Short title: Remineralization of artificial root caries by flavonoids

Effect of flavonoids on remineralization of artificial root caries

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ABSTRACT

Background: This study compared the effects of three flavonoids, including proanthocyanidin, naringin and quercetin on remineralization of artificial root caries.

Methods: Demineralized root fragments (n = 75) were randomly divided into five groups for treatment with the remineralizing agents for 10 minutes: (1) 6.5% proanthocyanidin; (2) 6.5% naringin; (3) 6.5% quercetin; (4) 1000 ppm fluoride; and (5) deionized water (control). The demineralized samples were pH-cycled through treatment solutions, acidic buffer and neutral buffer for eight days at six cycles per day. The remineralization effects were evaluated using Knoop microhardness, transverse microradiography (lesion depth and mineral loss) and confocal laser scanning microscopy. Microhardness at different lesion depths was analysed with two-way ANOVA and Tukey's test, while lesion depths and mineral loss were analysed with one-way ANOVA and Tukey's test.

Results: Artificial caries lesions treated with fluoride and flavonoids showed significantly greater hardness than the control group ($p < 0.05$). Both lesion depths and mineral loss of the flavonoid treated groups were significantly lower than the control group ($p < 0.05$), but significantly higher than the fluoride treated group. No significant difference in lesion depth and mineral loss was found among the three flavonoids ($p > 0.05$).

Conclusions: All three flavonoids showed positive effects on artificial root caries remineralization, which are significantly lower than that of 1000 ppm fluoride.

Keywords: Naringin, proanthocyanidin, quercetin, remineralization, root caries.

Abbreviations and acronyms: CLSM = confocal laser scanning microscopy; NR = naringin; PA = proanthocyanidin; QC = quercetin.

(Accepted for publication 14 August 2015.)

INTRODUCTION

With the improvement in oral health care, the rate of edentulism has reduced significantly over the years in Australia.[1] However, with the increased number of natural teeth present and in the elderly population, the risk and prevalence of caries have increased.[2] There is also a concomittent increase in the prevalence of periodontontal disease with the decreasing rate of edentulism in the elderly. Gingival recession resulting from the periodontal diseases leave the root surfaces exposed to the development of root caries, which has important implications for preventive and restorative treatments.[3]

In contrast to young people, early intervention and conservative treatment to arrest dental disease are preferred in the elderly as complex restorative treatments may be difficult for them to maintain with advancing age.[4] As root caries is very common among the elderly, oral health care providers should establish a preventive regime to address the problem using minimal intervention treatment tailored to the patient's needs.[5] The modern concepts of minimum intervention dentistry place strong emphasis on arresting and reversing incipient lesions, using appropriate topical remineralizing agents.[6] Although fluoride is a remarkable remineralizing agent of enamel, it is less effective on dentine.[7] This could be attributed to the presence of a highly organized collagen matrix with less mineral content in dentine, making it more susceptible to degradation by free- and collagen-bound proteases.[8,9] Thus, there is a need for development of novel strategies to preserve the organic dentine matrix and promote remineralization, which is required to reduce root caries progression.[10]

Flavonoids are a group of bioactive molecules that are capable of performing various biological processes that have antioxidant, antibacterial and anti-inflammatory properties.[11] They are consumed in high amounts in fruits and vegetables. Apart from having varying degrees of antioxidant, anti-inflammatory and anti-tumour effects, flavonoids can interact with proteins, such as collagen and receptor molecules or anions, like calcium and iron.[12-15]

Recently, the application of flavonoids in dentistry has become popular. Several flavonoids, such as hesperidin, green tea extract, genipin and proanthocyanidin have been proven beneficial in promoting oral health.[12,16,17] Hesperidin preserves dentine collagen and stimulates mineral deposition on root surface caries.[16] Green tea extract has been shown to reduce erosion and abrasion, and to facilitate preservation of the dentine matrix, *in vitro* and *in situ*.[18-20] Cranberry extract inhibits the formation of dental biofilm and reduces plaque formation.[21]

