Correlation between pain and hyalinization during tooth movement induced by different types of force

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ABSTRACT

Objectives: To evaluate the correlation between pain and tissue reactions during induced tooth movement (ITM).

Materials and Methods: Forty-two male Wistar rats (Rattus norvegicus; ~90 days of age, 300 g) were used. The animals were divided into seven groups of six rats each: one control group and six experimental groups subjected to ITM by continuous force (CF) or interrupted continuous force (ICF) for 1, 3, and 5 days. Hyalinization of the periodontal ligament (PL) and occurrence of pain were observed. Animal behavior (walking, climbing, immobile posture, resting/sleeping, and directed face grooming) and the presence of chemical mediators associated with nociception, cyclooxygenase-2 (COX-2), and interleukin-1 beta (IL-1 β) in the PL were analyzed.

Results: There was a moderate positive correlation between hyalinization and the presence of COX-2 ($r_s = 0.404$; P < .05) and IL-1 β ($r_s = 0.429$; P < .05). There was a moderate negative correlation between hyalinization and exploratory behaviors (walking, r = -0.586, P < .01; climbing, r = -0.573, P < .01), and a moderate positive correlation between hyalinization and resting/ sleeping (r = 0.467; P < .01).

Conclusions: The results suggest a correlation between pain and undesirable tissue reactions in ITM. (*Angle Orthod.* 2019;89:788–796.)

KEY WORDS: Tooth movement; Pain; Rats; Wistar; Behavior; Hyalinization

INTRODUCTION

Induced tooth movement (ITM) occurs as a result of forces applied to the teeth. Biologically appropriate forces allow ITM to occur with minimal side effects. However, inappropriate forces can generate excessive periodontal ligament (PL) stress, leading to the formation of hyalinized areas. Excessive stress on the PL and the formation of hyalinized areas can result in tooth movement delay, tooth mobility, bone loss, and root resorption.^{1–4}

The adverse effects of ITM can be controlled by achieving appropriate force intensity, distribution, and duration. However, application of the appropriate force is a challenge in clinical practice.^{1,5} The literature supports the use of light, interrupted, or intermittent, well-distributed forces.^{4–9} However, there are no clinical instruments that precisely measure the stress applied to different root, PL, or alveolar bone areas. Individual variability in tooth structures, PL, and alveolar bone hinders the establishment of clinical standards for ideal forces. Thus, the successful application of orthodontic

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Figure 1. Experimental groups.

force is still largely dependent on professional experience and skill.¹

A clinical parameter that can be useful during orthodontic treatment is pain perception. Pain associated with ITM results from mechanical stimuli and inflammatory reactions in PL compression and traction regions.¹⁰ However, the association between ITMrelated pain and inappropriate force application and, thus, undesirable tissue reactions, is unknown. Therefore, this study evaluated the relationship between PL hyalinization and pain in rats subjected to ITM via continuous force (CF) and interrupted continuous force (ICF). Pain was evaluated by the presence of chemical mediators associated with nociception in the PL, as well as by animal behavior.

MATERIALS AND METHODS

Experimental Groups

This study was approved by the Ethics Committee for the Use of Animals, Araçatuba Odontology University, Júlio de Mesquita Filho, São Paulo State University. Forty-two 90-day-old male Wistar rats (body weight, ~300 g) were used. The animals were housed in plastic cages in a colony room with a 12/12-hour light cycle at a temperature of 22 \pm 2°C with unrestricted access to food and water. After their arrival, the rats were allowed 1 week for acclimatization prior to study entry.

The animals were divided into seven groups of six rats each (Figure 1). The control group (C) was subjected to acclimatization, with a collection of behavioral data. A group of 18 animals underwent ITM with CF for periods of 1 (CF1), 3 (CF3), and 5 (CF5) days. The other group of 18 animals underwent ITM with ICF for periods of 1 (ICF1), 3 (ICF3), and 5 (ICF5) days.

