

## DISTRIBUTION OF CYCLIC-AMP IN MECHANICALLY-STRESSED PERIODONTAL FIBROBLAST CELLS

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**SUMMARY:** Adenosine 3', 5'-monophosphate (cAMP) is an intracellular mediator of the effects of external signals on target cells. Using immunohistochemistry, it has been observed that orthodontic forces alter the cAMP content in several cell types in the cat periodontal ligament (PDL). The objective of this study was to determine the effects of orthodontic forces on the intracellular distribution of cAMP in PDL fibroblasts. Twelve, one year old cats were treated by 80 g tipping force to one maxillary canine for 1, 7 and 14 days. Maxillary horizontal sections (5 m) were stained for cAMP. Cellular staining intensity was measured as percent light transmitted by computer aided microphotometry using the MAPS program (Zeiss). Both the nuclei and the cytoplasm of the cells on the treated side stained darker with respect to the control side, except for the nuclei at 1 day. The cytoplasm stained darker than the nuclei on the treated side at all time intervals. These results indicate that mechanical force affects the cAMP content and its intracellular distribution in periodontal cells.

**Key Words:** cAMP, orthodontic tooth movement, PDL cells.

**ÖZET:** ORTODONTİK KUVVET UYGULANMIŞ PERİODONTAL FİBROBLASTLARDA cAMP'İN DAĞILIMI. Hedef hücrelerde eksternal sinyallerin etkisinin hücre içi mediatörü Adenosine 3',5'-monofosfat'tır (cAMP). Kedilerin periodontal ligamenti'nde (PDL) mevcut çeşitli hücre tiplerinin ortodontik kuvvet altında cAMP miktarında değişiklik olduğu immunohistokimya yardımıyla gösterilmiştir. Bu çalışmanın amacı, ortodontik kuvvetlerin PDL fibroblastlarında hücre içi cAMP dağılımına etkisini ortaya koyabilmektir. Oniki adet, bir yaşında kedinin birer maksiller kaninine 80 gr. eğilme kuvveti 1, 7 ve 14 gün boyunca uygulanmıştır. Maksiller horizontal kesitler (5 m) cAMP için boyanmıştır. Hüccresel boyanma yoğunluğu, MAPS programı kullanılarak bilgisayar destekli mikrofotometri yöntemiyle geçen ışık yüzdesi olarak ölçülmüştür. 1 gün nukleus hariç, tedavi tarafında hücrelerin nukleus ve sitoplazmaları kontrol tarafına oranla daha koyu boyanmıştır. Tedavi tarafında, tüm zaman aralıklarında sitoplazma nukleus'a oranla daha koyu boyanmıştır. Bu sonuçlara göre, mekanik kuvvet periodontal hücrelerde cAMP miktarını ve hücre içi dağılımını etkilemektedir.

**Anahtar Kelimeler:** cAMP, ortodontik diş hareketi, PDL hücreleri.

### INTRODUCTION

Orthodontic treatment uses mechanical forces to move teeth to desirable positions in the oral cavity. These forces stimulate cells which are responsible for remodelling mineralised and nonmineralised tissues. What compels these cells to respond, or which local or systemic factors have a role in stimulating these cells to respond to mechanical forces, is still not fully understood.

For the purpose of localising these factors in the PDL during force-induced remodelling, it is feasible to use immunohistochemical methods. Microphotometric measurements, in terms of light transmitted through cells, is a useful tool for the evaluation and relative quantification of immunohistochemical staining. In this method, a microscope interfaced with a microphotometer and a computer can be utilised with supporting software. In previous studies (6-8), a software program called Photan (Zeiss, New York, N.Y.) was used to quantify and evaluate the staining intensity. That program is able to perform single measurements of circular areas of 0.5 m in diameter over each cell.

However, as the staining intensity varies within different areas of the cell, the operator must arbitrarily choose an area which best represents the overall staining intensity of that particular cell. Recently, a more reliable method for quantifying staining intensity in individual cells has become available by the use of a new software program called microscope Analysis with Photometric Scanning (MAPS, Zeiss, New York, N.Y.). This program enables the operator to scan an entire cell, as well as to divide it into its basic components (nucleus and cytoplasm) and to measure the average staining intensity of the nucleus and the cytoplasm individually.

