ARTICLE

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Validation of an *in vitro* model to estimate the amount of fluoride released from toothpaste during toothbrushing

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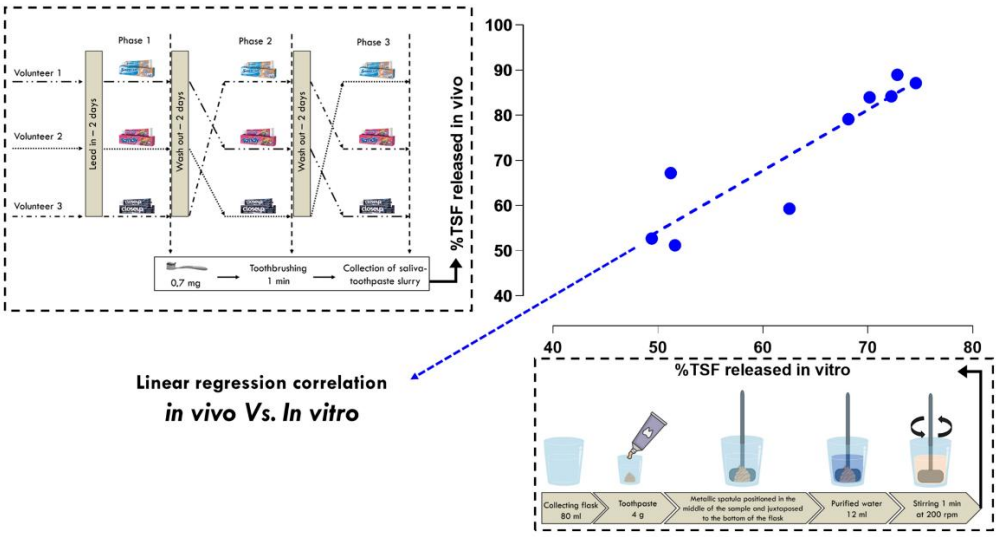
Toothpastes should ideally release most of their fluoride during toothbrushing; however, no clinically validated model currently exists to measure this release. In response, we developed an in vitro model to assess fluoride release from toothpaste, which was validated by comparing it to fluoride levels in saliva during toothbrushing. A crossover in vivo study was conducted with three participants, who brushed their teeth using one $Na_2FPO_3/CaCO_3$ toothpaste (1450 ppm F) and two NaF/SiO_2 toothpastes (1100 or 1450 ppm F). Fluoride release was measured in both in vivo and in vitro experiments using a fluoride ion-selective electrode. The in vitro test involved mechanically agitating three toothpaste samples, while rheological properties were evaluated using a rheometer to determine viscosity and hysteresis area. The in vivo test was conducted by the participants using a standardized toothbrushing technique. Linear regression analyses were performed to examine the relationships between in vivo and in vitro fluoride release, as well as viscosity metrics. A significant linear relationship was found between in vivo and in vitro total soluble fluoride (TSF = ions F^- + FPO_3^{2-}) release, as well as between in vivo TSF release and up-shear viscosity. These findings suggest that our validated in vitro test can effectively estimate fluoride bioavailability from toothpaste during toothbrushing in real-world scenarios.

Key Words: Toothpaste; Fluoride; Bioavailability; *In vitro*; rheology



Introduction

Fluoride toothpaste is regarded as the most effective method for utilizing fluoride, as it simultaneously disrupts the biofilm while releasing fluoride into the oral cavity to intervene in the development of carious lesions (1). Fluoride exerts a physicochemical effect on caries development by reducing demineralization and promoting the remineralization of dental minerals (1,2). For this effect to occur, fluoride must be chemically soluble and bioavailable in the mouth during toothbrushing (3,4).

Fluoride is chemically soluble in non-calcium-based toothpastes containing sodium fluoride (NaF), stannous fluoride (SnF_2), or amine fluoride (AmF) salts (5). Conversely, calcium-based toothpastes should use sodium monofluorophosphate (Na_2FPO_3), as the ion (FPO_3^{2-}) does not immediately react with calcium ions (Ca^{2+}) present in the abrasive (1). However, Na_2FPO_3/Ca-based toothpastes lack chemical stability over time, as the FPO_3^{2-} ion can hydrolyze, releasing fluoride ion (F^-) that forms insoluble salts with Ca^{2+} within the toothpaste tube (1,6). Previous research from our group demonstrated that the chemical solubility and bioavailability of fluoride during and after toothbrushing were compromised by the aging of Na_2FPO_3/$CaCO_3$-based toothpastes (4).

