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Fluoride dose-response of human and bovine enamel caries lesions under remineralizing conditions

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ABSTRACT: Purpose: To investigate the relative fluoride dose-response of human and bovine enamel caries lesions under remineralizing conditions and utilizing an established pH cycling model. **Methods:** Early caries-like lesions were formed in human and bovine enamel, characterized using Vickers surface microhardness (VHN) and assigned to five dentifrice treatment groups: 0/250/1100 ppm fluoride as sodium fluoride (F as NaF) formulation 1; 1100 ppm F as NaF formulation 2; 1000 ppm F as monofluorophosphate (MFP) formulation 3. The daily pH cycling regimen comprised: 4×1-minute dentifrice slurry treatments; 1×4-hour acid challenge and intermittent remineralization in a 1:1-mixture of pooled human/artificial saliva. After 20 days, VHN of specimens were measured again and changes from lesion baseline calculated (REM). Subsequently, enamel fluoride uptake (EFU) was determined using the microdrill technique and specimens were demineralized again to determine their acid resistance (DEM). Data were analyzed using two-way ANOVA (factors: enamel, dentifrice). **Results:** Both enamel type and dentifrice as well as their interaction affected REM and DEM. EFU was only affected by dentifrice. Human and bovine enamel showed a good fluoride dose-response for REM and correlated well. However, bovine enamel showed more remineralization than human enamel. There were good correlations between dentifrice-F concentration vs. REM and EFU, and between REM vs. EFU, regardless of enamel type. (*Am J Dent* 2012;25:000-000).

CLINICAL SIGNIFICANCE: Although many researchers consider human and bovine enamel to be interchangeable, the results from this study suggest bovine enamel may not be a true replacement for human enamel, as our knowledge of the tissues' responses to anticaries agents and de- and remineralization challenges is far from what can be considered comprehensive.

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Introduction

Our understanding of the structural and compositional differences between human and bovine enamel and their relative susceptibility to cariogenic challenges has evolved significantly over the last few decades: bovine enamel was shown to be more porous,[1] to exhibit higher carbonate,[2] but lower fluoride contents,[3] a different prism arrangement,[4,5] larger crystallites[6] but smaller prism diameters[7] than human enamel. Similar radiodensity between human and bovine enamel were reported,[8] although a later study[9] highlighted a greater presence of interprismatic substance and "fibril-like" structures around prisms in bovine enamel. These structural and compositional differences would suggest faster lesion initiation and progression in bovine than in human enamel.[10-14] These differences, however, were postulated to be "mainly one of degree"[15] thereby suggesting that bovine and human enamel behave very similarly. Consequently, researchers have considered bovine enamel a suitable surrogate for human enamel in caries research. Yet, to this day, our understanding of the respective fluoride dose-response of human and bovine enamel lesions under dynamic conditions typical for the caries process remains poor. This knowledge is essential in determining the suitability of bovine enamel in caries research.

Laboratory methods are one of the key tools in caries research as they allow e.g. for the assessment of the anticaries potential of novel agents under highly controlled and cost-effective conditions, and thereby provide researchers with

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valuable information ahead of often costly in situ and/or clinical research. However, as laboratory models have to be seen as bridges to in vivo caries,[16] these laboratory models must mirror clinical conditions, where de- and remineralization alternate constantly (i.e. pH cycling) and are only interrupted during the (often very short) application of anticaries agents, such as fluoride. Several pH cycling models have been developed over the years, which can, albeit crudely, be divided into models with either net re- or demineralization outcomes, with typical examples being the models developed by White[17] (remineralization) and Featherstone et al.[18] (demineralization). Fluoride provides its anticaries benefits through several modes, including enhancement of remineralization and inhibition of demineralization. As one of the key modes of action for fluoride is the enhancement of remineralization[19] – models with a net remineralization outcome can be considered suitable to investigate potential differences in the relative fluoride dose-response of different hard tissues. Furthermore, only two comparative pH cycling studies,[20,21] utilizing net demineralization models have been reported, highlighting the need for further research utilizing net remineralization models to further our understanding of the tissues' behavior.

