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ORIGINAL ARTICLE

Effects of rinsing with arginine bicarbonate and urea solutions on initial enamel lesions *in situ*

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OBJECTIVE: The aim of this study was to investigate the effects of rinsing with arginine or urea solution on initial enamel lesions *in situ*.
METHODS: Fourteen subjects who wore mandibular removable partial dentures embedded with bovine enamel blocks with artificial enamel lesions were included. The experiment included four 4-week rinsing periods with a 10-day washout period between each rinsing period. In each rinsing period, the subjects rinsed after meal or snack using water, or 2% arginine bicarbonate, or 1% urea, or 0.05% NaF solution, five times daily. The mineralization changes of the enamel lesions were assessed using quantitative light-induced fluorescence.
RESULTS: All groups except the water group showed a statistically significant decrease in the fluorescence loss after treatment, compared with their respective baseline. Although both the arginine group and urea group showed more decrease in fluorescence loss than that of the water group, the decrease was not statistically significantly different from that of the water group. The decrease in fluorescence loss of the NaF group was statistically significant than that of the water group, arginine group, and urea group.
CONCLUSION: Rinsing with arginine or urea solution offers limited remineralizing benefit to enamel lesions over a period of 4-week time.
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Keywords: arginine bicarbonate; enamel lesion; quantitative light-induced fluorescence; remineralization; urea

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Introduction

Caries is a multifactorial disease. The integrity of the hard tissues of teeth is closely associated with the microbial balance and the acid–base balance in dental plaque (Marsh, 1994), which undergoes acidification and alkalization cycles with the periodic oral dietary intake of an individual. When dietary carbohydrates are metabolized, the pH of dental plaque decreases to a low level and tooth demineralization is initiated when the pH is below 5.5. This is usually followed by an alkalization phase that is attributed to an increased pH caused by ammonia generation through bacterial metabolism of substances such as urea and arginine, and by the effects of saliva. This, therefore, triggers remineralization (Stephan, 1940; Burne and Marquis, 2000; Kleinberg, 2002; Bowen, 2013). Caries develops when this acid–base balance is disturbed. Much attention has been focused on the role of frequent and prolonged acidification of dental biofilm through the metabolism of excessive and frequent intake of fermentable carbohydrates in caries formation. However, recent studies suggest that a deficiency or loss in base formation is also an important risk factor for caries development. An association between a decreased potential of alkali formation from arginine or urea and increased caries activity was found in both adults and children (Shu *et al*, 2007; Nascimento *et al*, 2009; Gordan *et al*, 2010; Morou-Bermudez *et al*, 2011). Moreover, in the earlier studies, patients with chronic renal failure show elevated salivary urea and correspondingly less caries (Peterson *et al*, 1985); caries-free adults show higher levels of saliva arginine than that of the adults with a history of dental decay (Van Wuyckhuyse *et al*, 1995). These findings collectively suggest that alkali-producing substrates such as urea and arginine may contribute to caries prevention.

Urea or arginine can be metabolized by plaque bacteria to generate ammonia, which can considerably increase the plaque pH (Stephan, 1943; Kleinberg, 1967) and effectively reverse the carbohydrate-induced pH decrease (Imfeld *et al*, 1995). This effect on pH homeostasis may influence both the ecology of the biofilm and the chemical balance between the tooth mineral and dental plaque, which is believed to play an important role in the

inhibition of caries. However, the results of previous studies on the anti-caries potential of urea-containing gums were inconclusive (Fure *et al*, 1998; Itthagarun *et al*, 2005). Although a number of industry-supported clinical studies revealed that arginine-containing compound provides significantly greater benefits in arresting and reversing caries lesions compared with conventional fluoride toothpaste alone (Cummins, 2013; Li *et al*, 2016), more details remain to be understood. We also previously observed by indwelling electrode pH telemetry that 2% arginine bicarbonate or 1% urea rinses can effectively reverse the pH decreases induced by sugar consumption (Wang *et al*, 2012, 2015). However, whether this acid-neutralizing effect of arginine or urea could contribute to the clinical anti-caries benefits remains to be determined. Few studies regarding the effects of alkali generation from substrates such as arginine and urea on demineralization and remineralization of lesions *in situ* are available. Therefore, we postulated that the pH recovery effect of arginine and urea solutions could enhance remineralization of the initial enamel lesions *in situ*.

