Research Article

Solutions containing a statherin-derived peptide reduce enamel erosion *in vitro*

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Short title: Statherin-derived peptide reduce enamel erosion

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Abstract

The effect of solutions containing a statherin-derived peptide (Stn15pSpS) on the protection against enamel erosion *in vitro* was evaluated. Bovine enamel specimens were divided into 4 groups (n = 15/group): 1) Deionized water (negative control), 2) Elmex Erosion Protection™ (positive control), 3) 1.88×10^{-5} M Stn15pSpS and 4) 3.76×10^{-5} M Stn15pSpS. The solutions were applied on the specimens for 1 min. Stimulated saliva was collected from 3 donors and used to form a 2-h acquired pellicle on the specimens. Then, the specimens were submitted to an erosive pH-cycling protocol 4 times/day, for 7 days (0.01 M HCl pH 2.0/45 s, artificial saliva/2 h, and artificial saliva overnight). The solutions were applied again during pH cycling, 2 times/day for 1 min after the first and last erosive challenges. Enamel loss (µm) was assessed by contact profilometry. Scanning electron microscopy (SEM) was assessed using cold field emission. Data were analyzed by Kruskal-Wallis and Dunn's test ($p < 0.05$). The best protection against erosion was conferred by Elmex Erosion Protection that differed from all the other treatments, followed by 1.88×10^{-5} M Stn15pSpS that performed significantly better than 3.76×10^{-5} M Stn15pSpS and the negative control. These did not differ from each other. SEM images revealed changes in enamel topography after rinsing with the treatment solutions. The solution containing the lower concentration of Stn15pSpS protected against erosion *in vitro*, which should be confirmed using protocols that more closely resemble the clinical condition.

Introduction

Erosive tooth wear (ETW) is the loss of dental hard tissues provoked by exposure to acids without bacterial involvement (Schlueter et al., 2020). Some degree of ETW is regarded as physiological, occurring throughout life, but when this process becomes excessive, it can be considered pathological (Donovan et al., 2021). Due to the increase in the prevalence of ETW and to the progressive characteristic of the lesion, it is important to perform early diagnosis and implement preventive measures in due time (Carvalho et al., 2015, Simões et al., 2020).

The preventive measures to ETW are directed to fight the risk factors that may be related to nutrition or to the patient (Buzalaf et al., 2018). Among the patient-related factors, saliva is one of the most important. This fluid remineralises the tooth structure, providing calcium, phosphate and fluoride, and has the ability to remove erosion-inducing acids from the tooth surfaces, acting as a cleaning agent (Buzalaf et al., 2018). Saliva also provides most of the proteins that form the acquired pellicle, a bacteria-free biofilm that covers oral hard and soft tissues and is composed of glycoproteins, proteins and lipids. This integument provides protection against ETW, since in the short term, as acids must first pass through the film before coming into direct contact with the tooth surfaces. In other words, the film acts as a permeable and selective barrier that reduces demineralization (Hara and Zero, 2014). It has been shown that most of the protection is conferred by the proteins found in the basal layer of the acquired pellicle, which are not displaced even after severe erosive challenges (Hannig 1999). Thus, the reinforcement of the basal layer of the acquired pellicle with acid-resistant proteins has been suggested as a possible new strategy to protect against ETW (Carvalho et al., 2020; Pela et al., 2021a), which has been named “acquired pellicle engineering”.

