Enhancing predicted fluoride varnish efficacy and post-treatment compliance by means of calcium-containing gummy bears

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Short title: Fluoride varnish and calcium gummies–efficacy and compliance

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Abstract

Objectives: This study determined whether consumption of calcium-containing gummies prior to fluoride varnish application enhances plaque fluoride retention and compliance with post-varnish application instructions.

Methods: The present study followed a multi-center, parallel, randomized, and laboratory analyst-blind design. Following IRB approval, parent consent and child assent, 44 subjects (7-12 years), were randomized to either gummy or no-gummy study groups. A baseline plaque sample was obtained after a wash-out period. Fluoride varnish (5% NaF) was applied; subjects in the gummy group received two calcium-containing gummies prior to varnish application. Subjects were given two questionnaires to

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complete (subject and parent) to investigate adherence to post-treatment instructions. Three days later, a second plaque sample was obtained. Plaque was analyzed for plaque fluid and solid fluoride concentrations. Fluoride data were analyzed using Wilcoxon Rank Sum tests, questionnaire data using Pearson chi-square tests.

Results: Plaque fluid fluoride did not change pre- to post-treatment in the gummy group (mean±sd: 8.8±5.7µmol/l vs. 10.0±6.3µmol/l; p=0.265) or in the no-gummy group (8.1±4.4µmol/l vs. 16.1±20.0µmol/l; p=0.058). Groups were not different for plaque fluid fluoride pre-treatment (p=1.000), post-treatment (p=0.904), or change (p=0.904). Plaque solid fluoride did not change pre- to post-treatment in the gummy group (0.89±1.10µmol/g vs. 1.37±1.77µmol/g; p=0.073) or in the no-gummy group (0.68±0.77µmol/g vs. 2.01±5.00µmol/g; p=0.190). Groups were not different for plaque solid fluoride pre-treatment (p=1.000), post-treatment (p=0.466), or change (p=0.874). No significant differences were found between groups for questionnaire responses.

Conclusion: This study failed to demonstrate an effect of calcium-containing gummies in enhancing plaque fluoride retention.

Keywords: fluoride varnish; plaque; caries; calcium; gummies

Clinical Significance: The consumption of calcium-containing gummies prior to fluoride varnish application does not promote greater intra-oral fluoride retention or better adherence to post-treatment instructions.

Introduction

Several systematic reviews have concluded that fluoride varnishes are effective in preventing dental caries in children and adolescents [1-23]. However, recent caries clinical trials have demonstrated only marginal or no efficacy for fluoride varnishes in general and in particular in high-risk populations [4-567]. This highlights the need for interventions that are more efficacious and different approaches to utilize the wide array of caries-preventive agents.

Fluoride exerts its anti-caries effect primarily through small, but protracted elevations of fluoride in saliva and, in particular, in dental plaque, the biofilm covering the teeth [8,9]. While fluoride has very good substantivity in partially demineralized enamel, the overall amount that can be retained in this reservoir is low and depends on the diffusion of fluoride through plaque, which is restricted [10]. Fluoride's retention in plaque has been shown to depend strongly on the co-presence of calcium [11]. Thus not surprisingly, several studies were able to demonstrate that ionic calcium applied to the oral cavity prior to fluoride greatly enhances fluoride retention in saliva [12], plaque and plaque fluid [13]. The mechanism of action is obvious as plaque-bound calcium presents more retention sites for a subsequent fluoride application. Similarly, the combined application of calcium and fluoride, either mixed immediately before [14] or during application [15], has shown great potential. However, translational approaches have thus far been largely unsuccessful due to poor consumer acceptability (two-product approach is cumbersome) or increased manufacturing costs (dual-chamber/compartment delivery systems).

Fluoride efficacy also strongly depends on the subject's compliance with the provided (post-) treatment instructions. For fluoride toothpastes, the effects of rinsing behavior, brushing frequency and time have been shown to have marked effects on caries incidence [16-1718]. While no such data could be retrieved relating to professional fluoride interventions, such as fluoride varnishes, parallels to toothpastes can be drawn nonetheless. Premature removal of fluoride varnish, motivated by its poor taste and/or cosmetic appearance, will likely limit its efficacy.

