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Efficacy and safety of TiF_4 varnish in preventing erosive tooth wear in a rat animal model

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An animal model was applied to develop erosive tooth wear (ETW) and to evaluate the efficacy and safety of titanium tetrafluoride (TiF_4) in preventing ETW. Forty-eight male Wistar rats were divided into three groups ($n = 16$): TiF_4 (2.45% F^-), NaF (2.45% F^-) and placebo varnishes. Eight from each group were subjected to erosive challenges (Sprite Zero) and the other received tap water, both *ad libitum*. After twenty-eight days, the mandibles were resected for histopathological gingival analysis, clinical and microscopic tooth evaluation by 3D confocal laser microscopy, scanning electron microscopy (SEM-EDX) and micro-Raman spectroscopy (MRS). Organs were evaluated with respect to fluoride content. No significant difference was found in F content in tissues. No histopathological damage was seen in gingiva. ETW was clinically more aggressive in rats from placebo group consuming Sprite compared to water (Q^2=12.6, $p < 0.01$), in accordance with confocal images. TiF_4 was superior in reducing cross-section area loss ($0.036 \pm 0.01 \mu m^2$) compared to NaF and placebo, respectively ($0.044 \pm 0.01/0.063 \pm 0.01 \mu m^2$, ANOVA, $p < 0.0001$). Dentin exposure was detected by SEM in rats belonging to placebo consuming Sprite. Peaks compatible with typical apatite bands were visible. TiF_4 reduces the progression of ETW without causing any relevant side-effect and the rats' model was able to simulate ETW in vivo.

Keywords Erosion, Fluoride, Tooth erosion, Titanium

Erosive tooth wear (ETW) is a multifactorial condition stemming the exposure of the dental substrate to acids, not from microorganism, such as dietetic and stomach acids[1–5]. This injury has garnered significant attention from researchers and clinicians globally, given its escalating prevalence and clinical detection rates[2,6–8]. Estimates suggest that it affects approximately 30–50% of deciduous teeth and 20–45% of permanent teeth worldwide[9,10]. ETW has become increasingly common even in children and adolescents[8,10]. The prevalence of ETW in adolescents was considered high and associated with lifestyle, diet, sociodemographic and economic characteristics[8]. In adults, even higher ETW prevalence (about 75%) was found compared to the results showed in young[11].

The progressive loss of tooth structure due to ETW gives rise to undesirable consequences, including dentin hypersensitivity, functional impairment, pain arising from pulp exposure, deterioration of esthetic appearance due to discoloration and/or shortening of teeth, and even muscle pain resulting from the loss of vertical dimension in severe cases[2].

To control ETW, fluorides containing polyvalent metals, such as titanium tetrafluoride (TiF_4), have shown protective effect[1,3,5,12–14]. The protective effect of TiF_4 is attributed to the formation of a glaze-like layer rich in hydrated titanium phosphate, titanium oxide and calcium fluoride on enamel[15]. The glaze-like layer has shown to be more acid-resistant than CaF_2 produced by NaF application[3–5,15]. Consequently, TiF_4 has a promising effect in inhibiting ETW both in the form of varnish and as a solution for home application, in vitro and in situ, when compared to other type of fluoride as NaF[1,3,5,14].

Although the in vitro and in situ studies have shown positive results, few have been done in vivo. The protective potential of a solution containing TiF_4 and NaF was analyzed in vivo, after a short erosive challenge, by using

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Groups	Feed (g/day)	Liquid (mL/day)	Baseline weight (g)	Final weight (g)	Weight gain (g)
(1) TiF_4 + Water	59.20 ± 1.12	61.73 ± 7.00	332.50 ± 23.30[a]	403.25 ± 35.46	70.80 ± 18.99[b]
(2) TiF_4 + Soft drink	53.64 ± 1.85	61.99 ± 2.50	332.00 ± 18.80[a]	397.12 ± 36.19	65.10 ± 32.53[ab]
(3) NaF + Water	54.46 ± 6.17	64.67 ± 0.11	349.38 ± 17.15[a]	401.50 ± 21.92	52.10 ± 14.90[b]
(4) NaF + Soft drink	54.10 ± 1.45	57.92 ± 1.03	341.75 ± 30.29[a]	422.37 ± 37.53	80.60 ± 18.52[ab]
(5) Placebo + Water	53.46 ± 7.36	62.20 ± 8.25	345.00 ± 36.24[a]	416.50 ± 21.60	71.50 ± 26.27[b]
(6) Placebo + Soft drink	50.69 ± 1.63	54.53 ± 1.36	349.63 ± 14.07[a]	383.00 ± 20.20	33.40 ± 9.80[a]

Table 1. Mean ± standard deviation of food and liquid intake during the 28-day period for the different treatment and conditions groups ($n = 4$) and mean ± standard deviation of animal weight per treatment and condition group comparing the baseline and the final values with respect to weight gain during the experimental period ($n = 8$). * There was no significant difference between groups (ANOVA, $p > 0.05$ for feed (g/day), liquid (mL/day) and baseline weight (g)). Different lowercase letters in the same column indicate that there was a significant difference ($n = 8$, ANOVA/Tukey, $p = 0.0011$ for weight gain).

Treatments	Blood plasma [F] (µg/mL, ppm)	Kidney [F] (µg/g)	Intestine [F] (µg/g)	Stomach [F] (µg/g)	Liver [F] (µg/g)
TiF_4 varnish	0.038 ± 0.014[a]	0.073 (0.018)[a]	0.014 (0.005)[a]	0.302 ± 0.192[a]	0.036 (0.016)[b]
NaF varnish	0.030 ± 0.011[a]	0.102 (0.068)[a]	0.016 (0.011)[a]	0.260 ± 0.229[a]	0.029 (0.008)[ab]
Placebo varnish	0.035 ± 0.015[a]	0.069 (0.024)[a]	0.017 (0.006)[a]	0.260 ± 0.166[a]	0.025 (0.010)[a]

Table 2. Mean ± standard deviation or median (interquartile range) of fluoride content in blood plasma and different tissues for the treatment groups ($n = 16$). * Different lowercase letters in the same column indicate that there was a significant difference ($n = 16$). Blood Plasma: ANOVA, $p = 0.2252$. Kidney: Kruskal-Wallis, $p = 0.0684$. Intestine: Kruskal-Wallis, $p = 0.3300$. Stomach: ANOVA, $p = 0.8056$. Liver: Kruskal-Wallis, $p = 0.0145$ (Placebo x NaF, $p = 0.1922$; Placebo x TiF_4, $p = 0.0126$ and NaF x TiF_4, $p = 0.9606$).

calcium release as measurement of tooth demineralization[5]. However, it is very challenging to perform clinical studies in humans, since it is not ethical to induce tooth wear as well as the time for its natural development can take several years.