Quantitative light-induced fluorescence (QLF) is a sensitive and non-invasive method for the longitudinal assessment of initial caries. It has been employed in caries research and has a broad range of applications, including the assessment of *in vitro* and *in vivo* demineralization and remineralization (De Josselin De Jong *et al*, 1995; van der Veen and De Josselin De Jong, 2000; Feng *et al*, 2008). Comparative studies have shown a good correlation between the results of QLF and those of other techniques, including transverse microradiography, which is considered the gold standard (Hafstrom-Bjorkman *et al*, 1992; Ando *et al*, 2001; Gomez *et al*, 2014).

The purpose of the present study was to evaluate with QLF whether rinsing with arginine bicarbonate or urea solution after meal or snack could enhance remineralization of the initial enamel lesions *in situ*.

Subjects and methods

Subjects

Fourteen healthy patients (six men and eight women; mean age, 63 years; range, 42–77 years) who wore mandibular removable partial dentures were recruited for this study. The number of subjects was calculated on the basis of a pilot study of three subjects. The calculation was performed using the G*Power version 3.1.9.2 sample size package (http://www.gpower.hhu.de/) and was based on a one-way analysis of variance with four groups and an effect size of 0.63. The effect size of 0.63 was calculated based on the means ($\Delta F_{post} - \Delta F_{base}$) for rinsing with water (1.3), arginine (4.1), urea (6.3), and NaF (7.9), and a common standard deviation of 3.9 within groups, which was derived from the performing one-way analysis of variance for the data. With a 5% significance level and a power of 90% (usually ≥ 80%, Batterham and Atkinson, 2005), it was calculated that at least 10 subjects were required. Therefore, we totally recruited 14 subjects for possible dropouts and none of them dropped out during this study. Intraoral examination was performed to confirm the absence of untreated caries, periodontal disease, or other oral pathologies. All participants had a minimum of eight natural teeth and unstimulated and stimulated salivary flow rates of ≥0.2 and ≥0.8 ml min^{-1}, respectively (Zero, 1995), and none had consumed antibiotics and/or medications that could affect their salivary flow for at least 2 months before the study. Thorough prophylaxis was performed before experiment initiation. The study has been independently reviewed and approved by the Ethics Committee of Peking University Health Science Center (PKUSSIRB-201413038) and followed the ethical considerations of the Helsinki Declaration. Informed written consent was obtained from each subject before experiment initiation.

Experimental protocol

In this double-blind, randomized, cross-over study, each subject served as his or her own control and was randomly assigned to use one of the four different rinsing solutions while wearing a mandibular removable partial denture placed with artificially demineralized bovine enamel blocks. The whole experiment comprised four phases, each phase lasted 4 weeks and was separated by a 10-day washout period to minimize the risk of carry-over effects. During each phase, the subjects rinsed only one of the four solutions. Both the subjects and investigators were blinded to the order of solution use.

Two to three days following dental prophylaxis, the subjects were instructed to wear the dentures for the pre-experimental 10-day washout period without using any tested solution to check for irritation or discomfort. On the last day of this washout period, four enamel blocks with artificial initial enamel lesions were placed in the buccal flange areas of the partial dentures, with the side position (i.e., left or right) randomized among the subjects. The specimens were recessed 1 mm below the surrounding surface of the flange to facilitate plaque growth (Cochrane *et al*, 2012).

Four solutions were prepared in distilled deionized water: placebo (water), 2% arginine bicarbonate (Arg; TelSun Chemical and Scientific Limited, Jiangsu, China), 1% urea, and 0.05% sodium fluoride (NaF). The concentrations of urea and arginine solutions were determined mainly based on our previous studies, which demonstrate by indwelling electrode pH telemetry that 2% arginine bicarbonate or 1% urea rinses can sufficiently reverse the sucrose-induced pH decrease in plaque (Wang *et al*, 2012, 2015). The four solutions were identical in appearance and distributed in 200-ml plastic bottles with a cap calibrated up to 15 ml. The subjects were instructed to rinse with 15 ml of the assigned solution for 1 min, five times daily, after the following meals: breakfast, lunch, dinner, and two times of snacking between meals.

