Effect of acute administration of nicotine and ethanol on tooth movement in rats

Abstract: The aim of this study was to evaluate the effect of acute administration of nicotine and ethanol on tooth movement in rats. Two hundred rats were divided into eight groups: S: saline; N: nicotine; E: ethanol; NE: nicotine and ethanol; SM: saline with tooth movement; NM: nicotine with tooth movement; EM: ethanol with tooth movement; and NEM: nicotine and ethanol with tooth movement. All the solutions were applied for 32, 44, or 58 days, according to the subgroup. Orthodontic movement (25 cN) was initiated 30 days after solution administration in the groups with tooth movement. The rats were euthanized 2, 14, or 28 days after initiation of tooth movement. Tooth sections were stained using picrosirius and tartrate-resistant acid phosphatase (TRAP). The data were compared by ANOVA using Tukey’s HSD and Games-Howell. On day 28 of tooth movement, the NEM group had a lower percentage of type I collagen compared to the SM group (p = 0.0448), and the S group had a higher number of osteoclasts/μm² compared to the N group (p = 0.0405). Nicotine and ethanol did not affect the tooth movement rate, regardless of induction of orthodontic movement. Nicotine influenced the number of osteoclasts by decreasing their quantity when dental movement was not induced. When nicotine was associated with ethanol, it interfered in the maturation of collagen fibers during orthodontic movement.

Keywords: Nicotine; Ethanol; Tooth Movement Techniques; Orthodontics.

Introduction

Alcohol ranks first and nicotine abuse ranks third among the preventable causes of death in the United States, accounting for more than 480,000 and 80,000 deaths per year, respectively. Nicotine is one of approximately two thousand substances with toxic potential in cigarette smoke, and has been identified as the most important promoter of tobacco-induced pathogenic effects on the periodontium. As such, this substance is a risk factor for osteoporosis, and hinders the repair of bone fractures. Ethanol is the main component of alcoholic beverages, and is considered toxic to bones. It promotes several direct effects on the activity of bone cells, such as inhibition of mesenchymal cell differentiation into osteoblasts in the bone marrow, and the growth of osteoblastic cells.

Although the risks associated with smoking have been known for decades, there are an estimated 1.3 billion smokers worldwide.
indicate that nicotine plays an important role in bone metabolism. Sodagar et al.\textsuperscript{11} evaluated the rate of tooth movement in rats subjected to 28 days of nicotine application, and observed that nicotine accelerated the tooth movement of these animals after 14 days of movement, and had a dose-dependent effect. Bakathir et al.\textsuperscript{12} also applied nicotine for 28 days and performed orthodontic movement for 14 days, and found that nicotine accelerated orthodontic movement, causing an imbalance between reabsorption and bone neoformation. However, Shintcovsk et al.\textsuperscript{13} evaluated the effect of 2 mg/kg nicotine on tooth movement in rats in a histological study, and observed that nicotine affected bone remodeling, reduced angiogenesis, osteoclasts and Howship's lacunae, and also delayed collagen maturation in the bone matrix.

Alcohol abuse is a public health problem in the United States, and adolescent and young adult populations maintain dangerous drinking practices, highlighting that binge-pattern alcohol consumption is particularly common in these age groups.\textsuperscript{24,25} The National Institute of Alcoholism and Alcohol Abuse (NIAAA), a branch of the United States Department of Health, defines this pattern as promoting high serum levels of ethyl alcohol, which usually occur about 2 hours after 4 drinks for women and 5 drinks for men. Thus, this pattern is not only associated with a high serum concentration, but also with a short time period.\textsuperscript{16} A bibliographical review conducted by Barcia et al.\textsuperscript{17} on the effect of ethanol on orthodontics mentioned that there is only one study in the scientific literature on the effects of ethanol on tooth movement, despite several studies having indicated the potentially deleterious effects of ethanol on periodontium and alveolar bone. This singular study was conducted by Araujo et al.\textsuperscript{8} using a dose of 3 g/kg/day of 20% ethanol in rats that mimicked the binge pattern of ethanol consumption. The study evaluated this effect on orthodontic movement, and observed a decrease in bone resorption on day 28 of this movement.