The use of animal model is an alternative for clinical studies to help understanding the development of ETW and the efficacy of preventive measures. The first study done in animals performed an operation to force reflux of gastroduodenal contents in male Wistar rats and examined the teeth after 15 and 30 weeks, confirming, through this experimental model, the relationship between ETW and gastroesophageal reflux disease (GERD)[16]. The animal model was also applied in other works to investigate the influence of reduced salivary flow and a calcium-modified beverage on the progression of ETW[17], and to simulate ETW caused by extrinsic sources[18].

One of the advantages of the animal model is the possibility to analysis the local and systemic side-effect of the treatments. In human, such kind of protocol is not allowed, as for example, to offer acidic foods for some period in order to induce ETW and, thereafter, extract teeth for further microscopic analysis. In addition, before testing any new product in human, it is mandatory to know possible side-effects that cells culture methods do not provide adequately answer as animal model does. On this regard, few studies focusing on this issue were done using fibroblast cells cultures and both showed low cytotoxicity effect of TiF_4 varnish as well as, by asking volunteers about their felling when applying the product in situ[5,14,19,20].

Therefore, the present work aimed to apply an animal model to develop erosive tooth wear (ETW); evaluate the effect of titanium tetrafluoride (TiF_4) in preventing ETW and its possible toxic effect. The null hypotheses tested were: titanium tetrafluoride has no toxic effect on gingival tissues and does not increase fluoride content in different organs involved in its metabolism and excretion compared to the other varnishes (H1); titanium tetrafluoride cannot reduce the ETW development compared to the other varnishes (H2); and the applied animal model is not able to induce the development of ETW (H3).

Results

No visual alterations of the rats' behavior were seen during the study. Regarding the baseline body weight, no significant difference was found among the groups (ANOVA, $p = 0.54$). When the final weight was subtracted from the initial weight, to quantify weight gain during the experimental period, a significant difference between the groups was seen (ANOVA, $p = 0.0011$). Tukey test showed that rats consuming soft drink had lower weight gain compared to those from water groups in all three treatment groups (Table 1). There was no difference in food and liquid consume between the different groups to justify such difference in weight gain (ANOVA, $p = 0.06$ and $p = 0.23$ for food and liquid, respectively, Table 1).

Regarding blood plasma F, there was no statistically significant difference between the groups (ANOVA, $p = 0.2252$). The same was seen for fluoride content in kidney (Kruskal-Wallis, $p = 0.0684$), intestine (Kruskal-Wallis, $p = 0.3300$) and stomach (ANOVA, $p = 0.8056$). On the other hand, a significant difference was found in liver, when TiF_4 was compared to placebo (Kruskal-Wallis/Dunn test, $p = 0.0126$) (Table 2), but the overall amount detected was low.

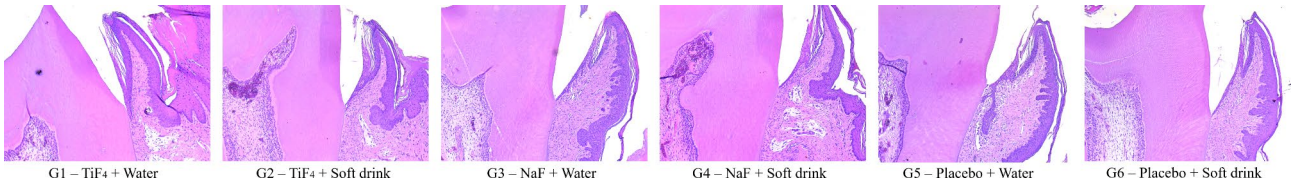


Fig. 1. Histological analysis of gingival tissue corresponding to all groups (magnification 10x).

Treatments	Conditions	Score 0	Score 1	Score 2
TiF_4 varnish	Water	16	8	0
	Soft drink	11	13	0
NaF varnish	Water	11	13	0
	Soft drink	6	16	2
Placebo varnish	Water	24	0	0
	Soft drink	14	10	0

Table 3. Number of rats´ teeth with different scores according to the groups and conditions ($n=24$). * Q^2 test (scores associated with water x soft drink): TiF_4, $Q^2=2.12$ and $p=0.146$; NaF, $Q^2=3.78$ and $p=0.151$ and Placebo, $Q^2=12.6$ and $p<0.01$. The effect of treatments and the scores were also associated under the condition soft drinks (erosion): TiF_4 x NaF x Placebo, $Q^2=8.55$ and $p=0.074$.

Groups	Cross-sectional loss area (μm^2)	Volume of tissue loss (μm^3)
(1) TiF_4 + Water	0.035 ± 0.01^{aA}	0.067 ± 0.01^{aA}
(2) TiF_4 + Soft drink	0.036 ± 0.01^{aA}	0.075 ± 0.02^{aA}
(3) NaF + Water	0.040 ± 0.01^{aA}	0.072 ± 0.01^{aB}
(4) NaF + Soft drink	0.044 ± 0.01^{aB}	0.077 ± 0.01^{aA}
(5) Placebo + Water	0.041 ± 0.01^{aA}	0.080 ± 0.01^{aC}
(6) Placebo + Soft drink	0.063 ± 0.01^{bC}	0.111 ± 0.01^{bB}

Table 4. Mean ± standard deviation of erosive tooth loss analyzed by 3D confocal microscopy, according to condition and treatment groups ($n=8$). * Different lowercase letters indicate significant differences between soft drink and water ("conditions") for each treatment. Different capital letters indicate significant differences between treatments (TiF_4, NaF and placebo) for each condition (two-way ANOVA and Tukey's test, $p<0.0001$ for conditions, treatments and interaction for cross-sectional area; two-way ANOVA and Tukey's test, $p=0.004$, $p<0.0001$ and $p=0.008$ for conditions, treatments and interaction between factors for volume analysis, $n=8$).

