THESES OF DOCTORAL (Ph.D) DISSERTATION

EXAMINATION OF THE EFFECTS OF PEROXIDES ON HUMAN ENAMEL STRUCTURE

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INTRODUCTION

Dentistry is aimed at reaching an ideal esthetic result. Several methods can be used to achieve this. One of them is prosthetic treatment that requires the removal of the hard tissues of the tooth. The desired esthetic result can be achieved by changing the color of the tooth, chroma or lightness, using bleaching methods without applying a fixed prosthesis.

Nowadays 'at-home' and 'in-office' tooth whitening methods are used widely for bleaching both vital and non-vital teeth. In 1989, Haywood and Heymann described a bleaching technique that allows patients to do treatment at home. The essence of their method was the use of low concentration peroxides, applied on tooth surface. Since the concentration is low the treatment takes longer, but patients should not stay in the office while bleaching is taking place. The technique, known as „at-home" tooth bleaching, involves the use of low concentration peroxides and the solution is formulated in gel, delivered in syringes. The active ingredient of the gel is 10% carbamide peroxide, which can dissociate into 3-5% hydrogen peroxide and urea. Possibly because they focused on peroxide systems and no new material has been introduced yet, innovations have not resulted in a breakthrough since Haywood and Heymann described their method. The only new development was the introduction of Whitestrips© in 2001. Whitestrips© contains 6% hydrogen peroxide which is applied on a celluloid layer which can stick to tooth surface. The concentration of the system (6%) not only reaches but increases the typical concentration of peroxides used in the at-home treatment.

In contrast with methods that utilize low concentration peroxides, it is possible to apply high concentration peroxides as whitening agents. These materials can solely be used at chair side in a dental office, e.g. hydrogen peroxide or carbamide peroxide are used in the in-office procedures. The concentration of the peroxides is between 30 and 38 percent. Such concentrated peroxide requires shorter treatment time but patients have to spend this time in the office or in the dental chair. Immediate soft tissue protection should be taken during treatment. The dissociation of peroxides can be increased by high energy light sources to make bleaching faster. Halogen or plasma lamps are used in clinical practice for this purpose.

To understand the whitening process the chemistry of dental stain formation must be understood as well. The etiology of tooth discoloration is diverse. There are several chemical agents and molecules that can result in the discoloration of teeth. This leads to an increased number of groups in classification. The viewpoints of classification are different: place of discoloration, chemical agents, point of time of discoloration, etc. One of the most widely

used classifications has been described by SA Nathoo who classified tooth discoloration as extrinsic and intrinsic. Extrinsic discoloration can be subdivided into groups known as *N1*, *N2* and *N3* of which *N1* and *N2* are the so called direct discolorations, since the chromogen causing the discoloration also has a visible color. In the case of the *N3*, a colorless chromogen results in the discoloration of the tooth. Although the chemistry of intrinsic discoloration is not well understood yet, intrinsic discoloration is of great importance since its treatment is difficult in clinical practice.

The mechanism of tooth whitening is a so called "non selective" oxidation on the surface and in the tooth. Hydrogen peroxide is a very unstable molecule. In contact with saliva, blood, protein, or other organic materials peroxides dissociate. The result of the dissociation is free radicals that can convert water into water and oxygen in a spontaneous process. One of the most important properties of free radicals is the low molecular weight, which allows their penetration into enamel and dentin. After penetration into tooth hard tissues free radicals can oxidize organic materials. Since chromogens that cause discoloration in or on the surface of tooth are organic oxidative treatment has reason for the existence. Nevertheless free radicals oxidize the organic materials in the dentin and enamel too. The whitening process in this respect is non selective to the chromogen molecules. The above mentioned penetration is essential in the bleaching process. The amount of the penetration in ideal circumstances spreads to the dentin pulp interface only, which is of clinical importance Most of the side effects of tooth whitening are associated with the pulpal penetration of peroxides and free radicals.

The side effects of tooth whitening are objective or subjective. The most frequent subjective side effect is tooth sensitivity during and after treatment. Approximately 60% of the patient complained of medium, while 20% reported severe tooth sensitivity. Moreover, tooth bleaching affects the sense of taste and causes soft tissue irritation, especially that of the gingiva. Objective side effects result from in vitro laboratory circumstances. Such side effects include the release of mercury from amalgam fillings, reduction of bond strength of composite filling materials or orthodontic braces, etc. Peroxides have their effects on the pulp as well although most of the side effects are observed in the hard tissues of tooth. The changes can be detected at several levels.

