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EFEITO DE DOSES CRÔNICAS OSCILANTES DE FLUORETO NO DESENVOLVIMENTO DE FLUOROSE DENTAL EM RATOS

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Odontologia, área de concentração em Saúde Coletiva.

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DEDICATÓRIA

Diante da atual conjuntura nacional, de inversão de moralidades, do triunfo das nulidades, do prosperar da desonra e do crescimento da injustiça e da famigerada corrupção, dedico aos meus pais **Sílvio José Catani** e **Maria de Lourdes Bonadia Catani**, que nos proporcionaram, a meu irmão e a mim, exemplos e princípios de moralidade e ética.

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“Algumas pessoas vêem as coisas como são, e perguntam: 'por quê ?' Eu sonho com as coisas que nunca existiram e me questiono: 'por que não ?'”

(George Bernard Shaw)

RESUMO

O efeito do fluoreto (F) no desenvolvimento de fluorose dental em animais expostos a doses crônicas constantes é bem conhecido, mas não há informações sobre o efeito de doses crônicas oscilantes, o que foi estudado neste trabalho. Além disso, não há estudos comparando diferentes métodos de quantificação de defeito de esmalte induzido pelo F, o que também foi avaliado. O estudo foi composto por três trabalhos. Foram utilizadas ratas fêmeas da linhagem Wistar com idade de 35 dias, as quais receberam água e ração *ad libitum* durante um período suficiente para permitir o completo crescimento dos incisivos (70 a 78 dias). Após, os animais foram anestesiados, o sangue foi coletado e os incisivos, mandíbulas e fêmures, removidos após a morte por hemorragia. A concentração de F no plasma, nos incisivos e nos ossos foi determinada usando um eletrodo íon específico. No primeiro estudo, 20 ratas foram divididas casualmente em 5 grupos: quatro grupos receberam água com concentrações constantes de F (0; 12,5; 25 ou 37,5 µg F/mL) e o outro grupo recebeu água contendo 12,5 e 37,5 µg F/mL, alternados a cada 72 h (média de exposição igual a 25 µg F/mL). Não foi observada diferença nas variáveis metabólicas e na gravidade de fluorose dental entre o grupo que recebeu doses oscilantes e o grupo que recebeu dose correspondente à média das oscilações ($p > 0,05$). No segundo estudo, foram utilizadas 58 ratas, as quais foram divididas casualmente em grupos expostos a diferentes concentrações de F na água (0 a 75 µg F/mL) constantes ou alternantes (12,5 e 75 µg F/mL) por períodos assimétricos (2 ou 8 dias). A exposição a doses oscilantes de F resultou em concentrações de F no sangue e ossos e fluorose dental semelhantes às observadas nos animais expostos à média das oscilações em função do tempo utilizado. No terceiro estudo, foram utilizadas 35 ratas, divididas casualmente em 7 grupos de 5 animais cada, que receberam água em concentrações crescentes de F: 0; 12,5; 25; 50; 62,5; 75 ou 100 µg F/mL. Os incisivos foram utilizados para avaliar 5 métodos de quantificação de fluorose:

radiomicrografia, microdureza, microscopia de luz polarizada, análise de fluorescência e análise da imagem da superfície do esmalte segundo o padrão de bandas claras e escuras presentes na superfície do esmalte fluorótico de ratos. Radiomicrografia, microdureza e microscopia de luz polarizada só foram capazes de detectar fluorose sob altas concentrações de F (≥ 50 µg/mL) e a análise de fluorescência não permitiu diferenciação entre os grupos. A análise da imagem da superfície do esmalte foi satisfatória para quantificar fluorose, mesmo nas menores concentrações de F utilizadas nesse estudo, e foi utilizada para quantificar fluorose nos estudo subsequentes. Os resultados dos estudos sugerem que o efeito biológico da exposição crônica a doses oscilantes de F reflete a média do efeito das doses oscilantes de acordo com o tempo de exposição. A análise da imagem da superfície do esmalte se mostrou um método adequado para quantificar fluorose em incisivos de ratos.

Palavras-chave: fluoreto, metabolismo, fluorose dental, imagem, microrradiografia, microdureza, microscopia, QLF, ratos, incisivos.

ABSTRACT

The effect of fluoride (F) on the development of dental fluorosis in animals chronically exposed to constant F doses is well known, but there is no information about the effect of chronic oscillating doses, and this was evaluated in the present study. Moreover, there are no studies comparing different methods of quantification of enamel defects induced by F, which was also evaluated. The study comprised of three experiments. Wistar female rats, aged 35 days, received water and food *ad libitum* during enough time to allow the full growth of the incisors (70 to 78 days). After, the animals were anesthetized, blood was collected and femurs, mandible bones and incisors were removed after their death by bleeding. F concentration in the blood plasma, bones and teeth was determined using ion-specific electrode. In the first study, 20 rats were randomly divided into 5 groups: four groups received water with constant F concentration at 0, 12.5, 25 or 37.5 μg F/mL and the other group received water containing 12.5 and 37.5 μg F/mL, which were alternated at each 72 h (mean exposure equals to 25 μg F/mL). No difference in metabolic variables and in severity of dental fluorosis between the group which received oscillating doses and the group receiving 25 μg F/mL was observed ($p > 0.05$). In the second study, 58 rats were randomly divided into groups which were chronically exposed to different F concentration in the water (0 to 75 μg F/mL), constant or oscillating (12.5 and 75 μg F/mL) for different periods of length (2 or 8 days). Exposure to oscillating F doses resulted in F concentrations in blood plasma and bones and enamel fluorosis similar to those observed in groups receiving F concentrations equal to the means of the oscillating range, according to the period length. In the third study, 35 rats were randomly divided into 7 groups of 5 animals, which received water at increasing F concentrations: 0, 12.5, 25, 50, 62.5, 75 or 100 μg F/mL. Incisors were used to assess fluorosis using five quantification methods: transverse microradiography, cross-sectional microhardness, polarized

bright microscopy, quantitative light-induced fluorescence and image analysis of the enamel surface based on the pattern of white and dark bands present on rat fluorotic enamel. Transverse microradiography, cross sectional microhardness and bright field microscopy were only able to detect the fluorotic defect in the rat incisor when high F concentrations (≥ 50 µg F/mL) were used and quantitative light-induced fluorescence analysis did not allow the differentiation among the groups. The image analysis method of the enamel surface was satisfactory to quantify dental fluorosis, even at low F concentrations, and was used to evaluate fluorosis in the subsequent studies. The present study suggests that the biological effect of F when an animal is exposed chronically to oscillating F doses may reflect the effect of the mean of the oscillating doses, according to exposure length. The image analysis of the enamel surface was a suitable method to quantify enamel fluorosis in rat incisors.

Key word: fluoride, metabolism, dental fluorosis, image analysis, microradiography, microhardness, microscopy, QLF, rats, incisors.

LISTA DE SIGLAS, SÍMBOLOS E ABREVIATURAS

BFM	Bright Field Microscopy
CA	Estado da Califórnia
Ca^{2+}	cálcio iônico
CDTA	ácido ciclohexilendinitrilo tetra acético
CSMH	Cross-sectional Microhardness
DFIA	Dental Fluorosis by Image Analysis
F	fluoreto
FL	Estado da Flórida
HCl	ácido clorídrico
IL	Estado de Ilinois
KHN	Knoop Hardness Number
MA	Estado de Massachussets
NaCl	cloreto de sódio
NaOH	hidróxido de sódio
NC	Estado da Carolina do Norte
NY	Estado de Nova York
QLF	Quantitative Light-induced Fluorescence

SD	standard deviation (desvio padrão)
SP	Estado de São Paulo
TISAB	Total Ionic Strength Adjustment Buffer
TMR	Transverse Microradiography
U.S.A.	Estados Unidos da América
#	número
%	porcentagem
<	menor
=	igual
>	maior
≥	maior ou igual
±	mais ou menos
µg	micrograma
µm	micrometro
µM	micromolar
cm	centímetro
cm^3	centímetro cúbico
g	grama
g	força de centrifugação

h	hora
kV	quilovolt
M	molar
mA	miliampere
mg	miligrama
mL	mililitro
mm	milímetro
mm^2	milímetro quadrado
n	número amostral
ºC	grau Célsios
p	probabilidade estatística
ppm	parte por milhão
s	segundo
vol	volume
α	erro alfa

SUMÁRIO

INTRODUÇÃO

Dentre os mais diversos agentes preventivos ou terapêuticos de sucesso em Odontologia, que causaram um impacto importante na saúde e qualidade de vida das pessoas, o fluoreto (F) se destaca, pois tem sido responsável pelo declínio na incidência de cárie dental em todo o mundo. Dentre os meios de utilização de F, destacam-se a água fluoretada, de abrangência coletiva, e dentifrício fluoretado, de uso individual. Em ambos os meios, o efeito do F é essencialmente local, diminuindo a desmineralização dental durante eventos de queda de pH no biofilme cariogênico, ou potencializando o efeito remineralizador da saliva em pH neutro. No entanto, ambos os meios podem resultar em exposição sistêmica ao F (compulsória, pela ingestão de água fluoretada ou alimentos preparados com ela, ou involuntária, pela ingestão de dentifrícios fluoretados por crianças de pouca idade), o que pode resultar no único efeito colateral pela exposição sistêmica a baixas doses de F, a fluorose dental.

Em termos de farmacocinética, após a ingestão, o F é absorvido pelo estômago e início do intestino delgado e atinge a corrente sanguínea, através da qual é distribuído para todo o organismo, sendo incoporado pelos tecidos mineralizados, como ossos e dentes. Nos dentes, pode promover um distúrbio do desenvolvimento do esmalte dental, conhecido como fluorose dental (Aoba & Fejerskov, 2002).

A fluorose dental consiste na hipomineralização do esmalte, produzida pela ingestão crônica de F durante seu período de desenvolvimento (Aoba & Fejerskov, 2002). Algumas hipóteses foram levantadas para descrever o mecanismo de ação do F no desenvolvimento da fluorose (Browne *et al.*, 2005). Há consenso que este efeito não ocorre no nível celular, mas que o F afeta o processo de remoção das proteínas da matriz do esmalte durante a maturação

(Aoba & Fejerskov, 2002). Em acréscimo, há dados sugerindo que o F por si só não inibe as proteases responsáveis pela degradação das proteínas (Gerlach *et al.*, 2000). Atualmente, há uma forte suspeita de que o F diminua a capacidade de ganho de mineral pelo esmalte durante o desenrolar do processo de maturação desse tecido. Acredita-se que este íon interfira na concentração de Ca^{2+} presente no fluido tecidual, da matriz do esmalte em formação, e deste modo na atividade das proteases cálcio-dependentes, o que resultaria no atraso da maturação (Aoba & Fejerskov, 2002). Isto, consequentemente, levaria a uma deficiência de mineralização da matriz do esmalte.

Não existe um limiar crítico para a ingestão de F abaixo do qual o efeito sobre o esmalte dental não irá se manifestar, considerando que a relação dose-resposta entre a ingestão de F e a gravidade da fluorose é claramente linear (Aoba & Fejerskov, 2002). O efeito clínico, no entanto, poderia ser evidenciado com maior ou menor intensidade, sendo que estudos experimentais indicam que a gravidade da fluorose dental é uma função não apenas da dose de exposição ao F, mas também da duração dessa exposição (Angmar-Månsson & Whitford, 1984; Ishii & Suckling, 1991). Assim, os aspectos clínicos desta alteração, em humanos, variam desde finas estrias esbranquiçadas, em geral horizontais e translúcidas, que seguem as periquimáceas do esmalte, até estágios em que o esmalte se rompe podendo incorporar pigmentação amarronzada (Thylstrup & Fejerskov, 1978).

O período crítico, em humanos, de exposição ao F com relação à fluorose dental em dentes anteriores (esteticamente comprometedora), segundo a Organização Mundial de Saúde (WHO, 1994) seria entre 18 e 32 meses de idade, pois corresponde a um estágio de transição entre o fim da fase secretória e início da fase de maturação dos incisivos permanentes. Particularmente, nos incisivos permanentes o período crítico parece estar compreendido entre 11 e 34 meses (Ishii & Suckling, 1991), porém, alguns autores estimam que o período de transição e maturação inicial do esmalte dental, em incisivos centrais superiores,

mais suscetível à fluorose está entre a idade de 15 e 30 meses (Evans & Stamm, 1991). Contudo, Bårdsen (1999) afirmou que a duração da exposição do F durante a amelogênese dental, mais do que períodos específicos de risco, parece explicar o desenvolvimento da fluorose no incisivo central superior permanente.

Fluorose dental em experimentos utilizando modelo animal

Modelos animais têm sido utilizados com êxito para investigar o mecanismo biológico da indução por F de lesões em esmalte e dentina (Aoba & Fejerskov, 2002). Por muitos anos acreditou-se que as doses orais administradas diariamente aos animais, por serem mais elevadas do que as doses associadas ao homem para induzir ao mesmo grau de alteração, não seriam relevantes para a situação humana (Creath *et al*., 1989; Smith *et al*., 1993). Em experimentos envolvendo suínos (Richards *et al*., 1986) e ovinos (Suckling *et al*., 1988), no entanto, foi provado que as características subsuperficiais da hipomineralização sem hipoplasia do esmalte, como visto no esmalte fluorótico humano, podiam ser produzidas após ingestão crônica de F. Além disso, as experiências anteriores com ratos (Angmar-Månsson & Whitford, 1982, 1984) e suínos (Richards *et al*., 1985) mostraram que a concentração plasmática de F associada a lesões de esmalte nos animais é da mesma ordem de grandeza que aquelas que podem ocorrer no homem.

As importantes conclusões sobre fluorose dental, obtidas a partir de estudos anteriores com modelo animal são: (1) os efeitos sistêmicos do F, por exemplo, distúrbios na homeostasia de cálcio, não estão necessariamente envolvidos com o esmalte fluorótico (Angmar-Månsson & Whitford, 1984; Andersen *et al*., 1986); (2) o aumento da gravidade da fluorose é proporcional à concentração de F no esmalte (Richards *et al*., 1985; Speirs, 1986; Angmar-Månsson & Whitford, 1990) e (3) qualquer alteração na síntese protéica durante a

fase secretória não é absolutamente necessária para o desenvolvimento de fluorose (Richards *et al*., 1986; Kierdorf *et al*., 1996).

