Fluoride Dose-Response of Human and Bovine Enamel Artificial Caries Lesions under pH-Cycling Conditions

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Abstract

Objectives This laboratory study aimed to: a) compare the fluoride dose-response of different caries lesions created in human and bovine enamel (HE/BE) under pH-cycling conditions, and b) investigate the suitability of Knoop and Vickers surface microhardness (K-SMH/V-SMH) in comparison to transverse microradiography (TMR) to investigate lesion de- and remineralization.

Materials and Methods Caries lesions were formed using three different protocols (Carbopol, hydroxyethylcellulose-HEC, methylcellulose-MeC) and assigned to 24 groups using V-SMH, based on a 2 (enamel types)×3 (lesion types)×4 (fluoride concentrations used during pH-cycling–simulating 0/250/1100/2800 ppm F as sodium fluoride dentifrices) factorial design. Changes in mineral content and structural integrity of lesions were determined before and after pH-cycling. Data were analyzed using three-way ANOVA.

Results BE was more prone to demineralization than HE. Both enamel types showed similar responses to fluoride with BE showing more remineralization (as change in integrated mineral loss and lesion depth reduction), although differences between tissues were already present at lesion baseline. Carbopol and MeC lesions responded well to fluoride, whereas HEC lesions were almost inert. K- and V-SMH correlated well with each other and with the integrated mineral loss data, although better correlations were found for HE than for BE, and for MeC than for Carbopol lesions. Hardness data for HEC lesions correlated only with surface zone mineral density data.

Conclusion BE is a suitable surrogate for HE under pH-cycling conditions.

Clinical Relevance The in vitro modelling of dental caries is complex and requires knowledge of lesion behavior, analytical techniques and employed hard tissues.

Keywords: dental caries, pH-cycling, human enamel, bovine enamel, transverse microradiography, surface microhardness

Introduction

Bovine enamel (BE) has often been considered a suitable substitute for human enamel (HE) in the study of dental caries [1]. BE has many obvious advantages over HE, e.g. it is more readily available, easier to obtain due to ethical aspects, its larger size allows for the preparation of specimens with a more uniform enamel thickness, and its lack of prior cariogenic challenges and topical and systemic fluoride exposure should, at least in theory, provide a tissue of lesser biological variation than HE. Several morphological and compositional differences between the two tissues have been reported and were summarized recently [2]: BE is more porous [3], softer [4] contains more carbonate [5], but less fluoride [6] than HE. Their prism arrangement differs [7, 8], especially in the inner third of the enamel [9], and crystallites are larger [10] but prism diameters smaller [9] in BE. Similar radio-densities were reported [11], and a subsequent study [12] showed not only a greater presence of interprismatic substance but also "fibril-like" structures around prisms in BE. Greater porosity, solubility and presence of interprismatic enamel would suggest that lesions progress faster in BE than HE, which, in deed, has been reported by many researchers [2, 13-17], although relative differences varied considerably between laboratories, highlighting that biological variation within tissues should not be overlooked and especially in larger studies [2]. However, little is known about potential differences in the tissues' susceptibility to fluoride and remineralization. A recent study [18] highlighted that BE may be more prone to remineralization than HE, although the study was somewhat limited in scope. No differences between tissues were found in their fluoride response under net demineralizing conditions in two other pH-cycling studies [19, 20], highlighting the need for a more comprehensive study.

Several interrogation techniques are being utilized in caries research to monitor changes in, for example, lesion mineral content or structural integrity, however, rarely in combination. Crudely, these techniques can be divided into destructive and non-destructive as well as being direct or indirect measures of mineral content. Among those techniques are the 'gold standard' transverse microradiography (TMR), Knoop and Vickers microhardness either used perpendicular (K-SMH, V-SMH) or parallel to the hard tissue surface, polarized light and confocal laser scanning microscopy (both used parallel to the surface), and quantitative light-induced fluorescence (used perpendicular to the surface). Researchers have either shown good [4, 21, 22] or poor correlations [23, 24] between TMR and SMH, depending on the extent of demineralization and lesion mineral distribution. However, previous

studies either utilized lesions created using only one protocol, or different lesions in the absence of any treatments, thereby providing scope for further research.

Consequently, the aims of the present in vitro study were two-fold: a) to compare the fluoride dose-response of different caries lesions created in HE and BE under pH-cycling conditions, and b) to investigate the suitability of K-SMH and V-SMH in comparison to TMR to investigate caries lesion de- and remineralization.

Materials and methods

Study Design

The present study followed a 2 (enamel types) × 3 (lesion types) × 4 (fluoride concentration) factorial design, thus resulting in a total of 24 experimental groups. Early caries lesions, utilizing three different lesion creation protocols (Carbopol, HEC, MeC), were created in enamel specimens prepared from human and bovine permanent enamel. Lesions were allocated, based on V-SMH, in order to have uniform baseline surface microhardness (demineralization) to four groups of different fluoride concentrations used during the 10 d pH-cycling phase. Changes in mineral content and structural integrity of the lesions were determined before and after pH-cycling by K-SMH, V-SMH and TMR.

Specimen Preparation

Enamel specimens were obtained from human permanent (predominantly molars and premolars, only buccal and/or lingual surfaces were used) and bovine incisor teeth (only buccal surfaces were used). Human teeth were extracted mainly for orthodontic reasons and were obtained from dental offices located in the State of Indiana, USA (water fluoridation at 1 ppm F). Bovine teeth were obtained from Tri State Beef Co. (Ohio, USA), from cattle with an average age of three years (range: 18 months to five years) and which stem from several states in the USA [personal communication with Tri State Beef Co.]. Both human and bovine teeth are received at the present, first author's

laboratories approximately monthly; however, determinations of origin, exact age and other characteristics of the donor (human or animal) are impossible due to the large number of teeth received.

Tooth crowns were cut into 4 × 4 mm specimens using a Buehler Isomet low-speed saw. The teeth were stored in deionized water containing thymol during the sample preparation process. Specimens were ground and polished to create flat, planar parallel dentin and enamel surfaces using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, Pa., USA). 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 until the enamel surface had a minimum of a 3 × 4 mm highly polished facet across the specimen. Resulting specimens had a thickness range of 1.7 – 2.2 mm. This polishing procedure ensured the removal of surface enamel (approx. 200 – 300 μm, depending on the natural curvature of the enamel surface) which may contain relatively high concentrations of artificially introduced trace elements (e.g. F, Sr) that would otherwise compromise the comparison between tissues. The specimens were assessed under a Nikon SMZ 1500 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.