Statherin is one of the salivary proteins found in the basal layer of the acquired pellicle. It has 43 amino acid residues, with a primary sequence similar to osteopontin and casein and calcium binding capacity. Its density of negative charges (due to the phosphorylation of serines 2 and 3) and helical conformation in the N-terminal region are important for its interaction with hydroxyapatite (Raj et al., 1992). A statherin-derived peptide containing 15 N-terminal residues with serines 2 and 3 phosphorylated (Stn15pSpS) has been employed in acquired pellicle engineering. Rinsing with this peptide increases acid resistant proteins within the acquired pellicle (Araújo et al., 2022; Carvalho, Araújo, et al., 2020; Taira et al., 2021) and protects against initial intrinsic erosion (Taira et al. 2020; Carvalho, Araújo, et al. 2020). Epidemiological data corroborate these findings, since in patients with dental erosion, the concentration of statherin in the EAP is reduced by 35% (Carpenter et al., 2014). These data together indicate that this peptide has great potential to resist to removal by intrinsic acids and, therefore, it could be incorporated into dental products, such as mouthwash solutions, to prevent

ETW. However, all the studies mentioned above evaluated to potential of Stn15pSpS to protect against initial erosion, using surface hardness as response variable.

Therefore, the aim of the present study was to evaluate the effect of solutions containing different concentrations of Stn15pSpS in the protection of bovine enamel against ETW *in vitro*, under prolonged erosive challenges, using profilometry as a response variable. The null hypothesis tested is that solutions containing Stn15pSpS, regardless of the concentration, do not protect enamel against ETW.

Materials and Methods

Ethical Aspects

This research was approved by the Animal Research Ethics Committee of Bauru School of Dentistry under number 008/2020 and by the Human Research Ethics Committee of Bauru School of Dentistry under number 40189220.6.0000.5417. The volunteers participated after signing an Informed Consent Form.

Production of statherin-derived peptide (Stn15pSpS)

The peptide containing the 15 N-terminal residues of statherin (DSSEEKFLRRIGRFG), with phosphorylated serines 2 and 3 (StN15pSpS), was synthesized at the Department of Biochemistry and Chemical Technology, Instituto de Química – UNESP, Araraquara. For this, the peptide was chemically synthesized by the solid phase method (Amblard et al., 2005; Merrifield, 1963), according to the standard protocol that employs the FMOC group as a protector of α-amino groups and t-butyl derivatives for the protection of the side chains of trifunctional amino acid residues (Chan and White, 2000). As a starting polymer, a Wang-type resin containing the first amino acid of the previously incorporated sequence was used, initially using Diisopropylcarbodiimide/1-hydroxybenzotriazole as condensing agents. The deprotection of the α-amino groups (removal of the labile Fmoc base group), after the entry of each amino acid, was performed using a solution of 4-methylpiperidine 20% (v/v) in dimethylformamide. Peptide purification was performed by High Performance Liquid Chromatography (HPLC) using a Jupiter Phenomenex C18 reversed phase column (250 x 10 mm; 10 µm; 300 Å) with a linear gradient of organic component (acetonitrile: water; 0 .04% TFA) variable, flow rate of 2.0 mL/min and detection at 220 nm. Analytical HPLC was performed on a Shimadzu chromatograph, using a Kromasil C18 reversed-phase column (25 x 0.46 cm; 5 µm; 300 Å), with a linear gradient from 5 to 95% solvent B (A: water, 0.045 % TFA; B: ACN, 0.036% TFA) in 30 min, flow rate 1.0 mL/min and detection at 220 nm. The characterization of the products after purification was carried out by molecular mass

determination, using a Bruker Amazon SL spectrometer coupled to a Shimadzu liquid chromatograph (LC-MS).

Preparation of enamel samples

60 enamel samples (12 mm^2 exposed area) were prepared from bovine incisors (Magalhaes et al. 2016) stored in 0.1% thymol solution (pH 7) for 30 days. The roots and crowns were separated using a precision cutter (Buehler, USA) and a diamond disc (Maruto). The crowns were placed in prefabricated silicone molds (Biopdi, São Carlos, Brazil) and embedded in self-curing acrylic resin (Jet, Clássico, Campo Limpo Paulista, Brazil). After polymerization, the samples were polished using 320, 600 and 1200 grit sandpaper (Buehler, Lake Bluff, IL, USA). A mark was made in the control area using a drill to facilitate the location of the first profile reading (baseline). In addition, two lines were produced using a diamond disc (American Burrs, SC, Brazil) on the tooth surface to separate the eroded area from the control area, thus allowing the comparison of baseline and final profiles. The baseline profile was then measured as described below, and the control areas were protected with nail polish. (Risqué, Taboão da Serra, Brazil) (Magalhaes et al., 2016).

