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CLINICAL STUDIES

Substantivity of zinc salts used as rinsing solutions and their effect on the inhibition of *Streptococcus mutans*

Margarita Burguera-Pascu*, Alberto Rodríguez-Archilla, Pilar Baca

Department of Oral Medicine, School of Dentistry, University of Granada, Granada, Spain

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Abstract

The antimicrobial efficacy of zinc (Zn) salts (sulfate and acetate) against *Streptococcus mutans* (*S. mutans*) present in the oral cavity was tested in this study. The substantivity of Zn salts was assessed by determining the concentration of Zn in whole, unstimulated saliva and by measuring the magnitude of suppression of salivary *S. mutans*, 2 h after rinsing. The concentration of Zn was measured by atomic absorption spectrometry (AAS) with electrothermal atomization (ET AAS) in saliva sampled before (basal) and 24 h after mouth rinsing with different concentrations of Zn (0.1%, 0.5% and 1%) administrated as sulfate and acetate. The estimation of Zn levels in samples collected 30, 60, 90 and 120 min after rinsing was carried out by AAS with flame atomization (FAAS). Immediately after rinsing, the concentration of Zn in saliva sharply increased with respect to the baseline values (0.055 ± 0.017 mg/L), followed by a sustained decrease, probably due to clearance of salivary flow or swallowing during sampling. A significant reduction ($>87\%$) in the total mean *S. mutans* counts was found 2 h after rinsing either with sulfate or acetate solutions, as evidence of the high substantivity and effectiveness of the Zn salts tested. A statistically significant inverse relationship ($p<0.001$ and the Pearson correlation coefficients between -34% and -50%) was found between Zn levels and the respective pH values measured in the samples collected 60 and 120 min after rinsing, sustaining the theory of bacterial glycolysis inhibition.

Keywords: Saliva zinc levels; Zinc salts; Substantivity; *S. mutans* inhibition

Introduction

The oral cavity houses diverse microbial communities which naturally populate its hard and soft tissues. They develop in an organized manner to form biofilms at different mouth sites [1]. When the equilibrium of the oral environment changes, the proliferation of various bacterial species may hazardously increase and act together to initiate and further aggravate certain oral diseases. The association of periodontal diseases [2], dental caries [3,4], abnormal test acuity [5,6] or halitosis [7–10] with the growth of the dental plaque.

Many investigative studies have documented the inhibition of plaque growth and the reduction of bacterial acid formation by the use of antibacterial agents added to mouthrinses or toothpaste preparations [11–18]. According to their chemical characteristics, mouthrinses commercially available contain cationic, anionic and nonionic active ingredients which, to a higher or lower extent, alter the bacterial membrane function. Among the cationic agents, chlorhexidine and some divalent metal ions like Cu^{+2}, Zn^{+2} and Sn^{+2}, are

*Corresponding author. C.C. Plaza Las Américas, Piso 1, Local 19, Av. Las Américas, Mérida, Venezuela. Tel.: +58 274 5111214.
E-mail address: margaburpas@hotmail.com (M. Burguera-Pascu).

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most widely used [19]. They are electrostatically attracted by oral surfaces known to carry negatively charged groups, thus increasing the residence time of the active ingredient in the oral cavity [1–4]. It has been demonstrated that in the presence of such substances, *Streptococcus mutans* (*S. mutans*), the oral organism nearly always used in such assays, is unable to acquire the nutrients necessary for its survival and reproduction [3,20]. Certain metal ions may alter the function of the cells membrane and the enzymatic activity within the cell, impairing the production of acids during the glycolysis process [11–17].

In order to be effective, the active ingredient (s) in a mouthrinse preparation must show a high substantivity (ability to maintain an effective concentration for prolonged periods of time) and to be able to interfere with the metabolism of targeted microorganisms [20–23]. Additionally, its bactericidal effect would last as long as the agent's active form is present at effective levels and should be harmless towards the oral mucosa and of low toxicity to humans, since certain volume of the substance may be swallowed during rinsing [22,23].

The low baseline levels of zinc in saliva and all oral hard and soft tissues are only enough to fulfill the nutritional requirements of certain oral bacteria and to maintain active those enzyme, which depend on traces of zinc, while higher concentrations of this element become toxic to the oral microorganisms [24,25]. Broad variations in the concentrations of zinc added to mouthrinses formulations as different zinc salts (lactate, acetate, sulfate, chloride) have been used in combination with chlorhexidine [7–9] and/or triclosán [16–18] to control oral halitosis, plaque and calculus growth, gingival bleeding, etc.

