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Title

Susceptibility of partially-desalivated rats to erosive tooth wear by calcium-supplemented beverages

Running Title

Erosive tooth wear in partially-desalivated rats

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Abstract

OBJECTIVES: To investigate the susceptibility of partially-desalivated rats to erosive tooth wear (ETW); the anti-erosive effect of a calcium-supplemented beverage; and the quantification of ETW by micro-computed tomography (micro-CT). METHODS: The study population consisted of thirty-eight rats, divided into partially-desalivated (n=19) and normal salivary flow (n=19). They were randomly allocated into 3 subgroups (n=6-7): A-diet soda, B-diet soda+calcium, C-water (control). Solutions were provided ad-libitum for 28 days, and the rats were euthanized afterwards. Each left hemi-mandible was scanned using micro-CT for enamel volume (3 molars) calculation. Visual analysis of photographs of the lingual surface of 1st molars was performed independently by 3 blinded examiners. Data were statistically analysed (α=0.05). RESULTS: Micro-CT revealed no significant differences between partially-desalivated or normal groups. Rats consuming A had more enamel loss than those consuming B or C, which did not differ from each other. For visual analysis, desalivation did not affect ETW. Rats consuming C showed the lowest ETW, followed by B and then A, for both partially-desalivated and normal rats. Spearman correlation between the two ETW quantification methods was -0.65. CONCLUSIONS: Partial desalivation did not increase ETW. Ca-containing beverage prevented ETW. Micro-CT quantified ETW, although it was not as sensitive as visual analysis.

Introduction

Erosive tooth wear (ETW) is a prevalent condition, affecting all age groups (Salas et al., 2015, Jaeggi & Lussi, 2014). Patients with particular risk for ETW are those with high consumption of acidic beverages, eating disorders and gastro-oesophageal reflux disease (Jarvinen et al., 1991, Holbrook et al., 2009, Schlueter et al., 2012, Schlueter & Tveit, 2014). Calcium-modified acidic drinks present lower erosive potential (Davis et al., 2007, Attin et al., 2005, Larsen & Nyvad, 1999, Scaramucci et al., 2011, Scaramucci et al., 2012), and constitute a suitable approach for the prevention of extrinsic ETW. Calcium compounds

increase the saturation level of acidic drinks in relation to the tooth structure, preventing further dissolution (Featherstone & Lussi, 2006).

In the process of ETW development, salivary flow has been described as the single most important biological modifying factor. Reduced salivary flow rates (hyposalivation) can have a negative impact on acid clearance, buffering and neutralization (Hara & Zero, 2014). It is also responsible for a reduction in minerals available to interact with the tooth surfaces, favouring demineralization over remineralization (Piangprach et al., 2009, Magalhaes et al., 2009). Hyposalivation can occur as a side effect of medications, radiotherapy of the head and neck, or in association to medical conditions such as Sjögren syndrome (Napenas et al., 2009). Hyposalivation has been associated with increased risk of ETW (Piangprach et al., 2009, Scaramucci et al., 2013, Borges et al., 2014, Jarvinen et al., 1991). Thus, it can be speculated that hyposalivation patients would benefit from the protective effects of calcium supplementation in erosive drinks.

Previous research testing the preventive properties of additives on ETW were performed mainly in-vitro or in-situ (Huysmans et al., 2011, Grippo et al., 2012, Almeida e Silva et al., 2011, West et al., 1998). These studies present inherent limitations, such as the difficulty of replicating the complex oral environment in the laboratory, or the high costs of an in-situ design. It would also be particularly challenging to investigate ETW under chronic hyposalivation in these models (Hall et al., 1999, Huysmans et al., 2011). Although the impact of hyposalivation on caries has been extensively studied using animal models in the past (Schwartz & Shaw, 1955, Muhler et al., 1959, Bowen et al., 1988, Rosen et al., 1959a), to the best of our knowledge, no attempts have been made to study ETW under such conditions. Animal models present controlled genetic, dietary and environmental conditions, providing more dependable results (West et al., 1998). In addition, the most commonly analytical methods used to quantify ETW, such as surface microhardness and profilometry, are impractical to apply in vivo. Previous efforts to evaluate ETW in animals have been made, but they rely on visual scoring, being therefore limited in their objectivity (Sorvari et al., 1996, Sorvari & Kiviranta, 1988, Mistry & Grenby, 1993). To overcome this limitation, one option would be the use of micro-computed tomography (micro-CT), which has now the sensitivity to measure small increments of change in the volume of tooth structure, something that was not achievable in the past (Sorvari et al., 1988, Neves Ade et al., 2010).

