

IN VITRO STUDY OF DOSE-RESPONSE RELATIONSHIP OF FLUORIDE WITH DENTAL ENAMEL*

ESTUDO IN VITRO DA RELAÇÃO DOSE-RESPOSTA DO
FLUORETO COM O ÉSMALTE DENTAL

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SUMMARY

In vitro models for evaluation of fluoride (F) reactivity should present dose-response effect. Thus, the aim of this study was to evaluate the fluoride dose-response present in aqueous solution with bovine dental enamel. A hundred and twenty bovine enamel slabs (5 × 5 × 2 mm), 60 sound and 60 with caries-like lesions, were subjected during 10 min to distilled and deionized water (negative control) and aqueous solutions containing 50, 100, 200 or 400 µg F/mL. Each experimental group received 12 sound and 12 carious slabs. Two consecutive layers of dental enamel were removed from all slabs, by acid etching, and fluoride extracted was determined with specific electrode. The results of fluoride incorporated were expressed in µg per g of removed enamel, considering the total amount of the two layers. Fluoride uptake by sound enamel showed a linear dose-response ($p = 0.0001$), while that by carious slabs showed a polynomial one ($p < 0.0001$). The results suggest that the *in vitro* model of reactivity used in the present study is appropriate to evaluate dose-response between fluoride present in aqueous solution and that incorporated by either sound or carious bovine dental enamel.

UNITERMS: fluoride; dental enamel; reactivity; dose-response; *in vitro* study.

RESUMO

Modelos *in vitro* para avaliação da reatividade do fluoreto (F) devem apresentar resposta dose-efeito. Dessa forma, o objetivo desse estudo foi avaliar a relação dose-resposta do fluoreto presente em solução aquosa com o esmalte dental bovino. Cento e vinte blocos de esmalte bovino (5 × 5 × 2 mm), 60 hígidos e 60 com lesão artificial de cárie, foram submetidos durante 10 minutos à água destilada e deionizada (controle negativo) e soluções aquosas contendo 50, 100, 200 ou 400 µg F/mL. Cada grupo experimental recebeu 12 blocos hígidos e 12 blocos com lesão artificial de cárie. Duas camadas consecutivas de esmalte dental foram removidas de todos os blocos dentais por meio de ataque ácido e o fluoreto extraído foi determinado com eletrodo específico. Os resultados de fluoreto incorporado foram expressos em µg por g de esmalte removido, considerando a quantidade total das duas camadas. A incorporação de fluoreto pelo esmalte hígido mostrou uma relação dose-resposta linear ($p = 0,0001$), enquanto que os blocos com lesão de cárie mostraram relação polinomial quadrática ($p < 0,0001$). Os resultados sugerem que o modelo *in vitro* de reatividade empregado no presente estudo é apropriado para avaliar a relação dose-resposta entre o fluoreto em solução aquosa e aquele incorporado pelo esmalte dental bovino hígido ou com lesão artificial de cárie.

UNITERMOS: fluoreto; esmalte dental; reatividade; dose-resposta; estudo *in vitro*.

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INTRODUCTION

The role of fluoride (F) in prevention and control of dental caries has been well described, interfering with de and remineralization process (Fejerskov,⁹ 2004). Among the systems of fluoride delivery (Clarkson et al.,⁶ 2000), a great importance has been attributed to topical fluoridated agents, such as mouthrinses (Ogaard et al.,¹⁸ 1994). Mouthrinses present efficacy in the treatment of patients with high caries risk (Petersson et al.,²⁰ 2002) and can maintain frequent fluoride concentrations in the mouth (Ripa,²³ 1992).

The maintenance of bioavailable fluoride in the oral cavity by mouthrinses carries out a fundamental role in the dynamic of caries process (Cury,⁷ 2001), since fluoride concentration in these oral hygiene products is related to the fluoride bioavailability in saliva (Duckworth,⁸ 1987). Thus, a decrease in bioavailable fluoride could impair its protective effect, for example, due to the interference with other components present in a formulation of the fluoridated products (Barkvoll et al.,³ 1988) and (Franco et al.,¹¹ 1994) or due to problems in the formulation of these products (Tabchoury et al.,²⁴ 2005).

In this context, *in vitro* models of reactivity may be useful to evaluate the anti-caries efficacy of fluoride and verify its uptake by dental enamel under controlled conditions (White,²⁶ 1995). These models have been widely used to verify if fluoride present in fluoridated products is bioavailable and able to react with dental surface (White,²⁶ 1995).

