CAIO SAMPAIO

Influência da quantidade de dentifrício e concentração de fluoreto na retenção intrabucal e ingestão de fluoreto por crianças: estudo *in vivo*

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Orientador: Prof. Associado Dr. Juliano Pelim Pessan
Coorientador: Prof. Titular Dr. Alberto Carlos Botazzo Delbem

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SBPqO - Sociedade Brasileira de Pesquisa Odontológica.

IADR - International Association for Dental Research.

DEDICATÓRIA

Dedico este trabalho

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SAMPAIO C. **Influência da quantidade de dentifrício e concentração de fluoreto na retenção intrabucal e ingestão de fluoreto por crianças: estudo in vivo.** 2018. 62 f. Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2018.

RESUMO

O dentifrício fluoretado se constitui na forma mais amplamente difundida de uso de fluoretos (F), sendo considerado sua melhor forma de utilização, uma vez que combina a remoção mecânica do biofilme com os efeitos terapêuticos do flúor. No entanto, as evidências quanto às recomendações do uso deste produto em crianças ainda são inconsistentes, considerando o balanço entre cárie e fluorose dentária. Sendo assim, o presente estudo teve o objetivo de avaliar as concentrações de flúor na saliva de crianças após escovação com dentifrícios contendo diferentes concentrações de flúor, aplicados em diferentes quantidades, bem como avaliar a ingestão de flúor a partir escovação. Para isso, crianças (*n*=18, 2-3 anos de idade) foram aleatoriamente distribuídas em seis grupos experimentais, de acordo com possíveis combinações de dentifrícios (0/550/1100 ppm F, como NaF) e quantidade aplicada na escova (0,04/0,16/0,32 g, correspondendo a um grão de arroz, grão de ervilha e técnica transversal, respectivamente). Os voluntários fizeram uso de um dentifrício placebo durante uma semana. No sétimo dia, amostras salivares foram coletadas antes (*baseline*) e 5, 15, 30 e 60 minutos após a escovação com uma das possíveis combinações de tratamentos. Todo dentifrício expectorado após a escovação foi coletado. As concentrações de flúor foram determinadas após tamponamento com TISAB III (saliva) e por microdifusão facilitada por hexametildisiloxano (conteúdo expectorado). Os dados foram submetidos a ANOVA ou teste de Kruskal-Wallis, seguidos dos testes de Fisher LSD ou Student-

Newman-Keuls, respectivamente ($p<0.05$). A escovação com 550 ppm F (grão de ervilha ou técnica transversal) aumentou os valores de área sob a curva (AUC) para níveis semelhantes aos alcançados a partir da escovação com 1100 ppm F (grão de arroz). O maior valor de AUC e níveis salivares de flúor 5 minutos após escovação foi obtido a partir do tratamento com 1100 ppm F (grão de ervilha), seguido por 550 ppm F (técnica transversal). Para a ingestão de flúor a partir da escovação, o maior valor foi observado para o dentifrício de 550 ppm F (técnica transversal), seguido pelo 1100 ppm F (grão de ervilha). Concluiu-se que a quantidade de dentifrício e a concentração de flúor no produto afetam significativamente as concentrações de F na saliva e a ingestão de flúor durante a escovação.

Palavras-chaves: Fluoretos. Saliva. Criança. Dentifrícios.

SAMPAIO C. **Influence of the amount of dentifrice and fluoride concentration on intraoral fluoride uptake and on fluoride intake by children: *in vivo* study.** 2018. 62 f. Dissertação (Mestrado – Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2018.

ABSTRACT

The use of fluoridated dentifrices is regarded as the best vehicle of fluoride (F) use, since it combines the mechanical removal of biofilms with the therapeutic effects of F. Nevertheless, the evidence for recommending such products to children are still inconsistent, given the balance between dental caries and fluorosis. Thus, the aim of the present study was to evaluate F concentrations in saliva of toddlers after brushing with dentifrices containing different F concentrations, applied in different quantities, as well as to estimate F intake from toothbrushing. Toddlers (*n*=18, 2-3 years old) were randomly assigned into six experimental groups, according to the possible combinations of dentifrices (0/550/1100 ppm F, as NaF) and amount applied on the toothbrush (0.04/0.16/0.32 g, corresponding to rice grain, pea-size and using the transverse technique, respectively). Volunteers used a placebo dentifrice during one week. On the 7th day, saliva samples were collected before (*baseline*), and at 5, 15, 30 and 60 minutes after toothbrushing with one of the possible treatment combinations. All toothpaste expectorated after brushing was collected. F concentrations were determined after buffering with TISAB III (saliva) and hexamethyldisiloxane-facilitated diffusion (expectorate). Data were submitted to ANOVA or Kruskal-Wallis test, followed by Fisher's LSD or Student-Newman-Keuls' tests, respectively ($p<0.05$). Brushing with the 550 ppm F toothpaste (pea-size or transversal technique) increased AUC values at similar levels to those attained by brushing

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with 1100 ppm F (rice grain). The highest AUC and salivary F at 5 min after brushing were achieved by brushing with 1100 ppm F (pea-size), followed by the 550 ppm F (transversal technique). As for F intake during toothbrushing, the highest values were observed for the 550 ppm F (transversal technique), followed by 1100 ppm F (pea-size). It was concluded that the amount of toothpaste and F concentration in the product significantly affected both salivary F concentrations and F intake during toothbrushing.

Keywords: Fluorides. Saliva. Child. Dentifrices.

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Amount of dentifrice and fluoride concentration influence salivary fluoride concentrations and fluoride intake by children

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Amount of dentifrice and fluoride concentration influence salivary fluoride concentrations and fluoride intake by children

ABSTRACT

Objectives: To evaluate fluoride (F) concentrations in saliva of toddlers after brushing with dentifrices containing different F concentrations, applied in different quantities, as well as to estimate F intake from toothbrushing.

Methods: Toddlers (*n*=18, 2-3 years old) were randomly assigned into six experimental groups, according to possible combinations of dentifrices (0/550/1100 ppm F, as NaF) and amount applied on the toothbrush (0.04/0.16/0.32 g, corresponding to rice grain, pea-size and using the transverse technique, respectively). Volunteers used a placebo dentifrice during one week. On the 7th day, saliva samples were collected before (*baseline*), and at 5, 15, 30 and 60 minutes after toothbrushing with one of the possible treatment combinations. All toothpaste expectorated after brushing was collected. F concentrations were determined after buffering with TISAB III (saliva) or after HMDS-facilitated diffusion (expectorate). Data were submitted to ANOVA or Kruskal-Wallis test, followed by and Fisher's LSD or Student-Newman-Keuls' tests ($p<0.05$).

Results: Brushing with the 550 ppm F toothpaste (pea-size or transversal technique) increased the area under the curve (AUC) values at similar levels to those attained by brushing with 1100 ppm F (rice grain). The highest AUC and salivary F at 5 min after brushing were achieved by brushing with 1100 ppm F (pea-size), followed by 550 ppm F (transversal technique). As for F intake during toothbrushing, the highest values were observed for the 550 ppm F (transversal technique), followed by 1100 ppm F (pea-size).