Experimental Protocol

The experimental protocol (Figure 2) included ankylosis induction, ITM, and behavioral data collection. The animals were euthanized when the experimental protocol was concluded. The procedures for ankylosis induction, ITM, and euthanasia were performed under general anesthesia by intramuscular injection of 80 mg/ kg of ketamine hydrochloride (Dopalen, Sespo Ind. e Com. Ltda., Jacareí, SP, Brazil), combined with 10 mg/ kg of xylazine hydrochloride (Anasedan, Agribrands do Brasil Ltda., Paulínia, SP, Brazil).

Ankylosis Induction

The rats' upper right incisors underwent ankylosis induction to interrupt the continuous eruption process,



Figure 2. Experimental protocol.

avoid ITM device displacement, and ensure standardization of the applied force. Ankylosis induction was clinically verified after 14 days by the absence of tooth eruption and mobility.¹¹

Induced Tooth Movement

A force of 50 cN was applied to move the upper right first molar mesially. The device recommended by Heller and Nanda¹² was used; it was modified by replacing the steel closed-coil spring by a nickeltitanium closed-coil spring (Sentalloy, GAC, New York, NY, USA). The springs were attached to the upper right first molar and upper right incisor, with a steel string measuring 0.22 mm in diameter (Morelli, Sorocaba, SP, Brazil). Composite light-cured resin (Z100, 3M, St. Paul, MN, USA) was applied to the incisor's buccal face to improve device retention.

To establish the CF, the springs were stretched to 3 mm and remained active during the experimental periods. To generate the ICF, the springs were stretched to 3 mm and deactivated and reactivated during the experimental periods. In ICF1, the springs were deactivated after 12 hours. In ICF3 and ICF5, the springs were deactivated or reactivated every 24 hours. During deactivation and reactivation of the springs, the animals were sedated by inhalation of halothane (Tanohalo, Cristália, Itapira, SP, Brazil).

Behavioral Data Collection

Once the experimental periods were completed, behavioral data were collected according to the method proposed by Yang et al.¹³ The animals were placed, one at a time, in a glass cage ($30 \times 30 \times 30$ cm) and allowed to adapt for 15 minutes. They were then video-recorded for three periods of 10 minutes, with 20-minute breaks between recordings. The recordings were made between 9 AM and 12 PM.

Euthanasia and Sample Collection

The animals then underwent transcardial perfusion with 100 mL of a solution containing 0.9% sodium chloride (Sigma, St. Louis, MO, USA) and 0.1% heparin (Heparin, Cristália), followed by 800 mL of a fixative solution containing 4% formaldehyde (Sigma) in phosphate-buffered saline (PBS; 0.1 M, 4°C, pH 7.4; Sigma). The animals' right jaws were dissected and subjected to postfixation in the same fixative solution for 48 hours.

Histological and Immunohistochemical Processing

The samples were demineralized in PBS (Sigma) with 10% ethylenediaminetetraacetic acid (EDTA; Sigma) for 30 days. They were subsequently subjected

to conventional histological processing and were embedded in paraffin (Merck Millipore, Darmstadt, Germany) and cut into 4- μ m-thick longitudinal sections from the cervix to the apex, using a microtome. Semiserial sections, which included the crown and mesiobuccal and distobuccal roots of the upper right first molars, were collected.

