The possibilities of reducing oral pathogen microorganisms, the dental use of chlorine dioxide

Doctorial theses

Herczegh Anna, DMD

Semmelweis University
Clinical Medicine PhD School

Consulents: Zsolt Lohinai, associate professor, Ph.D,
Ágoston Ghidán, researcher fellow, Ph.D.

Opponents: Krisztina Márton, associate professor, Ph.D,
Ákos Tóth, biologist professor, Ph.D.

Comprehensive exam committee president:
Péter Hermann Péter, professor, Ph.D.

Comprehensive exam members:
Noémi Rózsa, associate professor, Ph.D.
Márta Csire, laboratory director, Ph.D.

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Introduction

The need for new, effective, non-toxic antiseptic agents is continuous in all fields of dentistry. At birth the oral cavity is considered to be sterile, but a few hours later aerobe bacteria and fungi can colonize. Later anaerobe bacteria, protozoons, mycoplasma are also a member of the normal flora. The constantly changing equilibrium of these members form the oral microflora. Caries, gingivitis and parodontitis are infective oral diseases that manifest due to the disruption of the balance between the host and oral microflora.

The prevention and the treatment of oral disease is the main task of everyday dentistry. The most common and widespread method of maintaining oral hygiene is tooth brushing, although prevention against oral plaque, or in other words biofilm or if it formed its elimination from the hard-to -reach areas requires the use of other antibacterial agents besides toothpaste.

The results of a number research lead to a continuous perfection of the composition of toothpastes to contain effective antiseptic medication beside the appropriate abrasive, surfactant, stabilizing scent and taste agents. In the same way many researchers are looking for the ideal antiseptic oral rinse solution that is effective against pathogen microbes, but is not harmful for the human body at the same time.

The success of endodontic treatment beside effective hand and rotary instruments depends on the use of root canal irrigants, so a properly shaped, easily filled canal can be free from as many microbes as possible. It is proven that the long-term success of a root canal treatment is prolonged if the mechanical preparation is completed by the use of chemical agents, so we perform the so called chemo-mechanical preparation. The elimination of the harmful pathogen microbes from the root canal and dentin tubules during endodontic treatment alone does not insure a successful root canal treatment. The problem of maintaining the lowest number of microbes in the root canal and prevent the reinfection from outside of the canal and from the remained microbes is a critical point in endodontic treatment. While mechanical cleaning eliminates a significant amount of microbes from the root canal, remaining pathogens resting in areas unreachable for hand and rotary endodontic instruments may maintain or cause periodontal infection. The use of antimicrobial irrigants has an outstanding role in decreasing microbes to a number where the periapical tissue may heal.

The expectations of antibacterial agents are to inhibit the adhesion, the colonization and the metabolism of microorganisms and in this way to inhibit their growth. The efficacy of antiseptic agents may depend on their concentration and the length of time they are used for.

Chlorine dioxide is considered to be an ideal biocide. Its antimicrobial effect is outstanding amongst antiseptic agents. Its many advantageous properties (no resistance is formed against it, it reacts with few materials, it dissolves in polar and apolar solution, it is not toxic) and the possibility of using it in a pure, pollution free form (Solumium) gave us the chance to study its use in dentistry.

Aims

1. To study the effect of AmF and SnF_2 containing toothpaste and mouth rinse on *Streptococcus mutans* (*S. mutans*), *Lactobacillus acidophilus* (*L. acidophilus*), and *Candida albicans* (*C. albicans*) amount of the saliva *in vivo*.

2. To study the effect of milk as fluoride carrier, on the number of *S. mutans, L. acidophilus* and *C. albicans in vitro*. To examine the effect of NaF and Na_2PO_4F on the microorganism in milk and phosphate buffer in different concentration.

3. To study the relationship between the DNA pattern and the ability to cause disease of *S. mutans.*

4. To study the ability of D-amino acids to inhibit the forming of biofilm and to eliminate biofilm.

5. To compare the efficacy of hyper-pure ClO_2 to other well known, widely used mouthrinses and root canal irrigants on some selected oral pathogen microorganisms by means of phenol coefficient test *in vitro.*

6. To compare the oral biofilm eliminating ability of hyper-pure ClO_2 with other antiseptics *in vitro.*

7. To study the change in the number of *S. mutans* and the plate count in the saliva *in vivo* after a single rinse with hyper-pure ClO_2.