Adenosine 3', 5'-monophosphate (cyclic AMP, cAMP) is an intracellular mediator of the effects of extracellular signals on target cells (5, 13). Cells of mineralised and nonmineralised tissues respond to hormones such as PTH (3, 17) and Vitamin D (18) by demonstrating fluctuations in their cAMP content. Also, there is evidence for the involvement of cAMP in the response to mechanical forces in bone (4, 21, 23). Cyclic AMP is also involved in cell growth and proliferation (2, 14). Using immunohistochemistry, we had observed in the past that orthodontic forces alter the cAMP content in several cell types in the cat periodontal ligament (PDL) (5-7). The objective of

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this study was to determine the effects of orthodontic forces on the intracellular distribution of cAMP in fibroblast-like PDL cells in terms of mean percent light transmitted and thus derive new information with this kind of detailed evaluation.

## MATERIAL AND METHODS

Twelve female cats aged 9 to 12 months were used in this study. The cats were divided into three groups of 4 animals each. Distal tipping of one maxillary canine in each animal was performed by using a stainless steel closed coil spring (0.008x0.036 inch) which was precalibrated to generate 80 gm of tipping force. Grooves were cut into the canine and third premolars 1mm incisal to the gingival margin, and a precalibrated spring was attached to these grooves by stainless steel ligatures. The contralateral canine of each cat's maxilla served as the control, since no treatment was rendered to those teeth. The treatment period for each group was 1, 7 and 14 days, respectively. At the end of treatment, each cat was deeply anaesthetised with ketamine at a dose of 30 mg/kg (Kateset, Bristol Laboratories, Syracuse, N.Y.), and then euthanized. The heads were immediately frozen in liquid N<sub>2</sub>. Each maxilla was chiselled from the head while still frozen, and then embedded in a 2% methylcellulose aqueous solution. Undecalcified fresh frozen horizontal sections, 5<sup>o</sup> m thick, were cut at -20 C with a cryostat microtome, mounted on clear adhesive tape, and freeze-dried for 24 hours. Sections were collected at the level of the coronal third of the root, where compression of the PDL is observed at the distal side of the root and tension of the PDL is found on the mesial side.

Staining for cAMP was accomplished by the use of mouse anti-cAMP monoclonal antibody in delipidated ascites fluid (6). The antibody was diluted 1:40 and incubated overnight with the sections at 4 C. Following rinses in tris HCl buffer, pH 7.6, sections were incubated with goat anti-mouse IgG conjugated with horseradish peroxidase diluted 1:60 (antibody protein 26.1 mg/ml) and then with 3,3'-diaminobenzidine (DAB, 0.75 mg/ml) as described by Graham and Kanrovsky (10), to obtain the typical brown reaction product. A 0.1% solution of fast green was used for counter staining. Sections, still adherent to the adhesive tape, were mounted onto glass slides with Euparal.

For the evaluation of immunohistochemical staining, microphotometric measurement of percent light transmittance (PLT) through individual cells was performed using the MAPS program. This program enables the operator to scan square and rectangular areas on a tissue section and measure light transmittance at 0.25<sup>o</sup> m increments. In this manner, depending on the amount of light transmitted through every 0.25<sup>o</sup> m square, a graphic picture of the whole cell and its surrounding matrix was obtained. The procedure was performed as follows:

1) The microscope was focused on the cell of interest (Fig. 1).

2) A rectangle was constructed including the cell of interest in the given area by using the computer to mark the upper left and the lower right corners.

3) The computer then scanned the cell by driving the motorised stage, and a graphic image of the cell was obtained on the monitor with different colors demonstrating the amount of light transmitted, pixel by pixel (Fig. 2).

4) In the last stage, the operator extracted only the light transmitted through the cell (fig. 3), the nucleus (Fig. 4), and the cytoplasm (Fig. 5) within the area, by constructing a polygon around these components by the use of cursor keys, which then gave the average PLT of the components separately. Staining intensity (percent light absorbed) was defined as 100-PLT.

In this fashion, we measured staining intensity of cells in zones of compression and tension in the PDL, as well as in the corresponding control sites, in every maxilla. Ten PDL cells were measured in each zone, for a total of 480 cells.

Statistical evaluation of the data was made by a four-way repeated measures mixed analysis of variance. Three of the four ways were within animal differences: treated (force) sites versus control sites; tension versus compression sites; and nucleus versus cytoplasm. The three times of duration of force-induced tooth movement, 1, 7 and 14 days, constituted between animal differences. When the levels of a factor showed no substantial evidence of a differential cAMP response (as it did for tension versus compression), we combined the separate measurements and analysed the data by a three-way analysis of variance. Pairwise tests of significance were carried out using Tukey's procedure or the t-test, depending on the set of means to be compared.