Dentre os animais utilizados em pesquisas sobre fluorose dental, o rato tem sido o mais utilizado. Os incisivos de ratos apresentam duas peculiaridades; além de terem crescimento contínuo, a superfície do esmalte do incisivo erupcionado apresenta uma pigmentação marrom-alaranjada, que é causada pela incorporação de íon ferro (Pindborg, 1947; Halse, 1972a, b). Estudos histoquímicos e análises de microscopia eletrônica mostraram que o pigmento de íon ferro também é identificado nos ameloblastos durante a fase de maturação do esmalte (Pindborg, 1947; Stein & Boyle, 1959; Halse & Selvig, 1974). O íon ferro proveniente da corrente sanguínea, se incorpora aos ameloblastos em maturação e à superfície do esmalte completamente mineralizada, ao final do período de maturação (Karim & Warshawsky, 1984; Kubota, 1985). Neste peculiar sistema de transporte do íon ferro, presume-se que a incorporação desse pigmento aos ameloblastos e ao esmalte dental ocorre através do processo de digestão lisossomal da ferritina no período final da fase de maturação (Kallenbach, 1968, 1970; Takano & Ozawa, 1981).

Já o esmalte fluorótico, em ratos, apresenta um padrão típico de bandas alaranjadas e brancas repetidas, causado por uma alteração na deposição de íon ferro na superfície do esmalte alterado. Kato *et al*. (1987) avaliaram a distribuição do íon ferro no esmalte de dentes incisivos de ratos após a administração de diferentes quantidades de F, e sugeriram que, com aumento da concentração de F ingerido, o íon ferro penetra mais profundamente na região mais porosa, no tecido menos mineralizado e, portanto, a concentração de íon ferro na superfície do esmalte diminui.

Métodos de quantificação de fluorose dental

A necessidade de um método objetivo de quantificação de fluorose dental tem sido reconhecida por muitos anos (Ellwood *et al*., 1994). Alguns indicadores indiretos do grau de mineralização dental empregados para avaliar desmineralização devido à cárie dental têm sido utilizados para quantificação da gravidade da fluorose, já que esta se apresenta como uma hipomineralização do esmalte dental, e, portanto, semelhantes métodos de quantificação poderiam ser aplicáveis a ambos. Porém alguns deles só parecem ter efeito quando as alterações do esmalte são extensas, afetando propriedades físicas e mecânicas da estrutura do esmalte.

Dentre os métodos de quantificação do grau de mineralização descritos na literatura, destacam-se:

Radiomicrografia transversal (TMR)

A radiomicrografia transversal (transverse microradiography, TMR) é um método utilizado para avaliar des-re-mineralização em tecidos dentais duros em estudos *in vitro* e *in situ*, sendo altamente sensível para avaliar a morfologia e mudança no conteúdo mineral do esmalte e dentina. Na análise por radiomicrografia, amostras de dente são cortadas em fatias finas (cerca de 70 a 120 µm), as quais são expostas a radiação X. Os valores de concentração mineral podem ser calculados a partir da imagem feita na película radiográfica examinada por um microscópio (Ericsson, 1965). O método foi introduzido por Angmar *et al*. (1963) e, então, amplamente utilizado em pesquisa odontológica para determinar o porcentagem de mineral (%vol) no tecido dental, em função da profundidade da lesão de desmineralização (µm) (ten Cate & Duijsters, 1983). Arends *et al*. (1983) ampliaram o uso do método TMR, mostrando que é possível quantificar o volume

de perda mineral adquirida em amostras de tecido dental, depois de um processo de des-re-mineralização.

Assim, Shinoda (1975) ao verificar o efeito de doses crescentes de administração de F (0 a 113 ppm F) nas propriedades físico-químicas do esmalte dental de ratos, observou, microrradiograficamente, aumento da hipomineralização, na região da subsuperfície mais próxima a superfície do esmalte, proporcional à dose administrada.

Também, Angmar-Månsson & Whitford (1982 e 1984) utlizaram radiomicrografias para determinar o distúrbio de mineralização do esmalte dental devido ao F, correlacionando com a concentração deste íon no plasma sanguíneo. Os mesmos autores observaram através deste método que doses agudas de fluoreto de sódio (NaF) injetadas em ratos poderiam acarretar fluorose dental mesmo após os níveis altos de F no plasma terem retornado aos níveis basais, propondo que este íon poderia ser mobilizado do osso adjacente ao órgão dental (Angmar-Månsson & Whitford, 1985).

Hirasuna *et al*. (2008) consideram que este método pode ser usado potencialmente como uma ferramenta para avaliar a gravidade e extensão de defeitos no esmalte devido ao F.

Microdureza em secção transversal (CSMH)

A microdureza é um indicador indireto do grau de mineralização dental (Lazzari, 1976). A mensuração da dureza, expressa em Knoop Hardness Number (KHN), é representada como a relação da carga sobre a área onde é aplicada (Strange & Varshneya, 2001). A magnitude da carga a ser aplicada está em função da dureza do material em estudo, gerando uma impressão regular.

Vieira *et al*. (2005) verificaram a influência da concentração de F em dentes na fluorose dental através da análise de microdureza em dentina e concluíram que fatores genéticos (gravidade de fluorose) e ambientais (concentração de F no dente) influenciaram as propriedades mecânicas do dente (microdureza). Outros estudos também utilizaram este método de análise para avaliar defeitos de esmalte induzidos pelo F (Shinoda, 1975; Suckling *et al*., 1988; Brighenti *et al*., 2006; Mousny *et al*., 2008). A microdureza foi validada na avaliação do efeito do chumbo na formação do esmalte (Gerlach *et al*., 2002).

Quantitative Light-induced Fluorescence (QLF)

A análise de QLF utiliza o princípio da fluorescência para revelar o grau de mineralização do dente. O método é baseado na auto-fluorescência da estrutura dental (Angmar-Månsson & Bosch, 2001), quando submetida à luz azul de alta intensidade, a qual passa a emitir luz no espectro verde. A fluorescência dos tecidos dentais tem uma relação direta com o conteúdo mineral do esmalte.

Desta forma, Everett *et al*. (2002) usaram o QLF para testar a hipótese da influência de determinantes genéticos na susceptibilidade ou resistência ao desenvolvimento de fluorose dental em um modelo animal e observaram que camundongos de diferentes estirpes apresentaram diferentes níveis de fluorose. O mesmo foi observado por Vieira *et al*. (2005), quando comparou a gravidade de fluorose, usando QLF, com a microdureza de superfície.

Apesar de ser considerada uma ferramenta adequada para detecção de cárie em esmalte (Adeyemi *et al*., 2006), alguns autores acreditam que este método não é recomendável para avaliação de fluorose dental, pois a fluorose difere de cárie em esmalte, sendo geralmente caracterizada por uma hipomineralização difusa em toda a superfície dental (Ellwood & O'Mullane, 1995),

enquanto a lesão de cárie inicial em esmalte é caracterizada por uma hipomineralização subsuperficial localizada.

Efeito de doses oscilantes no desenvolvimento de fluorose

Tendo em vista que o grau de fluorose depende do tempo em que a matriz do esmalte em formação é exposta à determinada concentração de F, como visto anteriormente, a observação clínica do efeito no esmalte, quando há ingestão de água na concentração constante de 0,7 ppm F (considerada como ótima para o clima do Brasil), por exemplo, será o resultado da influência dessa concentração em todo o período de formação do dente. Porém, quando a concentração varia, por exemplo, de 0,3 a 1,1 ppm F (média igual a 0,7 ppm F) o efeito vai depender do tempo que estas concentrações forem mantidas constantes durante a formação do esmalte. Deste modo, uma concentração de 1,1 ppm F, portanto maior que a considerada como ótima, por um curto período de tempo, pode não resultar em grau de fluorose maior, dependendo da quantidade de esmalte formado sob esta condição.

Desta forma, Catani *et al.* (2007) verificaram fluorose dental em crianças de dois municípios do estado de São Paulo que por 10 anos foram expostas a duas condições distintas quanto à regularidade da concentração ótima de F na água de abastecimento público. No município o qual a concentração foi mantida no intervalo considerado ótimo (0,6 a 0,8 ppm F) a prevalência de fluorose foi aproximadamente 2 vezes maior do que aquela nas crianças da cidade que apresentava oscilações abaixo e acima dos limites considerados ótimos (0,3 a 1,1 ppm F). Embora este resultado possa parecer coerente com relação ao mais provável mecanismo de ação do F no desenvolvimento da fluorose, as diversas variáveis que interferem em estudos epidemiológicos impedem uma adequada avaliação do efeito das doses oscilantes de exposição ao F nesse desenvolvimento.

PROPOSIÇÃO

O presente estudo teve como objetivo analisar o efeito da oscilação nas doses de exposição crônica ao fluoreto no desenvolvimento da fluorose dental em ratos.

Os objetivos específicos deste estudo foram:

CAPÍTULO 1: Avaliar o efeito biológico de doses constantes de exposição ao fluoreto em relação a doses oscilantes assimétricas, porém com a mesma média de exposição ao fluoreto, no desenvolvimento de fluorose dental em ratos.

CAPÍTULO 2: Avaliar o efeito biológico de doses constantes de exposição ao fluoreto em relação a doses regulares oscilantes, porém com a mesma média de exposição ao fluoreto, no desenvolvimento de fluorose dental em ratos.

CAPÍTULO 3: Avaliar a capacidade quantitativa de diferentes métodos na determinação de fluorose em ratos.

Este trabalho foi realizado no formato alternativo, conforme deliberação CCPG/002/06, da Comissão Central de Pós-Graduação da Universidade Estadual de Campinas, que permite a inclusão de artigos científicos de forma a atingir o objetivo deste trabalho (Anexo 1).

CAPÍTULO 1

FLUOROSIS IN RATS EXPOSED TO OSCILLATING CHRONIC F DOSES

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Abstract

Since blood fluoride concentration varies according to fluoride exposure, and dental fluorosis is related to the thickness of enamel formed under a given fluoride dose, we hypothesized that fluorosis produced by an oscillating fluoride dose would reflect that caused by the mean of the oscillation during a given time. Rats received during 78 days water with fluoride concentrations constant at 0, 12.5, 25 or 37.5 μg F/mL, or oscillating between 12.5 and 37.5 μg F/mL every 72 h (mean exposure = 25 μg F/mL). Fluoride concentrations in plasma, bone and incisor, and dental fluorosis scores increased linearly for constant fluoride concentrations in water ($p < 0.0001$, r values = 0.82 to 0.98). The results of the oscillating group and that receiving 25 μg F/mL did not differ statistically ($p > 0.05$). The findings suggest that in animals exposed to symmetrical oscillating fluoride doses, the resulting dental fluorosis reflects the metabolic effect of the mean of the oscillation.

Keywords: fluoride, fluorosis, blood, bone, dental fluorosis, rats.

1. Introduction

Almost half century of research strongly supports that fluoride (F) exerts its effect on caries and on dental fluorosis by distinct mechanisms. Thus, while the effect on caries is local, based on maintenance of oral F concentration (reviewed by Cury and Tenuta, 2008), the effect on fluorosis is systemic, based on blood F concentration (Bronckers *et al*., 2009), which is influenced by innumerous metabolic variables.

For instance, it is recognized that blood F concentration is not controlled by homeostasis (Ekstrand, 1996), and thus the pattern of F use and how it affects plasma F levels may be of importance (Angmar-Månsson and Whitford, 1982). Furthermore, considering that enamel formation is a continuous process and that the clinically visualized defects are dependent on the thickness of enamel formed under exposure to a given F dose (Aoba and Fejerskov, 2002), it is expected that short peaks of plasma F concentrations due to an acute dose would have less importance than the continuous exposure to chronic lower F doses on fluorosis development (Angmar-Månsson and Whitford, 1982, 1984). In fact, in a city with a not well controlled system of community water fluoridation, fluctuations of water F concentration above the optimal concentration (0.7 ppm F) did not increased fluorosis prevalence (Catani *et al*., 2007). Actually, in the city where water F concentration oscillated above (higher than 0.8 ppm F) and below (lower than 0.6 ppm F) the optimum, the fluorosis risk was 8.3 times lower than in the another city with constant optimum water F concentration (Catani *et al*., 2007).

This epidemiological observation raised the question on how fluctuations in F exposure chronically would affect enamel fluorosis. Thus, we hypothesized that the systemic effect of F on fluorosis would depend on both the mean effect of each oscillating F dose and the average time of exposure of ameloblasts to them during enamel maturation, and a first experimental study was conducted in rats to

evaluate the effect of symmetrical oscillations in water F concentration on metabolic variables and fluorosis development.

2. Material & Methods

Experimental design

This blind study was approved by the Animal Ethics Committee of University of Campinas (UNICAMP, protocol # 1579-1). Twenty Wistar females rats, aged 35 days, were randomly divided into 5 groups of 4 animals each. Four groups received *ad libitum* during 78 days, enough time to allow the full renewal of rat incisors (Schour and Massler, 1971), water containing 0 (control), 12.5, 25 or 37.5 µg F/mL. During the same 78 days, the 5th group (oscillating) received alternately at each 72 h, water containing 12.5 or 37.5 µg F/mL (mean of 25 µg F/mL). The F solutions were prepared with NaF and the concentrations were checked. The rats were fed *ad libitum* with pellet chow (Labina, Purina®, Paulínia, SP, Brazil; total F concentration of 17 µg F/g). The amount of water ingested by each group was assessed daily and animals were weighed at the beginning and the end of the experiment. On the 79th day, the rats were anesthetized and blood was collected by disruption of the brachial plexus using heparin as an anticoagulant. One femur, mandible bone and mandible incisors of the animals were removed and coded for the analyses (blind study concerning the examiner). At the time of killing, animals of the oscillating group had been drinking water at 37.5 µg F/mL for the last 24 h.

Plasma fluoride determination

Plasma samples were obtained by centrifugation at 2,000 g for 10 min. F concentration in plasma was determined using an inverted F-specific electrode (Orion Research, model 94-09, Orion Research Incorporated, Cambridge, MA,

U.S.A.) and a micro-reference electrode, under microscope, after buffering with TISAB III (Vogel *et al*., 1997).

Fluoride determination in the femur (whole and surface), mandible (alveolar bone) and in the incisor tooth

The rat femur was used for F determination in whole bone and bone surface (Bezerra de Menezes *et al*., 2003). The femur was dried for 24 h at 60°C, sectioned transversally to obtain 2 slices measuring approximately 5 mm from the diaphysis region. For whole F determination, one slice was pulverized, the powder was sieved to obtain particles of 140 µm to 1,000 µm and left at 60°C for additional 24 h. Ten milligrams (± 0.01 mg) were weighed and transferred to plastic test tubes containing 1.0 mL of 0.5 M HCl. After 1 h, at room temperature and under agitation, 1.0 mL of TISAB II (1.0 M acetate buffer, 1.0 M NaCl, 0.4% CDTA, pH 5.0) containing 0.5 M NaOH was added.