Proanthocyanidin (PA), a plant flavonoid belonging to the flavanol group, has been reported to have a remineralization effect on artificial root caries.[12,17] PA is prevalent in pine bark, elm trees and grape seed.[18] PA is also commonly available in vegetables and fruits, but in lower concentrations. Quercetin belongs to the flavonol group and is found in citrus fruit. It has a collagen cross-linking effect and stimulates growth of bone mass.[19,20] Naringin is a flavonone glycoside, mainly present in apples and onions. It exhibits a strong affinity towards collagen and improves the quality of bone.[13,21] These three types of flavonoids share a common basic structural feature, mainly a benzopyrine ring, but differ according to the number and types of groups attached to the benzene ring.[22] The attachment of different functional groups in different positions can alter the biological effects of the flavonoids.[22]

Being an oligomeric molecule, PA presents with a larger molecular size, which may limit its penetration into demineralized dentine and therefore its remineralization potential. Furthermore, staining of dentine by PA also presents a clinical problem. Conversely, quercetin (QC) and naringin (NR) are both flavonoids, belonging to flavonol and flavonone glycoside groups. They have similar molecular structure to PA, but of smaller molecular sizes, which may facilitate their penetration into demineralized dentine and enhance the remineralization effect.

In our previous study, PA, QC and NR have been examined for their effects on the mechanical properties of dentine. Although not as effective as PA, with prolonged treatment (30 minutes to 1 hour), both QC and NR enhanced the ultimate tensile strengths and modulus of elasticity of demineralized dentine.[23] It has also been shown that PA acted simultaneously with CPP-ACP and CPP-ACFP to promote

remineralization of artificial root caries.[24] However, no studies have compared the remineralization effect of PA, QC and NR. Hence, this study compared the effect of three different types of flavonoids on remineralization of artificial root caries lesions by evaluating microhardness, lesion depth and mineral loss following treatment of the three flavonoids. The microhardness data is related to microhardness testing, while lesion depth and mineral loss to transverse microradiography, as an indirect measure of remineralization. The null hypothesis tested was that the tested flavonoids had no effect on remineralization of artificial root caries.

MATERIALS AND METHODS

Materials

Quercetin and naringin were obtained from Sigma-Aldrich with a purity of 95% and 93%, respectively. Proanthocyanidin was obtained from International Laboratory of USA, with a purity of >95% oligomeric proanthocyanidin. A 6.5% (w/v) solution of each flavonoid in phosphate buffer (0.025 M KH_2PO_4, 0.025M K_2HPO_4, pH 7.4) was used in this study. The chemical structures of the three tested flavonoids are shown in Fig. 1. Sodium fluoride solution (1000 ppm F, Fisher Chemical, USA) was used as a positive control, while deionized water was used as a negative control.

Preparation of dentine specimens

Forty extracted sound human third molars that had been stored in a 0.5% chloramine T solution at 4 ºC were used within one month after extraction. The teeth were collected after the patients' informed consent had been obtained under a protocol reviewed and approved by the Institutional Review Board, The University of Hong Kong (UW 11-242). The teeth were thoroughly cleaned. Seventy-five root fragments (5 mm x 5 mm x 5 mm) were obtained from the cervical root portions of the teeth. A window of 3 mm x 4 mm was created on the surface by applying acid resistant nail varnish (Revlon Corp., NY, USA) to the root surface.

Lesion formation

Lesion formation and pH-cycling were performed following the protocol used by Xie *et al*.[12] The root fragments were placed in a demineralizing solution (2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_2, 50 mM acetate, pH 4.6) for 96 hours at 37 ºC to create lesions 70–100 µm deep according to the previous study.[12] In order to maintain

the baseline lesion, the fragments were rinsed thoroughly with deionized water. One half of the window of each specimen was covered with the acid resistant nail polish (Revlon Corp., NY, USA) to maintain a reference baseline lesion.