In the histological evaluation of gingival tissue, no evidences of histopathological changes were observed. The qualitative analysis demonstrated the integrity of gingival epithelium, sulcular epithelium and junctional epithelium, as well epithelial thickness compatible with normality, as well as the absence of inflammatory cells and inflammatory infiltrate, suggesting the lack of local toxicity of fluorides (Fig. 1).

Table 3 shows the results of Q^2 when the clinical tooth wear scores were associated with the groups. ETW was clinically seen only for rats belonging to the placebo group consuming soft drink compared to those ingesting water ($Q^2=12.6$ and $p<0.01$), demonstrating that fluorides were able to contain the appearance of clinical wear (water x soft drink $Q^2p>0.05$). When treatments were compared, under soft drink consume, no statistical association was found between the type of treatment and the degree of ETW ($Q^2=8.55$ and $p=0.074$).

For 3D laser confocal microscopy of the 2nd molar, there was a significant statistical difference between rats consuming soft drink compared to water only in case of placebo varnish group either (ANOVA, $p<0.0001$), both for the area and volume analysis, in agreement with clinical data. In samples treated with fluorides, the soft drink was not able to cause significant loss of tooth tissue. When treatment groups were compared, differences were found for fluoride treatments compared to placebo (water), for both types of analysis (around 27–30% reduction in tooth wear for fluorides, ANOVA, $p<0.0001$). However, no significant differences were found between TiF_4 and NaF for volume analysis; but for area, TiF_4 was superior in reducing ETW compared to NaF (ANOVA, $p<0.0001$) (Table 4).

Figure 2 shows the obtained SEM images. Teeth from rats consuming soft drink without any F treatment (placebo group) showed dentin exposure through visualization of the dentinal tubules, which was not seen in rats consuming water. Therefore, the protocol was capable of inducing erosive tooth wear. Teeth samples treated with NaF showed deposits on the surface regardless of whether they were exposed to water or soft drink,

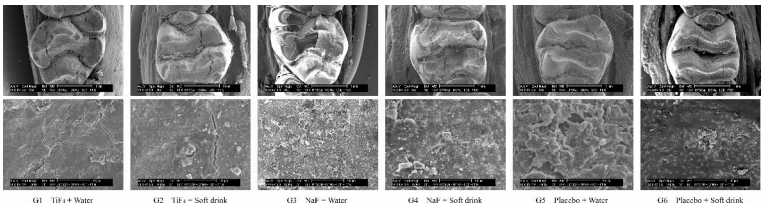


Fig. 2. Scanning electron microscopy (SEM) of a representative image per group at 35x and 1600x magnifications.

Groups	Carbon	Oxygen	Fluorine	Sodium	Magnesium	Phosphorus	Calcium	Titanium
(1) TiF_4 + Water	20.75 ± 5.41^{a}	29.10 ± 6.15^{a}	$0.17\ (0.07)^{a}$	0.53 ± 0.07^{a}	0.82 ± 0.23^{ab}	15.24 ± 3.14^{c}	37.48 ± 4.77^{b}	$0.01\ (0.03)^{a}$
(2) TiF_4 + Soft drink	41.09 ± 13.72^{b}	22.25 ± 4.81^{a}	$0.09\ (0.12)^{ab}$	0.55 ± 0.14^{a}	0.63 ± 0.18^{b}	11.39 ± 2.26^{ab}	26.75 ± 7.21^{a}	$0.01\ (0.05)^{a}$
(3) NaF + Water	20.87 ± 8.51^{a}	23.51 ± 5.10^{a}	$0.04\ (0.04)^{b}$	0.55 ± 0.17^{a}	0.77 ± 0.25^{ab}	14.38 ± 2.46^{bc}	40.94 ± 6.17^{b}	$0.01\ (0.01)^{a}$
(4) NaF + Soft drink	29.73 ± 7.75^{ab}	21.58 ± 6.65^{a}	$0.04\ (0.04)^{ab}$	0.54 ± 0.12^{a}	0.71 ± 0.22^{ab}	13.02 ± 2.75^{abc}	36.47 ± 9.32^{b}	$0.01\ (0.01)^{a}$
(5) Placebo + Water	25.61 ± 12.55^{a}	22.80 ± 7.72^{a}	$0.06\ (0.04)^{ab}$	0.51 ± 0.22^{ab}	0.99 ± 0.27^{a}	15.58 ± 2.90^{c}	38.80 ± 5.15^{b}	$0.02\ (0.03)^{a}$
(6) Placebo + Soft drink	39.65 ± 6.82^{b}	24.66 ± 8.56^{a}	$0.06\ (0.04)^{ab}$	0.31 ± 0.10^{b}	$0.63 \pm 0.25b^{b}$	10.00 ± 1.62^{a}	25.44 ± 5.93^{a}	$0.01\ (0.03)^{a}$

Table 5. Mean ± standard deviation or median (interquartile range) of chemical quantification for each element found on the teeth by EDX analysis ($n = 8$). * Different lowercase letters in the same column indicate that there was a significant difference. Carbon, Oxygen, Sodium, Magnesium, Phosphorus and Calcium: ANOVA, $p < 0.0001$. Fluorine: Kruskal-Wallis, $p = 0.0015$ and Titanium: Kruskal-Wallis, $p = 0.994$.

without major morphological differences, which is in agreement with the clinical analysis. Similar findings were observed in the case of teeth treated with TiF_4, revealing a layer with cracks.

Table 5 shows the EDX values. Higher percentage of carbon (ANOVA, $p < 0.0001$) was found on teeth from rat consuming soft drink compared to those consuming water, which may be due to the presence of carbonated apatite. On the other hand, the percentage of Mg^{2+}, Ca^{2+} and Na^{+} decreased on teeth from rat consuming soft drink compared to those from water group (ANOVA, $p < 0.0001$). No significant deposits of fluoride and titanium (Kruskal-Wallis, $p = 0.0015$ and $p = 0.994$, respectively) were found on the samples, regardless of the condition and the treatment.