Artificial Caries Lesion Creation

In vitro incipient caries lesions were prepared using three protocols.

For the Carbopol lesions, a modification of the method to create ‘low-R’ lesions as described by Lippert et al. [25] was used. Sound enamel specimens were immersed in a demineralization solution containing 0.1 M lactic acid, 4.1 mM $CaCl_2 \times 2\ H_2O$, 8.0 mM KH_2PO_4 and 0.2% w/v Carbopol 907 (BF Goodrich Co., USA), pH adjusted to 5.0 using KOH, for 6 d.

For the hydroxyethylcellulose lesions (HEC), a modification of the method described by Amaechi et al. [26] was used. An HEC gel was prepared by solubilizing hydroxyethylcellulose (Sigma 54290, Cellosize® QP-40,

80-125 cP) in a pH 4.5 (KOH) adjusted 0.05 M lactic acid solution at a ratio of 140 g HEC per liter lactic acid solution. The final pH of the HEC gel was approx. 5.5. Specimens were demineralized for 14 d.

For the methylcellulose lesions (MeC), a modification of the method used by 'Laboratory D' as described by ten Cate et al. [27] was used. Sound enamel specimens were demineralized at pH 4.6 and 37 °C in 8% methylcellulose (Sigma M0387, aqueous, 1,500 cP, 63 kDa) covered with an equal volume of 0.1 M lactic acid, pH adjusted with KOH, for 7 d.

For all protocols, demineralization was conducted at 37 °C and neither the gel nor the demineralization solution was replaced during the demineralization period. After lesion creation, specimens were rinsed with deionized water. Approx. half of the lesion surface was then covered using acid-resistant, colored nail varnish (Sally Hansen Advanced Hard As Nails Nail Polish, USA), protecting a lesion baseline area for subsequent TMR analysis. Specimens were stored at 100% relative humidity at 4 °C until further use.

Lesion Surface Microhardness

Specimens were mounted individually on 1-inch acrylic blocks using sticky wax. A total of eight lesion baseline indentations (2100 HT; Wilson Instruments, Norwood, Mass., USA) were placed in the uncovered lesion window of each specimen: four using a Knoop diamond indenter using a 50 g load (approx. 100 µm apart from each other), and four using a Vickers diamond indenter using a 200 g load (approx. 150 µm apart from each other and in close proximity to the Knoop indentations), each with a dwelling time of 11 s. The respective indentation loads and number of indentations per specimen were chosen based on standard operating procedures and reflect those typically employed in the main author's laboratories [22, 28]. Knoop and Vickers hardness numbers were derived from the respective indentation lengths (Knoop – length of the long diagonal, Vickers – mean of both diagonals) and recorded. Human and bovine enamel lesions were each allocated, by lesion type, to four groups each based on V-SMH so there was no significant difference in mean V-SMH between groups within enamel and lesion types. Although there were no inclusion/exclusion criteria per se, some enamel specimens had to be excluded from the study due to extensive demineralization or lack thereof after lesion creation. Hence, each experimental group (total of 24) contained between 13 and 15 specimens (n=15 was anticipated).

pH-cycling Phase

The present pH-cycling model was loosely based on that by Amaechi et al. [29]. The daily cyclic treatment regimen, which was repeated for 10 d and shown in Table 1, consisted of two 30-min acid challenges (50 mM acetic acid, 2.25 mM $CaCl_2 \times 2\ H_2O$, 1.35 mM KH_2PO_4, 130 mM KCl, pH adjusted to 4.5 using KOH) and four, 2-min sodium fluoride solution (0; 83; 367; 933 ppm F simulating 0; 250; 1100; 2800 ppm F dentifrices after 1:2 dilution) treatment periods with specimens stored in artificial saliva (2.20 g/l gastric mucin, 1.45 mM $CaCl_2 \times 2\ H_2O$, 5.42 mM KH_2PO_4, 6.50 mM NaCl, 14.94 mM KCl, pH adjusted to 7.0 using KOH) all other times. The pH-cycling phase was conducted at room temperature and without stirring. Fluoride concentrations were chosen to mimic 0 (placebo), 250, 1100 and 2800 ppm F dentifrices after 1:2 dilution.

Post pH-cycling Surface Microhardness

After pH-cycling, a second set of eight indentations were placed on each specimen, as described above and in close proximity to the lesion baseline indentations. V-SMH and K-SMH were recorded and changes vs. lesion baseline calculated for both Knoop and Vickers indenter types as follows: $\Delta SMH = SMH_{post} - SMH_{base}$. The authors refrained from calculating changes in indentation lengths as this is uncommon for Vickers and for the sake of clarity, although some previous studies [4, 13, 21, 22] highlighted linear correlations between Knoop indentation lengths vs. ΔZ and L.

Transverse Microradiography

One section per specimen, approximately 100 µm in thickness, was cut from the center of the specimen and across the baseline and post pH-cycling lesion windows using a Silverstone-Taylor Hard Tissue Microtome (Scientific

Fabrications Laboratories, USA). Sections which were found to be thicker than 120 μm (determined using drop gauge) were hand-polished using 2,400-grit silicon carbide paper to the required thickness. The sections were mounted, with an aluminum step wedge, on high resolution glass plates Type I A, Microchrome Technology Inc., San Jose, CA) and X-rayed at 20 kV and 30 mA at a distance of 42 cm for 65 min. The film was developed in Kodak d-19 developer for 3 min, placed in a stop bath (Kodak 146-4247) for 45 s, and then fixed (Kodak 146-4106) for 3 min. All plates were then rinsed in deionized water for 15 min and air-dried. Microradiographs were examined with a Zeiss EOM microscope in conjunction with the TMR software v.3.0.0.11 (Inspektor Research Systems BV, Amsterdam, The Netherlands). A window (approx. 400 × 400 μm), representative of the entire lesion area and not containing any cracks, debris or other alterations, was selected for analysis. Sound enamel mineral content was assumed not only to be equivalent between tissues [15] but also 87% v/v [30, 31]. Sectioning and initial TMR analyses (plate reading) were conducted by the same technicians. Final TMR analysis (actual determination of lesion parameters) was conducted by the present, first author. The variability of the repeated TMR analysis of sections was determined in a separate study. The standard deviation of the mean for any of the reported variables was found to be lower than 4 %.