Conclusions: The amount of toothpaste and F concentration in the product significantly affect both salivary F concentrations and F intake during toothbrushing.

Clinical significance: Lowering the amount of toothpaste used during toothbrushing significantly reduces salivary F concentrations and F intake from both toothpastes assessed, emphasizing the concept that the total treatment intensity (amount of dentifrice × F concentration) should be considered to balance risks and benefits.

Keywords: Fluorides. Saliva. Child. Dentifrices.

1. Introduction

The use of fluoride (F) has been attributed as one of the main reasons for the decline in dental caries prevalence worldwide [1]. Among the modalities of F delivery available, F dentifrices are currently regarded as the best vehicle, taking into account its mechanism of action combined to the mechanical removal of biofilms [2]. However, the cumulative and excessive F intake during the critical period of enamel formation may contribute to dental fluorosis development [3]. Given that 80% of the daily F intake is provided from dentifrices [4], strategies to minimize F intake from this source have been proposed, such as the use of low-fluoride dentifrices (LFD, 550 ppm F), or conventional dentifrices (CD, 1000-1100 ppm F) applied in reduced amounts. However, scientific evidence is still unclear and inconsistent to attest these recommendations, resulting in unknown risks to children related to both dental caries and dental fluorosis [5, 6].

Although the frequency of toothbrushing, rinsing habits and the amount of dentifrice applied on the toothbrush must be considered in order to maximize the effect of F on oral health [2, 7], scientific evidence on the influence of the amount of dentifrice is scarce. It has been shown that this variable significantly influences salivary fluoride concentrations and enamel demineralization, using *in situ* and *in vivo* protocols, in studies including only one strength of F in the product (1100 ppm F) in order to isolate the main study variable (*i.e.*, amount of dentifrice) [7, 8]. Recent studies have further addressed this issue, but also including the comparison of toothpastes with different F concentrations (LFD and CD, containing 550 and 1100 ppm F, respectively) [9, 10]. In an *in situ* study conducted with adult subjects, brushing with a LFD applied on all brush bristles led to significantly lower integrated loss of subsurface hardness compared with a CD applied in a pea-size amount, also having a significant impact on biofilm (solid and fluid phases) F concentrations [9]. In line with the above-mentioned study, brushing with a CD in a pea-size amount promoted significantly lower salivary F levels in 8-10 year-old children in comparison to a LFD applied by transversal technique [10]. The evidence from both studies clearly showed that the treatment intensity (*i.e.*, amount of dentifrice × F concentration in the product) was a more relevant parameter than merely observing the F content in the dentifrice.

Despite both aforementioned studies were well controlled, it was noteworthy that the differences in salivary F concentrations were not directly proportional to the intensity of the treatment [10]. Based on dose-response considerations, it was expected that the mean area under the curve (AUC) values resulting from the use of CD would be 2-fold higher than those of LFD within the same amount, but the increments in the AUC were shown to be directly related to the amount of product applied. Although the reasons for such a trend were not apparent, the authors hypothesized that F intake during toothbrushing might have contributed to the pattern observed, given that the amount of dentifrice had previously been shown to significantly influence F intake from this source [11].

Considering that none of the above-mentioned studies simultaneously assessed intraoral F uptake and F ingestion by children during toothbrushing, nor evaluated the impact of using very low amounts of toothpaste (*i.e.,* rice grain-size), it becomes clear that the issue of treatment intensity still demands further investigation. Most importantly, none of the above-mentioned studies enrolled children at the age risk for the development of dental fluorosis in the permanent incisors, so that it is possible that the pattern observed in the studies conducted by Hall et al. [10] and Paiva et al. [9] could not be directly extrapolated to younger children. Based on the above, the present study evaluated the effect of brushing with CD and LFD applied in different quantities on the resulting F concentrations in saliva, as well as on the F ingestion during toothbrushing, in 2-3 year-old children. The null hypothesis was that the amount of dentifrice used during the toothbrushing and the F concentration in the product would not influence the variables analyzed.

2. Materials and methods

2.1. Selection of the volunteers and ethical aspects

This study was approved by the IRB of the School of Dentistry, Araçatuba, (Unesp), Brazil (CAAE 69014017.9.0000.5420). There was an initial meeting with the parents in order to explain the aims and the methodological aspects of the study, as well as to emphasize the need for correctly following the protocol. Those who agreed with the study conditions read and signed an informed consent form. Only children who lived in the city of Araçatuba, São Paulo, Brazil (supplied with artificially fluoridated water, 0.6–0.8 mg F/L) [12], presenting good general and oral health and who were not using any medication that could interfere on the biofilm formation and on the salivary flow fulfilled the inclusion criteria. No restriction was established regarding dental caries status, which was evaluated using the ICCMS criteria [13].

Sample size was determined according to previous data [10], according to which 16 subjects would be required to detect significant differences in salivary fluoride concentrations when brushing with toothpastes containing 0 or 1100 ppm F, applied using the transversal technique (mean difference = 145.1 μg F/mL, standard deviation = 114.7), considering a power of 80% ($\alpha = 0.05$). Given the possibility of dropouts, and in order to achieve an equal number of children under each experimental condition (crossover protocol), 18 subjects were initially enrolled (*n* = 3 under each experimental condition).

2.2. Formulation and F determination of the experimental dentifrices

The experimental dentifrices were prepared in the laboratory of Pediatric Dentistry of São Paulo State University (Unesp), School of Dentistry, Araçatuba, with the following components: titanium dioxide; carboxymethyl cellulose; methyl p-hydroxybenzoate; sodium saccharine; oil peppermint; glycerin; silica abrasive; sodium lauryl sulfate; water and Sodium Fluoride (NaF, Merck®, Germany, 550 and 1100 µg F/g). A free-F dentifrice (placebo) was also produced. The dentifrices were packaged in identical tubes and coded by a researcher not involved in the experimental phase. Total and ionic fluoride concentrations were determined using a F ion-specific electrode (Orion 9409 BN, Orion Research Inc., Beverly, Mass., USA) coupled to an ion analyzer (SevenCompact S220, Mettler Toledo, Greifensee, Switzerland) previously calibrated with five standard solutions (0.125, 0.25, 0.5, 1.0, and 2.0 µg F/mL) [14]. The pH of the dentifrices was also analyzed [15].

2.3. Experimental design

The study followed a randomized, cross-over and double-blind protocol. The factors assessed were the amount of dentifrice applied on the toothbrush and the F concentration of the dentifrices, resulting in six combinations: 550 ppm F applied as a rice grain (0.04 g), pea-size (0.16 g) or by the transversal technique (0.32 g); 1100 ppm F applied as a rice grain (0.04 g) or pea-size (0.16 g); and Placebo (F-free dentifrice) applied by the transversal technique (0.32 g). A portable scale (precision 0.01-g, BEL Engineering, Monza, Italy) was used to guaranty accuracy during the interventions.