The medical literature contains only few papers devoted to the substantivity of Zn in the oral cavity and its antibacterial effect when used alone in mouthrinses solutions. Afseth et al. [26,27] studied the retention of copper and zinc in the oral cavity following rinsing with known volumes of the respective aqueous salts. Their study showed that a fraction of the metal ions (31% of copper and 15% of zinc) was retained after a single rinse. A similar study reported 12% retention of zinc from a mouthwash containing 0.20% zinc phenolsulphonate [28]. The concentration of zinc in saliva and dental plaque increased after rinsing and its effect lasted up to 6 h. Later on, Dobl and Nossek [29] studied the antibacterial effect of 0.2% and 0.4% zinc chloride mouthrinses on the total *Streptococcus* flora and dental plaque. Their results showed a significant decrease of colony developing units after a 7-day test. Since then, limited research has been carried out in vivo using zinc ions alone, although in vitro studies showed promising results [11,14].

The purpose of the present study was to obtain some information about the behavior of Zn in saliva, at different intervals (30, 60, 90, 120 min and 24 h) after rinsing with zinc salts (sulfate and acetate) at different concentrations (0.1%, 0.5% and 1%) and to test its bactericidal efficacy. The substantivity of Zn salts was assessed by evaluating the levels of Zn in whole, unstimulated saliva sampled at different intervals after rinsing and by determining the magnitude of suppression of salivary *S. mutans* 2 h after rinsing.

Materials and methods

Reagents

De-ionized and distilled water with specific conductance $<0.1\,\mu S/cm$, obtained in a Milli-Q system (Millipore, Bedford, MA, USA) and frequently tested to be zinc-free, was used for the preparation of rinsing solutions and of the working zinc standards. The chemicals used in this study, namely zinc sulfate ($ZnSO_4 \cdot 7H_2O$) and zinc acetate ($Zn(CH_3COO)_2 \cdot 2H_2O$) were at least of analytical-reagent grade from Guinama SL, Spain. A zinc stock solution of 1000 mg/L (Titrisol from Merck) was used to prepare the daily diluted standards for the analytical measurements. The diluent solution was prepared by diluting Triton *X*-100 (Sigma) in water to obtain a concentration of 0.02% v/v.

The following reagents were used for microbiological assays: agar MSB (mitis salivarius bacitracine), sucrose (PA-ACS, Panreac Química S.A., España), potassium telluride (bioMérieux SA, France) and bacitracine (Sigma chemical CO, Germany).

All standards and samples were stored in appropriate polystyrene screw-cup containers soaked overnight in 2% EDTA and then thoroughly washed with several portions of zinc-free water to remove any traces of zinc. The only glassware employed in this work consisted on few volumetric flasks used to prepare the calibration solutions.

Apparatus

Zn concentrations in saliva samples collected before and 24 h after rinsing were estimated by atomic absorption spectrometry (AAS) with electrothermal atomization (ET-AAS) [30–32]. The experiments were carried out using a Perkin–Elmer (PE) atomic absorption spectrometer, Model 4100 ZL with a transversally heated graphite atomizer and a Zeeman-effect background corrector. The instrument control and data processing was accomplished with an Epson personal computer (PC), Model EL 486UC through PE 4100PC software (version 7.3) under Gem Desktop (version Gem/3). A PE zinc hollow cathode lamp was operated at 15 mA and the resonance atomic zinc line of 213.9 nm was selected with a spectral bandwidth of 0.7 nm. L'vov

platforms and pyrolytic graphite-coated tubes were used throughout. Integrated absorbance values (peak area), peak profiles and statistical data were printed with an Epson LX-810 printer. Argon gas of 99.99% purity served as purge gas for the graphite furnace. An on-line dilution system was connected to the instrument. A single new graphite tube with integrated platform was used for all the experiments described in this work with no sign of loss in sensitivity.