The following variables were recorded for each specimen/section: ΔZ – integrated mineral loss (product of lesion depth and the mineral loss over that depth); L – lesion depth (83% mineral; i.e. 95% of the mineral content of sound enamel); SZ_{max} – maximum mineral density of the lesion surface zone; %R – percentage change in ΔZ values [32] which was calculated as $\%R = (\Delta Z_{base} - \Delta Z_{post})/\Delta Z_{base} \times 100$. Changes from lesion baseline were calculated as follows: $\Delta\Delta Z = \Delta Z_{base} - \Delta Z_{post}$; $\Delta L = L_{post} - L_{base}$; $\Delta SZ_{max} = SZ_{max,post} - SZ_{max,base}$.

Human and Animal Rights

The manuscript does not contain clinical studies or patient data. This study was of pure laboratory nature and did not involve human subjects or animals. Human teeth were obtained from dental offices located in the State of Indiana, USA, after obtaining informed consent from the adult donors. Bovine teeth were obtained from a local slaughterhouse (Tri State Beef Co., Ohio, USA) which adheres to the “Humane Slaughter Act” (United States Federal Law).

Statistical Analysis

The effects of enamel type, lesion type, fluoride concentration and their interaction on VHN, KHN, ΔZ, L, SZ_{max}, ΔVHN, ΔKHN, ΔΔZ, ΔL, ΔSZ_{max}, and %R were evaluated using three-way ANOVA. Where significant differences were indicated, the individual means were analyzed using Tukey's method to control the overall significance level at 5%. Pearson correlation coefficients were calculated to investigate associations between all study variables.

Results

Lesion Baseline

Table 2 provides the data and results of the statistical analyses for all lesion baseline variables for both HE and BE. The enamel type × lesion type interaction was statistically significant for all variables. Considering the TMR variables, lesions created in BE exhibited more demineralization, were deeper and, at least for HEC and MeC, also showed higher surface zone mineralization. However, relative differences between tissues varied depending on the lesion protocol. The hardness data showed good agreement between indenter and within lesion types; conversely, the overall findings for enamel types were not in agreement. For better visualization of differences between lesion and enamel types, Fig. 1 shows the mean mineral distributions for all baseline lesions. Differences between lesion types and to a lesser extent between tissues are apparent for all investigated lesion parameters.

Post pH-Cycling

Table 3 provides the data for all post-pH cycling lesion variables for both HE and BE. Online Resource 1 provides the results of the statistical analyses for all post pH-cycling lesion variables for both HE and BE. The enamel ×

lesion × fluoride interaction was only significant for ΔKHN. For all other variables, the lesion × fluoride interaction was significant. Only ΔL and ΔKHN showed interactions involving enamel type, whereas ΔΔZ was the only variable for which enamel type was a significant source of variation (BE showing more remineralization than HE). Both Carbopol and MeC lesions responded to different fluoride concentrations as evident by both the hardness and mineral loss data, whereas HEC lesions responded poorly considering TMR variables. In HEC lesions, HE appeared to show a better fluoride dose-response than BE. In Carbopol and MeC lesions, differences were less apparent between tissues and to some extent also depending on the interrogation technique. In Carbopol lesions, for example, the hardness data would suggest a stronger response for HE than for BE, whereas the TMR data suggests otherwise (ΔΔZ, ΔL). In MeC lesions, BE responded more predominantly than HE, although these differences were not apparent in the ΔVHN data. The post pH-cycling mineral distributions (not shown) were virtually identical between HE and BE. Both tissues showed remineralization in the lesion body and of the surface zone in Carbopol and MeC lesions, whereas HEC lesions showed very little change from baseline. Laminations were only obvious in the 2800 ppm F HE Carbopol group.

Correlations

Pearson correlation coefficients for baseline and post pH-cycling variables can be found in Table 4. At lesion baseline, there was little difference in Pearson correlation coefficients between HE and BE or Knoop vs. Vickers. For both indenter types, the strongest overall correlations were found in relation to $SZ_{max,base}$, although all were significant. However, there were differences between lesion types, as the hardness data correlated well with the ΔZ_{base} data for the Carbopol lesions. Considering the post pH-cycling data, correlations were somewhat stronger for HE than for BE. Again, hardness data correlated well with one another. The strongest correlations were observed for the hardness vs. ΔΔZ data and the weakest vs. the ΔL data. Again, there were differences between lesion types. Hardness/TMR correlations were strongest in MeC lesions and significant for all TMR variables, although strongest for ΔΔZ. In Carbopol lesions, only the hardness vs. $\Delta\Delta Z/\Delta SZ_{max}$ correlations were significant which were also weaker than for MeC lesions. In HEC lesions, hardness data correlated only with the $SZ_{max,base}/\Delta SZ_{max}$ data.

Discussion

The present lesion baseline observations which highlighted that BE is more prone to demineralization than HE are in agreement with the aforementioned findings by other researchers. Potential explanations lie in differences in structure (e.g. greater porosity of BE) and inherent solubility (e.g. greater carbonate content of BE) – the former explains differences in lesion depth, whereas the latter can be held accountable for differences in mineral loss. Differences between tissues within lesion types were, however, not consistent (Table 2). In Carbopol lesions, percentage differences in ΔZ_{base} between tissues (35.5%) were greater than those in ΔL (20.5%), whereas this relationship was reversed in HEC (18.7%/26.6%) and MeC (10.6%/21.4%) lesions. Similar differences were also observed for $SZ_{max,base}$. Lesion creation protocols were inherently different and may have contributed to the relative differences as diffusion was more retarded in the 'gel systems', HEC and MeC, thus artificially reducing differences in solubility (rate limiting step is diffusion of dissolution products away from the lesion surface) but somewhat enhancing dissimilarities in porosity (as acid will be 'forced' to penetrate deeper to dissolve enamel).

Differences in porosity would also suggest that BE is more prone to remineralization than HE. A recent study [18], albeit limited in scope in comparison to the present one, suggested that BE is more prone to surface rehardening and especially in the presence of elevated fluoride concentrations. The results of the present study, however, do not necessarily support these findings. In the statistical analyses (Online Resource 1), the factor 'enamel' was only found to be significant for the variable '$\Delta\Delta Z$' but not for '%R'. This variable was introduced to account for differences in ΔZ_{base} (Table 2) which was shown to be an important factor in remineralization (larger lesions will remineralize more) [17, 33]. There was, however, some indication that the 'fluoride response differential'; i.e. the numerical differences in %R between 0 and 2800 ppm F for Carbopol and MeC lesions (Table 3) were greater for BE than for HE. Likewise, changes in L were more pronounced with increasing fluoride concentrations in BE than HE, although these were not matched at baseline which makes comparisons difficult. Perhaps further studies using different lesions and with a broader range of $\Delta Z_{base}/L_{base}$ are required to better understand any potential differences in the tissues' respective fluoride dose-response.