The subjects wore their partial dentures throughout the whole day, except toothbrushing, during the four experimental periods. They were instructed to brush their teeth twice daily using a non-fluoridated dentifrice in a silica base without calcium and phosphates (Saky® toothpaste; Weimeizi Personal Care Articles Co., Guangzhou, China) 10 days before and during the experiment. No other oral hygiene procedures were performed using fluoride products; drinking green tea or chewing gum was also prohibited during the experimental periods. To help the development of plaque, touching or brushing the enamel block was prohibited during the experimental periods.

We took the following measures to maintain the compliance of the participants throughout the entire trial: at the beginning of each test period, we gave them a checklist and instructed them to keep a diary of times of meals, snacks, and rinsing. The participants were also instructed how to apply the solutions and how to clean their prosthesis (these instructions were also printed on the first page of each checklist). In addition, we called the participants or sent them text messages twice a week during the experiment for reminding. We also reviewed the dairy and checked the returned/used solution bottles to ensure the compliance when the participants were given new supplies of the solutions each time. The participants were also questioned on each visit time whether they had followed the instructions.

Changes in the mineral content of the enamel lesions were measured using QLF before and after the experimental periods. At the end of each experimental period, the plaque on each enamel block was first monitored using QLF as described in previous studies (Lee *et al*, 2013; Kim *et al*, 2014) to evaluate whether there was sufficient plaque developed on the specimen surface, and then, the mineral content of the enamel lesions was measured after the plaque residue was carefully removed from the specimens using a soft brush under running tap water.

Enamel specimen preparation

Each bovine incisor was cut into four parts (5 mm × 5 mm) using a water-cooled diamond saw. The enamel surfaces of the specimens were ground flat and polished using water-cooled sandpaper up to 4000 grit to remove approximately 300 μm of the outer enamel. The dentin side of the specimens was ground flat to a uniform thickness using 500-grit silicon carbide grinding paper. The resulting specimens had a thickness

approximately 2 mm and were assessed under the Nikon SMZ 1500 stereomicroscope at a magnification of ×20. Specimens with cracks or areas of hypomineralization (white spots) on the enamel surface were excluded. All enamel specimens were sterilized using ethylene oxide gas for 12 h. All specimen surfaces were coated with clear nail varnish (Revlon, NY, USA), leaving out a central experimental window measuring approximately 2 mm × 2 mm. The prepared specimens were stored in a relative humidity of 100% and a temperature of 4°C until further use.

Artificial enamel lesions

Enamel lesions were created at 37°C using a modified acid gel described in a previous study (Amaechi *et al*, 1998), which contained 3.0% hydroxyethyl cellulose (4500–6500 cps, Japan) and 0.1 mol l^{-1} lactic acid adjusted to pH 4.6. These artificially made enamel lesions were previously evaluated with microradiography to possess the characteristics of the initial enamel lesions (Wang *et al*, 2007). To examine what degree of demineralization of the enamel would be sensitively response to remineralization solution, the time for demineralizing the enamel blocks in the acid gel was optimized by a pilot experiment as follows: three groups (six specimens per group) of enamel specimens were demineralized in above acid gel for 12, 24, and 48 h, respectively. After rinsed thoroughly with distilled water, the enamel specimens were evaluated by QLF for mineral change. Demineralization of the specimens occurred significantly and proportionally with the treatment time. The specimens were immersed in a modified remineralization solution (1.5 mmol l^{-1} Ca, 0.9 mmol l^{-1} PO_4 and 10 ppm F^-, pH 6.5; Pearce and Nelson, 1988) for 7 days, and rinsed thoroughly with distilled water and evaluated with QLF again. Significant remineralization occurred for all of the demineralized specimens, and the specimens demineralized for 24 h was most responsive to the remineralization solution as measured by QLF (see Table S1). Therefore, we prepared the specimens with enamel lesions by demineralizing for 24 h for our next experiments. A total of 250 demineralized specimens were prepared and stored at 4°C in humid conditions until use.

