In vivo Experimental Study on the Influence of Sodium Fluoride and Amoxicillin/Clavulanic Acid on the Minerals Composition of Dental Enamel

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The aim of this study was to assess the morphological and chemical composition changes induced by the chronic intake of sodium fluoride (NaF) and Amoxicillin in mice enamel. 35 C57BL/6 adult male mice, were randomly divided into a control and 4 treatment groups (n = 7). After acclimatization, the experimental groups were simultaneously treated with 25 ppm (group 2 and 3) and 50ppm (group 4 and 5) of NaF, and 50mg/kg BW (group 2 and 4) and 100 mg/kg BW (group 3 and 5) of Amoxicillin as Amoxicillin/clavulanic acid (AMC). NaF was supplied through drinking water without restricting access, and AMC administered through subcutaneous injection, once per day, for 60 days. After harvesting, lower incisors' enamel was subjected to a scanning electron microscope (SEM) and to an energy dispersive X-ray analysis (EDX). In the treatment groups, SEM and EDX analysis in treatment groups showed an increasing trend of weight percentage (wt%) for C, N, O, F, Na and C/O, F/Fe ratio, and also a decreasing trend of wt% for P, Cl, Ca, Fe and Ca/P ratio. Morphological changes ranged from fissures and short grooves with pits-like appearance, in group 2 sometimes associated with limited demineralized areas looking like irregular scratches, up to demineralized areas extended in the outer enamel, which in group 5 gives the enamel the corroded look. The severity of the morphological changes in the mice enamel varied with the supplied dose of NaF and AMC, and had a uniform pattern in each experimental group. SEM analysis revealed a hypoplasia on the outer enamel and EDX analysis showed a hypomineralisation at the level of the outer enamel.

Keywords: teeth, fluorosis, hypomineralization, SEM, amoxicillin, sodium fluoride

Dental development is controlled by a sequence of inductive and reciprocal interactions between the epithelial and the mesenchymal cells. This process is a complex one and can be divided into morphogenesis, differentiation and dental cell secretion and mineralization of the specific dental matrix [1].

A systemic fluoride intake, higher than optimal one (≥0.07 mg/kg F/day) during critical periods of amelogenesis can lead to dental fluorosis [2-4]. Fluorine affects the functions of ameloblasts both in the secretory and maturation phase, having as results a poor mineralization and the formation of porous enamel, characterized by an increased dimension of the intercrystalline spaces that will be filled with proteins and water [5, 6].

Amoxicillin is the most prescribed antibiotic during childhood both in dentistry as well as in pediatrics [7, 8]; it is suggested that its use during early childhood is associated with Molar Incisor Hypomineralisation (MIH) syndrome and with defects resembling to dental fluorosis [9]. In the literature it is speculated that amoxicillin would interfere with the functions of ameloblasts during the secretory phase [10]. Also, the prevalence of the dental fluorosis and of the MIH syndrome increased worldwide, ranging from 2.8% to 44% for the MIH syndrome and from 2.9% to 42% for the dental fluorosis [11, 12].

Moreover, experimental studies have shown the existence of a linear relation between the severity of enamel defects and the administrated amoxicillin or fluorine dose, as well as the fact that these interfere with the dentine mineralization [2, 13].

Concerning the associated action of amoxicillin and fluorine on amelogenesis, there are only a few studies in the literature, with some methodological problems, indicating the necessity of additional studies in order to show the influence of this combination on dental enamel [9, 10, 14, 15].

The aim of our study was to assess in vivo the impact of the combined action effects of amoxicillin (as amoxicillin/clavulanic acid (AMC)) and sodium fluoride (NaF) on dental enamel of laboratory mice and to determine the morphological and compositional changes that occurred under the action of these two substances using a scanning electron microscope (SEM) and an energy-dispersive X-ray (EDX).

