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ORIGINAL SCIENTIFIC ARTICLES

Effect of *Galla chinensis* on the *In Vitro* Remineralization of Advanced Enamel Lesions

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Abstract

Aim The effect of *Galla chinensis* on de-/re-mineralization of advanced enamel lesions was investigated by using micro-CT in a prolonged *in vitro* experiment.

Methodology Baseline mineral contents of sound enamels were first analyzed. Then lesions were produced in an acidic buffer solution (2.2 mmol·L⁻¹ Ca(NO₃)₂, 2.2 mmol·L⁻¹ KH₂PO₄, and pH=4.5) for 21 days, with thrice daily three-minute treatments, divided into four groups: Group A, 4 000 ppm crude aqueous extract of *Galla chinensis* (GCE); Group B, 4 000 ppm gallic acid; Group C, 1 000 ppm F aq. (as NaF, positive control); Group D, deionized water (negative control). Next, the blocks were immersed in a remineralization solution (1.5 mmol·L⁻¹ CaCl₂, 0.9 mmol·L⁻¹ KH₂PO₄, 0.1 ppm F, and pH=7.0) for 200 days. Mineral loss (ML) in each region of interest

(ROI) and integrated mineral loss (IML) of the lesions were calculated (comparing with baseline mineral content of sound enamel) at different time points.

Results After 21 days demineralization, fluoride treatment showed a statistically significant demineralization-inhibiting effect among the four groups, and after 200 days of remineralization, mineral content recovery was ordered (lowest to highest) as A=C<B<D.

Conclusion GCE could slow down the remineralization of enamel in the surface layer and thereby facilitate ion transport into the lesion body. The mechanism of *Galla chinensis* in enhancing the remineralization of dental caries is different from fluoride.

Keywords demineralization, remineralization, advanced enamel lesion, *Galla chinensis*

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Introduction

Dental caries are one of the most prevalent chronic diseases worldwide. The carie-preventive effects of fluoride are now beyond doubt (ten Cate, 2004). In addition, however, many studies have considered different kinds of non-fluoride agents as supplementary methods for the prevention of dental caries.

Galla chinensis (*G. chinensis*) are a traditional Chinese herb investigated as an anti-cariogenic drug during recent years. Previous studies have indicated

that various chemical compounds of *G. chinensis* could inhibit demineralization and enhance remineralization of dental enamel (Liu et al., 2003; Chu et al., 2007). Zou et al. demonstrated the potential of the extract of *G. chinensis* (GCE) to affect demineralization under dynamic pH-cycling conditions (Zou et al., 2008). Cheng et al. indicated that GCE and gallic acid could enhance the remineralization of artificial early enamel caries. They also had combined effects with fluoride on enhancing remineralization of dental caries. Detailed investigations showed that gallic acid aided mineral

deposition predominately in the surface layer, while GCE encouraged more mineral deposition in the lesion body (Cheng *et al.*, 2008). Other studies showed that the chemical compounds of *G. chinensis* could regulate the demineralization/remineralization balance by influencing the morphology, structure and chemical content of enamel crystals, and the mechanism of *G. chinensis* seems to be different from fluoride (Cheng *et al.*, 2009). Previous studies, however, investigated the effect of *G. chinensis* on the initial enamel lesion, and none of them focused on the advanced enamel lesion in long-term studies. We have thus proposed a hypothesis that the chemical compounds of GCE could regulate the demineralization/remineralization balance in different regions of advanced lesions, and the mechanism of doing so was different from fluoride.

Micro-computerized tomography (micro-CT) is a new and developing technology that can be used to non-destructively map the distribution of mineral in teeth (Elliot, 1997). Efeoglu *et al.* developed a method for quantification of the mineral content of tooth specimens in three dimensions (Efeoglu *et al.*, 2005). We therefore applied micro-CT to study the possibility of remineralization of advanced enamel lesions *in vitro*, and to compare the effect of *G. chinensis* with fluoride on regulating demineralization/remineralization balance of advanced enamel lesions in a prolonged experiment.

Materials and Methods

G. chinensis sample

GCE, the extract of *G. chinensis*, was distilled according to previous studies (Cheng *et al.*, 2008). *G. chinensis* (1 kg) produced in the Sichuan province of China was dried in an oven at 60°C for 3 days, finely powdered, and added to 600 mL of distilled water. The mixture was stirred for 10 hours at 65°C and then filtered. The extract was re-extracted with distilled water under the same conditions. Then the extract was dissolved in 500 mL of ethanol (100%). After filtration and evaporation of the ethanol, the remaining extract was lyophilized to give a powder (*G. chinensis* extract, GCE) (yield, 160 g). The GCE preparation (160 g) was further fractionated by adsorption

chromatography using a Diaion HP-21 column (8 cm × 20 cm; Mitsubishi Chemical Industries, Tokyo, Japan). The column was eluted with deionized water (10 L), then with 30% ethanol (5 L), and finally with 100% acetone (5 L), and fraction GCE-B (2.7 g) was obtained. A portion of GCE-B was further purified by successive column chromatography with a Diaion HP-20 column (8 cm × 20 cm) and a Sephadex LH-20 column (3 cm × 120 cm; Pharmacia-LKB Biotechnology, Uppsala, Sweden). The column was eluted by acetone–water (2:8, 3:7, 4:6, V/V) and active compounds were obtained, characterized as gallic acid by spectroscopic methods including mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR).

