Impact of laminar flow velocity of different acids on enamel calcium loss

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ORIGINAL ARTICLE

Impact of laminar flow velocity of different acids on enamel calcium loss

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Abstract

Objective The aim of the study was to evaluate the impact of flow velocity under laminar flow conditions of different acidic solutions on enamel erosion.

Material and methods A total of 240 bovine enamel specimens were prepared and allocated to 30 groups ($n=8$ each). Samples of 18 groups were superfused in a flow chamber system with laminar flow behavior using 1 ml of citric acid or hydrochloric acid (HCl) of pH 2.0, 2.6 or 3.0. Flow rates in the sample chamber were adjusted to 10, 60 or 100 μl/min. To simulate turbulent flow behavior, samples of six groups were immersed in 1 ml of the respective solution, which was vortexed (15 min, 600 rpm). For simulating non-agitated conditions, specimens of the remaining six groups were immersed in 1 ml of the respective solution without stirring. Calcium in the solutions, released from the enamel samples, was determined using Arsenazo III method.

Results For acidic solutions of pH 2.6 and 3.0, erosive potential of citric acid was equivalent to that of HCl at a flow of 100 μl/min. The same observation was made for the samples subjected to turbulent conditions at pH 3. At all other conditions, citric acid induced a significantly higher calcium loss than HCl.

Conclusion It is concluded that under slow laminar flow conditions, flow rate variations lead to higher erosive impact of citric acid compared to hydrochloric acid at pH 2.0, but not at pH≥2.6 and increasing laminar flow or turbulent conditions.

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Clinical relevance Erosive enamel dissolution under laminar flow conditions is a complex issue influenced by flow rate and acidic substrate.

Keywords Enamel · Acid flow · Velocity · Calcium · Erosion

Introduction

There is some evidence that prevalence of dental erosion might be related to consumption of dietary acids [1–3], although this association is not clearly proved in every study [4]. Thus, focus has been applied on chemical characterization and alterations of acidic dietary substances to achieve a more comprehensive insight into the aetiology of dental erosions and their prevention [5–7]. Beside dietary acids, hydrochloric acid, originating from the stomach (gastric juice) and entering the oral cavity for example in case of vomiting or gastro-oesophageal reflux, is also held responsible for erosive tooth dissolution [8]. With regard to dietary acids, it was elucidated that the content of calcium and phosphate, in particular the saturation of the solution with respect to hydroxy apatite, the pH and amount of titrable acid, and also the kind of acid do have an impact on erosivity of a solution or beverage. However, besides the chemical aspects of substances, also the physical or physicochemical aspects, such as adhesiveness of the solution to the tooth surface, temperature, flow rate and agitation of the solution seem to have an influence on its erosive potential [9–11].

In the oral cavity, it might be assumed that during drinking or even swishing of a solution through the teeth, turbulences might occur, which lead to transport of unused H+-protons of the solution to the tooth surface, thus increasing tooth

demineralization. This hypothesis was corroborated in a laboratory study, in which enamel dissolution in an agitated solution was distinctly increased compared to a non-agitated solution [9].

Despite agitation or stirring of a solution, a semi-static layer of the liquid is formed immediately adjacent to solid surface, such as those of tooth samples immersed in a liquid. Within this so-called *Nernst* diffusion layer of about 1–10 μm thickness, diffusion alone controls the ionic transfer to the tooth surface. According to Fick's laws, the diffusion within the Nernst layer and in the bulk of a solution occurs down a concentration gradient [12]. At low liquid velocities, the layer becomes thicker and less undersaturated with respect to dissolved tooth mineral than under high velocities. The concentration of acidic or mineral compounds within the agitated part of the solution stays more or less constant, under the premise that the solution is applied in abundance. In an acidic solution, the Nernst layer is rapidly saturated with respect to tooth mineral, so that further demineralization is slowing down or even stopping, unless fresh acidic compounds are diffusing into the layer or the layer is completely replaced under agitated conditions or unless further dissociation of the acid is occurring within the layer.

However, during retention of an acidic substance in the oral cavity, also laminar flow of a solution might happen, so that the diffusion within the Nernst layer and the ability of the acid to produce further H^+-protons by dissociation might be of importance. Besides its capability to act as a chelating agent, citric acid as a weak trivalent acid ($pK_{a_1}=3.13$, $pK_{a_2}=4.76$, $pK_{a_3}=6.4$) also has the potential to further dissociate, when free protons have already been used up in the demineralization process. In contrast, strong monovalent acids, such as hydrochloric acid ($pK_a=-6$), are already completely dissociated, so that further delivery of protons in a closed or semi-closed system, such as the Nernst layer, only happens limitedly. Moreover, citric acid, as a weak acid, may act as an acidic buffer, especially at pH values which are close to one of the three different pK_a values. Thus, it might be speculated that even under assumption of same initial pH, a weak polyvalent acid might behave different and more erosive under laminar flow conditions as compared to a strong monovalent acid.