Zn concentrations in saliva samples collected 30, 60, 90 and 120 min after rinsing, were carried out by AAS with flame atomization (FAAS) using a PE 3100 spectrometer controlled by a Gem Desktop software also from PE. The instrument was equipped with a Zn hollow cathode lamp operated under the same conditions as for ET-AAS and an air/acetylene burner with an oxidant and fuel flow rates of 16.5 and 1.5 L/min, respectively. The nebulizer was coupled to a simple flow injection (FI) system which allowed the introduction of small aliquots into a carrier stream of water propelled by a peristaltic pump operated at a rate of 5.2 mL/min [33].

Appropriate amounts of zinc salts used to prepare the rinsing solutions were weighted with an analytical balance AND Model HM 202 from A&D Engineering Company, Melpins CA, USA.

A Vortex-type stirrer, Model Reax 1 from Heidolph, Germany was used to homogenize all saliva samples just before the analytical measurements and to breakdown the mucin filaments by vigorous shaking in the samples selected for microbiological assays.

A pressure pan (GENbaganaer. BioMerieux SA, France) was used for samples incubation under anaerobic conditions.

Saliva pH was monitored with a pH-Meter (micro-pH 2001, Crison, Spain). Its glass electrode was calibrated prior samples readings against standard buffers at pH 7.0 and 4.0.

Study population

The case–control study was chosen for this work because it involved healthy subjects with no signs of any oral disease and lasted a short-term evaluation period limited to few hours. Additionally, the case–control study provided a cheaper and quicker association between the variables studied in this work. A group of 44 healthy subjects, 15 men (34.1%) and 29 women (65.9%) with ages between 20 and 62 years and a mean age of 30.84 ± 9.42 years were selected among those who volunteered for this study. This population was randomly recruited among students, teachers and technicians of the Department of Oral Medicine, Faculty of Dentistry, University of Granada, Spain. Those selected, were randomly distributed in two experimental groups: 20 (7 men and 13 women) used Zn sulfate of 0.1% ($n = 7$), 0.5% ($n = 6$) and 1% ($n = 7$) and 18 (6 men and 12 women) used Zn acetate of 0.1% ($n = 6$), 0.5% ($n = 6$) and 1% ($n = 6$). There was also a control group ($n = 6$) who received distilled water as rinsing solution.

Before starting the sampling, each subject signed an informed consent form in accordance with regulations of University of Granada, Spain. The participants were also asked to complete a medical questionnaire and undergo an examination of the oral cavity.

Rinsing and sampling procedures

Saliva is often used for analytical evaluations and/or as a source of bacteria when testing mouthrinses [20]. Saliva sampling is of easy accessibility as it is a noninvasive, painless and fast technique. However, it is well known that the composition of saliva depends on a number of factors related to physiological, pathological and environmental changes [34,35]. An accurate standardization of sampling procedures is highly recommended in order to get a representative sample for analysis [36]. Therefore, the following precautions have been taken before, during and after sampling:

(1) Only those volunteers who showed lack of metal restorations and/or amalgam fillings, orthodontic appliances, fixed or removable dentures, healthy gums, carries-free and normal gustatory acuity were included in the study and were excluded those who were using Zn-containing dentifrice, antibiotics for the last 15 days, were pregnant or suffer any systemic illness.
(2) All saliva samples were collected between 8 and 9 a.m., after a fasting period of 12 h, and a professional tooth cleaning. Each subject was then instructed to rinse the mouth thoroughly with three portions of 10 mL each of pure water, to remain sited, maintain the head in a sub-horizontal position and keep the saliva in the vestibule zone of the oral cavity for 1 min without swallowing. The results obtained for these samples were regarded as baseline levels. The same procedure was repeated at 30, 60, 90, 120 min after rinsing for one minute with the unique 10 mL aliquot of the allocated zinc salt solution.
(3) For the collection of the sample 24 h after rinsing, the participants were asked to refrain from using their usual toothpaste for the next 24 h and resume the oral hygiene to mechanical tooth brushing only after meals, followed by repeated rinsing with water. This precaution minimizes the contamination of the sample with food debris.
(4) Portions of samples collected before and 120 min after rinsing were immediately transferred to

Ependorf-type tubes properly codified for microbiological measurements. Thereafter, sub-samples designated for Zn estimation were transferred to 1.5 mL cupped polyethylene conic-bottom tubes and frozen until Zn analysis was ready to be performed. Just before analysis, they were allowed to defrost at room temperature and were homogenized by vigorous shacking. This last operation was necessary as the different organic fractions of saliva, to which zinc might be bound, could have been stratified on standing by vertical gravitational fractionation [36].

