INFLUENCE OF UKRAIN AND STRONTIUM ON THE RAT TOOTH INTERTUBULAR DENTINE. II. ATOMIC FORCE MICROSCOPY STUDY

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Received for publication March 05, 2008

Abstract

The effects of different doses of Ukrain and/or strontium on the rat tooth intertubular dentine were analysed in cuts perpendicular to the dentinal tubes. The tooth surface and tooth cross-sections morphology and roughness were investigated with atomic force microscopy. The surface morphology and roughness of the dentine from seven groups of animals was examined with tapping-mode atomic force microscopy. The cross section surface of intertubular dentine was analysed by roughness and fractal parameters. Histograms were prepared for the typical samples from all the analysed groups of teeth. Dentine cross section surfaces showed significant and distinguishable differences between nano structures of the normal control rat teeth and those from animals administered with Ukrain and strontium.

Key words: rats, dentine, Ukrain, strontium, atomic force microscopy, surface images.

Ukrain is advertised by the manufacturer (Nowicky Pharmaceuticals, Austria) as a drug for alternative cancer cures with high activity against Ewing tumours. Recently, Lanvers-Kaminsky et al., concluded that Ukrain can be considered as a candidate for a subsequent drug development by xenograft studies followed by systematic clinical trials (17). The molecular mechanism of antineoplastic effects induced by Ukrain are not yet completely understood; however, there are some suggestions that the induction of apoptosis may be involved (16, 22). The investigations were concerning cells and soft tissues. Until now, the influence of Ukrain on the teeth was not investigated. The Lublin Medical University orthopaedic group investigated the influence of Ukrain on rat bone status. They found the anabolic effect of Ukrain on the femur in ovariectomised rats. However, its action could be slightly osteopoenic by affecting biomechanical bone properties. A decrease in the bone strength by 13% and in the bone mineral content was observed (13-15). Taking into account the effects of Ukrain on the bones, one can expect that also teeth could be similarly affected.

The aim of the study was to evaluate the effect of strontium and Ukrain on the rat tooth surface. To study the surface and tooth cross-sections, an atomic force microscopy (AFM) has become an indispensable method. The AFM’s ability to image surfaces with nanometer scale resolution in three dimensions has created a new nanometer-scale anatomy of tooth enamel, dentine, and dentine-enamel junction (7). In the last decade, numerous studies using AFM were done, mostly to evaluate the enamel surface morphology (1, 10) and its demineralisation/remineralisation abilities (8, 18, 19). The nanostructure of the enamel afflicted with hypoplasia was also investigated (2). The influence of bleaching agents on the enamel surface and etching process of teeth were successfully analysed by the AFM (11, 24, 26). The detailed studies of synthetic hydroxyapatite nanorods (4, 9) resulted in a new model for nanoscale enamel dissolution. It turned out that enamel solubility in acidic media depends on the ratio of pits to distance among them on the tooth surface, which is crucial to understanding the fewer caries lesion formation (25). Significantly less AFM studies were devoted to the dentine surface (23). The influence of such compounds as citric acid or hydrogen peroxide on the dentine nanosurface was investigated (3, 20). The AFM study of the enamel and dentine surface before and after application of new chemo-mechanical system for caries removal (Carisolv) showed that the using of the system resulted in smoother surface compared to conventional caries removal (21, 27).
Material and Methods

Forty-two adult rats of an approximate initial weight of 250 g were used. They were kept in separate cages and randomly allocated to one of six experimental groups. All the groups were given a standard rodent pellet diet ad libitum. The animals from the first group (the control group) received water ad libitum. The experimental animals from other groups also received water without limit, but with different additions. The second group was given water supplemented with strontium chloride in a concentration of 7.532 mmol/L. The animals from the third group received pure water but they were also injected with Ukrain (7 mg/kg) once a day. In the fourth group, the dose of Ukrain was twice higher (14 mg/kg). The rats from the groups 5 and 6 received once a day Ukrain (7 and 14 mg/kg, respectively) and were given water with 7.532 mmol/L of strontium chloride. The experiment lasted for six weeks and at the end of the experiment, the animals were decapitated. The full necropsy of the animals was done and individual bones and soft organs were collected for various examinations. The extracted teeth after removing the soft tissues with 25% formic acid and washing in distilled water were finally air-dried at 30°C. The crowns of all the teeth were sectioned latitudinally using a diamond saw. The cut surfaces were not polished. For the microscopic analysis, the intertubular dentine was used.

The surface morphology of the tooth samples was probed by atomic force microscopy Nanoscope III (Digital Instruments, USA). All samples were imaged in air using the tapping mode. Tapping mode is a patented technique (Vecco Instruments) that maps topography by lightly tapping the surface with an oscillating probe tip. The cantilever’s oscillation amplitude changes with sample surface topography and topography image are obtained by monitoring these changes with laser beam (5). A commercially available cantilever was used. Imaging was performed with standard geometry silicon nitride probes. Multiple images for each sample were obtained with three scan sizes: 1x1 µm, 3x3 µm, and 10x10 µm.

