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Anthocyanin Structure Determines Susceptibility to Microbial Degradation and Bioavailability to the Buccal Mucosa

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ABSTRACT: Anthocyanins are flavonoids with reported chemoprotective activities in the oral cavity. However, information about their stability, metabolism, and tissue uptake in the mouth is limited. Anthocyanin chemical structure was recently shown to affect their stability ex vivo in saliva, and it was hypothesized that structure may affect their availability in oral tissues in vivo. Here, 12 healthy individuals retained red grape or chokeberry juice in the mouth for 5 min. Anthocyanin stability, mucus binding, and uptake into epithelial cells were evaluated. Loss of delphinidin-3-glucoside in red grape juice exceeded that of other anthocyanin-glucosides, and lesser amounts of delphinidin- and petunidin-glucosides were associated with buccal scraping, suggesting the loss was due to degradation. In chokeberry juice, loss of cyanidin-3-xyloside exceeded that of other anthocyanins, whereas cyanidin-3-glucoside preferentially accumulated in epithelium cells. These results suggest that anthocyanin structure affects stability and buccal cell uptake and therefore the potential efficacy of anthocyanin-rich products for the promotion of oral health.

KEYWORDS: *anthocyanins, buccal cells, oral metabolism, chokeberry, Aronia melanocarpa, red grape, Vitis vinifera L.*

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) list oral cancer as one of the most common cancers in humans.[1,2] The National Cancer Institute estimated that 36540 people in the United States were diagnosed with oral cancer in 2010, and 7880 were projected to die as a result.[1] The estimated expense of treating oral diseases ranks fourth among all chronic diseases in developed countries, representing 5−10% of total public health expenditures.[3,4] Thus, there is an urgent need for cost-effective, accessible, and efficacious strategies to prevent and treat these diseases.

Whole-food-based chemoprevention is rapidly gaining recognition as one such economically sustainable and universally accessible option.[5] Epidemiological studies have shown an inverse relationship between the intake of fruits and vegetables and the incidence of oral cancer.[6−9] An emerging body of literature suggests that anthocyanins (ACNs), a class of flavonoids that are responsible for the orange, red, and blue colors of many fruits and vegetables, decrease cancer risk. For example, anthocyanin-rich extracts from strawberry,[10] blackberry, black raspberry, blueberry, cranberry, and red raspberry[11] each inhibited proliferation of KB and CAL-27 human oral cancer cell lines. Inclusion of 5% freeze-dried black raspberry in diet fed to hamsters reduced the number of chemically induced tumors in buccal mucosa.[12] Topical application of a gel treatment containing 10% (w/w) freeze-dried black raspberry to human subjects with oral intraepithelial neoplasia was similarly associated with decreased histological and molecular markers of carcinogenesis in lesional oral tissues.[13,14] Such results have provided impetus to incorporate ACNs in products such as bioadhesive gels and confectioneries[14,15] as a means of increasing the bioavailability of these compounds to oral tissues.

However, an important consideration in whole-food chemoprevention is source-related differences in anthocyanin structures. With more than 700 distinct ACNs in foods,[16] the relationship of ACN structure with the extent of metabolism and delivery of the ACNs or their products to oral tissues is of particular interest for developing dietary recommendations and food formulations aimed at promoting oral health. We recently reported that salivary microbiota degrade ACNs ex vivo and that the extent of degradation in saliva was affected by ACN structure.[17] The purpose of the present study therefore was to examine the effect of ACN structure on availability to the buccal mucus and epithelial compartments as well as their metabolism by oral microbiota by comparing two different juices with significantly different ACN profiles.

MATERIALS AND METHODS

Standards and Reagents. Unless otherwise indicated, all supplies and chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Fisher Scientific Co. (Pittsburgh, PA, USA).

Preparation of Juices. Red grapes (*Vitis vinifera* L.) were purchased from a local supermarket and frozen (−18 °C) until preparation of the juice. Red grapes were thawed (4 °C, overnight) and ground in a commercial blender for 10 min to produce a cold (<10 °C) slurry. Solids were removed by passing the slurry over nylon mesh (250 μm pore size, NITEX, Sefar Inc., Ontario, CA, USA). Pectin in the filtrate was degraded by incubation (0.6 mL/L juice, 3 h, room temperature) with food-grade pectinase (Enzeco Pectinase DV-2, Enzyme Development Corp., New York, NY, USA). Remaining solids were removed by centrifugation (100*g*, 5 min) to produce the

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clarified juice. Concentrated chokeberry juice was a gift from Artemis International (Fort Wayne, IN, USA). Concentration of ACN in chokeberry juice was normalized to 60 nmol Cy-3-glu equiv/mL by diluting with deionized (DI) water prior to storage in 20 mL aliquots at −20 °C until chemical analysis and use in the human trial. The qualitative and quantitative profile of ACNs was not altered during storage at −20 °C for 1 month.