Experimental part

The study was approved by the Institutional Ethical Committee of University of Medicine and Pharmacy Grigore T. Popa Iasi (Reg. Nr. 15488/30.VII.2013). For our study we used 35 C57BL/6 inbred strain adult male mice. The mice have been kept in conditions of constant temperature of 24±1°C, under a light-darkness cycle of 12h, in plastic cages and they had ad libitum access to food and distilled water. Their food was standard in the form of pellets (18.8% proteins, 2.3% fats, 6.1% fibers - Cantacuzino, Romania).

After 2 weeks of acclimatization, the mice have been randomly distributed in 5 groups: 1 control group (group 1) and 4 experimental groups (group 2, 3, 4 and 5), each group containing seven mice (n = 7). The administrated substances and the doses for each group were as follows:

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-group 1: received *ad libitum* distilled water for drinking and once per day subcutaneously (sc) 0.1 mL of the solvent (0.1 mL distilled sterile water);

-group 2: received *ad libitum* 25 ppm NaF in distilled water for drinking and once per day, sc 50 mg/kg BW of AMC;

-group 3: received *ad libitum* 25 ppm NaF in distilled water and once per day, sc 100 mg/kg BW of AMC;

-group 4: received *ad libitum* 50 ppm NaF in distilled water and once per day, sc 50 mg/kg BW of AMC;

-group 5: received *ad libitum* 50 ppm NaF in distilled water and once per day, sc 100 mg/kg BW of AMC;

NaF extra pure powder (Scharlau® Spain) was administrated through drinking water (distilled water as solvent) with *ad libitum* access for 60 days.

AMC extra pure powder for injections (1.2 g vials, Amoxiplus®, Antibiotice SA, Romania) was purchased and prepared extemporaneously with sterile distilled water used as solvent, and was administered through a subcutaneous injection (above the shoulders, in the loose skin behind the neck) in a single dose, for 60 days.

At the end of the experimental study, the mice were deeply anesthetized with Isoflurane and sacrificed. Subsequently, the mandibles were resected; surrounding tissues partially removed and the crowns of the lower incisors were sectioned in a mesio-distal direction, near the gingival edge, using a diamond disk. After fixation in glutaraldehyde and dehydration in ethanol, lower incisors' enamel was subjected to SEM and EDX analysis.

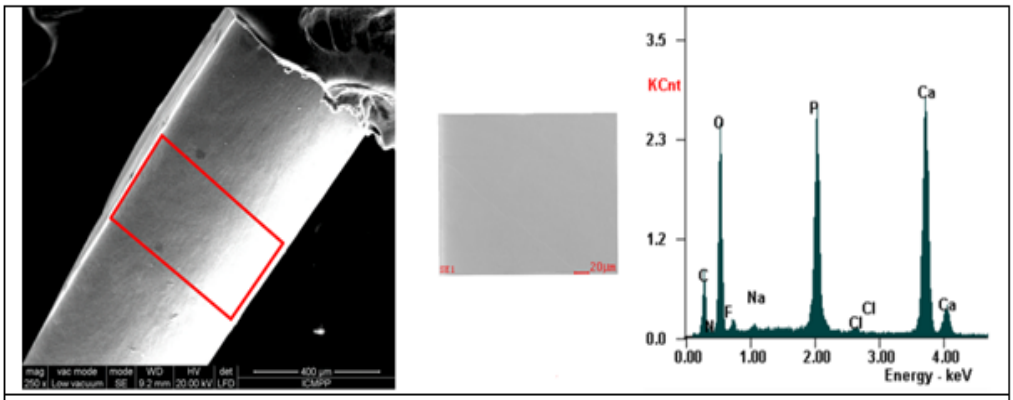
For both analysis (SEM and EDX), all the data were gathered by evaluating the middle third of the enamel (fig. 1). With the help of the scanning electronic microscope ESEM Quanta 200 (FEI, Eindhoven, Netherlands) equipped with an energy-dispersive X-ray device (EDAX), was assessed the outer enamel morphology and were determined the relative quantities of C, N, O, F, Na, P, Cl, Ca and Fe were determined; than were calculated the following ratios C/O, Ca/P and F/Fe.

The spectrums collection, the peaks identification and their quantification, were done using GENESIS Spectrum software.