For the histomorphometric analyses, the histological sections were stained with hematoxylin-eosin (HE; Sigma). For the immunohistochemical analyses, the histological sections were deparaffinized and hydrated. Antigenic recovery was performed by immersing the histological slides in citrate buffer (Diva Decloaker, Biocare Medical, Concord, CA, USA) in a pressurized chamber (Decloaking Chamber, Biocare Medical) at 95°C for 20 minutes. The slides were then immersed in 30% hydrogen peroxide (Sigma) for 1 hour and in 1% bovine serum albumin (Sigma) for 12 hours to block endogenous peroxidase and nonspecific sites, respectively. The slides were divided into two lots. Each lot was incubated with one of the following primary antibodies: anti-cyclooxygenase-2 (anti-COX-2) from a rat produced in a rabbit (mouse anti-COX-2, 1:200; SC-166475. Santa Cruz Biotechnology. Santa Cruz. CA, USA) and anti-interleukin-1 beta (anti-IL-1 β) from a rat produced in a rabbit (goat anti-IL-1β, 1:200; SC-1252, Santa Cruz Biotechnology). The sections were incubated with biotinylated secondary antibody for 2 hours and subsequently treated with streptavidin conjugated with horseradish peroxidase for 1 hour (Universal Dako Labeled HRP Streptavidin-Biotin Kit, Agilent Technologies, Santa Clara, CA, USA). At the end of each step, the slides were washed with 0.1 M PBS, pH 7.4 (Sigma).

The slides were developed using 3,3'-diaminobenzidine tetrahydrochloride as chromogen (DAB Chromogen Kit, Agilent Technologies). Counterstaining was performed with Harris hematoxylin (Sigma). The samples were subsequently dehydrated in ethanol (Sigma), diaphanized in xylol (Sigma), and covered with mounting medium (Permount, Fisher Scientific, San Diego, CA, USA) and glass slides (Knittel, Braunschweig, Germany). Negative control specimens were subjected to the procedures described previously, without the primary antibody.

Histomorphometric Analysis

For the histomorphometric analyses, the histological slide images were captured using a digital camera (JVC TK-1270 Color Video Camera, Long Beach, CA, USA) attached to a light microscope (Axiolab, Carl Zeiss, Oberkochen, Germany) and connected to a microcomputer (Dell, Round Rock, TX, USA) using Zen software (Axiolab, Carl Zeiss) and a magnification of



Figure 3. Histomorphometric analysis. (a) Red lines indicate the cervical and apical limits of the mesial face of the distobuccal root (MFDR); (b) the yellow line indicates the linear length of the MFDR; (c) green lines indicate the limits of the hyalinized area; (d) the blue line indicates the linear length of the hyalinized area; (e) $50 \times$ magnification.

50×. Quantitative analysis of the PL hyalinized area on the mesial face of the distobuccal root (MFDR) was performed using ImageJ software. Parallel lines were marked to determine the most cervical and apical portions of the MFDR. The total length of the MFDR was obtained by measuring the distance between the two lines. Subsequently, parallel lines delimitating the hyalinized region were marked and the linear length of the hyalinized region was obtained by measuring the distance between the two lines (Figure 3).

Immunohistochemical Analysis

Immunohistochemical analyses were performed on the PL of the mesiobuccal and distobuccal roots of the upper right first molars. The immunomarker patterns for COX-2 and IL-1 β were evaluated in all samples from group C to define the basal expression levels. The criteria for establishing the scores were as follows: 0, total absence of immunomarkers; 1, low immunomarker pattern, few immunoreactive cells (<10 cells/ area) and weak labeling in the extracellular matrix; 2, moderate immunomarker pattern, moderate number of immunoreactive cells (11-20 cells per area) and moderate labeling in the extracellular matrix; 3, high immunomarker pattern, large number of immunoreactive cells (\geq 21 cells per area) and strong labeling in the extracellular matrix. The areas of roots were analyzed at 400 \times magnification.

Behavioral Analysis

The following behaviors were considered:

- (a) Walking: when the animal walked inside the glass cage.
- (b) Climbing: when the animal climbed by placing one or both paws on the walls of the glass cage.
- (c) Immobile posture: when the animal remained still but awake.
- (d) Resting/sleeping: when the animal remained still in an apparent sleep state with its eyes open or closed.
- (e) Directed face grooming (DFG): when the animal rubbed its front paws against the mouth region.

The time spent in each of these behaviors was quantified with a chronometer. Each behavior was then calculated as a percentage of the total recording time.