Remineralization regimen

The demineralized root fragments were randomly divided into five groups (n = 15 per group) based on treatments: (1) 6.5% proanthocyanidin; (2) 6.5% naringin; (3) 6.5% quercetin; (4) 1000 ppm fluoride solution as NaF; and (5) control (deionized water). All specimens were pH-cycled through the treatment solutions (10 minutes), acidic buffer (50 mM acetate; 2.25 mM $CaCl_2.2H_2O$; 1.35 mM KH_2PO_4; 130 mM KCl; pH 5.0; 30 minutes) and neutral buffer (20 mM HEPES; 2.25 mM $CaCl_2.2H_2O$; 1.35 mM KH_2PO_4; 130 mM KCl, pH 7.0; 10 minutes). Six demineralization–remineralization cycles were performed each day and continued for eight days. A 250 mL polystyrene jar was used for the *in vitro* pH-cycling experiments. All solutions were freshly prepared just before use. The specimens were stored in a neutral buffer overnight at 37 ºC.

Microhardness test

After eight days of pH cycling, five specimens from each treatment group were rinsed in deionized water for 2 minutes and sectioned into two halves. One half of the specimens was embedded perpendicular to the demineralized surface in epoxy resin for microhardness measurement. The other half was used for transverse microradiography and confocal laser scanning microscopy (CLSM) analysis.

For the microhardness test, polishing of the embedded samples was performed on a water-cooled polishing unit (EcoMet 3000, Buehler, Lake Bluff, IL, USA) with abrasive papers (400-, 600- and 1200-grit) followed by polishing with 0.9, 0.6, 0.3 and 0.1 µm diamond suspensions (Metaldi Supreme, Buehler, Lake Bluff, IL, USA). The residue was removed by ultrasonically cleaning the samples for 2 minutes. Cross-sectional microhardness measurements perpendicular to the demineralized surface (Leica, Tukon 200, Germany) were performed at 20, 50, 80, 110 and 140 µm form the surface using Knoop hardness indentation at a 25 g load for 15 seconds in an ambient environment. Measurements were performed for all the samples at three different locations at each depth.

Transverse microradiography

The remaining halves of the specimens (n = 5 per group) were dehydrated using a series of ethanol solutions from 20% to 100%, followed by a transitional medium containing propylene oxide. The specimens were then embedded in epoxy resin and sectioned longitudinally through the lesion centre into 200 ± 20 µm thick sections (Isomet, Buhler Ltd., Lake Bluff, IL, USA). The 200 ± 20 µm thick sections were X-rayed together with an aluminium step wedge at 12 kV and 4 mA for 45 seconds. The reading of the step wedge was transformed into a mineral content number and used for calculating lesion depth and mineral loss. Microradiographic plates were processed (Eastman Kodak Co., Rochester, NY, USA) and the radiographic images were exported to the computer via a CCD camera from the microscope. Lesion depth was defined as the distance from the lesion surface to the site where the mineral content was more than 95% for the sound dentine. Mineral loss was evaluated by computing the area obtained by plotting the vol% mineral profile towards lesion depth in each dentine section, with the sound dentine set as 48 vol% mineral contents.[25]

Confocal laser scanning microscopy

The remaining specimens (n = 10 per group) were embedded in epoxy resin at the wet stage, sectioned into 200 ± 20 µm thick sections using a diamond wafering blade immediately after setting of the resin under water cooling (Isomet, Buhler Ltd., Lake Bluff, IL, USA) for CLSM evaluation. Prepared dentine sections were stained with a freshly prepared 0.1% Rhodamine B solution (Sigma-Aldrich Chemical Corp., Milwaukee, WI, USA) for 1 hour, and rinsed three times with deionized water. Samples were analysed with a CLSM (Zeiss LSM 510, Carl Zeiss Inc., Germany), using an argon laser with a 529 nm excitation wavelength. Areas were scanned between 10 and 50 µm below the cut surface to reduce the influence of the smear layer created during the cutting and polishing procedures.