In orthodontic practice, the prevalence of smokers in orthodontic treatment represents, on average, 26% of adults and 12% of adolescents.\textsuperscript{5} According to data from the American Association of Orthodontists the percentage of adults who used orthodontic appliances from 1994 to 2010 increased from 680,000 to 1.1 million per year (58%). According to Barcia et al.,\textsuperscript{17} many of them consumed ethanol at some point during orthodontic treatment. Since excessive consumption of alcoholic beverages is highly correlated with smoking,\textsuperscript{18} it is extremely important to know the effects of these two substances on orthodontic movement.

Therefore, the objective of this study was to evaluate the effect of nicotine and ethanol on tooth movement in rats, in regard to the following aspects: bone neoformation, bone resorption and tooth movement rate.

**Methodology**

The present study was prepared according to the guidelines of Animal Research: Reporting of In Vivo Experiments (ARRIVE)\textsuperscript{19}, and was approved by the Ethics Committee on Animal Use (#778/#779).

**Sample**

The initial sample consisted of 210 male *Wistar* rats (*Rattus norvegicus albinus*), approximately 9 weeks of age and weighing 300–350 g. The animals were provided and maintained by the vivarium of the university; all care followed the recommendations and ethical guidelines of the Canadian Council on Animal Care.\textsuperscript{20}

The animals were randomized into 8 groups (Figure 1). Group S had a smaller sample size due to issues involving the release of animals by the ethics committee. Twenty percent ethanol was administered intraperitoneally at a dose of 3 g/kg\textsuperscript{8,21} that mimicked the binge pattern of ethanol consumption: administration for 4 consecutive
days, followed by 3 days of abstinence.\textsuperscript{8,22} The nicotine (nicotine hemisulphate, Sigma-Aldrich Chemicals Pvt. Ltd., St. Louis, MO, USA) was previously prepared, and was diluted in 0.9% saline, homogenized, and administered subcutaneously at a dose of 1 mg/kg\textsuperscript{11,23,24,25} on the dorsa, every day until the day of euthanasia. In the groups in which ethanol and nicotine were applied, the same administration protocols were followed concomitantly. The saline solution was administered in a volume and at a periodicity similar to the groups receiving the experimental solutions. All the solutions were applied once daily for 32, 44, or 58 days, according to each subgroup. The orthodontic movement began after 30 days of application to the groups with tooth movement.

The rats were weighed weekly using a precision electronic balance (Gehaka-BG 4001, São Paulo, Brazil) to adjust the dose of the administered substances. The animals were euthanized, according to subgroup, after 32, 44, or 58 days of substance administration, and 2, 14, or 28 days of tooth movement in groups with orthodontic appliances. All the animals were euthanized by anesthetic overdose (270 mg/kg ketamine and 30 g/kg xylazine) administered intraperitoneally.

**Tooth movement**

Tooth movement was induced with an orthodontic device\textsuperscript{8,26,27} consisting of a nickel-titanium spring (Ultralight, 3mm, 25g, Dentsply GAC, Dentsply Sirona, Islandia, USA – Reference Number 10-000-26). A stainless steel 0.025-mm ligature wire (Dental Morelli Ltd., São Paulo, SP, Brazil) was used to attach the spring to the first right maxillary molar and central incisors, and to transmit a reciprocal force of 25 cN (Figure 2).\textsuperscript{26} The force was measured by a previously calibrated dynamometer (Haag-Streit 0 2468 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 Days Start of the application of the solutions Orthodontic device installation Euthanasia 2 Days Euthanasia 14 Days Euthanasia 28 Days

**Figure 1.** Flow chart showing distribution of the animals per group (initial sample and final sample) and experiment timeline.
AG, Koeniz, Switzerland). The tip of the ligature wire was fixed to the upper right incisor, and the ligature was attached to the tooth with composite resin (Charisma, Heraeus, Hanau, Germany). The upper left incisor was also coated with resin to increase the stability of the device. After initial activation, the spring was not reactivated during the experimental period. However, the positioning was checked daily.