The aims of this study were: 1. To test the effect of partial desalivation on the susceptibility of rats to ETW; 2. To investigate and compare the anti-erosive effect of a Ca-containing beverage in normal and partially-desalivated rats; and 3. To compare the ability of micro-CT to detect ETW compared to a visual assessment. The respective related hypotheses were: 1. Partial desalivation would increase the susceptibility of rats to ETW; 2. The erosive effect

of an acidic beverage would be reduced with calcium supplementation in normal and partially-desalivated rats; 3. ETW could be quantified by micro-CT.

Materials and Methods

Experimental design

The experimental factors were 1. Salivary gland condition, at two levels: normal and partially-desalivated); and 2. Erosive solution, at three levels: deionized water (control), diet soda (Sprite Zero, SZ), and diet soda with 20 mmol/l calcium (6 g/l of calcium lactate pentahydrate) (SZ + Ca) (Scaramucci et al., 2012, Attin et al., 2005). ETW was analysed quantitatively by means of micro-CT and qualitatively by visual grading. A secondary outcome was the amount of fluid consumption by the rats.

Animals

The Indiana University School of Dentistry's Institutional Animal Care and Use Committee reviewed and approved the study protocol (#DS0000946R). The study was performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). A total of 38 male inbred Fischer 344 rats, at three months of age, were used. Half the animals (n= 19) underwent partial desalivation by removing the sublingual and submandibular salivary glands as described previously (Schwartz & Shaw, 1955). The animals underwent a one week recovery and acclimation period, in which they were fed with a standard rodent diet (PMI Labdiet 5001, St. Louis, MO, USA) and drank deionized water ad-libitum with 12 h light/dark cycle in standard conditions.

Preparation of drinking fluids

Through our preliminary in-vitro testing, we found that the addition of 20 mmol/l of calcium to Sprite Zero (SZ) (The Coca-Cola Company, Atlanta, GA, USA) in the form of calcium lactate pentahydrate reduced its erosive potential by 50% (Table 1). This concentration also had the lowest impact on the drink´s taste, as subjectively evaluated by five blinded testers (Table 1). Thus, Sprite Zero with 20 mmol/l of calcium added (46.5 mmol/l calcium lactate pentahydrate) was the modified drink used in this study. Both beverages were degassed before dispensing, as rats have a gastroesophageal characteristic that limits their ability to expel gas (Montedonico et al., 1999). Deionized water was used for the control groups.

Both normal and partially-desalivated animals were randomly assigned into three groups (deionized water, SZ and SZ+Ca). All fluids were provided ad-libitum using 250 ml water bottles. Animals were weighed, amounts of consumed fluids were measured and fluids were changed 3 times a week. SZ+Ca was prepared fresh with each change. Animals were euthanized after 28 days from the start of the study and their left hemi-mandibles were dissected and fixed with 10% (w/v) phosphate-buffered formaldehyde for 7 days. The right hemi-mandibles were discarded.

Micro-computed tomography

Each left hemi-mandible was coated with paraffin and scanned using a high resolution Skyscan 1172 micro-CT (Skyscan, Kontich, Belgium) with the resolution set at 8.12 µm/pixel. The system was set at a source of 60 kV/165 µA, a rotation angle of 180°, with a rotation step of 0.70°. Raw image data was reconstructed using the NRecon software (Version 1.6.3.2; SkyScan). Dynamic range was set at 4253 and 4430 for all samples. The lower left three molars of each animal were used to calculate tooth enamel volume using CTan software (Version 1.10.1.0; SkyScan). For all scans, the enamel of the molars was segmented with a fixed threshold (greyscale range 100 – 255), and the enamel volumes were automatically calculated from the resultant binary datasets (Figure 1). Enamel volume was reported in cubic millimetres.

Visual grading of ETW

ETW was scored visually using a modified version of two indices used previously (Sorvari & Kiviranta, 1988, Higo et al., 2009). To enhance ETW visibility, dissected hemi-mandibles were immersed in 1% methylene blue, rinsed and photomicrographs were taken using Nikon SMZ1500 stereomicroscope and Nikon DXM1200f digital camera under five times magnification and standardized positioning and illumination. Three calibrated and blinded examiners independently graded the photomicrographs, which were projected on a screen in random order. Examiners graded the appearance of the left mandibular first molars against a chart (Figure 2). The median score for each sample was used in the statistical analysis.