Bovine dental enamel is an adequate dental substrate for caries models, because it is easier to obtain, presents less variable chemical composition and higher and more planified surface than human dental enamel (Mellberg,¹⁵ 1992). In addition, the absence of previous cariogenic challenges decreases the variability of bovine dental enamel to treatments (Mellberg,¹⁵ 1992). However, to evaluate fluoride bioavailability, these models with bovine enamel should confirm dose-response effect.

Thus, a validation of *in vitro* model of reactivity is necessary to evaluate dose-response relationship between fluoride present in aqueous solution with sound and carious bovine enamel.

MATERIALS AND METHODS

Experimental Design

One hundred and twenty slabs were obtained from bovine incisors and caries-like lesions were

induced (Paes Leme et al.,¹⁹ 2003) in half of them. The sound and carious enamel slabs were randomly submitted to the following treatment groups (n = 12): distilled and deionized water (negative control), aqueous solutions containing 50, 100, 200 or 400 $\mu\text{g F/mL}$, prepared with NaF (Merck, Darmstadt, Germany). The enamel slabs reacted with the treatment solutions during 10 minutes. All treatment solutions had their fluoride concentration determined; also, the pH of solutions was determined before and after the reactivity test. From all slabs, two consecutive layers of dental enamel were removed by acid etching and the fluoride content in the extracts was determined with F specific electrode. The results of fluoride were expressed in μg per g of removed enamel, considering the total amount of the two layers.

Preparation of the enamel slabs

One hundred and twenty slabs (5 × 5 × 2 mm) were obtained (Fushida et al.,¹³ 1999) from sound bovine incisor teeth that had been stored in 2% formaldehyde (Chemco, Campinas, Brazil) solution (pH 7.0) at room temperature for at least 30 days (White,²⁵ 1987). The dentin was flattened and the enamel surface was polished (Fushida et al.,¹³ 1999). During these procedures, the dental slabs were moistened with distilled and deionized water to avoid cracks in enamel. The surfaces of all the slabs were protected with a layer of acid-resistant varnish, except the vestibular surface. Then, the slabs were measured with a digital paquimeter (Mitutoyo, Suzano, Brazil) to determine the exposed area (mm²). Artificial carious lesions were produced in 60 slabs by immersion in 0.05 M sodium acetate buffer at 37°C, pH 5.0, 50% saturated in relation to bovine dental enamel (Paes Leme et al.,¹⁹ 2003), in a proportion of 2 mL solution/mm² of exposed enamel for 16 h. To prepare this solution, bovine enamel powder (0.074-0.105 mm) was kept in 0.05 M sodium acetate buffer, pH 5.0, (0.25 g/L) for 96 h at 37°C under agitation. All dental slabs were stored in a refrigerated environment (4°C).

Preparation of the fluoridated solutions

Fluoride concentration in the treatments solutions was determined after buffering 1:1 with TISAB II (1.0 M acetate buffer pH 5.0, containing 1.0 M NaCl and 0.4% CDTA). The analyses were made in triplicate using a specific electrode (Orion 96-06) and an ion analyzer (EA 940, Orion, Boston, USA), previously calibrated.

The pH of the treatment solutions was determined before and after the reactivity test, using a glass specific electrode and an ion analyzer (Procyon, São Paulo, Brazil) calibrated with standard buffers pH 4.0 and 7.0 (Orion, Beverly, USA).

Reactivity of fluoride with dental enamel

The slabs were immersed in the treatment solutions (proportion of 2 mL solution/mm² of enamel surface) at room temperature and under agitation (100 rpm). After 10 min the slabs were washed for 1 min with distilled and deionized water and stored at 4°C.

Two consecutive layers of enamel were removed from all slabs by acid immersion in 0.25 mL of 0.5 M HCl (Merck, Darmstadt, Germany) for two periods of 15 seconds under agitation, followed by buffering with the same volume of TISAB II pH 5.0 modified with an addition of 20 g of NaOH/L (Maia et al.,¹⁴ 2003) (Merck, Darmstadt, Germany). The fluoride concentration in the extracts was determined (Tabchoury et al.,²⁴ 2005) using an ion analyzer (EA 940, Orion, Boston, USA) and an ion specific electrode (Orion 96-09), previously calibrated with standards of 0.02 to 1.28 µg F/mL (Orion, Beverly, USA). The amount of removed enamel (g) during each acid attack in each layer was determined by the quantification of the inorganic phosphorus (Fiske et al.,¹⁰ 1925), considering the quantity of Pi in enamel as 17.4%. Then, the amount of F in the two layers was combined and the results were expressed in µg of F per g of removed enamel.

