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Original Article

Relationship between fluoride exposure and count of *Streptococcus mutans* in supragingival biofilm of mexican scholar children

B. Holguin-Loya [a], U. Soto-Barreras [a*], R. Martinez–Martinez [b], G. Martinez-Mata [a], L. Sanin [c], M. Nevarez-Rascon [a], R. Dominguez-Perez [d]

[a] *Facultad de Odontología, Universidad Autónoma de Chihuahua, Chihuahua, México*
[b] *Maestría en Ciencias Odontológicas, Universidad Autónoma de San Luis Potosí, SLP, México*
[c] *Facultad de Medicina, Universidad Autónoma de Chihuahua, Chihuahua, México*
[d] *Facultad de Medicina, Universidad Autónoma de Querétaro, Santiago de Querétaro, México*

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Background/purpose: The use of fluoride is known to reduce the risk of dental caries. There is limited information on the relationship between *Streptococcus mutans* (*S. mutans*) and fluoride exposure. This study investigated the association between the count of *S. mutans* on supragingival biofilm and fluoride exposure of scholar children.
Materials and methods: In this cross-sectional study, 56 children from 9 to 11 years of age were selected. Fluoride concentration in drinking water, urine and saliva of each participant were assessed. The count of *S. mutans* was estimated by calculating the DNA copy number through a quantitative real time polymerase chain reaction (qPCR) assay. Also, sociodemographic data, oral and general health information and variables related to caries risk were evaluated. A stepwise multiple linear regression was performed in all caries related predictor variables with the count of *S. mutans* as the dependent variable.
Results: The multiple linear regression analysis showed that the concentration of fluoride in saliva ($\beta = -3.029$, $p < 0.001$) and urine ($\beta = -2.057$, $p = 0.017$), time of last visit to the dentist ($\beta = 1.968$, $p = 0.001$), plaque index ($\beta = 1.637$, $p = 0.006$) and number of surfaces with codes 3–6 ($D_{3\text{-}6}MFS$) of ICDAS II criteria ($\beta = 0.283$, $p = 0.076$) were significantly associated with the count of *S. mutans* (Adjusted R square = 0.427, $p < 0.001$).

* Corresponding author. Circuito Universitario Campus I, Facultad de Odontología, Av. Universidad s/n, 31000, Chihuahua, México.
E-mail address: usoto@uach.mx (U. Soto-Barreras).

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Conclusion: Fluoride levels in urine and saliva were negatively associated with the count of *S. mutans* in supragingival biofilm. Plaque index, D_{3-6}MFS and time of last visit to the dentist showed a positive association.

Introduction

Dental caries is the most prevalent disease on the world and is considered a multifactorial disease.[1,2] The progression of caries results from a microbiome dysbiosis with the involvement of multiple cariogenic species, including: *Streptococcus mutans (S. mutans*), lactobacilli, *Scardovia wiggsiae*, and several *Actinomyces* species.[3] Although multiple biological factors contribute to the caries establishment, microbiome dysbiosis and cariogenic diet plays a critical role on the caries development.[4]

On the other hand, several protective biological or therapeutic factors for dental caries have been proposed, including: water fluoridation, fluoride dental varnish, calcium and phosphate dental products, chlorhexidine mouthwashes, xylitol gum and lifestyle habits.[2,5] The success of fluoride in the control of dental caries is well documented.[6] However, contemporary evidence that has evaluated the effectiveness of water fluoridation for the prevention of dental caries is still insufficient.[7] Some studies have shown an inverse relationship between fluoride exposure and caries experience.[8,9] Nonetheless, high concentrations of fluoride intake have been associated to adverse health effects.[10] It is important to evaluate the success of early exposure to fluoride on dental caries for the development of preventive public health programs.[11,12]

Potential advantages of fluoride exposure compared to other anti-caries agents have been described, such as reduction of the enamel solubility, inhibition of the production of acids in the biofilm, reduction of demineralization, promotion of remineralization and inhibition of cellular enzymes of oral bacteria.[13] However, the knowledge about the association between *S. mutans* and fluoride exposure is relatively uncertain.[14] Thus, the aim of this study was to evaluate the association of fluoride exposure and the count of *S. mutans* on supragingival biofilm from scholar children.