Figure 3 corresponds to the MRS graph that represents the data obtained through the analyses of all samples, grouped according to their respective treatment groups. The presence of peaks can be observed in the areas of approximately 450 cm^{-1}, 600 cm^{-1} and 950 cm^{-1}, all compatible with typical bands of phosphate and apatite[21] present in the composition of the tooth. Therefore, no specific formation containing titanium (such as titanium dioxide, hydrated titanium phosphate) and calcium fluoride could be identified, in agreement with the EDX data.

Discussion

In recent decades, literature indicates that in different parts of the world, such as Brazil[6,7], Colombia[22], Saudi Arabia[23], Norway[24], Sweden[25], Finland[26], Ukraine and Poland[27], the prevalence of ETW has been expanding[28] especially in a young population. Knowledge about predisposing factors, diagnosis, prevention and therapies for ETW is still a challenge for researcher and clinicians[29].

Most studies on ETW reported in the literature, as mentioned, were done in vitro or in situ. One of the objectives of this study was to develop a model that allows the evaluation of ETW development and the protective effect of preventive agent in a closer way to reality as done by few authors[16–18]. Sprite Zero was chosen since it has a significant erosive potential[17] and it does not contain sugar that could be attractive to other small animals or insects into the bioterium. Other point is that its pH value remains constant during the experiment, regardless of the presence of gas. The main advantage of animal model is the controlled genetic, dietary and environmental conditions, which it is not possible with human. Additionally, the animal model provides the opportunity to assess potential side effects of the treatment through the molecular and histological analysis of soft tissues, which justified the model applied in the present study. According to the results of clinical assessment and 3D confocal microscopy, the rats' model was able to induce ETW (H3 rejected).

It is important to note that the feed provided to the animals is standardized by the local bioterium (Nuvilab CR-1 irradiated). This rigid food has a maximum mineral content of 90 g/kg and a fiber content of 70 g/kg, which is crucial for the necessary stimulation for rodents. This rigid feed may have contributed to the development of ETW, especially in rats consuming soft drink. Although the varnish was applied on all teeth, the analyses were conducted on the posterior teeth (molars), excluding the incisors, which are worn down by gnawing and are in constant growth and change. Additionally, after the varnish was applied, the animals were kept without food or drink for 30 min, to simulate clinical conditions.

In this animal model, TiF_4 and NaF varnishes did not differ from each other in terms of tooth volume loss by clinical evaluation and 3D laser confocal microscopy analysis, in accordance with in vitro studies[12,13].

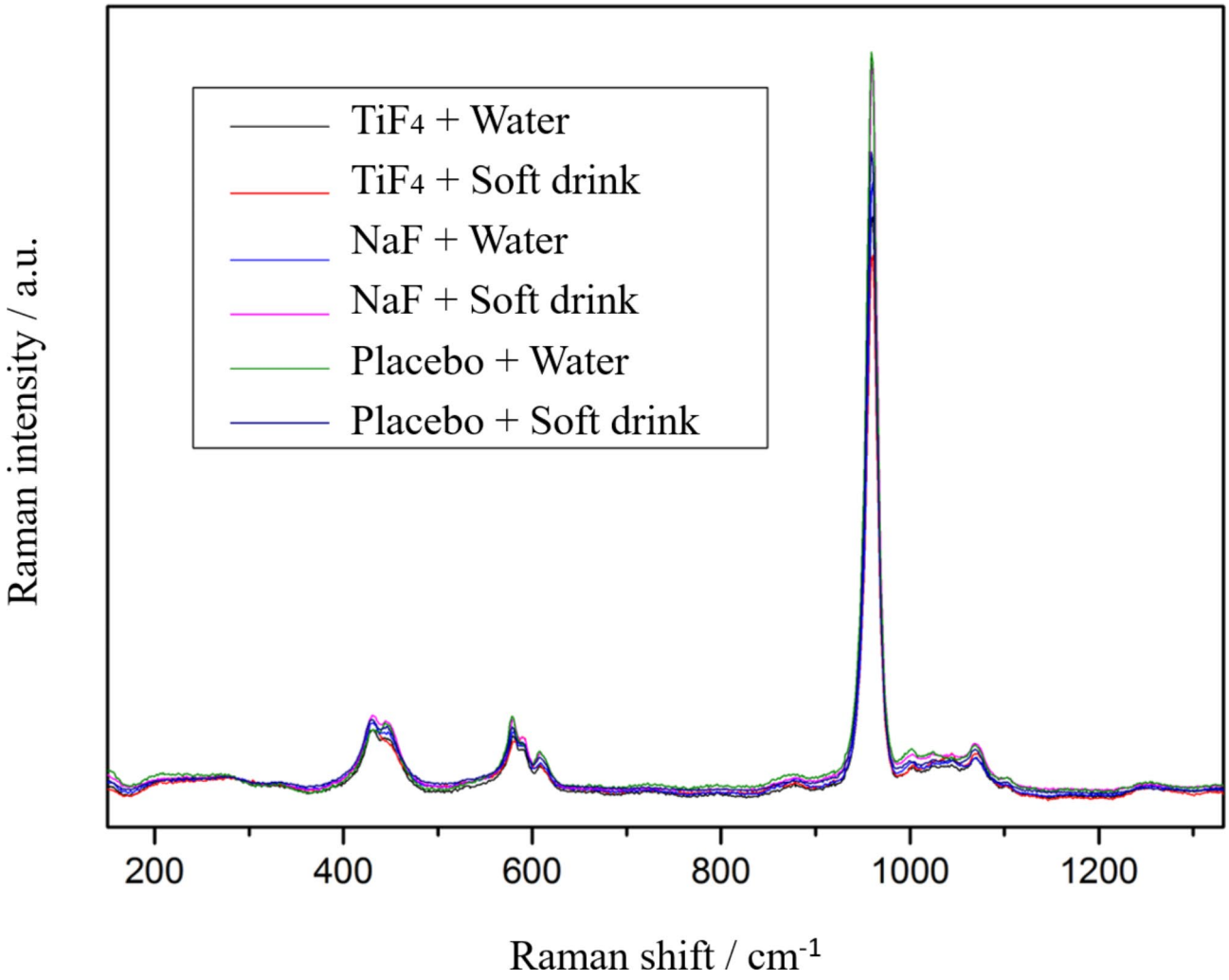


Fig. 3. Micro-Raman Spectroscopy (MRS) graph containing information from all groups.