Statistical Analysis

The data were analyzed using SPSS Statistics for Windows, Version 15.0 (SPSS Inc., Chicago, IL, USA). To calculate the methodological error, the evaluations were repeated with a randomly selected 10% of the samples. Comparisons between the analyses were conducted using interclass correlation and Kappa coefficients. The Shapiro-Wilk test was used to determine the normality of the data, and Levene's test was used to analyze the consistency between the variances. Groups were compared using parametric (one-way analyses of variance [ANOVA] and post-hoc Tukey's tests) and nonparametric (Kruskal-Wallis and post-hoc Mann-Whitney U-tests) analyses. Pearson's and Spearman's correlation coefficients were used to analyze correlations between the variables. A P-value of <.05 was considered significant. The sample size was based on previous studies where the same methodology was used. Sample size ensured study power greater than 80%.

RESULTS

Intraclass correlation coefficients for the histomorphometric and behavioral analyses and Kappa coefficients for the immunohistochemical analyses were >0.8, indicating excellent intra-examiner agreement. Figure 4 shows the histological slides from the different groups. Table 1 shows the results of the histomorphometric analysis. Hyalinization is shown as a percentage of the total linear length of the MFDR. Table 2 shows the results of the immunohistochemical analyses, and Table 3 shows the results of the behavioral analyses.

Hyalinization was significantly (moderately) positively correlated with the presence of COX-2 ($r_s = 0.404$; P



Figure 4. Histological slides from the different groups: distobuccal root. (a) A-CF 1; B-CF 3; C-CF 5; D-ICF 1; E-ICF 3; F-ICF 5; G-C; (b) pl indicates periodontal ligament; p, pulp; d, dentin; h, hyalinization; ab, alveolar bone; (c) $50 \times$ magnification.

< .05) and IL-1 β (r_s = 0.429; P < .05). Figure 5 shows scatter plots for the correlations between hyalinization and behaviors. Hyalinization was significantly (moderately) negatively correlated with walking (r = -0.586; P < .01) and climbing (r = -0.573; P < .01), and (moderately) positively correlated with resting/sleeping (r = 0.467; P < .01).

DISCUSSION

In search for clinical criteria to guide the application of orthodontic forces, this study aimed to explore the correlations between pain and tissue reactions caused by ITM. Two parameters were used to evaluate pain: the presence of chemical mediators in the PL and

Table 2. Immunomarker Pattern of Cyclooxygenase-2 and Interleukin-1 Betaa*

	Score		
Group	COX-2	IL-1β	
CF1	З А	З А	
ICF1	3 A	3 A	
CF3	2 в	З А	
ICF3	2 в	3 A	
CF5	2/1 в	2 в	
ICF5	2/1 в	2/1 в	
С	1 в	1 в	

^a CF 1 indicates Continuous Force 1 day; CF 3, Continuous Force 3 days; CF 5, Continuous Force 5 days; ICF 1, Interrupted Continuous Force 1 day; ICF 3, Interrupted Continuous Force 3 days; ICF 5, Interrupted Continuous Force 5 days; C, Control; COX-2, Cyclooxygenase-2; IL-1β, Interleukin-1 beta.

Scores followed by different letters indicate a significant difference (Kruskal-Wallis test; Mann-Whitney U-test for multiple comparisons with a Bonferroni correction; P < .0167).

animal behavior. Studies correlated pain intensity with the presence of IL-1 β^{14-16} and prostaglandin $E_2^{14,16}$ in the crevicular fluids of patients receiving ITM. The action of COX-2 on arachidonic acid is the primary mechanism of prostaglandin E₂ production.¹⁷ This evidence substantiated the investigation of IL-1 β and COX-2 in this research.