**Histotechnical processing**

After the animals were euthanized, the right hemimaxillae were removed, stored in 10% formaldehyde for 24 h, and demineralized with 5% ethylenediamine tetraacetic acid (EDTA) (Biotec Analytical Reagents, Pinhais, PR, Brazil) for 2 months. After demineralization, the specimens were processed and embedded in paraffin.

Six 4μm-thick transverse sections were removed from each tooth for staining of the cervical third of the mesiobuccal root of the upper first molar. The teeth were cut using a microtome, with the occlusal surface of the molar parallel to the microtome, and with a 60-μm interval between each section, totaling 1,200 sections for each staining. The sections were stained by picrosirius techniques28 and tartrate-resistant acid phosphatase29 (TRAP).

New bone formation was verified through picrosirius staining. An area of the bone adjacent to the side where the fibers were most tightly stretched was chosen for evaluation, since bone is deposited on the alveolar wall of the traction side during orthodontic movement. Images were taken using an Olympus BX-50 microscope (Olympus, Tokyo, Japan) with a polarized Olympus® U-Pot lens (Olympus, Tokyo, Japan), coupled to a Dinolite® microcamera (AmMo Electronics Corporation, New Taipei City 241, Taiwan) with 200X magnification. The images were edited with Adobe Photoshop® CS5 (Adobe Systems Incorporated, San Jose, USA); the periodontal ligament and the root were excluded from the images to allow for analysis of the bone tissue. The images were evaluated using the Image-Pro Plus 4.5 image analyzer (Media Cybernetics, Rockville, USA), which measured the percentage of the area of mature and immature collagen in the alveolar bone, using the “count and measure objects” tool.30 Type I collagen (mature collagen) presented as a red-orange color, and type III collagen (immature), as green-yellow.30 The percentage of collagen for each animal was calculated by taking the average of the 6 sections.

In the sections stained by TRAP, bone resorption was evaluated by the number of osteoclasts per square micrometer (μm²) in the periodontal ligament. This enzyme is considered a marker of osteoclastic cells, because it allows quantification of bone resorption. Accordingly, multinucleated TRAP-positive cells in the periodontal ligament adjacent to the alveolar bone were considered as functional osteoclasts and were quantified.33 For quantification purposes, images of all the periodontal ligament with TRAP-positive cells were captured using an Olympus BX-50 microscope coupled to a Dinolite® microcamera at 400X magnification. The mean of osteoclasts per
Araujo CM, Rocha AC, Araujo BMM, Johann ACBR, Pereira LF, Tanaka OM et al.

The area of the entire periodontal ligament was quantified using the “count and measure objects” tool of the Image Pro-Plus 4.5 (Media Cybernetics, Silver Spring, USA) morphometry program. The osteoclasts were quantified by analyzing the images in the Image Pro-Plus 4.5 morphometry program, with a grid used for counting osteoclasts. After obtaining the number of TRAP-positive cells in all the ligaments and area of the periodontal ligament, the mean number of osteoclasts/μm² in the periodontal ligament was calculated using the following formula:

\[
\frac{\text{Total number of TRAP-positive cells}}{\text{Periodontal ligament area}}
\]

Each variable was measured by a single trained and calibrated evaluator. Reproducibility of the measurements and intraexaminer calibration was confirmed by remeasuring 30% of the samples 21 days after the first measurement, using the Dahlberg error and the Student’s t-test. The result of the Dahlberg error was 1.36%, indicating that the measurement was reproduced reliably by the evaluator. To evaluate systematic error, the mean of the variables at two time periods was compared using the Student’s t-test for paired samples. The test result indicated that there was no statistically significant difference between the mean values of the variable at the two time points.

