The Impact of the Phosphoric Acid on Calcium Content of Dental Enamel - *in vitro* Examination

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This study is intended for testing the way and extent how various acids influence dental erosion. Impacted third molar teeth removed under surgical exploration have been used in our experiments. The test samples have been prepared based on the Semmelweis University Dental Care Clinic protocol. Then the organic material has been removed using incubation solutions. Every 6 hours, the calcium concentration has been measured using a Radelkis OP- 274 Ph-ionometer and an OP-Ca-0711P-S type electrode sensitive to calcium ions, strictly following the manufacturer's instructions. Data were statistically processed in Graphpad InStat 3 (Graphpad Software Inc, CA, USA) using paired T-test and ANOVA. The results contradict several articles published in the scientific literature dealing with this subject and describing the linear dependence between the dental erosion progress and time. Our study emphasized that the erosion process progressed in time, however it failed to show a linear dependence.

Keywords: dental erosion, acids, calcium concentration, dissolution, ion-selective electrode

In materials science, the term erosion is used for covering the concept of damage caused by the impact of the particles upon an object, while in dentistry the same is used for indicating the material loss due to chemical effects, such as the acid attack [1]. In dental medicine, the term erosion became widely used for describing the damage caused by acids in natural hard tissues, and is now extended to include the restorative materials degradation, as well [1]. The high prevalence and incidence of erosive lesions resulted in intensifying the research efforts in the field of dental erosion [2]. Changes in the dietary regimen, i.e. the inclusion of acidic foods and beverages, as well as the gastro-esophageal affections, are deemed to be the main cause of this phenomenon [3-5]. In this sense erosion can be defined as the dissolution of dental tissue under the effect of acids of non-bacterial origin [6].

In order to understand the dental erosion process, it is absolutely necessary to know the dentine and dental enamel structure, and, respectively, the demineralization mechanism which occurs under the action of various acids. The dental enamel and the dentine are made up of mineral substances, proteins, lipids and water [7, 8]. About 96% of the dental enamel mass is mineral substance, however, for accessing the crystals, the various molecules have to get by diffusion through the matrix made up of water, proteins, and lipids, which represent the remaining 4%.

The intact dental enamel contains hydroxyapatite, and in small quantities apatite substituents (for instance fluorine-, chlorine-, sodium-, magnesium apatite), octa calcium phosphate and defective hydroxyapatite [9]. Due to the substitutions, the dental enamel is more soluble in acid than if it were made up of pure hydroxyapatite (the critical pH value is of about 5.5 [10], and with orders of magnitude more soluble in acid than if it were made up of

fluorapatite only (the critical pH value is about 4.5) [11]. A comparative scanning electron microscope (SEM) study results showed different micromorphological changes depending on the duration of acid treatment [12]. Phosphoric acid demineralizes the enamel surface at depths ranging from 5 to 25 μm. SEM analysis can discover the earliest signs of human enamel demineralization and porous teeth surface [13].

For studying the dental enamel superficial softness and the dental matter loss due to dental erosion, there are several measurement and assessment possibilities. If we wish to monitor the superficial erosive alteration by time, securing the reproducible test piece in the measuring device becomes of an utmost importance. The smaller the matter loss, the more critical this approach becomes. Therefore, for data comparison purposes, we must as far as possible confine us only to quantitative methods, while the qualitative results just serve as a guide for the analyst during the assessment process. The in vitro measuring methods can be divided into two groups, from a methodological viewpoint. On the one hand, there are the methods used for analyzing the changes which occur at the surface level, in the superficial layers, while on the other hand there are the methods used for analyzing the loss incurred by the hard tissue matter [14].

With a view to veraciously simulate intraoral erosion, it is recommended to produce the erosive effect on a natural dental surface. Mainly, accurate executions and reference planes require polished surfaces. This means that the natural dental surface, which is rich in fluorine, has to be removed. Also, it must be taken into account that whenever intraoral erosions take place, the acid attacks remove the most exterior superficial layer of the tooth, as well. Thus a *quasi polished* surface is obtained [14].

The scanning electron microscope, the superficial micro roughness examination, the superficial profilometry and the microradiography are used for analyzing the remaining surface, however measuring the dissolved calcium/phosphate by atomic absorption spectroscopy and measuring calcium/phosphate through an ion-selective electrode, are two procedures relying upon the substances dissolved from teeth [15, 16].