Specimen preparation

Incisors from 4-year-old cows were obtained from the local slaughterhouse. Immediately after extraction the teeth were rinsed under tap water and stored at 4°C in water containing 0.05% thymol until required. A diamond-coated band saw (Struers Minitom; Struers, Copenhagen, Denmark) was used to separate root from crowns and to cut sections approximately 4 mm × 4 mm × 3 mm. The enamel surfaces were then ground flat and hand-polished using aqueous slurries of progressively finer grades of silicon carbide, up to 4000 grit (Struers, Copenhagen, Denmark), thereby removing about 150 µm from the original tooth surface, and creating access to dentinal tissue for the external solution (ten Cate and Duijsters, 1982).

Lesion formation

All surfaces of the sections were protected with acrylic resin except the polished enamel surface. The sections were immersed in the solution containing 2.2 mmol·L⁻¹ Ca(NO₃)₂, 2.2 mmol·L⁻¹ phosphate as KH₂PO₄, and 50 mmol·L⁻¹ acetic acid (pH=4.5). The solution was stirred at about 1 000 r·min⁻¹ and demineralization was performed at 37°C for 21 days. Each daily demineralization included thrice-daily three-minute applications with one of four treatments: Group A, 4 000 ppm GCE; Group B, 4 000 ppm gallic acid; Group C, 1 000 ppm F aq. (as NaF); Group D, deionized water.

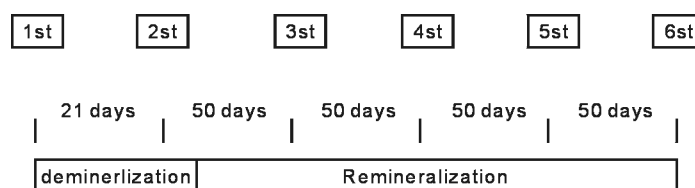


Figure 1 Time points of scanning samples by micro-CT

Remineralization

After lesion formation, the sections were equilibrated in deionized water overnight. The single sections were then immersed in a remineralization solution containing $1.5 \text{ mmol}\cdot\text{L}^{-1}$ CaCl_2 , $0.9 \text{ mmol}\cdot\text{L}^{-1}$ KH_2PO_4 , $130 \text{ mmol}\cdot\text{L}^{-1}$ KCl , $20 \text{ mmol}\cdot\text{L}^{-1}$ HEPES (N-hydroxyethylpiperazine-N-ethanesulfonate, pH=7.0) and 0.1 ppm F as NaF. The solutions contained sodium azide to prevent bacterial growth. Remineralization was done for 200 days at 37°C . The solutions were stirred and were refreshed once every week.

Micro-CT analysis

The mineral content of the tooth specimens was quantified at six time points (Figure 1) using a micro-CT scanner (μCT 80, Scanco, Switzerland). Thus, each specimen was scanned for six times. The same scanning parameters were applied in all scans. The X-ray source was set at 45 kVp, and $177 \mu\text{A}$. Integration time was 400 seconds. The entire thickness of the tooth rods were scanned at high resolution. The data were used to reconstruct images with a resolution of 2048×2048 pixels and with an isotropic voxel size of $25 \mu\text{m}$. A custom sample holder was used to position the specimens in the sample holder of the micro-CT scanner. During scanning, a damp sponge was placed in the sample holder and the holder was sealed with cling film to maintain a humid environment therefore preventing any cracks that might occur in a dry environment.

The evaluation software available in the workstation of the scanner was used to define seven regions of interest (ROI) per tooth rod. The thickness of each ROI was $60 \mu\text{m}$. Gray level median values for each ROI were converted to mgHA/ccm through calibrated by standard cylin-

drical hydroxyapatite block (Plasma Biotol Ltd., Buxton, UK), the mineral content of each ROI was then calculated. Then, the mineral content (MC) of specimens in different groups at different time point was recorded. Mineral loss (ML) was calculated as follows:

$$\text{ML} = \frac{\text{MC} - \text{MC}_0}{\text{MC}_0}$$

MC_0 is the mineral content of sound enamel sample before demineralization.

In addition, evaluations were carried out on each ROI at different time point.