Results and Discussion

In our study, we used the tapping mode AFM, which has an advantage of avoiding any surface etching effects. Intertubular dentine was chosen for the study because the presence of the tubules with various diameters could give a false picture of the surface roughness and fractal parameters. The AFM scans revealed more compact packing among the apatite grains in the control group than in the groups administered with strontium or with strontium and Ukrain. The influence of Ukrain and strontium on the dentine structure could be noticed. However, this influence was more obvious when we analysed the nano surface parameters with the WSxM programme. In the investigated cross-section of the teeth, the following area could be observed: enamel, dentine enamel junction, intertubular and peritubular dentine, and peritubular and intertubular junction. The influence of the Ukrain on the rat teeth was analysed on the basis of nano surface morphology changes in intertubular dentine. This relatively less mineral part of the tooth seems to be appropriate for these studies as it is more susceptible to substances transported through tubules than enamel. Although peritubular dentine is even more susceptible, the presence of tubules with various diameters could make false picture of the nano surface. Obtaining scans without tubules is not easy in the AFM method, so we did not take into consideration pictures with obvious tubules.

The analysed teeth cross sections were not polished. Polishing is a usual practice in electron microscopy, but polished interfaces examined by AFM do not disclose many details, mainly due to the destruction and smearing of soft surface texture that obscures the nano surface. It was shown that the diamond saw produced clean cross sections through interfaces (29).

Representative AFM image of cross-sectioned tooth from the control group intertubular dentine is shown in Fig. 1. Intertubular dentine was chosen for the study because the presence of the tubules with various diameters could screen the properties of the surface. Fig. 1a shows a top view of AFM tapping mode image of dentine from control group recorded in “height” mode known also as 2d mode, while on the Fig. 1b, the first derivative of this scan known as “deflection” mode is shown. Fig. 1c reveals 3d presentation of the same AFM scan, but emphasising the height and topography of the dentine in the analysed sample with 3x3 µm size. This scans clearly show the distribution, size, and shape of numerous closely packed grains of the outer dentine surface.

For the data interpretation, the free-ware programme WSxM was applied (12). Among other capabilities, the programme allows a user to perform 3D rendering, pseudocolour image representation, roughness analysis, and histograms with fractal analysis. AFM images processed with the WSxM programme are presented on the Figs 2 – 4. The surfaces of rat teeth cross sections scanned for every tooth in three perimeters (1x1 µm, 3x3 µm, and 10x10 µm) are shown for the teeth of animals from the control group (Fig. 2), from the group administered with strontium (Fig. 3), and from that administered with Ukrain and strontium (Fig. 4).

The cross section surface of intertubular dentine was firstly analysed by roughness parameters. Histograms were prepared for the typical samples from all the analysed groups of teeth. They are shown on the Fig. 5.

These plots demonstrate a size of particle distribution based on the height measure (in the nanometer scale). On the Y axis the number of events is plotted. This number describes how many measurements (“taps” in tapping mode AFM) in the scanned area have the same size of height. As a result, the peaks in plot determine the heights of the most popular grains in the
Fig. 5 clearly shows that the height distribution is different for the dentine for every analysed group of rats. The minimum “heights” for the control group is observed. Contrary, the biggest size is observed for the dentine of the animals administered with Ukrain (group 3) and Ukraine and strontium (group 5). The dentine from groups 4 and 6 (rats administered with double dose of Ukraine and the same dose of Sr) is located rather closely to control group, while dentine of animals administered only with strontium (group 2) is located in the middle of the size histograms. The plots received for the scanned area 10 by 10 µm are not so clear, as the intertubular surface is contaminated by the tubules. On the area 50 x 50 µm over 50 tubules could be seen, what was reported in the literature (23). In our 10x10 µm are scans usually two or three tubules are present and such scans are not useful for image data processing from the intertubular dentine surface point of view.

![Typical AFM images of rat tooth dentine cross section](image)

**Fig. 1.** Typical AFM images of rat tooth dentine cross section with the 3x3 µm size for the control group. The scans are shown in 2d “height” (a), derivative “deflection” (b) and 3d (c) modes.
Fig. 2. The AFM images of the intertubular dentine cross section received from the tooth of rat from control group.

Fig. 3. The AFM images of the intertubular dentine cross section received from the tooth of rat administered with strontium.
Fig. 4. The AFM images of the intertubular dentine cross section received from the tooth of rat administered with Ukrain and strontium.