Furthermore, the present study showed that the application of fluoride varnishes minimizes ETW development, not showing an additional tooth wear in rats consuming soft drink compared to those consuming water. The only parameter able to differentiate TiF_4 and NaF effects was tooth area loss, which deserves more attention in future studies using the 3D confocal microscopy. It is important to highlight that the tooth area measurement was obtained from a transverse line drawn from the highest point to the lowest point of occlusal area of the 2nd molar. The profile of the lesion below this line (cross section) was obtained for the purpose of measuring the amount of substance loss. Using the same software, the volume was obtained to determine the amount of substance loss, selecting the concavity area of each cusp. Therefore, the area seems to be better standardized than the volume analysis, considering that the width and height of the teeth concavities also presented considerable variations between the animals. However, both area and volume assessments are of great value for evaluating the effectiveness of fluoride varnishes with respect to ETW progression and shall be better explored in the future.

It is also important to emphasize the difficulty of this analysis because it is not possible to record the baseline situation of the animals' teeth, before applying treatments and exposing them to the erosive agent. Few studies in the literature applied 3D laser confocal microscopy to analyze ETW, taking as an example the work of Ren et al. (2009)[30] in which the action of fluorides on tooth enamel surface, subjected to the action of erosive agents, was evaluated by analyzing the surface profile of the third molars. In agreement with our results, 3D laser microscopy seems to be a good alternative to be applied in clinical studies[30]. More recently, a study showed that 3D laser microscopy is an excellent method to evaluate ETW due to its greater sensitivity in detecting the boundary between enamel and dentin compared to digital radiographs[31]. Taking together the results of clinical assessment and 3D confocal data, both fluorides have protective effect on ETW (H2 rejected).

Another important finding of the present study is the lack of toxicity of fluoride on gingival tissue supporting future clinical trials. In the literature there is consistent data showing that TiF_4 and NaF varnishes have a similar toxic effect on cell viability and morphology as well as on apoptosis event in fibroblasts[19,20]. To check the systemic effect, the amount of fluoride in different tissues and organs responsible for the absorption, metabolism and excretion of this compound was quantified. In fact, we could not detect high amounts of F in the tissues, showing low ingestion and absorption of fluoride (H1 accepted). Although a difference was found in the liver between

TiF_4 and placebo, the overall amount detected was low. However, one limitation of this study is the absence of baseline blood fluoride measurements. Additionally, the inherently small quantity of varnish applied at the day 0 may also contribute to the low fluoride levels observed in blood after 28 days.

Previous work showed the mean levels (± standard deviation) of fluoride in the liver of male Wistar rats for the control groups (0 mg/L F), 5 mg F/L and 50 mg F/L in drinking water of 0.042 (± 0.016), 0.034 (± 0.021) and 0.059 (± 0.006) mg/kg, respectively[32]. In the present study, the amount of fluoride found in the liver of animals treated with TiF_4 varnish was 0.036 (0.016) mg/kg, similarly to the values from animals which ingested 5 mg F/L (equivalent to 1 mg F/L in water for humans).

MRS offers the possibility of obtaining information about the structural chemical organization of different minerals, including tooth, and thus becoming an important tool for dentistry[21,33–35]. This is a non-destructive technique used to characterize organic and inorganic compounds by identifying vibration modes or characteristic bands[21,33,34]. It has currently been used to evaluate the degree of mineralization and the effect on the de/remineralization of carious lesions[21,33–36]. Our study detected typical bands of phosphate and apatite, which are present in the structure and composition of the dental element[21,33,35,36]. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is the main tooth mineral that can be dissolved by acids forming intermediated calcium phosphate compounds such as octacalcium phosphate (OCP) and dicalcium phosphate dihydrate (DCPD)[37]. This happens since hydrogen ions (H^+) present in the acidic environment react with the phosphate (PO_4^{3-}) and hydroxyl (OH^-) groups in hydroxyapatite, resulting in the release of Ca^{2+} and HPO_4^{2-} ions[37]. However, the amount of these intermediate compounds may be low, after acid washing and tooth wear, to be detectable in MRS analysis.

The importance of fluoride treatments, for example in the form of varnish, is well supported in the literature, for maintaining the integrity of tooth enamel, either by creating physical barriers, such as CaF_2 or other fluoride precipitates, that prevent demineralization[38] or by reducing the loss of essential minerals during exposure to acids[39] - due to chemical changes in the main component of enamel (hydroxyapatite), thus inducing chemical stability[38]. The applied fluoride is incorporated into the enamel crystals, replacing the hydroxyl ions, giving rise to fluoroapatite, $Ca_{10}(PO_4)_6F_2$[38,39], which improves the enamel's ability to partially resist an acid challenge. Fluoride ions are incorporated into the apatite network through precipitation and growth reactions[38,39].

F varnish and toothpaste application has shown, for example, decrease in the peak close to 950 cm^{-1}, corresponding to a substantially greater change in the enamel structure[35]. However, this difference was only possible to be detected, because the authors compared the baseline and final graphic. Since our study was carried out in animal, this analysis was limited to the end of study, reducing the possibilities to detect slight changes provoked by the treatments.

It was not possible to identify any specific peak correspondent to titanium (such as titanium dioxide, hydrated titanium phosphate) and calcium fluoride in the MRS analysis, which corroborates the data obtained in the EDX analysis. Differently, these compounds were found through X-ray diffraction analysis of enamel powder previously[15], and Ti, but not F, was detected on polished enamel samples from an in situ study by EDX[40], most likely due to the type of analysis and the study model, respectively. In our study, the F varnishes were applied only at the beginning of the study; therefore, it is expected that the compounds produced by their application on the teeth at day 0 may be lost during the daily erosive drink and rigid food consume, and thus not be founded at day 28.

Clinical analysis, 3D laser microscopy and SEM analysis were coherent. Obviously, microscopy analyses are more sensitive, since the animals' hemi-mandibles or hemi-maxillae are small and, thus, greater difficulty is encountered in visual assessment, even with magnification through photography. Despite challenging, clinical assessment was able to detect differences between teeth from rats consuming soft drink compared to water. Methylene blue staining has been applied for better clinical visualization[17], however, in our study it was not necessary. Besides, any stain could interfere in other analysis done.