As there is still considerable scope for introducing strategies to enhance the anti-caries efficacy of fluoride, the present in vivo study investigated whether consumption of calcium-containing gummy bears, a dietary supplement for children, prior to fluoride varnish application can enhance not only intra-oral fluoride retention but also increase compliance with post-treatment application instructions. The null hypothesis tested was that consuming calcium-containing gummy bears prior to fluoride varnish application does not

promote greater plaque fluoride retention and does not positively affect child compliance with post-fluoride varnish application instructions.

Subjects and Methods

Study Design

The present study followed a multi-center, parallel, randomized, laboratory analyst-blind design. Subjects (7-12 yrs) were randomized to either a gummy bear or no-gummy bear study group. A baseline plaque sample was obtained after a wash-out period. Fluoride varnish was applied to all teeth in both groups, with subjects in the gummy bear group receiving two calcium-containing gummy bears prior to varnish application. Subjects were given two questionnaires to complete (one for the subject, one for the parent – identical content, with the one for the parent serving as validation of their child's answers) to investigate adherence to post-treatment instructions. Three days after varnish application, a second plaque sample was obtained. Plaque samples were analyzed for plaque fluid and total plaque fluoride concentrations. Responses for each question in the questionnaire were summarized using basic frequencies and adherence to written instructions determined.

Ethical Aspects

The present study was conducted in accordance with the Declaration of Helsinki. The study protocol, forms, written instructions and questionnaires were reviewed and approved by the IUPUI Institutional Review Board, #1509237792. The study was conducted at schools and community centers in Indianapolis, IN (US) and at the Oral Health Research Institute (OHRI). Written parent consent and child assent (subjects) were obtained prior to screening. Subjects received oral soft and hard tissue examinations throughout the study.

Subjects

Forty-four subjects, aged 7-12 years, who met the inclusion criteria (good general and oral health; at least 16 teeth; no oral soft tissue lesions, no periodontal disease including severe gingivitis or cavitated carious lesions; understand, willing, able and likely to comply with study instructions) were enrolled. Exclusion criteria were known or suspected allergy or hypersensitivity to fluoride varnishes (e.g. pine nut allergy); taking fluoride supplements or other fluoride products for medical purposes except for fluoride naturally occurring in diet and toothpaste; taking any prescription antibiotics for any medical purpose. A

randomization schedule, provided by the biostatistician, was used to assign subjects to the two study groups at screening.

Study Products

The study products can be found in table 1. The gummy bears had a declared calcium content of 100 mg per gummy in the form of tricalcium phosphate, with a daily serving size of two gummies. Prior to the conduct of the present study, the ability of a variety of calcium-containing gummies to release ionic calcium was evaluated (see Supplemental Material). The chosen brand was selected due its superior ability to release calcium. The serving size of 200 mg calcium is somewhat comparable to the amount of calcium applied in a pre-rinse in the study by Vogel et al. [12] (20 ml of 150 mM calcium equates to 120 mg calcium).

Clinical Procedures

There were three visits for all subjects. At the screening visit, parental consent and child (subject) assent were secured and the inclusion/exclusion criteria reviewed. Subjects received wash-out toothpaste and toothbrush and were instructed not to brush in the morning of the second visit which was approximately 7 d after visit one. At the second visit, a pooled, baseline interproximal and buccal surface plaque sample was collected from all teeth in the maxillary right (1) and mandibular left (3) quadrants (see *Plaque Collection*). Both groups of subjects were informed about the purpose of the fluoride varnish application and received verbal and written post-treatment instructions (table 4). Subjects in the gummy bear group received two calcium-containing gummy bears and were asked to chew and suck the gummy bears until they dissolve rather than to just swallow them. Then, fluoride varnish was applied to all teeth (facial surfaces only). The teeth were not cleaned in any way prior to varnish application. The amount of varnish applied was standardized by the surface area of the teeth, as all facial tooth surfaces were covered by a single coating of varnish, similarly to a routine application would have been performed. Two compliance questionnaires were handed to the subjects, one for the subject to complete the next morning, the other for the parent or legal guardian to complete in the form of an interview with their child the next morning (validation of the subject's answers). Subjects were instructed not to brush their teeth in the morning of the test day (3 d later). At the third visit, the questionnaires were collected and another plaque sample collected, however from the maxillary left (2) and mandibular right (4) quadrants.