Statistical analysis

The microhardness data, lesion depth and mineral loss data were analysed using a statistical package (SigmaStat Version 16, SPSS, Chicago, IL, USA). As the normality (checked by Kolmogorow–Smirnoff test) and the equal variance assumptions (checked by modified Levene test) of the data were valid, two-way

ANOVA (remineralization treatments vs lesion depths) and *post hoc* Tukey multiple comparison test were used to examine the effect of various remineralization treatments on microhardness, while one-way ANOVA and *post hoc* Tukey multiple comparison test were used to examine the effect of various remineralization treatments on lesion depth and mineral loss. A level of significance of $\alpha = 0.05$ was set in all statistical tests.

RESULTS

Table 1 presents the cross-sectional microhardness measurements of the artificial caries lesion from the different treatment groups at different lesion depths from the surface. Results of two-way ANOVA indicated that the factors 'remineralization treatments' ($p < 0.001$) and 'lesion depth' ($p < 0.001$) had significant effects on microhardness. The interaction between the two factors was also significant ($p < 0.001$). Except the naringin treated group at 110 µm, artificial caries lesions treated with fluoride and flavonoids showed significantly greater microhardness values than the control group at all lesion depths ($p < 0.05$). No significant differences in microhardness values were observed among the fluoride and flavonoid treated groups up to 80 µm deep ($p > 0.05$), but fluoride group showed significantly higher hardness values at 110 µm and 170 µm ($p < 0.05$).

Table 2 summarizes the lesion depth and mineral loss obtained from transverse microradiography. The one-way ANOVA indicated the factor 'remineralization treatments' had a significant effect on lesion depth ($p < 0.001$) and mineral loss ($p < 0.001$). The fluoride group showed the lowest lesion depth and mineral loss, when compared to all the other groups ($p < 0.001$). No significant differences in lesion depth and mineral loss were observed among the three tested flavonoids ($p > 0.05$).

Figure 2 shows representative transverse microradiographic images of artificial root caries lesions from different treatment groups. The control group showed the deepest and least mineralized lesion (Fig. 2a). Both fluoride (Fig. 2b) and flavonoid treatments (Fig. 2c–e) reduced lesion depths. However, the fluoride treated dentine showed a thicker, well-defined surface mineralized layer (Fig. 2b), when compared to the flavonoid-treated dentine groups (Fig. 2c, 2d and 2e). Some mineral deposition

was observed throughout the body of lesions in the flavonoid-treated dentine groups (Fig. 2 c–e).

Figure 3 shows representative CLSM images of artificial caries lesions from the different treatment groups. The red fluorescent band representing the caries lesion was reduced in both fluoride and flavonoid treated groups, when compared to the control group, indicating that both fluoride and flavonoids enhanced remineralization of artificial root lesions. However, in the flavonoid treated groups, the red fluorescent band was less well-defined than the fluoride treated group. A thick dense band was observed on the surface of fluoride treated lesions.

DISCUSSION

Our study compared the effects of three flavonoids, PA, QC and NR, belonging to three different classes of flavonoids, flavanol, flavonol and flavonone glycoside, on remineralization of artificial root caries. The allocation of root fragments within groups was done randomly and there is a possibility that the root fragments from one tooth might be included in the same group. However, as only two root fragments were obtained from a single tooth for each group, the effect of data clustering is minimal.

Knoop hardness number values were used in the current study together with transverse microradiography and CLSM as indirect measurements of remineralization. According to the lesion depth and mineral loss measurements from the transverse microradiographs, all three tested flavonoids inhibited demineralization and promoted remineralization of artificial root caries lesions. The microhardness of the artificial caries lesion of the flavonoid treated groups was significantly higher compared to the control. Hence, the null hypothesis that the tested flavonoids had no effect on remineralization of artificial root caries was rejected. However, the remineralization effect of the flavonoids was less than fluoride.

Enamel caries is caused by acid dissolution of minerals by bacteria. Remineralization of enamel mainly occurs in an inorganic environment with homogenous hydroxyapatite crystal seeds. In contrast, dentine caries involves both demineralization and breakdown of the collagen matrix. Remineralization of dentine occurs in an organic environment within a demineralized collagen matrix. According

to Paven *et al*.,[17] the formation of a hypermineralized surface layer by fluoride prevents remineralization in deeper parts of the lesions. Hence, it is necessary to find alternative methods for dentine remineralization.