Two previous pH-cycling studies [19, 20] utilizing net de- rather than remineralizing models, did not highlight any differences between BE and HE in terms of their response to fluoride. This, together with other [2] and

especially the present observations perhaps highlights that while there are structural and chemical differences between BE and HE, these are easily overshadowed by the impact of differences in mineral distributions and extent of demineralization. Two of the chosen lesion protocols were previously shown to respond differently to fluoride under demineralizing conditions [34], and similar observations were made presently under net remineralizing conditions. HEC lesions do not appear to be suitable for short-term pH-cycling studies, and considerably longer periods are required to extract a fluoride response [29]. Carbopol and MeC lesions, on the other hand, seem more suitable, although they varied in their overall extent of remineralization and ability to differentiate between higher fluoride concentrations. These lesions showed considerable differences in their respective mineral distributions (Fig. 1) – MeC were more demineralized closer to the lesion surface and therefore more easily remineralized, which is in agreement with findings by Lynch et al. [35]. Naturally, this all begs the question what resembles most closely a clinically relevant lesion – all three lesion protocols are justifiable, but do they mimic in vivo caries lesions? Artificial caries lesions are created using continuous demineralization protocols and are formed over short periods of time. In vivo caries lesions, however, are subject to multiple cycles of de- and remineralization with fluctuations in calcium and fluoride concentrations and pH and form over a considerably longer period of time. No comparative studies have been conducted yet and most likely because of the difficulty in standardizing in vivo demineralization conditions, which, of course, vary considerably between individuals which introduces aforementioned confounding factors and thereby limits the value of any such comparison.

The present study has also shown that surface microhardness techniques could be used in the study of caries lesion de- and remineralization. However, this 'recommendation' cannot be generalized as significant correlations between ΔVHN/ΔKHN vs. ΔΔZ were only noted for Carbopol and MeC lesions, but not for HEC lesions (Table 4). In HEC lesions, only the hardness vs. (Δ)SZ_{max} correlations were significant, albeit weak. This highlights the importance of lesion mineral distribution (Fig. 1) rather than ΔZ or L, as HEC lesions were, on average, considerably less demineralized and somewhat shallower than Carbopol lesions, yet HEC hardness TMR correlations were weaker. Hardness does not necessarily measure mineral content or changes thereof, hardness measures "structural integrity" [36] which may be indicative of (changes in) the mineral content of a lesion or not. Hence, the mineral distribution of a lesion needs to be investigated first before any assumptions about the validity or lack thereof of hardness measurements can be made.

Two other aspects are worth noting: Correlations were typically stronger for HE than for BE. One explanation may lie in the mismatch of lesion baseline variables, as HE lesions were less demineralized and shallower, thus artificially favoring correlations over BE as previous research has shown the disconnect between hardness and L in the study of deeper lesions [13] for example. Furthermore, there was, at least for MeC lesions, a strong correlation for ΔKHN vs. ΔZ. Knoop indentations are very shallow in depth, approx. 1/30 of the length of the long diagonal. This would equate to an approx. indentation depth of 4.7 µm for a KHN of 36 (mean of all MeC lesions post pH-cycling) which in turn compares to L_{post} of 53.8 µm as measured by TMR. Considering that the hardness of a multi-layered material (such as a caries lesion) is affected by usually 7-10 times the indentation depth [37], it is perhaps not that surprising after all. Weaker correlations were shown for Carbopol lesions which one would expect given their greater extent of demineralization and depth in comparison to MeC lesions.

In conclusion, BE appears to be a suitable surrogate tissue for HE under pH-cycling conditions. Hardness techniques have to be used with caution in the study of caries lesion de- and remineralization as correlations to TMR data were generally weak. Ideally, hardness techniques are to be used in combination with TMR as lesion mineral distribution and extent of demineralization ultimately determine their suitability. Lesion mineral distribution appears to outweigh any potential differences between HE and BE in terms of their fluoride response under net remineralizing pH-cycling conditions.

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Conflict of interest The authors declare no conflict of interest.

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Table 1 Daily cyclic treatment regimen

Duration	Specimen Treatment
2 min	Fluoride Solution
30 min	Artificial Saliva
30 min	Acid challenge
2 min	Fluoride Solution
30 min	Artificial Saliva
2 min	Fluoride Solution
30 min	Artificial Saliva
30 min	Acid challenge
2 min	Fluoride Solution
(overnight)[1]	Artificial Saliva

[1]After the very last treatment or treatment day in week 1 (Friday), specimens were placed into artificial saliva for 30 min before being rinsed under running deionized water and stored at approx. 100% relative humidity at 4°C until the next treatment day (Monday) or until post-pH cycling hardness measurements commenced.

Table 2 Least square means ± standard error of the mean and results of the statistical analyses for all lesion baseline variables

Enamel	Lesion	n	VHN_{base}	KHN_{base}	ΔZ_{base} [vol%min×µm]	L_{base} [µm]	$SZ_{max,base}$ [vol%min]
Human	Carbopol	15	**49±2**[1]	**57±3**	2577±61	91.3±1.7	**56.0±0.8**
Bovine		15	28±2	30±3	**3492±59**	**110.1±1.7**	52.9±0.8
Human	HEC	13	104±2	107±3	1666±63	85.0±1.8	65.2±0.9
Bovine		15	**112±2**	**150±3**	**1977±66**	**107.6±1.9**	**71.3±0.9**
Human	MeC	14	17±2	13±3	2103±62	57.5±1.8	39.5±0.8
Bovine		15	19±2	15±3	**2325±60**	**69.8±1.7**	**47.6±0.8**
	Enamel		***0.009***[2]	***0.015***	***<0.001***	***<0.001***	***<0.001***
			human > bovine	bovine > human	bovine > human	bovine > human	bovine > human
	Lesion		***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***
			HEC > Carbopol > MeC	HEC > Carbopol > MeC	Carbopol > MeC > HEC	Carbopol > HEC > MeC	HEC > Carbopol > MeC
	Enamel × Lesion		***<0.001***	***<0.001***	***<0.001***	***0.013***	***<0.001***

[1]Significant differences within variables and lesion types but between enamel types are highlighted in bold. There were no statistically significant differences between fluoride concentrations within each group; hence groups are shown by enamel and lesion type only for better clarity.

[2]*p* values for each factor, interaction between factors and grouping information using Tukey method.