To determine F concentration on the bone surface, the other femur slice was sealed with acid-resistant nail varnish leaving a circular hole (2.0 mm diameter) exposed (Bezerra de Menezes *et al*., 2003). Three layers of bone were sequentially removed by immersing the slice in tubes containing 0.25 mL of 0.5 M HCl for 15, 30 and 60 s, under constant agitation. The extracts were neutralized and buffered with equal volumes of TISAB II, as described before.

For F determination on the surface alveolar bone where there was an incisor tooth, one hemi-mandible from each animal was dried for 24 h at 60ºC. The lingual blade of the alveolar socket was removed, thereby exposing the rest of the alveolar socket. All other regions were isolated with acid-resistant nail varnish. Using an automatic pipette, 0.1 mL of 0.5 M HCl was applied to the alveolar socket and the acid was flowed for 15 s by aspiration and injection. The extract was transferred to an assay tube and buffered as described before.

F concentration in the mandible incisors was determined at three transversal slices measuring approximately 1 cm from the apical, middle and incisal regions. These fragments were separately pulverized and the powder was dried at 60°C for 24 h. Five milligrams (± 0.01 mg) were weighed and transferred to plastic test tubes, to which 1.0 mL of 0.5 M HCl was added for F extraction as described for whole femur F determination.

F in all extracts was determined using an ion-specific electrode (Orion Research, model 94-09, Orion Research Incorporated, Cambridge, MA, U.S.A.) and a reference electrode (Flex-Ref, WPI Inc., Sarasota, FL, U.S.A.) both coupled to a potentiometer (Orion Research, model EA 940, Orion Research Incorporated, Cambridge, MA, U.S.A.), previously calibrated with standards with known F concentration, and prepared under the same conditions as the samples. All results were expressed as μg F/g dry weight and the amount of bone removed from the surface of femur or alveolar bone was calculated according to Bezerra de Menezes *et al*. (2003).

Dental fluorosis assessment

Due to the limitations of hardness and transverse microradiography to quantify dental fluorosis in rats exposed to low F concentrations (Shinoda, 1975; Angmar-Månsson and Whitford, 1982), we have developed a method of image analysis named dental fluorosis by image analysis (DFIA), based on the known effect of F changing the iron pigmentation of the rat incisor enamel surface, which was quantified. To acquire the input images, one mandible incisor from each animal was fixed with wax on a glass slide (Figure 1) with the enamel surface of incisal third of the crown upward parallel to a macro lens (Medical Nikkor, Nikon, Tokyo, Japan) attached to a vertical stand at a fixed distance of 7 cm above the teeth. An image was acquired with the digital camera (Nikon D70, Nikon, Tokyo, Japan) using standardized light intensity and focus. A central area of 690 x 170 pixels was cropped from the digital image and converted to grayscale (Figure 1).

For the cropped image, the DFIA index was obtained by difference between I_H (the average of the higher intensity pixel values, originally white bands) and I_L (the average of the lower intensity pixel values, originally orange bands). Higher values of DFIA index should correspond to higher fluorosis severity.

Statistical analysis

Linear regression analysis was used to correlate the administered constant F concentrations in water (independent variable) with F concentration in plasma, bones and incisor, and dental fluorosis found (SPSS software, version 12.0, SPSS Inc., Chicago, IL, U.S.A.). Using the linear equations, the estimated mean F concentrations to which the oscillating group (12.5/37.5 μg F/mL) would have been subjected during the all experiment was calculated. Analysis of variance was used to test the differences among groups, followed by Tukey test as a post-hoc test (SAS software, version 8.01, SAS Institute Inc., Cary, NC, U.S.A.). All analyses were performed with α level fixed at 5%.

3. Results

No difference in water intake (daily) and rats weights (either initial or final) was observed among groups ($p > 0.05$).

A statistically significant dose-response effect ($p < 0.0001$) was found between the F concentrations in the drinking water given constantly to the rats during 78 days and the dependent variables F concentrations in plasma, bone (whole, surface and alveolar) and incisor, and the fluorosis index DFIA , with correlations for the linear regression models between 0.82 and 0.98 (Table 1).

The mean F concentration to which the oscillating group (12.5/37.5 μg F/mL) would have been subjected during the experiment was calculated for each

dependent variable using the linear equations and varied from 21.3 to 31.8 µg F/mL (Table 1).

When the data were compared by ANOVA the group receiving oscillating doses did not differ statistically from the group receiving constantly 25 µg F/mL (Table 2).

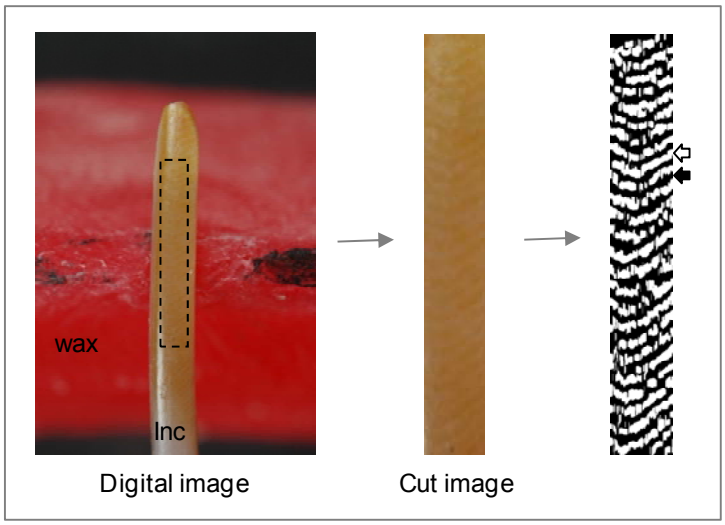

Figure 1. Schematic representation of the DFIA index for dental fluorosis quantification. Inc: mandible incisor; dashed line: cropped area; dark arrow: higher intensity pixels (white bands); white arrow: lower intensity pixels (orange bands). The difference between the means of higher and lower intensity pixels expresses fluorosis index (DFIA).

Table 1. Correlation (r) between fluoride concentration in rats plasma, bone and incisor, and dental fluorosis by DFIA index (y) as a function of constant fluoride concentration in water (x), linear equation and estimated F concentration in the water given to the rats of the oscillating group (12.5/37.5 µg F/mL).

y	X	r*	Equation	Estimated [F] in water for the oscillating group
Plasma	[F] in water	0.824	y = 2.260 + 0.138x	26.5 ± 5.6
Whole femur	[F] in water	0.981	y = 250.960 + 33.771x	24.6 ± 3.2
Femur surface (10.0 µm)	[F] in water	0.917	y = 764.653 + 30.427x	31.8 ± 3.7
Femur surface (24.7 µm)	[F] in water	0.941	y = 535.008 + 37.751x	31.8 ± 4.2
Femur surface (46.2 µm)	[F] in water	0.948	y = 458.152 + 31.762x	28.7 ± 5.9
Alveolar bone surface	[F] in water	0.973	y = 461.328 + 34.286x	25.7 ± 3.9
Apical (tooth)	[F] in water	0.976	y = 99.350 + 16.580x	26.0 ± 3.8
Middle (tooth)	[F] in water	0.979	y = 96.388 + 15.685x	26.0 ± 3.6
Incisal (tooth)	[F] in water	0.984	y = 89.387 + 14.401x	24.9 ± 2.7
Dental fluorosis	[F] in water	0.845	y = 0.928 + 0.087x	21.3 ± 7.8

* $p < 0.0001$ for all variables

Table 2. Mean ± SD (n=4) fluoride concentrations in rats plasma, bone and incisor, and dental fluorosis index as a function of fluoride concentration in water.[a]

[F] in water	Plasma	Whole femur	Femur surface (µg F/g)			Alveolar bone surface	Incisor (µg F/g)		
(µg F/mL)	(µM)	(µg F/g)	10.0 µm	24.7 µm	46.2 µm	(µg F/g)	Apical	Middle	Incisal
[b]0	2.1±0.3	249.2±91.5	736.8±213.4	550.6±35.1	482.5±32.8	448.7±77.5	114.1±29.1	109.1±27.2	100.6±25.6
12.5	4.3±0.5	714.9±41.8	1217.1±194.8	1014.9±270.8	829.1±187.5	921.8±125.3	312.9±31.9	300.2±22.8	274.8±17.8
25	5.5±1.7	1016.9±105.7	1464.7±218.2	1416.0±236.3	1231.2±262.9	1292.5±186.0	457.1±62.1	434.8±61.6	405.0±32.9
37.5	7.5±2.4	1555.7±112.2	1922.1±192.6	1989.9±232.3	1671.9±102.5	1753.7±99.4	756.9±45.0	717.8±24.3	657.2±33.2
[c]12.5/37.5	5.9±0.8	1082.7±108.6	1731.9±112.3	1734.3±156.9	1369.2±188.5	1343.9±133.9	529.8±69.1	503.8±55.7	447.5±39.5

[a]In order to fit the assumptions for this test, data of plasma fluoride and dental fluorosis were log-transformed.

[b]This group differed statistically from all groups for all variables ($p<0.05$).

[c]This group did not differ statistically from group received 25 µg F/mL for all variables ($p>0.05$).

4. Discussion

Rats have been extensively used as a model to study fluorosis because plasma F concentrations causing enamel fluorosis in these animals and in humans are similar (Angmar-Månsson and Whitford 1982, 1984), as well as the structure and formation of the enamel (Warshawsky *et al*., 1981).

In the present study, to estimate the effect on dental fluorosis of oscillating F doses to which rats were subjected chronically by alternately drinking water containing 12.5 and 37.5 µg F/mL, the dose-response effect of drinking constantly distilled water (control) or water containing 12.5, 25.0 or 37.5 µg F/mL was determined on some biological parameters related to F metabolism and fluorosis.

A significant increase in the value of all variables analyzed was observed according to the increase in F concentration in the water for the constant groups (Table 1), in agreement with other studies with rats (Ekstrand *et al*., 1981). Also, the effect of increasing levels of F exposure was satisfactory reflected ($r=0.85$) in increasing fluorosis severity quantified by the DFIA index method developed for this research.

The estimated water F concentration to which the rats were subjected by the oscillating concentrations was very close to 25 µg F/mL for all metabolic variables analyzed (Table 1), which is the expected mathematical mean of drinking water containing 12.5 and 37.5 µg F/mL given at symmetrically alternating periods. Indeed, the group subjected to the oscillating dose did not differ statistically from the group drinking constantly 25 µg F/mL in any biological variable analyzed and on fluorosis development (Table 2).

These findings are supported by the relationship between F concentration in blood and bone. It is known that there is no homeostatic

mechanism to maintain blood F concentration (Ekstrand, 1996), and that F will either be taken up from blood by bone or, when the F exposure is interrupted, the concentration in the blood could be maintained for some days due to the F coming from the exchangeable bone sub-compartment (Rao *et al*., 1995), but later will decrease (Ekstrand *et al*., 1981). Thus, F accumulated in the bone could contribute to increase fluorosis by sustaining, for a certain period of time, blood F concentration systemically or locally in fluids adjacent to maturating enamel. It has been hypothesized that after an acute F dose, the gradual release of F from the adjacent bone could be sufficient to cause enamel fluorosis (Angmar-Månsson and Whitford, 1985) and the similar F concentration on the surface of alveolar bone for the oscillating and 25 µg F/mL groups (Table 2) show some evidence favoring this hypothesis, even for chronic dose. Accordingly, F concentration in femur and other long bones would contribute to a steady sate plasma F concentration (Ekstrand *et al*., 1981) and the findings showed that the oscillating and the 25 µg F/mL groups did not differ concerning femur and plasma F concentrations (Table 2). Therefore, the same F concentrations in incisors teeth of oscillating and 25 µg F/mL groups (Table 2) could be explained by a local and systemic effect.

Moreover, the similar fluorosis observed for the oscillating and the constant 25 µg F/mL groups may also be explained by the timing of enamel formation under this metabolic condition. Therefore, the main effect of chronic low F dose occurs during enamel maturation (Denbesten *et al*., 1985) and in rats this phase takes approximately 10.5 days (Leblond and Warshawsky, 1979). Thus, enamel under maturation during a given period would partly be exposed to both the oscillating concentrations. As the rat mandible incisor is totally renewed in approximately 60 days, the clinical outcome on enamel would reflect the mean effect of the oscillating F concentrations given each 3 days during the 78 days of the experiment. Indeed, the same effect on fluorosis by oscillating group compared with the 25 µg F/mL is supported by F concentration in the incisor tooth, which was similar for both groups (Table 2).

The present experimental study gives support to the epidemiological observation that short fluctuations in F concentration in the water above the optimal, do not necessarily result in a higher fluorosis risk (Catani *et al.*, 2007). This could be explained by the small thickness of enamel formed during each period of exposure to a given dose, suggesting that the mean exposure to F is the one that adequately reflects the resulting enamel fluorosis. The lower fluorosis risk found in a city with oscillating water F concentrations could thus be explained by longer periods when the water F was maintained below the optimum, resulting in a lower average F dose when compared to a city in which water concentration was kept within the optimal range.

In summary, the findings suggest that the biological effect of F on animals chronically exposed to symmetrical oscillating F doses reflect the effect of the mean of the oscillating doses. However, limitations on the extrapolation of the present results should be taken into account considering the model used and the necessity to conduct another study to evaluate the effect of oscillating F doses during asymmetrical periods of exposure, which is under investigation in our lab.

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CAPÍTULO 2

EFFECT OF CHANGE OF EXPOSURE FLUORIDE CHRONIC DOSE IN THE DEVELOPMENT OF DENTAL FLUOROSIS

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Abstract

The effect of symmetrical oscillating fluoride (F) doses on the development of dental fluorosis has been shown to reflect the metabolic effect of the mean of the oscillation. However, the biological effect of erratic oscillations in the exposure to F is not known. In this study, approved by the Animal Ethics Committee of UNICAMP, 58 Wistar female rats, aged 35 days, were randomly divided into 7 groups which were exposed chronically to different F concentrations in the water, either constant at 0, 12.5, 25, 62.5 or 75 µg F/mL or oscillating between 12.5 and 75 µg F/mL for either 8 days or 2 days. After 78 days, rats were sacrificed and blood, femurs and mandible bones analyzed for F concentration, while incisors were analyzed for fluorosis using the dental fluorosis by image analysis method. A significant linear increase in the variables values was observed according to the increase in F concentration in the water for the constant groups ($p < 0.0001$). Exposure to oscillating F doses resulted in F concentrations and dental fluorosis similar to those observed by the means of the doses used, according to the period length. The results suggest that even for oscillating doses used during asymmetrical periods, the resulting biological effect may reflect the mean of the dose and exposure length.