Statistical analysis

A sample size calculation was performed prior to the study. The calculation assumed the standard deviation of enamel volume to be 0.15 mm^3. The study was designed to have 80% power to detect a difference of 0.27 mm^3 between groups with a sample size of 6 per group, using two-sided tests at a 5% significance level. The effects of fluid and desalivation on enamel volume, fluid consumption, and weight gain were evaluated using two-way ANOVA, with pair-wise tests between erosive solutions performed when the overall effect was significant. Differences in visual score were evaluated using Mantel-Haenszel tests for

ordered categorical responses. Pearson correlation coefficients were calculated to assess the associations among enamel volume, fluid consumption, and weight gain. A Spearman correlation coefficient was calculated to show the association between enamel volume and visual score. Statistical significance was set at 5%. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

All animals survived the duration of the study. The partially-desalivated rats had lower starting weights, as expected from the surgery (O'Connell et al., 1994). However, weight increase in the surgical groups resumed normal rates after surgery, similar to normal rats (Table 2). In addition to lower starting weights, at the end of the study, the partially-desalivated group consumed about 20% less fluids compared to the normal ($p<0.0001$).

Micro-CT measurement revealed no significant differences between enamel volumes of normal and partially-desalivated rats (Table 2). However, rats consuming SZ had significantly lower enamel volumes (more ETW) than rats consuming water ($p=0.0002$) or SZ + Ca ($p=0.0002$). No significant differences were found in enamel volumes between rats consuming water and SZ+Ca ($p=0.88$).

For the visual scores of ETW, the weighted kappa was ≥ 0.77 and ≥ 0.82 for the intra- and inter-examiner comparisons, respectively. Groups which consumed SZ or SZ+ Ca had significantly higher scores of ETW compared to groups which had water as the drinking fluid, except for the partially-desalivated group drinking SZ+Ca, which was no different from the normal rats that consumed water (Table 3). In addition, both groups consuming SZ+Ca had lower visual scores when compared to the groups drinking SZ only. However, there was no difference between partially-desalivated or normal animals drinking SZ only, or between partially-desalivated and normal groups drinking SZ+Ca. In the assessment of the severity of the enamel ETW, the visual scores were negatively correlated with Micro-CT enamel volume measurements (correlation=-0.65).

Discussion

The first hypothesis of this study stating that partial desalivation would increase the susceptibility of rats to ETW was rejected, as the removal of the sublingual and submandibular glands did not significantly affect the amount of ETW observed for the partially-desalivated rats when compared to rats which had not undergone surgery, either measured by the micro-CT or visually. This unexpected result can possibly be explained

when factoring in the amounts of acidic fluids consumed in both groups. Due to the difference in starting weights, it was observed that the partially-desalivated group consumed about 20% less erosive fluids and yet attained similar levels of ETW compared to the normal rats (Table 2). The lower starting weights of the partially-desalivated group, a limitation of this study model, would not be normalized by simply increasing the post-surgical recovery period, as these animals are known for their continuous weight gain until their 77th week of life (Solleveld et al., 1984). The type of fluid provided had no effect on the quantity of fluid consumed. However, there was a strong positive correlation between the animal weight and amount of fluid consumed ($r= 0.78$, $p<00001$). This indirectly suggests that partial desalivation increases the susceptibility to ETW, as the amounts of ETW were similar but the fluids consumed were significantly different ($p<0.05$).