The aim of the present study was to evaluate the erosive behaviour of a monovalent strong acid and a polyvalent weak acid under laminar flow conditions. Hydrochloric acid and citric acid were selected as representatives for a monovalent strong acid and a polyvalent medium-strong to weak acid, respectively, which also might be found in the oral cavity during gastro-oesophageal reflux (hydrochloric acid) or consumption of beverages (citric acid).

Material and methods

Specimen preparation

Sixty bovine permanent mandibular incisors from 2–3-year-old cattle were extracted. The crowns were separated from roots, cleaned of periodontal tissue residues by means of a scaler and stored in 0.5 % thymol solution for a maximum of 6 months at 5°C until used in the present study. Four enamel cylindrical specimens (diameter 3 mm) were prepared from each crown using a water-cooled trephine mill and embedded in acrylic resin blocks (6 mm in diameter, Paladur, Heraeus Kulzer, Germany). The enamel surface was ground plane and fine with water-cooled carborundum discs (1200, 2400 and 4000 grit, Water Proof Silicon carbide Paper, Struers, Erkrath, Germany).

Treatment of specimens

Then the specimens were randomly allocated to 30 groups ($n=8$). Samples were either treated with citric acid (Fluka, Buchs Swizerland) or hydrochloric acid (Merck, Darmstadt, Germany) dilutions with pH 2.0, 2.6 and 3.0. One hundred forty-four samples were fixed in a superfusion chamber; 48 were stored in 1 ml of an acidic solution without agitation, and 48 were immersed with agitation using a vortex (600 rpm, mini-Shaker, Fisher Scientific, Pittsburgh, USA). In the superfusion chamber system, the samples were rinsed with 1 ml of the respective acid. For adjustment of flow rate, the amount of liquid flowed through the chambers was collected and measured during intervals of 30 min, and the connected pump was set accordingly. The flow rate in the superfusion chambers was then adjusted to 10, 60 or 100 μl/min. Thus, the total contact time of the acidic solutions with the samples in the superfusion chambers was different for the specimens submitted to different flow velocities. Thus, the following groups resulted (Table 1).

Description of superfusion chamber system

In the superfusion chambers, each sample was fixed in a metal jig, which allows for exposure of the enamel samples surface to a small erosion chamber of 0.94 mm^3 volume (2 mm in diameter × 0.3 mm in height). For rinsing of the samples, the respective acidic solution was pumped from a reservoir outside of the chambers into the space of 0.3 mm height, which was located between the surface of the enamel specimen and the top of the chamber. The solution enters and leaves the chamber through two orifices of 0.4 mm diameter each. The distance between these orifices amounts to 1.6 mm, i.e. travelled length of the fluid. When adjusting the flow rate to maximum speed of 100 μl/min, the solution

Table 1 Flow rate/velocity of the solutions under the different erosive conditions, and duration of treatment for the different specimens subjected to citric acid or hydrochloric acid of pH 2.0, 2.6 and 3.0, respectively

Erosive conditions	Flow rate, velocity	Time [min]	Hydrochloric acid [pH] (*n*=8 per group)			Citric acid [pH] (*n*=8 per group)		
Immersion	0 μl/min	100.0	2.0	2.6	3.0	2.0	2.6	3.0
SC-10	10 μl/min	100.0	2.0	2.6	3.0	2.0	2.6	3.0
SC-60	60 μl/min	16.7	2.0	2.6	3.0	2.0	2.6	3.0
SC-100	100 μl/min	10.0	2.0	2.6	3.0	2.0	2.6	3.0
Vortex	600 rpm	10.0	2.0	2.6	3.0	2.0	2.6	3.0

SC: storage in superfusion chamber; immersion: storage without agitation; vortex: storage with agitation of the fluid on a vortex

runs over the specimen surface with a velocity of 212 mm/min (0.0035 m/s).

The Reynolds number is a dimensionless number in fluid mechanics. It expresses the ratio of inertial forces to viscous forces and is used to calculate whether a fluid movement is regarded as laminar or turbulent.

The Reynolds number [Re] of the above-described chamber system is calculated as $Re=(vd)/\nu$ (where d=travelled length of the fluid [meters], v=mean velocity of the fluid [meters per second] and ν=kinematic viscosity of the fluid, 1.002 m^2/s for water at 20°C).