The aim of this study is to evaluate the impact of the phosphoric acid on calcium content of dental enamel, and how calcium dissolution evolves in time after acid attack.

Experimental part

For our research, we used forty surgically removed interrupted teething third molars. On the occasion of the surgical intervention, the exact date, patient data (name, year of birth) and data concerning the removed molar (inferior/superior, side of removal) have been recorded. The so removed third molars have been put in distilled water in flasks labelled as per the ordinal number pertaining to each patient's data. These test samples have been kept in a refrigerator at 2-6°C.

Next, the actual preparation of the test samples followed. We separated the molars, removed the root, taking care to perform sectioning in a coronary position as to the enamel-cement limit. We took into account the degree of development featured by the molar roots used for the experiment and we involved only molars where the root was developed up to the apex. Wherever possible, we further divided the coronary portion into two parts, excluding in the same time molars which showed damages, scratches or losses of matter in the coronary portion due to the surgical removal procedure.

The subsequent work phase was aimed at removing the organic matter. The preparations have separately been kept in 5% sodium hypochlorite solution (Clorox; The Clorox Company, Oakland, CA, USA) for 20 min in order to dissolve periodontal ligaments and pulp remains. For the purpose of rinsing with a disinfecting solution, we applied incubation in distilled water for 10 min. In order to remove the remaining organic matter, we brushed the test samples using a soft toothbrush (Sensoyne Pronamel Soft; Glaxo SmithKline Brentford, Middlesex, United Kingdom) for half a minute and washed the test samples under running distilled water.

In the so cleaned molar portions we made inspection windows. On the enamel surface with the greatest convexity we applied a transparent self-adhesive foil previously perforated so as to have a 0.8 mm diameter and 2.01 mm² area hole. On all other surfaces outside the area of interest, we applied two layers of acid-resistant nail polish (Nagellack Kallos Nail Colour – Int. Lacquers Luxemburg), especially taking care to cover only the portion outside the inspection window of the self-adhesive film. We wished to ensure that the closure line from the critical edge along the self-adhesive film is absolutely surely covered since the said portion was supposed to decisively influence our results.

As soon as the nail polish was fully dry, incubation in acid followed. We used 10 mmol pH=2 hydrochloric acid solution for modeling the acids which act during dental erosion. We put the test samples in flasks containing 20 mL hydrochloric acid. Every 6 h, the preparations have been relocated into a new flask each filled with fresh solution. After 24 h, we reduced the incubation period to 12 h. By the end of the experiment, measuring solutions were taken at 0-6, 6-12, 12-18, 18-24 and 24-36 h were available for us. We took pictures of the test samples removed from the

last solution, at a 4x magnification in order to check the intactness of the nail polish. In the incubations solutions, we measured the Ca^{2+} concentration by the instrumentality of a Radelkis OP-274 pH-ionometer using a OP-Ca-0711P-S type electrode sensible to calcium, in fully keeping with the manufacturer's instructions. After exclusion of the outliers, statistical analysis was done with GraphPad Instat using paired T-test and ANOVA.

Results and discussions

For the purpose of the analysis, we used a total number of 40 enamel preparations. On the high magnification images of the enamel preparations nos. 1, 15, 17, 21, 26, 29, 30, 32 and 34, we found that the nail polish is not intact and therefore no calcium measurement has been performed in relation thereto. With test sample no. 25, the enamel was also damaged however we submitted it to the measurement. In the case of this very test sample, its damaged condition explains the calcium removal by dissolution in a significantly greater extent (see C25 in fig./3). Relying upon the 31 measurements, the concentration of the calcium removed by dissolution within 0-6 h was 2.46±0.3(SE) mmol/L; within 6-12 h 8.07±0.94 (SE) mmol/L; within 12-18 h 1.6±0.42 (SE) mmol/L; within 18-24 h 0.89±0.1(SE) mmol/L mand in the last period, encompassing 12 h, i.e. within 24-36 h, was 2.54±0.3(SE) mmol/L (fig. 1).
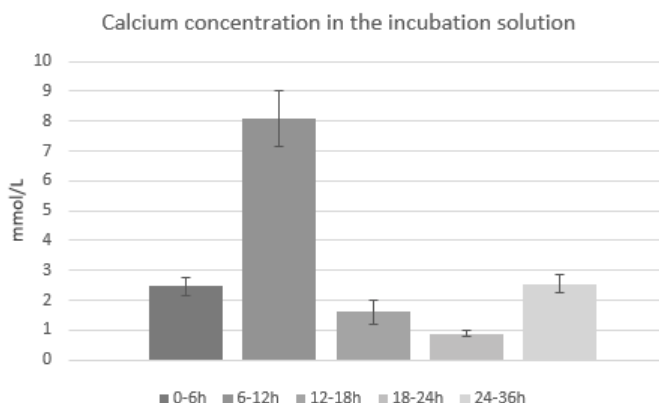