The organic phase of dentine consists of 90% type I collagen and 10% non-collagenous proteins. The remineralizaton process in dentine is controlled through the interactions of minerals crystallites with the collagen matrix; therefore, the organic matrix of dentine plays a major role in demineralization and remineralization.[12] Preservation of the demineralized dentine matrix, which acts as a template for mineral deposition, is essential for remineralization of dentine to occur. Inhibition of mineral loss in the flavonoid treated groups could be attributed to stabilization of the collagen matrix. PA is a well-known collagen cross-linker, the phenolic hydroxyl groups of PA can form hydrogen bonds with the amide carbonyl or hydroxyl groups of the collagen, enhancing the mechanical properties of collagen fibrils.[26] It also inhibits both soluble and matrix-bound matrix metalloproteinases and cysteine cathepsins in dentine, increasing the resistance of collagen fibrils to degradation by enzymatic digestion.[27] Furthermore, the stabilized collagen matrix acts as a mechanical barrier, which prevents ingress of acid and further loss of calcium and phosphate ions from the carious lesions.

It is likely that PA, QC and NR may all form complexes with calcium ions as they all contain hydroxyl groups in their molecular structure. Minerals are deposited on both the surface and subsurface layers of demineralized dentine. The mineral deposition in the subsurface layer may form nucleation sites for hydroxyapatite crystals. Furthermore, the negatively charged non-collageneous proteins in the organic matrix contain highly charged phosphorylated serine and threonine residues. These residues may also attract and trap calcium ions leading to nucleation and growth of hydroxyapatite. The hydroxyapatite residues in demineralized dentine could act as sites for apatite nucleation and growth of hydroxyapatite in the presence of calcium and phosphate, enabling remineralization and partial recovery of the mechanical properties of the demineralized dentine.[28]

Dentine consists mainly of an organic matrix with higher percentage of collagen and non-collagenous proteins.[29] The mineral phase is distributed in the extrafibrillar and intrafibrillar compartments of collagen. While extrafibrillar mineral is deposited in

interstitial spaces between collagen fibrils, intrafibrillar minerals are deposited within gap zones and microfibrillar spaces. Recent advancements in remineralization of dentine have shown the importance of a hierarchical assembly of minerals in collagen fibrils to enhance the mechanical properties of dentine.[9,30]

Although all the tested flavonoids inhibited demineralization and enhanced remineralization of dentine in the present study, there is no evidence of intrafibrillar mineral deposition. Intrafibrillar mineralization is necessary to recover the mechanical properties to remineralize dentine functionally.[30] Recently, the introduction of polyvinyl phosphonic acid to the collagen matrix has been shown to stimulate intrafibrillar mineralization.[31] Further studies will be conducted to stimulate intrafibrillar mineralization of flavonoid treated collagen fibril matrix with conjugation of various non-collagenous molecules and acidic groups.

CONCLUSIONS

Within the limits of this study, it may be concluded that all three flavonoids, proanthocyanidin, naringin and quecertin are equally effective in inhibiting demineralization and enhancing remineralization of artificial root caries lesions. It was also found that the remineralizing potential of the flavonoids was lower than fluoride.

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Table 1. Cross-sectional microhardness of the artificial caries lesions of different treatment groups