QLF measurements

The plaque and enamel lesions were evaluated using QLF system (QLF-D Biluminator™ system; Inspektor Research systems BV, Amsterdam, The Netherlands). The fluorescence images of plaque as well as enamel lesions were captured with a digital SLR camera (EOS 550D; Canon, Tokyo, Japan) using the following settings: shutter speed of 1/30 s, aperture value of 5.6, and ISO speed of 1600. The built-in software (C3 v1.16; Inspektor Research Systems BV) was used to capture and store all of the digital images into a personal computer (PC). The red fluorescence images of plaque were first captured, and then, the fluorescence images of the lesions were captured after the plaque was removed. For the fluorescence images capture of the lesions, all samples with removal of the nail varnish were dried with compressed air for 15 s immediately before images taken. The reposition tool of the software was used to ensure that the images were automatically captured when the correlation was higher than 0.90, to ensure consistent capture areas of lesions before and after treatment. The red fluorescence images of the plaque (see Figure S1) were analyzed for plaque score values by the built-in software (QA2 v.1.23; Inspektor Research). A value from 0 (no mature plaque) to 5 (high amount of mature plaque) was recorded after analysis by the built-in software (http://www.inspektor.nl/download/WhitepaperQLF-11.pdf). The built-in software (QA2 v.1.23) was also used to analyze the fluorescence loss (ΔF) of lesion in the same area of each enamel block. All analyses were performed by a single trained examiner.

Because the lesion window was identical in size for all specimens, only ΔF values were recorded with a threshold level of 5%, that is, minimum 5% fluorescence loss between sound and demineralized enamel. ΔF values were measured after artificial lesion creation as ΔF_{base} and after each treatment period as ΔF_{post}. The change in the fluorescence loss ($\Delta\Delta F$) was calculated using the following equation: $\Delta\Delta F = \Delta F_{post} - \Delta F_{base}$. Therefore, the positive $\Delta\Delta F$ values indicate remineralization, whereas the negative $\Delta\Delta F$ values indicate further demineralization. Subsequently, the overall change in mineralization for each test leg was calculated as described above. All data were presented as means ± s.d.

Statistical analysis

Statistical analysis was performed using SPSS Version 15.0 for Windows (SPSS, Chicago, IL, USA). Normality of the data was confirmed using normal probability plots and the Kolmogorov–Smirnov test. Homogeneity of variance was confirmed using Levene's test. The pre- and post-treatment data for each group were compared using the paired *t*-test. Comparisons among groups were performed using a one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

All participants completed the four treatment periods with good compliance. None complained that the solution had an unpleasant taste.

The fluorescence of the plaque

The plaque was visualized obviously on most of the enamel blocks. The plaque score of all specimens measured by QLF system was 3.75 ± 1.79, 4.12 ± 1.46, 3.23 ± 1.97, and 3.17 ± 2.06 for the water group, Arg group, urea group, and NaF group, respectively. No significant difference was observed among the four groups ($P > 0.05$).

The fluorescence of the enamel lesions

A total of 224 specimens used for the experiments, and among them, only 214 specimens were analyzed for mineral change by QLF system, due to 10 specimens missed or broken during the *in vivo* experimental time. The

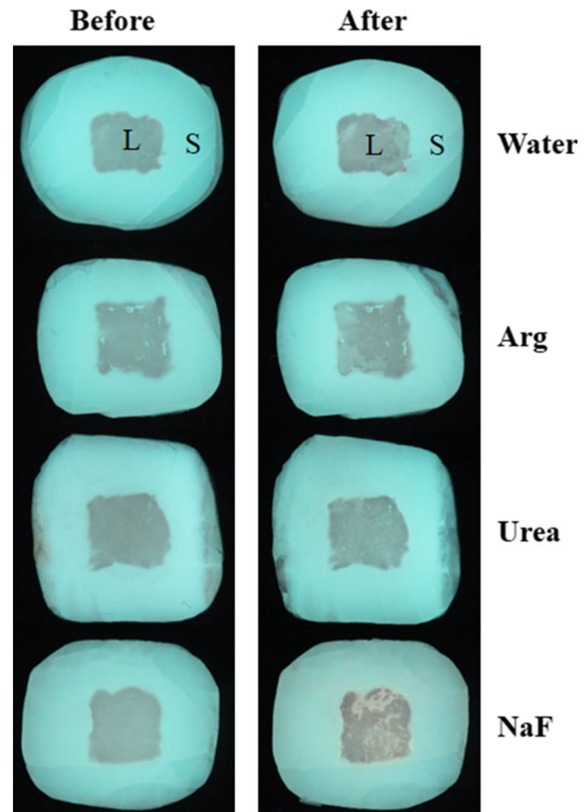

Figure 1 Representative QLF images of enamel lesions at baseline (before) and after treatment. L, lesion area; S, sound area [Colour figure can be viewed at wileyonlinelibrary.com]

representative QLF images of enamel lesions at baseline (before) and after different rinses are shown in Figure 1.