Table 3 Least square means ± standard error of the mean for all post-pH cycling variables

Enamel	Lesion	Fluoride concentration	ΔVHN	ΔKHN	%R	ΔΔZ [vol%min×µm]	ΔL [µm]	ΔSZ_{max} [vol%min]
HE	Carbopol	0	-15.5±3.9	-19.4±2.5	8.6±4.7	267±127	-8.6±2.9	0.7±1.7
		250	-7.4±2.3	-6.0±4.9	11.4±6.4	275±165	-11.9±4.2	-2.1±1.1
		1100	9.7±3.2	6.3±2.1	28.3±1.5	701±51	-8.3±1.6	1.3±1.2
		2800	28.2±2.9	25.5±3.5	42.6±3.2	1169±142	-13.1±5.5	7.3±2.6
BE		0	-2.8±1.7	-7.2±4.0	7.2±4.0	325±164	-6.6±3.0	0.3±1.5
		250	0.0±1.9	2.6±4.1	2.8±6.3	202±241	1.0±5.5	1.5±2.8
		1100	6.5±1.5	11.7±3.4	35.4±3.0	1160±108	-19.5±4.1	1.7±0.9
		2800	20.4±3.0	13.8±5.4	46.4±3.5	1586±136	-24.7±4.4	2.6±1.4
HE	HEC	0	-29.1±4.5	-49.7±6.3	3.7±5.0	111±80	-6.8±2.9	-5.6±2.9
		250	-3.3±4.7	-22.4±5.5	9.9±4.5	174±75	-6.7±2.9	0.3±2.1
		1100	7.3±5.0	2.8±5.3	15.3±4.7	272±89	-5.6±2.7	6.5±1.2
		2800	5.9±3.5	5.5±6.6	14.3±4.0	247±68	-4.8±4.4	5.6±1.3
BE		0	-27.5±5.2	-65.3±7.4	17.1±6.0	406±136	-12.9±4.8	3.2±1.8
		250	4.6±4.3	-29.7±14.0	14.0±7.5	376±173	-11.1±6.7	3.4±2.7
		1100	-5.4±7.2	-32.2±10.6	10.4±9.4	273±151	-3.8±5.3	1.0±2.0

		2800	11.5±5.3	-34.6±10.1	26.1±19.1	492±280	-17.7±9.4	6.1±3.3
HE	MeC	0	5.1±2.5	-2.6±1.5	12.2±4.4	322±101	-4.2±1.8	0.1±2.1
		250	16.0±2.2	6.7±1.8	27.7±2.5	552±72	-8.2±2.4	1.9±1.8
		1100	44.9±2.6	20.5±1.3	52.2±1.9	1051±41	-14.9±2.1	8.9±3.8
		2800	72.4±4.4	32.6±3.1	57.6±2.3	1186±84	-12.2±2.2	18.3±4.3
BE		0	2.1±1.6	0.8±1.5	-1.8±5.3	-18±122	-0.7±3.1	-1.1±2.5
		250	14.8±2.7	17.4±3.1	27.1±3.6	644±94	-2.4±3.4	5.3±2.4
		1100	45.7±3.0	41.5±6.8	59.7±2.2	1421±103	-17.5±3.0	8.4±3.0
		2800	72.4±7.0	57.5±6.1	65.9±1.6	1468±62	-18.1±1.5	16.7±3.7

Table 4. Pearson correlation coefficients for lesion baseline and post-pH cycling variables

Variable interaction		All	BE	HE	Carbopol	HEC	MeC
VHN_{base}	KHN_{base}	**0.92**	**0.94**	**0.92**	**0.64**	**0.55**	**0.55**
VHN_{base}	ΔZ_{base}	**-0.46**	**-0.48**	**-0.44**	**-0.64**	-0.10	**-0.28**
VHN_{base}	L_{base}	**0.35**	**0.36**	**0.50**	**-0.49**	0.03	**0.24**
VHN_{base}	$SZ_{max,base}$	**0.80**	**0.79**	**0.86**	**0.36**	**0.22**	**0.40**
KHN_{base}	ΔZ_{base}	**-0.42**	**-0.48**	**-0.39**	**-0.63**	-0.09	**-0.24**
KHN_{base}	L_{base}	**0.37**	**0.34**	**0.54**	**-0.55**	0.17	0.15
KHN_{base}	$SZ_{max,base}$	**0.80**	**0.78**	**0.88**	**0.28**	**0.43**	**0.33**
ΔVHN	ΔKHN	**0.68**	**0.68**	**0.74**	**0.63**	**0.49**	**0.74**
ΔVHN	ΔΔZ	**0.52**	**0.45**	**0.64**	**0.44**	0.15	**0.73**
ΔVHN	ΔL	**-0.18**	**-0.17**	**-0.21**	-0.17	-0.08	**-0.47**
ΔVHN	ΔSZ_{max}	**0.45**	**0.36**	**0.54**	**0.29**	**0.27**	**0.49**

ΔKHN	ΔΔZ	**0.47**	**0.41**	**0.61**	**0.39**	0.10	**0.74**
ΔKHN	ΔL	**-0.14**	-0.13	**-0.17**	-0.16	0.01	**-0.50**
ΔKHN	ΔSZ_{max}	**0.37**	**0.29**	**0.51**	**0.20**	**0.29**	**0.52**

