Mode of action studies of a new desensitizing mouthwash containing 0.8% arginine, PVM/MA copolymer, pyrophosphates, and 0.05% sodium fluoride

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ARTICLE INFO

Article history:
Received 19 July 2012
Received in revised form 30 October 2012
Accepted 1 November 2012

Keywords:
Mouthwash
Arginine
Tooth sensitivity
Dentine occlusion

ABSTRACT

Objective: The mode of action of an arginine mouthwash using the Pro-Argin™ Mouthwash Technology, containing 0.8% arginine, PVM/MA copolymer, pyrophosphates and 0.05% sodium fluoride, has been proposed and confirmed as occlusion using a variety of in vitro techniques.

Methods: Quantitative and qualitative laboratory techniques were employed to investigate the mode of action of the new arginine mouthwash. Confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM) investigated a hydrated layer on dentine surface. Electron spectroscopy for chemical analysis (ESCA), secondary ion mass spectroscopy (SIMS) and near-infrared spectroscopy (NIR) provided information about its chemical nature.

Results: CLSM was used to observe the formation of a hydrated layer on exposed dentine tubules upon application of the arginine mouthwash. Fluorescence studies confirmed penetration of the hydrated layer in the inner walls of the dentinal tubules. The AFM investigation confirmed the affinity of the arginine mouthwash for the dentine surface, supporting its adhesive nature. NIR showed the deposition of arginine after several mouthwash applications, and ESCA/SIMS detected the presence of phosphate groups and organic acid groups, indicating the deposition of copolymer and pyrophosphates along with arginine.

Conclusion: The studies presented in this paper support occlusion of the dentine surface upon the deposition of an arginine-rich layer together with copolymer and phosphate ions from an alcohol-free mouthwash containing 0.8% arginine, PVM/MA copolymer, pyrophosphates and 0.05% sodium fluoride.

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1. Introduction

Tooth sensitivity has been recognized as one of the earliest maladies of the mouth reported in history. Tooth sensitivity is a common name for dentine hypersensitivity or root sensitivity. It is estimated that up to 57% of the population in the United States suffers from dentine hypersensitivity. Dentine hypersensitivity is characterized by short, sharp pain arising from exposed dentine in response to stimuli, typically...
thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other dental defect or pathology.2 Diverse in-office treatments have been reported to address dentine hypersensitivity, such as desensitizing pastes, fluoride varnishes, and restorative resins.3–13 Over-the-counter products that treat dentine hypersensitivity are normally toothpastes formulated with potassium ions or dentine occlusion agents.14–21 The mode of action of desensitizing products can be divided into two main groups: (1) nerve desensitization by potassium ions and (2) decrease in dentine permeability by occlusion agents. Historically, marketed anti-hypersensitivity toothpastes were recommended for a minimum of two weeks use, in order to allow the accumulation of an effective concentration of potassium ions to desensitize the nerve endings. The over-the-counter occlusion treatment approach has been traditionally reported as the deposition of solid particles or mineralized crystals, such as stannous, strontium and oxalates.4,5 More recently, a breakthrough occlusion technology containing arginine and calcium carbonate has been formulated into toothpaste and in-office products.7,20–24

In vitro investigations of dentine occlusion systems are commonly carried out via Passey’s hydraulic conductance method25–29 and surface analysis techniques.1,23 Also, Kolker et al.30 studied the in vitro performance of different synthetic resins, showing effective reduction of hydraulic conductance in the range of 40–60%, for different coatings with diverse chemical compositions. In contrast to mineral solids occlusion, the mechanism of action of adhesive coatings depends on the physical adsorption of organic materials, their diffusion through porous dentine, the chemical bonding or curing and electrostatic interactions. The viscosity and contact time of the polymeric layer are also important to allow the coating effect (film-forming) and ensure enough cohesive forces to increase the life-time of the coating. Most recently, hydraulic conductance studies have demonstrated a significant reduction in fluid flow for a toothpaste31 and for a mouthwash.31