In addition to ensuring chemical compatibility between fluoride and abrasive salts, toothpaste formulations must maintain fluoride bioavailability during toothbrushing (3), meaning fluoride must be effectively released from the toothpaste. This release may be influenced by the rheological properties of the toothpaste (7). Rheology involves the study of how materials deform and flow under external forces (7). Toothpastes should flow easily when a force is applied and return to their original structure once the force is removed (7). Well-formulated toothpastes need to be fluid during extrusion and toothbrushing, while remaining stable on the toothbrush to ensure effective delivery to the teeth (8).

During brushing, toothpaste must undergo structural breakdown to reduce its viscosity (7,8). This transition to a fluid state enables the release of therapeutic agents, such as fluoride, into the oral cavity. This fluid state is essential and should persist long enough to maximize the availability of therapeutic agents (7,8). However, the rheological behavior of the toothpaste can significantly influence this process. A formulation with higher viscosity may resist breakdown during toothbrushing and return to a semi-solid state more quickly, thereby limiting the time that fluoride remains bioavailable (7,9,10). Additionally, the short duration of toothbrushing (11, 12) may affect the complete homogenization of the toothpaste, which is crucial for the bioavailability of therapeutic agents.

Fluoride is well-established as an effective anticaries agent; thus, maximizing its release from toothpaste during toothbrushing is essential (5,13). However, the release of fluoride can vary among commercial toothpastes due to differences in rheological modifiers and their concentrations (8,14). Given the critical role of fluoride in preventing caries, evaluating the fluoride release capacity of toothpaste formulations should be considered a fundamental pre-test for assessing their anticaries potential (3).

While some efforts have been made to estimate fluoride bioavailability during toothbrushing (3,15,16,17,18), existing tests often lack methodological standardization and correlation with *in vivo* data. To address the need for a pre-test to evaluate the anticaries potential of toothpastes, we developed and standardized an *in vitro* test that correlates with *in vivo* fluoride bioavailability during toothbrushing with various commercial toothpastes. Furthermore, we assessed the rheological behavior of these toothpastes and their relationship with fluoride bioavailability during toothbrushing.

Material and methods

Experimental design

A blinded, crossover, three-phase *in vivo* study and an *in vitro* study were conducted. In both studies, the same tubes of three commercial toothpastes, purchased from a store in Santa Bárbara do Oeste, SP, Brazil, were used: SDB: Sorriso Dentes Brancos (Na_2FPO_3/$CaCO_3$, 1,450 ppm F (mg F/kg); batch: 9154BR121J, expiration date: June/2021), T: Tandy (NaF/SiO_2, 1,100 ppm F (mg F/kg); batch: 9078BR122C, expiration date: March/2022), and CFA: Close-up Fresh Action (NaF/SiO_2, 1,450 ppm F (mg F/kg); batch: T3C10, expiration date: April/2022). These toothpastes were selected because they demonstrated varying fluoride release levels in a preliminary *in vitro* study (19). The *in vivo* study was approved by the Research Ethics Committee of (*Place blinded for reviewers*) (CAAE: 19842819.0.0000.5418). Furthermore, the rheological properties of these toothpastes were assessed using a rheometer to measure their stepped-shear flow curves.

In vivo test

The study was conducted at the Oral Biochemistry Laboratory FOP UNICAMP in the morning. Prior to the experiment, participants were instructed not to use any oral hygiene products or consume food for at least two hours. Additionally, for two days before the start of the experiment (lead-in period) and between phases (washout period), volunteers used a non-fluoride toothpaste (20). They were instructed to avoid fluoride-rich foods, such as *green tea (Camellia sinensis*). In the laboratory, they brushed their teeth for one minute (11, 12) using 0.7 g of one of the test toothpastes (4). All the toothpaste-saliva slurry produced during toothbrushing was collected, and its volume was measured (Figure 1). The samples were then centrifuged (16,000 *g* for 10 min), and the supernatant was analyzed for total soluble fluoride (TSF = ions F^- + FPO_3^{2-}).