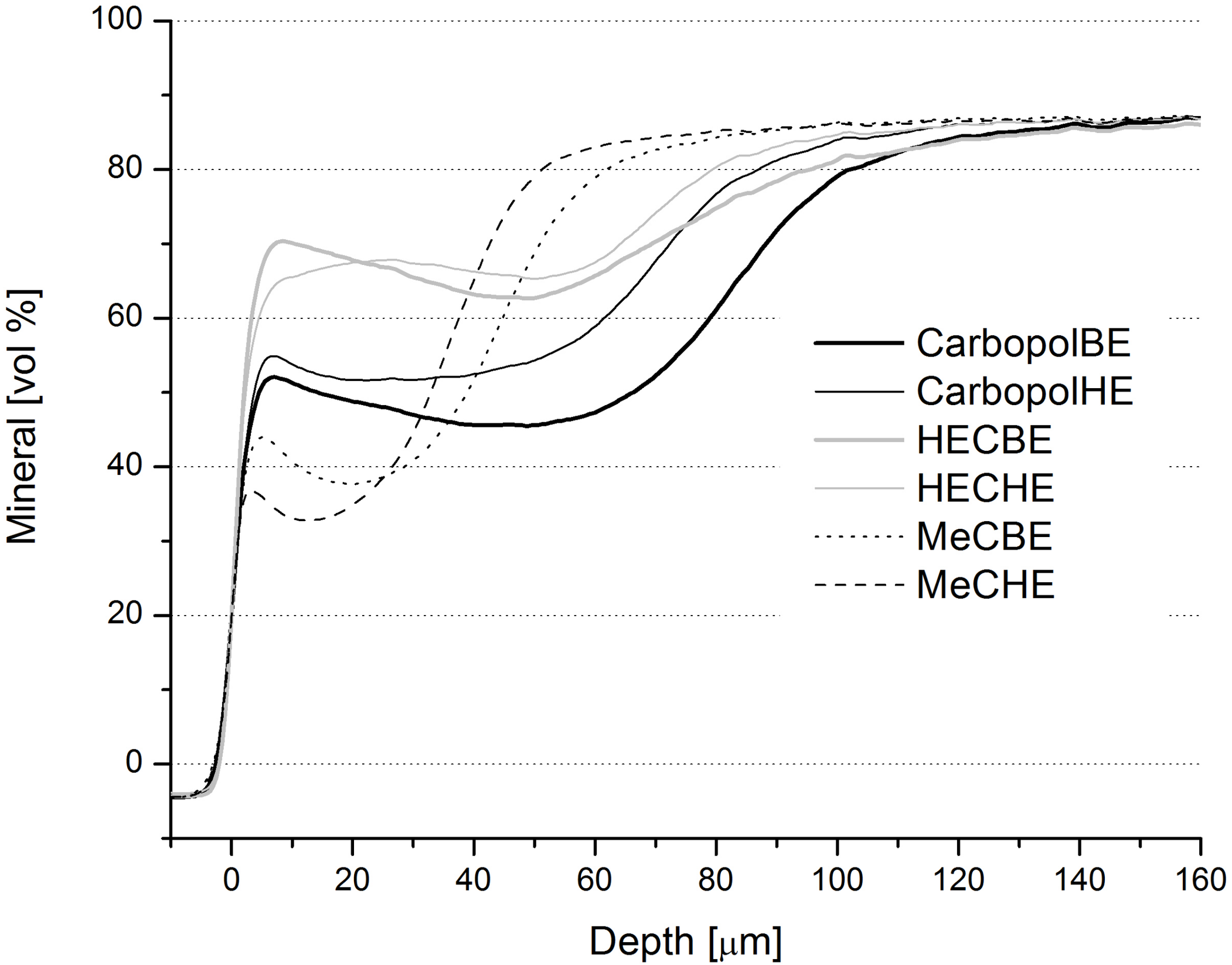
[1]Significant interactions (p<0.05) are highlighted in bold.

Fig. 1 Mean mineral distribution graphs of human (HE) and bovine enamel (BE) Carbopol, hydroxyethylcellulose (HEC) and methylcellulose (MeC) baseline lesions.

Fig. 2 Means and standard deviations for all post-pH cycling variables by enamel type for Carbopol lesions.

Fig. 3 Means and standard deviations for all post-pH cycling variables by enamel type for hydroxyethylcellulose lesions.

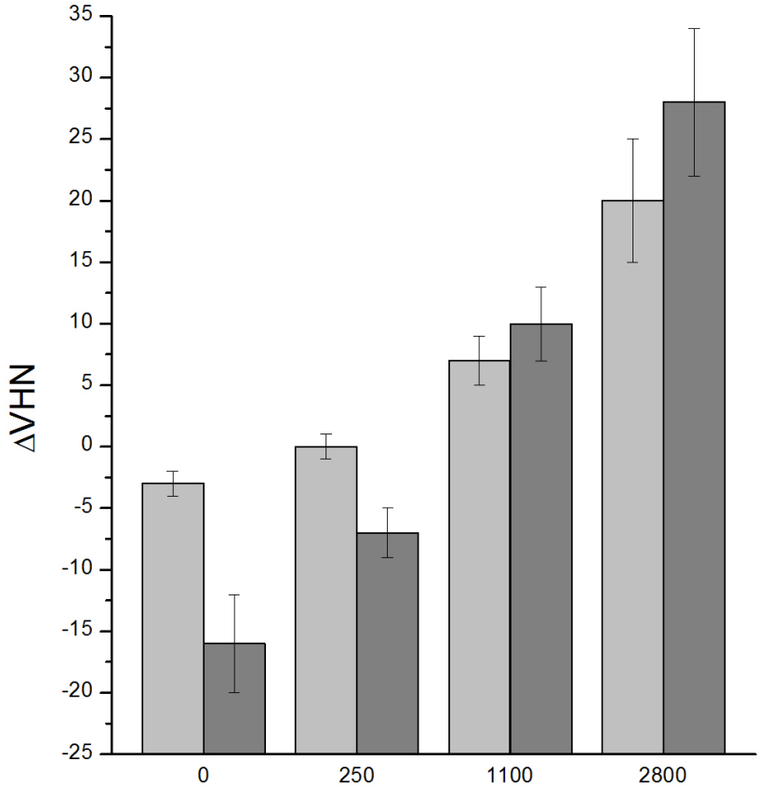
Fig. 4 Means and standard deviations for all post-pH cycling variables by enamel type for methylcellulose lesions.


100
80
60
40
20
0
Mineral [vol %]
0
20
40
60
80
100
120
140
160
Depth [μm]
CarbopolBE
CarbopolHE
HECBE
HECHE
MeCBE
MeCHE