Sample collections were conducted on the premises of two kindergartens of the city of Araçatuba (SP), beginning at 7 a.m., aiming to avoid influences of the circadian rhythm on salivary flow [16]. Prior to and between each experimental phase, the volunteers were submitted to a *wash out* period of one week, in which a F-free dentifrice was used at home and at the kindergarten.

2.4. Saliva collections

Prior to toothbrushing, unstimulated saliva was collected using a Salivette® (Sarstedt, Germany) [17, 18], for 3 minutes, and was used as *baseline*. The children brushed their teeth during 1 minute, with or without the assistance of an adult (depending on the child's habit). They were then instructed to expectorate the resulting dentifrice slurry into a plastic vessel with wide opening, and to rinse their mouth with 50 mL of deionized water, which was also expectorated. The toothbrush was washed into the same vessel, and one aliquot of this mixture (expectorate + rinse + remaining on the toothbrush) was frozen for posterior F analysis. From that point onwards, four saliva samples were collected, at 5, 15, 30 and 60 after brushing (as described for *baseline* collection), totaling five samples per child [7].

2.5. Analysis of F concentrations in saliva

Saliva samples were extracted from the Salivette by centrifugation (5500 × *g*, 10 min, 4º C) [18], and was further centrifuged in order to separate desquamated epithelial cells, food particles, and bacteria from the saliva (12000 rpm or 15471 × *g*, 5 min) [19]. Next, the supernatant was buffered with TISAB III (Orion), and placed on the membrane of an ion-specific electrode (Orion 9409). A reference electrode (Orion Reference Electrode Double Junction 900100) was used to close the circuit. The electrodes were calibrated with standard solutions of known concentrations of fluoride, also buffered with TISAB III. The values obtained in millivolt (mV) were converted into µM F using the Microsoft Excel software (Version 2010, Microsoft Corp., Redmond, Wash., USA), allowing to determine the analytical parameters (linearity, slope, and coefficient of variation). All samples and standards were read in triplicate.

2.6. Analysis of F concentrations in the expectorate

Fluoride content from the expectorate was analyzed using an ion-specific electrode (Orion 9409 BN, Orion Research Inc., Beverly, Mass., USA) coupled to an ion analyzer (SevenCompact S220, Mettler Toledo, Greifensee, Switzerland) after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion, according to Taves [20], as modified by Whitford [21].

For diffusion, an aliquot each sample was placed into the bottom of nonwettable diffusion dish (Falcon, 1007), together with deionized water. Then, 50 µL sodium hydroxide (0.05 M) were placed on the inside surface of the lid, in five equal drops. The Petri dishes was sealed with petroleum jelly (Cinord Sudeste Química, Serrana – SP – Brazil) on the inside periphery of the lid and sealed to the bottom, and 2 mL of 3 M sulfuric acid saturated with HMDS was added through a small hole previously made in the lid of the diffusion dish. The orifice was sealed immediately after this step with petroleum jelly, to preclude acid evaporation.

Petri dishes were gently swirled on a rotatory shaker (Nova Técnica, NT 145), and the diffusion process occurred overnight at room temperature. On the next day, the dishes were opened, the lid was inverted, and 25 µL acetic acid (0.2 M) was pipetted on the sodium hydroxide. Then, the total volume was adjusted to 75 µL by the addition of deionized water. The drop that contained all fluoride from the sample was analyzed using the aforementioned equipment.

A set of standards ranging from 0.01 to 0.16 µg F was diffused in the same way, using serial dilution from a 100 mg F/L stock solution (model no. 940907, Orion Research). In addition, nondiffused fluoride standards were prepared with the same solutions (0.05 M NaOH, 0.20 M acetic acid, plus NaF) that were used to prepare the diffused standards and samples. The nondiffused standards were prepared to have exactly the same fluoride concentrations as the diffused ones. Comparison of the millivolt readings demonstrated that the fluoride in the diffused standards had been completely trapped and analyzed. The millivoltage potentials were converted to µg F using a standard curve with a correlation coefficient of $r \geq 0.999$. All the samples were read in triplicate, and the mean of the three readings was used.

2.7. Estimation of F ingestion from the dentifrices

The amount of fluoride applied on the toothbrush was obtained by multiplying the weight of the toothpaste on the toothbrush by the F concentration in the toothpaste (A). The amount of F not ingested (expectorate + rinse + remaining in the toothbrush) was obtained from the analysis of the expectorate (B), allowing the determination of the amount of F ingested (C), using the formula: $C = A - B$. [11].

Brushing frequency was considered in the determination of daily fluoride intake from the dentifrice. In addition, children were weighed in order to determine their daily F intake from this source (mg F/kg body weight/day) [22].

2.8. Statistical analysis

The area under the curve (AUC) for salivary fluoride clearance was calculated using the individual points (trapezoidal rule). Data of AUC (raw data) passed normality (Shapiro-Wilk) and homogeneity tests (Bartlett), and were submitted to 1-way, repeated measures ANOVA, followed by Fisher's LSD test. Data of fluoride ingested from toothbrushing (µg F) and estimation of daily fluoride intake from toothbrushing (mg F/kg/day) did not pass normality and homogeneity tests, and were analyzed by Kruskal-Wallis and Student-Newman-Keuls' *post hoc* test. Data on salivary fluoride concentrations at each individual time point also failed normality and homogeneity tests and were submitted to 2-way, repeated measures ANOVA, followed by Fisher's LSD test. Statistical analysis was conducted using SigmaPlot 12.0 software (San Jose, California, USA), adopting $p<0.05$.

3. Results

All subjects completed the six experimental phases of the study. Mean (SD) age of the children was 38.5 (6.3) months. Eleven boys and seven girls comprised the study population. Mean (SD) total and ionic F concentrations in the dentifrices were 21 (2) and 15 (1), 519 (17) and 539 (14), and 1037 (41) and 1105 (39) ppm F, respectively for the Placebo, 550 and 1100 ppm F. Mean pH of the dentifrices was 7.5, ranging from 7.4 to 7.6. Regarding dental caries status, no caries lesions were observed through the examination, so that the study comprised a caries-free population.

Figure 1 presents data on the AUC calculated for each treatment. A dose-response relationship was observed between the amount of toothpaste used during toothbrushing and the resulting AUC values within each toothpaste. Brushing with the 550 ppm F toothpaste at the lowest amount did not significantly increase the AUC values compared to the Placebo. However, the use of this toothpaste applied as a pea-size or using the transversal technique increased AUC values at levels similar to those attained by brushing with the 1100 ppm F applied as a rice grain. The highest AUC was achieved by brushing with a pea-size amount of the 1100 ppm F toothpaste.