The ΔF before and after treatment, and the $\Delta\Delta F$ are shown in Table 1. There were no statistically significant differences in ΔF values at baseline among the four groups ($P > 0.05$). After the 4-week rinsing period, the ΔF values decreased significantly compared with their corresponding ΔF values at baseline in the Arg, urea, and NaF groups ($P < 0.01$), but not in the water group ($P > 0.05$). Although the $\Delta\Delta F$ (as an indicator of degree of mineral change) of both Arg group and urea group were higher than that of the water group, the difference was not statistically significant ($P > 0.05$). There was also no statistical difference between $\Delta\Delta F$ of Arg group and that of urea group ($P > 0.05$). However, the $\Delta\Delta F$ of NaF group was statistically significantly higher than that of Arg group or urea group as well as the water group ($P < 0.05$).

Examination of the statistical power after study

To examine whether our study had enough power to adequately test the hypothesis, we calculated the actual power after the study using G*Power version 3.1.9.2 based on a one-way analysis of variance, for the post hoc power analysis, with a 5% significance level, the total sample size 56 (for four groups, 14 each group), and effect size of 0.46. The effect size of 0.46 was calculated by means ($\Delta F_{\text{post}} - \Delta F_{\text{base}}$) for rinsing with water (0.61), Arg (2.52), urea (2.67), and NaF (5.86) (Table 1), and a common standard deviation of 4.1 within groups, which was derived from the performing one-way analysis of variance for the data. The actual power was calculated as 0.80, which is the minimum acceptable (Batterham and Atkinson, 2005).

Discussion

In the present study, we expectedly observed that the remineralization of initial enamel lesions was significantly increased after rinsing with 2% arginine bicarbonate or 1% urea solution, but not with water, after meal and snack during a 4-week period. However, unexpectedly, the degree of this remineralization (reflected by $\Delta\Delta F$) after rinsing with arginine or urea was not statistically significantly higher than that after rinsing with water. As a positive control, NaF rinse resulted in statistically significantly higher remineralization of the enamel lesions than the water group, and also higher than the arginine and urea groups.

The enhancement of remineralization of initial enamel lesions by rinsing with arginine or urea during a relative short period was limited. Our previous studies showed that after sucrose challenge, rinsing with 2% arginine bicarbonate solution or 1% urea can effectively and quickly reverse the plaque pH from 4.3 to above 5.7, otherwise the plaque pH remains below 5.7 for at least 80 min (Wang *et al*, 2012, 2015). It occurred to us that rinsing with arginine or urea after meal or snack might significantly decrease demineralization period or relatively increase remineralization for the teeth. Therefore, we designed the present study and asked the subjects to rinse with 2% arginine or 1% urea after meal or snack in order to counteract the acids resulting from carbohydrate fermentation in the plaque. The results were only some expected; that is, arginine or urea rinse could increase remineralization of the initial enamel lesion, as the fluorescence loss (ΔF) in the lesions was significantly less than that before rinse ($P < 0.01$); water rinse failed to result in less ΔF than that before rinse ($P > 0.05$). These results, on the one hand, seem to imply that rinsing with 2% arginine or 1% urea would be superior to rinsing with water after meal and snack with respect to enhancement of *in vivo* remineralization of the initial enamel lesions. Unexpectedly, $\Delta\Delta F$, an important parameter reflecting the degree of remineralization, in the arginine and urea groups, was not statistically different from that of the water group, although the $\Delta\Delta F$ for the arginine group (2.52 ± 3.54) and the urea group (2.67 ± 3.39) were both higher than that for the water group (0.61 ± 4.30). These results, on the other hand, seem to imply that rinsing with 2% arginine or 1% urea failed to be superior to rinsing with water after meal and snack with respect to the degree of *in vivo* remineralization of the initial enamel lesions. However, the $\Delta\Delta F$ of the NaF group did show statistically higher than that of the water group as well as that of the arginine or urea group ($P < 0.05$), implying that after meal and snack rinsing with 0.05% sodium fluoride could better induce *in vivo* remineralization of the initial enamel lesions than rinsing with water, and also better than rinsing with 2% arginine or 1% urea. The enhancement by NaF of remineralization of initial enamel lesions was well consistent to the results in the previous studies (Chow *et al*, 2000; Songsiripradubboon *et al*, 2014). Therefore, after meal and snack rinsing with 2% arginine or 1% urea could still be a little more favored than rinsing with water, but was inferior to rinsing with 0.05% NaF regarding to *in vivo* remineralization of initial enamel lesions.