Materials and methods

Study population

In this cross-sectional study, nine public elementary schools were randomly selected from a total of 73 schools in Chihuahua, Mexico. A total of 161 children aged 9—11 years were consecutively examined. This study was approved by the clinical and ethical research committee of the Faculty of Dentistry of the Autonomous University of Chihuahua (reference number: FOIP-004-17). Written and voluntary informed consent was obtained from the parents or guardians and the assent of each of the eligible participating children. The risks and benefits of the study were explained before the clinical examination, according to the Helsinki Declaration of Ethical Principles 2008. The selection criteria included children with fully erupted permanent first molars with International Caries Detection & Assessment System (ICDAS II) detection codes $\leq$ 03 (enamel carious lesions less to 0.5 mm). Children with special care needs, orthodontics or orthopedics appliances, use of hyposalivants, application of topical fluoride on the last six months or use of antibiotics on the last three months were excluded from the study. Only 56 children met the selection criteria.

Medical record and oral examination

The parents or caregivers fulfilled a medical record questionnaire with the following data from the children: general and demographic information, pathological history, use of medications, nutrition, socioeconomic condition and caries risk factors. The data concerning of nutritional status were obtained with the body mass index according to sex and age of children, based on the Center for Disease Control growth charts. The socioeconomic status was estimated using a region-specific and household survey of the Mexican Association of Market Research and Public Opinion Agencies. The time of last visit to the dentist of children was registered as follows: six months or less ($\leq$6 months), more than 6 months ($>$6 months) or the child has never had a consultation with the dentist (never). The caries risk was determined by the Caries Risk Assessment tool for Infants, Children, and Adolescents.[15]

A single calibrated examiner carried out the dental examinations assisted by a collaborator as a recorder. The inter-examiner and intra-examiner reliability in regard to diagnosis of dental caries and dental fluorosis were analyzed by intraclass correlation coefficient, obtaining scores above 0.85. Dental plaque was measured with Silness and Löe index, using a sterile explorer from vestibular and lingual surfaces. Dental caries was measured using ICDAS II.[16] Activity of caries lesions were assessed according to the appearance of the lesion, its location, and tactile sensation upon careful probing. Dental fluorosis was assessed on vestibular, occlusal and lingual surfaces in accordance with the Thylstrup-Fejerskov index. Also, a volume of 2.5 ml of non-stimulated whole saliva was collected at least 1.5 h after the last food intake in 15 ml falcon tubes at two different days. The salivary pH was determined with a pH meter (Orion 710A, Thermo Orion, Beverly, MA, USA).

Fluoride assessment

Fluoride exposure was based on the fluoride concentration in drinking water, urine and saliva of each child evaluated. Drinking water and urine samples were collected in acid-washed 50 ml ultra-cleaned polyethylene bottles, following the sampling instructions of the Environmental Protection Agency[17] and the National Institute for Occupational Safety and Health standard method[18] respectively. All samples were kept at −20 °C until testing. Samples were mixed on a magnetic stirrer at room temperature with total ionic strength adjustment buffer II (TISAB, Sigma–Aldrich, Steinheim, Germany) at the same volume ratio of 10 ml. The whole saliva samples were centrifuged at 6000 rpm for 10 min. Then, an aliquot of 1 ml of the saliva sample was mixed at room temperature with 1 ml of the TISAB II solution. The analysis was carried out by triplicate with the ion selective electrode (Orion 9609BNWP, Thermo Scientific, Waltham, MA USA) standardized in the range of 0.01–100 μg/ml. Standardization was controlled by running the standard solutions with every 10 specimens to maintain quality assurance.