Many studies have evaluated pain in rats through behavioral analysis and grooming was one of the primary activities observed.¹⁸⁻²⁰ Grooming is a regular behavior in rodents, consisting of prolonged episodes of highly organized fur care in which the paws, tongue, and incisors are frictioned against different parts of the body. The friction between the front paws and the face is called face grooming. Grooming is associated with cleaning, thermoregulation, and pheromone distribution for social signaling purposes. This behavior also occurs in response to painful stimuli, typified by its higher intensity, lower duration, and targeted direction toward irritated or painful areas.13,19 In addition to grooming, Vos et al.20 reported that rats subjected to painful stimuli exhibited a decrease in normal exploratory behavior.

Behaviors associated with pain have also been observed in rats subjected to ITM. Yozgatian et al.²¹ and Shibazaki et al.22 observed both a reduction in exploratory behavior and an increase in face grooming. Yang et al.¹³ identified directed face grooming (DFG)

Table 1. Hyalinization of the Mesial Face of the Distobuccal Roota*

	Group							
	CF1	CF3	CF5	ICF1	ICF3	ICF5	С	
Mean (%) \pm SD	$49.48\pm6.36~\text{a}$	$53.25\pm4.02~\text{a}$	51.75 ± 8.80 a	$59.39\pm5.98~\text{a}$	57.12 ± 15.15 a	$52.11\pm9.47~\text{a}$	0.00 ± 0.00 в	

^a CF 1 indicates Continuous Force 1 day; CF 3, Continuous Force 3 days; CF 5, Continuous Force 5 days; ICF 1, Interrupted Continuous Force 1 day; ICF 3, Interrupted Continuous Force 3 days; ICF 5, Interrupted Continuous Force 5 days; C, Control.

Means followed by different letters indicate a significant difference (one-way analysis of variance; Tukey's test for multiple comparisons; P < .05).

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Group			Behavior, Mean (%) \pm SD		
	Walking	Climbing	Immobile Posture	Resting/Sleeping	DFG
CF1	3.19 ± 2.11 A	0.32 ± 0.12 A	12.26 ± 3.73 A	63.44 ±12.69 A	4.93 ± 3.60 Å
CF3	5.91 ± 5.71 A	0.78 ± 1.03 A,B	45.38 ± 33.55 A	32.20 ± 42.25 A	5.26 ± 6.75 /
CF5	6.45 \pm 4.60 A	0.72 ± 1.14 A,B	31.42 ± 23.14 A	42.15 ± 31.26 A	4.45 ± 3.85 /
ICF1	$6.15~\pm~5.85$ A	0.47 ± 0.51 A	26.44 ± 20.11 A	53.85 ± 13.71 A	4.77 ± 3.13 /
ICF3	10.46 ± 5.81 A,B	1.38 ± 1.08 A,B	30.51 ± 17.89 A	46.89 ± 25.69 A	6.75 ± 5.32 /
ICF5	10.02 ± 5.61 _{А,В}	1.80 ± 1.49 A,B	26.81 ± 20.78 A	29.85 ± 26.32 A	7.50 ± 4.64 /
С	21.41 ± 12.99 в	4.69 ± 4.16 в	38.31 ± 29.94 A	9.57 ± 13.28 A	4.43 ± 3.29

Table 3. Time Spent Performing Each Behaviora*

^a CF 1 indicates Continuous Force 1 day; CF 3, Continuous Force 3 days; CF 5, Continuous Force 5 days; ICF 5, Interrupted Continuous Force 1 day; ICF 3, Interrupted Continuous Force 3 days; ICF 5, Interrupted Continuous Force 5 days; C, Control; DFG, Directed Face Grooming.
* Means followed by different letters indicate a significant difference (one-way analysis of variance; Tukey's test for multiple comparisons; P <

as an indicator of pain caused by ITM in rats. This relationship was also reported by Yang et al.^{23} and by Gao and Duan.^{24}

In this study, PL hyalinization occurred in all groups receiving ITM. There was no significant difference between the groups, even when the type of force applied and the experimental periods varied. Burstone²⁵ highlighted that it was difficult to achieve tooth movement without pathological damage. Hyalinization occurs due to excessive stress in the PL. Although it is difficult to perform tooth movement without the occurrence of hyalinization, this phenomenon is an undesirable side effect of tooth movement.^{1–4} Thus, in the present study, any level of hyalinization was considered as an undesirable tissue reaction of ITM.

