ATTEMPT TO MODIFY THE CHEMICAL MODEL OF ENAMEL DEMINERALIZATION USED IN MICROINVASIVE DENTISTRY

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ABSTRACT

INTRODUCTION: Developments in minimally invasive dentistry result both in the advancement of methods used in early diagnosis and of the therapy of carious lesions.

OBJECTIVES: The aim of the study is to compare the efficiency of the demineralization process (which caused artificial caries lesions) controlled with a chemical model on the surface of enamel of extracted healthy human teeth. Two demineralizing solutions were used: one based on methylenehydroxydiphosphonate (MHDP) and another based on methylenediphosphonate (MDP).

MATERIAL AND METHODS: The material (16 healthy human teeth) was divided into two parts, and exposed to two acidic solutions (MHDP or MDP): 3 mM calcium chloride dihydrate, 3 mM dipotassium phosphate, 50 mM acetic acid and either 6 μM MHDP or 6 μM MDP. Both solutions had an established pH of 5 (with a constant temperature of 37°C, and a constant composition). Then the specimens were dissected along their long axis, and prepared for scanning electron microscopy.

RESULTS: The analysis showed no statistically significant quantitative changes in calcium and phosphorus at the measurement points a and b in both groups (MHDP and MDP). Results obtained in area S for both groups showed smaller discrepancies of Ca and P values, which suggests that the course of decalcification obtained at a distance of 60 μm down the surface area is similar in both groups. Moreover, the variance of the content of phosphorus and calcium is always lower in the samples with the MHDP formulation.

CONCLUSIONS: Use of MDP instead of MHDP in the buffer bath does not change properties of the environment which affect its ability to cause artificial caries lesions in hard tissues of teeth.

KEY WORDS: artificial caries, scanning electron microscope, microinvasive dentistry, chemically induced demineralization, subsurface lesion.

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INTRODUCTION

Developments in minimally invasive dentistry result both in the advancement of methods used in early diagnosis and of the therapy of carious lesions. The main goal is to diagnose demineralization as early as possible and apply such therapy which will stop the progression of the decalcification process in the hard tooth tissues and therefore development of a carious lesion [6]. Successful diagnosis of demineralization at its early stage and within the enamel requires the dentist to have a very good eye or to use specialist equipment for the diagnosis of dental caries. Those devices feature high objectivity of measurement and have an ability to precisely establish quantitative changes in the enamel tissue and reach a satisfactory compromise between sensitivity and specificity of a given method [6, 13]. Apart from advances in diagnostics, developments in minimally invasive dentistry also bring improvements in therapeutic techniques of tissues affected with the initial dental caries, that is remineralization and infiltration [19, 22]. The purpose of infiltration is to deliver a low viscous resin (infiltrant) as deeply as possible into the demineralized tissue of enamel, and to cut off early pathways for bacteria and their toxins and acids which may move there from the environment of the oral cavity (dental plaque) [6, 18].

Research aimed at improvement of materials used for infiltration requires availability of the test materials, which in this case should preferably be human teeth. In many cases *in vitro* research has an even more focused purpose, and is aimed at obtaining demineralized hard tooth tissue (enamel specimens with subsurface white spot lesions). To obtain human teeth with (standardized) subsurface white spot lesions developed naturally in the oral cavity is considered extremely difficult, even if some research has described such use [18, 22]. This is due to the fact that teeth at that stage of lesions are rarely extracted because preventive treatment is the established recommendation in such cases. Therefore simultaneous experiments to improve controlled demineralization techniques of human enamel are conducted using *in vitro* conditions.

Demineralizing solutions used to originate artificial subsurface lesions have been described in the available literature [1, 13]. Originally, this method was described by Buskes *et al*. in 1985 [2]. Accordingly, teeth are soaked for four several days (up to weeks, depending on the designated lesion depth) in an acidic buffer with constant composition (with the main ingredients being acetic acid (50 mM) with addition of MHDP (6 μM), $CaCl_2$ 2 H_2O × (3 mM) and KH_2PO_4 (3 mM)). The buffer's pH should be controlled regularly (at approximately 5), and the temperature of the process should be 37°C [2].