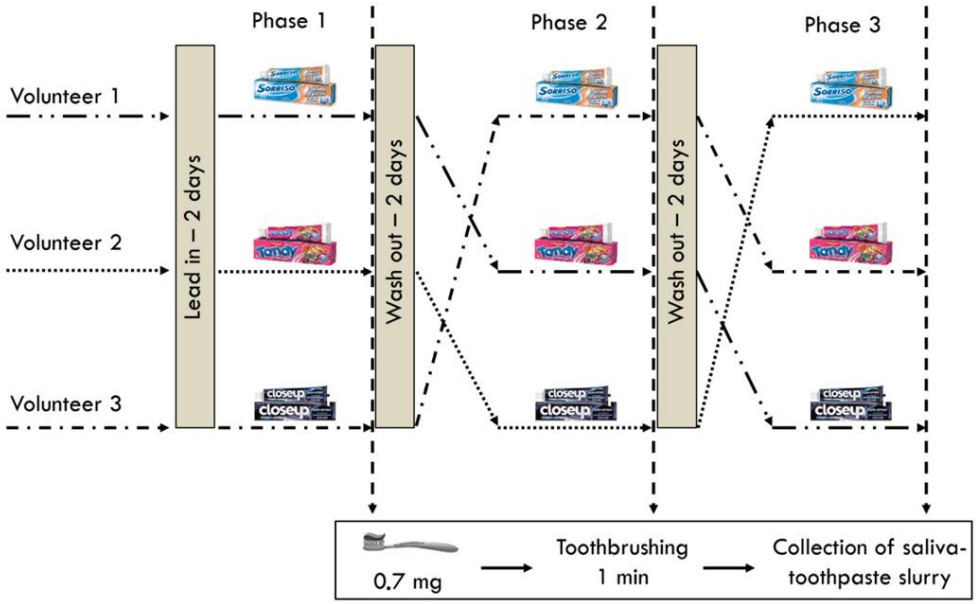


Figure 1. Outline of the *in vivo* study experimental design.

In vitro test

An *in vitro* study was conducted using the same tubes of three toothpastes (n = 3) (19). A 4 g sample of each toothpaste (3,15,16,17,18) was placed at the bottom of a collecting flask (lower external diameter: 45.0 mm; upper external diameter: 50.9 mm; height: 57.1 mm; volume: 80 ml). A metallic spatula for stirring was positioned in the center of the toothpaste, adjacent to the bottom of the flask (Figure 2). Next, 12 ml of water was added to the flask containing the toothpaste sample, followed by mechanical stirring for 1 min at 200 rpm. The collecting flask was then inverted to collect the material that had been mechanically released into the water. Immediately afterward, the samples were centrifuged (16,000 *g* for 10 min), and the supernatant was analyzed for TSF.

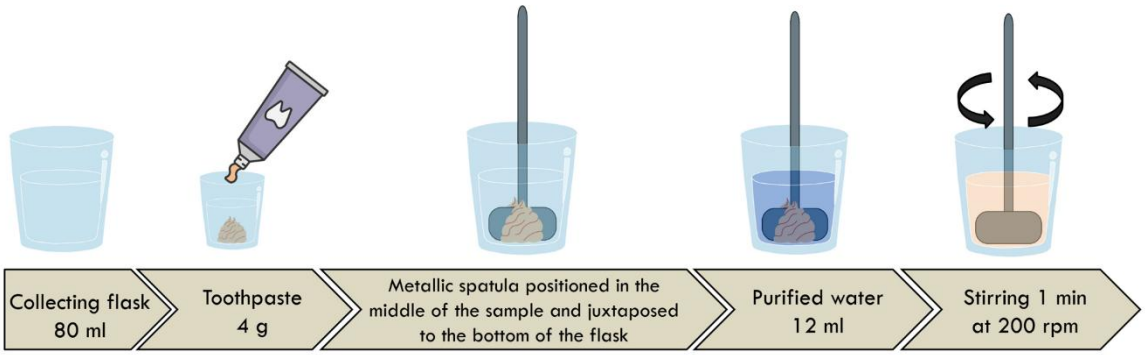


Figure 2. Outline of the *in vitro* study experimental design.