Keyword: fluoride, metabolism, blood, bone, dental fluorosis, rats.

1. Introduction

The mechanisms of fluoride (F) effect on caries and dental fluorosis are clearly distinct. While the effect on caries is local, based on maintenance of oral F concentration (reviewed by Cury and Tenuta, 2008), the effect on fluorosis is systemic, based on maintenance of blood F concentration (Bronckers *et al*., 2009), which is influenced by innumerous metabolic variables.

For instance, it is recognized that blood F concentration is not controlled by homeostasis (Waterhouse *et al*., 1980; Ekstrand, 1996), and thus the pattern of F use and how it affects plasma F levels may be of importance. Relatively constant plasma levels produced by the daily intake of fluoridated water might have a different effect on enamel fluorosis than higher but erratic F concentration peaks observed after inadvertent ingestion of F dentifrice. Furthermore, enamel formation is a continuous process and the clinically visualized defects are dependent on the thickness of enamel formed under exposure to a given F dose (Aoba and Fejerskov, 2002).

It was recently shown that the biological effect of F when an animal is chronically exposed to oscillating F doses during brief and symmetrical periods may reflect the effect of the mean of F exposure (Catani *et al*., 2010). However in humans the pattern of oscillation in F exposure is never symmetrical, and increases in the F concentration might happen for a short period of time. Thus, in the present study, the effect of asymmetrical periods of oscillation in the dose of F exposure on metabolic variables and fluorosis development in rats was studied.

2. Material & Methods

Experimental design

This blind study was approved by the Animal Ethics Committee of University of Campinas (UNICAMP, protocol # 1579-1) and it was conducted during 78 days, enough time to allow the full renewal of rat incisors (Schour and Massler, 1971).

Fifty eight Wistar females rats, aged 35 days, were randomly divided into 7 groups. Five groups with 5 animals each received *ad libitum* during 78 days water containing constant F doses at 0, 12.5, 25, 62.5 or 75 µg F/mL. During the same 78 days, the 6th and 7th groups (oscillating) received for either 8 days or 2 days water containing 12.5 and 75 µg F/mL, according to the flow chart in Figure 1 (mean of exposure during the experiment equals to 25 and 62.5 µg F/mL for 6th and 7th groups, respectively). The F solutions were prepared with NaF and the concentrations were checked. In the oscillating groups, animals were killed at different intervals after dose change (Figure 1). The rats were fed *ad libitum* with pellet chow (Labina, Purina®, Paulínia, SP, Brazil; total F concentration of 17 µg F/g). The amount of water ingested by each group was assessed daily and animals were weekly weighed. On the 79th day, the rats were anesthetized and blood was collected by disruption of the brachial plexus using heparin as an anticoagulant. One femur, mandible bone and mandible incisors of the animals were removed after their death and coded for the analyses (blind study concerning the examiner).

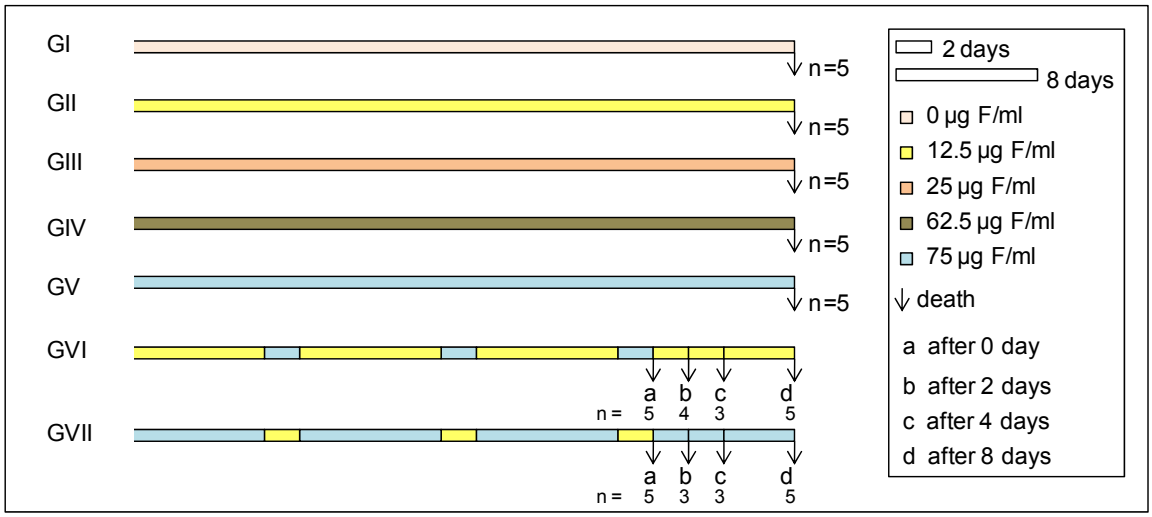

Figure1. Flowchart of experimental design.

Plasma fluoride determination

Plasma samples were obtained by centrifugation at 2,000 *g* for 10 min. F concentration in plasma was determined using an ion-specific electrode (Orion Research, model 94-09, Orion Research Incorporated, Cambridge, MA, U.S.A.) and a reference electrode (Flex-Ref, WPI Inc., Sarasota, FL, U.S.A.) both coupled to a potentiometer (Orion Research, model EA 940, Orion Research Incorporated, Cambridge, MA, U.S.A.), previously calibrated with standards with known F concentration, after buffering with total ionic strength adjustment buffer (TISAB III).

Fluoride determination in the femur (whole and surface) and mandible (alveolar bone)

The rat femur was used for determination of whole bone and bone surface F concentrations (Bezerra de Menezes *et al.*, 2003). The femur was dried for 24 h at 60°C, sectioned transversally to obtain 2 slices measuring approximately 5 mm from the diaphysis region. For whole F determination, one slice was pulverized, the powder was sieved to obtain particles of 140 µm to 1,000 µm and left at 60°C for additional 24 h. Ten milligrams (± 0.01 mg) were weighed

and transferred to plastic test tubes containing 1.0 mL of 0.5 M HCl. After 1 h, at room temperature and under agitation, 1.0 mL of TISAB II (1.0 M acetate buffer, pH 5.0 with 1.0 M NaCl and 0.4 % cyclohexanediaminetetraacetic acid) was added. To this TISAB solution NaOH was added at concentration of 0.5 M to neutralize the HCl.

To determine F concentration on the bone surface, the other femur slice was sealed with acid-resistant nail varnish leaving a circular hole (2.0 mm diameter) exposed (Bezerra de Menezes *et al*., 2003). Three layers of bone were sequentially removed by immersing the slice in tubes containing 0.25 mL of 0.5 M HCl for 15, 30 and another 30 s, under constant agitation. The extracts were neutralized and buffered with equal volumes of TISAB II, as described before.

For F determination on the surface alveolar bone where there was an incisor tooth, one hemi-mandible from each animal was dried for 24 h at 60ºC. The lingual blade of the alveolar socket was removed, thereby exposing the rest of the alveolar socket. All other regions were isolated with acid-resistant nail varnish. Using an automatic pipette, 0.1 mL of 0.5 M HCl was applied to the alveolar socket and the acid was flowed for 15 s by aspiration and injection. The extract was transferred to an assay tube and buffered as described before.

F in all extracts was determined as described for the plasma, but the F standards were prepared under the same conditions as the samples. All results were expressed as µg F/g dry weight and the amount of bone removed from the surface of femur or alveolar bone was calculated according to Bezerra de Menezes *et al*. (2003).

Dental fluorosis assessment

Dental fluorosis was determined by dental fluorosis by image analysis index (DFIA) according to Catani *et al*. (2010). To acquire the input images, one mandible incisor from each animal was fixed with wax on a glass slide with the

enamel surface of incisal third of the crown upward parallel to a macro lens (Medical Nikkor, Nikon, Tokyo, Japan) attached to a vertical stand at a fixed distance of 7 cm above the teeth. An image was acquired with the digital camera (Nikon D70, Nikon, Tokyo, Japan) using standardized light intensity and focus. A central area of 690x170 pixels was cropped from the digital image and converted to grayscale. For the cropped image, the DFIA index was obtained by difference between I_H (the average of the higher intensity pixel values, originally white bands) and I_L (the average of the lower intensity pixel values, originally orange bands). Higher values of DFIA index should correspond to higher fluorosis severity.

Statistical analysis

Linear regression analysis was used to correlate the administered constant F concentrations in water (independent variable) with F concentration in plasma, bones and dental fluorosis found (SPSS software, version 12.0, SPSS Inc., Chicago, IL, U.S.A.). Using the linear equations found, the estimated mean F concentrations to which the oscillating groups (12.5/75 and 75/12.5 µg F/mL) would have been subjected during the all experiment was calculated. Analysis of variance was used to test the significance of differences among groups, followed by Tukey test as a post-hoc test (SAS software, version 8.01, SAS Institute Inc., Cary, NC, U.S.A.). Data which did not fit the assumptions of normal distribution of errors and equality of variances were transformed (Box *et al.*, 1978). All analyses were performed with α level fixed at 5%.

3. Results

No difference in water intake (daily) and rats weights (either initial or final) was observed among groups ($p > 0.05$).

A statistically significant dose-response effect ($p < 0.0001$) was found between the F concentrations in the drinking water given constantly to the rats during 78 days and the dependent variables F concentrations in plasma and bone (whole, surface and alveolar), and the fluorosis index DFIA (Table 1), with correlations for the linear regression models between 0.72 and 0.98 (Table 1).

The mean F concentration to which the oscillating groups (12.5/75 and 75/12.5 µg F/mL) would have been subjected during the experiment was calculated for each dependent variable using the linear equations (Table 1). These estimated varied from 15.7 to 31.7 µg F/mL and from 59.2 to 118.2 for groups received 12.5/75 and 75/12.5 µg F/mL, respectively (Table 1).

When analysis of variance (ANOVA) was used to compare the groups, no significant difference could be observed between the groups received oscillating doses and the groups received doses corresponding to the mean of the oscillation (Table 2).

Figure 2 shows F concentration in plasma of groups that received oscillating doses of F after different periods of animals' death. After 2 days, plasma level had returned to low levels after decreasing from the high to the low F concentration exposure and had increased after increasing the F dose.

Table 1. Correlation (r) between fluoride concentration in rats plasma and bone, and dental fluorosis by DFIA method (y) as a function of fluoride concentration in water (x), linear equation and estimated F concentration in the water given to the rats of the oscillating groups (12.5/75 and 75/12.5 µg F/mL). Values correspond to the sampling at the end of the 8-day period at the same dose (Figure 1, "d").

y	x	r*	Equation	Estimated [F] in water for the group received 12.5/75 µg F/mL	Estimated [F] in water for the group received 75/12.5 µg F/mL
Plasma	[F] in water	0.919	y = 0.688 + 0.076x	26.9 ± 17.7	118.2 ± 61.5
Whole femur	[F] in water	0.980	y = 163.662 + 23.437x	23.5 ± 1.9	59.2 ± 11.6
Femur surface (4.5 µm)	[F] in water	0.872	y = 916.763 + 24.327x	25.0 ± 10.3	59.9 ± 22.0
Femur surface (12.3 µm)	[F] in water	0.945	y = 561.862 + 24.489x	31.7 ± 16.8	67.2 ± 14.5
Femur surface (19.3 µm)	[F] in water	0.929	y = 454.874 + 28.353x	22.8 ± 4.9	65.5 ± 12.9
Alveolar bone surface	[F] in water	0.961	y = 391.795 + 26.273x	25.1 ± 2.0	64.9 ± 7.8
Dental fluorosis	[F] in water	0.722	y = 1.526 + 0.051x	15.7 ± 5.8	61.3 ± 36.3

* $p < 0.0001$ for all variables

Table 2. Mean ± SD (n=5) of fluoride concentrations in rats plasma and bone (whole, superficial and alveolar), and dental fluorosis index as a function of fluoride concentration in water. Values for the oscillating groups correspond to the sampling at the end of the 8-day period at the same dose (Figure 1, "d").[a]

[F] in water (µg F/mL)	Plasma (µM)	Whole femur (µg F/g)	Femur surface (µg F/g)			Alveolar bone surface (µg F/g)	Dental fluorosis (DFIA index)
			4.47 µm	12.28 µm	19.28 µm		
[b]0	0.8 ± 0.2	161.4 ± 17.8	906.4 ± 411.9	418.8 ± 84.0	382.6 ± 89.1	296.5 ± 76.5	1.3 ± 0.1
12.5	1.8 ± 0.5	441.8 ± 52.9	1028.0 ± 183.5	853.8 ± 171.7	767.2 ± 151.0	722.7 ± 98.4	2.1 ± 0.3
25	2.4 ± 0.6	770.5 ± 159.9	1801.6 ± 302.4	1368.9 ± 186.3	1254.6 ± 159.1	1209.7 ± 371.5	2.9 ± 0.2
62.5	5.0 ± 0.6	1632.7 ± 225.5	2356.8 ± 247.9	2242.6 ± 264.7	2508.0 ± 461.3	1948.4 ± 230.7	5.4 ± 3.1
75	6.8 ± 2.0	1913.5 ± 193.0	2748.2 ± 686.0	2210.8 ± 294.0	2323.9 ± 449.5	2379.4 ± 202.5	4.7 ± 1.2
[c]12.5/75	2.7 ± 1.3	714.8 ± 44.8	1523.8 ± 250.1	1337.4 ± 410.7	1100.3 ± 138.9	1050.1 ± 51.2	2.3 ± 0.3
[d]75/12.5	9.7 ± 4.7	1550.0 ± 270.9	2373.0 ± 534.9	2207.5 ± 335.7	2311.0 ± 366.9	2097.9 ± 205.7	4.7 ± 1.9

[a]In order to fit the assumptions for this test, data of fluoride concentration in plasma, whole bone and bone surface were log-transformed and data of dental fluorosis were transformed to inverse.

[b]This group differed statistically from all groups for all variables ($p<0.05$).