Dental plaque collection and quantitative polymerase chain reaction assay

Supragingival biofilm samples were obtained from the permanent first molars. The samples were collected using a sterile Gracey curette (Hu-Friedy. Chicago, IL, USA) at five locations around each tooth (mesial, distal, vestibular, lingual and oclusal surfaces), and placed in a micro-centrifuge tube with 1 mL phosphate-buffered saline. All samples were packed and transported in coolers with ice and were stored at −20 °C until molecular analysis. All samples were processed aseptically to prevent contamination from the environment, the DNA extraction was performed in accordance with the protocol for Gram-positive bacteria with a commercial kit (Wizard Genomic DNA Isolation Kit, Promega Corporation, Madison, WI, USA).

To quantify the number of copies of *S. mutans*, a quantitative real time polymerase chain reaction assay (qPCR) was carried out. A mixture of 20 μL containing 50 ng of genomic DNA, 1 × TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.5 mL of Custom TaqMan Gene Expression Assay for *S. mutans* (Applied Biosystems) was placed in each well of a 48-well plate. The amplification and detection were performed using the StepOneTM System (Applied Biosystems) with the cycling profiles indicated in the manufacturer's instructions. Standard curves were prepared using plasmidic DNA cloned into a vector in *Escherichia coli* competent cells by a 10-fold dilution series. The qPCR results were expressed as the number of copies of *S. mutans* DNA per milligram of dental plaque.

Statistical analysis

Concentrations in qPCR are expressed in the logarithmic scale (log-10) to improve normality of distributions. Descriptive statistics for quantitative variables are expressed as mean, median, standard deviation and range. Qualitative data are expressed as frequency and percentage. A stepwise multiple linear regression was performed in all caries related predictor variables with the log-10 count of *S. mutans* as the dependent variable. A criterion of 0.1 was used for inclusion and permanence of independent variables. Dummy coding was used for the analysis and interpretation of categorical variables. Each level was compared to the reference level. The normality, heteroscedasticity and independence of the residuals were verified, as well as the collinearity by means of the variance inflation factor (VIF). All data was analyzed using SAS software version 9.0.

Results

In this study, 56 children from ages 9–11 years were included. Table 1 shows descriptive statistic of quantitative variables. It was reported a mean of 4.2 meals and 2.2 snacks per day. The mean fluoride concentration in urine and saliva rounded at average normal levels. However, a wide variation in their distribution was observed. Furthermore, high values of fluoride in drinking water were found. It was found a considerable proportion of obese children (19.6%) (Table 2). But, most of children were at normal BMI range. Caregivers reported that 69.6% of the children had never gone to the dentist, and no children were found in high socioeconomic level status. Furthermore, low risk was the most predominant level of Caries Risk Assessment (75.0%) followed by the high risk (17.9%).

Table 3 describes the mean surface and the activity of carious lesions according to ICDAS II. It was observed more activity of dental caries when carious lesions were established in dentine. The multiple linear regression analysis (Table 4) showed that five variables explained significantly the variance of the DNA copy number of *S. mutans* on supragingival biofilm ($P < 0.0001$, adjusted R square = 0.4276). The final model included the following variables: time of last visit to the dentist (never), plaque index (grade 2), urinary fluoride, fluoride in saliva and number of surfaces with codes $D_{3\text{-}6}$MFS of ICDAS II. Fluoride variables demonstrated a negative association with *S. mutans*. In return, the other variables presented a positive contribution.

Discussion

Dental caries is considered one of the most frequent chronic infectious diseases of childhood and the most common noncommunicable disease worldwide.[19] *S. mutans* represents a primary etiologic agent of dental caries and its presence is common on the so-called dental plaque, which constitutes a multi-species biofilm formed on hard surfaces of the tooth.[20] In the present study, we assessed whether fluoride exposure (fluoride levels in saliva, urine and drinking water) and other predictor variables had an effect on the amounts of *S. mutans* in supragingival biofilm. Our findings indicated that the time of last visit to the dentist, plaque index, number of surfaces with codes $D_{3\text{-}6}$MFS of ICDAS II and the concentration of fluoride in saliva and urine variables were significantly associated with the count of *S. mutans*. There are numerous factors that might affect the *S. mutans* count in dental biofilms, including, increased

Table 1 Distribution of quantitative predictor variables.