Statistical analysis

Differences between the groups were tested for significance at the $P < 0.05$ level by ANOVA followed by Duncan's multiple range test using SPSS software (version 11.0).

Results

Integrated mineral loss at different time point

The average integrated mineral loss (IML) in different groups at different time point is shown in Figure 2. After demineralization, enamel samples in the fluoride treatment group (Group C) showed the least IML, and the largest demineralizations were observed in Group D (deionized water). Most of remineralization occurred in the first 50 days in all the groups. From 50 days to 200 days, slight remineralization still occurred in Group A and Group C, but there was no difference in IML between the two groups. After 200 days of remineralization, mineral content recovery occurred in the order (lowest to highest) $A=C < B < D$.

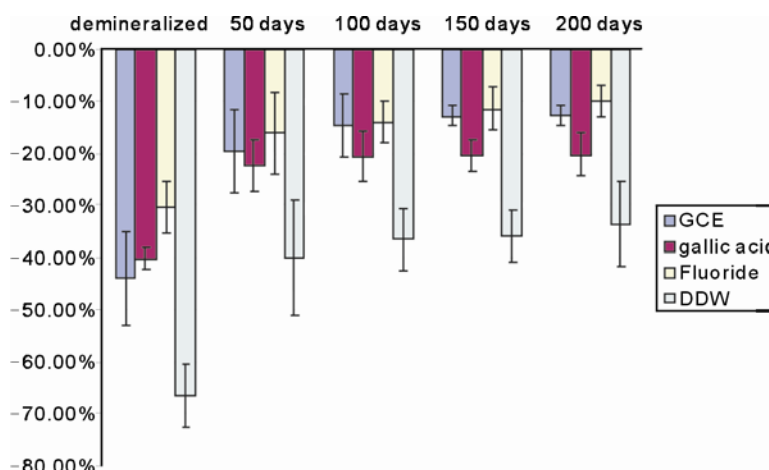


Figure 2 IML of different groups at six time points

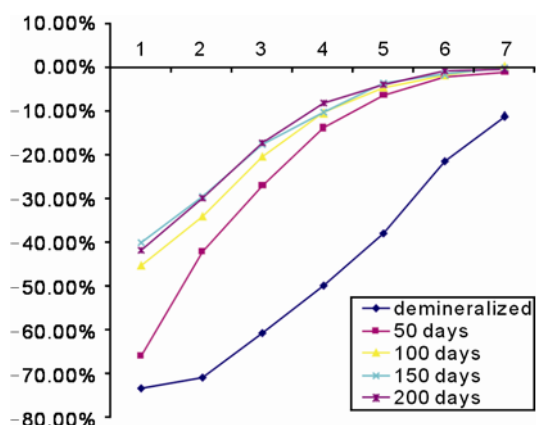


Figure 3 ML of GCE treatment group at different time points

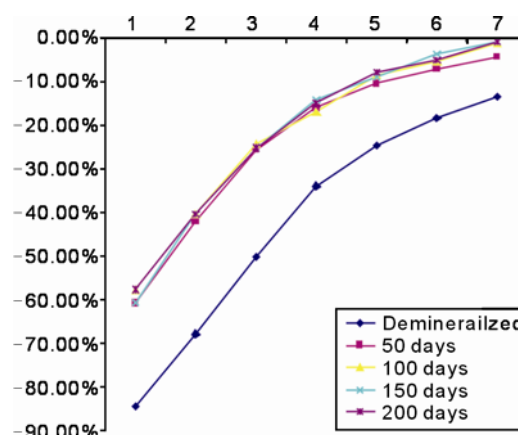


Figure 4 ML of gallic acid treatment group at different time points

Mineral loss in each group at different time point

In the GCE treatment group (Figure 3), obvious remineralization occurred in the ROI 2-5 during the first 50 days. Then, during the second 50 days, more mineral deposited in the ROI 1; and during the third and fourth 50 day periods, slight remineralization was observed in all ROIs.

In gallic acid treatment group (Figure 4), remineralization occurred in all of ROIs during the first 50 days. However, little remineralization was observed from 50 days to 200 days, even after 200 days' remineralization, ML of ROI 1 remained about 60%, compared with 40% in GCE treatment group. In the fluoride treatment group (Figure 5), fluoride aided more ion deposition on the surface layer, so remineralization was more obvious in

ROI 1-2 during the first 50 days. In deionized water treatment group (Figure 6), remineralization of ROI 4-5 was more obvious and the ML of ROI 1-3 was more than 50% even after 200 days of remineralization.