[c]This group did not differ statistically from group received 25 µg F/mL for all variables ($p>0.05$).

[d]This group did not differ statistically from group received 62.5 µg F/mL for all variables ($p>0.05$).

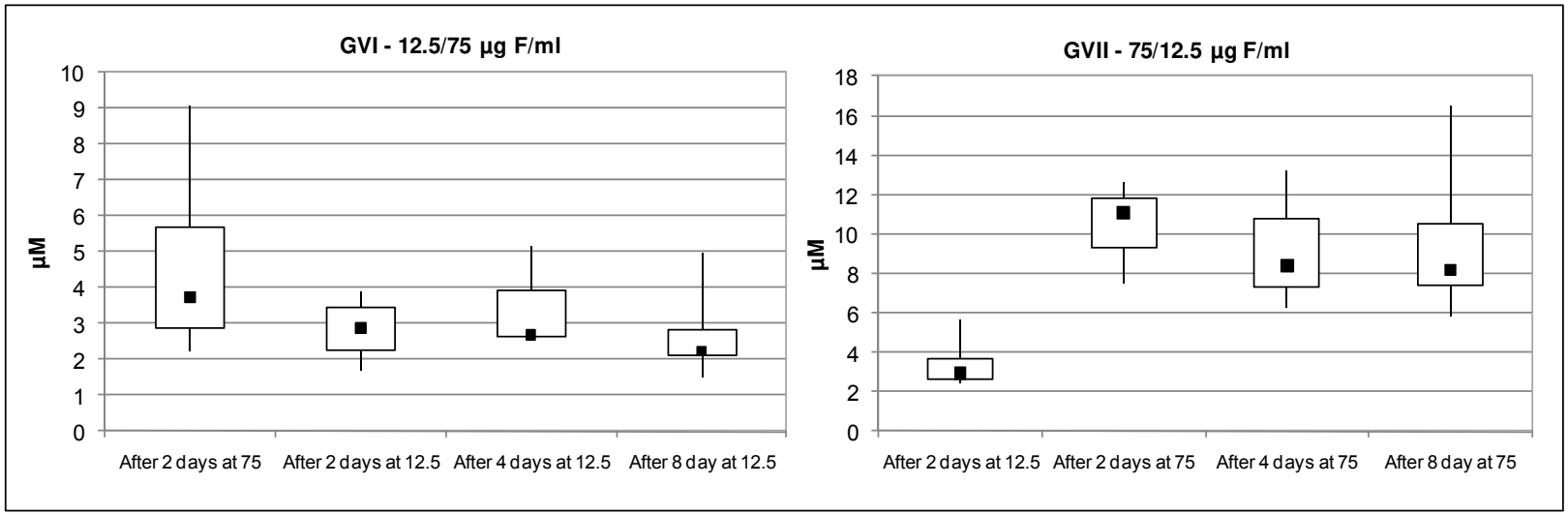

Figure 2. Box plots of fluoride concentration in plasma (µM) of groups that received oscillating doses (µg F/mL).

4. Discussion

Since there is no homeostatic mechanism to maintain F concentrations in the blood, plasma F concentrations will vary upon F exposure (Waterhouse *et al*., 1980; Ekstrand, 1996). In a previous study, Catani *et al*. (2010) showed that the biological effect of F when an animal is exposed chronically to oscillating F doses during symmetrical and brief periods reflects that caused by a constant F dose corresponding to the mean of the oscillating range. The results of the present study showed that the effect of ingesting a higher or lower F dose, during few days, still seems to reflect the effect of a mean exposure to the oscillating doses considering the time of exposure. This could be explained by the small thickness of enamel formed during each exposure period, suggesting that the mean exposure to F during a given time is the one that adequately reflects the resulting enamel fluorosis. Others experimental studies indicate that the severity of dental fluorosis is a function of both the dose and the duration of exposure to F (Angmar-Månsson and Whitford, 1984). Thus, considering that enamel formation is a continuous process and that the fluorosis degree depends on the length of exposure of ameloblasts to F during the maturation phase (Aoba and Fejerskov, 2002), the clinically visualized defects are dependent on the thickness of enamel formed under exposure to a given F dose. Thus, when there is change in the concentration (oscillating doses), the effect on the enamel will depend on the time that these concentrations are maintained constant during the enamel formation. Therefore, the resulting effect on F accumulation would be the mean effect of the concentrations range.

The plasma levels in rats also show a linear dose-dependent increase in plasma F levels, from 0.8 μM (no F in water) to 6.8 μM, with increases in F in drinking water from zero to 75 μg F/mL. In animals receiving oscillating doses, plasma F levels increased significantly after two days receiving high F dose (75 μg F/mL). Also the plasma level decreased only 2 days after discontinuation of a high

F regimen, or increased 2 days after increasing the F dose. This agrees with other studies, which observed that plasma levels decreased after removing moderate to high doses of F (50 to 100 ppm) from the drinking water of rats, but remained slighted elevated (Ekstrand *et al*., 1981; Larsen *et al*., 1981). In fact, the mean values of plasma F while rats receiving the oscillating doses were drinking 12.5 µg F/mL were slightly higher than those of rats receiving constantly 12.5 µg F/mL (Table 1, Figure 2). These slightly elevated values could be the result of continuous remodeling of bone, which would have incorporated higher F concentrations due to the high F dose used in the oscillating groups (Robinson *et al*., 1996).

The results of the image analysis method of the enamel surface show great variability of degree of the enamel defects among animals of the same groups, specially for the high F concentrations used (higher than 62.5 µg F/mL). Studies on the effects of F on ameloblast modulation (Denbesten *et al.,* 1985; Smith *et al.,* 1993) showed that even when a constant 100 ppm F dose is used in the drinking water, with similar plasma F levels, some rats in the F-exposed group (approximately 15%) seemed profoundly affected by the treatment, while others showed quantitative parameters that were almost within the normal range. Although the DFIA showed promising results to quantify dental fluorosis in rats when doses of up to 37.5 µg F/mL were used (Catani *et al*., 2010), it seems to be influenced by the greater variability of the high doses used in the present study.

5. Conclusion

The results suggest that the biological effect of F when an animal is exposed chronically to oscillating F doses, regardless of time of oscillation or of the difference between the exposures doses, may reflect the mean effect of the duration period of exposure to the oscillating doses.

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CAPÍTULO 3

COMPARISON OF DIFFERENT METHODS TO EVALUATE DENTAL FLUOROSIS IN RATS

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Abstract

Rat models have been successfully used to study enamel defects caused by chronic exposure to fluoride (F), but the quantification of these defects, especially when mild alterations are present, is still a challenge. The aim of this study was to compare 5 different methods to quantify dental fluorosis in rats. The study was approved by the Animal Ethics Committee of UNICAMP and 35 Wistar female rats, aged 35 days, were randomly divided into 7 groups of 5 rats, which received water *ad libitum* at increasing F concentrations: 0, 12.5, 25, 50, 62.5, 75 or 100 µg F/mL. After 70 days, rats were sacrificed, F exposure was determined by the F concentration in the blood and femurs; and dental fluorosis was analyzed on the incisors. Transverse microradiography, cross sectional microhardness and bright field microscopy were only able to detect the fluorotic defect in the rat incisor when high F concentrations (≥ 50 µg F/mL) were used; image analysis method based on the pattern of dark and white bands of the fluorotic rat incisor enamel was satisfactory to quantify dental fluorosis, even at low F concentrations; and quantitative light-induced fluorescence analysis did not allow the differentiation among the groups. The present study suggests that among the evaluated methods, the dental fluorosis by image analysis method is the only one able to quantify small alterations caused by F on rat enamel.

Key words: fluoride, dental fluorosis, quantitative analysis, image, microradiography, microhardness, bright field microscopy, QLF, rats, incisors.

1. Introduction

Rat models have been successfully used to investigate the metabolism of fluoride (F) and its effect at chronic doses on the development of dental fluorosis (Aoba and Fejerskov, 2002).

To determine the degree of mineralization of these hypomineralized areas and thus quantify the severity of fluorosis in rats, some indirect indicators of the degree of dental mineralization employed to assess demineralization due to dental caries have been used, such as: transverse microradiography (TMR), cross-sectional microhardness (CSMH), bright field microscopy (BFM) and quantitative light-induced fluorescence (QLF). However, there is no information in the literature about the sensitivity of the different methods to detect enamel defects induced by F at increasing levels in the rat incisors.

Also, the vestibular surface of normal rat incisor enamel has an orange color caused by iron deposition at the end of enamel maturation (Pindborg, 1947). However, fluorotic rat incisor enamel shows a typical pattern of pigmented (orange) and white strains on the enamel (Kato *et al*., 1988), and there is evidence that the white bands represent hypomineralized areas of the fluorotic enamel (Saiani *et al*., 2009). This pattern of white bands on rat fluorotic enamel, which are present even at exposure to low F concentrations, could be used to quantify the severity of the defect. Based on this, we have developed a method to quantify dental fluorosis by image analysis (DFIA) of the enamel surface.

Thus, the aim of this study was to compare five methods (TMR, CSMH, BFM, QLF and DFIA) on the quantitative evaluation of dental fluorosis in rats.

2. Material & Methods

2.1. Experimental design

In the blind study, previously approved by the Animal Ethics Committee of University of Campinas (UNICAMP, protocol # 1579-1), 35 Wistar females rats, aged 35 days, were randomly divided into 7 groups with 5 animals each, which received water at increasing F concentrations: 0, 12.5, 25, 50, 62.5, 75 or 100 µg F/mL. The F solutions were prepared with NaF and the concentrations were checked. The rats were fed *ad libitum* with pellet chow (Labina, Purina®, Paulínia, SP, Brazil; total F concentration of 17 µg F/g). The amount of water ingested by each group was assessed daily and animals were weekly weighed. After 70 days, enough time to allow the full renewal of rat incisors (Schour and Massler, 1971), the rats were anesthetized and blood was collected by disruption of the brachial plexus using heparin as an anticoagulant. One femur, mandible bone and incisors of the animals were removed after their death and coded for the analyses (blind study concerning the examiner).

2.2. Fluoride analyses

Plasma fluoride determination

Plasma samples were obtained by centrifugation at 2,000 *g* for 10 min. F concentration in plasma was determined using an ion-specific electrode (Orion Research, model 94-09, Orion Research Incorporated, Cambridge, MA, U.S.A.) and a reference electrode (Flex-Ref, WPI Inc., Sarasota, FL, U.S.A.) both coupled to a potentiometer (Orion Research, model EA 940, Orion Research Incorporated, Cambridge, MA, U.S.A.), against F standards, after buffering with total ionic strength adjustment buffer (TISAB III).

Fluoride determination in the femur (whole bone) and mandible (alveolar bone)

The analysis for whole bone F determination was done according to Bezerra de Menezes *et al*. (2003). The femur was dried for 24 h at 60°C, sectioned transversally to obtain one slice measuring approximately 5 mm from the diaphysis region. This slice was pulverized, the powder was sieved to obtain particles of 140 µm to 1,000 µm and left at 60°C for additional 24 h. Ten milligrams (± 0.01 mg) were weighed and transferred to plastic test tubes containing 1.0 mL of 0.5 M HCl. After 1 h, at room temperature and under agitation, 1.0 mL of TISAB II (1.0 M acetate buffer, pH 5.0 with 1.0 M NaCl and 0.4 % cyclohexanediaminetetraacetic acid) was added. To this TISAB solution NaOH was added at concentration of 0.5 M to neutralize the HCl.

For F determination on the surface of incisor alveolar bone, one hemi-mandible from each animal was dried for 24 h at 60ºC. The lingual blade of the alveolar socket was removed, thereby exposing the rest of the alveolar socket. All other regions were isolated with acid-resistant nail varnish. Using an automatic pipette, 0.1 mL of 0.5 M HCl was applied to the alveolar socket surface and the acid was flowed for 15 s by aspiration and injection. The extract was transferred to an assay tube and buffered as described before.

F in all extracts was determined as described for the plasma, but the F standards were prepared under the same conditions as the samples. The amount of bone removed from the surface of incisor alveolar socket was calculated by determining inorganic phosphorus (Fiske and Subbarow, 1925) in the acid extracts and assuming that its concentration in dry bone is 13.5%. The bone surface layer removed (µm) was estimated assuming a bone density of 2.14 g/cm^3. All results were expressed as µg F/g dry weight.

2.3. Dental fluorosis assessment

Transverse Microradiography (TMR)

One mandible incisor from each animal was worn off to give planoparallel specimens of 80 µm (± 5.6) thickness from the middle portion of the crown using a disc (Multiprep™ System, Allied Hight Tech Products, Inc., Rancho Dominguez, CA, U.S.A.). The specimens were mounted on a microradiographic plate-holder bearing an aluminium stepwedge (25 mm steps). The microradiographs were taken with a 10 min exposure time on Kodak high-resolution plates (Type 1A, Eastman Kodak Co., Rochester, NY, U.S.A.), using a Cu(Kα) X-ray source (Philips BV, Eindhoven, The Netherlands) operating at 25 kV and 10 mA at a focus–specimen distance of 30 cm. The plates were developed using standard techniques according to the manufacturer's recommendations. The microradiographs were examined using a Leica DMRB microscope (Leica, Wetzlar, Germany). The image was captured at a magnification of 20 x/0.40 via a CCD video camera (Sony, Tokyo, Japan) connected to a computer (Viglen PC, London, U.K.). The degree of deficient enamel mineralization (fluorosis) was calculated from the integrated % of volume mineral versus enamel depth (µm) by a two-step image analysis technique (Amaechi *et al.*, 1998) using a software package (TMRW v.1.22, Inspektor Research System BV, Amsterdam, The Netherlands) based on the work described by de Josselin de Jong *et al*. (1987).

Cross-sectional Microhardness (CSMH)

One upper incisor from each animal was embedded in acrylic resin with its long-axis plane-parallel to the surface (Arotec EMB-30, Arotec Ind. Com. Ltda., Cotia, SP, Brazil) and abraded (Arotec APL-4, Arotec Ind. Com. Ltda., Cotia, SP, Brazil) with a 600-grit paper until the center of the incisors was exposed. Polishing was done using a 1,200-grit paper and a 1-µm diamond particles polishing paste. Microhardness was evaluated by means of a Knoop microhardness tester (HMV-2000, Shimadzu Corp., Nakagyo-ku, Kyoto, Japan) at 3 different regions (apical,

medial and incisal) (Figure 2a). A Knoop diamond was used under a 25 g load applied for 5 s, with the long axis of the diamond being parallel to the outer enamel surface. Three indentations were made at 10 μm from the enamel surface and one at 30, 50 and 70 μm from the enamel surface (Figure 2a).