8. To compare the effect of hyper-pure ClO_2 with standard root canal irrigants sodium hypochlorite (NaOCl) and chlorhexidine gluconate (CHX) in the elimination of the

intracanal *Enterococcus faecalis* (*E. faecalis)* biofilm. To prove infection or its absence by scanning electron microscopy. To study the effect of the gas phase of the same solutions on the growth of *E. faecalis in vitro*.

9. To study the effect of dentin powder on the antibacterial properties of ClO_2 *in vitro.*

10. To study the tissue dissolving effect of ClO_2 on bovine pulp *in vitro.* To compare the result with the tissue dissolving effect of NaOCl and CHX.

11. To study the interaction between ClO_2 and CHX and between ClO_2 and EDTA.

Methods

1. The test group of 20 individuals used toothpaste and mouthrinse containing AmF and SnF_2 (each being 750 ppm F^- together 1500 ppm F^-) for the toothpaste and each being 125 ppm F^-, together 250 ppm F^- for the mouthrinse, the control group of 24 individuals used toothpaste containing 1500 ppm NaF and mouthrinse containing 250 ppm F^-.

   The participants brushed their teeth with standardized toothbrush twice a day. Following the tooth brushing they rinsed also twice a day. We determinated the amount of *S. mutans, L. achidophilus* and *C. albicans* number in stimulated saliva at the beginning of the test period, three months later and at the end of the test period (five months).

2. We examined the effect of NaF and Na_2PO_4F (MFP) on *S. mutans, L. achidophilus, C. albicans* strains after adding 1.5 % pasteurized milk or phosphate buffer (PBS) on pH 6.5 and on pH 5.5. We observed the effect of the two fluorides in 1, 5, 10 and 50 ppm concentrations. After an incubation time of 0, 60, and 120 minutes we prepared serial of tenfold dilution. After inoculation to appropriate plates and incubation we determined the colony forming units (CFU).

   To be able to study the actual effect of fluorides (to exclude the possible influence of milk itself), we performed phenol coefficient test. We used 1, 5, 10, 50, 100 and 500 mg/L concentration of the fluorides.

We studied the growth rate of microorganisms using different concentrations of fluoride (0.875- 500 mg/L NaF, MFP)) by Bioscreen biophotometer.

3. We collected 28 *S. mutans* strains from the plaque of 100 participant child. We performed identification by API rapid ID 32 Strep diagnostic test and raffinose fermentation test (BioMérieux, Marcy I'Etoile, France). The percipients were divided into caries free (DMFT = 0, GI= 0-1), caries active (DMFT ≥ 5, GI = 0-2) and gingivitis (G I≥ 2 DMFT =0) groups.

   The DNA analyses of the strains were done by Pulsed-Field Gel Electrophoresis (PFGE). The DNA patterns were studied by Bionumerics program (Applied Math, Belgum) and dendogramms were drawn. When determining the degree of relationship we set the WARD's (squared Euclidean distance, variables normalized using z-scores) parameters.

4. We studied the biofilm inhibiting effect of D-amino acids on mixed populations of microorganisms. The plaque samples were kept in 50 % glycerine solution on - 20°C in 1.5 mL Eppendorf tubes. After melting it in 37°C water bath for 2 -3 hours the bacteria were incubated. The plaque samples were placed in one-one 24 wells tissue plate. We added sterile broth, bacteria and D-tyrosine or D-methionine or D-tryptophan or combination of these (Daa) to the wells in appropriate concentrations. We incubated them for five days in 37°C.

   To measure ability of biofilm elimination we prepared the massive 7 days old biofilm in a 96 well microtiter plate and added a certain amount of amino acids. The measurement was done by crystal violet staining.

   The absorbance of the solutions was performed by ELISA reader (Bio-Rad, PR Z100, Reader, Redmond, WA, USA) ON 590 nm. We used physiological solution as control.

   The change in the amount of biofilm was given in per cent compared to the control.

