Egyptian
Orthodontic Journal

QUANTIFICATION OF OSTEOCYTE APOPTOSIS DURING EXPERIMENTAL TOOTH MOVEMENT AS REVEALED BY IMMUNOHISTOCHEMISTRY OF CASPASE 3

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ABSTRACT:

Objective: *To investigate the time course of osteocyte apoptosis in a rat model of orthodontic tooth movement as revealed by the caspase-3 activation pathway.* ***Materials and Methods:*** *Fifteen male Sprague Dawley rats (6–9weeks old) were loaded with an orthodontic appliance. A spring delivering 10–12 g of force was placed between the maxillary first molar and the incisor to displace the first molar mesially. The animals were equally divided into three time points: 24 hours, 3 and 7 days of orthodontic loading. Five animals served as 0 day group. Caspase 3 immunostaining, was performed on histologic sections of the first molars. The labeling was quantified in osteocytes on the mesial and distal sides of the alveolar bone in each group.* ***Results:*** *Caspase 3 labeling at the mesial side significantly increased at 1, 3 and 7 days after orthodontic loading compared to the control group. No peak increase was observed. There was no significant difference in caspase 3 labeling at the distal side.* ***Conclusions:*** *Osteocyte apoptosis during orthodontic tooth movement could be revealed by caspase 3 immunostaining. This suggest that caspase 3 apoptotic pathway is involved in osteocyte death during orthodontic tooth movement.*

Keywords: *Osteocyte, Apoptosis, Caspase 3, Orthodontic Tooth Movement*

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INTRODUCTION

Orthodontic tooth movement is a process of alveolar bone modeling in response to an applied mechanical force that results in areas of pressure and tension in the periodontal ligament.[1]

Osteocytes may play a significant role as a mechanical sensory unit for regulating bone modeling and remodeling.[2,3] Osteocyte apoptosis have been proposed to play a specific role in recruiting osteoclasts to remodeling sites.[4]

It has been reported that both the intrinsic and extrinsic pathways regulating apoptosis in bone converge at the level of caspase 3,[5] which is required for the formation of apoptotic bodies, a typical hallmark of apoptosis.[6] Caspase-3 is thus considered a marker of apoptosis in bone studies, and its localization marks the initiation of the apoptotic process.

A few in vivo studies[7,8] reported that osteocytes could undergo apoptosis before osteoclast recruitment, the results however, were based on morphologic changes in nuclei,[7] or detection of DNA degradation by TUNEL[8] which is plagued with false positive results.[9]

There is a paucity of studies that investigate osteocyte apoptosis during orthodontic tooth movement using markers of the apoptotic pathway. Moin et al.[10] investigated osteocyte apoptosis in a mouse model of orthodontic tooth movement using immunohistochemistry of caspase 3. Elevated caspase-3 labeling was noted at 12, 24, and 72 hours and 7 days after loading, although the increase was not significant, casting doubt on the role of caspase 3 pathway in osteocyte apoptosis.[10] However, this stands in disagreement with a large body of evidence from investigations in other bone models and despite the greater specificity and sensitivity of caspase 3 assay to osteocyte apoptosis. [9]

This study aimed to investigate the temporal expression osteocytes apoptosis by caspase 3 immunostaining of paraffin sections in a rat model of orthodontic tooth movement in an attempt to validate this assay for osteocyte apoptosis for future studies.

MATERIALS AND METHODS

Study Design:

The experiment was performed under an institutionally approved protocol for the use of animals in research as part of a project for the doctorate thesis of the first author. In total, 20 male six to nine week old Sprague Dawley rats were used for the study. Fifteen animals were equally divided into 3 groups: 1, 3, and 5 days of orthodontic tooth movement. Five animals served as a 0 day control group. An orthodontic spring was placed in all animals between the maxillary left first molar and the maxillary incisors incisor. The rats were housed under normal laboratory conditions and weighed before the placement of the appliance and at the time of sacrifice. They were fed powdered chow and water ad libitum.

Application of Orthodontic Appliance:

Orthodontic appliances were placed under general anesthesia according to a protocol similar to that by Olson et al.[11]. The length of the coil spring was adjusted to deliver a force in the range of 10 – 12 g as measured by a Correx gauge (Haag-Streit, Koeniz, Switzerland). The appliances were checked daily to ensure their integrity.