Bright Field Microscopy (BFM)

For qualitative evaluation, the same specimen used in TMR analysis was immersed in distilled water, mounted on a glass microscope slide and examined under bright field using a Nikon Optiphot® light microscope with rotating stage, polarizer and analyzed at a magnification of 450 x (Nikon, Tokyo, Japan). The thickness of the hypomineralized bands observed by bright field microscopy was measured (Figure 3a, letter H).

Quantitative Light-induced Fluorescence (QLF)

One mandible central incisor from each animal was used in this analysis. First, the fluorescence of the iron banding area on the surface of the enamel was analyzed. However, due to the iron deposition, the fluorescence of dentin through enamel could not be observed. Thus, the more apical area of immature enamel, preceding the iron deposition area, was analyzed. To acquire the input images, the incisors were fixed with wax on a glass slide with the area to be analyzed parallel to the QLF camera (RGB) attached to a vertical stand at a fixed distance of 4 cm above the teeth. A black cardboard was kept under the glass slide to eliminate and standardize the background fluorescence. An image was acquired with the camera connected to a controlbox (Inspektor Research Systems BV, Amsterdam, The Netherlands) and analyzed by QLF Patient software, version 2.00d.

Dental Fluorosis by Image Analysis Index (DFIA)

In order to determine dental fluorosis, we proposed a new image analysis method (DFIA) of digital pictures of the enamel surface, which is based on the effect of F changing the iron pigmentation of the rat incisor enamel surface resulting in a typical pattern of orange and white bands (Figure 5a). To acquire the input images, one mandible incisor from each animal was fixed with wax on a glass slide (Figure 4) with the enamel surface of incisal third of the crown upward parallel to a macro lens (Medical Nikkor, Nikon, Tokyo, Japan) attached to a vertical stand at a fixed distance of 7 cm above the teeth. An image was acquired with the digital camera (Nikon D70, Nikon, Tokyo, Japan) using standardized light intensity and focus. A central area of 690x170 pixels was cropped from the digital image and converted to grayscale (Figure 4). For the cropped image, the DFIA index was obtained by difference between I_H (the average of the higher intensity pixel values, originally white bands) and I_L (the average of the lower intensity pixel values, originally orange bands). Higher values of DFIA index should correspond to higher fluorosis severity.

2.4. Statistical analysis

Linear regression analysis was used to correlate the administered F concentrations in water with the respective measured F concentration in plasma and bones, and dental fluorosis (SPSS software, version 12.0, SPSS Inc., Chicago, IL, U.S.A.). Analysis of variance was used to test the significance of differences among groups, followed by Tukey test as a post-hoc test (SAS software, version 8.01, SAS Institute Inc., Cary, NC, U.S.A.). Data which did not fit the assumptions of normal distribution of errors and equality of variances were transformed (Box *et al.*, 1978). All analyses were performed with α level fixed at 5%.

3. Results

No difference in water intake (daily) and rats weights (either initial or final) was observed among groups ($p > 0.05$).

A significant increase in the F concentrations in plasma and bone (whole femur and surface of incisor socket) was observed according to the increase in F concentration in the water (Table 1). The correlations for the linear regression models lied between 0.89 and 0.98, with $p < 0.0001$ (Table 2).

Table 1. Mean ± SD (n=5) of fluoride concentrations in rats plasma and bone as a function of fluoride concentration in water.

[F] in water (µg F/mL)	Plasma (µM)	Whole femur (µg F/g)	Alveolar bone surface (µg F/g)
0	0.8 ± 0.2 ^{a}	161.4 ± 17.8 ^{a}	296.5 ± 76.5 ^{a}
12.5	1.8 ± 0.5 ^{b}	441.8 ± 52.9 ^{b}	722.7 ± 98.4 ^{b}
25	2.4 ± 0.6 ^{b}	770.5 ± 159.9 ^{c}	1209.7 ± 371.5 ^{c}
50	5.3 ± 1.8 ^{c}	1276.0 ± 136.1 ^{d}	1769.8 ± 237.4 ^{cd}
62.5	5.0 ± 0.6 ^{c}	1632.7 ± 225.5 ^{de}	1948.4 ± 230.7 ^{d}
75	6.8 ± 2.0 ^{c}	1913.5 ± 193.0 ^{ef}	2379.4 ± 202.5 ^{d}
100	8.8 ± 2.9 ^{c}	2181.2 ± 172.6 ^{f}	2390.1 ± 218.9 ^{d}

Distinct letters follow means which differed by ANOVA ($p>0.05$). In order to fit the assumptions for this test, data of plasma fluoride and alveolar bone surface fluoride were log-transformed and data of whole bone fluoride were transformed to the square root.

Using TMR a significant decrease in degree of mineralization (represented by the deficient mineralization area) could be observed only in groups that received high F concentrations (≥ 50 µg F/mL), when compared to the lower concentration groups and the negative control ($p < 0.01$) (Figure 1). Although the correlation for the linear regression models was 0.95, with $p < 0.0001$ (Table 2), no

deficient mineralization was observed in groups received lower F concentration (< 50 µg F/mL) (Figure 1). The highest deficient mineralization was observed for group 100 µg F/mL when compared to the others ($p < 0.05$). However, no difference in radiopacity could be observed between the areas presenting different surface banding pattern (white or pigmented striae).

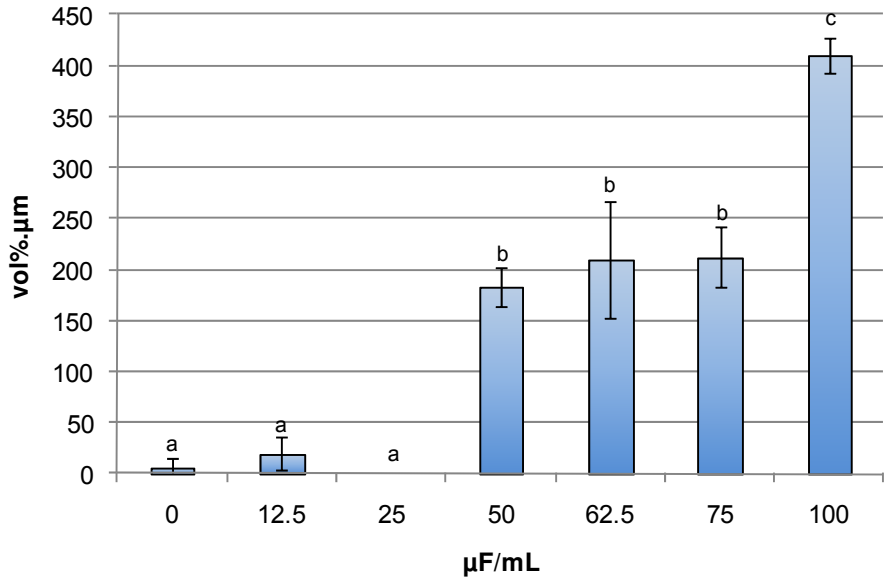
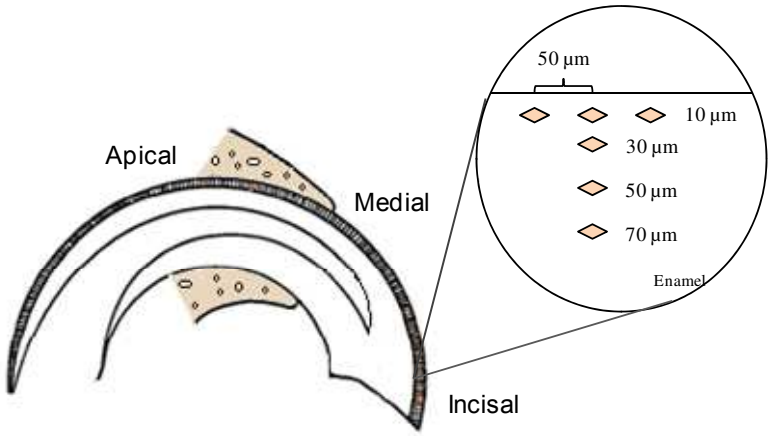


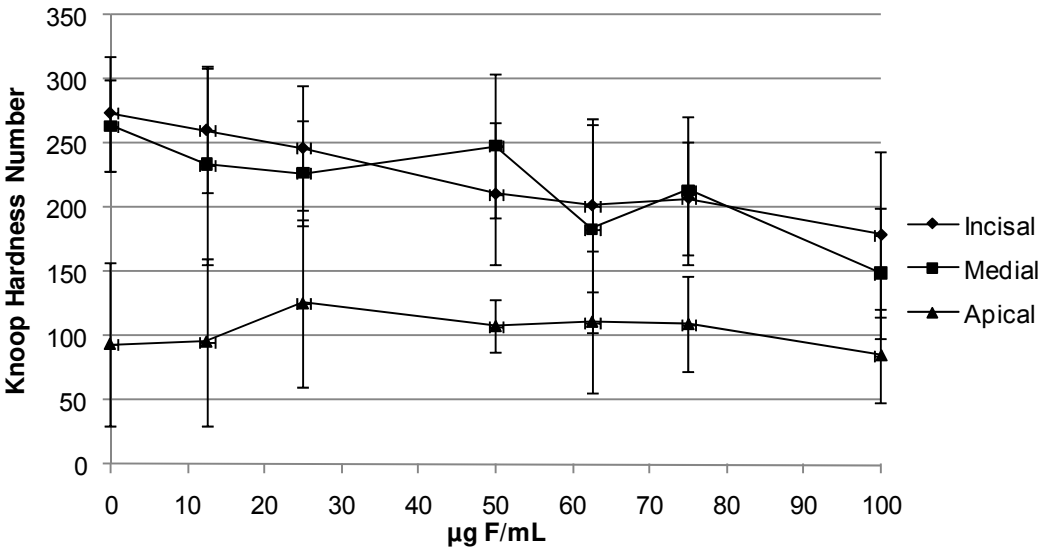
Figure 1. Mean and SD (n=5) of the degree of deficient enamel mineralization (vol%µm) by TMR analysis as a function fluoride concentration in water (µg F/mL). Note the decrease in degree of mineralization in groups received high fluoride concentrations (≥ 50 µg F/mL). Distinct letters (a-c) follow means which differed by ANOVA ($p > 0.05$).

The results of CSMH analysis showed no difference in hardness among groups at 30, 50 and 70 µm from the surface ($p > 0.05$). However, a tendency for decrease in hardness at the very superficial layer of enamel (10 µm) in the medial and incisal regions of the rat incisor crown was observed (Table 2; Figure 2b), although no statistically significant difference among the groups was observed

(Figure 2b). Also, at the apical region, all groups presented lower hardness values, compatible with a lower degree of mineralization.



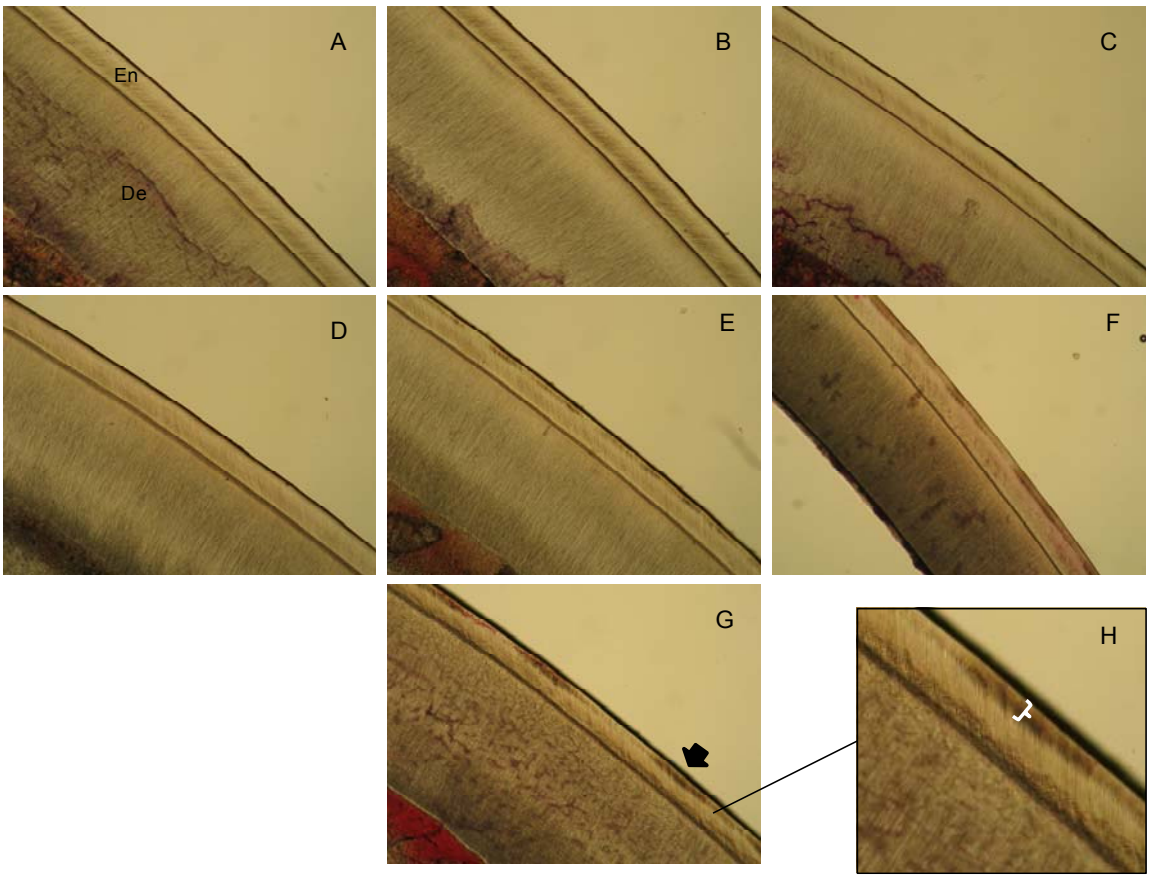
2a



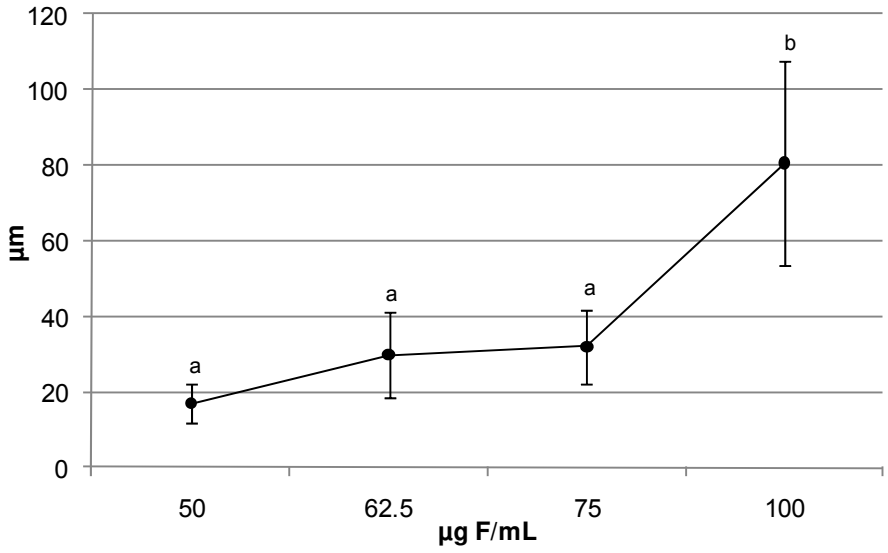
2b

Figure 2. (a) Schematic representation of the measured points of microhardness of enamel. (b) Mean and SD (n=5) of the enamel microhardness values (kg/mm^2) at 10 µm from enamel surface by Cross-sectional Microhardness as a function of fluoride concentration in water (µg F/mL). Note a tendency for decrease in hardness in the medial and incisal regions of the rat incisor.