5. We compared the antiseptic effect of 5.25 % NaOCl, 0.2 % CHX, Listerine and 0.03% ClO_2 by phenol coefficient test. When diluting the antiseptics we compared to the 100% active agent concentrations of the solutions. The tested microorganisms were: *S.*

mutans, L. acidophilus, E. faecalis, Veillonella alcalescens, Eikenella corrodens, Actiomyces odontolyticus and C.albicans.

After a 5 and 10 minutes contact time with the microorganisms the aerobes were inoculated onto blood agar and incubated for 2 days on 37°C. The anaerobes were inoculated onto Columbia agar and were incubated in Gas-Pack (Becton Dickinson Microbiology system, Cockeysviele, Md. USA) for 5 – 6 days on 37°C.

Distribution of the raw data was checked with Shapiro-Wilk normality test and repeated measures ANOVA was used with Scheffe's post hoc test for further statistical analysis. Significance was determined at $p<0.05$. We used the computer program Statistica 8.0, StatSoft Inc., Tulsa, OK, USA.

6. We made massive 4 days biofilm from the plaque of healthy adult volunteers'. Following treatment of the *in vitro* gained biofilm by antiseptics, the change in its amount was demonstrated by crystal violet staining. The biofilm was treated 1 or 5 minutes by 0.2 % CHX or Listerine or 0.03% ClO_2 solutions. The absorbance was measured on 590 nm by ELISA reader (Bio-Rad, PR Z100, Reader, Redmond, WA, USA). Physiological saline was used as control.

   Distribution of the raw data was checked with Shapiro-Wilk normality test and repeated measures ANOVA was used with Bonferroni post hoc test for further statistical analysis. Significance was determined at $p<0.05$. We used the computer program Statistica 8.0, StatSoft Inc., Tulsa, OK, USA.

7. We collected 1 mL unstimulated saliva from volunteer patients (25 individuals) whose oral hygiene was poor (DMF$\geq$ 10, minimum 5 active caries, PSR $\geq$ 2). The patients rinsed with the 20 fold dilution of Soluminum Oral (0.03 % ClO_2) mouthrinse for 1 min, and then we collected 1 mL saliva 5 minutes later. Listerine Total Care was used for control. Following dilution and incubation we determined the number of total colony forming units and *S. mutans* before and after rinsing.

   Distribution of the raw data was checked with Shapiro-Wilk normality test and Wilcoxon matched pair nonparametric test was used for further statistical analysis. We used the computer program Statistica 8.0, StatSoft Inc., Tulsa, OK, USA.

8. Forty single, straight-rooted, human teeth previously extracted because of periodontal disease were decoronated. Root canals were prepared up to the same size, autoclaved

and afterwards infected by *E. faecalis.* The canals were enlarged up to 40 K-file and then rinsed with 0.12 % ClO_2 (0.5 ml 0.12 % ClO_2, 2 mL 20 fold diluted ClO_2) or 5.25 % NaOCl or 2 % CHX. The solution flow rate and the time spent in the canals was the same (2.5 mL solutions for 2 minutes). The study was repeated 2 and 5 days later to examine reinfection. Scanning electron microscope pictures were taken from the hemi sectioned teeth. Filter-paper soaked in solutions was placed in the cover of an upside-down Petri dish and we examined the inhibition of inoculated *E. faecalis* growth.

For the normality test we used the Shapiro-Wilk test, two-way Repeated Measures ANOVA and then Bonferroni post hoc test was used. The difference was determined signicant at $p<0.05$. For the statistical analysis computer program was used (Statistica 8.0, StatSoft Inc. Tulsa, OK, USA).

9. We studied the effect of dentin powder on the antibacterial activity of root canal irrigants. For this we used a modified in vitro dentin powder model.
Initially we studied the effect of root canal irrigants on *E. faecalis* without dentin powder: 2% CHX, 2,5 % NaOCl, 0,12 % ClO_2 and $Ca(OH)_2$, control: sterile saline solution. 50 µL bacterial suspension (*E. faecalis* 10^8), 50 µL of physiological saline, 50 µL of antibacterial agent was mixed. We made a serial dilution after 1, 10 and 60 minutes of interaction. Incubation was performed on blood agar on 37°C for 24 hours. When evaluating the results, the control was always 100%.

As a second step, the study without dentin powder was modified and we mixed 28mg of dentin powder into 50 µL of physiological saline. The dentin powder was gained from human extracted teeth.