Mean salivary fluoride concentrations at each individual time point are displayed in Table 1. Significant differences were observed among the treatments (F = 8.25, $p<0.001$), time points (F = 101.54, $p<0.001$), and for the interaction between these variables (F = 8.59, $p<0.001$). Regarding F concentrations as a function of time, all fluoridated toothpastes promoted significant increases 5 min after brushing, returning to baseline values at the subsequent time points. The only exception was observed for the 1100 ppm F toothpaste applied as a pea-size, which also promoted a significant increase at 15 min after brushing compared with the baseline. As for salivary F concentrations at each individual time-point, the higher the amount of toothpaste used, the higher the resulting salivary fluoride concentration. Brushing with 1100 ppm F applied as a pea-size resulted in the highest salivary F levels among all groups, followed by the 550 ppm F applied using the transversal technique, which resulted in significantly higher salivary F concentrations compared with 550 ppm F applied at lower amounts and 1100 ppm F applied as a rice grain.

Tables 2 and 3 show the estimated amount of fluoride ingested during toothbrushing (µg F) and daily fluoride intake from toothbrushing (mg F/kg/day), respectively. A clear influence of the treatment intensity (*i.e.*, amount of dentifrice × fluoride concentration) was observed both in the resulting toothpaste/saliva slurry expectorated after brushing, and on the amount of fluoride ingested. Regarding the amount of fluoride ingested, significant differences were observed among all groups, with the highest values observed for the

550 ppm F applied by the transversal technique, followed by 1100 ppm F toothpaste used as a pea-size.

4. Discussion

A systematic review from the Cochrane Collaboration provided strong evidence on the use of dentifrices containing 1000 ppm F or above for caries control in the permanent dentition [5]. Due to concerns on excessive fluoride intake from this source, especially by toddlers, several scientific societies around the World have endorsed the use of such formulations, but using reduced amounts of the product [23, 24]. Given that such recommendations are not supported by scientific evidence [9, 10], the present study was conducted in order to better understand the complex interplay between fluoride concentration in the toothpaste and the amount used during toothbrushing on salivary fluoride concentrations and on fluoride intake during toothbrushing, in a population in which the use of low-fluoride toothpastes might be beneficial considering both risks and benefits. Since both independent variables (amount of dentifrice and F concentration in the product) significantly influenced the response variables analyzed, the study's null hypothesis was rejected.

As previously mentioned, two recent studies demonstrated that the total intensity of the treatment (amount of toothpaste × F concentration) is more closely related to the resulting effects on intraoral F concentrations (saliva, biofilm and biofilm fluid) and on the development of enamel subsurface lesions *in situ* than the F concentration in the dentifrice itself [9, 10]. In line with previous observations, the present study also showed a trend of dose-response relationship between the treatment intensity and F levels in saliva (both AUC and individual point at 5 min after brushing), so that using higher amounts of a 550 ppm F toothpaste (*i.e.*, pea-size or transversal technique) was able to promote similar or superior salivary F concentrations in comparison with the 1100 ppm F toothpaste applied at the lowest amount (*i.e.*, rice grain). It is noteworthy, however, that salivary F concentrations at 5 min after brushing and the mean AUC achieved by brushing with the 1100 ppm F toothpaste applied as a pea-size was significantly higher than the corresponding values of the 550 ppm F toothpaste applied using the transversal technique, despite both treatments provided the same amount of F to the oral environment. A similar trend had also been observed in a previous study using a similar protocol in older children (8-10 years old) [10], demonstrating that not only the treatment intensity plays a role on the resulting intraoral fluoride concentrations.

Two factors might have influenced the pattern observed. Firstly, it is safe to assume that the initial brushing strokes occur without a marked dilution of the toothpaste in saliva, so that the higher concentration in the 1100 ppm F toothpaste would result in more F being delivered the oral surfaces exposed at the beginning of toothbrushing (compared with the 550 ppm F toothpaste). Following this rationale, F would be retained to tooth surfaces, dental biofilm and oral mucosa, and then slowly released into saliva, thus contributing to the highest salivary F levels observed for the 1100 ppm F toothpaste applied as a pea-size. Another hypothesis is related to F intake during toothbrushing. The present data showed that brushing with 550 ppm F toothpaste applied using the transversal technique led to significantly higher F intake in comparison to 1100 ppm F applied as a pea-size. Given that both treatments provided the same amount of F, significant differences in F intake during toothbrushing would not be expected. However, a well-controlled study assessing several variables related to F intake during toothbrushing concluded that the amount of toothpaste applied on the toothbrush directly affects F intake from this source

[11], a trend that had also been previously observed in an observational study [4]. Thus, the lower salivary F levels resulting from brushing with 550 ppm F toothpaste applied by the transversal technique (compared with a pea-size amount of 1100 ppm F) may be attributed to the higher F intake during the toothbrushing.

As for the estimation of daily F intake (DFI) from toothbrushing, this parameter was directly related to the treatment intensity, with a dose-response relationship more evident than that seen for salivary F concentrations. Considering that the so-called optimum DFI ranges from 0.05 to 0.07 mg F/kg/day [3, 25], and given that the highest contribution to DFI by 2-3 year-olds results from brushing with fluoridated dentifrices [4], it was noticeable that even the highest treatment intensities did not reach the aforementioned upper limit. This clearly shows that the use of small amounts of F dentifrice by younger children is indeed an effective measure to reduce F intake from this source. Nonetheless, considering the mean age of the study population enrolled in the study, it could be assumed that most of children presented a more evolved swallowing reflex in comparison with younger ones, implying that the present estimates of DFI from toothpaste may still not be fully representative to all children at the age risk for development of dental fluorosis in the permanent incisors (first 3 years of life) [3, 26]. This reinforces the recommendations of several Pediatric Dentistry associations on the need for the close monitoring of children during toothbrushing, especially regarding the expectoration of the product after brushing.

It must be emphasized that the present data was obtained from caries-free children, what may have clinical practical implications. Based on the inverse relationship between the fluoride levels in the oral environment and the prevalence of dental caries, [27, 28] the trend observed in this study might not be completely extrapolated to cariesactive children, so that this important variable (*i.e.*, caries status) should be taken into account in future investigations addressing the influence of treatment intensity on both intraoral F retention and on F ingested from toothpaste.

Based on the above, it was possible to conclude that the treatment intensity significantly affects both salivary F concentrations and F intake during toothbrushing, in line with previous findings demonstrating that lowering the amount of toothpaste used during toothbrushing significantly reduces several parameters related to intraoral F retention *in vivo* and the development of caries lesions *in situ* [7-10]. Considering the limitations of our short-term study protocol, it is not possible to make any clinical recommendation for the use of toothpastes to young children, given that this study used a surrogate endpoint that might not be directly related to the development of caries lesions under clinical conditions. However, this study adds to the body of evidence that the current recommendations by scientific and professional societies regarding the use of very low amounts of toothpaste by young children has an empirical basis and, as such, should be re-evaluated, in order to provide the best treatment option considering risks and benefits, and taking into account professional's and parent's personal preferences.