Determination of zinc in saliva

Given its high selectivity and sensitivity as well as its relatively low cost, ET AAS was used for the determination of salivary zinc baseline concentrations.

In order to bring the samples within the working range of the instrument, the defrosted and homogenized saliva samples were diluted 1:20 with a surfactant solution (0.02% Triton X-100) in an on-line system, directly connected to the ET-AAS, which was described elsewhere [32]. Ten microliters of the diluted saliva samples or zinc standards were injected onto the platform of the graphite furnace. This aliquot was submitted to two drying steps (at 90 and 130 °C), followed by pyrolysis and atomization at 700 and 1700 °C, respectively. The integrated absorbance (A) used for calculations, was the average of three determinations and its linear relationship ($r = 0.9984$) with [Zn] (zinc concentration from 0 to 6 μg/L) followed the equation $A = 0.00132 + 0.0397$ [Zn].

The concentration of zinc in the samples collected within 2 h after rinsing is high enough to be determined by FAAS. The limited volume of sample available demanded the connection of a single channel FI system to the spectrometer through a 10 cm long capillary tubing. A home-made chromatographic-like injector allowed the introduction of 40 μL of untreated sample or zinc standard into the carrier stream which transported it to the nebulizer [33]. The linear calibration ($r = 0.9998$) obeyed the equation $A = 0.00293 + 0.03764$ [Zn] where r is the correlation coefficient, A is the mean absorbance of three measurements and [Zn] is the concentration of the analyte in the range 0–4 mg/L.

Microbiological assay

The samples reserved for microbiological measurements were dispersed and diluted (10^1–10^2) with phosphate buffer at pH 7.3. Volumes of 100 μL of these samples were disseminated in Petri dishes containing the following culture medium: agar MSB supplemented with 15.0% sucrose and 1.0 mL of each of the following sterilized solutions: 1.0% potassium telluride and 0.2 U/mL bacitracine. This solid artificial medium is selective for *S. mutans* and intends to reproduce the bacteria natural environment, to cover all its nutritive requirements and to allow its growth and proliferation under laboratory conditions. Then, the samples were incubated for 48 h at 37 °C under anaerobic conditions, and finally, the colony forming units (CFU/mL) were counted.

Evaluation of the astringent sensation

The use of zinc salts as mouthrinses may produce a metallic-like taste, which could last for certain periods of time in the mouth. The participants were asked about the astringency sensation and its duration, and were finally classified as no sensation, up to 1 h, up to 3 h, up to 6 h, more than 6 h.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) was used to analyze the data. ANOVA was used to check for significant differences between mean values and for inter-subjects and inter-groups comparisons. The Pearson correlation coefficients were also used to test for significant relationships between zinc concentrations, pH values and bacterial counts at each evaluation time. Results were expressed as mean ± standard deviation (mg/L) and the level of significance selected was of $p < 0.05$ for all the statistic tests in this study.

Results

All 44 volunteers participated until the end of the study. Neither serious nor minor side-effects occurred, except for the astringent taste experimented by 88.6% of the participants. There were highly significant differences between men and women when referring to the duration of such sensations: About 77% of the men felt that the astringency lasted for only 1 h, while more than 60% of the women complained for up to 3 h after rinsing. There were only slight differences between the salts type (apparently sulfate is more unpleasant than acetate) and the volunteers could not relate the taste to the salts concentrations. All the controls but one could recognize the water as their rinsing solution and therefore no comments about the taste were made in this group. No staining of any oral tissues was observed at the end of this study.

Zn levels in saliva after a unique mouth rinse with Zn salts

The baseline Zn values (0.055 ± 0.017 mg/L) obtained for the 44 participants showed an intrinsic individual

variation from 0.020 to 0.100 mg/L, with a relatively large coefficient of variation of 30.9%, thus the three experimental groups were not statistically different with respect to their Zn baseline values in saliva. Considering the whole population, a significant decrease of Zn levels was observed with increasing age; there was no apparent effect of sex on Zn concentration. Any statistical comparison with respect to age and sex was limited by the small number of subjects selected in each group. An immediate increase of Zn concentrations in saliva was observed from the mean baseline values to 2.58 ± 1.21 and 3.59 ± 2.00 mg/L at 30 min after rinsing with sulfate and acetate, respectively. This was followed by a rapid loss to 1.08 ± 0.62 and 0.078 ± 0.034 mg/L and to 1.33 ± 0.72 and 0.082 ± 0.035 mg/L at two and 24 h after rinsing with sulfate and acetate, respectively.