As a third step, the disinfectant was preincubated with dentin powder for 1 hour, then the bacterial suspension was added to it. The given times, the dilution, the inoculation, the incubation and determining the CFU was performed the same way as in the first step.

For the normality test we used the Shapiro-Wilk test, two-way Repeated Measures ANOVA and then Fisher LSD post hoc test was used. The difference was determined significant at $p<0.05$. For the statistical analysis computer program was used (Statistica 8.0, StatSoft Inc. Tulsa, OK, USA).

The next step was gradually decreasing the amount of dentin powder from 28mg to 3.5mg to find out which dentin powder amount does not influence the antibacterial effect of ClO_2.

The effect of dentin powder on ClO_2 was also examined by titration.

10. Dental pulps collected from bovine lower incisors were divided into equal portions and were measured. The effect of the following solutions on dental pulp was examined: 5.25% NaOCl, 2.5 % NaOCl, (pH 11), 0.12 % ClO_2 (pH 6), 2% CHX (pH 6). Our control was 0.9 % NaCl. The solutions were left on the tissue for 10 times 2 minutes to exert their effect, with a continuous change of the solutions. Their weight was remeasured. The change of weight was given in percent, having 100% at the initial stage.

    Statistical analysis was done according to the Kruskal-Wallis test, because the distribution was non-parametric.

11. The interaction of ClO_2 and CHX was determined by HPLC (High Performance Liquid Chromatography). The initial UV spectrum of CHX was recorded.

    The identification of the low peaks on the CHX chromatogram, which were likely to represent para-chloroanilin (PCA), was done by dissolving 1 mg PCA in 1000 µL water and by placing 5 µL of it in HPLC/MS.

    Following this we added 50 µL concentrated, undiluted, high purity 0.12% ClO_2 to 1000 µL 1 % CHX solution in a closed container. After 20 and 40 minutes of reaction time on room temperature, we separated the mixture by HPLC to determine whether a new product has been produced or has the amount of PCA risen.

    We studied whether PCA has oxidized due to ClO_2 or not. For this 1 mg PCA was dissolved in water and 50 µL concentrated high purity 0.13 % ClO_2 was added to it in a closed container. Its reaction time was 4 hours on room temperature. The spectrums were recorded by Agilent 1100 series LC/MSD.

    The interaction between ClO_2 and EDTA was determined by Nuclear Magnetic Resonance (NMR), by the method of ^{1}HNMR, which studies the chemical change of protons in solutions. When preparing the solutions, the determination of the concentrations was done by taking into consideration the amount necessary for oxidation. The final concentration of EDTA and ClO_2 was 3 mM and 16 mM, respectively.

^{1}HNMR spectroscopy was done by Bruker Avance III 500 MHz spectrometer in a solution of H_2O/D_2O with a ratio of 9:1, by recording it in a 1 dimension spectrum. We used Bruker Topspin 3.0 software to evaluate the spectrums.

Results

1. There was no significant change in the number of *S. mutans, L acidophilus and C. albicans* in any of the groups. The value of *S. mutans* increased in five cases, in two cases it remained unchanged, and in eight cases it decreased in the test group. The value of *Lactobacillus* increased in 6 cases, remained unchanged in five cases and decreased in nine cases. There was a light decreasing tendency in the number of the two bacteria. The CFU was unchanged in the case of *C. albicans*.

2. NaF and MFP in the tested concentration during the tested period did not cause a considerable decrease in the viable number of microbes, either in phosphate buffer or in milk.

   The results of the phenol coefficient investigation showed that neither concentration of NaF and MFP was adequate to kill the microbes of the three different species (*S. mutans*, *L. acidophilus*, *C. albicans*) during the 5 and 10 minutes of examination period. While 1:100 dilution of phenol was necessary to destroy the cells of both *S. mutans* and *L. acidophilus* within 5 minutes, the 1:80 dilution was enough to kill *C. albicans.*

   NaF influenced the growth dynamics of microorganisms. The exponential phase of *S. mutans* flattened out at the highest 500 mg/L NaF concentration. MFP behaved indifferent on the growth dynamics of microorganisms.