STATISTICAL ANALYSIS

Fluoride concentration and the pH of the solutions were descriptively analyzed. The results of fluoride in enamel and thickness of layers were submitted to statistical analyses. An exploratory analysis of data was carried out and those variables that did not present homogeneous variances and normal distribution were transformed (Box,² 1978). The results of thickness of layers were submitted to ANOVA, and the comparisons were made within sound or carious slabs (data not shown). The results of total fluoride in sound and carious dental enamel were transformed, respectively, in log₁₀ and square root and submitted to ANOVA, followed by linear regression for sound slabs and quadratic polynomial regression for carious slabs. For these analyses, SAS software (SAS program, version 8.2, SAS Institute

Incorporation, Cary: NC, 2001) was used and the significance limit was established at 5%.

RESULTS

Table 1 shows F concentration and pH of treatment solutions. Fluoride concentration was close to the expected values (from 49.2 to 395.7 µg F/mL) and the mean variance coefficient was -1.6%, -1.1%, -1.6% and 1.1%, respectively for 50, 100, 200 and 400 µg F/mL solutions. The initial pH ranged from 6.10 to 6.34, and the final pH, considering sound and carious enamel, ranged from 5.96 to 6.25.

TABLE 1 – Means (± sd) of fluoride concentration (µg/mL) in the solutions and their pH before (initial) and after (final) the reaction with dental slabs.

Treatment Solutions	F (µg/mL)	pH		
		Initial	Final	
			Sound Enamel	Carious Enamel
Negative control	0.036 ± 0.005	6.10	6.08	5.99
50 ppm F	49.2 ± 0.1	5.95	6.13	6.08
100 ppm F	98.9 ± 0.2	6.13	6.00	6.24
200 ppm F	196.7 ± 0.4	6.24	6.14	6.23
400 ppm F	395.7 ± 0.8	6.34	6.25	5.96

Table 2 shows the total F in sound and carious dental enamel. The data of thickness of enamel layers were not statistically different among the treatment groups, either for sound or carious slabs, showing a mean of 18.2 and 14.7 µm for all groups for sound and carious slabs, respectively.

TABLE 2 – Means (± sd; n = 12) of total fluoride incorporated (µg/g) in sound and carious enamel according to the treatments.

Treatments	Total F (µg/g)	
	Sound ¹	Carious ²
Negative control	153.1 ± 81.6	306.7 ± 115.4
50 ppm F	139.2 ± 105.7	914.7 ± 109.5
100 ppm F	194.7 ± 113.7	1023.1 ± 240.6
200 ppm F	190.7 ± 121.7	1338.5 ± 314.6
400 ppm F	288.3 ± 133.6	1543.0 ± 516.0

¹Linear regression; R² = 0.8779; p = 0.0001.

²Quadratic polynomial regression; R² = 0.9097; p < 0.0001.

There was an increase in F uptake by dental enamel, either in sound or carious slabs, in response to increasing F concentration in treatment solutions. Figure 1 shows that there was a linear relationship between total F in sound enamel

and F concentration in treatment solutions. This linear regression shows $R^2 = 0.8779$ and is statistically significant ($p = 0.0001$). Figure 2 shows dose-response relationship between total F present in carious enamel and F in treatment solutions. This relationship is quadratic polynomial, with $R^2 = 0.9097$ and is also statistically significant ($p < 0.0001$).

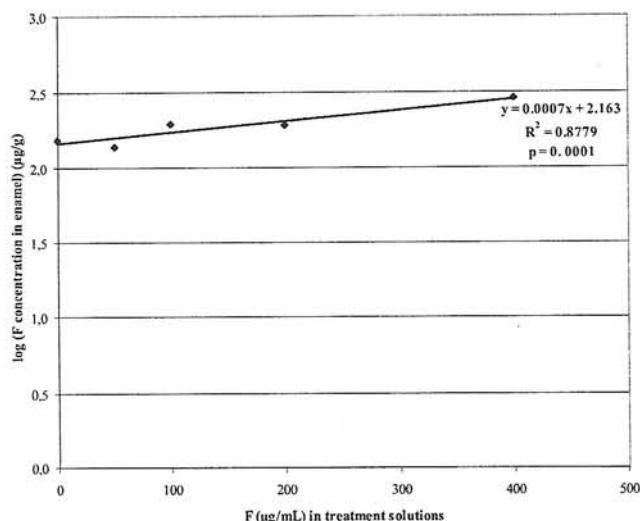


Figure 1 – Dispersion graphic and regression equation adjusted to data of total F concentration ($\mu\text{g/g}$) in sound enamel related to F concentration in treatment solutions.

