Chemical Content Variations and Morphological Changes Evaluation of Incisors Enamel Induced by Chronic Fluoride Intoxication

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The aim of this study was to assess the morphology and chemical composition changes induced by chronic intoxication with low doses of sodium fluoride (NaF) in mice enamel. Twenty-one C57BL/6 male mice of the same age were randomly divided into a control group and 2 experimental groups (n = 7). The experimental groups were treated with 25ppm and 50ppm respectively of NaF. NaF was supplied through drinking water, without restricting access for 60 days. After fixation in glutaraldehyde and dehydration in ethanol, lower incisors enamel was subject to scanning electronic microscope (SEM) and energy dispersive X-ray (EDX) analysis. The elemental composition analysis in both experimental groups showed higher wt% (percentage by mass) of mean values for N, O, F, Na, P, and F/Fe ratio, and a decrease of mean values for C, Cl, Ca, Fe, and C/O, Ca/P ratios. SEM analyses showed morphological changes which ranged from small, isolated enamel pits with regular margins, to extensive and deep loss of incisors enamel with irregular margins, as well as multiple fissures in the enamel surface.

Keywords: mice, dental fluorosis, scanning electron microscopy, energy dispersive X-ray spectroscopy, sodium fluoride

Fluoride is a common element in the earth crust and is widely used in many industrial processes [1]. It is known that fluoride ions participate actively in the enamel remineralization process and inhibit the cariogenic bacteria activity from the oral biofilm [2]. In the last 60 years, fluorides have played a central role in promoting oral health and remain an important ally in the fight to reduce caries experience. However, systemic intake of fluoride higher than the optimal dose (≥ 0.07 mg F/kg/day) during critical periods of amelogenesis may lead to dental fluorosis (DF) [3-6]. DF is defined as the developmental defect of dental enamel, caused by amelogenin proteins retention determined by fluoride ions and it is characterized by enlargement of the intercrystalline spaces that will be filled with proteins and water [7]. Those changes causes enamel hypomineralization, increased porosity in outer and inner enamel, loss of enamel translucency and increase of its opacity [7, 8]. Fluoride affects the ameloblasts function during the early secretory and the transitional phase in acute fluoride overexposure, and during the maturation phase in chronic fluoride overexposure [9-16].

Effects of fluoride on dental enamel development have been studied in a wide range of species and type of teeth. In most studies, rat incisors and molars were used, but there are enough studies that used mice, sheep, pigs, rabbits, hamsters and zebra fish [11]. Small rodents were found to be good models for the study of human dental fluorosis, because they develop fluorotic lesions at plasma F levels similar to humans (min. 2 μmol/L) [1, 11].

The aim of this study was to assess the morphology and compositional changes induced by chronic intoxication with low doses of sodium fluoride (NaF) in mice enamel.

Experimental part
The research project was fully approved by the Research Ethics Committee of the “Grigore T. Popa” University of Medicine and Pharmacy Iasi, Romania (Reg. No. 15488/30.VII.2013). From the Baneasa Station, unit of the “Cantacuzino” National Institute in Research and Development in Microbiology and Immunology (Bucharest, Romania) we obtained twenty-one C57BL/6 inbred strain weanling male mice (8 to 10 weeks old). All mice were housed within the Center for the Study and Therapy of Pain, „Grigore T. Popa” University of Medicine and Pharmacy Iasi, Romania. Mice were kept at 24±1°C in boxed caging and allowed ad libitum access to food and water. They were fed a standardized laboratory rodent diet (18.8% proteins, 2.3% fats, 6.1% fibers) and they were given permanent access to distilled water.

After one week of acclimatization, mice were randomly divided into a control group and 2 experimental groups (group 2 and 3), which consisted of 7 mice each. Fluoride was supplied as NaF (Sodium fluoride, extra pure, Ph Eur, BP, USP, Scharlau® Spain) through drinking water, for 60 days. Control group received only distilled water, group 2 received distilled water supplemented with 25 ppm fluoride, and group 3 received distilled water supplemented with 50 ppm fluoride. After 60 days of fluoride treatment, the mice were deeply anesthetized with isoflurane (Anestefan, Rompharm Company, Romania) using anesthetic machine (Komesaroff, Medical Developments Australia) and sacrificed by decapitation (using sharp scissors). Lower incisors crowns were cross-sectioned in the mesiodistal direction near the cervical margin, using a diamond cutting disc adapted to dental laboratory
induction micromotor (Marathon Multi 600, Megadental GmbH) without water cooling. Each tooth was washed with distilled water, fixed for 24 h in glutaraldehyde 2% and dehydrated in ascending ethanol concentrations (70%, 85%, and 99% for 1 day for each concentration). Finally, each lower incisor was mounted on an aluminum stub for outer enamel structural defects examination by scanning electron microscopy (SEM) (FEI Quanta 200, Eindhoven, Netherlands) operating at 20 kV in low-vacuum mode for secondary electron imaging. The Quanta 200 SEM was equipped with an energy dispersive X-ray spectroscopy (EDS) system for qualitative and quantitative analysis and elemental mapping. To acquire information for elemental content of enamel, spectra were collected across selected areas in the middle third of the incisor enamel surface. All obtained spectra were analyzed using EDAX inc. Genesis Spectrum SEM Qanta ZAF Software (version 6.10). The relative amounts of C, N, O, F, Na, P, Cl, Ca and Fe were encoded into a spreadsheet program (Excel, Microsoft, Seattle, WA) and descriptive statistics was applied. Statistical analysis was performed with SPSS version 21.0 using the Kruskal-Wallis and Mann-Whitney test with a significant difference level set to $p < 0.05$ for comparison of the chemical composition of the above elements on the outer enamel layer, as well as C/O, Ca/P and F/Fe ratios.