Acknowledgements

The authors thank the volunteers and the kindergartens for taking part of the study. The authors also acknowledge the São Paulo Research Foundation (FAPESP, grant #2017/07424-1) for the concession of a scholarship to the first author, which played no role in the study design, data collection and analysis, decision to publish, or

preparation of the manuscript. There is no conflict of interest that might affect the manuscript's judgment.

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Table 1. Mean (SD) salivary fluoride concentrations (μM) according to the treatments at each individual collect point

Dentifrices	Amount (g)	Time after brushing				
		Baseline	5 min	15 min	30 min	60 min
Placebo	0.32	3.2^{Aa}	3.4^{Aa}	3.4^{Aa}	2.9^{Aa}	3.3^{Aa}
		(1.3)	(1.5)	(1.2)	(1.4)	(1.5)
550 ppm F	0.04	3.1^{Aa}	7.6^{Bb}	3.6^{Aa}	3.0^{Aa}	3.2^{Aa}
		(0.8)	(2.1)	(1.1)	(1.1)	(1.2)
	0.16	3.2^{Aa}	8.2^{Bb}	4.0^{Aa}	3.8^{Aa}	2.8^{Aa}
		(1.4)	(3.9)	(1.5)	(1.3)	(0.6)
	0.32	4.1^{Aa}	11.2^{Cb}	4.6^{Aba}	3.3^{Aa}	3.2^{Aa}
		(1.8)	(4.2)	(1.2)	(1.2)	(1.0)
1100 ppm F	0.04	3.2^{Aa}	8.5^{Bb}	4.2^{Aba}	4.0^{Aa}	3.1^{Aa}
		(1.1)	(4.0)	(1.5)	(1.6)	(1.3)
	0.16	2.8^{Aa}	15.1^{Db}	5.5^{Bc}	3.9^{Aa}	3.1^{Aa}
		(0.9)	(9.3)	(2.7)	(1.4)	(1.1)

Different superscript upper letter reports significant difference among the treatments, while different superscript lower case indicates significant difference among the collect times assessed after brushing (Fisher's LSD test, $p<0.05$, $n = 18$).

Table 2. Fluoride concentrations (µg F) ingested during toothbrushing according to the experimental groups of dentifrices and quantities.

Toothpastes	**Placebo**	**550 ppm F**			**1100 ppm F**	
Amount on the toothbrush	**0.32 g**	**0.04 g**	**0.16 g**	**0.32 g**	**0.04 g**	**0.16 g**
Median	5.1^{A}	11.4^{B}	46.5^{C}	106.4^{D}	22.2^{E}	87.8^{F}
Interquartile range	0.7	7.3	22.7	67.5	8.5	46.0

Different superscript letters indicate significant difference among the experimental groups (Student-Newman-Keuls' test, p<0.05, n = 18).

Table 3. Daily fluoride intake (mg F/kg/day) from dentifrice according to the experimental groups

Toothpastes	**Placebo**	**550 ppm F**			**1100 ppm F**	
Amount on the toothbrush	**0.32 g**	**0.04 g**	**0.16 g**	**0.32 g**	**0.04 g**	**0.16 g**
Median	0.0005[A]	0.0016[B]	0.0046[C]	0.0107[D]	0.0024[E]	0.0084[F]
Interquartile range	0.0003	0.0016	0.0036	0.0056	0.0011	0.0088

Different superscript letters indicate significant difference among the experimental groups (Student-Newman-Keuls' test, $p<0.05$, $n = 18$).

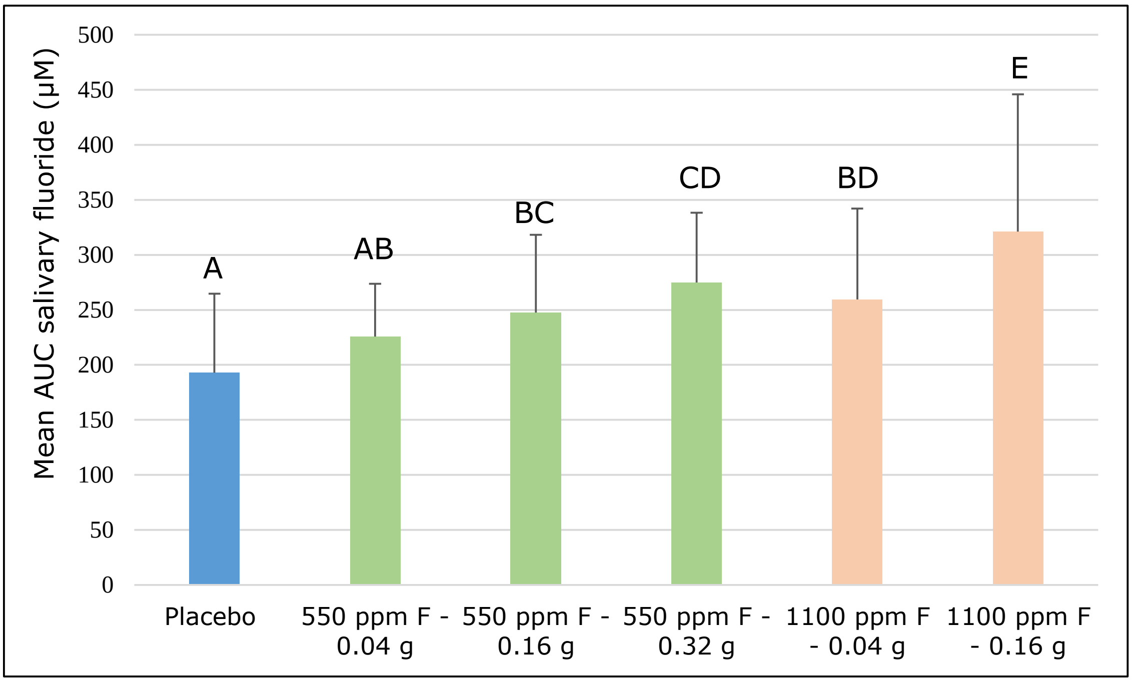

Figure 1. Mean (SD) area under the curve (AUC) fluoride concentration in saliva collected over 60 minutes after toothbrushing according to the experimental groups of dentifrices and quantities. Different letters indicate significant difference among the groups (Fisher's LSD test, $p<0.05$, $n = 18$; bars indicate standard deviation).