Discussion

Micro-CT was applied in this study to analyze mineral content of enamel specimens. Although there are many different techniques available to assess the mineralization of enamel in previous investigations (Arends and ten Bosch, 1992), quantitative measures of mineral content were possible only if direct chemical and radiographic techniques were used (White et al., 1992). Transversal microradiography (TMR) and cross sectioned

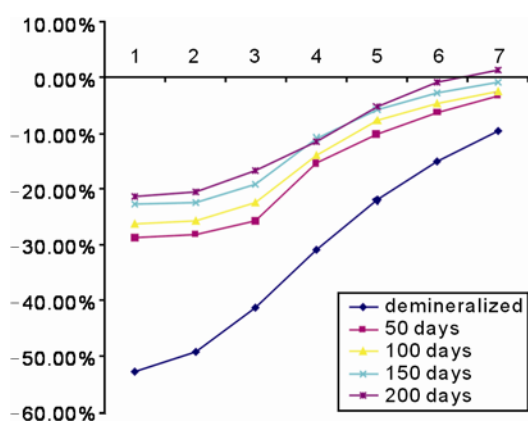


Figure 5 ML of fluoride treatment group at different time point

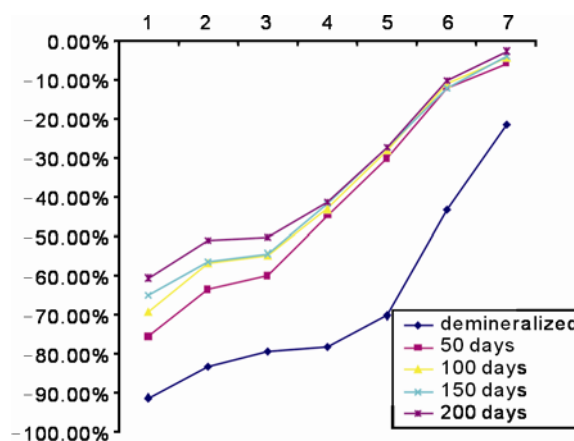


Figure 6 ML of deionized water treatment group at different time point

microhardness methods are commonly used for determination of mineral gain and loss (Kielbassa *et al.*, 1999); however, both methods are destructive on enamel. And in the present study, advanced enamel lesions with fragile surfaces were formed. Thus, it was very difficult to prepare the sections without destroying the surface layer. Micro-CT showed its advantage in evaluating mineralized enamel samples nondestructively in three dimensions (Elliot, 1997). But the main drawback of the technique is that a large amount of time is required to reconstruct and analyze the data. Efeoglu *et al.* described a new *in vitro* micro-CT method to determine the mineral content of dental enamel (Efeoglu *et al.*, 2007). We applied this method in our experiments, except for modifying the thickness of ROI.

The present results showed that mineral uptake and loss occur – at least partly – at different depths within the specimens. There was no fluoride in the demineralization solution, thus the surface layer in negative control group indicated more significant demineralization. Compared with the negative control group, fluoride and chemical compounds of *G. chinensis* could inhibit demineralization and then influence the remineralization of advanced enamel caries. And fluoride treatment showed a statistically significant demineralization-inhibiting effect compared to the other groups. Relatively highly mineralized surface layer (ROI 1-2) was formed after demineralization in the fluoride treatment group. Previous studies indicated that fluoride could affect the mineral deposit in the outer

enamel, but did not significantly affect precipitation of mineral in the inner enamel and dentine (ten Cate, 2001). The present experiment showed that advanced enamel lesions, with very low mineral contents, could still be remineralized under *in vitro* conditions. But the demineralization/remineralization balance of ROIs in different groups indicated different characteristics. Detailed investigation indicated that in the beginning of the remineralization, GCE aided more mineral deposit in ROI 3-6 compared with gallic acid and fluoride, which was similar with the results of short-time remineralization experiments (Cheng *et al.*, 2008). But the remineralization process of surface layer was relatively slow. This implies that the mechanism of *G. chinensis* on regulating the demineralization/remineralization balance of dental enamel might be different from fluoride. Thus, we propose that some component of GCE might combine with the enamel crystals of surface layer and inhibit the demineralization of enamel. According to previous studies, if the mineral structure was destroyed to the extent that reprecipitation of mineral on remaining hydroxyapatite crystallites was no longer possible, remineralization could not be achieved (ten Cate, 2008). On the one hand, combination of GCE with the enamel surface could reduce the demineralization of dental enamel. After demineralization, the mineral structure of the surface layer was not destroyed too greatly, so that deposit of mineral on remaining hydroxyapatite would occur slowly on the surface layer. On the other hand, the remineralization of enamel crystals

on the surface would also be slowed by the combination with GCE. And thus, more calcium and phosphate could enter into the lesion body but would not first precipitate in the layers closest to the surface.

Conclusion

GCE could slow down the remineralization of enamel in the surface layer and thereby facilitate ions transport into the lesion body. The mechanism of *G. chinensis* in enhancing the remineralization of dental caries is different from that of fluoride.

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