Figure 3a shows water-immersed transversal sections of control and fluorotic enamel analyzed by BFM. White (normal) and dark (hypomineralized) areas corresponding to the iron banding observed at the enamel surface were observed in groups receiving 50 µg F/mL or more. Figure 3b shows the results of the thickness of hypomineralized (dark) regions in groups' drinking water with 50 µg F/mL or more. The thickness of hypomineralized area of the fluorotic incisors was higher for the rats receiving 100 µg F/mL in the water when compared to the other three groups (Figure 3b) ($p < 0.001$). Since no hypomineralized bands could be visualized and measured using BFM in enamel exposed to F concentrations lower than 50 µg F/mL, this method was not evaluated by linear regression.



3a



3b

Figure 3. (a) Examples of bright field microscopy images of transversal sections from control and fluorotic mandible rat incisors. A: control group; B: 12.5 µg F/mL; C: 25 µg F/mL; D: 50 µg F/mL; E: 62.5 µg F/mL; F: 100 µg F/mL. En: enamel; De: dentine; arrow: hypomineralized region (bands); brace: thickness of hypomineralized bands. (b) Mean and SD (n=5) of the thickness (µm) of hypomineralized bands according to groups received 50, 62.5, 75 and 100 µg F/mL in water by BFM analysis. Note the thickness of hypomineralized area of the fluorotic incisors was higher for the rats receiving 100 µg F/mL. Distinct letters follow means which differed by ANOVA ($p > 0.05$).

Using QLF it was not possible to observe significant differences among the groups receiving water with different F concentrations (0 to 100 µg F/mL) ($p > 0.05$). Also, the correlation for the linear regression models was not observed (0.13, $p = 0.45$, Table 2).

The images of rats mandible incisors of control group (0 µg F/mL) show that normal enamel displays an even orange coloration, while groups exposed to higher F concentrations in the drinking water (12.5, 25, 50, 62.5, 75 and 100 µg F/mL) show a succession of white and pigmented bands, with increase of lack of iron pigmentation in enamel surface according to the increase in F concentration

(Figure 5a). Also, using DFIA was possible to observe that fluorosis severity increased with the increase in F concentration in the water (Figure 5b). The correlation for the linear regression model was 0.77, with $p < 0.0001$ (Table 2).

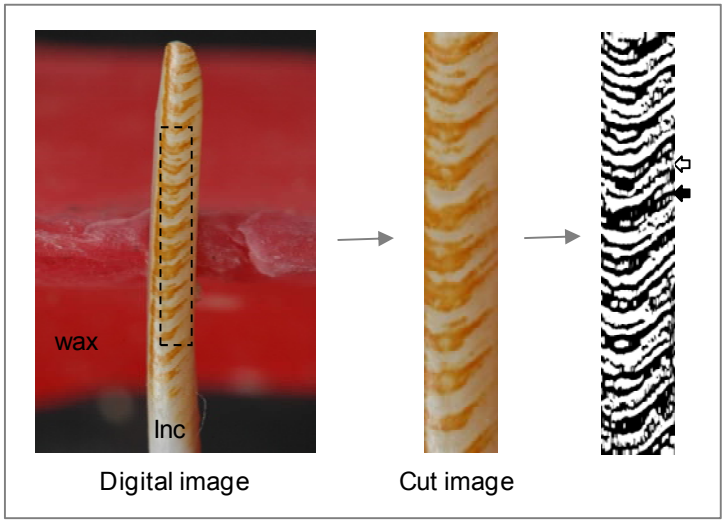
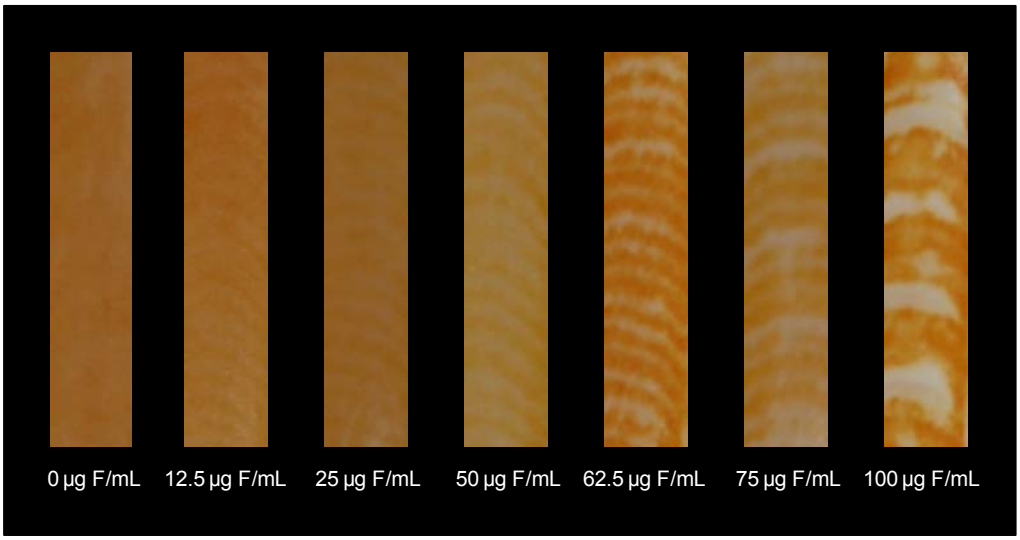
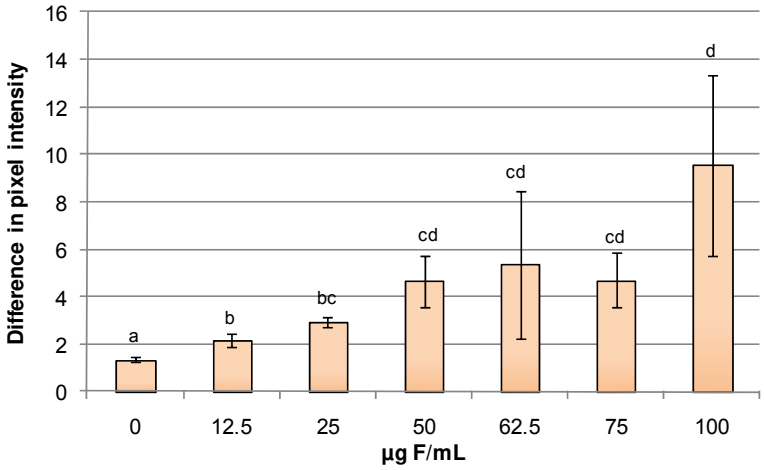


Figure 4. Schematic representation of the DFIA index for dental fluorosis quantification. Inc: mandible incisor; dashed line: cropped area; dark arrow: higher intensity pixels (white bands); white arrow: lower intensity pixels (orange bands). The difference between the means of higher and lower intensity pixels expresses fluorosis index (DFIA).



5a



5b

Figure 5. (a) Cut photographs images of rat incisors enamel surface of group received water with fluoride concentration in water at 0, 12.5, 25, 50, 62.5, 75 and 100 µg F/mL. Normal enamel displays an even orange coloration and fluorotic enamel is characterized by a succession of orange and white bands. (b) Mean and SD (n=5) of the fluorosis score (difference in pixel intensity) by DFIA method as a function of fluoride concentration in water (µg F/mL). Distinct letters follow means which differed by ANOVA ($p > 0.05$). In order to fit the assumption for this test, data were log-transformed.

Table 2. Correlation (r) between fluoride concentration in rats plasma and bone, and dental fluorosis by TMR, CSMH, QLF and DFIA (y) as a function of fluoride concentration in water (x).

y	x	r	Equation
Plasma	[F] in water	0.889 (p < 0.0001)	y = 0.684 + 0.079x
Whole femur	[F] in water	0.975 (p < 0.0001)	y = 214.887 + 21.147x
Alveolar bone	[F] in water	0.935 (p < 0.0001)	y = 512.309 + 21.940x
TMR	[F] in water	0.947 (p < 0.0001)	y = -39.754 + 4.049x
CSMH Incisal	[F] in water	0.554 (p = 0.001)	y = 269.637 - 0.942x
CSMH Medial	[F] in water	0.499 (p = 0.004)	y = 261.844 - 0.935x
CSMH Apical	[F] in water	0.043 (p = 0.814)	y = 106.309 - 0.059x
QLF	[F] in water	0.134 (p = 0.452)	y = 70.028 + 0.043x
DFIA	[F] in water	0.774 (p < 0.0001)	y = 1.068 + 0.071x

4. Discussion

A linear dose-dependent increase in plasma and bone was observed according to the increase in F concentration in the water, what was expected, considering that the higher the level of F exposure, the greater the concentration of F in the plasma and, therefore, incorporated in the mineral tissues (Ekstrand *et al*., 1981).

The results of the TMR analysis showed a lower degree of the enamel mineralization with an increase in the F concentration in the water, which was clearly observed only for groups exposed to 50 µg F/mL in the water or more. This suggests that the TMR may not be a sensitive method to detect dental fluorosis in rats exposed to lower F concentrations. Using thinner rats incisors sections (30 to 35 µm of thickness), however, Shinoda (1975) could observe that the radiolucency subsurface area expanded to inner layer when high F concentration (≥ 68 ppm F) in drinking water was administered.

The results of the microhardness on the incisal and medial regions (at 10 μm) of the rat incisor suggest that fluorotic lesions are shallow. Also, there is a trend of loss of hardness according to the increase in F concentration in the water, although no statistically significant difference between the groups used in this study was observed. Microhardness studies have indicated that subsurface areas are softer with increasing F concentration in drinking water (Shinoda, 1975; Suckling and Thurley, 1984). It is suggested that F may change crystal size, number, shape, or quality by interfering with their formation (Eanes and Hailer, 1998). However, this trend was not observed in the apical region, which presented lower hardness than mature regions. This might be due to porosity of the rats incisor enamel in this region because the apatite crystals have not yet increased in width (Selvig and Halse, 1972).

In addition, the data suggest that both TMR and CSMH, conventional methods used to assess hard tissue alterations, can only be used to quantitatively evaluate the fluorotic defect in the rat incisor if extensive alterations in enamel structure (mechanical and physical) are already present, in accordance to Shinoda (1975) and Angmar-Månsson and Whitford (1982).

Using BFM, it was possible to see, in groups' drinking water with 50 μg F/mL or more, an area with hypomineralized enamel, corresponding to the regions of white bands seen on the surface of the incisors. The thickness of this hypomineralized area was higher in the rats receiving 100 μg F/mL, suggesting a deeper defect in enamel mineralization in this group. Considering the thickness of these areas observed by BFM, it is possible to infer that this method is more sensitive than CSMH to study enamel alterations caused by F, since the hardness was only decreased in the upmost 10 μm of enamel. On the other hand, the lower degree of mineralization observed using TMR in groups receiving water with 50 μg F/mL or more agrees with the BFM results, but with the latter, the pattern corresponding to the surface banding of enamel could be clearly observed. BFM analyzes aspects of the molecular organization of the structure, while TMR is an

indicator of the degree of dental mineralization. Thus, the results suggest that microscopy analysis is more sensitive to mineral loss than microradiography and may be a very useful for researchers trying to better characterize these defects under high F concentration. In fact, the polarized light microscopy was successfully used by Saiani *et al*. (2009) to study the characteristic of fluorotic lesions in the rat incisors.

It has been shown that the QLF method is a sensitive and reproducible method for quantification of enamel lesions on smooth surfaces (Tranaeus *et al*., 2005), although some factors can influence the measurements (Angmar-Månsson *et al*., 2000, Al-Khateeb *et al*., 2002). The method depends on the presence of sound enamel surrounding the lesion, and although useful to quantify localized areas of mineral loss due to caries, in fluorosis the deficient mineralization is usually diffuse across the entire tooth surface (Ellwood and O'Mullane, 1995). Thus, this technique used in the assessment of carious mineral loss may be inappropriate to accurately measure enamel fluorosis (Pretty *et al*., 2006). In the present study, the area of mature enamel could not be used for QLF evaluation since the iron banding blocked the fluorescence of the dentin. At the pre-banding, apical area of enamel, however, no difference in the fluorescence through enamel could be observed among groups. Taking into account that the CSMH data of the apical region of the incisor also did not show a difference between groups, it could be argued that it would not be possible to see enamel alterations in this region of enamel using QLF. Interestingly, an increase in fluorescence has been described in fluorotic area of the mice incisors (Everett *et al*., 2002; Carvalho *et al*., 2009). This suggests that further studies are necessary to understand the effect of fluorotic alterations on enamel on the resulting fluorescence evaluated by QLF.