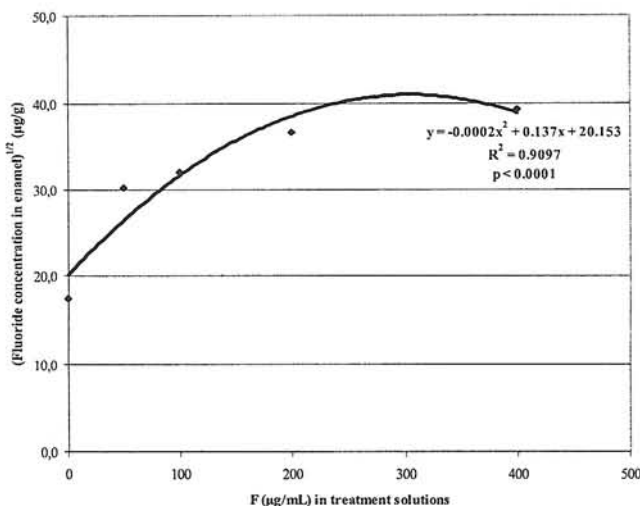


Figure 2 – Dispersion graphic and regression equation adjusted to data of total F concentration ($\mu\text{g/g}$) in carious enamel related to F concentration in treatment solutions.

DISCUSSION

According to (Proskin,²¹ 1992), the validity of models for studying the caries-preventive effect of fluoride is related to their capacity of response to

the experimental conditions imposed during the study. In this way, the *in vitro* model of reactivity was used in this study to verify the behavior of dental enamel exposed to solutions with increasing fluoride concentrations.

In an attempt of validating this *in vitro* fluoride reactivity model, a dose-response relationship was evaluated. Data of F in enamel (Table 1 and Figures 1 and 2) show a positive and statistically significant correlation between F concentration in treatment solutions and F uptake, as much for sound bovine enamel as for artificial carious lesions slabs, suggesting that the *in vitro* model of reactivity proposed is adequate for studying fluoride bioavailability.

Figures 1 and 2 show that there was a direct proportional relationship between fluoride present in treatment solutions and that present in dental enamel, either for sound bovine enamel or for artificial carious lesions slabs, showing a dose-response effect. For sound enamel, there was a linear dose-response effect and, thus, it is possible that there would be a higher fluoride uptake if the dental slabs were submitted to an aqueous solution with F concentration higher than $400 \mu\text{g F/mL}$. For artificial carious enamel slabs, the dose-response relationship was quadratic polynomial: the fluoride uptake by enamel increased according to the F concentrations in treatment solutions until a maximum of $300 \mu\text{g F/mL}$, approximately. It suggests that the peak of fluoride uptake by artificial carious enamel was reached before $400 \mu\text{g F/mL}$ of treatment solution. The higher area of reaction present in carious enamel could be responsible for the higher fluoride uptake when compared to sound enamel, which may explain why in carious enamel a higher F concentration would not result in greater F uptake.

The dose-response relationship between fluoride and dental enamel is reported in the literature. Negri et al.¹⁷ (2002) showed a dose-response relationship in terms of F concentration on enamel surface when it was submitted to dentifrices with different fluoride concentrations (275, 550 and $1100 \mu\text{g F/g}$). Fu et al.¹² (1999) showed a linear relationship between F uptake by bovine sound enamel and that present in dentifrices (250, 675 and $1100 \mu\text{g F/g}$). In clinical terms, this linearity in fluoride uptake by dental enamel reflects a decrease on dental demineralization or an increase on dental remineralization, as previously reported (Chon et al.,⁵ 2002), (Argenta et al.,¹ 2003) and (Rapozo-Hilo et al.,²² 2005). However, it is important to emphasize that a direct comparison

between the present study and those from Negri et al.¹⁷ (2002), Argenta et al.¹ (2003) and Rapozo-Hilo et al.²² (2005) is difficult, since the treatment (solutions versus dentifrices) and fluoride concentrations used were different.

According to Table 1, there is a higher reactivity of F with artificial carious enamel than with sound bovine enamel. This behavior was previously observed (White et al.,²⁷ 1990, Chan et al.,⁴ 1991) and reflects the fact that the artificial carious enamel has a higher contact surface, enabling higher reaction area. Although, it is important to emphasize that in the present study these data were not statistically compared.

According to Mellberg et al.¹⁶ (1974), F uptake by bovine enamel should be evaluated with caution, since it could not show a real situation, once bovine enamel uptake is higher when compared to human enamel. However, the homogeneity of results obtained by bovine enamel is assured, since these teeth were not submitted to a previous cariogenic challenge, as occur with human teeth (Mellberg,¹⁵ 1992). Besides, in spite of the differences existing between bovine and human dental enamel, they probably are not relevant when anti-caries agents were evaluated (Mellberg,¹⁵ 1992).

CONCLUSIONS

The results suggest that the *in vitro* model of reactivity used in the present study is adequate to evaluate a dose-response relationship between F present in aqueous solution and bovine sound enamel or with artificial carious lesion.

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