Carbopol 907 Lesions

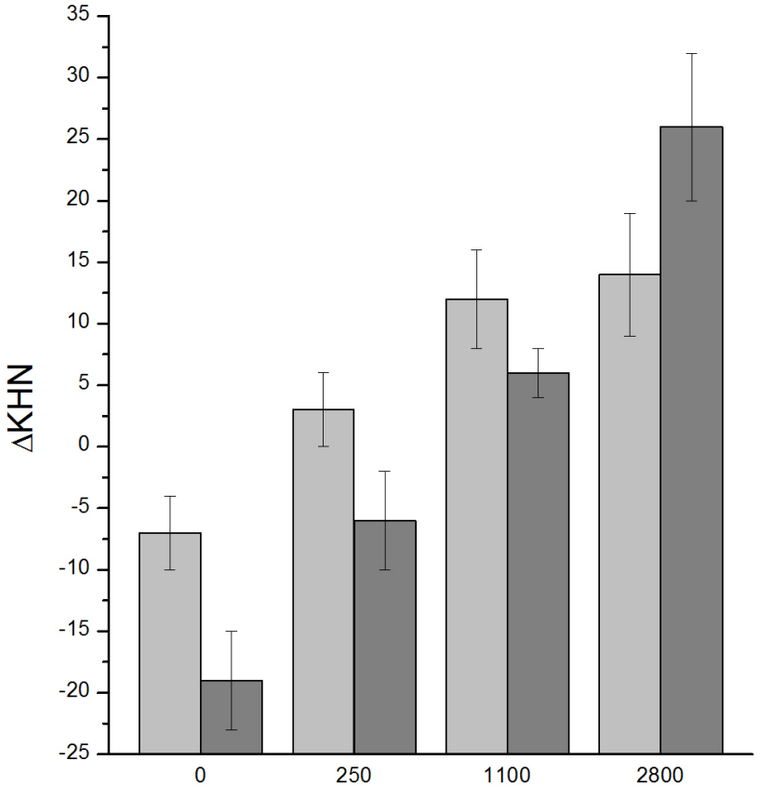
Bovine
Human

ΔVHN



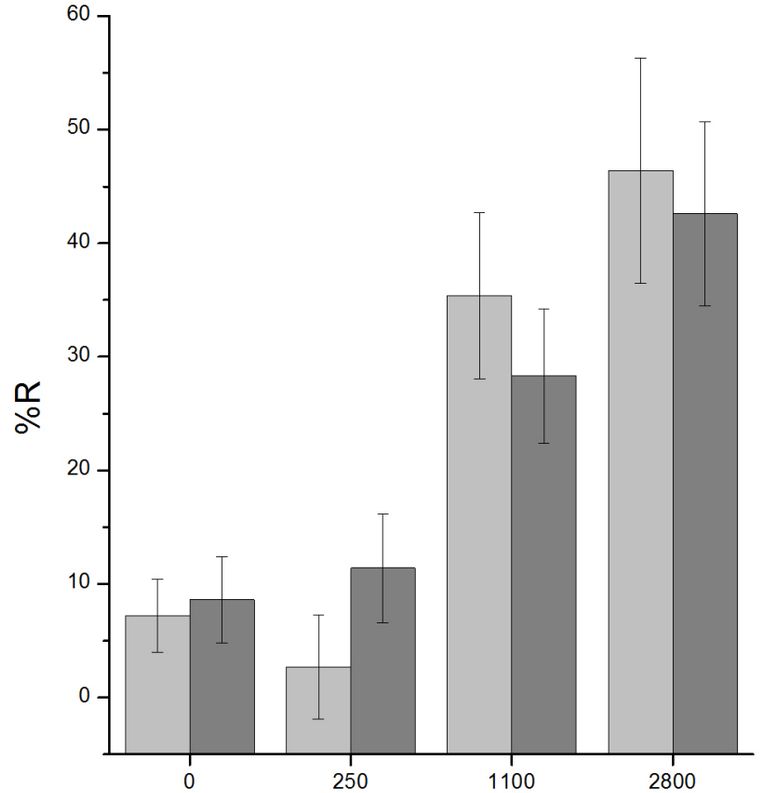
Fluoride concentration [ppm]

ΔKHN



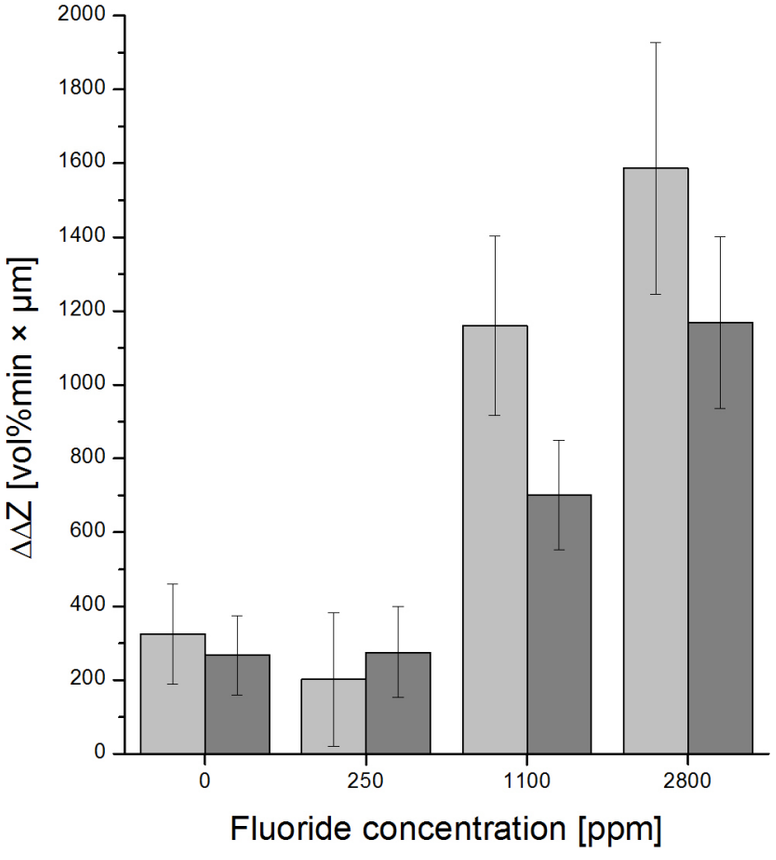
Fluoride concentration [ppm]

%R



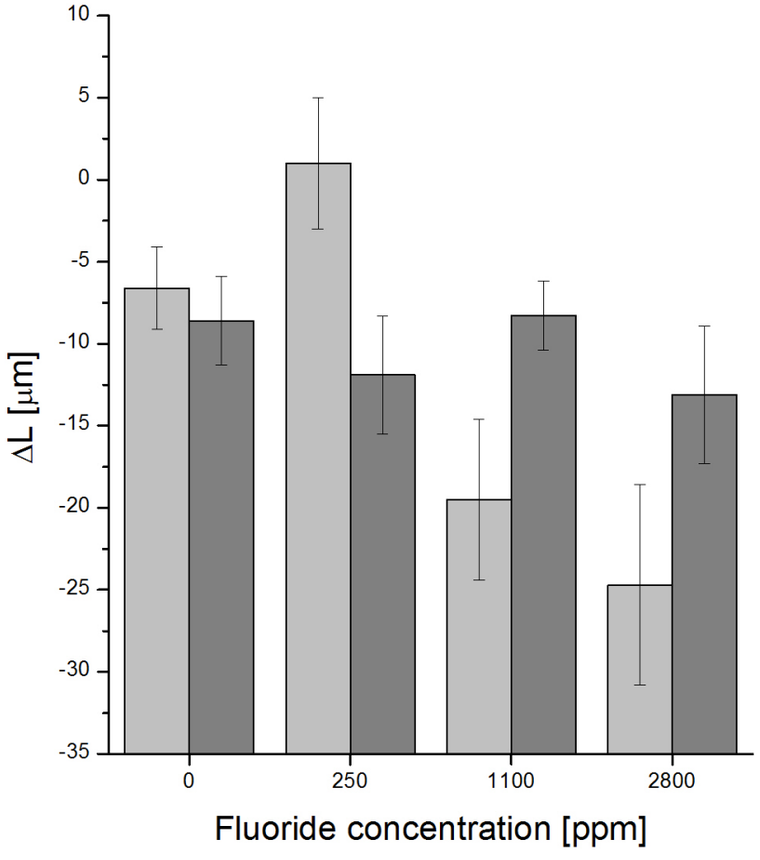
Fluoride concentration [ppm]