The last ingredient, MHDP (methylhydroxydiphosphonate, oxidronic acid, hydroxymethylenediphosphonate, hydroxymethanediphosphonic acid, HMDP) [12, 24], acts as an inhibitor of surface dissolution, therefore causing only subsurface demineralization. Its aim is to gain slow precipitation of a calcium/MHDP phase on the specimen's surface. Through this process we obtain surface changes caused by the MHDP-containing model system. The surface changes caused by the MHDP-containing model system are confined to a general enlargement of the intercrystalline spaces resulting from partial dissolution of the peripheries of the individual crystals without direct dissolution of the outer surface [6, 11].

MDP (phosphomethylphosphonic acid, methylene diphosphonate, medronic acid, methylene diphosphonic acid) is a chemical compound with a structure similar to MHDP and definitely cheaper and easier to obtain [9]. This will allow its wider use during in vitro studies in the field of microinvasive dentistry.

The structural difference is that two phosphonate groups are bound by a hydroxymethylene group (–CHOH–) in the case of MHDP, while in the case of MDP it is a methylene group (–CH_2–) (Figure 1).

It was hypothesized that due to similarity in the chemical compounds MDP and MHDP, the efficiency in demineralizing baths will also be similar.

AIM OF THE STUDY

The aim of the study is to compare the efficiency of the demineralization process (which caused artificial caries lesions) controlled with a chemical model on the surface of enamel of extracted healthy human teeth. Two demineralizing solutions were used: one based on methylenehydroxydiphosphonate (MHDP) and another based on methylenediphosphonate (MDP).
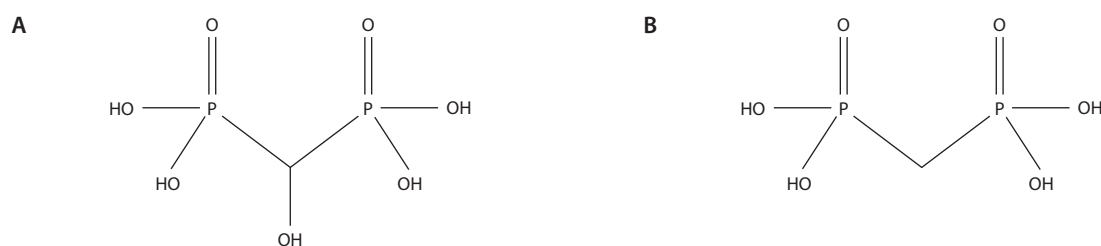
FIGURE 1. Structures of compounds: **A)** MHDP, **B)** MDP

MATERIAL AND METHODS

CLINICAL AND LABORATORY PART

This study used 20 healthy human teeth, recently carefully extracted for orthodontic, periodontal, or prosthetic reasons. Prior to processing, all teeth were stored in chloramine solution (NH_2Cl; Bochemie, Katowice, Poland), carefully cleaned of soft tissues and calculus using an ultrasonic dental scaler (Satelec Acteon, Merignac, France). Before starting with the experiments, the teeth were washed thoroughly in distilled water (twice for 5 min), and were stored in distilled water for 24 h to completely remove remnants of chloramine. Before being dried (the enamel was wet) and after being dried (the enamel was dry) the enamel was independently evaluated by two examiners to make sure that the enamel was healthy. Scoring was carried out without using optical magnification aids, and only the clinically visible appearance was rated.

Creation of subsurface demineralization of enamel in vitro in the chemical model was based on rules described by the team of Buskes et al. In 1985 [2, 10, 13, 14, 24]. The teeth were assigned randomly to two groups (with $n = 10$ teeth each), and stored in two acid solutions.

The first solution included:
- 3 mM of $CaCl_2 \times 2\ H_2O$ (calcium chloride dehydrate; Reachim; Moscow; Russia),
- 3 mM of KH_2PO_4 (potassium dihydrogenphosphate; POCh; Gliwice; Poland),
- 50 mM of CH_3COOH (acetic acid; CHEMPUR; Piekary Śląskie; Poland),
- 6 μM of MHDP (HMDP – hydroxymethylene diphosphonate; ABX; Radeberg; Germany).