The failure for the arginine or urea group in being much superior to the water group might be mainly due to the mechanisms of actions of arginine and urea. First, the acid-counteract effect of arginine and urea could mainly effectively hinder the further demineralization rather than remineralize the enamel lesions. The acid-neutralizing effect of arginine and urea might provide the basic environment that can favor remineralization, but without combining with other cariostatic elements such as fluoride, calcium, and phosphate, it might not be sufficiently effective on promoting the net remineralization. For example, dentifrices containing 1.5% arginine, an insoluble calcium compound, and fluoride provide superior remineralizing effect than that still containing the insoluble calcium compound and fluoride but without arginine in an *in situ* study

Table 1 The fluorescence change (mean ± s.d.) before and after rinsing with different solutions

Groups	ΔF_{base}	ΔF_{post}	$\Delta\Delta F$
Water	−22.60 ± 1.51	−21.99 ± 4.58	0.61 ± 4.30
Arg	−20.68 ± 2.39	−18.16 ± 3.20*	2.52 ± 3.54
Urea	−19.70 ± 2.32	−17.03 ± 3.67*	2.67 ± 3.39
NaF	−20.17 ± 2.24	−14.31 ± 4.15*	5.86 ± 4.56**

*$P < 0.01$ *vs* baseline.
**$P < 0.05$ *vs* Water group or Arg group or Urea group.

(Cantore *et al*, 2013). Urea gum containing calcium and phosphate significantly reduces the lesions depth and increases the mineral content than urea gums without calcium and phosphate (Itthagarun *et al*, 2005). Therefore, alkali substrates combining with fluoride, calcium, and phosphate may be suggested for better caries prevention. Second, the 4-week period might not be long enough for reversing pH in the plaque by arginine or urea to induce significant remineralization of the initial enamel lesions. Therefore, longer period of using arginine or urea might be more effective to enhance remineralization; in addition, longer period might bring about an ecological advantage, that is, microbiology compositional and metabolic changes of oral biofilms to significantly affect demineralization and remineralization balance (Liu *et al*, 2012). Significant caries preventive effects of arginine using for much longer period (1–2 years) have been observed in clinical studies (Acevedo *et al*, 2005, 2008). Third, our sample size might not be large enough. The reason why the degree of remineralization ($\Delta\Delta F$) in arginine and urea groups was not statistically significantly higher than that in water group might also be, to some extent, due to large individual variations. The larger the individual variations, the more subjects are demanded. In fact, we calculated the actual power after study and it was just 0.80, which is the minimum acceptable (Batterham and Atkinson, 2005). Although our sample size of 14 subjects per group was more than the minimum required size of 10 subjects per group, which is calculated from the pilot study by G Power, it appeared still not large enough. If the samples size would have been larger than the current one, it would be possible that $\Delta\Delta F$ in arginine and urea groups would be statistically significantly higher than that in water group. Expectedly, the sample size of 14 subjects per group appeared to be adequate for examination of the effect of fluoride on remineralization of the initial enamel lesions in the present study, as its $\Delta\Delta F$ was statistically significantly higher than that of water group. Similar studies with larger population of subjects may be needed to further evaluate the effect of arginine or urea on remineralization of enamel lesions.

