FLUORIDE CONTENT IN SUPERFICIAL ENAMEL LAYERS OF HUMAN TEETH FROM ARCHEOLOGICAL EXCAVATIONS

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SUMMARY: The aim of this study was to evaluate the content of fluoride in three superficial enamel layers of human teeth obtained from archaeological excavations. Comparative analysis concerned the teeth from burial sites dated to the third millennium BC and 13–15th centuries AD. Enamel samples were collected by acid etch biopsy with the use of perchloric acid and, in the obtained biopsies, fluoride content was determined by potentiometry using a fluoride ion-selective electrode. It was found that the fluoride contents were significantly higher, in each of the three layers of enamel, in the teeth which were older in terms of historical age. This diversity is most likely to have occurred due to the *post-mortem* fluoride sorption from the surrounding environment, mainly from the soil. The research presented in this paper confirms the possibility of using fluoride determinations in hard tissues to assess the historical age of human remains from archaeological sites.

Keywords: Archeological excavations; Enamel acid etch biopsy; Fluoride; Teeth.

INTRODUCTION

Acid etch biopsy, as a method of collecting microsamples of enamel in order to determine its mineral composition, was used for the first time in the 1970s.[1,2] Its development was associated with the need to improve the previously used methods: abrasive biopsy (with low repeatability) and the aliquot method (in which it is necessary to grind a tooth or part of a tooth).[3] An equally important reason for the invention of acid etch biopsy was the need to find a method that would enable *in vivo* research on the mineral composition of enamel.[1]

Since its first use, acid etch biopsy of enamel has been repeatedly modified.[4-17] The changes consisted mainly in selecting the appropriate type of acid and its concentration and amount, as well as the optimization of time required to etch samples. The prevailing view is that the optimal procedure for *in vitro* research is the use of perchloric acid at a concentration of 0.5 M for 30 seconds, while *in vivo* the acid should be applied at the same concentration but only for 3 seconds.[18-23] Thanks to its advantages, especially the possibility of performing research on extremely thin layers of enamel, acid etch biopsy has also been applied in studies on the enamel mineral composition of teeth obtained from archaeological sites.[24]

This study used acid etch biopsy to determine the fluoride content in three superficial enamel layers of human teeth from archaeological sites, taking into account their historical and individual age, and the depth of etching.

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MATERIAL AND METHODS

MATERIAL

The study material consisted of two groups of teeth taken from the skulls from burial sites at two sites in Poland: Group 1, 85 teeth from Złota near Sandomierz, a neolithic burial ground dated to the 3rd millennium BC; and Group 2, 128 teeth from Gródek near Hrubieszów, a burial ground dated to the 13th to the 15th century AD. The individual age of the skeletons (age at death) was determined on the basis of cranial sutures obliteration and dental crowns' attrition. The analysis involved the teeth of *adultus* individuals, i.e. aged 20–35 years at the time of death, and *maturus* individuals, in the age range of 35–55 years at the time of death.

METHODS

1. *Acid etch enamel biopsy:*

Prior to biopsy the teeth were thoroughly purified and then repeatedly washed with deionized water. After drying, the buccal surface of the teeth was degreased with a cotton swab soaked with ether and then bilateral adhesive tape was used to paste aluminum foil to the surface. The site of biopsy was uncovered by cutting a 2.4 mm^2 area in the foil. The enamel surface was digested for 30 seconds with the use of 3 μL of perchloric acid at a concentration of 0.5 M from an Eppendorf pipette. The enamel biopsy was aspirated and placed in a plastic tube. Next, using the same pipette tip, the etched enamel surface was moistened twice with the use of 3 μL of deionized water, in order to collect the remaining biopsy material. In this way, the total volume of the sample placed in the Eppendorf tube was 9 μL. Biopsies were taken in an analogous manner from two successive layers of enamel, thereby yielding material from three superficial enamel layers.