ANEXO A – CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA EM SERES HUMANOS

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Influência da quantidade de dentifrício e concentração de fluoreto na retenção intrabucal e ingestão de fluoreto por crianças: estudo in vivo

Pesquisador: Caio Sampaio

Área Temática:

Versão: 2

CAAE: 69014017.9.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.161.990

Apresentação do Projeto:

Com o projeto de pesquisa pretende-se avaliar as concentrações de fluoreto (F) na saliva, bem como analisar a ingestão de flúor após a escovação com dentifrícios contendo diferentes concentrações de F. Os voluntários (n=24, 2-3 anos de idade) serão aleatoriamente divididos em 6 grupos experimentais, de acordo com as possíveis combinações de dentifrícios e quantidades. As crianças farão uso de um dentifrício placebo (sem F) durante uma semana. No sétimo dia, amostras de saliva não estimulada serão coletadas antes da escovação (baseline) e 5, 15, 30, 60 e 120 minutos após escovação com uma das possíveis combinações de tratamento (quantidade de dentifrício x concentração de F no produto), seguindo um protocolo cruzado e duplo-cego. A quantidade de F ingerida durante a escovação também será avaliada, por meio da análise da concentração de F presente na espuma formada na boca e restante na escova. As amostras serão analisadas usando eletrodo íon-específico, após difusão facilitada com hexametil-disiloxano.Os resultados serão analisados quanto a normalidade (Kolmogorov-Smirnov) e homogeneidade (Bartlett); caso o uso de testes paramétricos seja possível, os dados serão analisados por ANOVA e teste de Tukey. Caso contrário, testes não paramétricos serão utilizados.

Objetivo da Pesquisa:

Avaliar o efeito da escovação com dentifrícios contendo diferentes concentrações de fluoreto em

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diferentes quantidades ("grão de arroz", "tamanho de uma ervilha" e "técnica transversal") sobre as concentrações de fluoreto na saliva, bem como sobre a ingestão de F a partir da escovação, em crianças na faixa etária de risco para o desenvolvimento de fluorose dental.

Avaliação dos Riscos e Benefícios:

A participação nesta pesquisa não infringe as normas legais e éticas havendo apenas o desconforto durante a escovação (a pesquisa possui riscos mínimos). Os procedimentos adotados nesta pesquisa obedecem aos Critérios da Ética em Pesquisa com Seres Humanos conforme Resolução nº. 466/12 do Conselho Nacional de Saúde. Com relação aos benefícios, os responsáveis pelas crianças receberão instruções de higiene bucal e correto uso de dentifrícios fluoretados. Além disso, será oferecido tratamento odontológico às crianças que apresentarem lesões de cárie. Por fim, o estudo trará como benefício o estabelecimento de novos protocolos da utilização de dentifrícios fluoretados por criança nesta faixa etária (2-3 anos).

Comentários e Considerações sobre a Pesquisa:

Trata-se de estudo que visa avaliar a concentrações de fluoreto (F) na saliva, bem como analisar a ingestão de flúor após a escovação com dentifrícios contendo diferentes concentrações de F.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram anexados adequadamente.

Recomendações:

Não Há

Conclusões ou Pendências e Lista de Inadequações:

Após a avaliação da metodologia proposta bem como dos documentos anexos somos favoráveis à execução do mesmo uma vez que a metodologia apresentada atende as normas da Resolução 466/12.

Considerações Finais a critério do CEP:

O CEP acata o parecer do relator. Informamos ao(a) senhor(a) pesquisador(a) que de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 10/01/2018. O CEP reitera a necessidade de entrega de uma via (não cópia) do TCLE ao sujeito participante da pesquisa e solicita ao pesquisador responsável leitura da carta circular 003/2011 CONEP/CNS

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antes do
início do projeto.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situaçã
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_917200.pdf	14/06/2017 10:00:45		Aceit
Declaração de Instituição e Infraestrutura	secretariadaeducacao.pdf	14/06/2017 09:20:26	Caio Sampaio	Aceit
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	14/06/2017 09:18:39	Caio Sampaio	Aceit
Projeto Detalhado / Brochura Investigador	Projeto_Mestrado_Caio_Sampaio_12_04_17_FINAL.docx	16/05/2017 11:20:03	Caio Sampaio	Aceit
Folha de Rosto	Folha_de_rosto.PDF	16/05/2017 10:57:57	Caio Sampaio	Aceit

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARACATUBA, 07 de Julho de 2017

Assinado por:
Aldiéris Alves Pesqueira
(Coordenador)

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ANEXO B – PROCEDIMENTOS EXPERIMENTAIS

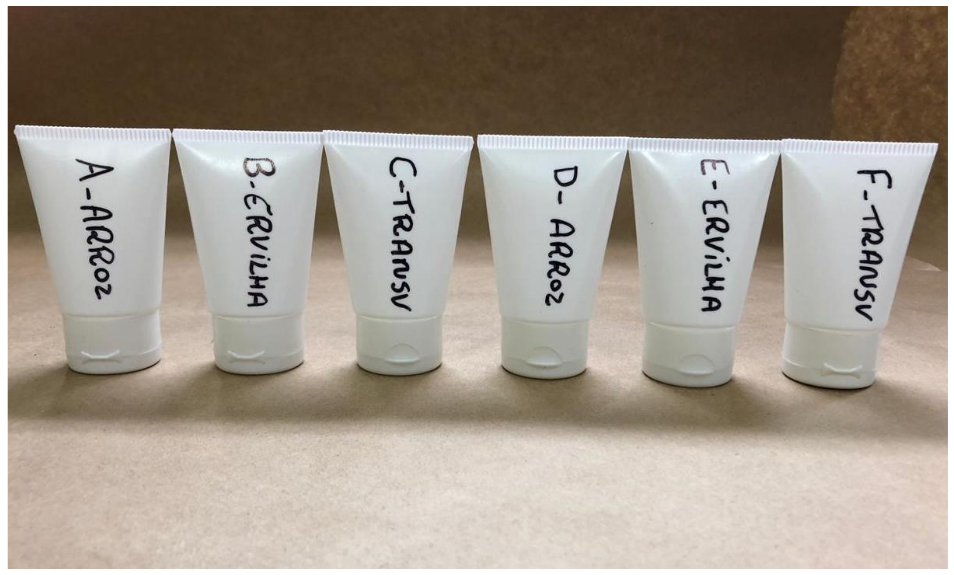

Figura 1. Dentifrícios experimentais.

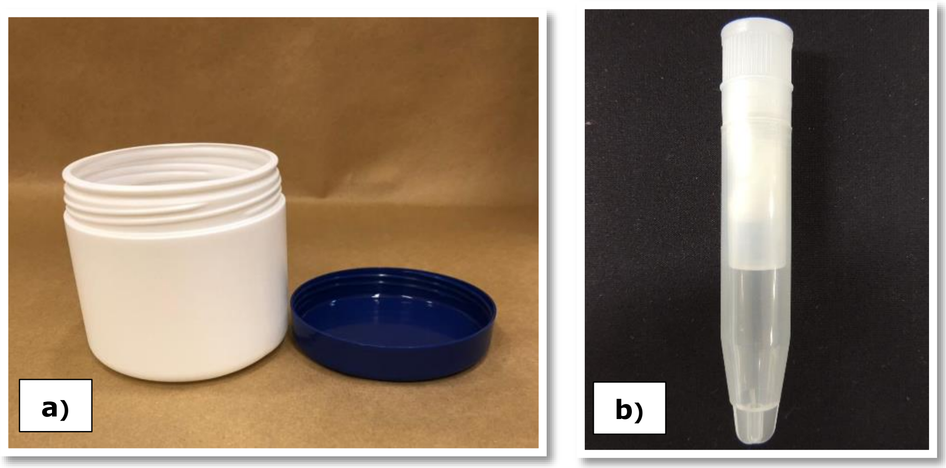

Figura 2. Recipiente coletor do conteúdo expectorado (a) e coletor Salivette (b).