This laboratory study investigated and compared the relative fluoride dose-response of early human and bovine enamel caries lesions under remineralizing conditions and, using an established pH-cycling model, which overall response to different fluoride dentifrices was shown to correlate well with in situ observations.[22,23]

Materials and Methods

Specimen preparation - Enamel specimens were obtained from human permanent molars (only buccal and/or lingual surfaces were used) and bovine incisors (only buccal surfaces were used). Human teeth were obtained from dental offices located in the USA. Bovine teeth were obtained from a slaughterhouse in Ohio, USA, from cattle with an average age of 3 years (range: 18 months to 5 years) and which stem from several states in the USA. Both human and bovine teeth are received unidentified at the Oral Health Research Institute, Indiana University School of Dentistry on a weekly and monthly basis, respectively; therefore, determinations of origin, exact age and other characteristics of the donor (human or animal) are impossible due to the large number of teeth being received.

Tooth crowns were cut into 3 × 3 mm specimens using a low-speed saw (Isomet[a]). The teeth were stored in deionized water saturated with thymol during the sample preparation process. Specimens were ground and polished to create flat, planar parallel dentin and enamel surfaces using a Rotopol 31/Rotoforce 4[b] polishing unit. The dentin side of the specimens was ground flat to a uniform thickness with 500-grit silicon carbide grinding paper. The enamel side of the specimen was serially ground using 1,200, 2,400 and 4,000 grit paper. The specimens were then polished using a 1 µm diamond polishing suspension on a polishing cloth. Resulting specimens had a thickness range of 1.7 – 2.2 mm. The specimens were assessed under a Nikon SMZ 1500[c] stereomicroscope at ×20 magnification for cracks, hypomineralized (white spots) areas or other flaws in the enamel surface that would exclude them from use in the

study. Prepared specimens were stored at 100% relative humidity at 4°C until use. All specimens were prepared by the same, well-trained technicians using standard operating procedures. Eighteen specimens per dentifrice treatment group and per enamel type were used for this study.

Lesion formation - Artificial caries lesions were formed in the human and bovine enamel specimens by immersion into a solution of 0.1 M lactic acid and 0.2% Carbopol C907 which was 50% saturated with hydroxyapatite and adjusted to pH 5.0 with KOH.[17] Initial demineralization was performed at 37°C and at a ratio of 10 ml solution per specimen. Human enamel specimens were demineralized for a period of 116 hours and bovine enamel specimens for 51 hours to ensure they met the study inclusion criteria. Demineralization periods were chosen based on prior experience and to create lesions with comparable VHN_{base}.

Lesion baseline characterization - Initial hardness of the demineralized specimens was determined using a Vickers microhardness indenter at a load of 200 g for 15 seconds.[17] The average specimen surface microhardness (VHN_{base}) was determined from four indentations on the surface of each specimen. Only specimens with a mean VHN_{base} between 25 and 45 were accepted. Specimens were then assigned to groups and subgroups following a stratified randomization procedure, based on their VHN_{base}.

No sound enamel baseline hardness measurements were performed in the present study as these data were not needed in the calculation of remineralization efficiency and fluoride dose-response.

Test dentifrices - A total of five test dentifrices (all provided by GlaxoSmithKline[d]) utilizing three inherently different formulations were employed in the present study. Their details can be found in Table 1. NaF-1 dentifrices were exploratory formulations, whereas 1,100F NaF-2[e] and 1000F-MFP[d] were commercial products. Study technicians were blinded to the treatments which were labeled A to E. Dentifrices with formulation 1 were used to establish a F dose-response and therefore served as primary human vs. bovine enamel comparison. Dentifrices 1100F-NaF-2 and 1000F-MFP were merely included to provide additional information regarding the different enamel types' responses to different dentifrice formulations and a different fluoride salt. Fluoride bioavailability (i.e. free F) was in agreement with manufacturers' claims of total F content (determined using ion-selective electrode, data not shown).

pH cycling model - A modified version of the pH cycling model developed by White[17] was employed in the present study. The daily cyclic treatment regimen consisted of a 4-hour acid challenge in the lesion forming solution and four 1-minute dentifrice slurry treatment periods with specimens stored in a 1:1-mixture of pooled human/artificial saliva all other times. The treatment schedule as outlined in Table 2 was followed daily over a period of 20 days.

A 1:1-mixture of human saliva (wax-stimulated and from at least five healthy volunteers, pooled and refrigerated until use) and artificial saliva (2.20 g/l gastric mucin, 1.45 mM $CaCl_2 \times 2H_2O$, 5.42 mM KH_2PO_4, 6.50 mM NaCl, 14.94 mM KCl) was used as the remineralization medium. Fresh saliva

mixture was used each day (changed during the acid challenge period).