Some studies could not reveal significant alterations neither on the surface nor in the inner structure of enamel. However, numerous studies showed the opposite. Rotstein et al. found the dissolution of Ca^{++} and $PO4^{2-}$ ions from enamel after hydrogen peroxide treatment. A study by McCracken et al. reported changes in the Ca/P ratio after a 7-day bleaching

regimen. Increased porosity of tooth hard tissues was found by Arwill et al. in their study after treatment with hydrogen peroxide for 6 hours. Attin et al. studied enamel and found significant reduction of microhardness of the enamel after the intervention. Peroxides also affect the enamel surface. Scanning electron microscopic studies by Ernst et al. found little change, but Bitter et al. reported severe alterations of the enamel surface.

It can be stated that, although tooth whitening is supposed to be easy, there are several questions in this field. In my theses I want to answer questions associated with the changes in enamel after tooth whitening and give a more detailed view of this novel technique.

AIMS

- Examination of the effects of peroxides on human enamel surface
  - Comparison of the effects of two tooth bleaching agents with 30% hydrogen peroxide solution on human enamel surface.
  - Comparison of the effects of different concentration of hydrogen peroxide solutions on human enamel surface.
- Examination of the changes in enamel structure treated with hydrogen peroxide
  - Assessment of alterations in the organic phase of human enamel.
  - Assessment of alterations in the inorganic phase of human enamel.

MATERIALS AND METHODS

Examination of the effects of peroxides on human enamel surface

Twenty seven teeth were used in AFM studies. A custom made stand alone type atomic force microscope was used for surface imaging. Images were made in contact and tapping mode in air. The head of the microscope is tripod system standing on a custom made aluminum stage with the sample on it. Cantilevers with Si_3N_4 integrated pyramidal tips were used. Scanning was made by PZT crystal tubes moving the tips above the sample. The diameter of the tips was between 10-30 nm, the average spring constant was around 0,06N/m. Different sample surface dimensions were scanned in different directions: 10x10 to 20x20 µm (x-y) and 4µm (z). Images of 500x500 pixels were collected and processed using software including plane fitting and x – y flattening.

Comparison of the effects of two tooth bleaching agents with 30% hydrogen peroxide solution on human enamel surface

In our examination the effects of two tooth bleaching agents (*Oplaescence* ©, *Nite White*©) were compared to the effects of 30% hydrogen peroxide (Sigma Chemical Corporation, Product No. H1009) solution on enamel surface. Teeth (n=15) were stored in 0.5% Chloramine-T solution after extraction until use to prevent dehydration. To prepare samples, the root and the lingual half of the crown of each tooth were cut. The samples were randomly divided in three groups of five. The samples in each group were treated with one of the three used materials. First of all, the samples were imaged without cleaning their surfaces. Five, non-overlapping areas were scanned per sample. After this, the samples were cleaned with cotton wool moistened with 5.25% sodium hypochlorite, washed and imaged again. These images served as negative control for each sample. After this the samples were treated with the peroxide containing materials. Total treatment time was 28 hours (in seven individual 4-hour treatments). Treatment was made at room temperature. The treatment procedure was the same as with the 30% hydrogen peroxide solution. AFM images were made after the 28-hour treatment procedure.

Comparison of the effects of 10-20-30% hydrogen peroxide solutions on human enamel surface

The effects of three hydrogen-peroxide solutions of different concentration (10-30%) on human enamel surface were studied. Teeth (n=12) were prepared by cutting the root and lingual half of the crown with a high speed rotary instrument using water air spray. The specimens were randomly divided into four groups of three. Group 1 was used as negative control. The samples in the control group were stored in isotonic salt solution for 1 hour then imaged with AFM. The specimens in Groups 2, 3 and 4 were treated with 10, 20 and 30% hydrogen peroxide (Sigma Chemical Corporation, Product No. H1009), respectively. First, the specimens were imaged without cleaning. After cleaning with 5.25% sodium hypochlorite, the enamel surfaces were imaged again with AFM. Treatment with peroxide solution lasted for 1 hour and it was performed in closed Petri dishes. The samples were washed for one minute with water air spray after treatment and stored in isotonic salt solution until AFM imaging.