	Knoop hardness numbers				
Depth from surface (μm)	Control	Fluoride	Proanthocyanidin	Naringin	Quercetin
30	$3.2\pm0.55^{A,a}$	$8.66\pm1.83^{A,b}$	$8.99\pm2.27^{A,b}$	$8.85\pm3.53^{A,b}$	$8.98\pm4.2^{A,b}$
50	$7.42\pm1.5^{B,a}$	$11.50\pm4.75^{A,b}$	$11.98\pm2.85^{A,b}$	$11.03\pm3.40^{A,b}$	$10.43\pm2.54^{A,b}$
80	$14.52\pm3.69^{B,a}$	$26.95\pm2.61^{B,b}$	$25.72\pm6.26^{B,b}$	$23.28\pm7.39^{B,b}$	$25.15\pm9.37^{B,b}$
110	$26.02\pm4.33^{D,a}$	$49.97\pm14.06^{C,c}$	$38.92\pm6.72^{C,b}$	$35.76\pm16.61^{C,a,b}$	$37.81\pm14.81^{C,b}$
140	$36.58\pm2.81^{E,a}$	$57.97\pm19.10^{C,b}$	$49.53\pm11.43^{D,b}$	$47.86\pm17.53^{D,b}$	$48.09\pm14.76^{D,b}$
170	$44.85\pm7.38^{F,a}$	$73.62\pm12.02^{D,d}$	$66.29\pm16.24^{E,b,c}$	$62.29\pm19.94^{E,b}$	$69.14\pm8.44^{E,b,c}$

Values are means and standard deviations.

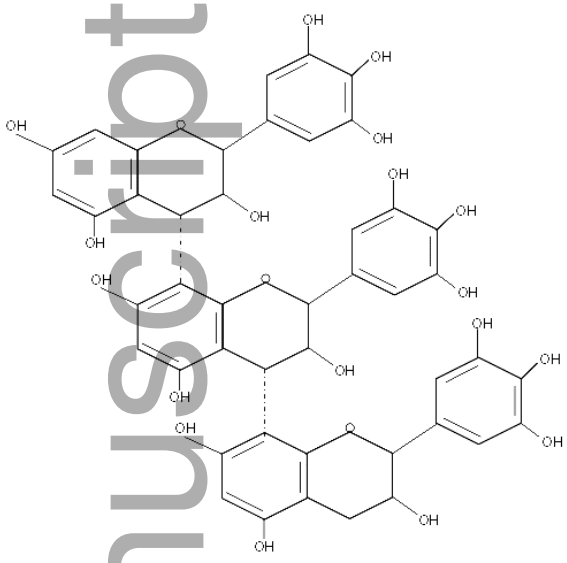
Upper case indicates statistically significant differences between depths ($p < 0.05$) and lower case indicates statistically significant differences between treatments ($p < 0.05$).

Table 2. Effect of remineralization treatments on mineral loss and lesion depth of the artificial root caries lesions evaluated using transverse microradiography

Remineralization treatments	Mineral loss (ΔZ, vol%)	Lesion depth (μm)
Control	4214.07 ± 707.42[a]	164.36 ± 40.63[a]
Fluoride	1206.66 ± 396.53[b]	62.53 ± 15.84[b]
Proanthocyanidin	2132.84 ± 755.53[c]	101.52 ± 16.58[c]
Naringin	1825.61 ± 797.20[c]	90.95 ± 27.09[c]
Quercetin	1793.38 ± 606.02 [c]	93.73 ± 23.09 [c]

Values are means and standard deviations.

Groups identified by different superscripts are significantly different ($p < 0.05$).



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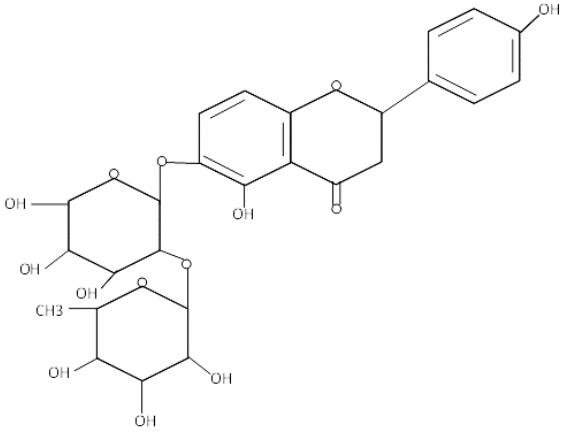