In this study, partial desalivation was performed by surgically removing the submandibular and sublingual salivary glands of the rats. The complete removal of the all major salivary glands in rats (parotid, submandibular, and sublingual) would result in approximately 90% decrease in salivary flow (Stricker, 1970), with severe hyposalivation and pronounced feeding difficulties, an undesirable effect that was not within the purpose of this study. Salivary flow was not measured in this study; however, it has been previously established that it significantly decreases after bilateral surgical removal of the submandibular and sublingual salivary glands (Rosen et al., 1959a, Ooshima et al., 1990). The parotid gland has been reported to provide only about 35% of the saliva in rats, and its removal was not able to increase the caries incidence rates, as opposed to rats with only the submandibular and sublingual glands removed or ligated, which presented substantially more caries, despite the preservation of the parotid gland (Rosen et al., 1959a, Rosen et al., 1959b, Shafer et al., 1958). In humans, it remains unclear if the protective effects of saliva is mainly through simulated or unstimulated salivary flow (Zwier et al., 2013, Mulic et al., 2012), and no attempt to differentiate between the two was done in this study. A sham group (rats undergoing sham surgery, involving surgical exposure and closure with no salivary gland removal) was not included in this study, as it has been shown in previous investigations that sham surgery has no effect on salivary flow, fluid consumption or weights of the animals when compared to non-surgical controls (Bowen et al., 1988, Schwartz & Shaw, 1955). In this study, all rats received standard rat feed in pellet form. Normally, signs of abrasive wear of molars show up in rats as young as 25 days old and continue to increase with age (Nishijima et al., 2007). Abrasion from food pellets in this study may have contributed to the loss of dental tissues in addition to ETW. Milled rat feed may have reduced the effect of food on ETW, but it would not likely affect the overall outcome of this study.

Rats consuming the calcium-supplemented beverage (SZ+Ca) showed less ETW than the rats that ingested the regular beverage (SZ), therefore, accepting the second hypothesis for both normal and desalivated rats. This result is consistent with previous investigations (Scaramucci et al., 2011, Scaramucci et al., 2012, Beiraghi et al., 1989), and it is related the common ion effect, where the driving force of tooth dissolution is reduced by increasing the saturation state of the drink with respect calcium ions (Grenby, 1996); in addition to the small increase in pH (of about 0.7) after calcium supplementation. Although one may suggest that the lower erosive potential of SZ+Ca may be due to its higher pH, a previous study has showed that a Ca-modified drink sustains lower erosive potential than the original drink even when its pH was adjusted to the original values (Scaramucci et al., 2011).

Although it was demonstrated that high calcium concentrations can almost completely inhibit the erosive effect of acidic drinks (West et al., 1999, Scaramucci et al., 2012), taste alteration and increased susceptibility to microbiological spoilage could be associated drawbacks (Grenby, 1996). To investigate changes in taste, a preliminary screening test was performed to determine the optimal concentration of the calcium additive. In this test, we observed that all the concentrations evaluated could reduce hydroxyapatite dissolution in the range of 44% - 94%. A marked dose-response pattern was noted until the concentration of 30 mmol/l, beyond which higher concentrations showed no additional benefit. The concentration of 20 mmol/l was chosen because it was able to reduce hydroxyapatite dissolution in 77%, with less taste alteration, as observed in the taste test. In this test, 40% of the volunteers noted a difference in the taste of the drink modified with 20 mmol/l of calcium when compared to the original drink. For the concentration of 30 mmol/l, this percentage increased to 80%. No differences in the amounts of fluid consumption were found between the animal study groups, indicating that taste was less of a factor. Calcium lactate pentahydrate was used because it is a non-toxic and food-approved compound (van der Hoeven, 1985). The choice of Sprite Zero as the erosive drink was due to the absence of both caffeine and fermentable carbohydrates. The baseline pH of Sprite Zero is comparable to many carbonated and juice drinks available in the market.

This was the first study to demonstrate the ability of micro-CT to quantify enamel volume loss caused by ETW. The parameters of the micro-CT scanning, reconstruction and measurements were standardized and executed in an automated fashion, thus eliminating the risk of examiner bias. A limitation of this approach was that it did not allow for longitudinal comparisons with the baseline sound tooth condition. Instead, the enamel volume in experimental groups was compared to independent control groups. While there may be slight variations in tooth size and morphology, they were minimized by the use of an inbred strain of rats with genetic uniformity (Sprott & Ramirez, 1997). It is worthwhile

mentioning that the existence of live animal, in-vivo micro-computed tomography facilities may allow for future longitudinal studies of ETW in animal models. This opens new possibilities for the quantitative investigation of anti-erosive agents, gastro-oesophageal reflux disease medications, etc. which may not be easily studied in in vitro or in situ conditions.