Dentifrice slurries were prepared by adding 5.0 g of dentifrice to 10 ml of fresh saliva mixture in a beaker with a magnetic stirrer. Fresh slurry was prepared for each subgroup just prior to each treatment. Dentifrice slurry and saliva treatments were stirred at 350 rpm, whereas the demineralization treatment was not. After each treatment, the specimens were rinsed briefly under running deionized water. All specimens were then placed back into the saliva mixture. The experimental phase was conducted at room temperature.

Post-pH cycling lesion characterization - The mean VHN_{post} of each specimen was determined, as described above, from four indentations on the surface of each specimen, next to the baseline indentations. The change in VHN vs. lesion baseline was calculated as follows: $REM^* = VHN_{post} - VHN_{base}$

*REM> 0 indicates the ability of a treatment to enhance remineralization after 20 days of treatments.

Enamel fluoride uptake (EFU) - After the VHN_{post} measurements, the fluoride content of each enamel specimen was determined using the microdrill technique to a depth of 100 µm. The diameter of the drill hole was determined. The enamel powder from the drill hole was collected, dissolved (20 µl of $HClO_4$ + 40 µl citrate/EDTA buffer + 40 µl deionized water) and analyzed for fluoride by comparison to a similarly prepared standard curve. Fluoride data was calculated as µg F/cm^3; i.e. ppm.

Determination of acid resistance - The resistance of the pH-cycled enamel specimens to a subsequent acid challenge was determined by placing the specimens into the lesion formation solution for one 2-hour period. Following this acid challenge, the mean VHN_{dem} of each specimen was determined, as described above, from four indentations on the surface of each specimen, next to the baseline indentations. The change in VHN vs. lesion baseline was calculated as follows: $DEM^* = VHN_{dem} - VHN_{base}$

*DEM > 0 indicates the ability of a treatment to enhance remineralization and prevent demineralization after 20 days of treatments.

Statistical analysis - The data were tested for normal distribution (Shapiro-Wilk test). The variables VHN_{post}, REM, VHN_{dem}, DEM and EFU were calculated for each specimen and analyzed using a two-way ANOVA with factors for 'enamel type' and 'dentifrice' and their interaction. REM was the primary variable. Where significant differences were indicated, the individual means were analyzed by the Student Newman-Keuls test. The significance level for the analyses was set at 5%. Correlation coefficients (Pearson) were calculated to evaluate the associations among the variables.

Results

VHN_{base} means were virtually identical between enamel types and between dentifrice treatment groups (Table 3). These data highlighted a rate of lesion progression between human and bovine enamel of approximately 1:2, assuming similar VHN of sound enamel, which were found in previous

studies (data not shown). Variability between enamel types at lesion baseline were similar – standard deviations of the mean of VHN_{base} for all specimens were 5.6 (human) and 4.9 (bovine).

The results and statistical analyses for all study variables are shown in Table 3. The Figure shows the F dose-response data for both enamel types with corresponding linear fits. The enamel × dentifrice interaction was significant for all but the EFU variable. Both enamel types responded to F (dentifrice formulation 1 data) in a linear dose-response manner and separated 0, 250 and 1100 ppm F with $r = 0.87$ for human and $r = 0.93$ for bovine enamel, respectively. Bovine enamel, however, exhibited a steeper gradient (0.065) than human enamel (0.042). Furthermore, bovine enamel showed not only more remineralization than human enamel ($P< 0.001$; REM variable), but was also more responsive to higher fluoride concentrations as highlighted by the observed differences within dentifrices 1100F-NaF-1 and -2 (both enamel comparisons were $P< 0.001$). However, human enamel differentiated between 1100F-NaF-1 and 1100F-NaF-2 ($P= 0.041$), whereas bovine enamel did not ($P= 0.095$). The DEM data mirrored REM observations as similar responses were noted. The EFU data were unaffected by enamel type ($P= 0.214$) and mirrored the F dose-response found in the human enamel REM data. No differences between enamel types were observed in relation to their response to 1000F-MFP for any variable, which was found to be equivalent to 250F-NaF-1 under the chosen conditions. Human and bovine enamel exhibited similar variability for any study variable and dentifrice (data not shown).