Figura 3. Coleta salivar realizada previamente à escovação e 5, 15, 30 e 60 minutos após as escovações.

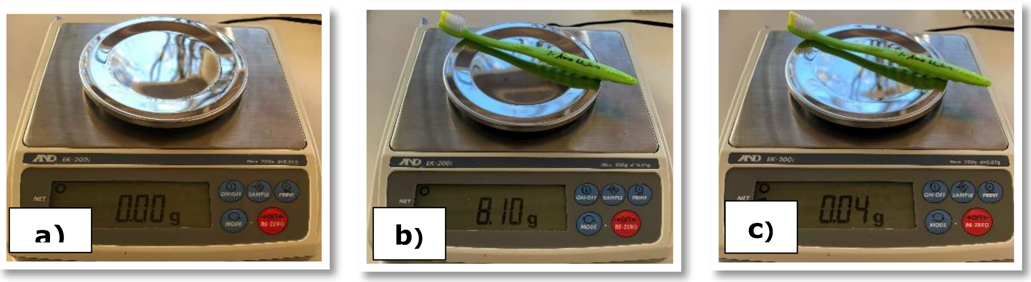

Figura 4. Pesagem do dentifrício previamente à escovação: balança de alta precisão (a); pesagem da escova isoladamente para exclusão do seu peso (b); pesagem do dentifrício de acordo com as combinações (c).

Figura 5. Escova de dente, copo com água deionizada (50 mL) e recipiente coletor do conteúdo expectorado.

Figura 6. Escovação por 1 minuto, com ou sem a ajuda de um adulto, de acordo com o hábito da criança.

Figura 6. Expectoração do dentifrício proveniente da escovação de acordo com o hábito da criança.

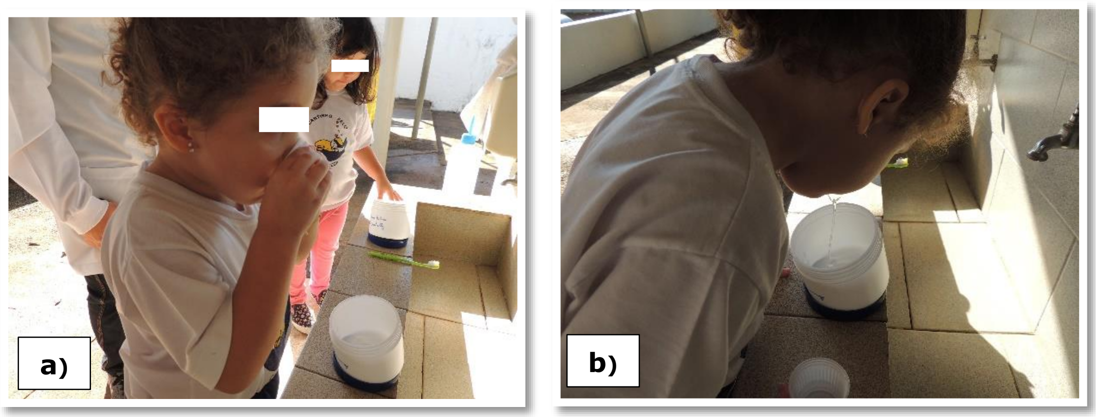

Figura 7. Enxágue com água deionizada (a) e expectoração deste conteúdo (b), de acordo com o hábito do voluntário.

Figura 8. Centrifugação das amostras de saliva (5500 x *g*, 10 minutos, 4º C) para posterior transferência para microtubos de centrifugação.

Figura 9. Transferência das amostras de saliva para microtubos de centrifugação.

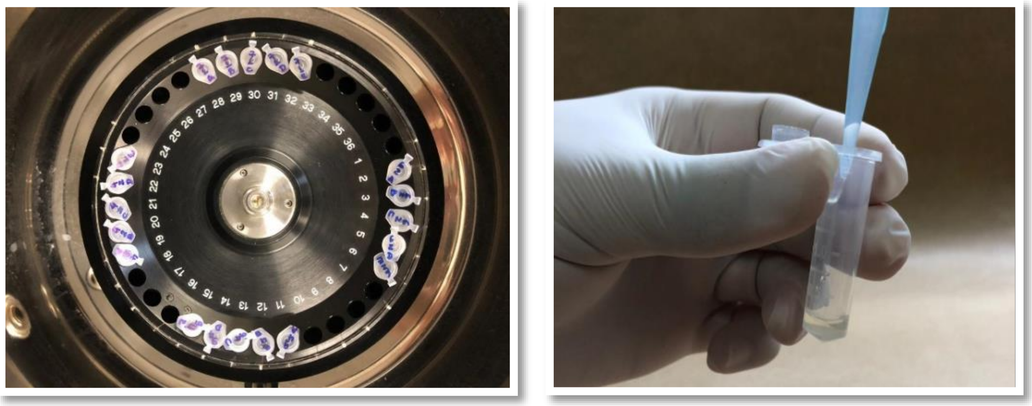

Figura 10. Transferência das amostras de saliva após centrifugação (15471 × *g*, 5 min, 25 ºC) para separação de restos epiteliais, células descamadas e partículas alimentares.

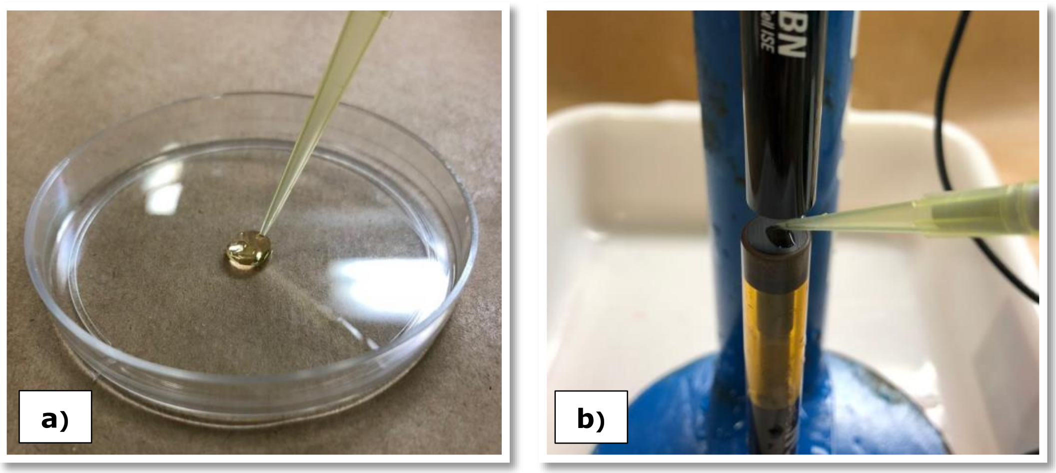

Figura 11. Análise da amostra de saliva. Tamponamento da saliva pela adição de TISAB III (a); conteúdo sendo dispensado sobre a membrana de um eletrodo íon específico para fluoreto (b).