Fig. 1. Calcium concentration during various periods of incubation

We have found significant difference between all consecutive measurements. Dissolution was gradual, showing statistically significant increase every six hours (p<0,01). In the last six hours, the extent of dissolution was lower, but the difference was still statistically significant (p<0.01).

The figure above illustrates the dynamics of the calcium removal by dissolution. It outlines that during the first 6-hours period, the process advances slowly, during the second 6-hours period the dissolution rate sharply increases and then the removal by dissolution is even slower than in the first period.

The total removal by dissolution from the beginning of the experiment is shown in figure 2. In this graphical presentation, the gradual difference between the individual value columns shows the dynamics of the hard tissue dissolution.

The graphical presentation in figure 3 separately shows the test sample dissolutions by time. Mention must be done about the fact that the differences among the individual test sample solubility values are manifestly kept during the subsequent test periods, as well.

In dental caries research, using bovine dental enamel as a model for the human dental enamel is a widely acknowledged method [17]. Nevertheless, for in vitro
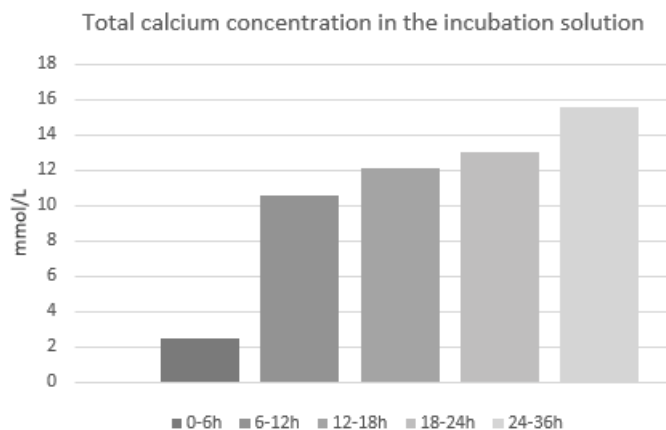
Fig. 2. Total calcium removed by dissolution from the beginning of the incubation
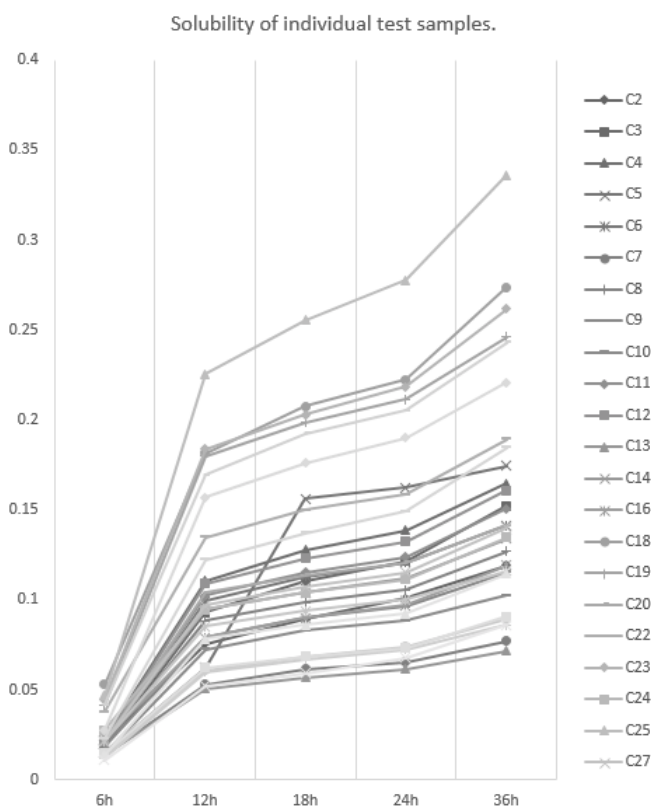