Examination of changes in enamel structure treated with hydrogen peroxide

Assessment of alteration in the organic phase of human enamel by revealing thiol groups

Seventeen teeth were used to test interaction between peroxides and enamel proteins, and changes in the enamel. The enamel specimens were broken into minute particles in a ceramic pound and divided into four groups. The samples in Group 1 served as control and were stored in isotonic salt solution. Groups 2, 3 and4 were treated with 10, 20 and 30% hydrogen peroxide solution (Sigma Chemical Corporation, Product No. H1009) for 1 hour, respectively. Each group was divided into two subgroups according to the dialization buffer. One specimen of each subgroup was dialyzed in 0.1 M phosphate buffer, pH 7.27, and another one in phosphate buffer containing 6 M guanidine chloride for 6 hours. Guanidine chloride unfolded proteins thus thiol groups, buried inside the proteins, could be tested with anions produced in Ellman reaction. Positive nitrogen tension was maintained by gently bubbling nitrogen through the dialysis medium. It is required because anions produced in Ellman reaction can be oxidized by hydrogen peroxide, which would yield an inaccurate result. The number of free thiols was determined with Ellman reaction. The samples were incubated with 1 ml Ellman reagent containing 5’5’-dithiobis (2-nitrobenzoic acid) in sterile tubes at room temperature for 1 hour. The 5’5’-dithiobis (2-nitrobenzoic acid) reacts with

thiols and thionitrobenzoic acid is released in the reaction. The amount of the thionitrobenzoic acid is proportional to the number of free thiol groups. The absorbance of the reagent was then determined at 412 nm in a spectrophotometer using the stock buffer as reference. The amount of free thiol groups was expressed as the number of free thiols per mg enamel.

Assessment of alteration in the inorganic phase of human enamel with FT-IR

Thirty teeth were used for infrared spectroscopy analysis. Samples made from human tooth crown were embedded in resin. The vestibular surface of the block was cut to get a flat enamel surface. Treatment was performed in 10-20-30 % hydrogen peroxide solutions (Sigma Chemical Corp. Product Code: H1009) in closed airtight dishes. The total treatment time was 120 minutes. First, spectra were taken before treatment, thus each specimen served its own negative control. Further spectra were taken after 30, 60 and 120 minutes. These samples were stored in isotonic salt solutions for two weeks. At the end of the 2-week incubation period, another set of infrared spectra was taken to test spontaneous reversibility of changes in the enamel.

Testing was performed in a SPECTRUM-ONE infrared spectrometer, equipped with a Universal ATR unit. The instrument was operated under the following conditions: 4000-650 cm^{-1} range, 4 cm^{-1} resolution, 4 scans co-addition, and room temperature. The quantitative analysis of the results was performed by calculating the area of the spectra between the selected wavelengths.

RESULTS AND DISCUSSION

Examination of the effects of peroxides on human enamel surface

Comparison of the effects of two manufactured tooth bleaching agents with 30% hydrogen peroxide solution on human enamel surface

It was the first time that atomic force microscopy was used to test enamel surface alterations after peroxide treatment. The enamel surface was smooth and shiny macroscopically after treatment with bleaching gels. The AFM images of treated and untreated samples showed great differences. The AFM image of the enamel before sodium hypochlorite treatment showed an unidentified layer on it. The thickness of this layer ranged between 175-250nm. Because of this layer the underlying enamel was invisible. After cleaning the samples with sodium hypochlorite, the AFM images showed characteristic enamel surface morphology. Grooves looking like scratches and running in different directions were present on the surface. By size the grooves were 14-70 nm deep and0.25-1.0µm wide. Groves were also present after treatment with Opalescence gel for 28h, but their width and depth ranged between 0.1-0.75µm, and 30-120nm, respectively. Moreover, the base and sides of the grooves became irregular after treatment. The changes of grooves after treatment with Nite White gel were not as explicit as in the case of the Opalescence gel. The parameters of the grooves after Nite White treatment were as follows: width: 0.1-0.3µm; depth: 20-80nm. Compared to the effects of the two carbamide peroxide-containing gels, there were distinct changes on enamel surface after treatment with 30% hydrogen peroxide solution. The AFM images showed intense widening (1.0-1.5µm) and roughening of the grooves. Depth alterations were not very expressed (90-350nm). The severe alteration images after 30% hydrogen peroxide treatment could contribute to the increase in peroxide concentration. Besides concentration, the Carbopol contents of the two gels account for another difference between the substances used in the study. The effect of Carbopol is not recognized in surface morphology. The effect of the two gels was not as expressed as that of the concentrated solution'. According to examinations, 10-15% carbamide peroxide released 7-10% urea and 3-5% hydrogen peroxide after dissociation. In our study the slow release of peroxide from the gels did not result in the same alteration on enamel as the high concentration solution. Considering the mechanic cleaning effects of hydrogen peroxide and

that peroxides react with enamel during the nonselective oxidation, peroxides could have caused the alteration described in our study.