## RESULTS

Overall examination of the data showed that tension versus compression sites did not differ significantly. Therefore, data belonging to compression and tension sites were pooled together for both the treated and the control sites. Table I shows the pooled data of the investigation. It demonstrates that the staining intensity was greatest at day one, in PDL cells of both control and treated canines. At control sites, for both cell cytoplasm and nucleus, the decline in cAMP staining intensity over time from day one values range from 6.69% to 7.56% by day 7 and 8.80% to 9.89% by day 14. These declines were all significant beyond the p<0.05 level. For treated sites, there was a smaller, but again a significant decline beyond the 0.05 level, for cAMP staining intensity in the cytoplasm of the cells at day 7 and 14, when compared to day one values (4.83% and 4.75% respectively). However, no

Table I: Percent staining intensity for cAMP of nucleus and cytoplasm of PDL cells in 3 groups of 4 cats each, subjected to a tipping force to one maxillary canine for 1, 7 and 14 days.

	Control		Treated	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
	$\bar{x}\pm SD$	$\bar{x}\pm SD$	$\bar{x}\pm SD$	$\bar{x}\pm SD$
<b>1Day</b>	48.42±1.77	47.04±1.53	47.35±2.75	50.51±2.40
<b>7 Days</b>	40.86±1.33	40.35±2.32	44.35±1.33	45.68±0.84
<b>14 Days</b>	38.53±0.77	38.24±1.13	45.04±1.86	45.76±1.80

significant declines from day 1 values were observed over time for staining intensity in the cell nucleus. Here, differences averaged 3.00% at day 7 and 2.31% at day 14.

When the data was evaluated according to the differences between cytoplasm and nucleus (Fig. 6), at day 1 control cytoplasm and nucleus showed a significant difference of 1.38% ( $p=0.038$ ), with the nucleus staining darker. At this same time period, treated cytoplasm and nucleus showed a significant difference as well, but here the pattern was reversed to that of the control: the cytoplasm stained darker than the nucleus ( $X=-3.16\%$ ,  $p=0.040$ ). At day 7, the difference between cytoplasm and nucleus at the control site was negligible ( $X=0.51\%$ ,  $p=0.337$ ). At the treated site, the staining intensity difference between the two components of the cell was -1.33% ( $p=0.059$ ). At day 14, the difference at control sites was 0.29% ( $p=0.297$ ), and the difference at the treated sites was -0.72% ( $p=0.052$ ) (Fig. 6).

When the components of the cells were evaluated by comparing control and treated sites (Fig. 7), except for the nucleus at day 1, the treated site stained darker than the control at all time periods. At day 1, the difference between the control and the treated nucleus was 1.07% ( $p=0.418$ ). For the cytoplasm, treated and control staining intensity showed a difference of -3.47% ( $p=0.056$ ). Staining intensity difference at day 7 between control and treated nuclei was -3.49% ( $p=0.039$ ); at the cytoplasm the difference was -5.33% ( $p=0.013$ ). At day 14, the difference between the nuclei was -6.51% ( $p=0.001$ ) and the difference between cytoplasms was -7.52% ( $p=0.001$ ).

## DISCUSSION

These results demonstrate that, except for the nucleus at day 1, at each time period, orthodontically treated

sites showed significantly elevated staining intensity for cAMP. In general, PDL cells respond to the application of mechanical force by elevations of their cAMP level (5, 7). Ortez (15, 16) has shown that the intensity of staining for cAMP is related to its cellular concentration; as the level of cAMP increases in the cell, so does the staining intensity, in a proportional order. Hence, it appears that after the application of force to teeth, the difference in cAMP staining intensity between control and treated site cells gradually increases with time.

At the control side, the nuclei and the cytoplasm stained with the same intensity at all time periods without any differences. However, the staining intensity appears to have decreased significantly as a function of time. It may be that the anesthesia administered to the animal at the time of appliance application may have caused the fluctuation of the cAMP level in the cells. But whatever the reason for this may be, we assume that it would affect both the control and the treated sites in the same manner. When we examine the treated site, we see the same trend on this side as well, which is a decrease in staining intensity as a function of time, and probably the difference between the control and treated site remained unchanged in terms of staining intensity. Therefore, the significant differences observed between control and treatment could be attributed primarily to the force application.