Although a variety of dental products have been employed successfully to address dentine hypersensitivity, there is still an absence of effective mouthwash products and only a few scientific papers have been published.32–35 Herein we present the in vitro evaluation of a new mouthwash designed to effectively reduce dentine hypersensitivity through the occlusion of the dentine surface via a clinically proven formulation of arginine, PVM/MA copolymer and pyrophosphates.36,37 Polyvinylvinyl ether/maleic acid (PVM/MA) copolymer and pyrophosphates have been associated with dentine occlusion in toothpaste formulations by Miller et al.38 and Mason et al.39 based on in vitro measurements of hydraulic conductance and surface analysis techniques. The mechanism of occlusion presented here differs from Miller et al.38 due to several factors, especially the presence of arginine, other formula ingredients, pH, and delivery in the absence of brushing (toothpaste vs mouthwash). The mode of action of the new mouthwash containing the Pro-ArginTM Mouthwash Technology is based on occlusion of the dentine tubules from the deposition of arginine, PVM/MA copolymer and pyrophosphates on the dentine, which after repeated application, decreases dentine permeability and reduces pain-causing stimuli. The mechanism of action was investigated via several laboratory techniques, namely confocal laser scanning microscopy (CLSM), atomic force microscopy (AFM), electron spectroscopy for chemical analysis (ESCA), secondary ion mass spectroscopy (SIMS) and near-infrared spectroscopy (NIR).

2. Materials and methods

2.1. Test products

The test mouthwash ("arginine") consisted of 0.8% arginine, polyvinylmethyl ether/maleic acid (PVM/MA) copolymer, pyrophosphates and 0.05% sodium fluoride (Colgate-Palmolive Company, New York, NY) in an alcohol-free base. The control mouthwash (negative control) (Colgate-Palmolive Company, New York, NY) did not contain any of the ingredients mentioned above. In addition, a commercial fluoride toothpaste (Colgate-Palmolive Company, New York, NY) was employed during one of the evaluations.

2.2. Dentine disc preparation

Dentine discs used in all tests were prepared from extracted human molars. An average of 3 discs per molar were cut and prepared accordingly to each to be used in different experiments. At least 6 discs were randomly assigned per sample in all tests. Specimens, 0.8 mm thick, were obtained using a slow-speed saw turning a diamond wafering blade (Isomet, Lake Bluf, USA). Afterwards, discs were sanded 600 grit paper discs (Carbimet, Buehler, Lake Bluf, USA) and polished with soft plane cloth (P4000, Silicon Carbide using a Variable Speed Grinder-Polisher EcoMet® 3000, Buehler, Lake Bluf, USA). Each dentine disc was sonicated in deionized water and dipped into 6% (w/v) citric acid for 1 min to remove the smear layer.28

2.3. Surface and chemical characterization

2.3.1. Confocal laser scanning microscopy (CLSM)

A Leica TCS SP confocal laser scanning microscope equipped with a spectral detection system was used to visualize the dentine surfaces. The 488 nm line from an argon laser along with a PLO APO 50× objective was used in all experiments. Dentine discs were prepared according to the procedure described above. Images were taken with a 50× objective and optical zoom of 2×. Distinct treatment procedures were developed for each detection mode in order to assess the relevant aspect of the coating mechanism, i.e. a thicker coating to allow the tubule blocking visualization (10 applications) and a thinner coating (2 applications) for the fluorophore detection inside the tubules.

Reflectance mode: on dentine discs pre-conditioned in phosphate buffer saline (PBS), 15 μL of mouthwash was applied 10 times for 1 min, with intervals of 10 min between each application.

Fluorescent mode: fluorescein–cadaverine (molecular probes) was added to both mouthwash samples prior to treatment, to a final concentration of 0.4 μM. A mouthwash aliquot of
15 \mu L was added to the dentine surface and left to dry for 10 min. The same procedure was repeated one more time followed by a gentle rinse with PBS.