It is of interest to point out that the net area under the curve (netAUC) values [37] also show the previously described tendency (Fig. 1), suggesting that, in the samples of treated subjects, Zn concentrations are more variable within the time ranging from 30 to 120 min. The netAUC values for control subjects remained constant (0.05 ± 0.02 mg h/L) for the entire period of sampling. These calculated values include all incremental area under the curve for a given time interval: e.g. the netAUC value for zinc (as sulfate) at 1 h after rinsing represents the increase on zinc concentration between 30 and 60 min after rinsing.

Zn levels in the samples of the participants using acetate as rinsing solution were higher at all times after rinsing, but not significantly different from those rinsing with sulfate. However, the metal levels in saliva collected 24 h after rinsing, remained significantly higher ($p < 0.001$) than the baseline Zn concentrations. No significant differences ($p > 0.05$) were found between the saliva Zn concentrations with respect to the concentration of Zn salt used at any of the sampling times. Therefore, in subsequent comparisons, the effect of salt type was analyzed using the mean obtained for the three concentrations of the rinsing solutions. As expected, with small variations, control subjects maintained the baseline levels during the experiment and were significantly different ($p < 0.001$) from those of the subjects who rinsed with Zn salts at any time after rinsing.

Bacteria counts

The microbiological experiments carried out in this work show that both rinsing products were able to cause significant reduction in the bacterial number when compared to water. The individual *S. mutans* counts before rinsing widely varied from 0 to 3000: these values significantly decreased ($p = 0.0039$) 2 h after rinsing with either salt. No significant variations in the CFU/mL were found for the individuals grouped as control ($p = 0.920$).

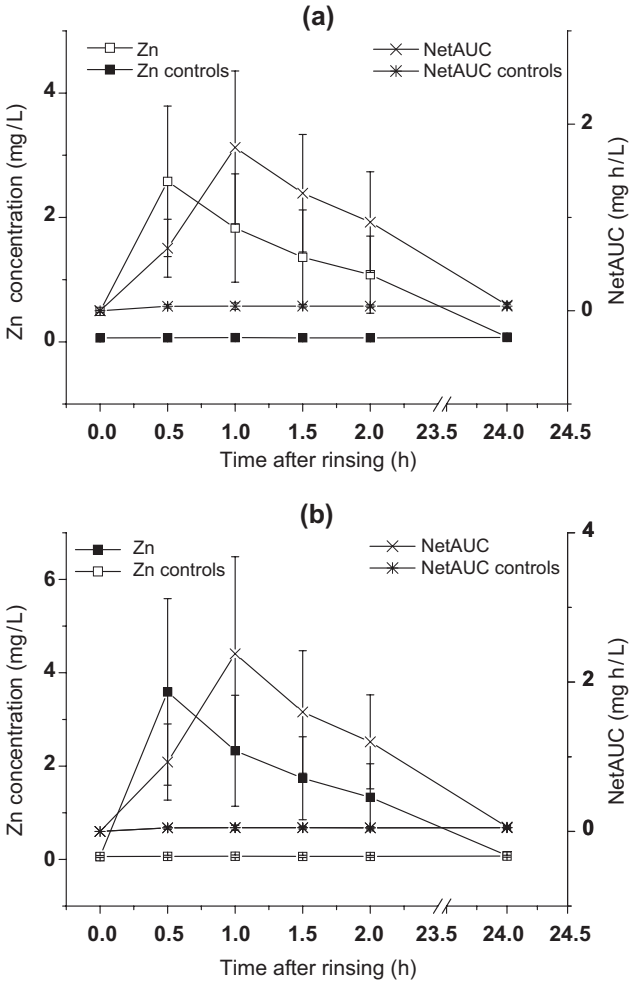

Fig. 1. Effect of the time after rinsing on the concentration of Zn in saliva and the corresponding net AUC values for (a) sulfate and (b) acetate.