Fig. 5. Height distribution plot obtained from AFM images, showing size distribution of surfaces particles of rat intertubular dentine. Cross sections of control groups were compared with those of animals treated with Ukrain and strontium.
### Table 1
Image processing results

<table>
<thead>
<tr>
<th>Sample series</th>
<th>Roughness</th>
<th>Flooding</th>
<th>Fractal analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RMS (nm)</td>
<td>Average height (nm)</td>
<td>Min–max height (nm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scanned area 1x1 µm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (control)</td>
<td>1.4</td>
<td>-0.003</td>
<td>14</td>
</tr>
<tr>
<td>Group 2 (Sr)</td>
<td>6.3</td>
<td>-0.009</td>
<td>70</td>
</tr>
<tr>
<td>Group 3 (U)</td>
<td>8.9</td>
<td>0.03</td>
<td>99</td>
</tr>
<tr>
<td>Group 4 (2 x U)</td>
<td>5.4</td>
<td>-0.0006</td>
<td>38</td>
</tr>
<tr>
<td>Group 5 (Sr + U)</td>
<td>15.1</td>
<td>-0.05</td>
<td>123</td>
</tr>
<tr>
<td>Group 6 (Sr+2 x U)</td>
<td>7.6</td>
<td>0.03</td>
<td>81</td>
</tr>
<tr>
<td><strong>Scanned area 3 x 3 µm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (control)</td>
<td>10.9</td>
<td>-0.01</td>
<td>85</td>
</tr>
<tr>
<td>Group 2 (Sr)</td>
<td>19.4</td>
<td>-0.03</td>
<td>177</td>
</tr>
<tr>
<td>Group 3 (U)</td>
<td>57.4</td>
<td>0.4</td>
<td>395</td>
</tr>
<tr>
<td>Group 4 (2 x U)</td>
<td>19.7</td>
<td>-0.03</td>
<td>158</td>
</tr>
<tr>
<td>Group 5 (Sr + U)</td>
<td>35.4</td>
<td>-0.16</td>
<td>379</td>
</tr>
<tr>
<td>Group 6 (Sr+2 x U)</td>
<td>21.0</td>
<td>0.14</td>
<td>191</td>
</tr>
<tr>
<td><strong>Scanned area 10 x 10 µm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (control)</td>
<td>6.3</td>
<td>-0.01</td>
<td>79</td>
</tr>
<tr>
<td>Group 2 (Sr)</td>
<td>26.6</td>
<td>0.003</td>
<td>266</td>
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<tr>
<td>Group 3 (U)</td>
<td>96.98</td>
<td>-0.04</td>
<td>724</td>
</tr>
<tr>
<td>Group 4 (2 x U)</td>
<td>45.72</td>
<td>-0.25</td>
<td>569</td>
</tr>
<tr>
<td>Group 5 (Sr + U)</td>
<td>88.1</td>
<td>0.30</td>
<td>566</td>
</tr>
<tr>
<td>Group 6 (Sr+2 x U)</td>
<td>58.8</td>
<td>-0.34</td>
<td>563</td>
</tr>
</tbody>
</table>

RMS – root-mean-square roughness, sometimes called the interface width

Similar results are seen from the other surface analysis parameters listed in Table 1. The RMS (root-mean square roughness) parameter, which expresses deviation from the mean plane, is a statistically valuable parameter since it is the standard deviation of height distribution in nanometer. However, RMS varies with the interval range and to estimate its influence in the next column average height is added. This parameter shows the mean height of intervals. The next column in Table 1 entitled Min-max (in nanometer) describes maximal peak-to-valley distance within the measurement. This parameter with values usually doubling the values of flooding height from fractal analysis is also useful in the characterisation of nano surfaces. All these parameters reveal the compact packing of the “grains” in dentine from control group of teeth. The highest values of these parameters are obtained for the groups administered with Ukrain, while those for the rats administered with strontium and Ukrain are close to the control group values. The different results are found from the fractal analysis. Performing a fractal analysis will give information about auto similarity of the shapes of the different objects in the image. In all measurement α is below 1, which means that in dentine we have so called mass fractal (6). The highest value of α parameter suggest that the highest space density occurs in the samples from the control group. The high space density usually occurs in the specimens closely packed with many branches. The dentine of rats administered with strontium or Ukrain possess the lower α value, while for the group 5 (administered with Ukrain and strontium at the same time) a small increase in fractal parameter α could be observed. This can be explained by the change of hydroxyapatite structure during the replacement of calcium by strontium.

AFM visualisation of the intertubular dentine cross section surfaces shows significant and distinguishable differences between nano structures of the normal control rat teeth and those from animals administered with Ukraine and strontium. In the normal tooth from control group, the dentine is closely packed and significant increase in RMS parameter from 1.4 nm to 8.9 and 15.1 nm for the teeth of animals administered with strontium and Ukrain in addition to strontium is observed. The results of the AFM investigations provide evidence that administration of Ukrain as well as strontium changes, described in Part I of the paper (28), the hydroxyapatite crystallites structure of the intertubular dentine.
References