2.3.2. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) is a high-resolution, non-destructive laboratory technique that allows imaging of nanostructures by mapping topography and/or other properties using a tip with a nanoscale apex. When working with soft samples such as polymer, biological and dental materials, the AFM is typically operated in 'AC' mode, where the probe is vibrated near a specimen such that they measurably interact \sim 10,000–300,000 times per second. A constant interaction amplitude (and thus force gradient) is then maintained by raising or lowering the tip as needed, which when scanning the sample thus provides images of the surface height with sub-nanometer resolution in the x, y, and z directions.

For the specimens considered here, the arginine mouthwash was coated on tooth substrates with a spin coating technique to create uniform layers. Each layer was spun with 3000 rpm for 30 s. The samples were then mounted on a glass slide and kept at 37 °C until the images were taken.

2.3.3. Near infrared (NIR)

Baseline spectra were taken prior to arginine mouthwash. Samples were evaluated in triplicate. Treatment was performed as follows:

1. 10 \mu L of mouthwash was applied to disc surface and left for 10 min on a hot plate at 37 °C.
2. Disc was rinsed with PBS buffer (3\times dipping for 1 s).
3. Buffer excess was removed with air stream parallel to the disc surface and taken immediately to the NIR instrument.
4. Following spectra acquisition, a new mouthwash treatment was applied to the disc.

2.3.4. Electron spectroscopy for chemical analysis (ESCA) and secondary ion mass spectroscopy (SIMS)

All dentine discs were analysed prior to treatment to obtain baseline compositions. For ESCA, three separate areas per disc were analysed, while for SIMS one area encompassing most of the disc was analysed. The same discs were analysed by both ESCA and SIMS. For the untreated dentine, the ESCA results represent the average compositions for the four discs studied, while the SIMS values are the averages for two of the discs studied. The ESCA and SIMS results shown for the treated discs represent the average values for two discs of each treatment regimen.

The Test treatment consisted of the commercial fluoride toothpaste and the arginine mouthwash, and the control treatment consisted of the commercial fluoride toothpaste and the negative control mouthwash.

Treatments were performed as follows:

1. Dentine surface was wetted with PBS.
2. Surface was gently brushed with toothpaste for 30 s.
3. Disc was air dried for 2 min, and then moved to a beaker containing PBS with mild agitation for 1 min.
4. Disc was removed from PBS, tapped dry on the back (untreated surface) to remove excess fluid and then placed in beaker containing mouthwash for 2 min under mild agitation.
5. Disc was removed from the mouthwash and air dried for 2 min. Excess mouthwash was gently removed with a small brush.
6. Two drops of PBS was placed on the surface and the excess of liquid was gently removed from the surface with a small soft brush. Film was air dried for 30–40 min. Steps 4–6 were repeated once.

3. Results

3.1. CLSM

Images shown in Figs. 1 and 2 represent dentine discs after ten 1-min treatments using the arginine mouthwash and the negative control mouthwash, respectively. There was a 10-min interval between each treatment. Reflectance mode (Fig. 1a and b) was used to assess the surface effect while fluorescent mode (Fig. 2a and b) investigated the penetration of the Arginine mouthwash into open tubules. Fig. 1a shows a continuous image after treatment with the arginine mouthwash, indicating complete coverage of the open dentine tubules. The solid line of the xz view indicates the surface effect, i.e. the light source cannot penetrate the tubules. Fig. 1b shows the open tubules before and after treatment with negative control mouthwash. A non-continuous line on the xz view confirms the openness of the tubules.

Fig. 2a shows the fluorescence images taken after treatment with the arginine mouthwash. The bright points indicate the presence of the arginine mouthwash throughout the surface. The side view shows the penetration of the arginine mouthwash inside the tubule walls, indicating the affinity of the mouthwash to the dentine surface. The same experiment was performed with the negative control mouthwash shown in Fig. 2b. In contrast to the arginine mouthwash, very little evidence of mouthwash on the surface (xy image) in the tubules (xz image) could be visualized for the negative control mouthwash. These images prove that the arginine mouthwash penetrates inside the tubules as well as deposits on the surface.