Plaque Collection

Immediately before dental plaque collection, the subject was instructed to swallow all remaining saliva and cotton rolls were placed to keep their mouth dry. They were instructed to keep their mouth open. The clinical examiner then collected interproximal and buccal plaque samples. Approximately 1 mg of dental plaque was collected from the interproximal and buccal surfaces of teeth of two of the four quadrants at each visit as described above. Plaque samples were collected using a standardized protocol [19]. Pooled plaque samples were collected using a stainless steel periodontal scaler (S. McCall 17/18, Hu-Friedy, Illinois, USA) and transferred to a pre-weighed plastic strip. The plaque containing strip was then placed into specially made centrifuge tubes constructed by heat sealing 10 µl micropipette tips that were filled with heavy mineral oil (Mineral Oil, Heavy (USP/ FCC) Fisher Chemical, Fisher Scientific, USA), then placed into a sealable sample vial (Eppendorf tube), stored on ice, and frozen (-20°C) upon arrival at OHRI for later analysis.

Plaque Fluid Fluoride Analysis

Plaque fluid fluoride was measured using an inverted fluoride electrode as described by Vogel et al. [20]. Samples were centrifuged at 10,000 rpm for 10 min at 4°C. Plaque fluid was then extracted from each sample vial via a micropipette, dispensed in triplicate with TISAB III onto the surface of a mineral oil-covered inverted fluoride half-cell electrode (Thermo Orion, 9409BN). The tip of a reference electrode was touched to each sample and millivolt (mV) readings were measured by an electrometer (World Precision Instrument, FD223a) and recorded by an American Dental Association Foundation plot program. Prior to sample analysis, a similarly prepared set of fluoride standards were analyzed by the aforementioned method to produce a standard curve to which sample mV readings were compared.

Plaque Total Fluoride Analysis

After plaque fluid recovery, total fluoride concentration were analyzed by inverted fluoride electrode as described by Vogel et al. [20]. Five µl of 1 M $HClO_4$ were added to each sample vial, mixed and then allowed to rest for at least 1 h. Five µl of 1 M NaOH/20% TISAB III were added to each sample vial, mixed and centrifuged at 10,000 rpm for 10 min at 4°C. Samples were then recovered and analyzed by the same method as described above. Prior to sample analysis, a similarly prepared set of fluoride standards were analyzed by this method to produce a standard curve to which sample mV readings were compared.

Power Calculation

Based on the study by Vogel et al. [13], the coefficient of variation was estimated to be 0.9. With a sample size of 21 per group the study had 80% power to detect 2-fold difference between the two groups, assuming two-sided tests conducted at a 5% significance level.

Statistical Methods

Wilcoxon Rank Sum tests were used to compare the two groups for differences in plaque fluid fluoride and total fluoride concentrations prior to treatment with the fluoride varnish (visit 2), after treatment with the fluoride varnish (visit 3), and for the change from pre- to post-treatment. Wilcoxon Signed Rank tests were used to test for significant changes in fluoride pre- to post-treatment within each group.

Pearson chi-square tests (when the "don't know" option was included) and Mantel-Haenszel chi-square tests for ordered categorical responses (when the "don't know" option was excluded) were used to compare the groups for differences in the questionnaire responses. Agreement between the child and parent responses to the questionnaire was evaluated using two-way contingency tables, percent agreement, and kappa statistics.

Results

Demographics

The demographics of the per-protocol population can be found in table 2. Data from eight of the 44 enrolled subjects were not included in the analysis due to missing or incomplete data points: two subjects withdrew from the study after visit 1, four after visit 2, and two subjects did not have a baseline sample. Furthermore, data from two subjects were excluded from the plaque fluid analysis as their plaque samples did not have recoverable fluid in the post-treatment plaque samples.

Plaque Fluoride

The plaque fluoride data can be found in table 3. Plaque fluid fluoride did not change significantly pre- to post-treatment in the gummy bear group (p=0.265) or in the no-gummy bear group (p=0.058). The two groups were not significantly different for plaque fluid fluoride pre-treatment (p=1.000), post-treatment (p=0.904), or change (p=0.904). Plaque total fluoride did not change significantly pre- to post-treatment in the gummy bear group (p=0.073) or in the no-gummy bear group (p=0.190). The two groups were not significantly different for plaque total fluoride pre-treatment (p=1.000), post-treatment (p=0.466), or change (p=0.874).