Figura 12. Pesagem dos recipientes coletores antes das coletas.

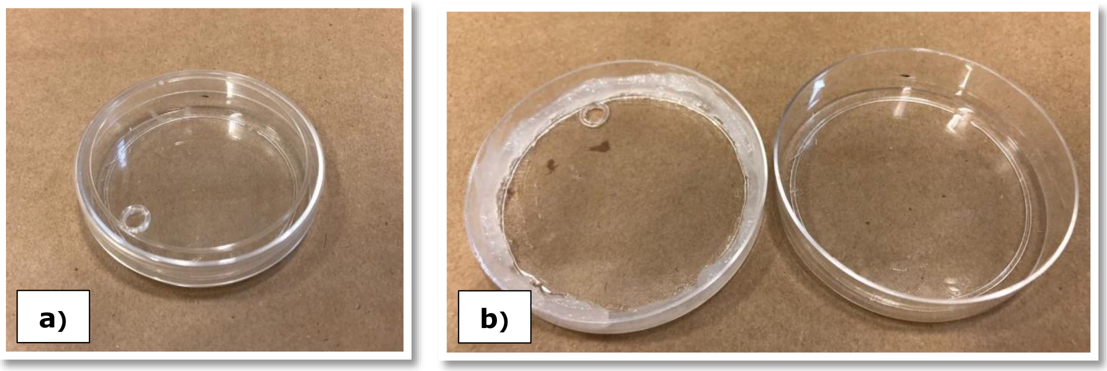

Figura 13. Placas de Petri com as tampas previamente furadas (a) e vaselinadas (b).

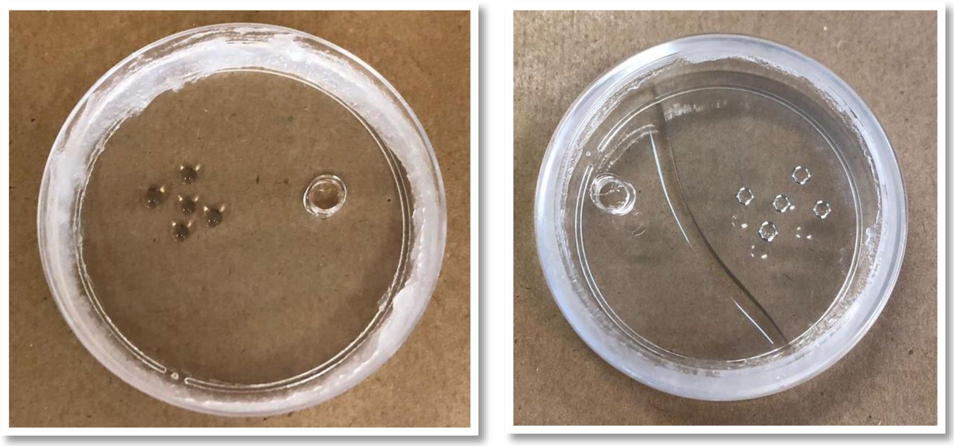

Figura 14. NaOH 0,05 M pipetado na tampa das placas.

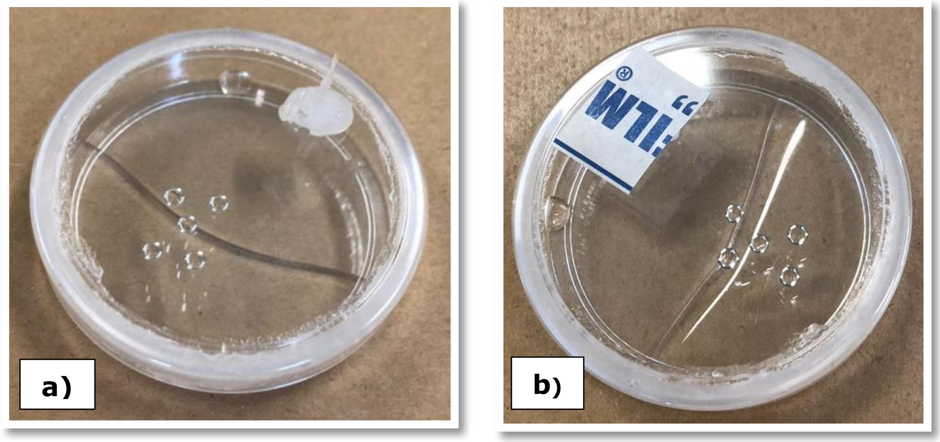

Figura 15. Orifício selado com vaselina (a) e parafilme (b).

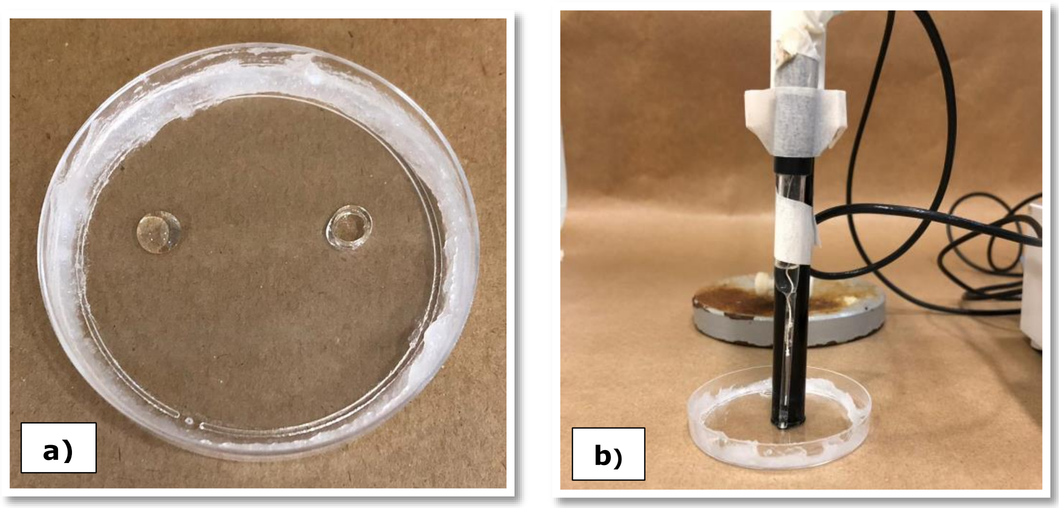

Figura 16. Tamponamento da gota com ácido acético 0,2 M (a), e leitura da gota tamponada com eletrodo ion-específico (b).

ANEXO C – NORMAS DE FORMATAÇÃO DO PERIÓDICO JOURNAL OF DENTISTRY

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INTRODUCTION
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[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/ xwj98nb39r.1.

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