Variation of tooth movement

Variation of tooth movement was evaluated by molding the upper dental arch of the rats before orthodontic device installation and at the end of the experiment, shortly after euthanasia. The molds were prepared as follows: the animal was anesthetized with sodium pentobarbital (Syntec, Cotia, Brazil) and its arch was molded with polydimethylsiloxane-based condensation silicone (Vigodent Coltene, Rio de Janeiro, Brazil).

The amount of molding material was carefully calculated to provide a base that would be thick enough to ensure no distortion of the mold. The models were made with type IV gypsum (Durone IV Gesso Pedra, Dentsply Sirona, Petrópolis, Brazil) (Figure 3).

Given the continuous eruption of incisors in rats, a fixed point of measurement was chosen. The distance between the most palato-cervical point of the upper right incisor and the most mesiocervical point of the upper right molar was measured using a digital caliper.

![Figure 3](image_url). Photograph of the molding procedure of the animal with condensation silicone (A), making of molds (B) and measuring the distance from the palatal face of the upper right incisor to the mesial surface of the first upper right molar, using a digital caliper (C).
of the first right upper molar was measured using a digital caliper (Absolute, Mitutoyo, Kawasaki-Shi, Japan). Tooth movement was calculated using the following formula:

\[
\text{Variation of tooth movement} = \text{Initial Distance} - \text{Final Distance}
\]

The researcher was blinded to all the research outcomes measurements by classifying the animals by number, to ensure that he did not know the animal's group. Blinding precluded any bias in the selection of results or influence in the outcomes evaluated.

**Statistical analysis**

Statistical analysis was performed using SPSS for Windows (Version 23.0, IBM Corp., Armonk, USA). A p-value < 0.05 was considered significant.

The normality of the data was tested using the Shapiro-Wilk test. Then, the homogeneity of the variances among the different treatments was tested using the Levene variance homogeneity test. Once the groups showed a normal distribution (p > 0.05), the comparison of the mean values according to group and time was performed using the two-way ANOVA parametric test with a full factorial model. When ANOVA indicated a difference, and when the treatments presented variance heterogeneity (p < 0.05), the comparison of 2 to 2 treatments was made using the parametric multiple comparisons test for heterogeneous Games-Howell variances. Tukey's multiple comparisons test was used for homogeneous variances. The power observed for each factor and for the group vs. time interaction (power test) was calculated for each of the dependent variables according to group vs. time.

**Results**

**Bone neoformation**

On day 28 of tooth movement, the NEM group presented a lower percentage of type I collagen than the SM group (p = 0.0448) (Table 1, Figure 4).

![Figure 4](image-url)
Bone resorption

In the comparison of groups without tooth movement, it was verified that group S had a greater number of osteoclasts on day 28 than group N (p = 0.0405) (Table 2, Figure 5).

Rate of tooth movement

There was no statistically significant difference in tooth movement variation in the group vs. time relationship (p > 0.05) (Table 3).

Discussion

The present study evaluated the following aspects regarding the effects of ethanol and nicotine on orthodontic movement: bone neoformation, bone resorption and tooth movement rate. Differences were observed in bone neoformation (SM × NEM) and bone resorption (S × N).

Approximately 90–95% of the organic matrix of the periodontal ligament is composed of type I

Table 2. Comparison of number of osteoclasts per μm² in relation to group vs. time (mean and standard deviation).