TSF determination in the toothpastes

TSF concentrations were measured in the toothpastes to calculate the amount of fluoride present in the toothpaste samples used in both *in vitro* and *in vivo* tests, using the formula below:

$$\text{AFD (µg F)} = \text{TSW (g)} \times \text{FCT (µg F/g)}$$

Where:
AFD = amount of fluoride in the dentifrice sample used *in vivo* or *in vitro* (µg F)
TSW = Toothpaste sample weight: 4 g or 0.7 g of toothpaste
FCT = Fluoride concentration determined in the toothpastes (µg F/g)

To prepare the toothpaste samples for fluoride determination, duplicates of 90-110 mg of each dentifrice were suspended in 10 ml of water (21). The suspensions were then centrifuged (3,000 *g* for 10 min), and duplicate aliquots of 0.25 ml of the supernatant were used for TSF analysis. All samples were treated with 0.25 mL of 2 M HCl for one h at 45 °C to hydrolyze the ion FPO_3^{2-}. After neutralization with 0.5 ml of 1 M NaOH and buffering with 1.0 ml of TISAB II, the fluoride concentration was determined using an ion-selective electrode (Orion 96-09; Orion Research, Cambridge, MA, USA) coupled to an ion analyzer (Orion Star A214; Orion Research, Cambridge, MA, USA), calibrated with fluoride standards prepared under the same conditions as the samples.

TSF determination in the *in vivo* and *in vitro* study

Initially, *in vivo* and *in vitro* samples were diluted 15-fold and 10-fold, respectively, to ensure that the expected fluoride concentrations fit the calibration curve limits. All samples were treated with 2 M HCl for 1 h at 45 °C, then neutralized and buffered with TISAB II (which contains 1 M NaOH) to determine fluoride concentration, as previously described. The amount of fluoride released was calculated based on the final volume of the saliva-toothpaste slurry for *in vivo* samples, while the volume of added water was considered for the *in vitro* test samples.

The formula used was:

$$\text{AF released (µg F)} = \text{CFE (µg F/ml)} \times \text{VS (}\textit{in vivo}\text{) or VW (}\textit{in vitro}\text{) (ml)}$$

Where:
AF released = amount of fluoride released *in vivo* or *in vitro* (µg F)
CFE = Fluoride concentration found in the *in vivo* or *in vitro* samples (µg F/ml)
VS = volume of the saliva-toothpaste slurry (ml) for *in vivo* samples
VW = volume of water added in the *in vitro* test (ml)

Using the amount of TSF released in both *in vivo* and *in vitro* studies, along with the amount of TSF present in the toothpaste samples, the percentage of fluoride release was calculated using the following formula:

$$\%\text{TSF released} = (\text{AF Released (µg F)} \div \text{AFD (µg F)}) \times 100\%$$

Where:
AF released = Amount of fluoride *in vivo* or *in vitro* (µg F)
AFD = amount of fluoride in the dentifrice sample used *in vivo* or *in vitro* (µg F)

Rheological measurements

All measurements for each toothpaste (n = 3) were conducted using a strain-controlled rheometer (AR 1500 ex model; TA Instruments, New Castle, USA), equipped with a 40 mm diameter roughened steel cone-plate geometry at a controlled temperature of 37 °C. Stepped-shear flow curves (Pa.sec) were obtained by gradually increasing the shear rate from 0.1 to 30 sec^{-1} (up-shear flow curve) and then returning to 0.1 sec^{-1} (down-shear flow curve). The sampling rate was set at 15

sec per point, collecting 10 points per decade (8). The apparent viscosity was determined by averaging the final 5 sec of each step (8). The hysteresis area (Pa/sec) was calculated by subtracting the area under the up-shear flow curve from the area under the down-shear flow curve (10).

Statistical analysis

Data were assessed for normal distribution using the Kolmogorov-Smirnov test. Linear regression analysis was performed to examine the relationship between %TSF released *in vivo* and %TSF released *in vitro*, as well as apparent viscosity (from both up- and down-shear flow curves) and hysteresis area. The %TSF released *in vitro* and *in vivo* was compared among the different toothpastes using one-way ANOVA, followed by Tukey's test for post hoc analysis. A significance level of 5% was established. All analyses and graphs presented here were generated using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com).

Results

Table 1 indicates that SiO_2-based toothpastes contained all fluoride in a soluble form, whereas the $CaCO_3$-based toothpaste exhibited 8.6% of insoluble fluoride relative to the total fluoride declared by the manufacturer. Regarding rheological modifiers, all tested formulations included cellulose gum as a ligand. However, for humectant ingredients, the SiO_2-based formulations contained various types of polyethylene glycol, while the $CaCO_3$-based formulation contained glycerin.