The second solution was similar to the first one, the only difference was that MHDP was replaced with MDP (methylene diphosphonate; Sigma-Aldrich; Saint Louis; USA):
- 3 mM of $CaCl_2 \times 2\ H_2O$ (calcium chloride dehydrate; Reachim; Moscow; Russia),
- 3 mM of KH_2PO_4 (potassium dihydrogenphosphate; POCh; Gliwice; Poland),
- 50 mM of CH_3COOH (acetic acid; CHEMPUR; Piekary Śląskie; Poland),
- 6 μM of MDP (methylene diphosphonate; Sigma-Aldrich; Saint Louis; USA).

Each of the solutions had a stable pH (5), which was checked daily and regulated by addition of acetic acid (CH_3COOH; CHEMPUR; Piekary Śląskie; Poland) or potassium hydroxide (KOH; STANLAB; Lublin; Poland). The jars with solutions and immersed teeth were put in a heater with a temperature of 37°C to reflect the environmental conditions of the body. This process lasted for 4 weeks [2, 11].

PREPARATION OF SAMPLES FOR MICROSCOPIC ANALYSIS

The teeth immersed in the demineralizing solutions were then washed twice with distilled water (30 s × 2) and dried with oil-free, compressed air (30 s). Next, they were sectioned along their longer axis using dental diamond drills (VERDENT; Łódź; Poland), smoothed and polished (Soft-Lex; Finishing and Polishing Strips system; 3M ESPE) to obtain a smooth surface necessary for observation and microscopic analysis. All samples were prepared by one operator, and the diamond drills and polishing Soft-Lex were renewed for every specimen. After that the polishing surfaces were washed and dried with oil-free air-water spray (30 s). We decided to reject 4 specimens (MDP group), because of damage during preparation.

After that, specimens were sputtered with carbon and then examined by means of a scanning electron microscope (Hitachi S-4200, equipped with an EDS Thermo Scientific NSS7 system for X-ray microanalysis; manufacturer, city, country) (Institute of Material Sciences, Silesian University of Technology, Katowice, Poland). Examination of the microstructures of the enamel was conducted by means of the secondary electron imaging technique. Tests were made on longitudinally cross-sectioned teeth. Content of two basic elements found in teeth, calcium and phosphorus, was examined. This analysis was performed in two measurement areas: in the extremely lateral spot of the enamel (a) and in a spot towards the center of the tooth (b), which was located at the distance of 60 μm down the surface area. An evaluation of the chemical composition was made in each area (a and b) in points from 6 to 10. The analysis time for each point was 100 s. Morphological investigation and X-ray microanalysis were carried out with the accelerating voltage of 15 kV. All samples were analyzed by one operator.

In order to conduct this research, an application was filed with the Bioethics Committee of the Medical University of Silesia in Katowice. The Committee issued an approving motion no. KNW/0022/KB1/168/12.

STATISTICAL ANALYSIS

Normal distribution of the content of calcium and phosphorus (Ca and P) in each of the analyzed measurement area (a and b) was found using the Shapiro-Wilk test. Prior to the main test for the two diameters, which was carried out using the Snedecor F-test, another examination for two variances was conducted. In all cases, the value of probability p was lower than the assumed level of significance (0.05). This means that the H_0 hypothesis assuming the equality of variance should be rejected and an alternative hypothesis should be assumed. Therefore, Satterwhite's assay should be used to compare mean values [8].

RESULTS

The overall summary of Ca and P concentrations indicates a significantly high scattering in each of the measurement areas (a and b) (Figure 2). The highest scatter-

ing can be observed in the subsurface layer of the MDP sample. That region has spots where the content of calcium is both the highest and the lowest of all tested samples. Average results for each area presented in Figure 3 confirm this, with standard deviation columns indicated on the graph.