Experimental Groups

Enamel specimens were randomly divided into 4 groups (n=15/group) according to the solution to be used: 1) Negative control (deionized water); 2) Positive control (Elmex Erosion Protection rinse solution, containing 800 ppm Sn^{+2} from $SnCl_2$, 500 ppm F from amine fluoride and sodium fluoride, pH 4.5, Colgate); 3) Solution containing 1.88 X 10^{-5} M Stn15pSpS and 4) Solution containing 3.76 X 10^{-5} M Stn15pSpS.

Human saliva collection

Total saliva was collected from 3 healthy volunteers between 9 and 11 am under masticatory stimulation using Parafilm. Samples were centrifuged at 14,000 g for 20 min at 4°C. The supernatants were collected to constitute a pool of saliva, which was be stored in the freezer at -80ºC until use.

The inclusion criteria were nonsmokers, good oral health (without caries, gingivitis, periodontitis, and other oral conditions that could affect the composition of oral fluid), good general health, no restorative treatment on the buccal surface of the teeth, and normal salivary flow (stimulated flow higher than 1 mL/min and unstimulated flow higher than 0.25 mL/min). Patients with systemic diseases and using chronic medication, as well as pregnant women, were not eligible to participate.

Treatment and pH-Cycling

The treatment solutions (25 µL/specimen) were applied for 1 minute, with a volumetric pipette (twice a day), covering the surface of the specimens and the excess solution was removed with absorbent paper, after the first and the last erosive challenges. Then, the specimens were incubated with pooled human saliva (300 µL per specimen) for 2 h at 37°C, for the formation of the acquired pellicle (Cheaib and Lussi, 2011), only on the first day of treatment. After the formation of the acquired pellicle on the first day, the specimens were subjected to erosive pH-cycling 4 times per day, for 7 days. Each cycle (Magalhaes et al., 2008) consisted of: immersion of specimens in 0.01 M HCl (pH 2.0) for 45 seconds (30 mL/specimen) at 25ºC, washing in deionized water for 5 s, remineralisation by immersion in artificial saliva (Klimek et al. 1982) for 2 h (pH 6.8, 30 mL/specimen) and washing with deionized water for 5 s. The specimens were immersed in artificial saliva overnight, completing each 24 h of cycling. Enamel loss was assessed using contact profilometry after 7 days of pH-cycling.

Contact profilometry

Enamel surface profiles were obtained with a contact profilometer (MahrPerthometer, Göttingen, Germany) before (baseline) and after the experimental period. The control areas were marked with a diamond disc (American Burrs, SC, Brazil) to allow the exact positioning of the cosmetic nail polish. Additionally, a small cavitation was performed in the samples with a 1/4 round bur, to enable the positioning of the profilometer tip in the first scan of each reading. In each reading, five scans (length 3 mm) were performed at the center of the sample surface at a distance of 250 µm each. To determine the change in the surface profile of the sample, after the experimental phase, the cosmetic nail polish was removed with an acetone solution (1:1 – acetone: water), and 5 final readings were performed on the same areas as the initial readings. To enable the correct repositioning of the samples during the readings, we will use a device by which we will standardize the position of the samples on the x, y and z axes. Initial and final profiles were performed and compared using the MarhSurf XCR20 software and the average wear for each sample was calculated (µm). The detection limit is 0.5 µm. The experimental design is shown in Fig. 1.

Scanning electron microscopy

The images were collected using a cold field emission scanning electron microscope (FE-SEM; JEOL model 7500F) with the following operating conditions: accelerating voltage of 2 kV, emission current of 10 uA, current probe of 9, work distance at 7.7 mm, secondary electron image (SE) mode, and 10,000x magnification (1 pixel = 0.935 nm) (Barreto, Tita, Orlandi, 2019).