All statistical analysis was performed using SPSS Statistics 21.0 software (IBM Corp., Chicago, IL, USA). Obtained data were represented as mean values ± standard deviation (SD) and were analyzed using Mann-Whitney and Kruskal-Wallis tests (statistical significance for $p < 0.05$).

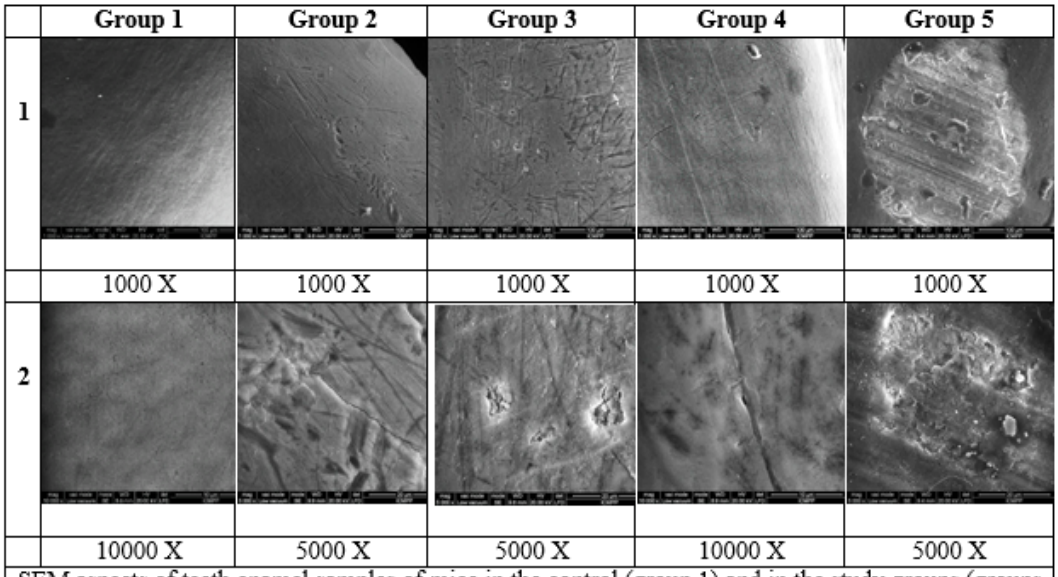
Results and discussions

The analyses of the images for the lower incisor enamel from the control group accomplished with SEM, showed homogeneous, smooth, regular surfaces with several scarcely visible point-like pits (fig. 2 – 1.1 and 2 - 2.1). In the experimental groups, enamel SEM aspects ranged from fissures and pits with aspect of fossae, sometimes associated with demineralized limited areas looking like irregular *scratches* in group 2 (fig. 2 – 1.2 and 2 - 2.2); in group 3,were detected point areas without external enamel, enamel demineralization which confers a *speckled* appearance (fig. 2 – 1.3 and 2 - 2.3); in group 4 deep fissures, with paths through fossae and with unstable



For the analysis an area from the middle third of the buccal face of the incisors was selected.

Fig. 1. Schematic representation of the selected area for SEM and EDX analysis



SEM aspects of teeth enamel samples of mice in the control (group 1) and in the study groups (groups 2-5) observed at different magnifications.

Fig. 2. Enamel SEM aspects in the control and study groups

images due to electrostatic charges (fig. 2 – 1.4 and 2 - 2.4); in group 5 changes up to demineralized areas extended in the external enamel, which gives to the enamel a *corroded* look (fig. 2 – 1.5 and 2 - 2.5).In the groups treated with AMC and NaF, elemental composition of the outer enamel evaluated using an EDX detector showed an increasing tendency of wt% values for C, N, O, F, Na and C/O, F/Fe ratio, and a decreasing tendency of wt% values for P, Cl, Ca, Fe and Ca/P (table 1). According to the EDX analysis, the mean values of the wt%, for the assessed elements and ratios, varied between the control group and the experimental groups. However, statistically significant differences were detected for Fe (between groups 1 and 3, 1 and 4, 2 and 4, its value decreased), for F (between groups 2 and 3, 2 and 5 4 and 5, its value increased), for N (between groups 2 and 3, its value increased) and for F/Fe ratio (between groups 1 and 3, 1 and 4, 1 and 5, 2 and 3, 2 and 4, 2 and 5, its value increased) (table 2).