The effects of arginine and urea on remineralization appeared to be inferior to that of the conventional 0.05% sodium fluoride in the 4-week period. This phenomenon could be less likely due to the reason that the concentration of arginine and urea used in the present study was insufficient, for the concentrations were based on our previous study, in which 2% arginine bicarbonate and 1% urea rinsing can effectively reverse sucrose-induced decrease in plaque pH in less than 10 min. This phenomenon was in agreement with previous studies, in which fluoride is a more important driving factor than pH for remineralization, and even a small increase in fluoride levels can compensate for relatively large pH differences under conditions resembling plaque fluid (Lynch *et al*, 2006; Lippert *et al*, 2011). Furthermore, in the present study, the fluoride group significantly remineralized enamel lesions, also demonstrating that our *in situ* model was responded well to 0.05% fluoride.

It should be mentioned that even the water group did not show net demineralization after treatments. This phenomenon might attribute to the increased clearance of sugars from the mouth and acids in the plaque by rinsing water after meal or snack, therefore contributing to prevent the enamel lesions further from demineralization. However, rinsing water did not show any enhancement on remineralization of the initial enamel lesions.

The subjects in the present study were asked to rinse after meal and snack, and all the subjects completed the four treatment periods with good compliance. In addition, in the present study, a 1.0-mm-deep recess was made to allow plaque formation on the enamel surface and a thickness-controlled biofilm was developed as QLF analysis confirmed that no significant differences of plaque scores were found among the groups. Therefore, the large variation of our data might be less likely related to the formation differences of biofilm, but be more likely related to the individual variations in saliva composition, chewing side, and enzymes in the bacteria with different ability to metabolize arginine and urea individually (Shu *et al*, 2007; Nascimento *et al*, 2013).

We designed an intensive applying frequency of five times daily to hopefully obtain the maximum effects of arginine or urea rinse on remineralization of the enamel lesions within 4 weeks for this *in situ* study. However, using a rinse five times daily would be difficult for long-term compliance. The reason why this study used a rinse five times daily was due to the purpose of this study to test the hypothesis as described in Introduction. We hoped that rinsing arginine or urea five times daily after meal and snack could serve the purpose that immediately neutralize the carbohydrate-induced decrease in plaque pH as far as possible. In addition, to obtain the maximum effect, using a rinse five times daily was also applied in previous *in situ* studies (Itthagarun *et al*, 2005; Hassan *et al*, 2015). Based on the results of the present study that the effect of arginine or urea rinse on remineralization of initial enamel lesion was limited, the previous clinical caries prevention effects by arginine might suggest the necessity of long-term use thus to have an ecological advantage, that is, the shift of microbiology compositional and metabolism changes of the biofilm in the long term (Liu *et al*, 2012). Therefore, we would suggest using arginine or urea rinse one or two times daily, instead of five times daily, for better compliance.

The washout period is usually from 1 to 2 weeks for fluoride related *in situ* studies (Zero, 1995; Sung *et al*, 2014). However, it is concerned that the use, especially long-term use of urea or arginine may induce microbial changes on plaque microbiota. It could be a potential weak point of our study that we did not test whether the 10-day washout period in our study was enough to eliminate any carryover effect of arginine or urea among our experimental phases. A very recent study showed that there is no real shift of the plaque microbiota after using toothpaste containing 8% arginine for 8 weeks (Koopman *et al*, 2017). It could be, to some extent, speculated that our 4-week use of arginine or urea might also not induce significant shift of the plaque microbiota, and the chance for carryover effect therefore would be less.

In conclusion, our results suggested that rinsing with arginine and urea after meal and snack provided limited

benefits for caries prevention during a relative short time period.

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Conflict of interests

None to declare.

Author contributions

Xiaoling Wang and Ye-Hua Gan designed the study. Yang Yu and Chuoyue Cheng performed the clinical examinations for recruitment of research subjects and Chunling Ge designed the prosthesis for each subject and Bing Wang manufactured the prosthesis for the subjects. Yang Yu and Xiaoling Wang performed the QLF measurements and statistical analysis. Yang Yu, Xiaoling Wang and Ye-Hua Gan wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative red fluorescence images of plaque on enamel blocks at the end of the four different test periods.

Table S1. The fluorescence change (means ± s.d.) before and after treatment with remineralization solution for the enamel blocks demineralized with different time.