Enamel prism termination was not visible on AFM images before and after treatment, which could be explained by enamel development. Prisms do not terminate on the outer surface of enamel but 5-100µm beneath it. The enamel prisms are covered with a prism free-enamel that arises in the last phase of development. This layer is present mainly on deciduous teeth but can be found on permanent ones too. It is present on surfaces (buccal, proximal, and cervical) that are not exposed to wear during use. This layer can show the absence of prism termination on AFM enamel. An important property of prism-free enamel is its high organic contents that are why it attracts hydrogen peroxide. It is supposed that alterations imaged by AFM represent changes in this enamel layer.

Comparison of the effects of 10-20-30% hydrogen peroxide solutions on human enamel surface

On non-treated enamel surfaces shallow grooves were present in irregular directions. Their depth and width varied between 28-84 nm and 100-250 nm, respectively.

Treatment with 20% and 30% solutions further increased the width and depth of the grooves. After treatment with 20% solution, their average width and depth ranged between 136-544 nm, and 100-150 nm, , respectively. Treatment with 30% solution had an even more significant effect on the parameters of the grooves (width: 710-2000 nm, depth: 50-250 nm).

Several studies have investigated tooth hard tissues, especially enamel. According to the literature, changes take place on the enamel surface and in enamel after peroxide treatment. These alterations have their impact on both organic and inorganic enamel. It is important to examine enamel as a complete system because this can simulate the in vivo state. Alterations in enamel after peroxide treatment can be attributed to peroxide penetration.

According to the literature it can be concluded that, in some cases, changes caused by peroxides can be reduced if fluorides are applied. *Seghi* and *Denry*, however, did not show reduction in hardness but fracture toughness, while resistance to abrasion decreased significantly. It is caused by the structural changes of the enamel. *Shannon* et al. found significant reduction in enamel microhardness after two weeks of treatment. Scanning electron microscopic investigations revealed morphological changes on enamel. *Titley* et al. used concentrated hydrogen peroxide (35%) and showed the formation of precipitation on enamel surface after 60 minutes. *McGuckin* et al. studied enamel using profilometri and found

roughened and wavy surface structures. *Goldberg et al.* and *Arends et al*. studied the role of organic and inorganic phases of the enamel in alterations found after tooth whitening.

Examination of changes in enamel structure treated with hydrogen peroxide

Assessment of alteration in the organic phase of human enamel by revealing thiol groups

The amount of the total free thiols was found to be 4.2±0.3 x 10^{12} and 1.01±0.12 x 10^{12} per mg enamel in the presence and absence of 6.4 M guanidine chloride, respectively. This result suggested that only 25% of the thiol groups were easily accessible by the 5'5'-dithiobis (2-nitrobenzoic acid). Hydrogen peroxide at a concentration of 10% was enough to completely oxidize the freely accessible thiol groups suggesting that these thiols were freely accessible for the hydrogen peroxide as well. Treatment with 10% hydrogen peroxide had no effect on the amount of buried thiols implying that these thiols were not accessible for low-concentration hydrogen peroxide either. The 20% hydrogen peroxide, on the other hand, reduced the percentage of buried thiols by one third. Further increase in the concentration of hydrogen peroxide up to 30% did not reduce the amount of free thiols significantly. The reduced number of the SH groups was proportional to the peroxide concentration. The generalization of the result is hard because SH-containing amino acids are not the most frequent ones in enamel proteins, but they can be a good marker of changes caused by oxidation.

Assessment of alteration in the inorganic phase of human enamel with FT-IR

Human enamel specimens showed a characteristic infrared spectrum with two distinct peaks, representing the hydroxiapatite structure i.e. biological PO4 ν1 at 996 cm^{-1} and biological PO4 ν2 between 1410 and 1460 cm^{-1} wavelength before hydrogen peroxide treatment. At 886 cm^{-1} a secondary peak was seen, which represented the carbonate apatite (ν2 CO3) phase of enamel. The characteristic shape of the biological PO4 ν2 was doubled at 1410 and 1460 cm^{-1}. These findings correlated with the results in the literature published by Bohic et al. and Eliades et al.