If we ignore standard deviation values, another aspects start drawing attention: huge differences in the content of calcium and phosphorus at the surface of teeth when compared to the areas situated 60 units from it in MDP samples. In the MHDP group, results obtained from the S layer are more homogeneous than in the case of the P layer.

To confirm the validity of these observations, the results were subjected to statistical tests. The main aim was to show that there are significant differences in the average content of phosphorus and calcium in the areas examined for a given sample (area a and b) and between samples (MDP and MHDP). The results are shown in Table 1. Similarity of the obtained results in both groups (MDP and MHDP) allows us to state that there are no differences in the effect of the two solutions.

DISCUSSION

The main objective of the study was to compare the efficiency of the *in vitro* demineralization process (which caused subsurface demineralization) controlled with a chemical model of Buskes on the surface of enamel of extracted healthy human teeth. Two demineralizing solutions were used – one, standard, based on methylenehydroxydiphosphonate (MHDP), and the second, modified, based on methylenediphosphonate (MDP) – because it was hypothesized that due to the similarity in the chemical compounds MDP and MHDP, the efficiency in demineralizing baths would also be similar.

Unfortunately, based on the available literature, no one has yet used MDP in the field of microinvasive dentistry, so these studies are pilot.

As reported by the literature, addition MHDP produced lesions similar to natural caries (subsurface demineralization of human teeth enamel). Without MHDP, the enamel surface was directly dissolved [4].

Research often uses artificially, in vitro, induced enamel demineralization, especially in the area of minimally invasive dentistry. Ability to obtain test material in the form of extracted human teeth with demineralized surface, which has similar properties to initial caries, significantly expedites research tests in minimally invasive dentistry [16]. This relates both to tests of the structure of hard tooth tissue surface (enamel) and to mechanisms which regulate the process of non-invasive measures (i.e., remineralization or infiltration concepts) [3, 5, 16, 22, 23].
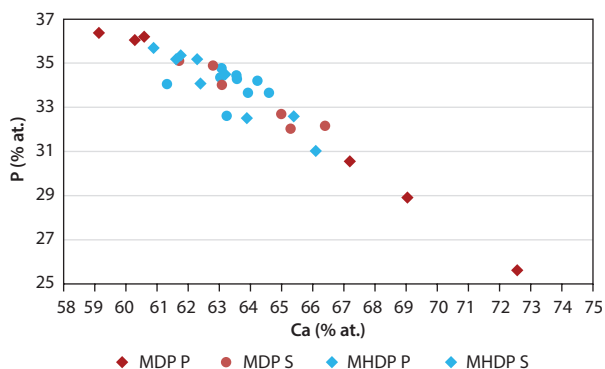

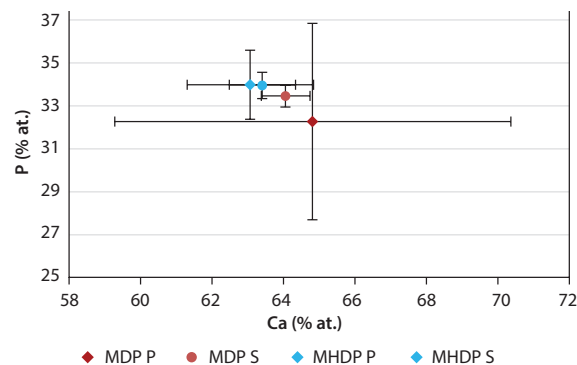**FIGURE 2.** Ca and P concentrations in each of the measurement areas

FIGURE 3. Ca and P concentrations in each of the measurement areas

TABLE 1. Concentration of Ca and P depending on localization of samples application

Element	Sample 1	Sample 2	Mean (1)	SD (1)	Mean (2)	SD (2)	*p*
Ca	MDP[a]	MDP[b]	64.81	5.55	64.05	1.79	0.7609
Ca	MHDP[a]	MHDP[b]	63.06	1.76	63.40	0.93	0.6252
P	MDP[a]	MDP[b]	32.30	4.57	33.50	1.37	0.5653
P	MHDP[a]	MHDP[b]	34.03	1.62	34.02	0.63	0.9791
Ca	MDP[a]	MHDP[a]	64.81	5.55	63.06	1.76	0.4896
Ca	MDP[b]	MHDP[b]	64.05	1.79	63.40	0.93	0.4414
P	MDP[a]	MHDP[a]	32.30	4.57	34.03	1.62	0.4143
P	MDP[b]	MHDP[b]	33.50	1.37	34.02	0.63	0.4232