When compared to the baseline counts, *S. mutans* did not show statistically different behavior in the presence of any of the salts used as mouthwashes solutions ($90.44 \pm 7.63\%$ with sulfate and $85.42 \pm 3.30\%$ with acetate), neither with respect to their concentrations (0.1%, 0.5% and 1%). In the control group, only a $5.9 \pm 0.40\%$ reduction was obtained. Apparently, the age and sex had no significant effect on the bacterial counts. Fig. 2 shows the mean *S. mutans* counts assessed after rinsing with zinc salts and water, compared to the baseline values (given the huge variations of the initial counts, the variability bars could not be shown in the figure). Instead, the mean reduction (%) in the bacteria counts caused by the rinsing with the salts is shown.

pH values

The individual baseline pH values obtained for the whole population under study varied from 6.44 to 7.70,

with a mean of 6.98 ± 0.66. No significant differences ($p > 0.05$) were found between the baseline salivary pH values and those obtained at any time after rinsing with any solution (zinc salts at any concentration or water). As a result, no discrimination between the pH values with respect to salt type and concentrations was made for subsequent considerations. This observation is further confirmed by the obtained netAUC values which only varied from 3.5 to 3.6 pH.

A highly significant negative correlation ($p < 0.001$) was found between zinc concentration and pH, exactly for the same time interval (60 min up to 120 min after rinsing). The corresponding the Pearson correlation coefficients vary from −34% to −50%, as shown in Table 1. Significant differences ($p < 0.027$) between the baseline salivary pH values and the age of the volunteers were also found. Curiously, the pH values for the participants older than 40 years of age are lower than 7, while for those younger than 40, all pH values are superior to 7. Gender however had no effect on the salivary pH values.

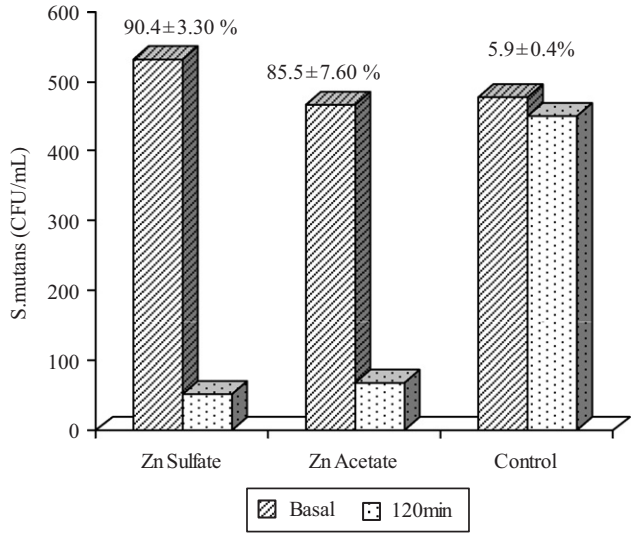

Fig. 2. Reduction of *S. mutans* counts 2 h after rinsing with zinc salts.

Discussion

This investigation was aimed to compare two different zinc-based mouthrinse formulations (sulfate and acetate) with regard to their substantivity and to their capacity to reduce the number of *S. mutans* in saliva, certain time after rinsing. The methodology was applied only to healthy volunteers, and the sampling protocol was cautiously followed for all subjects in order to obtain a representative sample for analysis. Besides these precautions, high individual variability of zinc concentrations and *S. mutans* counts was obtained, probably due to the intrinsic individual behavior or to parameters which have not been taken into account, like the type of food ingested, salivary flow rate or the presence of any infections which could not be detected at the beginning of the trial. The high coefficients of variation (31–57%) definitely suggest heterogeneity among individual behavior. However, the overall results obtained, have shown the efficacy of these antimicrobial formulations for oral use.

The direct determination of the amount of an active agent bounded somehow to the oral tissues after rinsing is methodologically impossible, unless radioactive markers are used [26,27,38]. As this methodology is not easily available, the simplest way to follow the substantivity of any rinsing solution would be to evaluate the concentration released into the salivary flow before and at certain time after rinsing. AAS technique, either ET-AAS or FAAS proved appropriate for the purpose of this work (evaluate Zn concentrations in saliva before and after rinsing with Zn salts).