Even if most dental dystrophies have included in their etiology also a genetic component, some predisposing or etiological factors can be easily avoided by simply respecting some indications for preventing their occurrence. From the perspective of dental dystrophies prevention, we decided to test the effects of the combined systemic and chronic intake of NaF and amoxicillin (as AMC) on enamel morphology of mice lower incisors.

The main role of the dental enamel is to withstand the masticatory forces and the chemical attacks generated by the diet and bacterial flora [16]. The enamel is the most mineralized tissue in mammals and it is composed in a proportion of over 97% by minerals, mostly in the form of calcium phosphates. Ca/P ratio in healthy enamel varies between 1.64 and 1.8, similar to that of apatite [17, 18].

Some authors utilizing a semi-quantitative analyses by EDX spectrometry on extracted MIH teeth, showed a reduction of mineral composition at the enamel level [19] and a decrease by approximately 19% in the mineral density in comparison with the healthy enamel [20-22]. The literature reports also a decrease in the Ca/P ratio of the enamel [23, 24] correlated with an increase of C content [25]. In our study, the EDX analysis on enamel showed a Ca/P ratio of 2.07 in the control group; this varied insignificantly in the experimental groups (between 1.97 and 2.20). From quality point of view, this fact suggests

Table 1

THE CHEMICAL COMPOSITION OF DENTAL ENAMEL FROM MICE LOWER INCISORS, OBTAINED BY EDX ANALYSIS AND THE ASSESSMENT OF THE STATISTICAL SIGNIFICANCE OF THIS VARIATION BETWEEN THE CONTROL GROUP AND THE EXPERIMENTAL GROUPS

Element/ report	Group 1	Group 2	Group 3	Group 4	Group 5	*p**
C	20.96±3.91	(+)23.25±4.99	(+)23.81±6.23	(+)25.21±8.00	(+)22.11±4.86	0.791
N	1.35±0.59	(+)1.40±0.41	(+)1.85±0.36	(+)1.55±0.50	(+)1.53±0.68	0.482
O	30.96±5.26	(-)28.30±8.38	(+)31.70±6.43	(+)33.00±7.33	(+)31.59±5.07	0.807
F	0.59±0.34	(-)0.45±0.13	(+)0.70±0.32	(-)0.56±0.20	(+)0.90±0.27	0.041
Na	0.48±0.06	(+)0.51±0.14	(+)0.54±0.12	(+)0.53±0.15	(+)0.53±0.13	0.884
P	14.32±1.18	(-)14.0 ±0.95	(-)13.50±2.00	(-)12.92 ±1.81	(-)13.93±1.61	0.668
Cl	0.41±0.08	(-)0.36±0.04	(-)0.38±0.08	(-)0.36±0.09	(-)0.36±0.03	0.740
Ca	29.79±4.73	(+)30.83±4.34	(-)26.89±4.41	(-)25.50±5.14	(-)28.43±4.12	0.427
Fe	1.23±0.41	(-)0.84±0.35	(-)0.62±0.33	(-)0.32±0.11	(-)0.59±0.32	0.007
C/O	0.69±0.18	(+)0.94±0.49	(+)0.80±0.41	(+)0.83±0.47	(+)0.73±0.28	0.995
Ca/P	2.07±0.23	(+)2.20±0.37	(-)2.00±0.28	(-)1.97±0.25	(-)2.04±0.24	0.571
F/Fe	0.56 ± 0.38	(+) 0.65 ± 0.33	(+) 1.51 ± 1.25	(+)2.08±1.35	(+)2.20±1.84	0.004

Note: Data represent mean + standard deviation values of percentage by mass (wt %) of 7 mice per group. (+) or (-) indicates an increase or decrease compared to the control group; *p**- *p* value from Kruskal-Wallis test, statistically significant differences ($p<0.05$).