According to this calculation, the Reynolds number for the system is $Re \ll 1$. This means that the fluid movement in the chamber is regarded as having laminar flow behaviour, since laminar flow is defined to occur when $Re < 2000$.

Prior to the acid exposure, distilled water was rinsed through the chamber to ensure a bubble-free flow. Due to the small height of the space, continuous flow of the acid without generation of bubbles resulted. Each of the eight chambers was connected to a single tube of a multichannel pump (Ismatec, Glattbrugg, Switzerland). The fluid flowed over the specimens and the solutions used for immersion and vortexing were analysed for calcium released from the enamel samples.

Calcium analysis

Calcium release into the acidic solutions was assessed by colorimetric analysis, using Arsenazo III method (Fluitest, Ca-A-II, analyticon, Lichtenfels, Germany) [13]. Arsenazo III reacts with calcium in an acidic solution forming a blue purple complex, whose intensity developed is proportional to the calcium concentration in the solution. Absorption can be determined at λ=650 nm. Reagent for determination of calcium was composed of 100 mmol/l Imidazol buffer (pH 6.5) and 0.12 mmol/l Arsenazo III.

Individual standard curves were obtained by admixing diluted samples of standardized calcium solutions to 1–20 μl of the respective acid at pH values of 2.0, 2.6 and 3.0. The lower limit of quantification (LLoQ) to detection of calcium in citric acid using this method amounts to 15 nmol Ca/ml and

was known from previous studies as the range of expected enamel calcium release [13–15]. This procedure of achieving calibration curves allowed for measuring calcium in citric acid, although chelation of calcium with the citric acid anion is supposed to happen.

For determination of the calcium content in the solutions, 100 μl of the reagent was added to each of the 96 wells of a microplate and mixed with standards or 1–20 μl of the respective acid solution, which has been flowed over the specimens. Absorbance was read within 30 min after mixing of the solutions. The measurements were performed at room temperature.

In order to validate the data from the calcium analysis and to make sure that the two acids did not impair calcium analysis, a phosphate analysis of the solutions was also performed. For this, the malachite green procedure to detect minimal amounts of phosphate dissolved in acidic solutions was used [16]. These data were further used to check whether the ratio of calcium and phosphate released from the enamel samples was constant and comparable for the two acidic solutions. A constant calcium/phosphate ratio is able to indicate the reliability of the data.

Statistical analysis

Descriptive statistics (mean, SD) of the differences between the acids were computed. Normal distribution of the data was checked with Kolmogorov–Smirnov and Shapiro–Wilk tests and was not found in two of 30 groups. In order to disclose the differences in the measurements between acid groups, non-parametric Mann–Whitney U tests were applied for each combination of pH and flow rate separately.

Results of the statistical analysis with p-values below 0.05 were considered to be statistically significant. Comparisons between the different flow conditions within one acid were not applicable, since the contact times of the 1-ml acid solutions to the samples were different due to the setup of the experiment. Thus, only comparisons of samples treated with flow rate of 0 vs. 10 μl/min (100 min contact) and 100 μl/min vs. vortex (10 min contact) were performed.

Results

In Table 2, the calcium release from the enamel samples into 1 ml of the respective acid is given for all experimental groups. Due to clarity reasons, data of phosphate analysis are not shown, but for all groups the findings of the phosphate determination were in agreement with the results of the calcium analysis. Calcium/phosphate ratios (C/P ratio) in all analyzed solutions were stable in a range of 1.98± 0.48, with a C/P ratio of 2.02±0.37 for HCl and 1.99±0.57 for citric acid. These findings indicated reliability of the calcium data measured under the different pH conditions in the different acidic solutions.

Samples treated with flow rate of 0 vs. 10 μl/min (100 min contact) showed an increase in calcium release for the specimens subjected to the respective acid in the flow chamber irrespective of the acid and pH applied. Immersion in vortex led to significantly higher calcium loss than under flow conditions at 100 μl/min. For acidic solutions of pH 2.6 and 3.0, erosive potential of citric acid was equivalent to that of HCl at flow of 100 μl/min. The same outcome was observed for the samples subjected to turbulent conditions in the vortex at pH 3. In the immersion group at pH 3.0, hydrochloric acid led to higher enamel calcium loss than citric acid. However, under all remaining conditions, citric acid induced a significantly higher calcium loss as compared to HCl.