<table>
<thead>
<tr>
<th>Element</th>
<th>Group 1 control</th>
<th>Group 2 25 ppm NaF</th>
<th>Group 3 50 ppm NaF</th>
<th>p* Group 1-3</th>
<th>p* Group 1/2</th>
<th>p* Group 1/3</th>
<th>p** Group 1-3</th>
<th>p** Group 1/2</th>
<th>p** Group 1/3</th>
<th>p** Group 2/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>20.96 ± 3.91</td>
<td>19.42 ± 2.45</td>
<td>18.66 ± 1.78</td>
<td>0.420</td>
<td>0.749</td>
<td>0.199</td>
<td>0.391</td>
<td>0.199</td>
<td>0.391</td>
<td>0.391</td>
</tr>
<tr>
<td>N</td>
<td>1.35 ± 0.59</td>
<td>1.49 ± 0.42</td>
<td>1.46 ± 0.52</td>
<td>0.916</td>
<td>0.688</td>
<td>0.775</td>
<td>0.886</td>
<td>0.775</td>
<td>0.886</td>
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<tr>
<td>O</td>
<td>30.96 ± 5.26</td>
<td>33.16 ± 3.20</td>
<td>35.07 ± 4.58</td>
<td>0.358</td>
<td>0.433</td>
<td>0.199</td>
<td>0.391</td>
<td>0.199</td>
<td>0.391</td>
<td>0.391</td>
</tr>
<tr>
<td>F</td>
<td>0.59 ± 0.34</td>
<td>0.63 ± 0.17</td>
<td>0.99 ± 0.38</td>
<td>0.088</td>
<td>0.936</td>
<td>0.086</td>
<td>0.046†</td>
<td>0.936</td>
<td>0.086</td>
<td>0.046†</td>
</tr>
<tr>
<td>Na</td>
<td>0.48 ± 0.06</td>
<td>0.56 ± 0.11</td>
<td>0.60 ± 0.08</td>
<td>0.079</td>
<td>0.173</td>
<td>0.022†</td>
<td>0.568</td>
<td>0.022†</td>
<td>0.568</td>
<td>0.022†</td>
</tr>
<tr>
<td>P</td>
<td>14.32 ± 1.18</td>
<td>14.85 ± 1.26</td>
<td>15.48 ± 1.36</td>
<td>0.827</td>
<td>0.522</td>
<td>0.775</td>
<td>0.775</td>
<td>0.775</td>
<td>0.775</td>
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</tr>
<tr>
<td>Cl</td>
<td>0.41 ± 0.08</td>
<td>0.32 ± 0.06</td>
<td>0.37 ± 0.10</td>
<td>0.259</td>
<td>0.078</td>
<td>0.474</td>
<td>0.473</td>
<td>0.474</td>
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<td>0.473</td>
</tr>
<tr>
<td>Ca</td>
<td>29.79 ± 4.73</td>
<td>28.83 ± 2.18</td>
<td>27.77 ± 4.29</td>
<td>0.872</td>
<td>0.873</td>
<td>0.876</td>
<td>0.75</td>
<td>0.876</td>
<td>0.75</td>
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<tr>
<td>Fe</td>
<td>1.0 ± 0.41</td>
<td>0.70 ± 0.36</td>
<td>0.46 ± 0.29</td>
<td>0.029</td>
<td>0.092</td>
<td>0.015†</td>
<td>0.198</td>
<td>0.015†</td>
<td>0.198</td>
<td>0.198</td>
</tr>
<tr>
<td>C/O ratio</td>
<td>0.69 ± 0.18</td>
<td>0.59 ± 0.12</td>
<td>0.53 ± 0.07</td>
<td>0.154</td>
<td>0.262</td>
<td>0.063</td>
<td>0.391</td>
<td>0.262</td>
<td>0.063</td>
<td>0.391</td>
</tr>
<tr>
<td>Ca/P ratio</td>
<td>2.07 ± 0.23</td>
<td>1.95 ± 0.23</td>
<td>1.89 ± 0.11</td>
<td>0.262</td>
<td>0.337</td>
<td>0.116</td>
<td>0.475</td>
<td>0.337</td>
<td>0.116</td>
<td>0.475</td>
</tr>
<tr>
<td>F/Fe ratio</td>
<td>0.55 ± 0.38</td>
<td>1.09 ± 0.53</td>
<td>2.69 ± 1.61</td>
<td>0.003†</td>
<td>0.109</td>
<td>0.003†</td>
<td>0.015†</td>
<td>0.109</td>
<td>0.003†</td>
<td>0.015†</td>
</tr>
</tbody>
</table>