Interesting was that SEM analysis showed dentinal tubules exposure in teeth belonging to rats consuming soft drink compared to water, in case of placebo varnish. While for rats treated with fluoride varnishes no morphological tooth differences were evident in the comparison between soft drink and water. Samples treated with NaF showed deposits on the surface regardless of whether exposed to water or soft drink, without major morphological differences. The same was seen for teeth treated with TiF_4, which even showed a layer with cracks similar to the glaze-like layers seen in laboratory study[15]. These findings are in line with what was reported yet, in which only enamel specimens treated with TiF_4 showed the formation of a protective film; furthermore, enamel samples treated with TiF_4 showed less damage on their surface than those treated with NaF[40].

The first null hypothesis was accepted, since TiF_4 did not exhibit any toxic effects on gingival tissues and did not significantly increase fluoride content considering all tissues together. The other two null hypotheses were rejected, as the applied animal model was capable of inducing the development of ETW, and TiF_4 was effective in reducing ETW progression.

It can be concluded that TiF_4 varnish reduces the progression of ETW without causing any significant side effects in this animal model, reinforcing the findings of in vitro and in situ studies, and supporting further research. Furthermore, the rats' model was able to simulate ETW in vivo.

Material & methods

Ethical aspects, experimental groups and animal management

The Local Ethics Committee for Animal Experiments approved all experimental protocols (#010/2019). Also, this study was carried out in accordance with ARRIVE guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals. The sample size was based on a previous study with a similar research protocol[17], considering $\alpha = 0.05$ and $\beta = 0.8$, in which the remaining enamel volume was 1.89 (0.05) mm^3 for rats drinking water and 1.76 (0.06) mm^3 (7% volume loss) for those consuming a soft drink ($n = 3$). Forty-eight male *Wistar Hannover* rats (90 days old) were randomly divided into three experimental groups ($n = 16$/group): 4%

TiF_4 varnish (2.45%, 24.500 ppm F^-, pH 1); NaF varnish (2.45%, 24.500 ppm F^-, pH 5) (positive control); and Placebo varnish (pH 5) (negative control).

All experimental varnishes had the same basic composition (except by the presence and type of fluoride): resin, synthetic resin, thickening polymer, essence, artificial sweetener and ethanol (FGM-Denstcare, Joinvile, Brazil)[1,3]. The animals were kept in an environment with controlled temperature and humidity, with a 12-hour light-dark cycle, with access to food and drink *ad libitum*.

To carry out the treatments, the animals were immobilized, their mouth was opened with the aid of an adapted mouth opener and 0.1 g of the respective varnish was applied on all teeth using a microbrush for one minute, only at the beginning of the experiment. Thereafter, the animals remained without eating or drinking for 30 min[12].

Half of the animals in each group ($n=8$) were subjected to erosive challenges by offering degassed soft drink (Sprite Zero - pH 3.3 without gas) *ad libitum*[17], while the other half of the animals drank tap water *ad libitum*. The amount of liquid and food (Nuvilab CR-1 irradiated) consumed was monitored throughout the experimental period, three times a week. The protocol was maintained for 28 days in order to induce the development of ETW[17].

Initial and final body weight data and food and liquid consume were analyzed and subjected to statistical comparison (ANOVA/Tukey test), using Graph Pad Prism software, version 10, https://www.graphpad.com, USA ($p<0.05$).

Then, the rats were euthanized by an overdose of anesthesia (ketamine: Dopalen 240 mg/kg and xylazine: Anasedan 30 mg/kg). The mandible and maxilla of each animal were desiccated for further analysis. In addition, samples of blood and organs involved in F metabolism and excretion (liver, kidney, stomach and small intestine) were collected for analysis. Figure 4 shows the flowchart of the study.

Fluoride analysis of blood plasma and organs

The blood samples were centrifuged at 3000 rpm and 4 °C for 15 min to separate the plasma and the red series. A pre-diffusion was carried out to eliminate CO_2 from blood. The volume of blood plasma used for the analysis was 0.5 mL, the organs were homogenized in a proportion of 0.2 g of the organ to 0.5 mL of deionized water[32]. Fluoride (F) measurements were determined in duplicate after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion as described by Taves (1968)[41], modified by Whitford (1996)[42], using a specific F^- electrode (Orion, model 9409), coupled to a reference calomel electrode (Accumet, #13-620-79), and to a potentiometer (Orion, model EA 940). The data obtained in mV were converted into mg/mL or mg/g, according to the standard curve performed with known concentrations of F, with $r^2>0.99$. The data were subjected to statistical analysis by ANOVA/Tukey or Kruskal-Wallis/Dunn test, considering $p<0.05$ (Graph Pad Prism version 10, https://www.graphpad.com, USA).

Histological analysis of gingival tissue

One hemi-mandible of each animal was fixed in 10% neutral buffered formalin. Subsequently, the pieces were demineralized in a solution with pH 7.2, containing 4.13% Titriplex III (Merck) and 0.44% sodium hydroxide, at a temperature of 2 to 8 °C, for an approximate period of 40 days, with weekly changes of demineralizing solution and monitoring through radiographic analysis.

After demineralization, the hemi-mandibles were washed in running water and immersed in distilled water for 24 h to completely remove EDTA. Each of them was then dehydrated in increasing ethanol solutions and afterwards subjected to diaphanization using xylene solutions. Subsequently, the hemi-mandibles pieces were processed and embedded in paraffin[43,44].

The blocks were subjected to semi-serial longitudinal Sect. 5 µm thick, with an interval of 500 µm, using a microtome (Leica Jung RM 2045). The sections were then stained with Harris' Hematoxylin and Lison's Eosin[45]. The histological slides were analyzed using a binocular optical microscope (Olympus, model CH-2), with a 4x, 10x and 40x magnification objective and photomicrographs were taken for a qualitative analysis.