Table 1. Information of the commercial toothpaste formulation regarding rheological modifiers, abrasive and fluoride salts declared by the manufacturer, as well as total fluoride (TF) declared and total soluble fluoride (TSF) found.

Toothpastes	Ligand	Humectant	Abrasive	Fluoride compound	*TF declared	**TSF Found
Sorriso Dentes Brancos	Cellulose Gum	glycerin	$CaCO_3$	Na_2FPO_3	1450	1324.5 ± 30.8
Tandy	Cellulose Gum	PEG-12	SiO_2	NaF	1100	1130.2 ± 87.2
Close-up Fresh Action	Cellulose Gum	PEG-32	SiO_2	NaF	1450	1494.2 ± 26.4

* ppm F (mg F/kg); ** mean ± SD in ppm F (mg F/kg).

Figure 3 shows the percentage of total surface free energy (TSF) released by the tested toothpastes in both in vitro and in vivo studies. In vitro, all toothpastes differed statistically ($p<0.05$). In the *in vivo* study, the group using Sorriso Dentes Brancos showed a statistically significant difference from the group using Close-up Fresh Action ($p<0.05$). However, they did not differ from the Tandy group($p>0.05$).

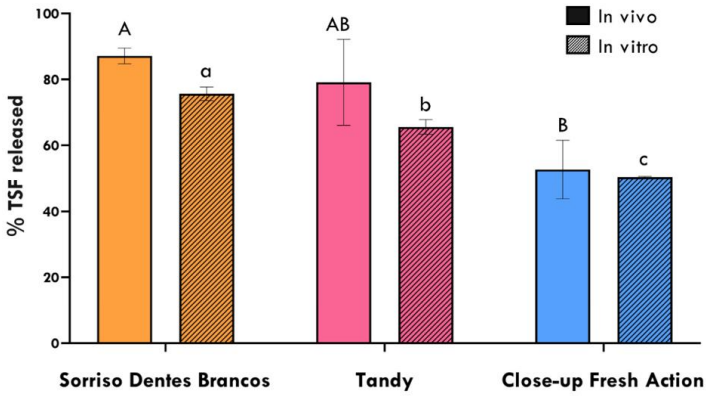


Figure 3. Percentage (mean ± SD; n = 3) of TSF released (%TSF) *in vivo* and *in vitro*, according to the tested toothpastes.

*Distinct capital and small letters represent, respectively, statistical differences among toothpastes for *in vivo* and *in vitro* studies.

Figure 4 compares the flow curves of the three different commercial toothpastes, highlighting considerable differences in their shear-thinning rheological behavior (Figure 4A). Close-up Fresh Action exhibited the smallest decrease in viscosity with increasing shear rate, but it quickly regained viscosity when the shear rate decreased. In contrast, Tandy and Sorriso Dentes Brancos both experienced a loss of viscosity with increasing shear rate; however, Tandy recovered its viscosity more quickly, while Sorriso Dentes Brancos did not fully recover (Figure 4A). When averaging the apparent viscosity (Pa · s) over the last 5 seconds of the up-shear flow curve, significant differences were observed among all toothpastes, with Close-up Fresh Action showing the highest apparent viscosity and Sorriso Dentes Brancos the lowest (Figure 4B). Tandy exhibited intermediate apparent viscosity during the up-shear flow curve. However, it was not significantly different from Close-up Fresh Action during the last 5 seconds of the down-shear flow curve. For the down-shear flow curve, Close-up Fresh Action and Sorriso Dentes Brancos again displayed the highest and lowest apparent viscosities, respectively (Figure 4B).