Tissue Preparation for Histologic Analysis:

Upon completion of the time course, the rats were euthanized under anesthesia. The maxillae were hemisected, dissected, and placed in 10% formalin for 7 days at 4°C. After fixation, maxillae were decalcified in 10% formic acid at 4°C, processed with a series of ethanol concentrations, and embedded in paraffin. Next, 5 μm serial cross sections were cut using a Leica RM 2125R7 (Leica Microsystems, Nussloch GmbH). Serial transverse sections of the first molar region were cut from the inter-radicular alveolar bone toward the root apex.[12]

Immunohistochemistry of Caspase-3 Immunostaining for caspase-3:

Sections were deparafinized in xylene and rehydrated in graded concentrations of ethanol. Heat-mediated antigen retrieval was done by boiling 10mM citrate buffer (pH 6.0) for 10 – 20 min followed by cooling

at room temperature for 20 min. Subsequently, sections were washed with phosphate buffered saline and blocked with H_2O_2 Block for 5 min followed by UV Block for 5 min at room temperature. Sections were incubated with primary rabbit polyclonal anti-caspase 3 antibody Caspase 3 (CPP32) Ab-4 at a concentration of 5 – 10 µg/ml for 30 min at room temperature. To develop the signal, sections were then incubated with Quanto Amplifier for 10 min, rinsed then incubated in Quanto Polymer for 10 min, and then rinsed and incubated in DAB

Area of observation:

The cross sections from the mid-buccal root of the left maxillary first molar were used for observation and quantification. For every specimen, 3 sections for each assay were taken at 100 µm intervals. Images were taken with an Olympus BX51 light microscope (Diagnostic intrusments Inc, Sterling Heights, Mich.) equipped with a Nikon DX100 digital camera (Nikon, Tokyo, Japan) and stored in 24-bit true-color JPEG format.

The images at x 200 were imported to Adobe Photoshop CS image processing software (Adobe Systems Incorporated). An overlay was layered over the image to designate the area used for measurement.

Measurement of Apoptotic osteocyte percent:

To estimate the number of apoptotic osteocytes, the number of caspase-3 positive osteocytes were counted in the alveolar bone in the designated areas. The number of caspase-3 positive osteocytes was expressed by a percentage of the total osteocyte number. Counting was done by one operator on the computer image and confirmed by simultaneously examining the sections under the microscope at higher magnification as needed. Empty osteocyte lacunae were counted as viable osteocytes to avoid overestimation of the total osteocyte count due to osteocyte loss from lacunae during processing. For each animal, the apoptotic osteocyte percent was calculated as the average of the percent of caspase-3 positive osteocytes measured in 3 sections approximated to one-decimal point.

STATISTICAL ANALYSIS

Statistical analysis was performed by the software SPSS Statistical Package for Social Sciences (SPSS for windows, version 16). Comparisons between the mesial and distal sides were carried out by paired t test. Mean differences among groups were compared by one-way analysis of variance (ANOVA) tests with a Tukey HSD post-hoc test. Significance was set at $P < 0.05$.

RESULTS

Representative images of caspase 3 immunostained sections are shown in Figure 1.

At the mesial side, the mean (S.D.) caspase 3 positive percent in the control group was 22.4% (4.21); whereas it was 48.2% (6.28) at day 1, 51.7 (4.88) % at day 3, and 45.4 (3.39) % at day 5 of orthodontic tooth movement. At the distal side, apoptotic osteocyte percent was 20.4% (2.70) in the control group; 22.2% (2.67), 22.9% (6.32), and 21.0% (5.75) at day 1, day 3 and day 5 respectively. Table 1

The mean difference (S.E.) of apoptotic osteocyte percent between the pressure side and tension side in the control group was 2% (1.99) which was not statistically significant ($P = 0.360$). On the other hand, there was a statistically significant difference between the two sides; 26.0% (1.97), $P = 0.001$; 28.8% (1.95), $P = 0.001$; and 24.3% (2.88), $P = 0.001$ at day 1, day 3 and day 5 respectively. Table 1