<table>
<thead>
<tr>
<th>Group/Day</th>
<th>S (Mean ± SD)</th>
<th>N (Mean ± SD)</th>
<th>E (Mean ± SD)</th>
<th>NE (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td>0.000154 ± 0.000094 ^</td>
<td>0.000086 ± 0.000024 ^</td>
<td>0.000207 ± 0.000122 ^</td>
<td>0.000219 ± 0.000122 ^</td>
</tr>
<tr>
<td>14 days</td>
<td>0.000069 ± 0.000031 ^</td>
<td>0.000081 ± 0.000058 ^</td>
<td>0.000142 ± 0.000080 ^</td>
<td>0.000086 ± 0.000046 ^</td>
</tr>
<tr>
<td>28 days</td>
<td>0.000216 ± 0.000047 ^</td>
<td>0.000102 ± 0.000031 ^ b</td>
<td>0.000314 ± 0.000113 ^ ab</td>
<td>0.000109 ± 0.000054 ^ ab</td>
</tr>
</tbody>
</table>

Two-way ANOVA full factorial design: p < 0.05; *Significance level of the Games-Howell test was p < 0.05; Different letters in the same line indicate statistically significant differences.

Figure 5. Photomicrograph blade of the periodontal ligament of the buccal mesial root of the first right upper molar. Image of groups S (A), N (B) E (C) and NE (D) on the 28th day after installation of the orthodontic device. Observe the lower number of osteoclasts per μm² in group N compared with S. AB, alveolar bone; PL, periodontal ligament. Black arrows indicate TRAP-positive cells. (TRAP, 500X magnification).

Table 3. Comparison of the varying tooth displacement rate (mm) in relation to group vs. time (mean, standard deviation and p value).

<table>
<thead>
<tr>
<th>Group/Day</th>
<th>SM (Mean ± SD)</th>
<th>NM (Mean ± SD)</th>
<th>EM (Mean ± SD)</th>
<th>NEM (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td>0.6833 ± 0.5845</td>
<td>0.4538 ± 0.5141</td>
<td>0.9571 ± 0.6579</td>
<td>0.6933±0.6649</td>
</tr>
<tr>
<td>14 days</td>
<td>0.3000 ± 0.2529</td>
<td>0.3000 ± 0.3012</td>
<td>0.5444 ± 0.6984</td>
<td>0.4400±0.4949</td>
</tr>
<tr>
<td>28 days</td>
<td>0.5399 ± 0.5176</td>
<td>0.3642 ± 0.3152</td>
<td>0.3889 ± 0.2713</td>
<td>0.5818±0.4792</td>
</tr>
</tbody>
</table>

Two-way ANOVA full factorial design: p > 0.05; *Significance level of the Tukey’s Test was p < 0.05.
collagen. Tooth movement causes bone resorption and consequent degrading of this matrix, which is subsequently repaired by the deposition of immature type III collagen fibers. In turn, these immature fibers are remodeled into type I collagen. In our study, the NEM group showed a significant decrease in type I collagen in the alveolar bone, compared with the SM group (p < 0.05). Our findings corroborate those by Soares et al., who observed that administration of a 10% ethanol solution and 1.25 mg/kg nicotine for 4 weeks had a negative effect on osteogenesis around the implants of the study animals. However, ethanol associated with nicotine intensified the effects.

Araujo et al. evaluated only the effect of ethanol (3 g/kg) on the collagen matrix in rats subjected to tooth movement and did not observe the differences in the percentage of type I collagen in the alveolar bone (p ≥ 0.05), suggesting that ethanol did not influence the deposition process of collagen fibers. Shintcovisk et al. evaluated the effect of nicotine (2 mg/kg) on the collagen matrix; they observed that nicotine delayed maturation of collagen fibers deposited in the bone matrix. However, this study used twice the dosage of the present study. Ghanem et al. performed a systematic review to evaluate the role of nicotine on the osseointegration of implants. They found that 62.5% of the studies reviewed showed no significant influence of nicotine on the healing process around implants. In the present study, our evaluation of the interaction of ethanol with nicotine suggests that nicotine associated with ethanol delayed the maturation of collagen and/or increased the degradation of type I collagen.