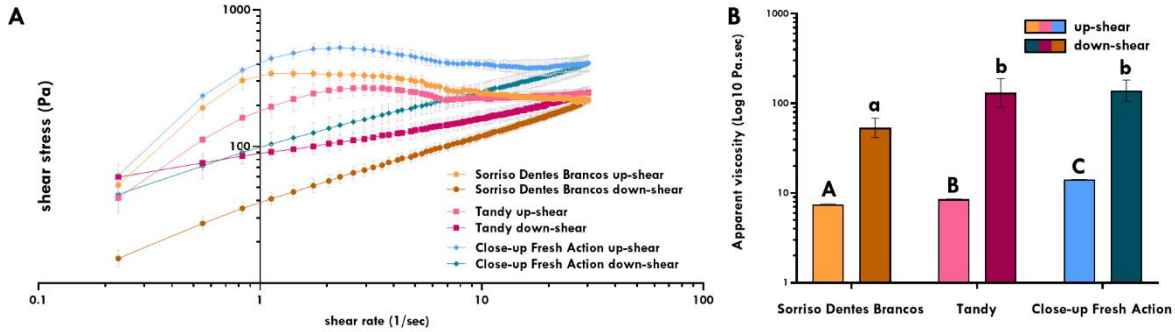


Figure 4. Rheological behavior of the different toothpastes tested. A. Stepped-shear flow curves tests at increasing (up-shear) and decreasing (down-shear) shear rate. B. Apparent viscosity by averaging the last 5 seconds of each flow curve.
*Different capital and small letters represent, respectively, statistical differences for apparent viscosity from up and down-shear flow curves among toothpastes.

A statistically significant linear correlation was found between %TSF released *in vivo* and %TSF released *in vitro* (Table 2), as well as with the apparent viscosity from the up-shear flow curve. However, no correlation was found between the percentage of TSF released and the apparent viscosity from the down-shear flow curve or the hysteresis area.

Table 2. Linear regression coefficients (r) and significance values (p) for the correlation between % Total Soluble Fluoride (%TSF) released *in vivo* and the response variables.

Variables	r	p
% TSF released *In vitro*	-0.68	0.0060
Apparent viscosity Up-shear (Pa.sec)	-0.68	0.0062
Apparent viscosity Down-shear (Pa.sec)	-0.03	0.6528
Hysteresis area (Pa/s)	-0.22	0.1985

Discussion

The total soluble fluoride (TSF), as ion F^- or FPO_3^{2-}, released by a toothpaste formulation during toothbrushing, is a critical factor in evaluating its anticaries potential (1,4). In this study, we developed an *in vitro* test to assess fluoride bioavailability during toothbrushing, which was validated through correlation with *in vivo* data. Additionally, we confirmed our hypothesis that the rheological properties of toothpaste formulations can influence the release of TSF.

All tested toothpastes were fresh and contained more than 1,000 ppm of TSF (Table 1), indicating their anticaries potential due to fluoride's ability to interfere with the caries process (4, 21). However, distinct fluoride release levels were observed when participants brushed their teeth for 1 minute with the evaluated toothpastes (Figure 3). For example, Sorriso Dentes Brancos (SDB) released over 80% of its TSF, while Close-up Fresh Action (CFA) released approximately 50%. Given the established dose-response relationship between fluoride concentration in toothpastes and decay prevention (22), the varying fluoride release levels suggest that different toothpastes may exhibit different anticaries potentials.

Thus, an in vitro test that reflects fluoride bioavailability during toothbrushing can serve to estimate the anticaries potential of commercial toothpastes (3,23). Our *in vitro* test is promising, as it demonstrated a statistically significant linear correlation between *in vivo* and *in vitro* %TSF (Table 2), thereby validating its applicability. The proposed test also features standardized procedures, particularly concerning mechanical agitation at a defined rotation speed to ensure consistent homogenization of the toothpaste sample. Currently available tests (3, 15, 16, 17, 18) lack validation regarding *in vivo* fluoride bioavailability during tooth brushing. Furthermore, these tests often rely on manual homogenization, which can introduce variability in the speed and force applied by different operators. Additionally, they do not differentiate between total fluoride and total soluble fluoride, and it is very well known that only the latter possesses anticaries efficacy (4,24).

Fluoride bioavailability can be influenced by the rheological properties of toothpaste formulations (7,14). All tested toothpastes contained cellulose gum as a standard rheological modifier, while humectants such as glycerin and polyethylene glycol (PEG) were included in the SDB formulation and the other two toothpastes, respectively (Table 1). Abrasive agents also significantly impact the rheological behavior, which can affect the release of fluoride. For instance, the T and CFA formulations contained hydrated silica (SiO_2) as the abrasive agent, which interacts with humectants and other components to create a polymeric matrix that agglomerates ingredients and alters the product's viscosity (7). In contrast, calcium carbonate ($CaCO_3$) in the SDB formulation is typically used at higher concentrations and also contributes to viscosity (7,25). However, unlike the more chemically stable matrix formed by silica, $CaCO_3$ is a salt that disperses easily in water and saliva, which helps explain the results observed in our study.