ΔΔZ [vol%min × µm]



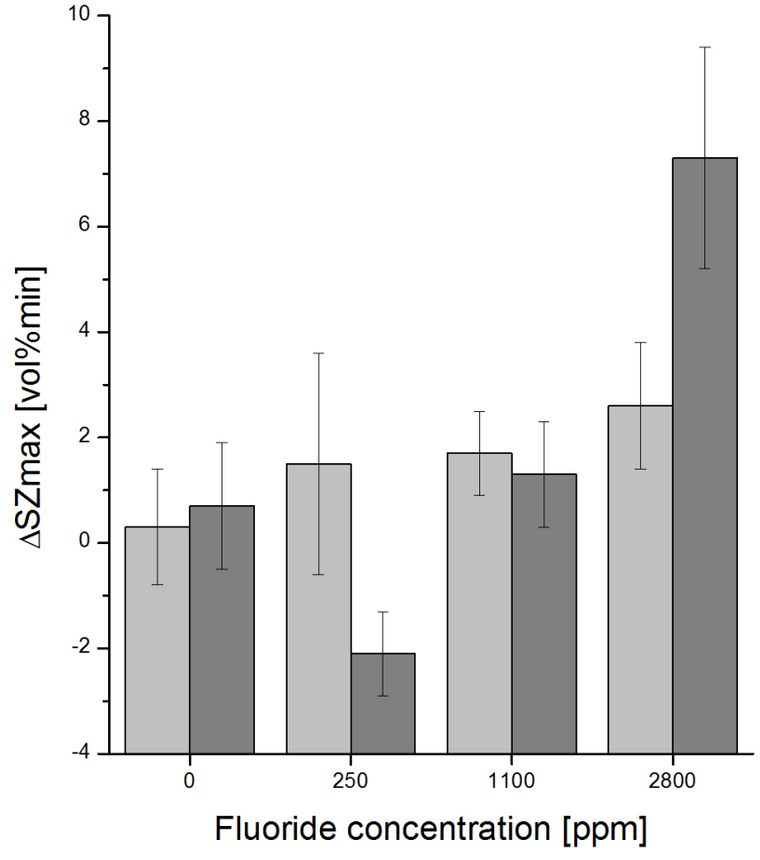
Fluoride concentration [ppm]

ΔL [µm]



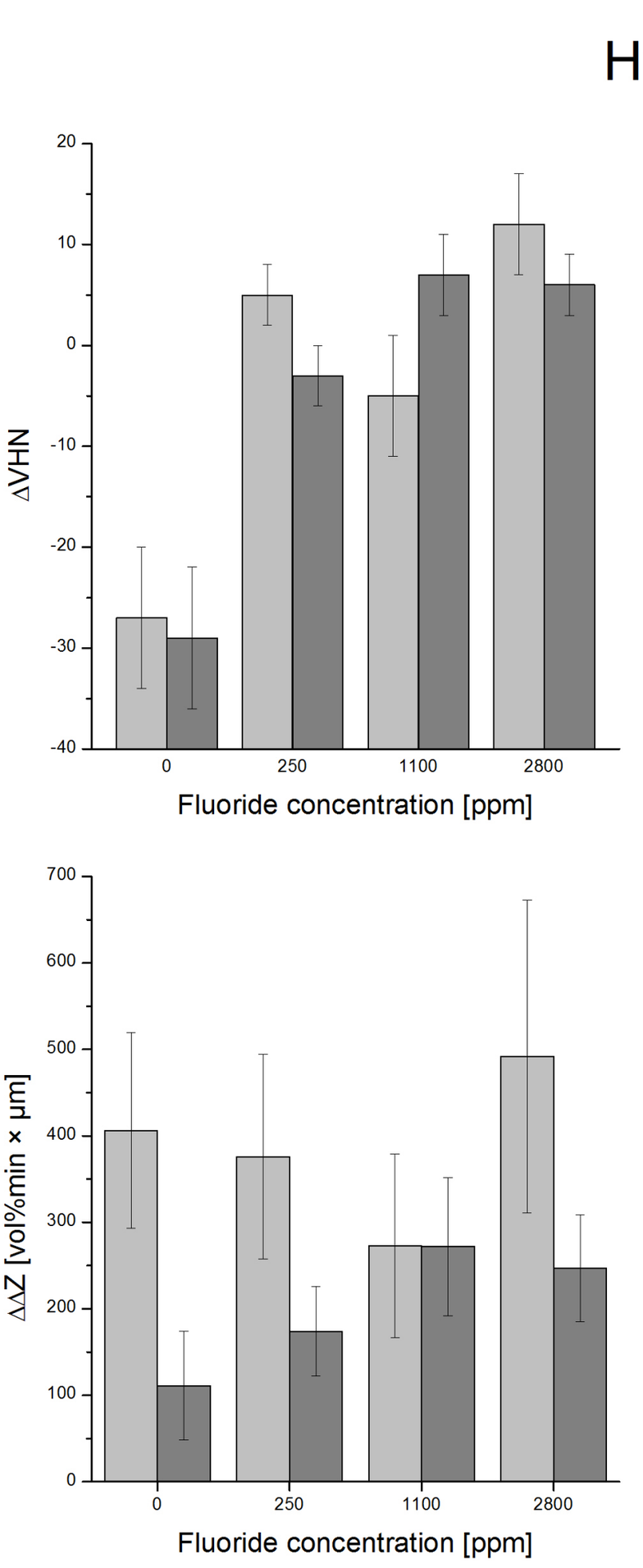
Fluoride concentration [ppm]

ΔSZmax [vol%min]



Fluoride concentration [ppm]

Hydroxyethylcellulose Lesions



Methylcellulose Lesions