2. *Determination of fluoride and calcium contents in enamel biopsies:*

The content of fluoride was determined by potentiometry using a fluoride ion-selective electrode (Orion 96–09). To determine the calcium concentration in the sample it was necessary to calculate the mass of the etched enamel using atomic absorption spectroscopy (Philips 9100X spectrometer). The mass of the collected enamel was calculated assuming a constant calcium content in the enamel (37.4%).[25-27] Then, based on the calculated enamel mass, at the assumed constant enamel density of 2.95 g/cm^3, and assuming a cylindrical shape of the etched area,[18,19] the thickness of each etched layer was calculated according to the following formula:

$$d = \frac{\text{enamel mass}}{2.95 \times \pi r^2}$$

d = depth of penetration (biopsied layer thickness); 2.95 = density of enamel (g/cm^3); r = radius of digestion (0.0875 cm)

On the basis of the thickness of the etched enamel layers, the depth of etching was calculated. The depth of the first etching of enamel was calculated by dividing the thickness of the first layer by 2, the depth of the second etching of the enamel was calculated by adding the thickness of the first layer to the half of the thickness of the second layer, and the depth of the third etching was the sum of the first, second, and the half of the third layer.

3. *Statistical analysis:*

The statistical significance of differences between the three groups of dependent variables (fluoride contents in three enamel layers) was measured by the Friedman ANOVA and Wilcoxon tests. The statistical significance of differences between the two groups of independent variables (teeth from sites in Złota and Gródek) was measured by the Mann-Whitney U test or Student's t-test. Differences were assumed to be statistically significant at $p<0.05$.

RESULTS

The test results are shown in Tables 1–5.

Table 1. Enamel mass, enamel thickness, and the depth of etching in three superficial enamel layers of teeth in Group 1 (85 teeth from a neolithic burial ground at Złota near Sandomierz dated to the 3rd millennium BC)

		Enamel layer			Significance of differences
		1 Outer	2 Middle	3 Inner	
Enamel mass (mg)	n	85	85	85	
	min. – max.	0.0074 – 0.0793	0.0076 – 0.0914	0.0081 – 0.0952	1/2 p<0.001
	$Q_1 – Q_3$	0.0255 – 0.0369	0.0283 – 0.0401	0.0291 – 0.0399	1/3 p<0.001
	median	0.0301	0.0331	0.0335	2/3 p>0.04
	mean ± SD	0.0330 ± 0.0144	0.0350 ± 0.0153	0.0358 ± 0.0158	
Enamel thickness (μm)	n	85	85	85	
	min. – max.	1.04 – 11.21	1.07 – 12.91	1.15 – 13.45	1/2 p<0.001
	$Q_1 – Q_3$	3.60 – 5.21	4.00 – 5.67	4.11 – 5.63	1/3 p<0.001
	median	4.26	4.68	4.73	2/3 p<0.04
	mean ± SD	4.66 ± 2.04	4.94 ± 2.17	5.06 ± 2.23	
Depth of etching (μm)	n	85	85	85	
	min. – max.	0.52 – 5.60	1.62 – 17.02	3.18 – 29.84	1/2 p<0.001
	$Q_1 – Q_3$	1.80 – 2.61	5.65 – 7.99	10.23 – 13.63	1/3 p<0.001
	median	2.13	6.60	11.26	2/3 p<0.001
	mean ± SD	2.33 ±1.02	7.13 ± 3.00	12.13 ± 5.03	

Table 2. Enamel mass, enamel thickness, and the depth of etching in three superficial enamel layers of teeth in Group 2 (128 teeth from a burial ground at Gródek near Hrubieszów dated to the 13th to the 15th century AD)

		Enamel layer			Significance of differences
		1 Outer	2 Middle	3 Inner	
Enamel mass (mg)	n	128	128	128	
	min. – max.	0.0059 – 0.1211	0.0056 – 0.0861	0.0112 – 0.0877	1/2 p<0.001
	$Q_1 - Q_3$	0.0283 – 0.0385	0.0301 – 0.0409	0.0319 – 0.0416	1/3 p<0.001
	median	0.0341	0.0370	0.0371	2/3 p<0.02
	mean ± SD	0.0334 ± 0.0115	0.0351 ± 0.0105	0.0363 ± 0.0103	
Enamel thickness (μm)	n	128	128	128	
	min. – max.	0.83 – 17.10	0.79 – 12.17	1.58 – 12.39	1/2 p<0.001
	$Q_1 - Q_3$	3.99 – 5.43	4.25 – 5.78	4.50 – 5.88	1/3 p<0.001
	median	4.82	5.23	5.24	2/3 p<0.02
	mean ± SD	4.71 ± 1.62	4.96 ± 1.48	5.13 ± 1.45	
Depth of etching (μm)	n	128	128	128	
	min. – max.	0.42 – 8.55	1.26 – 20.13	2.47 – 25.47	1/2 p<0.001
	$Q_1 - Q_3$	1.99 – 2.72	6.17 – 8.37	10.79 – 13.97	1/3 p<0.001
	median	2.41	7.40	12.74	2/3 p<0.001
	mean ± SD	2.36 ± 0.81	7.19 ± 2.14	12.21 ± 3.27	