Comparing time points at the mesial side, there was a statistically significant difference in the apoptotic osteocyte percent at days 1, 3 and 5 compared to the control group ($P = 0.001$). The mean differences (S.E.) were 25.8% (2.78) at day 1 ($P = 0.001$), 29.3% (2.77) at day 3 ($P = 0.001$) and 22.9% (2.70) at day 5 ($P = 0.001$). However, at the tension side, there was no statistically significant difference between the time points and the control group ($P = 0.797$), or the heavy force groups and the control group ($P = 0.688$). Table 2

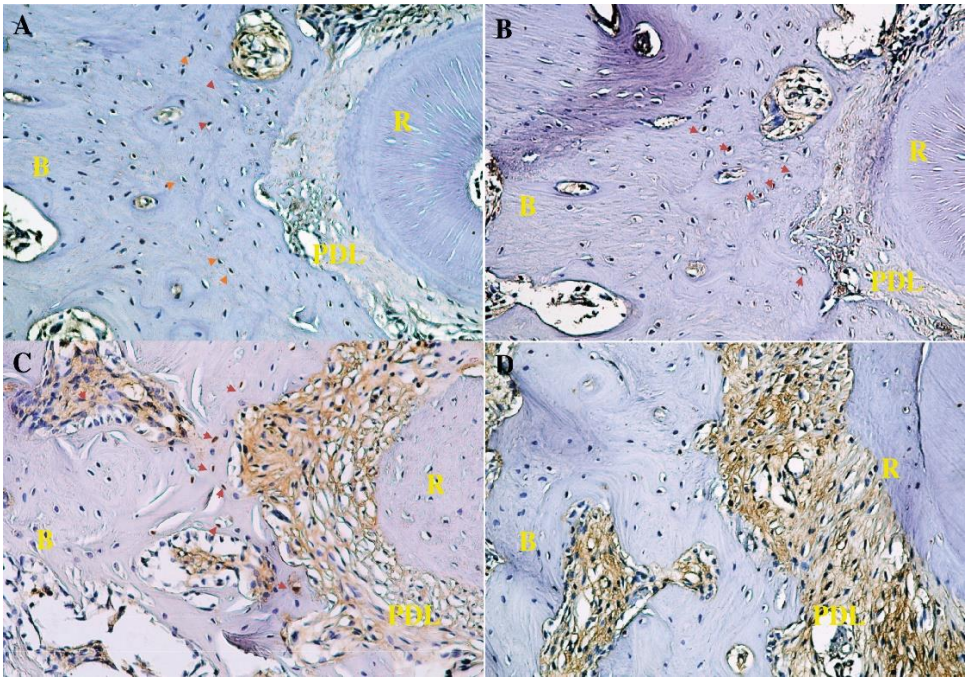

Figure 1. Photographs of immunostained sections. Positively stained osteocytes are shown in arrows. Control groups shows mostly negatively stained osteocytes. Micrograph of the mesial side. A. 200x. B. 400x. R Root; PDL Periodontal ligament; B Bone. A. 1 day; B. 3 days; C. 5 days D. Control group. (Caspase 3 x 200)

Table 1. Apoptotic osteocyte at the mesial and distal sides of each group.

	Apoptotic osteocyte percent (%)						
	Mesial Side		Distal side		Difference		
Study groups	Mean	S.D.	Mean	S.D.	Mean difference	Standard error	P value†
0 day (n=5)	22.4	4.21	20.4	2.70	2.0	1.99	0.360
1 day (n=5)	48.2	6.28	22.2	2.67	26.0	1.97	0.001*
3 days (n=5)	51.7	4.88	22.9	6.32	28.8	1.95	0.001*
5 days (n=5)	45.4	3.39	21.0	5.75	24.3	2.88	0.001*

† Based on paired samples t-test
* P ≤0.05

Table 2. Comparison of apoptotic osteocyte percent between groups at the mesial side and distal side.

	Difference of apoptotic osteocyte percent (%)					
	Mesial side†			Distal side†		
Comparison groups	Mean difference	Standard error	P value‡	Mean difference	Standard error	P value†‡
1 day – 0 day	25.8	2.78	0.001*	1.8	1.55	0.677
3 days – 0 day	29.3	2.77	0.001*	2.5	2.8	0.816
5 days – 0 day	22.9	2.70	0.001*	0.6	2.59	0.995

† Multiple comparison by ANOVA. P = 0.001, 0.757, respectively

‡ Post hoc comparison based on Tukey HSD test; equal variance assumed; significance level at P ≤0.05

* P ≤0.05

DISCUSSION

This study used an animal model of orthodontic tooth movement to investigate the responsiveness of osteocytes to different mechanical stress through the apoptotic pathway.