Statistical analyses

Data were analyzed using GraphPad InStat and GraphPad Prism softwares (GraphPad Software Inc.). The Kolmogorov-Smirnov test was performed and the data did not pass the normality test. Thus, they were analysed by Kruskal-Wallis followed by Dunn's test ($p < 0.05$).

Results

Enamel loss

There was a significant difference between the groups (KW = 35.537, $p<0.0001$). The best protection against ETW was conferred by Elmex Erosion Protection (positive control) that differed from all the other treatments, followed by 1.88×10^{-5} M Stn15pSpS that performed significantly better than 3.76×10^{-5} M Stn15pSpS and the negative control (water). These did not differ significantly from each other ($p>0.05$) (shown in Fig. 2).

Scanning electron microscopy

Enamel treated with water (negative control) showed several surface cracks (shown in Fig. 3. a). On the other hand, specimens treated with Elmex (positive control) presented a coating layer and some precipitates on the surface (shown in Fig. 3. b). Specimens treated with 1.88×10^{-5} M Stn15pSpS solution had only a slight demineralization, as shown in Fig. 3. c, where it is still possible to see the delimitation of the prismatic areas. Fig. 3. d shows that the 3.76×10^{-5} M Stn15pSpS solution did not protect against demineralization, since the prismatic structure of enamel is lost, with the presence of microcracks. According to the SEM images, Elmex and 1.88×10^{-5} M Stn15pSpS solutions presented a surface different from the other groups, with fewer microcracks and without pores (shown in Fig. 3. b and 3. c).

Discussion

The immobilization of proteins in the acquired pellicle has been proposed as a new measure for the prevention of ETW (Carvalho et al., 2020; Santiago et al., 2017; Pela et al., 2021a; Pela et al., 2021b; Taira, et al. 2020; Santos et al., 2021; Martini et al., 2020). Among the potential candidates to be employed for this purpose, the peptide derived from statherin, Stn15pSpS, has been evaluated, due to its high affinity to hydroxyapatite (Taira et al., 2020). The idea behind the use of this peptide is, due to its negative charges (Raj et al., 1992), to promote its binding to hydroxyapatite in great extension. This is able to dramatically change the proteomic profile of the acquired pellicle formed in the short-term, which contains high amounts of the peptide and less amounts of other proteins, with a much less complex proteome. In the long term, the proteome of the pellicle becomes more diverse

decreased (Taira et al., 2021), with increase in other acid-resistant proteins, like hemoglobin, cystatins, and proline-rich proteins. This helps to explain the protective effect of the peptide against demineralization (Carvalho et al., 2020).

Despite promising results have been obtained in *in vitro* (Taira et al., 2020) and *in vivo* (Carvalho et al., 2020) studies that evaluated statherin for the prevention of erosive demineralization, the protocol of these studies involved only initial erosion. In the *in vitro* study, the challenge was performed with 0.01 M HCl (pH 2.0) for 30 s in total (Taira et al., 2020), while in the *in vivo* one, the challenge was performed with 1% citric acid, pH 2.5 for 10 s only (Carvalho et al., 2020). The present study constitutes an additional step to support the use of products containing Stn15pSpS for the prevention of ETW, since we performed prolonged erosive challenge (0.01 M HCl, pH 2.0, for a total of 21 min). Even under this prolonged time of intrinsic erosive challenge, the lowest concentration of Stn15pSpS protected against ETW, but the highest concentration did not. This led us to partially reject our null hypothesis.