Nevertheless, there was only a moderate negative correlation between the results of micro-CT and of the visual scoring of ETW (r_S=-0.65). Both methods of measurement detected enamel ETW and demonstrated the protective effect of calcium supplementation. However, only visual scoring was able to detect a difference between groups consuming the calcium-modified drink and the groups consuming water. Visual scoring also showed a tendency towards an increase in protection of calcium supplementation in the partially-desalivated animals. In the visual method, a photomicrograph of the mandibular left first molar showing the lingual surface was used for scoring. It has been shown that the lingual surfaces of the mandibular molars in rats demonstrate the highest levels of ETW (Sorvari & Kiviranta, 1988). However, this method may overestimate the overall amount of enamel ETW due to the reliance on the most susceptible surface of the tooth. On the other hand, the micro-CT measured the remaining enamel volume on all surfaces of all three molars in the left hemi-mandibles, including the buccal surfaces, which were relatively unaffected by ETW. Although the inclusion of all molar surfaces in the quantification gave a more accurate representation of the overall surface loss, it weakened its ability to detect significant treatment differences. Isolating only the lingual surfaces of the molars in the micro-CT measurement could overcome the problem; however, this required operator input in determining the border limits of the lingual surface on a three-dimensional scan of the molars, which was highly subjective. From the results obtained from the microcomputed unit used in this particular study, we can infer that the micro-CT method was less sensitive than the visual method, because it considered the enamel volume of all surfaces of the three teeth, while the visual analysis was performed only on the lingual surface of the first molar. Perhaps the sensitivity of the method may increase if more generalized or advanced ETW is simulated. Also, the ability of micro-CT to detect ETW changes is limited by the micro-CT unit resolution and computing power. The progressive advancements of this technology would enable better sensitivity than the demonstrated by our instrumentation with resolution scans higher than the 8.12 μm/pixel used in this investigation.

Considering the limitations of this study, it can be concluded that, similar ETW was found for normal and partially-desalivated rats, despite the lower amount of beverage consumed in the desalivated group. This may indirectly indicate higher erosive risk caused by the reduced salivary flow as previously discussed, although further confirmation is required. The addition

of 20 mmol/l calcium lactate significantly reduced the erosive potential of the acidic drink, and this was shown in both evaluation methods, visual analysis and micro-CT. As tested, micro-CT was able to detect and quantify ETW, although it was not as sensitive as the visual method.

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Conflict of interests

The authors declare that they have no conflict of interests.

Author contributions

M. Aldosari participated in the study design, implementation, data acquisition, analysis of data, and in writing the manuscript. T. Scaramucci participated in the study design, implementation, and writing the manuscript. S.S-Y. Liu participated in the study design and data acquisition. J.M. Warrick-Polackoff contributed to the study design and implementation. G.J. Eckert participated in the statistical data analysis and interpretation. A.T. Hara participated in the study design, implementation, analysis of data and writing of the manuscript. The final manuscript was reviewed and approved by all the authors.

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Figure Legends

Figure 1. The process of enamel segmentation of a rat mandibular molar using CTan software (Version 1.10.1.0; SkyScan).

Figure 2. Visual scoring chart (adapted from Sorvari and Kiviranta, 1988).

Figure 3. Representative photomicrographs of left mandibular first molars stained with 1% methylene blue dye showing ETW grades 0 through 3.

Tables

Table 1. Erosive potential indicators and taste test of the beverage with five concentrations of added calcium lactate.

Groups	[Ca]**, mmol/l	pH	Titration	pH-stat	Taste test***
SZ*	0	3.35 (0.03)	10.4 (0.30)	2.30 (0.11)	1/5
SZ+10	10	3.88 (0.02)	8.30 (0.10)	1.30 (0.03)	2/5
SZ+20	20	4.04 (0.01)	8.60 (0.20)	0.53 (0.09)	2/5
SZ+30	30	4.16 (0.01)	8.20 (0.10)	0.15 (0.03)	4/5
SZ+40	40	4.22 (0.00)	8.20 (0.10)	0.23 (0.03)	4/5
SZ+50	50	4.28 (0.01)	7.80 (0.10)	0.25 (0.07)	3/5

** Sprite Zero,™ The Coca-Cola Company, Lot # 2012-0503. Composition: carbonated water, citric acid, potassium citrate, natural flavors, potassium bezoate, aspartame, acesulfame potassium.*