Table 1

**Results and discussions**

For our study we used mice because rodent incisor teeth grow continuously and exhibit all stages of tooth formation [17]. This characteristic makes them ideal models for the study of enamel formation. In mice, the enamel cover only the labial part of the incisors and are divided into 2 main layers, inner enamel and outer enamel. In the inner enamel, prisms are arranged in single layered rows, which were oriented transversely to the long axis of the tooth. Prisms of adjacent rows were inclined in opposite directions (mesially and laterally), and consequently crossed each other (decussation). In the outer enamel all prisms became parallel with each other, and ran in an incisal direction [18]. The enamel contains hydroxyapatite crystals as inorganic material (96% by weight and over 86% by volume), remnants of proteins from the period of development, water (3.5% by weight) and organic matter (0.6% by weight) [2, 19].

There are many in vivo and in vitro studies that evaluate different mechanisms and cell functions involved in dental fluorosis, but there is lack of data focused on morphological and chemical content changes in outer enamel induced by fluoride overexposure. In our study, EDX analysis on the enamel in both experimental groups showed an increase of mean values in N, O, F, Na, P, and F/Fe ratio, and a decrease of mean values in C, Cl, Ca, and F/Fe ratios compared with the control group (table 1).

EDX analysis between the experimental group 2 and experimental group 3 showed the same results with the following differences: an increase of mean value for Cl, and a decrease of mean values for N, P. Even if all mean values of the wt% (percentage by mass) for evaluated elements varied between control and experimental groups, statistically significant differences were found only in Fe, and F/Fe ratio on the enamel in both experimental groups and control group; Na, and F/Fe ratio on the enamel in experimental group 3 and control group and F, and F/Fe ratio on the enamel in experimental group 2 and experimental group 3 (table 1).

SEM observations of the incisors enamel in the control group showed a homogeneous, regular, smooth surface with several scarcely visible punctiform pits (fig.1A-D), which can correspond to Tomes' processes of ameloblasts [20].
In the experimental group 2, treated with 25 ppm NaF, we found the following several defects: occasional regular and superficial depressions localized in outer enamel with a rough base and with variable size (fig. 2A); irregular scratched pattern in all examined enamel surfaces (fig. 2A-D); isolated enamel loses with variable size and deep, with almost round to irregular margins, which extends from the outer to inner enamel (fig. 2B, C); conglomerate of irregular outer enamel loses with variable size, shape and depth giving a furrowed appearance to the affected areas (fig. 2D).

We found the same defects in the experimental group treated with 50 ppm NaF, but also new defects such as: demineralized outer enamel with splotchy appearance (fig. 3A); deep, narrow and elongated pits in outer enamel (fig. 3A); large outer and inner enamel loses with irregular margins, base and stair-stepped appearance (fig. 3B); isolated or multiple outer enamel cracks with irregular shape and antiparallel orientation (fig. 3C); conglomerate of irregular outer enamel loses with variable size, shape and depth associated with several enamel pits giving a rough appearance to the affected areas (fig. 3D).

It is known that fluoride induces mineralization disturbances in a dose and time dependent manner. The previous studies have reported that the lowest dose of fluoride in drinking water that induces visible and lasting defects in fully mature rat incisor enamel is 25-30 ppm F\(^{-}\) [11]. In mice, the normal rate of eruption is approximately 2.8 mm/week for the lower incisors. This results in a turnover of the entire tooth in 35-45 days [21]. It means that our experimental study protocol is reliable. Also, chronic exposure to fluoridated drinking water reduces or abolishes the typical orange pigmentation (caused by a pigment that contains iron) in mice incisors [22]. In our study, the iron content decrease in experimental groups was statistically significant, which is in concordance with previously reported data.

Conclusions

Selected C57BL/6 inbred strain mice were suitable for the purpose of the study (sensitive strain to dental fluorosis development).

The time period chosen to supply NaF was enough to induce lesions with uniform pattern in each experimental group. The severity of the morphological changes in mice enamel varied with the supplied dose of NaF: Thus, more severe changes in enamel surface morphology were found in experimental group treated with 50ppm NaF.

EDX analysis confirmed the compositional changes specific to dental fluorosis.

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References


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