The concentrations of Stn15pSpS evaluated were based in previous studies (Kosoric et al., 2007; Shah et al., 2011; Taira et al., 2020). The lowest concentration (1.88×10^{-5} M) that was able to protect enamel, also was shown to provide the best protection in our previous in vitro study involving initial erosion, in which a range of concentrations between 0.94 – 7.52 X 10^{-5} M was employed (Kosoric et al., 2007; Shah et al,. 2011; Taira et al., 2020). This concentration corresponds to the mean range of statherin concentrations found in saliva (Kosoric et al., 2007). On the other hand, the highest concentration despite was similar to the lowest one, did not significantly differ from the negative control. The lack of dose-response effect is intriguing. It should be considered that the reduction on demineralization depends not only on the adsorption of peptide on the enamel surface, but also on the amount of available free Ca^{+2} in the surroundings (Anderson et al., 2001). It is possible that higher concentrations of the peptide make Ca^{+2} unavailable, thus impairing the protective effect.

The best protective effect against ETW was provided by the positive control (Elmex), a commercial solution containing a combination of tin and fluoride ions that is one of the most efficient products against ETW (Huysmans et al., 2014). In the present protocol, the protection conferred by Elmex was significantly higher than that given by the lowest concentration of the peptide (shown Fig. 2). The mechanisms of action of both solutions are completely different. In the case of Elmex, we can see in Fig. 3. b, the deposition of an aprismatic layer on enamel, which confers protection. On the other hand, the SEM images of the specimen treated with the lowest concentration of the peptide (shown Fig. 3. c) still shows the delimitation of the prismatic structure, due to the action of the peptide reinforcing the acquired pellicle that, in turn, acquired as a barrier to the acid. The same was not the case for the highest concentration of the peptide. The specimen treated with this concentration

showed extensive demineralization, with loss of the prismatic structure (shown in Fig. 3. d). It should be highlighted that the broad use tin-containing products, such as Elmex, is impaired by the fact that they may provoke discoloration of the tooth surface and astringent sensation (de Souza et al., 2018).

Therefore, the findings of the present study pave the way for future *in situ* and clinical studies that will provide broad support for the use of Stn15pSpS-based products for the prevention of intrinsic ETW. Based on the study model adopted, the lowest concentration of Stn15pSpS presented a protective effect against ETW and has potential to be included in dental products for the prevention of this condition.

Statements

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Statement of Ethics

The volunteers participated after signing an Informed Consent Form. This research was approved by the Animal Research Ethics Committee of Bauru School of Dentistry under number 008/2020 and by the Human Research Ethics Committee of Bauru School of Dentistry under number 40189220.6.0000.5417.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

F.N.R.: performed the experiments and wrote the manuscript; M.M.F.: performed the experiments; N.D.G.S.: performed the experiments; V.T.P.: performed the experiments; J.V.F.C.: data curation; J.S.T.: performed the experiments; H.M.H.: data curation; E.C.: produced the statherin-derived peptide; R.M.: produced the statherin-derived peptide; M.A.R.B.: analyzed the data and wrote the manuscript. All authors revised and approved the paper.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Figure Legends

Fig 1. Experimental design of the study. a, b. Separation of crown and root of the bovine tooth c. Inclusion in acrylic resin discs, d. Mark in the control area, e. Sample polishing, f. Baseline profile scans, g. 2/3 of the surfaces were protected with nail varnish, h. Experimental groups and solutions were applied, i. Saliva collection, j. Acquired pellicle formation, k. Erosive pH-cycling, l. Application of test solutions during pH-cycling, m. Final profile measurement.

Fig. 2. Median (interquartile interval) enamel loss after treatment with solutions containing 1.88×10^{-5} M Stn15pSpS, 3.76×10^{-5} M Stn15pSpS, Elmex Erosion Protection (position control) or water (negative control) for 1 min, followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges (0.01 M HCl pH 2.0 for 45 s) 4 times/day for 7 days. Treatments were applied twice/day, after the first and last erosive challenges. Data were analyzed by Kruskal-Wallis and Dunn´s test ($p<0.05$). n=15. Means followed by different letters are significantly different.

Fig. 3. SEM images of enamel samples at 5.000x magnification, bar = 1µm. a. Water (negative control), b. Elmex Erosion Protection (positive control), c. 1.88×10^{-5} M Stn15pSpS, d. 3.76×10^{-5} M Stn15pSpS.