Table 3. Fluoride content (mmol/kg) in three superficial enamel layers in both groups (Group 1, 85 teeth from a neolithic burial ground at Złota near Sandomierz dated to the 3rd millennium BC, and Group 2, 128 teeth from a burial ground at Gródek near Hrubieszów dated to the 13th to the 15th century AD)

		Fluoride content in enamel layer (mmol/kg)			Significance of differences
		1 Outer	2 Middle	3 Inner	
Group 1 Złota	n	85	85	85	
	min. – max.	19.17 – 864.75	21.21 – 319.76	18.27 – 347.51	1/2 p>0.22
	$Q_1 - Q_3$	36.52 – 85.78	35.20 – 84.27	38.67 – 85.40	1/3 p>0.44
	median	55.53	48.47	46.89	2/3 p>0.42
	mean ± SD	83.82 ± 102.23	73.41 ± 60.24	76.68 ± 67.80	
Group 2 Gródek	n	128	128	128	
	min. – max.	9.99 – 192.44	18.11 – 152.10	13.46 – 109.82	1/2 p<0.001
	$Q_1 - Q_3$	34.31 – 61.90	29.27 – 54.41	30.48 – 50.73	1/3 p<0.001
	median	43.43	39.37	37.00	2/3 p<0.002
	mean ± SD	51.18 ± 27.62	46.46 ± 24.87	41.46 ± 16.81	
Comparing Groups 1 and 2:		p<0.002	p<0.001	p<0.001	

Table 4. Fluoride content (mmol/kg) in three superficial enamel layers in Group 1 (85 teeth from a neolithic burial ground at Złota near Sandomierz dated to the 3rd millennium BC) in relation to the individual age (*adultus*, aged 20–35 yr at the time of death, and *maturus*, aged 35–55 yr at the time of death)

		Fluoride content in enamel layer (mmol/kg)			Significance of differences
		1 Outer	2 Middle	3 Inner	
Group 1 *adultus*	n	47	47	47	
	min. – max.	26.74 – 174.96	21.21 – 233.05	24.29 – 347.51	1/2 p>0.10
	$Q_1 – Q_3$	36.38 – 83.75	33.44 – 83.42	36.56 – 66.34	1/3 p>0.19
	median	54.10	44.71	43.87	2/3 p>0.61
	mean ± SD	69.26 ± 40.64	63.83 ± 47.33	67.65 ± 62.29	
Group 1 *maturus*	n	38	38	38	
	min. – max.	19.17 – 864.75	25.04 – 319.76	18.27 – 320.01	1/2 p>0.89
	$Q_1 – Q_3$	38.02 – 123.05	38.64 – 111.50	40.74 – 125.99	1/3 p>0.84
	median	56.06	52.93	54.87	2/3 p>0.52
	mean ± SD	101.84 ± 145.15	85.27 ± 72.06	87.85 ± 73.35	
Comparing *adultus* and *maturus*:		p>0.88	p>0.14	p<0.06	

Table 5. Fluoride content (mmol/kg) in three superficial enamel layers in Group 2 (128 teeth from a burial ground at Gródek near Hrubieszów dated to the 13th to the 15th century AD) in relation to the individual age (*adultus*, aged 20–35 yr at the time of death, and *maturus*, aged 35–55 yr at the time of death)