Irrespective of the enamel type, good linear correlations were found for dentifrice-F concentration (formulation 1) vs. REM ($r = 0.88$) and EFU ($r = 0.92$), and for REM vs. EFU ($r = 0.84$).

Discussion

Fluoride does exert its anticaries action in a dose-response manner.[25] Thus, laboratory models which are useful tools to study the behavior of different enamel types (as in the present study), or e.g. to evaluate the anti-caries potential of oral care products[26] or experimental solutions,[27] have to show not only selectivity but also sensitivity to fluoride. These criteria were satisfied in the present study as a clear fluoride dose-response was shown and for both enamel types.

Bovine was found to be more prone to remineralization than human enamel; i.e. it responded more strongly to a fluoride treatment as highlighted by the steeper gradient of the fluoride dose-response curve (Figure); and differences between the tissues reached significance at the highest fluoride concentration tested (Table 3). These differences in response to fluoride are difficult to explain, and can perhaps be attributed to earlier reported dissimilarities in porosity.[1] As caries lesions progress more rapidly in bovine than in human enamel, it seems logical bovine lesions can also be remineralized more easily. However, it must be borne in mind that early caries lesions were created and matched for VHN before the experimental phase began; thereby minimizing any differences in porosity and solubility as lesions were virtually identical in terms of their surface microhardness (SMH). The SMH test is less prone to

variability than the current "gold standard" technique, transverse micro-radiography, when studying de- and remineralization of the presently employed early caries lesions, which was shown in several previous pH cycling studies,[17,28] and recent observations in the present authors' laboratories (data not shown). To continue, the observed differences in fluoride response can therefore only be explained by 'residual' structural differences between the tissues, a greater presence of interprismatic substance[9] and consequently greater porosity which allows faster ion influx and remineralization.

Do these observed differences give rise to over-interpretation of data generated solely on bovine enamel? Yes and no. A steeper fluoride dose-response curve would suggest easier discrimination between products with either different fluoride concentrations or similar fluoride concentrations but different bioavailability. The latter, however, was not the case in the present study as human enamel was able to highlight differences in effectiveness between dentifrices 1100F-NaF-1 and 1100F-NaF-2, whereas bovine enamel was not. Nevertheless, these differences should not be over-interpreted given the similarity of the P values (0.041 for human; 0.095 for bovine enamel).

No differences between enamel types in response to MFP were observed in the present study. As the experimental design did not involve enzymatic breakdown of the MFP ion, the release of ionic fluoride by phosphatases in dental plaque is responsible for MFP's anticaries activity,[29] a comparison to NaF under the chosen condition is invalid. Nonetheless, MFP was found to be equivalent to 250F-NaF-1 rather than the placebo, which can be explained by the free fluoride found in the MFP dentifrice (154 ppm), which is somewhat in agreement with previous observations.[30]

To the authors' knowledge, only two pH cycling studies[20,21] comparing human and bovine enamel have been reported. Net demineralization rather than remineralization models were employed and no differences were observed between enamel types with regards to fluoride dose-response or rates of demineralization. These observations were rather surprising, especially considering earlier reports of relatively large differences in the tissues' responses to demineralization challenges.[11] These in combination with present results highlight that biological variation within is perhaps larger than between tissues and that only a ring-experiment, i.e. a multi-site study, would allow to determine the relative susceptibility of human and bovine enamel to de- and remineralization challenges.

In summary, considerably more research is necessary, especially utilizing in situ models, to further our understanding of potential differences between human and bovine enamel. Likewise, the enamel types' response to other fluoride salts, such as stannous and amine fluoride, should be investigated. Given the increasing scarcity of human teeth suitable for caries research, appropriate substitutes for human enamel and dentin will eventually need to be found. Based on the results of the present study, bovine enamel may not be suitable as a true replacement for human enamel, as our knowledge of the tissues' responses to anticaries agents and de- and remineralization challenges is far from what can be considered comprehensive.

a. Buehler, Lake Bluff, IL, USA.
b. Struers Inc., Cleveland, PA., USA.
c. Nikon, Tokyo, Japan.
d. GlaxoSmithKline, CITY, UK.
e. Procter & Gamble, Cincinnati, OH, USA.

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Table 2. Daily treatment schedule for the pH cycling study.