3.2. NIR

Arginine has a unique fingerprint in the NIR region (~1530 nm), allowing its identification on the dentine surface. Fig. 3 shows the spectra obtained on the dentine surface before treatment (baseline) and after 3, 7 and 13 treatments with the arginine mouthwash. The presence of arginine molecules was identified by the 3rd treatment, and their concentration increased upon deposition with subsequent treatments.

3.3. AFM

The ability of the Arginine mouthwash to deposit onto dentine surfaces has also been explored by AFM. The high resolution of AFM even allowed for imaging the fine structure in the vicinity of a single dentine tubule (Fig. 4). The
5 μm x 5 μm images of single tubules, before and after coating, indicate that the arginine mouthwash is deposited onto the dentine surface. The occlusion occurs by a buildup mechanism of the arginine mouthwash in the tubules after several applications.

3.4. ESCA/SIMS

ESCA/SIMS assessed the chemical composition on dentine after treatment with a mouthwash in conjunction with a commercial fluoride toothpaste, in order to mimic human use. As shown in Table 1, the compositions of the discs before treatment were consistent with those for demineralized, untreated dentine: high surface protein with low calcium (Ca) and phosphorus (P) levels. After treatment, the sodium (Na) levels for the Test discs were greater than those for Negative Control discs. Potassium (K) was detected for Test discs only. The P/Ca ratio was also greater for Test discs compared to negative control discs. The Na, K and P/Ca results indicate the deposition onto the dentine surface of the tetrapotassium pyrophosphate and tetrasyodium pyrophosphate (pyrophosphates) present only in the arginine mouthwash. Elevated carbon (C) levels with reduced N, Ca, and P were observed for the Test discs compared to the negative control discs. This is indicative of the presence of an organic material on the Test discs. Also, the nitrogen (N) spectra for the Test discs differed.
in line shape from those for the negative control discs, because of the greater amount of charged nitrogen, N⁺. This difference is reflected in the N⁺/N ratios shown in Table 1. Higher N⁺ is consistent with that observed in data for arginine reference films, and indicates the presence of arginine on the surfaces of the Test discs. SIMS analysis (Table 2) of the treated dentine revealed the presence of a mass peak at 175 amu after treatment with the Test treatment. This peak was not

Fig. 2 – (a) CLSM images (fluorescence mode) of dentine surface treated with arginine mouthwash. Image scale 100 μm × 100 μm. (b) CLSM images (fluorescence mode) of dentine surface treated with the negative control mouthwash. Image scale 100 μm × 100 μm.

Fig. 3 – NIR spectra of dentine surface treated with Arginine mouthwash.
observed on untreated dentine or on dentine treated with the negative control mouthwash. The mass peak at 175 amu is consistent with that for the arginine ion and confirms deposition of arginine on the surfaces of the Test treated discs. SIMS analysis of PVM/MA copolymer reference films revealed mass peaks from fragments of the copolymer at 45 and 59 amu. SIMS analysis of the Test treated discs revealed an increase in the 45/43 and 59/43 peak intensity ratios, compared to the negative control treated discs. The increases in these ratios above the background values for the baseline suggests PVM/MA copolymer deposition.

4. Discussion

A new alcohol-free mouthwash, with 0.8% arginine, PVM/MA copolymer, pyrophosphates, and 0.05% sodium fluoride has been designed to alleviate dentine hypersensitivity by occlusion. To the best of our knowledge, this is the first time a mouthwash has been effective in addressing dentine hypersensitivity via occlusion mechanism with a unique combination of a complex formed by copolymer/aminoacid/pyrophosphate salts. Laboratory studies indicate the formation a micro-adhesive layer on the dentine surface comprised arginine, PVM/MA copolymer and pyrophosphates. Arginine and the PVM/MA copolymer form an adhesive complex, which deposits onto the dentine surface. The pyrophosphates promote the complex’s stability in solution and are deposited together onto dentine surface, leading to a coherent layer after multiple mouthwash applications to dentine specimens. The overall result is an arginine/copolymer/phosphate deposit on the demineralized dentine surface. Experimental challenges of characterizing a hydrated polymeric layer have been addressed by using a range of qualitative and quantitative techniques already established to investigate dentine occlusion as shown in the work of Petrou et al.34.