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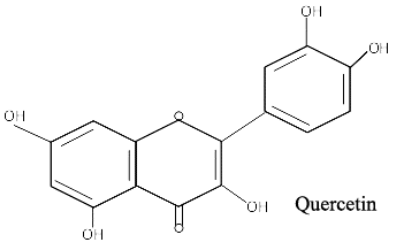

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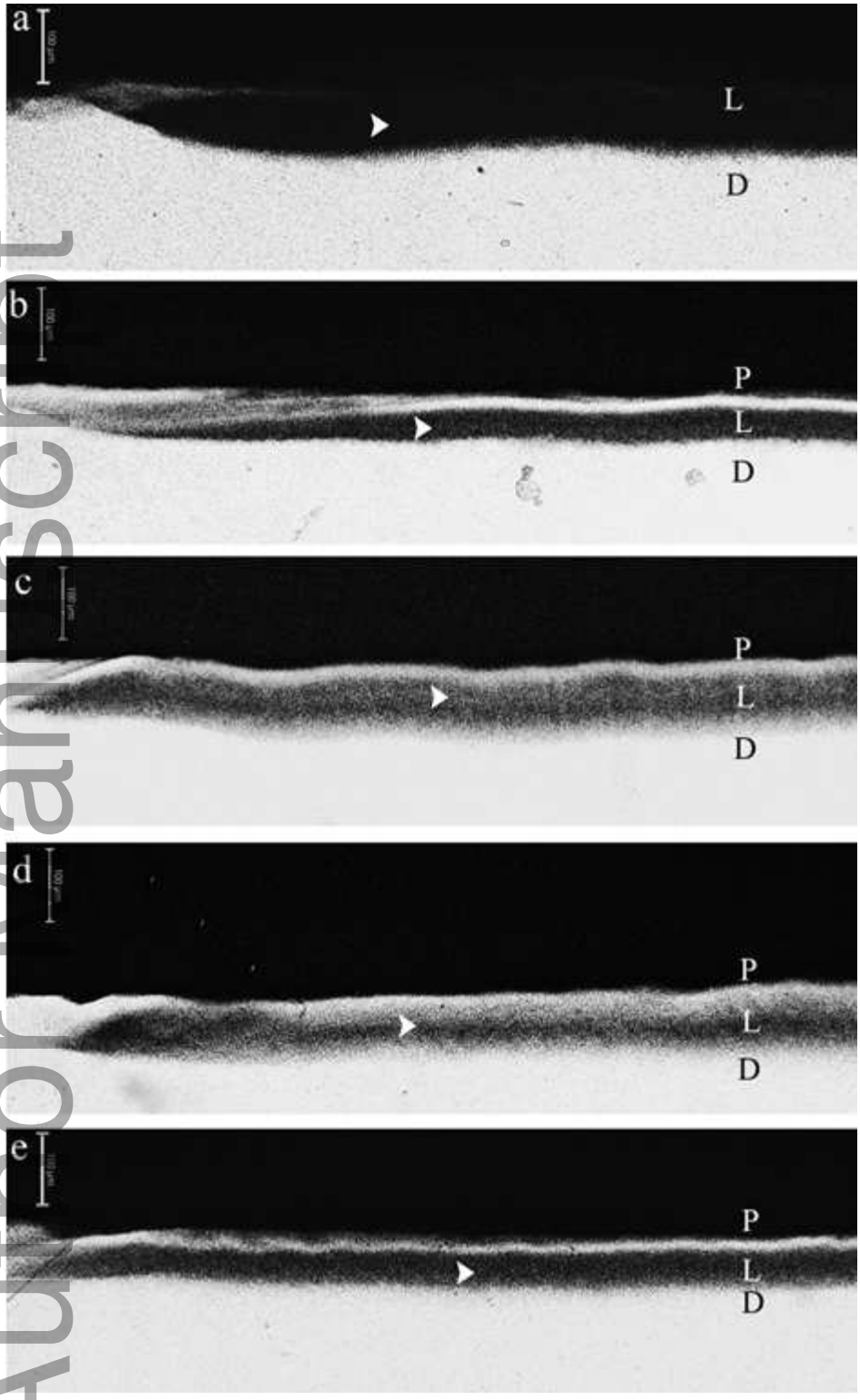