Step No.	Time frame	Treatment
1	8:00-8:01 a.m.	Dentifrice*
2	8:01-9:00 a.m.	Saliva
3	9:00-9:01 a.m.	Dentifrice
4	9:01-10:00 a.m.	Saliva
5	10:00 a.m.-2:00 p.m.	Demineralization
6	2:00-3:00 p.m.	Saliva
7	3:00-3:01 p.m.	Dentifrice
8	3:01-4:00 p.m.	Saliva
9	4:00-4:01 p.m.	Dentifrice
10	4:01 p.m.-8:00 a.m. (day 2)	Saliva

*The first day this treatment was not given. The study started with the saliva treatment to allow an artificial pellicle-like layer to form.

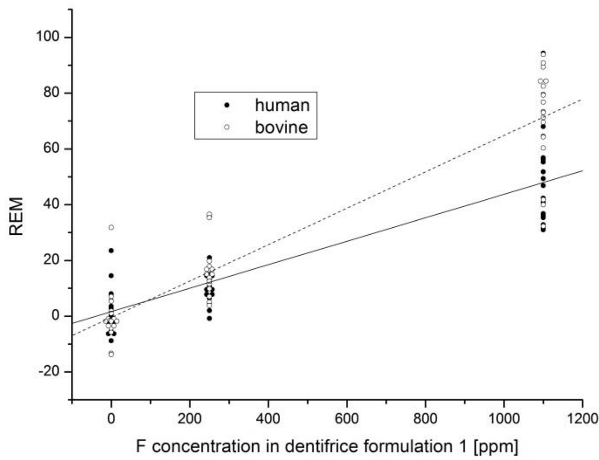

Figure. Fluoride dose-response data for human and bovine enamel with corresponding linear fits (straight for human, dashed for bovine enamel). All individual data points are shown, and offset when overlapping.

Table 1. Test dentifrices.

Code	Formulation	Fluoride salt	Fluoride concentration	Abrasive	Detergent
0F-NaF-1	1	n/a	0 ppm	Silica	Sodium dodecyl sulfate
250F-NaF-1		Sodium fluoride	250 ppm		
1100F-NaF-1		Sodium fluoride	1100 ppm		
1100F-NaF-2	2	Sodium fluoride	1100 ppm		
1000F-MFP	3	Sodium monofluorophosphate	1000 ppm	Calcium carbonate	

Table 3. Least square means, standard error of the least square means and results of the statistical analyses for all study variables.

Enamel	Dentifrice	VHN_{base}	VHN_{post}	REM	VHN_{dem}	DEM	EFU (ppm)
Human	0F-NaF-1	37.4	38.8C[1]	1.4 D	30.0 D	-7.4 D	124 D
	250F-NaF-1	37.5	49.9 B	12.4 C	46.0 C	8.5 C	1415 C
	1100F-NaF-1	37.6	**85.4** A	**47.8** B	**76.4** B	**38.8** B	4078 B
	1100F-NaF-2	36.8	**93.7** A	**56.9** A	**84.2** A	**47.4** A	4614 A
	1000F-MFP	37.6	45.2 BC	7.6CD	42.6 C	5.0 C	1421 C
Bovine	0F-NaF-1	38.2	38.0 c	-0.2 c	33.2 c	-5.0 c	132
	250F-NaF-1	38.2	53.6 b	15.4 b	48.8 b	10.6 b	1314
	1100F-NaF-1	38.2	**109.6** a	**71.4** a	**94.1** a	**55.9** a	3968
	1100F-NaF-2	38.8	**117.5** a	**78.7** a	**100.9** a	**62.1** a	4267
	1000F-MFP	38.8	50.5 b	11.7 b	46.6 b	7.8 b	1176
SEM[2]		1.3	3.3	3.1	2.8	2.8	142
P-values	Enamel	0.195	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.214
	Dentifrice	0.998	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	Enamel × dentifrice	0.984	**<0.001**	**<0.001**	**0.007**	**0.007**	0.918

[1]Significant differences between dentifrices within enamel types are highlighted by different letters (capital letters for human, small letters for bovine enamel), and differences between enamel types within dentifrices in bold. As EFU was only affected by dentifrice, the results and statistical analysis are irrespective of the enamel type and are therefore only presented once. Individual means for EFU by enamel type and VHN_{base} are presented for information only.
[2]Standard error of the mean