3. In the caries-active group, seven strains were examined and three types of different PFGE patterns were found. In the caries-free group, 10 strains were examined and three different PFGE patterns were found. We examined 11 strains in the gingivitis group. There were three types of different patterns identified. In this group two identical strains were found. Analysing the bands of all strains, three pairs of identical patterns were found in the eight major PFGE pattern groups and only one originated from the same tested group.

4. Reduction of the biofilm formation was observed on the plaque flora in case of D-methionine and D-tryptophan application.

   Evaluating the massive biofilm elimination by ELISA reader showed that during the test period and concentrations D-amino acids were almost ineffective in reducing the amount of biofilm. The reduction in biofilm volume compared to the control was an average of 10% in case of D-methionine and 8% in case of D-tryptophan. Increasing the concentration of amino acids did not alter efficacy.

5. All investigated antiseptic agents were significantly more effective than phenol itself in every case. High purity ClO_2 solution is significantly more effective than other currently used disinfectants in case of aerobic, facultative anaerobic bacteria (*E. faecalis, S. mutans*) and Candida. In case of anaerobically incubated facultative anaerobes (*V. alcalescens, A. odontolyticus*) and the anaerobe (*E. corrodens*), the efficiency is similar to that of CHX, but both agents were significantly more effective than NaOCl or Listerine.

6. All tested oral rinses significantly decreased the amount of *in vitro* formed biofilm compared to saline control at both investigated time points. There was no significant difference between the 1 and 5 minutes *in vitro* biofilm dissolving activity of the tested solutions. The biofilm dissolving effect of high purity ClO_2 was significantly higher compared to Listerine after 1 and 5 minutes as well, but to CHX only after 5 minutes. There was no significant difference in this respect between Listerine and CHX, either after 1 or after 5 minutes.

7. Solumium Oral significantly reduced the total plate count of saliva, while the use of Listerine Total Care did not change it significantly after 1 minute rinsing. The number of *S. mutans* was reduced by both solutions significantly. Considering the significance levels, the impact of Solumium solution was stronger.

8. All root canals were diagnosed infected by *E. faecalis* after 14 days of incubation. Only the control physiological saline treated group had a detectable number of bacteria right after the application of the chemo-mechanical procedure. No bacteria

could be detected in any of the irrigant administered groups. All test solutions reduced reinfection significantly after 2 days, compared to the massive reinfection of the control group, but there was a significant difference between NaOCl and CHX, as well as between NaOCl and ClO_2. No difference was observed between CHX and ClO_2. After the fifth day of reinfection there were significant differences among all investigated irrigants. ClO_2 was the most powerful in eliminating reinfection. Furthermore, there was a significant increase in reinfection between the 2nd and 5th day in case of the NaOCl group, but not in the CHX or the ClO_2 treated groups. The least reinfection CFU count was found in the ClO_2 treated group both on day 2 and 5. It is interesting to note, that from the investigated eight roots, in the ClO_2 irrigated group, in five cases no reinfection was detected at all, either after the 2nd or after the 5th day.

The Scanning electron microscopic images of the canal surface confirmed the results of the CFU/mL counts.

After comparing the antibacterial effect of the gas phase of 0.12 % ClO_2, 5.25 % NaOCl and 2 % CHX solutions, we observed that NaOCl and ClO_2 inhibited the bacterial growth of *E. feacalis* completely on the blood agar, in opposite to the absorbent paper. In the presence of CHX, the blood agar remained highly infected with bacteria.

9. Without dentin powder, high purity ClO_2 and $Ca(OH)_2$ completely killed all *E. faecalis* after 1 minute and 60 minutes, respectively. In case of CHX and NaOCl a small amount of bacteria survived in the investigated periods (1, 10, 60 minutes), the number of which reduced later in time, however these decreases were not significant.

When dentin powder was added to the tested agents, the survival percentages of *E. faecalis* with ClO_2 and $Ca(OH)_2$ treatment significantly increased after 10 minutes compared to results found without dentin powder involvement. This increase was even greater after 60 minutes using $Ca(OH)_2$, but not when using ClO_2. The dentin powder did not cause significant reductions during the tested periods in the antibacterial effect of CHX and NaOCl.

One-hour preincubation with dentin powder significantly reduced the antibacterial efficacy of all tested disinfecting agents.