Zn concentrations naturally present in saliva are too low to have an inhibitory effect over the oral bacteria; on the contrary, it is present at a level which is essential

Table 1. Association between Zn concentrations and the corresponding pH values in saliva at different times after rinsing

pH /(Zn)		(Zn) baseline	(Zn) 30 min	(Zn) 60 min	(Zn) 90 min	(Zn) 120 min	(Zn) 24 h
pH baseline	*r*	0.0255	−0.197	−0.325	−0.503	−0.503	−0.121
	p	0.879	0.2344	0.046	0.0013	0.0013	0.467
pH 30 min	*r*	−0.006	−0.181	−0.407	−0.161	−0.147	0.066
	p	0.970	0.276	0.0113	0.296	0.341	0.671
pH 60 min	*r*	−0.193	−0.186	−0.344*	−0.393**	−0.250	−0.170
	p	0.209	0.227	0.022	0.008	0.101	0.269
pH 90 min	*r*	−0.323*	−0.255	−0.421**	−0.481**	−0.370*	−0.322*
	p	0.032	0.095	0.004	0.001	0.014	0.033
pH 120 min	*r*	−0.334*	−0.227	−0.455**	−0.498**	−0.394**	−0.292
	p	0.026	0.139	0.002	0.001	0.008	0.054
PH 24 h	*r*	−0.107	0.092	0.030	−0.120	−0.068	−0.136
	p	0.491	0.554	0.847	0.438	0.659	0.378

r: the Pearson correlation coefficient; *p*: significance; *$p < 0.05$; **$p < 0.01$. min: minutes.

Table 2. Basic levels of zinc in saliva

Sample type	Zinc concentration (mg/L)			
	Mean ± SD[a]	Range	n[b]	Ref.
Parotid	0.051 ± 0.014	0.027–0.082	34	[5]
	0.112 ± 0.048	NR[c]	24	[38]
Stimulated	0.080 ± 0.043	NR[c]	35	[6]
Not stimulated	0.036 ± 0.017	0.028–0.070	6	[36]
	0.102 ± 0.056	0.046–0.170	5	[37]
	0.055 ± 0.017	0.022–0.098	44	This work

[a]Standard deviation.
[b]Number of subjects.
[c]Not reported.

for the survival and growth of *S. mutans* [24,25]. Only few studies have reported zinc baseline values in whole saliva. Table 2 shows that our values may be considered to be within "normal" zinc levels or are similar to those zinc baseline levels reported in the literature for parotid [5,39] and whole stimulated [6] and unstimulated [36,40] saliva.

The individual baseline Zn concentrations found in our samples could be compared directly with those of the controls (their values remained in the range of the baseline level for the entire period of time studied). The rapid increase in zinc salivary concentration during the first 30 min and the slow decrease up to 24 h after the single rinse (Fig. 1), most probably represents a clearance of loosely bound metal ions by salivary flow during sampling. Our results show that either zinc concentration determined in saliva sampled at a given time after rinsing or the netAUC values calculated over a period of time can be recommended to assess the affectivity of rinsing with either zinc salt in order to inhibit *S. mutans* growth.

The presence of statistically higher concentrations of zinc in saliva 24 h after the rinse is an evidence of the existence of a tightly bound metal fraction with a longer oral residence time. Accounting for the substantivity of zinc, this last observation might be of greater importance from the clinical point of view than the amounts of zinc released immediately after a rinse because it proves the long-term presence of Zn ions in the mouth. Such strong bounds would prevent the adherence of new bacteria to the receptors sites. Further studies have to be performed to determine the retention mechanism, although it may be speculated that the metal binds to oral mucosa and certain chemical structures in the oral cavity as previously discussed in the literature [26,27].

It is also important to state that none of the subjects exhibited zinc-related staining of any oral tissues, as reported for silver nitrate [41], tin chloride [42] or chlorhexidine [43]. The insoluble zinc sulfide, which might be formed with the volatile sulfur compounds solubilized in saliva has a pale yellowish color which is washed away by the salivary flow [10].

The number of *S. mutans* were significantly reduced after rinsing with either zinc salt: this reduction ranged from 12.5% to 100% (85.42 ± 7.30%) and from 71.4% to 96.7% (90.44 ± 5.63%) 2 h after rinsing with acetate and sulfate, respectively (Fig. 2). Given the important inter-subjects variability before rinsing, the lesser antimicrobial effect of zinc acetate solution compared to sulfate could not be statistically proved. The distilled water used as rinsing solution in the control group only produced a reduction in the bacteria counts of 5.9%, confirming the validity of the present study.