Generally, at the treated sites, we observed dark cellular staining for cAMP, except for the nuclei at day 1, indicating that cAMP levels increase in PDL cells exposed to mechanical stress. In this study, cAMP levels were found to be higher in the cytoplasm than in the nuclei at the early periods of tooth movement. But as time progressed, the difference in the staining intensity decreased between these two basic components of the cell.

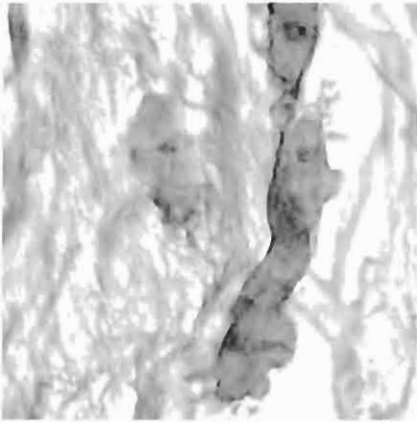


Fig. 1: A PDL cell on the tension side to be scanned for percent light transmitted.

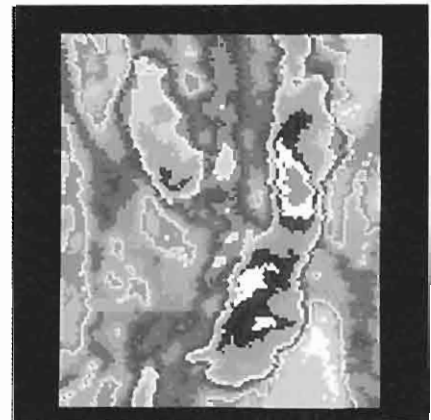


Fig. 2: Graphic image of the cell and surrounding areas in Figure 1.



Fig. 3: Graphic image of only the cell.



Fig. 4: Graphic image of only the nucleus.

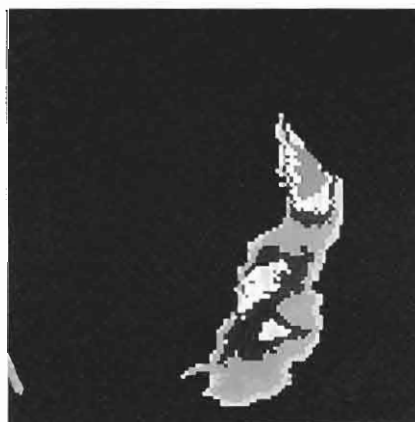


Fig. 5: Graphic image of only the cytoplasm.

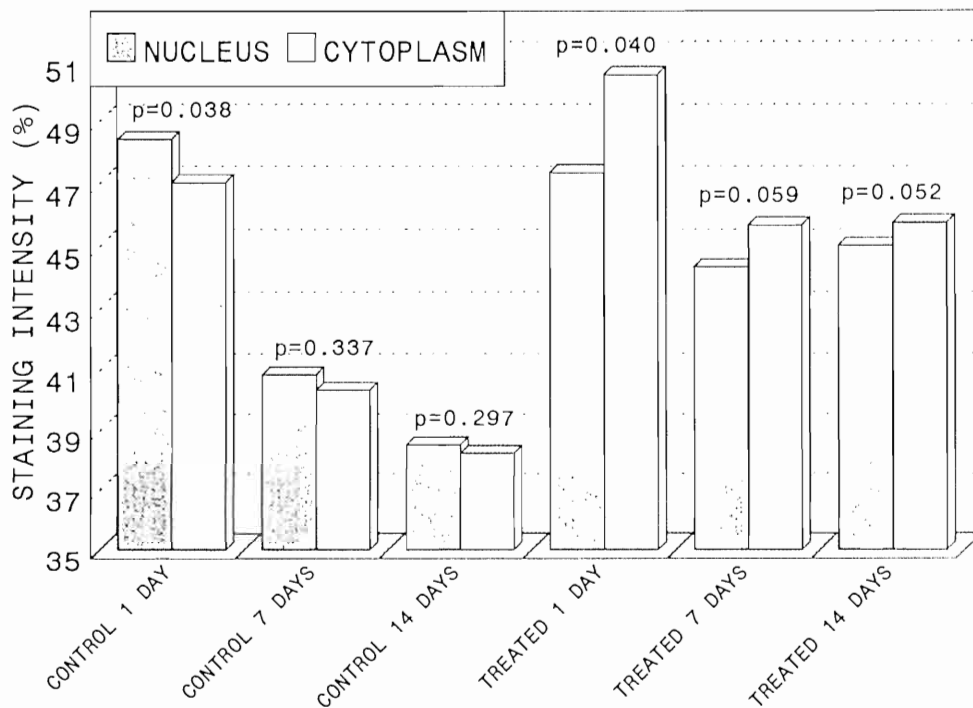


Fig. 6: Percent staining intensity for cellular cAMP comparing nucleus and cytoplasm in control and treated sites as a function time.