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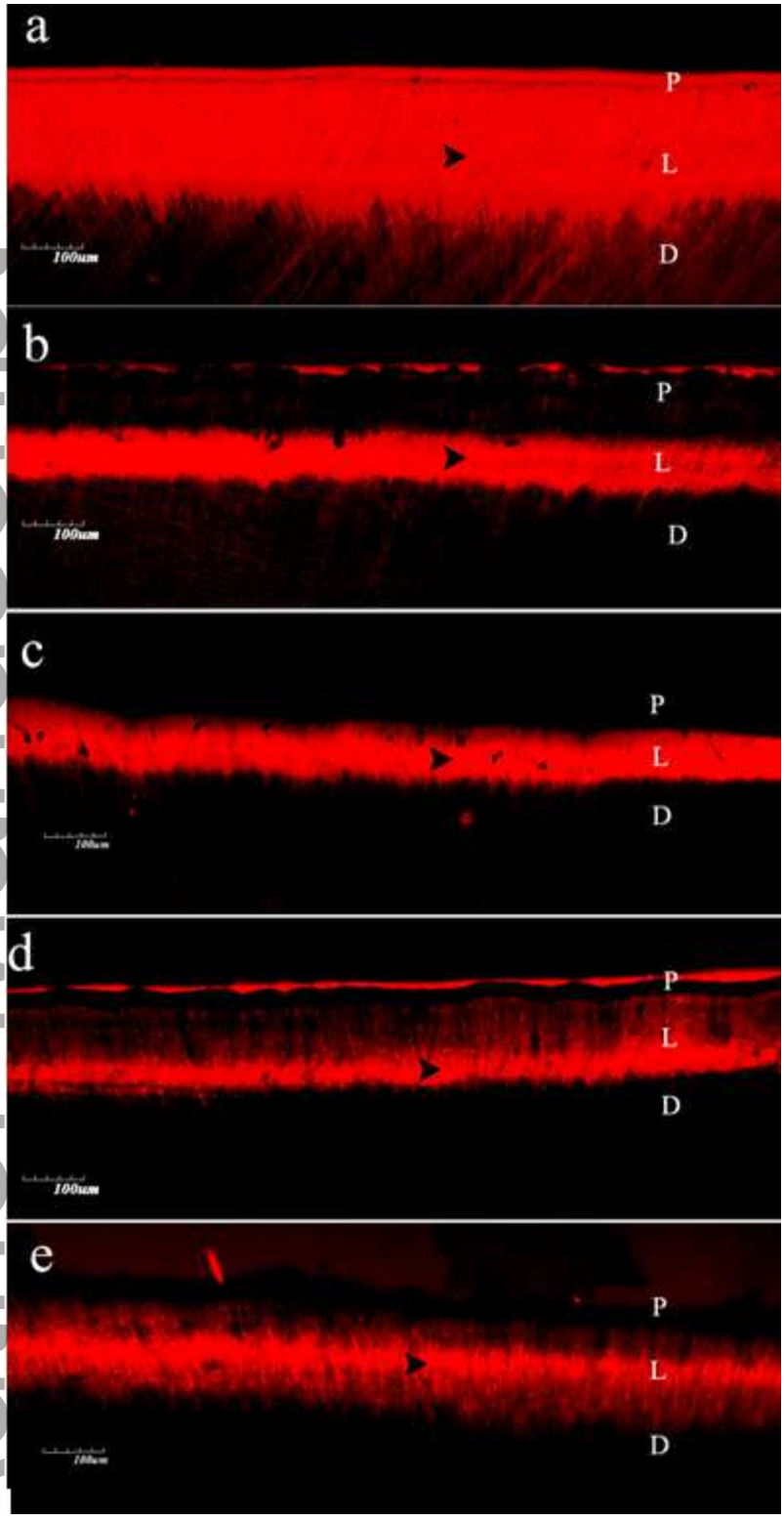

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