*** DC Calcium Lactate powder (Dee Cee Lab., White House, TN, USA)*

**** Five subjects were instructed to compare the taste of the original drink (SZ) with the modified drinks (given in randomized order) by holding 10 ml of each drink in the mouth for 15 s and expectorating. In between testing the two products, the subjects had to swish 10 ml of drinking water for 15 s and expectorate in order to clean their palate. After the test, subjects had to answer yes or no whether they noticed any difference in the taste of the two test beverages.*

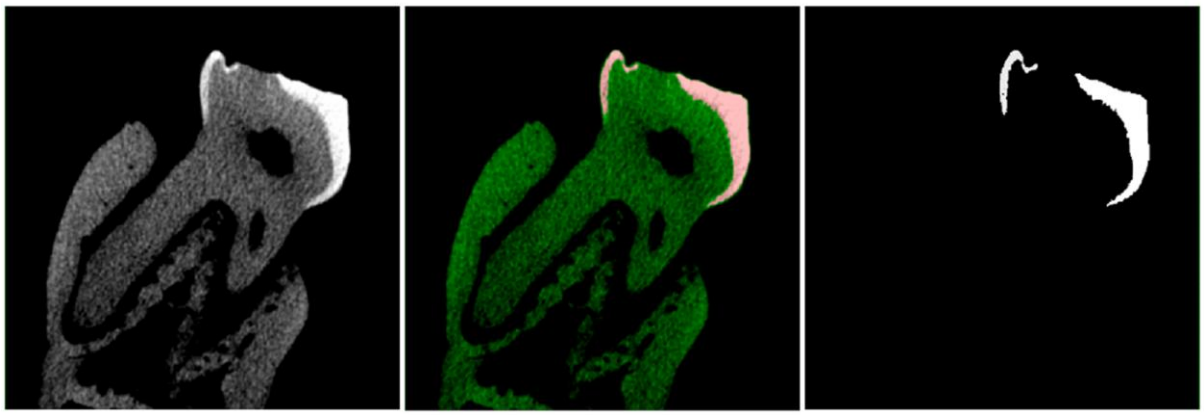
Table 2. Animal weight gains, percentage of weight gains and enamel volume as measured using micro-computed tomography (*standard error in parenthesis).*

Group	N	Fluid	Weight gain (g)	Weight gain (%)	Fluid consumption (ml/week)	Enamel vol (mm^3)
Partially-desalivated	6	water	60.5 (5.5)[a]	25.0 (2.3) [a]	64.6 (2.2)[a]	2.04 (0.07)[a]
	6	SZ	55.7 (3.7)[a]	24.5 (1.4) [a]	66.2 (0.9)[a]	1.65 (0.07)[b]
	7	SZ+ Ca	58.3 (2.3)[a]	25.1 (1.3) [a]	70.7 (3.3)[a]	1.96 (0.05)[a]
Normal	6	water	71.8 (3.2)[b]	28.3 (1.3) [a]	81.1 (1.5)[b]	1.89 (0.05)[a]
	6	SZ	71.8 (5.0)[b]	28.3 (2.1) [a]	84.9 (3.9)[b]	1.76 (0.06)[b]
	7	SZ+ Ca	64.1 (4.6)[b]	25.2 (1.9) [a]	84.5 (2.8)[b]	1.95 (0.04)[a]

[a, b] *Different letters denote significance ($p<0.05$).*

Table 3. Results of visual scoring of mandibular left first molars.

Group	Partially-desalivated	Fluid	Grade 0	Grade 1	Grade 2	Grade 3
1	Yes	water	6 (100%)	0 (0%)	0 (0%)	0 (0%)
2	Yes	SZ	0 (0%)	0 (0%)	2 (33%)	4 (67%)
3	Yes	SZ +Ca	3 (43%)	4 (57%)	0 (0%)	0 (0%)
4	No	water	5 (83%)	1 (17%)	0 (0%)	0 (0%)
5	No	SZ	0 (0%)	0 (0%)	4 (67%)	2 (33%)
6	No	SZ + Ca	1 (14%)	6 (86%)	0 (0%)	0 (0%)



Grade 0 — No erosion (no enamel erosion with smooth cusps)

Grade 1 — Initial signs of enamel erosion (reduced cusp height and/ or irregular scalloped cusps and or/ erosion effecting less than 1/3 of enamel surface area)

Grade 2 — Moderate erosion (erosion effecting more than 1/3 of enamel surface area)

Grade 3 — Advanced erosion (erosion effecting more than 1/3 of enamel surface area with dentin exposure)