The results revealed a high COX-2 concentration 1 day after the onset of ITM, with progressive decreases as experimental time increased. IL-1 β concentrations after 1 day were the same as after 3 days, but decreased by day 5. These results suggested that the rats experienced higher pain during the earlier ITM periods, regardless of the type of force applied.

Both DFG and behaviors related to higher (walking, climbing) and lower (immobile posture, resting/sleeping) exploratory activity were evaluated. No significant differences were observed for DFG. However, there was a reduction in exploratory behaviors of groups subjected to ITM when compared to Group C, suggesting pain in ITM-treated animals. This reduction was significant for the walking behavior between groups C and CF1, CF3, CF5, and ICF1, and for the climbing behavior between groups C and CF1 and ICF1. Although there were no significant differences in resting/sleeping, the time spent performing this behavior was noticeably lower in the control group (Group C) when compared to that of the ITM-treated groups, confirming the reduction in exploratory activity in the latter groups. Additionally, the time spent performing this behavior was considerably higher in Group CF1 and increased in Group ICF1, suggesting that the animals subjected to ITM with CF experienced more pain than those subjected to ITM with ICF, and that the 1-day experimental period was highly related to the occurrence of pain.

The results of this study indicated that hyalinization was positively correlated with the presence of chemical mediators associated with nociception in the PL, negatively correlated with exploratory behaviors (walking and climbing), and positively correlated with opposing resting/sleeping behavior. These findings suggested that the occurrence of pain with ITM was associated with the formation of hyalinized areas in the PL. Thus, the results revealed a correlation between pain and undesirable tissue reactions in ITM, suggesting that patient-reported pain could be a useful clinical criterion for determining an appropriate system of orthodontic forces to mitigate tissue damage and improve patient comfort.

Behaviors associated with pain were more notable during the early ITM periods, and the presence of chemical mediators associated with nociception decreased throughout the experimental periods, while hyalinization remained unaltered. Therefore, further studies with longer experimental periods are necessary to determine the temporal relationship between pain and hyalinization reduction. This study showed that pain, either due to orthodontic mechanics or to occlusal interference, indicated unfavorable biological recovery of the periodontium. It also showed that pain can be decreased, even when normal PL conditions have not been restored.

The optimal orthodontic force produces a maximum rate of tooth movement. Forces above optimal lead to the formation of hyaline areas and prevent frontal resorption of the alveolar bone, thus leading to reduction in the rate of tooth movement.¹ Theoretically, the reduction in the rate of tooth movement is accompanied by a higher level of pain due to the associated biological events. However, the relationship between less efficient tooth movement and higher levels of pain remains unclear. This is another point that deserves to be elucidated with further research.

.05)



Figure 5. Correlations between hyalinization and rat behavior.

In this study, the aim was to evaluate the pain arising from tooth movement. However, some level of pain may have occurred due to the incisor ankylosis or to the presence of the intraoral device. This was a limitation of the research. To overcome it, further research should be performed with groups of animals submitted only to ankylosis of the incisor and to the installation of the intraoral device, and not to tooth movement.

This study approaches an issue of great clinical importance that has been little explored. The results

indicate that orthodontic pain can be used as a clinical parameter to guide the application of adequate orthodontic forces, thus contributing to minimize the deleterious effects of tooth movement.

CONCLUSIONS

 The experience of pain is more intense in the initial ITM period. CF seems to be more closely associated with the occurrence of pain than ICF. The experience of pain during ITM was associated with the formation of hyalinized areas in the PL. Therefore, there may be a correlation between pain and undesired tissue reactions during ITM.

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