Table 2

p VALUES OBTAINED WITH MANN-WHITNEY TEST USED TO ASSESS THE STATISTICAL SIGNIFICANCE CONCERNING THE COMPOSITION DIFFERENCES BETWEEN STUDY GROUPS

Element/ raport	Group 1/2	Group 1/3	Group 1/4	Group 1/5	Group 2/3	Group 2/4	Group 2/5	Group 3/4	Group 3/5	Group 4/5
C	0.391	0.391	0.253	0.475	0.949	0.848	0.749	0.565	0.655	0.565
N	0.830	0.116	0.568	0.475	0.040	0.654	0.565	0.482	0.607	0.949
O	0.567	0.886	0.568	0.475	0.406	0.277	0.482	0.655	0.949	0.565
F	0.474	0.886	0.886	0.086	0.040	0.277	0.006	0.609	0.125	0.025
Na	0.830	0.224	0.519	0.316	0.848	0.654	0.949	1.000	0.701	0.949
P	0.389	0.568	0.116	0.775	0.748	0.336	0.949	0.565	0.749	0.338
Cl	0.313	0.617	0.316	0.221	0.561	1.000	0.699	0.564	0.521	0.796
Ca	0.668	0.568	0.153	1.000	0.179	0.084	0.482	0.655	0.565	0.338
Fe	0.252	0.046	0.003	0.063	0.306	0.004	0.224	0.064	0.949	0.110
C/O	0.775	0.886	0.886	0.886	0.949	0.654	0.749	0.949	0.949	0.565
Ca/P	0.567	0.317	0.391	0.886	0.277	0.110	0.482	0.749	0.655	0.482
F/Fe	0.567	0.032	0.015	0.015	0.018	0.013	0.006	0.482	0.482	0.898

Note .Red font = statistically significant differences ($p < 0.05$).

that the structure of the enamel has been insignificantly modified; however, it allows the possibility of significant differences in terms of its quantity. Moreover, the C/O ratio value of 0.69 for the control group, ranging between 0.73 and 0.94 in experimental groups, alongside with increased wt% values for C (20.96 in the control group and ranging between 22.11 and 25.21 in the experimental groups) and for O (30.96 in the control group and ranging between 28.30 and 33.00 in the experimental groups), can suggest excessive formation of carbonate hydroxyapatite $Ca_5(PO_4,CO_3)_3(OH)$. Carbonate hydroxyapatite being less dense can contribute to the occurrence of post eruptive defects, such as the *scratched* look of the enamel that we observed.

Increased values for wt% of F in the study groups 3 and 5 (0.70 respectively 0.90), can suggest the subsequent formation of carbonate fluorapatite $Ca_5(PO_4,CO_3)_3F$. It's considered that the presence of fluorine in the dental enamel contributes to its resistance against acidic attacks, however it does not contribute to its mechanical resistance, and it even undermines it [18]. This aspect can explain the presence of fissures in enamel observed through the evaluation of SEM images. Moreover, enamel roughness clinically observed in the groups treated with AMC and NaF, can be associated with its inadequate development [17].

In his study regarding the mass spectrometry of secondary ions and the X-ray microanalysis of hypomineralised enamel, Jalevik et. al. [26] concluded that an increased content of Na towards the surface of the enamel can suggest hypomineralization; however the variations of Cl content have had no correlation with the degree of demineralization. In our study, the wt% values of Na from experimental groups have been slightly increased (0.51 - 0.54) in comparison with the control group (0.48), an aspect the can be considered as being another sign of hypomineralization. Concerning Cl, its quantitative values were constant in the experimental groups (0.36 - 0.38) and remained roughly similar to the one from the control group (0.41); its variations have not been related to the administration of NaF and AMC.

Conclusions

The chronic administration of NaF through the drinking water along with AMC through subcutaneous injections in various doses in C57BL/6 mice, leads to the occurrence of defects in lower incisors enamel with a uniform distribution within the experimental groups. The microstructural changes observed using SEM showed hypoplasia at the level of outer enamel. The assessment of the chemical composition using EDX analysis showed hypo-mineralization.

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