Discussion

In the present study a superfusion chamber system was used for rinsing enamel samples with the two acids in a laminar flow situation. The chambers of the systems have a small volume of about 0.94 mm^3, in which the liquid is flowing over the samples. Due to this small volume, it is supposed that a distinct portion of the solution flowing on the samples comes into contact and might react with the superfused enamel. The applied colorimetric method to record mineral loss from the enamel samples allows for determination of minimal amounts of calcium in small acid fractions [14]. The precision of the method was described previously [13]. To confirm the results

of the calcium analysis, the acid samples had also been analysed for phosphate, showing that results of phosphate and calcium determination were in agreement.

The samples were rinsed with hydrochloric acid and citric acid at pH 2.0, 2.6 and 3.0. The two acids represent a very strong monovalent (hydrochloric acid) and a medium-strong to weak polyvalent (citric acid) acid. Moreover, citric acid was chosen as a typical representative of acidic compounds present in acidic beverages. The chosen pH values of 2–3 exhibit a range of pH which was reported to be found at tooth surfaces for a short time period after taking a sip of 1 % citric acid [17]. Hydrochloric acid was chosen as it is the acidic compound of gastric juice. Data of measurements of intra-oral pH in patients suffering from reflux or during vomiting attacks with the exposure of gastric acid (HCl) in the oral cavity are not present in literature. In studies dealing with this topic, only length of time span with pH < 4 in the oral cavity is reported [18–20]. The minimum pH is not given in those studies. Fasted gastric fluid has a pH of about 1.3, with an increase to pH 4.9 during meal and a drop to pH 2 4 h after having a meal [21]. Thus, it can only be speculated that the intra-oral pH after vomiting and reflux might also be in the range of the pH chosen for the two acidic solutions.

In the oral cavity two mechanisms might appear within such acidic solutions during drinking. Assuming that the liquid is kept in the oral cavity with very little agitation, saturation with respect to hydroxy apatite accompanied by an increase of pH might occur within the Nernst layer of the solution. In contrast, when the solution is highly agitated in the oral cavity, the Nernst layer will be replaced by fresh acidic compounds, thus keeping both undersaturation and pH in the Nernst layer low. Since the contact of teeth in the oral cavity with solutions is influenced by various parameters and presents a very complex system, it remains a speculation which of these two mechanisms might prevail during drinking of beverages or vomiting.

Bovine samples were used as substrate, as they are widely used in erosion research and their chemical and mechanical properties are similar to human dental hard tissues. However, it has to be taken into account that the susceptibility to erosion

Table 2 Mean (standard deviation) calcium loss [nmol/ml] in enamel samples treated with hydrochloric and citric acids of pH 2.0, 2.6 and 3.0, and subjected to different erosive conditions

*Values indicated by an asterisk are not statistically significantly different with respect to comparison of hydrochloric acid vs. citric acid at same pH

Erosive conditions	Hydrochloric acid			Citric acid		
	pH					
	2.0	2.6	3.0	2.0	2.6	3.0
Immersion	490 (177)	78 (13)	60 (6)	1577 (155)	137 (25)	35 (10)
SC-10	909 (69)	223 (17)	64 (8)	2137 (642)	330 (30)	84 (12)
SC-60	390 (64)	86 (9)	29 (6)	889 (102)	135 (23)	35 (3)
SC-100	398 (70)	68 (15)*	27 (8)*	501 (46)	63 (29)*	26 (2)*
Vortex	682 (141)	166 (9)	75 (13)*	1274 (99)	232 (20)	67 (4)*

might slightly differ between bovine and human substrates [22, 23]. A salivary pellicle was not formed on the enamel surface, in order to minimize the variables in the study and to allow focussing on the acids' behaviour only. However, for extrapolation of the present results to the situation in the oral cavity, it should be borne in mind that absence or presence of salivary pellicle might influence the erosive destruction induced by various acids [15, 24–26].

In the experiment, flow rates of 10–100 μl/min were chosen, which were possible to apply in the superfusion chamber system with Reynolds numbers distinctly below 1. Thus, these values represent very low rates, assuring laminar flow conditions. As written in the "Introduction", the study aimed to investigate the erosive behaviour of two different types of acid under non-turbulent conditions, which might occur during slow drinking or holding of an acidic compound in the oral cavity. On the other hand, turbulent conditions were simulated by immersing the enamel samples in vortexed solutions, in which turbulences were obviously visible. However, it should be noticed that to the knowledge of the authors, no information about the exact flow velocities of solutions in contact with tooth surfaces is available.