Variable	Mean (SD)	Median	Min-Max	Confidence interval
Age (years)	9.34 (0.55)	9	9—11	9.19—9.48
Nutrition				
Weight for age percentile (kg)	63.57 (31.99)	75	5—99	55.18—71.96
Meals per day	4.23 (1.03)	4	2—6	3.96—4.5
Sugary drinks and snacks[a]	2.23 (0.85)	2	0—5	2.01—2.46
Fluoride exposure				
Water fluoride (mg/L)	1.36 (1.02)	1.45	0.03—3.11	1.09—1.63
Urinary fluoride (mg/L)	0.58 (0.32)	0.45	0.06—1.42	0.50—0.67
Salivary fluoride (mg/L)	0.25 (0.32)	0.11	0.01—1.43	0.16—0.33
Oral health				
Diary toothbrushing frequency	1.73 (0.86)	2	0—3	1.51—1.96
Salivary pH	7.23 (0.51)	7.28	5.57—8.08	7.09—7.36

[a] Average daily consumption frequency reported by parents.

caries rates,[21] oral hygiene, salivary conditions,[22] acidic environments[23] and cell to cell interactions.[22] In this study some confounding variables were controlled by restriction in the study design or by statistical analysis. For instance, biofilm samples were obtained only from children with permanent first molars without carious lesions greater than 0.5 mm in enamel. Other variables, such as, sex, sugary products intake, socioeconomic status, salivary pH and oral health were adjusted in the model.

Table 2 Frequency and percentage of categorical predictor variables.

Variable	Frequency	Percentage
Sex		
Female[a]	27	48.2
Male	29	51.8
BMI for age percentiles		
Underweight	1	1.8
Normal weight[a]	36	64.3
Overweight	8	14.3
Obese	11	19.6
Socioeconomic status		
Low	17	30.4
Middle[a]	39	69.6
High	0	0
Time of last visit to the dentist		
Never	39	69.6
>6 months	6	10.8
≤6 months[a]	11	19.6
Caries Risk Assessment		
Low risk[a]	42	75.0
Moderate risk	4	7.1
High risk	10	17.9
Silness & Löe plaque index		
Grade 0[a]	14	25.0
Grade 1	17	30.4
Grade 2	22	39.2
Grade 3	3	5.4

[a] Reference level.

A variety of mechanisms have been implicated by which saliva plays an important role in the activity and composition of the oral microbiota, such as, the flow rate, buffer ability and antimicrobial agents.[24,25] However, there is a lack of clinical studies regarding the relationship between fluoride levels in saliva and oral bacteria. Some studies suggest that fluoride affects the physiology of microbial cells in several ways.[26,27] Furthermore, previous *in vitro* reports had shown inhibitory effects of fluoride, such as, acid production, acid tolerance and glucosyltransferases production on *S. mutans* biofilm models.[28,29] In addition, an *in vitro* study indicated that the inhibitory effects of fluoride on *S. mutans*, could suggest its potential use as an effective measure to control dental biofilms.[28] Nevertheless, so far there are no clinical studies assessing the potential impact of fluoride exposure on the amount of *S. mutans* in dental plaque and its possible interaction with biofilm formation. Therefore, it is crucial to recognize the possible effects that fluoride could have at the level of the microbial biofilm.

The recommended community water fluoridation is estimated to range from 0.7 to 1.0 ppm with a maximum contaminant level of 1.5 mg/L.[30] Besides, fluoride contents in saliva and urine in children are positively correlated with the fluoride levels in drinking water.[31,32] In this research, it was found that 39% of the drinking water samples presented fluoride levels above 1.5 mg/L. In addition to drinking water, other sources of fluoride have

Table 3 Decayed, missing and filled surfaces and caries activity by type of dentition.