Fig. 3. Solubility of various test samples

studies, it has been shown that the bovine dental enamel preparations are less resistant than those made of human dental enamel both in terms of the erosive effect and in terms of abrasive combinations [18]. Relying upon this result, we decided to involve test samples of a human origin. For standardization purposes, we opted for interrupted teething molar test samples thus excluding the exposure to uncontrolled acidic effect before the examination.

We recorded the patients' names for cases of any possible subsequent examinations and have always kept their personal data in keeping with the relevant provisions concerning medical ethics and research. The year of birth supports the documentation of the tooth bud assessment. We have chosen hydrochloric acid for this study because our aim was to create standard circumstances. In no case was our wish to opt for an acid which features chelating properties, since any effect to produce chelates could have altered in an uncontrolled manner the study performed by us using an ion-selective electrode [14]. Moreover,

hydrochloric acid – as the main component of the gastric acid – excellently exemplifies the dental erosion of an intrinsic origin, as well. In selecting the measurement methodology, we took into account several considerations. We chose to use an ion-selective electrode due to several reasons: the method is suitable for in vitro measurements and is supported by the reference literature, as well [14,19]; enables a quantitative assessment; can be used for non-polished native surfaces, as well; is suitable for detecting the tiniest quantities of matter removed by dissolution, which is an important aspect especially in the incipient phases of the erosion study; the same test sample can be measured several times, since it is not destroyed during the measuring process; although using this method requires a lot of time and an exquisite routine, it is a relatively cheap procedure.

In order to provide a thorough image of the study, the way how dental matter losses can be deducted from the calcium concentrations measured by us must be discussed, too. In this respect, we use the following formula [19,20,21]:

Mineral substance loss (µg) = [concentrations of the studied element (mmol/L) x volume of the incubation solution (mL) x relative atomic mass] / quota in hydroxyapatite

In our experiments, the volume of the incubation solution was always 20 mL. The relative atomic mass of calcium is 40.08. As already shown on several occasions, the percentage ratio by mass in hydroxyapatite is about 0.39% by mass. From the mineral substance loss, the dental issue loss by erosion can be obtained using the following formula [22-24]:

Dental tissue loss by erosion (µm) = mineral substance loss / [hydroxyapatite density x examined surface (mm²)]

The hydroxyapatite density is 3.15 g/cm³ and in our experiments the size of the inspection window was 2.01 mm². The searched value may be calculated relying upon this.

As far as we know, there is no methodology included in the reference literature to concord with the study protocol designed by us. Using interrupted teething third molars, actual unpolished surfaces and hydrochloric acid is a rare combination in the field of in vitro research.

Our results contradict quite a lot of articles in the reference literature which processes this subject matter [25-27] and describe the linear correlation between the dental erosion evolution and time. Although the erosive process evolved in time during our study, it featured no linear correlation, as shown in figure 3. Just one article in the reference literature questions linearity [22,28,29] however even this fails to thoroughly deploy the above aspects and rather confines itself to barely mention them as a related experimental result.

It is worthwhile to further emphasize our graphic presentation which indicates the calcium removed by dissolution from the individual test samples (fig. 3). One can clearly see how the curves representing the removal by dissolution according to the various test samples show a parallel variation. One single curve (C5) is of a nature to intersect other curves. We are not aware what causes this phenomenon. Relying upon all the other curves, we can draw the conclusion that the relative solubility of the individual test samples among themselves is not changed in the various phases of dilution. We are going to dedicate our future research work to finding out why enamel preparations resist to acids differently.

Conclusions

Ion-selective electrode method is suitable for in vitro measurements and enables a quantitative assessment of small amounts of dissoluted materials. Due to acid attack the Ca dissolution process progressed in time but showed no linear correlation. Solubility remains unchanged in the various dilution phases, enamel preparations resist to acids differently.

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