Rat fluorotic enamel shows macroscopically a pattern of repeated pigmented (orange) and white bands on the labial surface of incisors and there is evidence that the white bands of fluorotic enamel represent hypomineralized areas of the enamel (Saiani *et al*., 2009). In the present study, this typical pattern of

orange and white bands was used to quantify enamel fluorosis using a digital image analysis of the enamel surface, the dental fluorosis by image analysis (DFIA). In accordance to the visual examination, an increase of scores of dental fluorosis by DFIA according to increasing F concentration in water was observed, even when low F concentration are used. The data showed that processing the digital images of enamel surfaces and computing the average difference in pixel intensity can be satisfactorily be used to quantify the fluorotic defects and may be a suitable method to quantify enamel fluorosis in rat incisors, because the images of the enamel surfaces contains useful features to characterize the groups and that this information can be extracted and evaluated using simple image analysis methods. In additional, the results suggest that the DFIA may be a promising tool as an objective, potentially blinded system, to evaluate dental fluorosis in epidemiologic studies, however, further work is required to determine this potential.

5. Conclusion

The present study suggests that among the methods employed to evaluate rats' dental fluorosis, the dental fluorosis by image analysis (DFIA) was the simplest, sensitive and may be a suitable method to quantify mild alterations caused by F in rat incisors.

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CONSIDERAÇÕES GERAIS

O estudo dos fatores relacionados ao desenvolvimento de fluorose dental é imprescindível para a adequada compreensão dos mecanismos que levam a ocorrência desse defeito no esmalte e sua gravidade. Embora muito já se saiba sobre prováveis mecanismos envolvidos, o estudo da fluorose dental ainda é desafiador para pesquisadores.

O modelo animal tem sido usado com sucesso na avaliação do desenvolvimento da fluorose (Aoba & Fejerskov, 2002), e nesse sentido o rato apresenta a vantagem de permitir a realização de estudos em curto período de tempo, com delineamentos dinâmicos e flexíveis devido ao crescimento contínuo do incisivo. Neste estudo, a fluorose dental foi estudada em ratos expostos a água com diferentes concentrações de F, sendo avaliado o efeito da manutenção do estímulo ou da oscilação nas doses de exposição no desenvolvimento de fluorose.

Assim, foi observado um aumento nas concentrações de F nas variáveis metabólicas de acordo com o aumento na concentração de F na água, o que era esperado, considerando que quanto maior a concentração de F exposta cronicamente, maior a concentração depositada nos tecido (Ekstrand *et al*., 1981). Ao mesmo tempo, o efeito do incremento da concentração de F exposta foi refletido na gravidade da fluorose dental (Aoba & Fejerskov, 2002).

No entanto, não foi observada diferença significativa entre grupos que receberam doses alternantes de F na água e grupos que receberam doses constantes correspondentes à média das oscilações. É conhecido que não há um mecanismo homeostático para manter a concentração de F no sangue (Waterhouse *et al*., 1980; Ekstrand, 1996), mas que o F pode retornar ao sangue através de sub-compartimentos do osso que sofrem constantes remodelações

(Pendrys & Stamm, 1990). Portanto, quando a exposição ao F é interrompida, a concentração no sangue pode diminuir, como também, nos sub-compartimentos ósseos (Rao *et al*., 1995), e este declínio pode demorar alguns dias (Likins *et al*., 1956; Zipkin *et al*., 1956). Foi observado que após 2 dias já se observa tal redução na concentração plasmática de F. E o oposto também foi observado para o efeito do incremento da concentração de F exposta. Assim, o efeito biológico do F quando um animal é exposto a concentrações alternantes, por períodos curtos e simétricos ou por períodos discrepantes, pode corresponder à média do efeito do tempo de oscilação das doses.

Com relação aos métodos quantitativos de fluorose dental, os resultados do presente estudo, sugerem que métodos convencionais usados para avaliar alterações de tecidos duros, como radiomicrografia, microdureza e microscopia de luz polarizada, só conseguem quantificar defeitos de esmalte hipomineralizado (fluorótico), em ratos, quando alterações extensas, que afetam as propriedades físico-mecânicas da estrutura do esmalte, estão presentes (Shinoda, 1975; Angmar-Månsson & Whitford, 1982; Brighenti *et al*., 2006; Mousny *et al*., 2008). Já a fluorescência induzida por luz quantitativa pode ser inapropriada para mensurar fluorose dental em incisivos de ratos (Pretty *et al*., 2006).

A análise da imagem da superfície do esmalte, através da mensuração da média da diferença da intensidade de pixels, pôde quantificar satisfatoriamente fluorose dental em ratos, especialmente nos graus mais brandos quando os outros métodos ainda são insensíveis para detectar o defeito, e pode ser um adequado método para este tipo de modelo de estudo de fluorose.

CONCLUSÃO

O presente estudo sugere que o efeito biológico do F quando um animal é exposto a concentrações alternantes, por períodos curtos e simétricos ou por períodos discrepantes, pode refletir a média do efeito durante o tempo de oscilação das doses.

As alterações induzidas pelo F no esmalte dental de ratos resultam em um tecido menos mineralizado e com propriedades mecânicas diminuídas. No entanto, tais alterações são quantificáveis por métodos consagrados na avaliação mineral apenas quando a exposição ao F resulta em significativa alteração da estrutura mineral. Por outro lado, a análise do padrão de pigmentação da superfície do esmalte do incisivo do rato se mostrou um método adequado para quantificar fluorose em incisivos de ratos.

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* De acordo com a norma da UNICAMP/FOP, baseada na norma do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviaturas dos periódicos em conformidade com o Medline.

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APÊNDICE 1 - Dados referentes ao estudo do Capítulo 1.

Quadro 1. Média e desvio padrão da massa corporal dos animais (g), dos grupos experimentais, aferida após 30 dias do início do experimento e ao final do exeperimento.

	Grupo I - água		**Grupo II - 12,5 µg F/mL**		**Grupo III - 25 µg F/mL**		**Grupo IV - 37,5 µg F/mL**		**Grupo V - 12,5/37,5 µg F/mL**	
	30 dias (g)	Final (g)	30 dias (g)	Final (g)	30 dias (g)	Final (g)	30 dias (g)	Final (g)	30 dias (g)	Final (g)
Animal 1	261.52	300.13	244.01	280.73	240.37	282.72	247.06	283.05	220.11	254.82
Animal 2	204.49	241.87	233.22	270.77	242.09	271.21	253.46	292.33	203.22	235.89
Animal 3	245.56	311.28	256.22	286.13	228.36	263.22	244.81	276.72	251.74	304.60
Animal 4	212.94	247.61	230.34	265.71	239.00	283.10	229.35	257.16	223.40	260.13
Média	**231.13**	**275.22**	**240.95**	**275.84**	**237.46**	**275.06**	**243.67**	**277.32**	**224.62**	**263.86**
DP	26.91	35.57	11.76	9.28	6.19	9.63	10.23	14.89	20.13	29.08

Início do experimento: 03/09/2008

Final do experimento: 19/11/2008

Quadro 2. Média e desvio padrão (n=4) do volume de água ingerido diariamente pelos animais dos grupos experimentais.

Grupos	**Vol Total**	
	Média (ml)	dp
GI - água dest	112.81	8.61
GII - 12,5 µg F/mL	112.98	11.68
GIII - 25 µg F/mL	107.10	9.54
GIV - 37,5 µg F/mL	117.98	10.12
GV - 12,5/37,5 µg F/mL	112.63	8.56

Quadro 3. Média e desvio padrão da temperatura (ºC) diária durante o período do experimento.

	Externa (ºC)	Interna (ºC)
Média	29.77	24.82
dp	3.65	1.85

Concentração de fluoreto na ração = 17.02 µg F/g

APÊNDICE 2 - Dados referentes ao estudo do Capítulo 2.

Quadro 1. Média e desvio padrão da massa corporal dos animais (g), dos grupos experimentais que receberam doses constantes de fluoreto, aferida no início e ao final do experimento.

	Grupo I - água		Grupo II - 12,5 µg F/mL		Grupo III - 25 µg F/mL		Grupo IV - 62,5 µg F/mL		Grupo V - 75 µg F/mL	
	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)
Animal 1	117.07	222.26	116.88	208.26	142.12	234.86	132.56	236.44	116.38	266.73
Animal 2	129.27	247.44	123.48	219.35	128.13	221.38	141.46	254.92	100.82	233.06
Animal 3	124.15	248.85	122.22	243.16	135.57	250.75	124.64	247.32	94.04	262.23
Animal 4	125.39	238.62	110.34	204.34	133.74	237.29	133.97	248.57	106.51	297.47
Animal 5	120.38	239.56	136.80	248.87	123.81	226.19	128.53	252.08	94.45	216.37
Média	**123.97**	**239.29**	**118.23**	**218.78**	**134.89**	**236.07**	**133.16**	**246.81**	**104.44**	**264.87**
DP	5.09	12.22	5.99	17.45	5.77	12.03	6.89	7.67	9.45	26.36

Quadro 2. Média e desvio padrão da massa corporal dos animais (g), dos grupos experimentais que receberam doses oscilantes de fluoreto, aferida no início e ao final do experimento.

	Grupo VI - 12,5/75 µg F/mL		Grupo VII - 75/12,5 µg F/mL	
	Inicial (g)	Final (g)	Inicial (g)	Final (g)
Animal 1	132.82	251.78	144.95	249.74
Animal 2	136.73	245.33	128.37	236.61
Animal 3	133.65	261.51	138.15	252.16
Animal 4	129.14	229.48	137.29	233.23
Animal 5	123.76	221.03	140.05	258.08
Animal 6	140.55	236.43	148.41	242.70
Animal 7	146.48	236.53	132.64	246.91
Animal 8	138.51	233.47	139.62	240.94
Animal 9	125.61	223.39	134.61	241.64
Animal 10	131.33	222.34	134.28	226.71
Animal 11	141.02	250.57	146.15	263.09
Animal 12	146.43	254.87	150.32	284.51
Animal 13	130.43	226.93	139.59	270.66
Animal 14	137.22	228.23	140.24	251.71
Animal 15	136.85	250.71	133.61	258.13
Animal 16	150.56	246.45	135.90	241.91
Animal 17	122.92	214.86	.	.
Média	**133.09**	**247.03**	**137.19**	**242.94**
DP	3.12	13.46	6.81	9.41

Quadro 3. Média e desvio padrão do volume de água ingerido diariamente pelos animais dos grupos experimentais.

[F] na água (µg F/mL)	Média	DP
0	120.39	8.40
12,5	114.80	12.99
25	139.07	14.97
62,5	120.83	8.01
75	122.23	9.30
12,5/75	121.86	10.22
75/12,5	128.55	17.77

Concentração de fluoreto na ração = 17.04 µg F/g

APÊNDICE 3 - Dados referentes ao estudo do Capítulo 3.

Quadro 1. Média e desvio padrão da massa corporal dos animais (g), dos grupos experimentais, aferida no início e ao final do experimento.

	Grupo I - água		Grupo II - 12,5 µg F/mL		Grupo III - 25 µg F/mL		Grupo IV - 50 µg F/mL		Grupo V - 62,5 µg F/mL		Grupo VI - 75 µg F/mL		Grupo VII - 100 µg F/mL	
	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)	Inicial (g)	Final (g)
Animal 1	117.07	222.26	116.88	208.26	142.12	234.86	123.24	224.17	132.56	236.44	116.38	266.73	129.14	254.50
Animal 2	129.27	247.44	123.48	219.35	128.13	221.38	141.77	257.05	141.46	254.92	100.82	233.06	135.16	247.95
Animal 3	124.15	248.85	122.22	243.16	135.57	250.75	134.35	235.83	124.64	247.32	94.04	262.23	128.14	232.72
Animal 4	125.39	238.62	110.34	204.34	133.74	237.29	120.93	210.04	133.97	248.57	106.51	297.47	157.75	265.10
Animal 5	120.38	239.56	136.80	248.87	123.81	226.19	136.79	254.62	128.53	252.08	94.45	216.37	146.68	270.53
Média	**123.97**	**239.29**	**118.23**	**218.78**	**134.89**	**236.07**	**130.07**	**231.77**	**133.16**	**246.81**	**104.44**	**264.87**	**137.55**	**250.07**
DP	5.09	12.22	5.99	17.45	5.77	12.03	9.75	19.88	6.89	7.67	9.45	26.36	13.82	13.55

Quadro 2. Média e desvio padrão (n=5) do volume de água ingerido diariamente pelos animais dos grupos experimentais.

[F] na água (µg F/mL)	Média	DP
0	120.39	8.40
12,5	114.80	12.99
25	139.07	14.97
50	125.00	9.74
62,5	120.83	8.01
75	122.23	9.30
100	140.33	14.27

Concentração de fluoreto na ração = 17.04 µg F/g

ANEXOS

ANEXO 1

INFORMAÇÃO CCPG/002/06

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III, IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

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§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **1579-1**, sobre **"Efeito da variação da dose de exposição crônica de fluoreto no desenvolvimento da fluorose dental"**, sob a responsabilidade de **Prof. Dr. Jaime Aparecido Cury / Danilo Bonadia Catani**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **05 de agosto de 2008**.

C E R T I F I C A T E

We certify that the protocol nº **1579-1**, entitled **"Effetc of change of exposure fluoride chronic dose in the development of dental fluorosis"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **August 5, 2008**.

Campinas, 05 de agosto de 2008.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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ANEXO 3

JDR JOURNAL OF DENTAL RESEARCH®
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Manuscript #	10-0035
Current Revision #	0
Submission Date	2010-01-22
Current Stage	Under Consideration
Title	Fluorosis in Rats Exposed to Oscillating Chronic F Doses
Running Title	Oscillating chronic F doses and fluorosis
Manuscript Type	Research Report
Special Section	N/A
Category	Biological
Manuscript Comment	Number of words in the abstract: 150 Number of words in the abstract and text: 2555 Number of tables and figures: 3 Number of cited references: 17
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Abstract	Since blood fluoride concentration varies according to fluoride exposure, and dental fluorosis is related to the thickness of enamel formed under a given fluoride dose, we hypothesized that fluorosis produced by an oscillating chronic fluoride dose would reflect that caused by the mean of the oscillation during a given time. Rats received during 78 days water with fluoride concentrations constant at 0, 12.5, 25 or 37.5 μg F/mL, or oscillating concentrations of 12.5 and 37.5 μg F/mL every 72 h (mean exposure=25 μg F/mL). Fluoride concentrations in plasma, bone and incisor, and dental fluorosis scores increased linearly for constant fluoride concentrations in water ($p<0.0001$, r values=0.82-0.98). The results of the oscillating group and that receiving 25 μg F/mL did not differ statistically ($p>0.05$). The findings suggest that in animals chronically exposed to symmetrical oscillating fluoride doses, the resulting dental fluorosis reflects the metabolic effect of the mean of the oscillation.
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Manuscript Items

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