Compliance

The questionnaire questions, answer ranges and post-treatment instructions can be found in table 4. The questionnaire data and results of the statistical analyses thereof can be found in the supplementary material (tables 3-4). No significant differences were found between groups for the questionnaire responses. The gummy bears were well liked by the subjects (mean response ± standard deviation: 1.2±0.5). The varnish was neither liked nor disliked by the subjects (4.3±1.8), and the consumption of gummy bears did not alter this perception (p=0.30). Compliance with post-treatment instructions was generally good (59-76%) with the exception of the instruction to not eat or drink anything for at least 1 h after varnish application, which more than half of the subjects did not follow (53% non-compliance). The data regarding the subject and parent agreement for questionnaire responses can be found in the supplementary material (table 5). There was generally a good agreement, ranging between 78-97%.

Discussion

The present study failed to demonstrate that consumption of calcium-containing gummy bears can enhance intra-oral fluoride retention and improve compliance with post-application instructions. Therefore, we failed to reject the null hypothesis. There are several possible explanations, which will be discussed briefly. The present study was motivated by research which showed that application of ionic calcium prior to fluoride can greatly enhance intra-oral fluoride retention [12,13]. However, these studies utilized calcium lactate, which presents a source of bioavailable calcium. Such rinses cannot be utilized in child populations due to their unacceptable organoleptic properties (e.g. chalky taste is difficult mask). Furthermore, the present study aimed to utilize a dietary supplement designed for children to not only overcome this issue, but also to offer potentially a route for enhancing efficacy and compliance while translating research findings into practice. Calcium-containing gummy bears, however, contain tricalcium phosphate, which is less soluble than calcium lactate. Although we demonstrated prior to the conduct of the present study (see Supplementary Material) that the chosen brand of gummy bears releases 80% of its calcium content as ionic calcium into human saliva during a two-minute period in vitro, the co-presence of phosphate may have resulted in less bioavailable calcium than what could have been expected. The study of intra-oral calcium retention as a function of different calcium salts and preparations could provide a rationale for the present findings and define future research studies. Likewise, calcium lactate-containing gummy bears may be a suitable alternative for studies on fluoride rinses and toothpastes.

A further explanation for the present findings is that fluoride varnishes may not enhance plaque fluoride concentrations longitudinally, although they are believed to exert their anticaries effects due to enhanced intra-oral fluoride retention. Indeed, it has been shown in several studies that a single fluoride varnish application can lead to elevated surface enamel fluoride concentrations that persist for several weeks [21,22]. This approach was avoided due to obvious ethical issues and the questionable validity of surface enamel fluoride concentration as a biomarker for caries experience [23]. Little information, however, is available on intra-oral biofiolms. A study, concluded during the conduct of the present study, demonstrated that fluoride varnishes do not result in elevations of plaque fluid fluoride concentrations for more than 24 h [19]. The present data would suggest that this also true for total plaque biofilm (solids). However, a study on orthodontic patients [24], which arguably present different plaque retention profiles than the present study population, suggested that a conventional 5% sodium fluoride varnish can enhance plaque fluoride concentrations for at least 3 d. The present data would suggest that while enamel may be an important reservoir for fluoride from fluoride varnishes, biofilms are perhaps not. Elevations in biofilm fluoride concentrations appear to be only transient and perhaps of lesser importance for fluoride varnishes than for daily interventions, such as fluoride toothpastes and rinses, which constantly replenish lost fluoride. Again, further clarifying research is warranted and to define the still ill understood mode of action of fluoride varnishes.

The present study also failed to demonstrate that consumption of calcium-containing gummy bears can enhance adherence to post-treatment instructions (see supplementary data tables 3-4). The questionnaires were designed to obtain information about child compliance with post-fluoride varnish treatment instructions and to what extent the gummy and varnish were liked by the children. The parent served as validator of the child's answers as the parent had to complete a second, content-identical questionnaire in the form of an interview with the child. The response rate was considered very good with 34 out of 36 subjects (94%) completing the study also returning the questionnaires, although not all questions were answered by all subjects and not all parents completed their questionnaire. Nonetheless, the questionnaire data can be considered representative. Whilst compliance with post-treatment instructions was generally good, more than half of the children either ate or drink within 1 h after fluoride varnish application, despite being instructed otherwise. The consumption of gummy bears, although liked by the children, did not affect this either. A possible explanation may lie in the finding that the gummy bears did not affect the perception of the varnish, as subjects in both study groups were indifferent about the varnish. Perhaps a stronger cue than a gummy bear would be needed to alter this emotion. Furthermore, it is currently unknown for how long fluoride varnishes need to remain on the teeth

undisturbed to exert their full anti-caries potential. Present recommendations do not only vary between manufacturers [25], they are also being provided without any scientific evidence to support them. In other words, these are merely 'common sense' recommendations and highlight that further research is necessary to provide more evidence-based post-treatment instructions. However, only five subjects reported they brushed in the evening of the same day the varnish was applied and none reported to have removed it immediately after application. This would imply that the varnish remained on the teeth for an adequate amount of time to provide its benefits [19]. Lastly, the presently observed good agreement between child and parent responses can potentially be used as a justification to omit parent questionnaires in future studies. This would reduce the burden on participants and can in turn increase their motivation to participate in research.