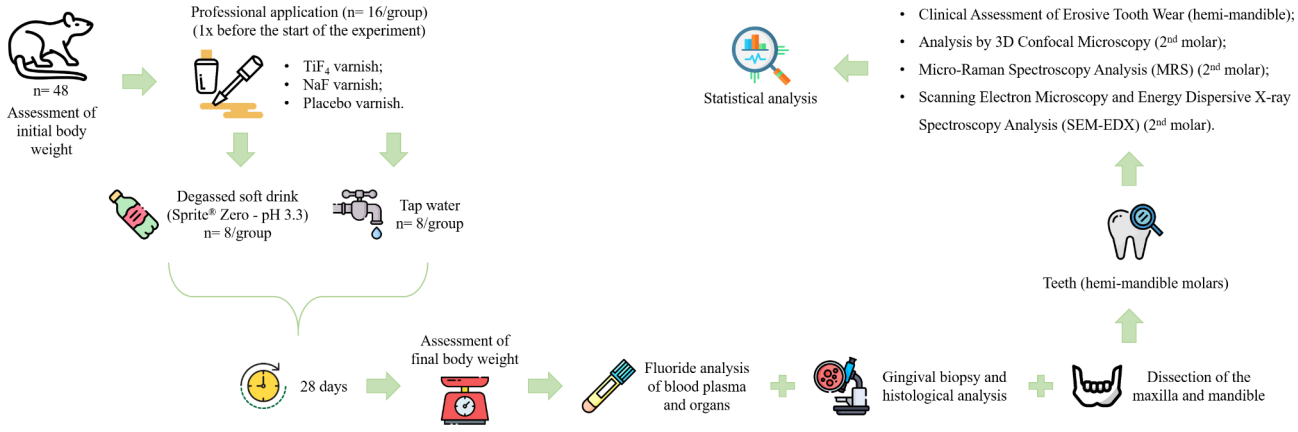


Fig. 4. Flowchart of the study.

Clinical assessment of erosive tooth wear (ETW)

Photos of the hemi-mandible of each animal were taken (CANON T5i Camera with 100 mm macro lens) for visual analysis of the severity of ETW on the occlusal surface of 3 molars by two independent and blindly calibrated examiners. The severity of ETW was determined according to the comparative clinical aspects with the "negative control" (the group treated with placebo varnish and received water throughout the treatment period)[17].

The scores were assigned as follows: 0- normality parameter referring to "negative control"; 1- enlargement of pattern zero but without union between the worn areas; 2- widening of the worn areas so that there is a union between two of them; 3- enlargement of the worn areas so that there is a union between more than two of them. The scores were defined after analyzing the anatomy of the occlusal surface of molars' rats, in which some physiological wear is expected, as seen in the rats belonging to the negative control group. Also, the score applied was based on the study by Aldosari et al. (2018)[17], which in turn adapted the visual score proposed by Sorvari & Kiviranta (1988)[46]. The analyzed hemi-mandibles were then classified according to the indexes of the three molars. The Kappa test was performed to evaluate intra-examiner ($\kappa = 1.0$) and inter-examiner ($\kappa = 0.89$) agreement and statistical analysis was performed using the Q^2 test, considering number of rats' teeth ($n = 24$, 8 rats x 3 molars per group) and $p < 0.05$ (Graph Pad Prism version 10, https://www.graphpad.com, USA).

Analysis by 3D confocal microscopy

For the quantitative analysis of the tooth wear and topographic characterization of the molar surfaces of the hemi-mandibles, a 3D laser confocal microscope (VK-X3000, Keyence, Neu-Inseburg, Germany) was applied on the 2nd hemi-mandible molar only. A standardized area of 3×4 quadrants was scanned on each occlusal surface of the tooth using a 20x magnification objective. Subsequently, all images of each tooth were analyzed with compatible software (Multi-File Analysis Application Ver. 3.3.1, Keyence, Germany), including assessment of volume (μm^3) as an indicator of tissue loss, and also the area of surface loss (μm^2), related to the differences between the top of the cusps and the deepest point in the central fissure using the cross-sectional area (Fig. 5A and B). The height resolution of the microscope using the confocal laser was 0.1 nm. The data were subjected to two-way analysis of variance (2-way ANOVA) followed by the Tukey test, to compare the conditions (soft drink vs. water) and treatments (TiF_4, NaF and placebo) with respect to the severity of the metrics of cross-sectional area (μm^2) and volume loss (μm^3). The significance level was set at $p < 0.05$ (Graph Pad Prism version 10, https://www.graphpad.com, USA).

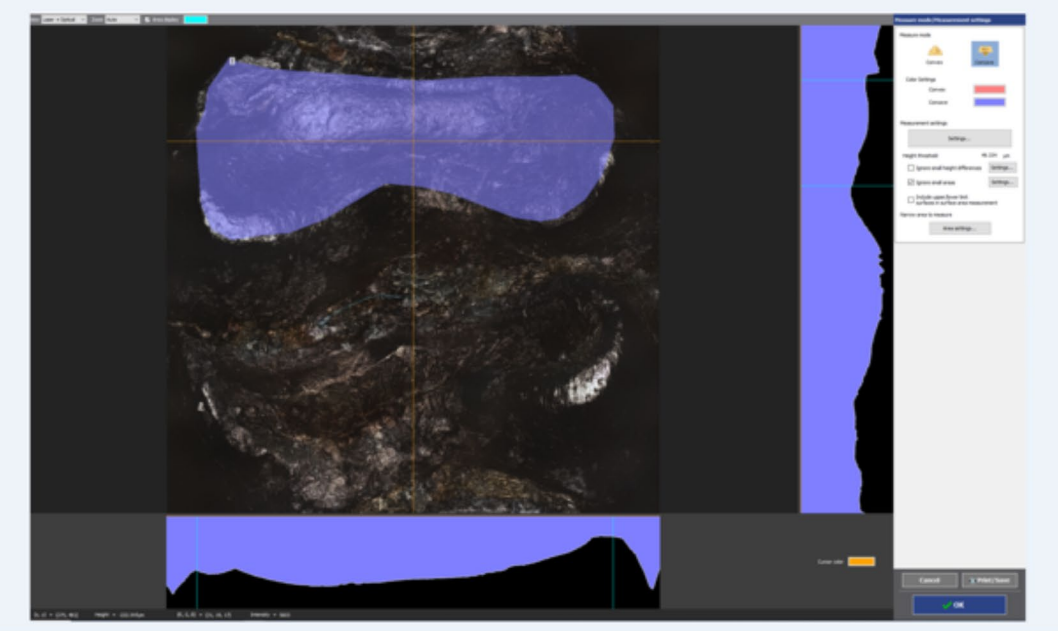
Scanning electron microscopy and energy dispersive X-ray spectroscopy analysis (SEM-EDX)

The hemi-mandibles were subjected to a dehydration process by immersion in an ascending series of ethanol solutions at 50, 60, 70, 80, 90 and 100% for one hour in each bath. After that, they were also dried in a vacuum desiccator for a continuous period of two days and then covered with a layer of gold to allow the samples to be evaluated[12,15]. Scanning electron microscopy (SEM) Phillips XL-30 FEG (FEI Company, Hillsboro, OR, USA) was used to take image the surface, at 35x and 1600x magnifications and at 10 or 30 kV.