Alterations in the IR spectra were found after hydrogen peroxide treatment in all cases (i.e. peroxide concentration and treatment time). The biological PO4 ν1 became wider and distorted after treatment. These changes were directly proportional to the hydrogen peroxide concentration. The distortion of the biological PO4 ν2 could also be observed on spectra. The biological PO4 ν2 had been distorted to such a degree after the treatment with 20% hydrogen peroxide that only a part of the band could be seen at 1460 cm^{-1}. At 30% hydrogen peroxide concentration, the biological PO4 ν2 was missing. These changes of the spectra indicated destruction of the hydroxiapatite phase. It was surprising since apatite-containing minerals are very stable chemically.

Treatment time or the kinetics of hydrogen peroxide resulted in similar alterations of the spectra like those of the different concentrations. The kinetics of the process can be best demonstrated at 10% hydrogen peroxide concentration because at 20% concentration the biological PO4 ν2 is dissolved and it is impossible to test kinetics. In case the treatment time is increased, the biological PO4 ν1 becomes wider and distorted. The characteristic shape of the biological PO4 ν2 is detectable after 30 minutes of treatment but only a single peak can be observed after 60 minutes, and it becomes indistinguishable after 120 minutes.

The quantitative description of the effects of hydrogen peroxide on enamel can be made by calculating the area of the biological PO4 ν1. When the spectral integral of biological PO4 ν1 is plotted against hydrogen peroxide concentration a graph with offset can be drawn. This means that distortion of the biologic PO4 ν1 and the degradation in enamel are apparent if the concentration is less than 10%. In contrast with this, apparent changes can be found between 10 and 20 % of concentration. Between 20 and 30% of concentration the changes have not been apparent. The practical aspect of this is that 10% hydrogen peroxide does not cause considerable degradation of apatite. The widening of the biological PO4 ν1 is not explicit compared to negative control thus whitening treatment should be performed using low concentrated hydrogen peroxides.

Plotting the spectral integral of biologic PO4 ν1 against treatment time showed a linear graph. The alteration of the biologic PO4 ν1 was linear in time. As far as treatment time is concerned, the above suggest that whitening treatment should not take longer than 30-60 minutes in one session, because the biologic PO4 ν1 becomes significantly wider The dissolution of the biologic PO4 ν2 peak was visible on spectra after 60 minutes* of treatment.

The widening of the biologic PO4 ν1 and dissolution of the biologic PO4 ν2 caused degradation in apatite structure. As mentioned earlier, it is surprising since apatite is a chemically resistant mineral. The reaction between hydrogen peroxide and apatite is not well

understood. The most probable reaction between PO4 in apatite and peroxides is not a red ox reaction; instead it is a complex chemical interaction, which can serve as a possible explanation to the detected spectral changes.

The position of peroxides in apatite is hard to define. Hydrogen peroxide is located between 3500 and 4000 cm^{-1} range of the infrared spectrum. It is hypothesized that hydrogen peroxide, which can form a so-called diperoxo (H_4O_4) format, is capable of changing the apatite structure and the PO4 is replaced by the diperoxo ligand to form a new complex. The other substitution of the coordinated ligand in hydroxiapatite can happen when the complex is formed through metal ions (e.g. ferric) or when the CO_3 is replaced by the ●OH released from H_2O_2. The OH band can be found in the IR spectra between 3000 and 3700 cm^{-1}. This region is variable with very little consistency. It is supposed that the enamel is hydrated during peroxide treatment or bleaching. The interpretation of this inconsistency can explain for the possible alteration of OH-binding in the crystal. This bond can become weaker and stronger. In other words, the crystal can release the OH or bind in a stronger way. A change in OH binding can also take place in the possible ligand. It is supposed that these substitutions are weak and the process can be reversed by applying fluoride. Treatment with desensitizing fluoride gels or preventive pastes can induce the build-in of fluoride. If this 'remineralization' is fast (high concentration of F^-), fluoride may not be able to penetrate into the deep structures of enamel and it takes the peroxide effect a long time to happen. The spontaneous reversibility of changes in enamel was tested in the second part of the study. The samples were stored in isotonic salt solutions for two weeks. Storage in fluoride would not be physiologic. Neither the biologic PO4 ν1, nor the biologic PO4 ν2 bands showed alterations that could be interpreted as reversible ones.

CONCLUSIONS

1. Hydrogen peroxide and the used carbamide peroxide containing bleaching agents have effects on human enamel surface.
2. Alterations on human enamel surface are directly proportional to hydrogen peroxide concentration.
3. Alteration can be detected in the organic phase of enamel.
4. The alterations in the inorganic phase of enamel are directly proportional to the treatment time and the hydrogen peroxide concentration.
5. Irreversible changes were detected in our studies.
6. Lower peroxide concentration and shorter treatment time can be advantageous in the degradation processes in human enamel.

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