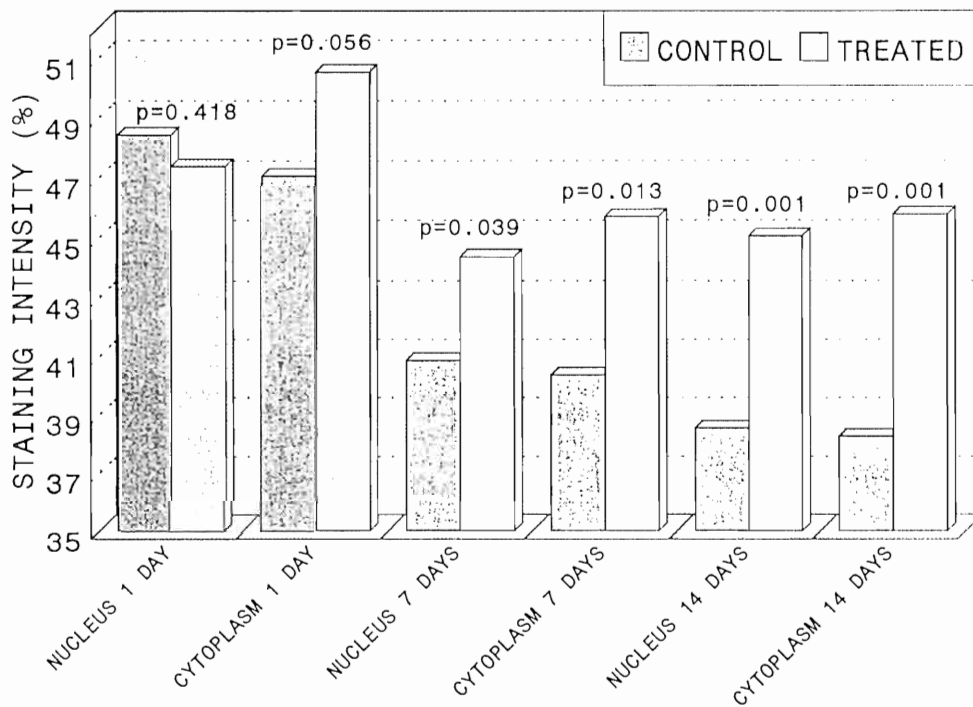


Fig. 7: Percent staining intensity for cellular cAMP comparing control and treated sites for nucleus and cytoplasm as a function of time.

As stated earlier, at 24 hours the nuclei of the cells at treated sites stained lighter than the control. Cyclic AMP has been shown to decrease during cell proliferation (9, 22). Low levels of intracellular cAMP are associated with the initiation of proliferation (2, 14). Some cells in the PDL might enter a proliferation process shortly after the application of force (19, 20), and this might show itself as decreased staining for cAMP in the nuclei. The cytoplasm stains darker than the nuclei also at 7 days. The reason for the difference in staining intensity between the nucleus and the cytoplasm may partly lie in the protein kinase content of the cells. Cyclic AMP-dependent protein kinase mediates the effects of cAMP (1, 2, 11, 24), and this protein kinase may have a different distribution in terms of content in the nucleus and cytoplasm (12). During the immunohistochemical staining, free cAMP molecules do not remain in the tissue section because of their water solubility. Therefore, the cAMP detected during staining is that which has bound to the regulatory subunit of protein kinase. Thus, the level of bound cAMP in different components of the cell, as detected by immunohistochemistry, may be related to content and location of the protein kinase in the cells.

It is of specific interest that the use of the MAPS scanning program, as reported in this paper, has eliminated observer bias by taking the mean staining intensity of whole cells or cell components, instead of single readings of PLT through cells. We, therefore, conclude that this program is a useful tool to obtain information about the detailed molecular nature of the response of PDL cells to applied mechanical stresses.

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