Future studies may want to consider utilizing a different calcium source, such as calcium-containing toothpastes or gels, use a professional fluoride rinse (2% NaF) instead of fluoride varnish, and/or utilize a different biomarker (e.g. salivary fluoride) altogether. While the present study was not successful for above-mentioned reasons, there is still a need to improve the anticaries properties of fluoride as fluoride on its own has been shown to be of limited efficacy recently [4-7].

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Declaration of Interest

Declarations of interest: none

Legends

Table 1. Study products

Table 2. Study demographics

Table 3. Plaque fluoride data

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Table 4. Questionnaire codes, questions, answer ranges and post-treatment instructions

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Figure Caption

Table 1. Study products

Treatment	Product Name	Manufacturer
Fluoride Varnish	CavityShield 5% Sodium Fluoride	3M ESPE
Calcium-containing Gummy Bear	Gummy Cuties Calcium with Vitamin D	Natural Dynamix
Toothbrush	Oral-B P40 Medium	Procter and Gamble
Fluoride-free Wash-out Toothpaste	Natural Fluoride Free Toothpaste for Children, Silly Strawberry	Tom's of Maine

Table 2. Study demographics

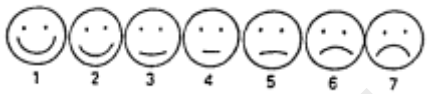
		All	Gummy Bear	No Gummy Bear
	n	36	19	17
Sex	female	18 (50%)	11 (58%)	7 (41%)
	male	18 (50%)	8 (42%)	10 (59%)
Age [y]	mean (sd*) range	8.9 (1.1) 7-12	8.8 (1.2) 7-11	9.1 (1.1) 8-12

*standard deviation

Table 3. Plaque fluoride data

		All		Gummy Bear		No Gummy Bear	
Plaque fluoride	time	n	mean (sd)	n	mean (sd)	n	mean (sd)
fluid [µmol/l]	pre	34	8.5 (4.1)	18	8.8 (5.7)	16	8.1 (4.4)
	post	34	12.9 (15.7)	18	10.0 (6.3)	16	16.1 (20.0)
	change	34	4.4 (15.9)	18	1.2 (5.3)	16	8.0 (19.1)
total [µmol/g]	pre	36	0.79 (0.99)	19	0.89 (1.10)	17	0.68 (0.77)
	post	36	1.68 (4.05)	19	1.37 (1.77)	17	2.01 (5.00)
	change	36	0.89 (3.84)	19	0.49 (1.21)	17	1.34 (4.81)

Table 4. Questionnaire codes, questions, answer ranges and post-treatment instructions

Code	Question	Answer range	Illustration/Answers
Q1[1]	Did you like the gummy bears?	1 to 7	
Q2	Did you like the special tooth vitamin paint?	1 to 7	
Q3	After we painted your teeth: a) When did you eat or drink again?	1 to 5	1 - within 30 min 2 - within 1h **3 - within 4h[2]** **4 - at least 4h later** 5 - I do not remember
Q4	b) When did you eat hard food such as candy or crispy food again?	1 to 5	1 - within 30 min 2 - within 1h 3 - within 4h **4 - at least 4h later** 5 - I do not remember
Q5	c) When did you drink hot drinks such as hot chocolate or tea again?	1 to 5	1 - within 30 min 2 - within 1h 3 - within 4h **4 - at least 4h later** 5 - I do not remember
Q6	d) When did you brush your teeth again?	1 to 5	1 - immediately after we applied it 2 - in the evening the same day we applied it **3 - the next morning** 4 - the next evening 5 - I do not remember

[1]This question was omitted from the questionnaire for subjects in the no-gummy bear group.

[2]Answers which display adherence to post-treatment instructions are highlighted in bold.