To understand how the rheological behavior of the formulations under study influences fluoride release, we conducted rheological tests. CFA exhibited a more viscous formulation and the lowest %TSF release, whereas SDB had lower viscosity and the highest %TSF release. These data suggest that viscosity may have affected fluoride release (10,14), as supported by a statistically significant linear correlation between the percentage of total soluble fluoride (TSF) released *in vivo* and the apparent viscosity from the up-shear flow curves (Table 2). This finding implies that measuring viscosity could serve as an alternative method for evaluating %TSF release (10). Interestingly, no correlation was found between the percentage of TSF released *in vivo* and the apparent viscosity for down-shear flow curves or the hysteresis area. This may be attributed to the rapid recovery observed in Tandy toothpaste (Figure 4), resulting in apparent viscosity and hysteresis area values similar to those of CFA. Thus, it appears that the disruption of the toothpaste structure during flow, indicated by viscosity loss, is a more reliable predictor of %TSF release than its recovery.

This study has limitations that should be noted. First, the *in vivo* study involved only three volunteers; however, it was still possible to statistically differentiate the toothpastes evaluated based on the study's outcomes. Additionally, the in vitro test was conducted using only three different commercial toothpastes, despite the wide variety of products available on the market. Future studies should include a larger sample size to enhance the validity of the findings. Furthermore, the reproducibility of our in vitro test should be validated by other laboratories.

In conclusion, the validated in vitro test developed in this study provides a reliable and straightforward method to estimate fluoride bioavailability from toothpaste during toothbrushing. It has the potential to serve as a screening tool for manufacturers and regulatory agencies, supporting the development and approval of effective fluoridated dentifrices.

Resumo

Os dentifrícios devem, idealmente, liberar a maior parte do fluoreto durante a escovação, mas atualmente não existe um modelo clinicamente validado para estimar essa liberação de fluoreto. Em resposta a essa lacuna, desenvolvemos um modelo in vitro para avaliar a liberação de fluoreto por dentifrício, o qual foi validado em comparação com os níveis de fluoreto disponibilizados na saliva durante a escovação. Um estudo cruzado in vivo foi conduzido com três voluntários, que escovaram os dentes utilizando um dentifrício com $Na_2FPO_3/CaCO_3$ (1450 ppm de F) e dois com NaF/SiO_2 (1100 ou 1450 ppm de F). A liberação de fluoreto foi determinada tanto nos experimentos in vivo quanto nos in vitro utilizando um eletrodo específico para fluoreto. O teste in vitro envolveu a agitação mecânica de amostras dos dentifrícios, enquanto as propriedades reológicas foram analisadas em um reômetro para medir a viscosidade e a área de histerese. O teste in vivo foi realizado pelos participantes usando uma técnica de escovação padronizada. Análises de regressão linear foram realizadas para examinar as relações entre a liberação de fluoreto in vivo e in vitro, bem como com as métricas de viscosidade. Foi observada uma relação linear significativa entre a liberação de fluoreto solúvel total (TSF = íons F^- + FPO_3^{2-}) in vivo e in vitro, assim como entre a liberação de TSF in vivo e a viscosidade em regime ascendente (up-shear). Esses achados sugerem que nosso teste in vitro foi validado para estimar de forma eficaz a biodisponibilidade do fluoreto de dentifrício que ocorre in vivo durante a escovação dentária.

Acknowledgements

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Statement of ethics

The participation of the volunteers to toothbrush with different commercial toothpastes, as well as the collection of toothbrushing residues and saliva were all approved by the local Research and Ethics Committee of the Piracicaba Dental School, University of Campinas (protocol number 19842819.0.0000.5418). Parts of this manuscript were reviewed by ChatGPT-4o mini for clarity while maintaining the original meaning as closely as possible.

Conflict of interest statement

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Conceived and designed the experiment: JAC and APRF. Performed the experiment: MXQ. Analyzed the data: MXQ, APRF and JAC. Wrote the paper: MXQ, APRF and JAC.

Data availability statement

All data generated or analyzed during this study are included in this article and are available in UNICAMP repository. Further inquiries can be directed to the corresponding author.

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