Ca – calcium, P – phosphorus, [a] – extremely lateral spot of the enamel, [b] – spot towards the center of the tooth

That kind of demineralization is similar to the process observed at the moment when a white spot carious lesion appears in the course of initial caries in the natural environment of the oral cavity. In order to conduct research in minimally invasive dentistry it is crucial to obtain subsurface decalcification similar to the natural conditions. In the environment of the oral cavity, a natural subsurface carious lesion consist of four different layers being visualized with polarized light microscopy (pseudointact surface layer, body of the lesion, dark zone, and translucent zone) [16, 18]. The surface layer is located most externally, and continuously has contact with the environment of the oral cavity. The width of the surface layer is 20-50 μm, with higher mineral content (and decreased pore volume) compared to the body of the lesion. The percentage volume of pores is 1%, just like the loss of mineral substances, and constitutes a barrier for infiltrant-based solutions and prevents their penetration to the central layer below it, where the loss of mineral content in natural conditions can be as high as 25% [16, 18]. Therefore, the surface of enamel is etched with hydrochloric acid immediately before the infiltration in order to enable easier penetration of the enamel pores by the resin [24].

In vitro models allow one to maintain stability and control over the environment and guarantee repeatability of the results. It is also easier to study pH of the environment in which the test takes place and to maintain stable conditions.

With the present study, the scanning electron microscope has proven to be an effective tool for observation and analysis of quantitative changes of surface (area a) and subsurface (area b) calcium and phosphorus values of demineralized human enamel, investigated using a chemical model.

First area (a), subjected to microscopic analysis, was located on the surface of the enamel. The second area (b) analyzed for calcium and phosphorus content was located at the distance of 60 μm down the surface area. Two factors were taken into consideration when a decision to use such distance from the surface was made. The first factor was related to the layer structure observed in a cross section of a white spot carious lesion. The body of the lesion (the layer with high mineral loss and high pore volume) is located directly beneath the surface layer. Thus, the lesion body is deeper than 20-50 μm [7, 16]. The second factor was derived from the available literature, which suggests that the infiltration process is considered to be efficient if the infiltrant penetrates depths of at least 60 μm. Only then does it seem able to block further demineralization of enamel [6]. Due to the fact that the current study may in future be used for research in the field of infiltration, such a decision seems right.

In the present investigation we compared content of phosphorus and calcium in the examined areas (a and b) in both groups (MDP and MHDP). The analysis shows no statistically significant quantitative changes in calcium and phosphorus at the measurement points a and b in both groups (MDP and MHDP).

Table 1 shows that $p > \alpha$ in all compared areas. Therefore, there is no basis to reject the hypothesis of the equality of concentration of the analyzed elements in these areas. From this point of view, the effect of both formulation used in the studies is the same.

It should also be noted that the area located away from the surface of the teeth (area b) is more homogeneous in its chemical composition than in proximity of the surface (area a). Such occurrence was observed in the case of all examined teeth.

Results obtained in area S for both groups (MHDP and MDP) showed smaller discrepancies of Ca and P values, which suggests that the course of decalcification obtained at a distance of 60 μm down the surface area is similar in both groups (MHDP and MDP). Moreover, the variance of the content of phosphorus and calcium is always lower in the samples with the MHDP formulation. The analysis shows that quantitative changes in calcium and phosphorus in both demineralized groups of teeth (MDP and MHDP) are statistically insignificant.

Whether this is due to individual features of the examined teeth or different nature of the used formulations requires further study.

CONCLUSIONS

Use of MDP instead of MHDP in the buffer bath does not change the properties of the environment which affect its ability to cause artificial caries lesions in hard tissues of the teeth.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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