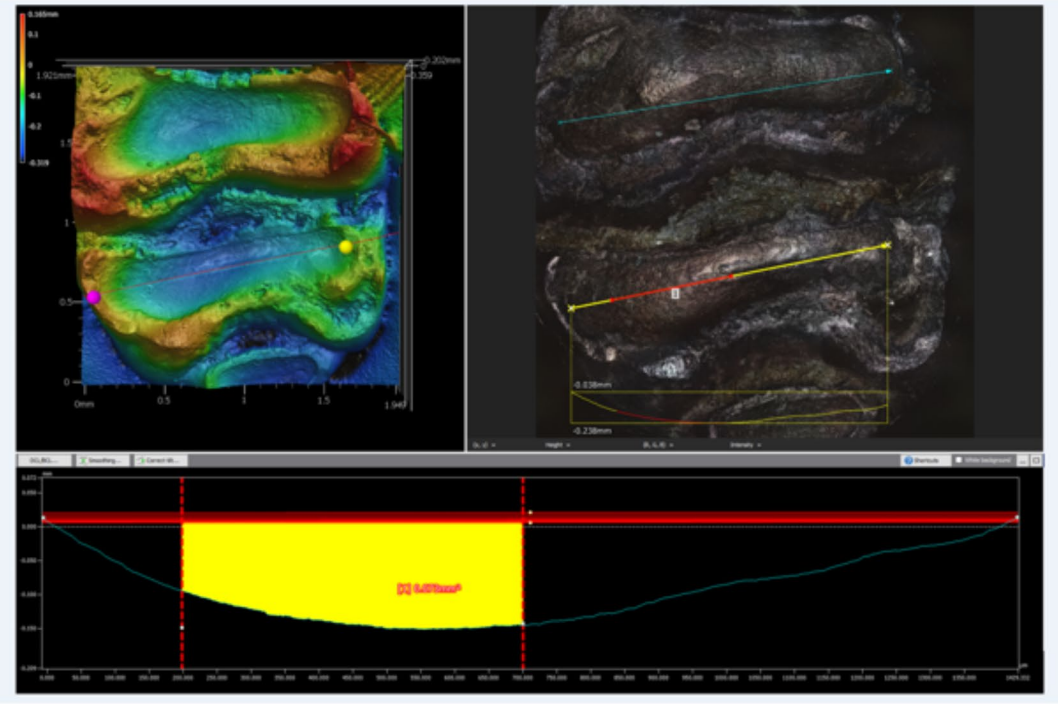
Chemical characterization was carried out by quantifying the elements in percentage using an EDX microanalysis detector (energy dispersive X-ray spectroscopy) coupled to the SEM. The EDS equipment was calibrated according to a standard reference stainless steel material − 1155 NIST (National Institute of Standards & Technology), and the results were collected using a voltage of 25 kV for 100 s (live time) in 2 areas of 350 μm^2. The microanalysis was carried out in two different regions of the surface, with a magnification of 200x and 20 kV, using quantitative analysis with ZAF correction[12,15]. Carbon, oxygen, sodium, magnesium, phosphorus and calcium were statistically evaluated using the ANOVA/Tukey test, while for the elements fluorine and titanium non-parametric Kruskal-Wallis/Dunn test was used, considering $p < 0.05$ (Graph Pad Prism version 10, https://www.graphpad.com, USA).

Micro-raman spectroscopy analysis (MRS)

Hemi-mandibles were subjected to micro-Raman Spectroscopy to evaluate possible formation of compounds on the tooth surfaces. The equipment is always calibrated with silicon before taking measurements, using the band in the region of 520 cm^{-1}. Static scanning was performed using a 50x objective lens, 785 nm laser (500 mW) at 50% power, 1200 line diffraction grating, acquisition time of 5 s and 10 accumulations, varying in the range of areas peak from 120 cm^{-1} to 1400 cm^{-1} of the Renishaw inVia Reflex device (UK). For data analysis, three spectra from different regions of each sample (occlusal region of the 2nd molar) were collected, all of which showed high similarity. From these spectra, an average spectrum was generated using an R script. The Fityk 1.3.1 software was then employed to remove the baseline from the average spectrum. Subsequently, the OriginLab 8.6 software was used to plot the Raman spectrum, which shows the intensity of scattered light as a function of frequency shift (wave number). The intensity shows peaks at specific frequencies, which provides information about the chemical bonds of the compounds present on the occlusal areas of the 2nd molar.



(A)



(B)

Fig. 5. (**A**) Assessment of volume (μm³) as an indicator of tissue loss, throught selecting the concavity area of each cusp; (**B**) Assessment of area of surface loss (μm²), related to the differences between the top of the cusps and the deepest point in the central fissure using the cross-sectional area. In the image, the color variation between red and blue refers to the height of the tooth structure, being higher and lower, respectively.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files), more informations ou doubts can be directly resolved with the corresponding author.

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Author contributions

A.C. Magalhães, M. Vertuan, G.P. Garlet, S.H. Niemeyer and M. Esteves-Oliveira designed the experiment. M. Vertuan and J.F. Silva got a scholarship to develop this project. M. Vertuan, J.F. Silva, A. Dionizio, B.M. Souza, V. Mosquim, T. Martini and A.C. Magalhães performed the experiment. M. Vertuan and A.C. Magalhães wrote the paper. All authors contributed to the paper.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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