodontic tooth movement. Since no differences in PI, GI, and BOP were found throughout the study, the periodontal conditions did not influence the GCF CT levels.

The highest intensity of dental pain was reached at 24 h after the beginning of orthodontic treatment, which is similar to the results of previous studies.[25,29,30] As a novelty, we introduced a functional task in the evaluation of perceived dental pain: that of clenching with a cotton roll. In this study, a negative association was found between the percent CT levels and VAS pain score changes in the experimental tooth over the period from 24 h to 15 d. These results showed that, as CT levels increase, dental pain decreases, what may indicate an analgesic effect of CT in orthodontics-induced dental pain, although the mechanism remains unclear. Several hypotheses have been proposed to explain this powerful analgesic effect of CT: a central action,[9,10] inhibition of PGE_2 and thromboxane synthesis,[11,12] and stimulation of β-endorphin release.[7,11,13] In other studies assessing orthodontic-related dental pain, the initial intensity of pain (at 1 h) was associated with increased GCF PGE_2 and IL-1β levels.[25,29] It will be therefore interesting to determine whether the increase in CT levels reported in this investigation would correlate with the β-endorphin and PGE_2 levels in GCF.

This prospective clinical investigation has shown that CT levels in the GCF significantly increased in the compression site after the short term after application of orthodontic forces and these changes were negatively associated with the perceived patient's dental pain during the period from 24 h to 15 d after the application of orthodontic forces.

Funding

This study was funded by an investigation grant from The ''Fundación Investigación Médica Mutua Madrileña''.

Competing interests

All authors state that they have no conflicts of interest.

Ethical approval

The parents of all patients were informed on the characteristics of the study and agreed for their children to participate by signing an EC-approved informed consent form.

Acknowledgement

This study was funded by an investigation grant from The Fundación Investigación Médica Mutua Madrileña.

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