The flow rate in the superfusion chamber was very low as compared to previous studies also investigating the impact of liquid flow velocity on enamel erosion. Shellis et al. [11] applied flow rates of 26–126 ml/min, which were distinctly higher as compared to the flow rates of 10–100 μl/min used in the present study. Moreover, they have used a circulating system to pump citric acid (pH 3.2) over enamel samples. A circulating system bears the risk of saturation of the solution due to long-term contact of the solution with the samples. This is in contrast to the present study, in which different types of acids with different pH only ran once over a sample before determining calcium in this solution. Despite those differences in flow rate and experimental setup, they also found that for a given exposure time, enamel erosive loss increases with an increasing flow velocity. The same increase in enamel calcium loss with increase in flow velocity results was seen in the present study, when the groups with same exposure times and different flow rates (immersion vs. SC-10, vortex vs. SC-100) were analysed.

Laminar flow conditions and their impact of erosion were also tested in a single experimental group in a study by Eisenburger and Addy [10]. It was found that enamel loss was equivalent to a group subjected to unstirred conditions. However, in this study only a single laminar flow velocity (225 mm/min) and a single acidic solution (citric acid pH 3.2) was tested, thus not allowing to estimate the behaviour of different acidic compounds and pH values under different laminar flow conditions.

We decided to keep the exposure liquid volume constant and to vary the exposure time by varying the laminar flow velocities. This was done to mimic the situation of a given volume in the oral cavity during drinking of a sip. In order to allow for further comparisons, the time of exposition in the non-agitated group was set equivalent to the duration in the group with the lowest liquid velocity (SC-10). Moreover, exposition time in the vortex group was the same as in the group with the highest velocity (SC-100). This setup allowed for directly estimating the influence of velocity, showing that an increase in velocity results in an increase of calcium dissolution for both acidic compounds. This finding corroborates the results of previous studies [9–11].

Our experiment also shows that under the premise of same pH, citric acid might lead to more erosive mineral loss than hydrochloric acid. This was true for nearly all conditions except for groups SC-100 and vortex, in which no difference between the two acids was recorded at pH 3.0 (for both conditions) and 2.6 (for SC-100 only), and except for the immersion groups with pH 3.0. The reason for the more pronounced erosive potential of the citric acid as compared to hydrochloric acid might be explained by the different degrees of dissociation of the two acids at the respective pH values. In a pilot and unpublished titration experiment, we found that hydrochloric acid is completely dissociated at pH 2.0, 2.6 and 3.0, whereas at those pH values the trivalent citric acid is only dissociated to about 3.1 %, 8.8 % and 17.4 %, respectively. This means that especially at lower pH, citric acid exhibits a great potential for further dissociation and delivery of H^+-protons. With increasing flow velocity and decreasing contact time of the acid with the enamel and increase of pH, this particular effect is decreasing or even completely diminished, thus leading to a more or less similar behaviour of the monovalent hydrochloric acid and the trivalent citric acid. This behaviour might be accompanied by a removal and exchange of the Nernst diffusion layer, diffusion of unused acidic compounds into this layer or reduced further dissociation of citric acid within this layer. Additionally it is important to notice that citric acid has chelating properties, which allow binding of calcium ions released from the enamel. This effect supports the undersaturation of the solution and is a driving force for further enamel dissolution. Moreover, citric acid, as a weak acid, may show some buffer properties. According to the Henderson–Hasselbalch equation, citric acid is therefore able to keep the pH constant, especially at pH ranges close to one of its pKa values. Thus, it is should be noted that citric acid with pKa$_1$=3.13 acted as a buffer under pH 2–3 as chosen in the experiment. This effect may also explain its greater erosive potential compared to hydrochloric acid at the same pH values.

The study also shows that the amount of un-dissociated and titrable acid seems to be only important under static

conditions and very low flow laminar velocities, and under the premise that a high portion of un-dissociated acid compounds are available.

At higher flow velocities, as they are expected during drinking, and a lower portion of un-dissociated acid, i.e. pH $\geq$ 2.6 for citric acid, the pH seems to be the more important driving force for enamel dissolution.

The results of the present study exhibit that demineralization of enamel samples in a flow model is very complex and does not merely follow the rules of solution equilibrium, which allows calculation of supposed enamel demineralization referring to pH, concentration of mineral salts and temperature (and others) in a static fluid model. Extrapolating the results of this theoretically orientated basic research study to the situation in the oral cavity, during drinking of a beverage, should be done with caution.

In conclusion, the experiment shows that under laminar flow conditions with very low liquid flow velocities, citric acid is more erosive as compared to hydrochloric acid. However, an increase of laminar flow velocity and pH might induce a similar behaviour of these two acidic compounds with respect to enamel dissolution.

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