When the amount of dentin concentration was stepwise reduced by dilution down to 3.5 mg, the antibacterial activity of ClO_2 did not change even after only one minute contact time.

Titrations showed that dentin powder depleted the ClO_2 amount to 70 % by the end of the first minute. This means, that minimum 40 ppm ClO_2 remained of the 66 ppm. The reduction of ClO_2 did not stop at any intermediate stage, but continued until chloride, till the end of the reaction.

10. Treatment of the bovine pulp tissue with root canal irrigants gave the following results. The weight loss of the control solution was 4 %, the loss of ClO_2 was 5%. The use of 5.25 % NaOCl reduced the weight of the pulp tissue by 79%. The remaining tissue was separated into several small pieces. In some cases we could not perform measurement, because the tissue was completely dissolved. In case of 2.5 % NaOCl the weight change was 35 %. The effect of 2 % CHX caused a 10 % weight loss.

11. The contaminants that appear in the CHX UV spectrum given by HPCL are PCAs, the concentration of which is less than 0.3 %.

    No interaction occurred between CHX and the added ClO_2. The spectrum of CHX did not change during the tested period. ClO_2 did not oxidize PCA, the putative PCA peak did not increase.

    The two main peaks featuring the chemical structure of EDTA, showing the oxidation of EDTA on ^{1}HNMR spectra, moved away from each other due to the effect of ClO_2 (0.12%). A slow degeneration, oxidation occurred as a result of the chemical reaction.

Conclusions

1. The short period use of AmF and SnF_2 containing toothpastes and mouthwashes does not reduce the amount of *S. mutans, L. achidophilus,* and *C. albicans* significantly.

2. NaF and Na_2PO_4F do not show an immediate effect on *S. mutans, L. achidophilus,* and *C. albicans* microorganisms in the applied concentrations and duration; the presence of milk does not change this. NaF has an effect on microbial growth curve, while MFP has an indifferent behaviour on it.

3. The closely related *S. mutans* strains can cause various dental diseases, but may be part of the normal flora as well.

4. D-methionine, D-tryptophan reduce the formation of biofilms formed from a mixed oral flora but do not have any virtual effect in maturated biofilm elimination.

5. High purity ClO_2 solution is significantly more effective than CHX, NaOCl and Listerine in case of aerobic and facultative anaerobic bacteria and *Candida*. In case of anaerobically cultured bacteria its efficiency is similar to that of CHX, but both are significantly more effective than NaOCl or Listerine.

6. Biofilm eliminating effect of high purity ClO_2 *in vitro* compared to the ability of Listerine after 1 and 5 minutes is significantly higher and after 5 minutes its effect is significantly stronger than that of CHX.

7. *In vivo*, high purity ClO_2 solution significantly decreases the salivary *S. mutans* and total bacteria count even after one rinse.

8. The high purity ClO_2 solution and its gas phase significantly eliminate the experimental *E. faecalis* infection from the root canal system. It is more effective against the reinfection than NaOCl or CHX disinfectants.

9. Reducing of dentine powder from the root canal is necessary to ensure adequate antibacterial activity of ClO_2.

10. The high purity ClO_2 has no pulp tissue dissolving effect.

11. ClO_2 does not interact with the CHX and does not increase the amount of toxic PCA found in CHX, so their simultaneous use is secure in the root canal. EDTA is oxidized by ClO_2, thus it loses its stability and activity.

12. Summarizing the results of experiments with high purity chlorine dioxide, we found that chlorine dioxide is suitable for preventive and therapeutic uses in oral hygiene and in endodontic treatments.

List of own publications

Publication in connection with the dissertation

1. **Herczegh A,** Gyurkovics M, Ghidan A, Megyesi M, Lohinai Z. (2014) Effect of dentin powder on the antimicrobial properties of hyperpure chlorine-dioxide and its comparison to conventional endodontic disinfecting agents DOI: 10.1556/AMicr.61.2014.2.10 **(IF: 0,646)**

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6. **Herczegh** A, Gyarmati I, Nász I, Bánóczy J. (1991) Néhány fotopolimerizációs tömőanyag és üvegionomer cement baktériumállóságának vizsgálata. Fogorv Sz, 84: 151-154.