Immunostaining for caspase 3 showed that there was a significant increase in apoptotic osteocyte percent at day 1. Similarly, Sakai et al.[8] demonstrated a significant increase in osteocyte apoptosis starting at 6 hours using the TUNEL assay. Hamaya et al.[7] found that osteocytes exhibiting histological signs of apoptosis in H & E sections are significantly increased at 6 hours.

On the other hand, Moin et al.[10] found an increase in caspase 3 positive osteocyte starting at 12 hours, similarly trending thereafter with no statistically significant difference with the control values. In their study, TUNEL assay, however, showed a statistically significant increase at 12 hours trending till day 3. The difference in the caspase 3 assays between the current study and the results of Moin et al.[10] can be attributed to different reasons. Moin et al.[10] used the unloaded contralateral side as control. It can be noticed that the caspase 3 positive osteocyte percent kept fluctuating all along the time points which may suggest a distant effect of the apoptotic pathway. This claim can be supported by their

finding that the osteoclast number increased in the contralateral non-compression side.[10] In addition, CD-1 mice used in the study of Moin et al.[10] are significantly smaller in size compared to Sprague Dawely rats used in this study whereas the force used to move the maxillary molar in their study was similar to that of the light force group in the current study. This may have resulted in more osteocyte necrosis rather than apoptosis which is reflected in the difference in their results between the caspase 3 assay and TUNEL assay.

In the present study, there was no statistically significant difference in the apoptotic osteocyte percent at all the points. Moin et al.[10] reported in the mouse model that caspase 3 positive osteocyte increased slightly at day 1 and at day 3 and to a lesser extent at day 7 with no statistically significant difference among the time points. In contradiction, Moin et al.[10] found a peak increase in osteocyte apoptosis using the TUNEL assay at 24 hours. However, Sakai et al.[8] reported a peak increase at 12 hours using TUNEL. It is plausible to say that the current study missed the peak of osteocyte apoptosis at 12 hours as demonstrated by Sakai et al.[8] The inclusion of additional time point in the first few hours following the application of orthodontic force could have revealed a peak in osteocyte apoptosis. The conflict between the results of this study and TUNEL assay findings reported by Moin et al.[10] and Sakai et al.[8] may be explained by the inherent limitation of the TUNEL assay in differentiating osteocyte apoptosis and necrosis [13] in addition to false positive reaction due to DNA nicking during tissue processing.[9] Still, Hamaya et al.[7] detected a peak increase in osteocyte apoptosis at day 1 where apoptotic osteocytes were counted by examining H & E stained sections for signs of chromatin condensation and nuclear fragmentation. TUNEL assay was used only qualitatively to confirm their findings. This may have resulted in erroneous detection of osteocyte apoptosis especially that they detected chromatin condensation and nuclear fragmentation in osteocytes at 6 hours following the application of orthodontic force whereas the osteocyte did not show a positive TUNEL reaction till day 1 or day 2 despite that the PDL was TUNEL positive as early as 6 hours.

In the current study, the apoptotic osteocyte percent remained significantly elevated at day 5 compared to the control group. Moin et al.[10] did not study osteocyte apoptosis at day 5. However, they found that increase in caspase 3 positive osteocytes remained to a lesser extent at day 7 despite no significant difference between the increases along the time points. Sakai et al.[8] and Hamaya et al.[7] found levels to return to baseline at day 3 and day 4 respectively in contrast to the results reported by the present study. This may be attributed to differences in the animal models used and assays used to detect apoptosis.

In conclusion, the present study detected a significant increase in caspase 3 positive osteocyte in the mesial side considered to be a site of compression compared to the distal side considered to be a tension side in this animal model and an increase of caspase 3 positive osteocyte starting at day 1 and sustained till day 5. This suggests that caspase 3 immunohistochemistry can be a valid assay for the quantification of osteocyte apoptosis during orthodontic tooth movement.

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