	Mean	SD	Median	Min-Max	% of active lesions
$d_{1\text{-}2}$mfs	0.45	1.01	0	0—6	60.71
$d_3$mfs	1.20	1.90	0	0—8	51.67
$d_{4\text{-}6}$mfs	1.05	2.38	0	0—11	92.96
$D_{1\text{-}2}$MFS	0.48	0.89	0	0—4	55.56
D_3MFS	1.04	1.54	0	0—6	58.62
$D_{4\text{-}6}$MFS	0.16	0.56	0	0—3	66.67

$d_{1\text{-}2}$mfs, $d_3$mfs, $d_{4\text{-}6}$mfs (deciduous dentition); $D_{1\text{-}2}$MFS, D_3MFS, $D_{4\text{-}6}$MFS (permanent dentition).

Table 4 Stepwise multiple linear regression of the log-10 concentration of *S. mutans* on categorical and quantitative predictor variables.

Variable	Coefficient	Standard error	Standardized coefficient	*p* value	VIF
Constant	7.8849	0.7334		<0.001	0
Urinary fluoride (mg/L)	-3.0296	0.7381	-0.4485	0.0002	1.0430
Plaque index (grade 2)	1.6376	0.5688	0.3212	0.0061	1.0869
Salivary fluoride (mg/L)	-2.0570	0.8337	-0.2677	0.0175	1.0287
Time of last visit to the dentist (never)	1.9686	0.5782	0.3698	0.0016	1.0629
$D_{3\text{-}6}$MFS[a]	0.2830	0.1563	0.1976	0.0768	1.0400

Model summary: Adjusted R square = 0.4276, P <0.0001,
[a] D3-6MFS: Decayed (cavitated enamel or dentin), missing and filled surfaces on permanent dentition; VIF: variance inflation factor.

been described such as, fluoridated toothpastes, processed beverages and foods, salt, and other dental products.[33,34] Although several factors may influence the metabolism and excretion of fluoride,[35] urinary fluoride has been described as one of the most useful biomarkers for exposure to fluoride, since it is the most important metabolic route for its elimination.[36] Accordingly, the inverse relationship observed between the levels of fluoride in urine and the count of *S. mutans* in the supragingival biofilm, suggests that exposure to fluoride could play an important role against the microorganism.

There is conflicting evidence regarding the relationship of counts of *S. mutans* and dental caries experience.[21,37] Moreover, other cariogenic bacteria can modulate the pathogenic potential at different stages of caries development.[38] Some of the limitations of this research include not evaluating other bacterial species such as *S. sobrinus* and lactobacilli, the sample size and not including children from other geographic areas where fluoride levels in drinking water are significantly lower. Also, the cross-sectional design of the study limited the measurement of variables at one point in time, which could lead to chronological bias. Hence, these results should be interpreted with discretion. Despite these limitations, a rigorous control was carried out in the measurement and analysis of caries related variables. In addition, a reliable and efficient quantification of *S. mutans* was performed using qPCR. Even though the role of fluoride in the prevention of dental caries has been widely studied, there is little evidence in the literature evaluating the clinical effects of fluoride exposure on *S. mutans* in dental biofilm.

In conclusion, our results suggest that fluoride levels in saliva and urine are negatively associated with the count of *S. mutans* in supragingival biofilm. On the other hand, plaque index (grade 2), time of last visit to the dentist (never) and number of dental surfaces at ICDAS II cut-off points $D_{3\text{-}6}$MFS had a positively influence in the count of *S. mutans*. Further studies are necessary to analyze and confirm the clinical effects of different sources of fluoride exposure on the levels of *S. mutans* in the biofilm.

Declaration of competing interest

The authors declare that they have no conflict of interest due to any relationship or financial support received.

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