		Fluoride content in enamel layer (mmol/kg)			Significance of differences
		1 Outer	2 Middle	3 Inner	
Group 2 *adultus*	n	63	63	63	
	min. – max.	21.31 – 160.13	18.11 – 151.82	13.46 – 89.02	1/2 p<0.001
	$Q_1 – Q_3$	33.04 – 66.90	28.56 – 55.46	26.10 – 54.37	1/3 p<0.001
	median	43.29	37.69	34.86	2/3 p<0.02
	mean ± SD	51.48 ± 25.64	46.31 ± 26.02	40.10 ± 17.19	
Group 2 *maturus*	n	65	65	65	
	min. – max.	9.99 – 192.44	19.81 – 152.10	18.44 – 109.82	1/2 p<0.004
	$Q_1 – Q_3$	35.56 .– 54.58	30.36 – 53.03	32.25 – 49.64	1/3 p<0.003
	median	43.68	39.49	38.66	2/3 p<0.05
	mean ± SD	50.89 ± 29.61	46.61 ± 23.90	42.77 ± 16.47	
Comparing *adultus* and *maturus*:		p>0.96	p>0.62	p>0.17	

DISCUSSION

As follows from the analysis of the statistical data presented in Tables 1 and 2, the most externally located enamel layer was the most resistant to etching with perchloric acid. The calculated depth of etching averaged 2.13 μm in Group 1 and 2.41 μm in Group 2. After the second etching these depths amounted to 6.6 μm and 7.4 μm, and after the third etching the depths reached 11.26 μm and 12.74 μm, respectively. These data are in agreement with the results of Jakubowska[19] and Chlubek et al.[24] who reported the greatest resistance to acid etching in the outermost layers of the enamel. This is due to the fact that the hardness of the enamel in human teeth is greatest in the surface layers and decreases toward the enamel-dentine junction.[28-31] Based on the analysis of results on the depth of enamel etching, it should be noted that the lack of significant difference in this respect between the two tested groups (Tables 1 and 2) allowed for an objective comparative evaluation of fluoride content in the individual enamel layers of the tested teeth.

The data on fluoride content in the individual layers of tooth enamel in both groups clearly show that in each layer the contents were significantly higher in the teeth which were older in terms of historical age (Table 3). It is worth noting that earlier studies on bones from different historical periods show similar variation.[32,33] This can be explained by the dynamic *post-mortem* enrichment of bone structures with fluoride, especially in earth burials and when the soil showed a relatively high content of fluoride. Therefore, bone material with a considerably different historical age (in thousands of years) also differs significantly in fluoride content.

Based on the results obtained in this study, it appears that *post-mortem* fluoride sorption from the soil occurs also in the case of enamel, a strongly mineralized and integral tissue. It should be emphasized that individuals belonging to both groups lived in times when unnatural exposure to fluoride was impossible. Caries prevention with the use of fluoride preparations and anthropogenic environmental pollution with fluorine compounds were non-existent. Therefore, the only reasonable explanation for the observed differences is the *post-mortem* sorption of fluoride from the soil. Penetration of fluoride into the successive layers of enamel is a very slow and thus a long-term process, requiring thousands of years. This is evidenced by the fact that differences in fluoride content between the different layers of the enamel showed statistical significance only in the Group 2 (teeth from 500–700 years ago) (Table 3). In the case of Group 1, these differences got blurred due to the much longer duration of fluoride penetration into the deeper layers of the enamel (about 4500 years).

Analyzing the content of fluoride in the teeth of both groups, taking into account the distinction between the *adultus* and *maturus* categories (individual age), one may observe a lack of significance of differences between the individual layers of enamel (Tables 4 and 5). This may mean that the life-span of several dozen of years is too short to result in different contents of fluoride in the superficial layers of enamel, or that these differences are eliminated by the influence of sorption processes in skeletons remaining in burial sites for hundreds or thousands of years. The latter reason seems to be closer to the truth as contemporary subjects generally exhibit some differences in the contents of fluoride between their individual enamel layers, with the fluoride content decreasing with the depth of etching.[19-21,34] It should be emphasized again that these differences arise from processes occurring during life, associated with the use of active fluoride for the prevention of tooth decay or environmental pollution with fluorine compounds. These factors cannot be taken into account in the analysis of teeth from distant historical periods.

To sum up, the results of this analysis of the fluoride content in the archaeological material showed that regardless of the individual age, the superficial enamel layers of the teeth which were older in terms of historical age (remaining longer in the place of burial) were significantly richer in fluoride compared with the historically younger teeth.

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