The insignificant pH changes observed during the first hour after rinsing (Fig. 3) might be produced by the rinsing solutions (their pH was <4), which probably modify the saliva buffer capacity [44]. The slight increase in the pH values during the second hour after rinsing might sustain the theory of bacterial glycolysis inhibition. Such effects are more strongly evidenced by in vitro studies [11–14]. The data in Table 1 indicate that as salivary Zn concentrations decrease, pH values increase. Apparently, the antibacterial process, do not start immediately after rinsing; in fact, our preliminary microbiological experiments failed to demonstrate any reduction in the bacteria counts 30 min after rinsing. This inverse correlation has also been reported by Afseth et al. [27], although the authors do not discuss such findings. In our opinion, this is an additional evidence of the affectivity of the rinsing solutions used, meaning that the portion of Zn retained on the oral surfaces is capable to exert the antibacterial effect for a long period of time. It is believed that the regular use of such preparations can lead to a build-up of antibacterial agents that continue to work for even longer periods of time. In order to confirm their inhibitory effect on bacterial glycolysis, a larger population needs to be studied.

The significant correlation between age and saliva pH found in this work was also recently reported by Aframian et al. [45]. Their mean pH value of 6.78 ± 0.04 obtained for 50 healthy adults also falls within our range. Study of a larger population is needed in order to be able to give a suitable explanation why the pH values are below 7 for the participants older and higher than 7 for the younger than 40 years of age. No gender effects were noted by these authors either.

Conclusions

The case–control study adopted in this work and the reliable analytical methods used, contribute to the assessment of baseline zinc levels in whole, unstimulated saliva, and can easily be applied for routine testing and

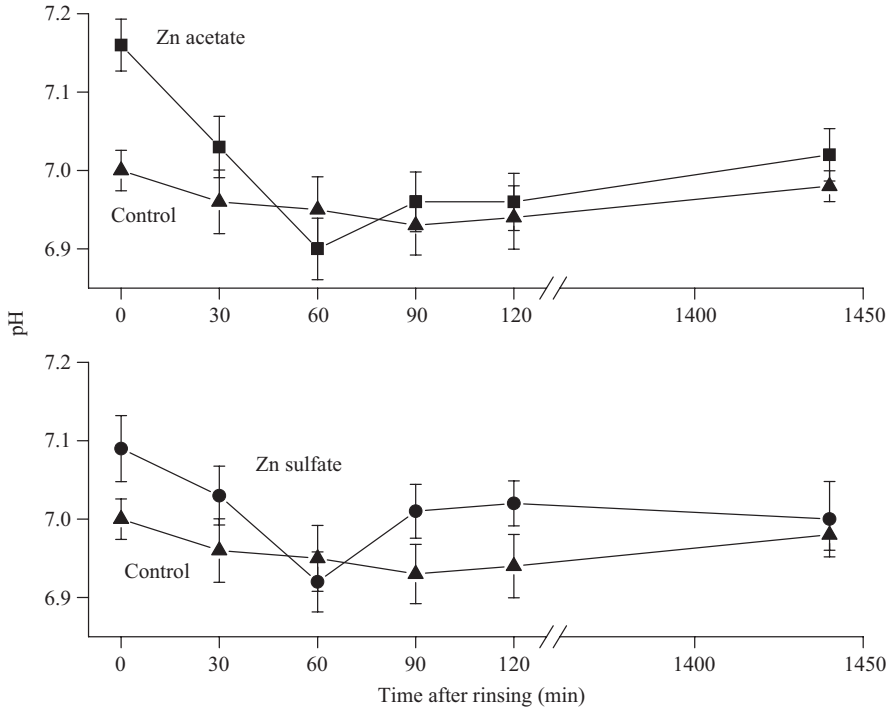

Fig. 3. Variation of salivary pH values with time after rinsing.

further development of these and other Zn-containing mouthrinses. The highly significant Zn concentrations still present in saliva 24 h after rinsing are sufficient testimony for the high substantivity of Zn in the oral cavity. Both zinc salts were found to be equally active against *S. mutans* at any concentration tested. Such mouthrinses may effectively complete the mechanical tooth brushing for a long-lasting oral hygiene. The only adverse effect related to the use of zinc ions in mouthrinses is their unpleasant astringent taste, which could be easily improved by the addition of appropriate additives. Additionally, the prolonged use of zinc salts may have the least tendency to stain the oral tissues, when compared to other active ingredients like Ag, Sn or chlorhexidine.

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