The scientific literature is unanimous in concluding that different force vectors create different stresses along the root. No tooth has the ideal shape and proportion, and linearity assumptions about the distribution of force in hard and soft tissues are problematic. For this reason, the use of mean osteoclasts per square micrometer (TRAP) and the choice of what bone should be evaluated based on the location where the fibers are most tightly stretched (picrosirius) avoids measurement bias. This is because the distribution of tension and compression forces varies according to the height assessed on the long axis of the root.

Regarding bone resorption, in the present study, we observed that group N had a lower number of osteoclasts on day 28 than group S. Although the nicotine dosage administered by Shintcovisk et al. was different from that used in the present study, they observed that animals receiving 2 mg/kg nicotine and subjected to orthodontic movement had a reduced expression of osteoclastic cells and Howship’s lacunae. However, when tooth movement was performed in the present study, it did not result in any statistical difference in number of osteoclasts. In constrast, Bakathir et al. used three doses (0.37 mg/kg, 0.57 mg/kg, and 0.93 mg/kg) and observed a higher frequency of osteoclasts in the groups that received nicotine – the authors presented no quantification of histological data, only qualitative results. The method of measuring bone resorption may have influenced the results. In our study, the mean osteoclasts per square micrometer were measured to avoid the risk of bias relative to the choice of the compression side in the periodontal ligament and alveolar bone. The mean of all ligaments has become a more reliable model to avoid a false-positive or false-negative result. Thus, in the present study, the variables, including bone resorption, had convergent results, thus increasing the strength of the evidence.

Studies on the effect of ethanol on bone tissue have used concentrations ranging from 5% to 20% for 4 to 12 weeks. Sampson et al. evaluated the effect of 5% ethanol on rat bone conditions and observed increases in bone mineral density. On the other hand, Callaci et al. administered 20% ethanol in rats, in a binge-pattern, and found decreased mineral density and compressive strength in the vertebrae. In the present study, ethanol did not influence the bone metabolism, independent of the movement of the teeth. Although there have been advances in understanding the complex actions of alcohol on bone, much remains to be determined. Limited evidence points to age, the skeletal site evaluated, and the duration and pattern of drinking as important variables, and may explain the conflicting results between studies.

To our knowledge, no scientific study in the literature has evaluated the effect of ethanol and nicotine on the variation of tooth movement. There
are only isolated reports of the influence of these substances. Sodagar et al. evaluated the effects of three doses of nicotine (0.5 mg/kg, 0.75 mg/kg, or 1 mg/kg) on tooth movement in rats, using a force of 60 g/f. Bakathir et al. also used three different doses of nicotine associated with orthodontic movement (30 g/f), and concluded that, after 14 days of movement, nicotine accelerated the rats’ tooth movement, and had a dose-dependent effect. However, there were differences in the methodological designs of their studies in comparison with our study, such as the methods of measurement and the time of administration of nicotine prior to orthodontic movement. Regarding the measurement method, Bakathir et al. used a digital caliper and Sodagar et al. used an interproximal thickness gauge. In the present study, we opted to make molds and gypsum models before and after the movement period to provide a more reliable result. Our method ensured that the soft tissues of the animal’s mouth did not interfere with the measurement.

Based on our findings, nicotine and ethanol can interfere in bone metabolism, leading to reduced bone neoformation when dental movement is induced, and a reduced number of osteoclasts when not induced. Extrapolating to a clinical situation, the delay in maturation or the increased degradation of type I collagen may indicate to the orthodontist that a longer interval is needed between visits for these patients. However, although rats are a well-established model for evaluating the effect of these substances on bone metabolism, animal studies provide only preliminary evidence that points toward the need to limit use of these substances, and that strongly recommends future studies to be conducted on humans to strengthen the weight of the already found results.

Conclusion

Nicotine and ethanol did not affect the rate of tooth movement, regardless of the induction of orthodontic movement. Nicotine influenced the number of osteoclasts by decreasing their quantity when dental movement was not induced. When nicotine was associated with ethanol, it interfered in the maturation of collagen fibers during orthodontic movement.

References

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