In addition, hydraulic conductance studies have shown that this new mouthwash provides a significant reduction in fluid flow, consistent with an occlusion mechanism.31 The occlusion of dentine tubules with this arginine mouthwash is the basis of the dentine hypersensitivity relief demonstrated in two clinical studies.36,37 In this paper, CLSM confirmed the ability of the Arginine mouthwash to form a layer on the dentine surface (reflectance) as well as within the internal walls of the dentine tubules (fluorescence). Based on the fluorophore chemistry, which is designed to interact with carboxylic groups, the fluorescence detected is due to arginine and copolymer deposition inside the dentine tubules as well as on the dentine surface. One important aspect of the CLSM study was the ability to study the deposition of the arginine mouthwash onto dentine in open air and after a relatively short time after treatment (30 min). Arginine is drawn to the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gantrez peak intensity ratios</th>
<th>Arginine peak (amu)</th>
</tr>
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<tbody>
<tr>
<td>Untreated dentine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10, 11 Avg.</td>
<td>0.098</td>
<td>0.164</td>
</tr>
<tr>
<td>Fluoride toothpaste/Negative Control MW – 20 treatments 2, 10 Avg.</td>
<td>0.069</td>
<td>0.041</td>
</tr>
<tr>
<td>Fluoride toothpaste/Arginine MW – 20 treatments 3,11 Avg.</td>
<td>1.823</td>
<td>0.514</td>
</tr>
</tbody>
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PVM/MA copolymer – fragment positive ions at 45 and 59 amu; hydrocarbon reference peak at 43 amu. Arginine – molecular positive ion at 175 amu.
dentine surface, serving as the anchor for subsequent copolymer deposition. Studies using NIR spectroscopy confirm arginine deposition on the dentine surface upon application of the arginine mouthwash. As seen using AFM, the images of a single tubule indicate that the arginine mouthwash is present on the dentine surface, and occlusion happens by a buildup mechanism of the arginine mouthwash in the tubules after several applications. This may explain the lack of a hypersensitivity effect within the first 30 min of using the arginine mouthwash for the first time.  

Finally, the ESCA and SIMS results indicate the presence of an organic material on the discs with the Test treatment. Also, the nitrogen spectra for these discs differed in line shape from those for the negative control treated discs. The discs treated with the Test treatment containing the arginine mouthwash exhibited a greater amount of charged nitrogen than the Negative Control treated discs.

5. Conclusion

A new hypersensitivity mouthwash based on the Pro-Argin™ Mouthwash Technology containing 0.8% arginine, PVM/MA copolymer, pyrophosphates and 0.05% sodium fluoride has been developed. In vitro studies confirm the mechanism of action as occlusion. CLSM captured images of the hydrated layer on the dentine surface. Fluorescence studies confirmed penetration of the hydrated layer in the inner walls of the dentine tubules. ESCA confirmed deposition of arginine and phosphates onto the dentine surface. AFM confirmed this observation, even identifying tubules that are spanned by the mouthwash. SIMS analysis confirmed the presence of arginine and organic groups which are indicative of copolymer deposition. NIR confirmed the presence of arginine on dentine surface upon treatment with the arginine mouthwash. The occlusion from the arginine mouthwash has been achieved due to a formula design that allows for the deposition of arginine and phosphates embedded in a thin polymeric matrix. This unique formulation is an effective system to address dentine hypersensitivity.

Conflicts of interest

None declared.

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