Characterization of Juices. Content of monomeric anthocyanins of each juice was measured by pH differential method.[18] The total polyphenolic content of each juice was analyzed by using the Folin−Ciocalteu assay and reported as gallic acid equivalents.[19] Soluble sugar and pH were measured by portable refractometer (Fisher Scientific, 13-946-21, 0−32 °Brix) and by pH meter (Accument XL15, Fisher Scientific), respectively. The ACN profile of each juice was analyzed by HPLC as described below.

Experimental Design. The human study protocol was approved by the Institutional Review Board of The Ohio State University (IRB#20090058). Thirteen systemically and dentally healthy subjects (as evidenced by clinical attachment levels ≤2 mm and probing depths ≤3 mm and a low DMF (decayed, missing, filled teeth) index) over the age of 20 were recruited for the study. Subjects who had antibiotic therapy or professional cleaning within the previous 3 months, used immunosuppressant medications, bisphosphonates, or steroids, reported pregnancy, or a history of diabetes or HIV were excluded from this study to minimize variations in salivary secretion and oral bacterial profiles.[20,21]

A crossover study design with washout was used to investigate the role of oral microbiota on the metabolism of ACNs in the oral cavity. Subjects were instructed to refrain from consumption of ACN-containing foods, coffee, and tea for 24 h prior to the study. On the day of study, subjects brushed their teeth after awakening, avoided use of antibacterial mouthwash, and did not consume breakfast until arrival at the clinic. Control buccal cells were collected by brushing the left buccal mucosa 10 times with a toothbrush, followed by rinsing with 20 mL of phosphate-buffered saline (PBS), and used to determine baseline ACNs. Subsequently, chokeberry juice (20 mL in preweighed polypropylene tube) was retained and swished around the mouth for 5 min. Samples were not smelled to minimize the effects of smell on salivary production. Chokeberry juice remaining in the tube was measured gravimetrically. The retentate was expectorated and collected along with subsequent oral rinse (20 mL deionized water) in preweighed tubes to determine total amount of juice and saliva expectorated. A new toothbrush was used to collect buccal cells from the right buccal mucosal surface followed by saline rinse as for collection of control buccal cells. Tubes were reweighed to estimate the amount of saliva in retained juice and oral rinse. After a 1 week wash-out period, subjects were asked to follow the same procedures prior to arrival at the clinic, where they received professional oral cleaning to remove the oral bacterial biofilms, followed by rinsing with 15 mL of 0.12% chlorhexidine gluconate (Colgate-Palmolive, New York, NY, USA) for 1 min and followed by a 5 min wash-out period. Subjects retained chokeberry juice in their mouth using the same protocol of the previous week. Control buccal scrapings, retained chokeberry juice, and postjuice buccal scrapings were collected and processed using the same procedures as described above.

To determine the effect of ACN structure on stability and delivery to buccal tissue, juices from chokeberry and grape were assigned to subjects in a randomized crossover design with a 1 week wash-out period between juice treatments. Human subjects (n = 12) were instructed to abstain from consuming ACN-containing foods and beverages after 10 p.m. on the evening before testing. On the day of study, subjects brushed their teeth and consumed breakfast before arrival at clinic. Each subject served as their own internal control. Control buccal cells were collected prior to retention of juice, followed by postretention collection of buccal cells from the opposite cheek as described above. Data from one subject who reported accidental swallowing of a portion of the chokeberry juice were excluded.

Sample Processing. Retained juices and aqueous oral rinse were separately weighed and acidified with 88% formic acid (final concentration = 5% by adding 1.2:20 v/w) within 30 min after sample collection. Both solutions were combined, filtered (0.22 μm pore size), and stored at −20 °C until analysis. Buccal scrapings were centrifuged (130g, 5 min, 4 °C) to separate soluble mucus from buccal cells. Cell pellets were washed three times with 10 mL of cold sodium phosphate buffer (0.1 M, pH 6.0) and centrifuged to separate extracellular ACNs and mucin from buccal cells.[22] Washed cell pellets were resuspended in sodium phosphate buffer and stored at −20 °C. Pooled supernatant containing mucus from each subject also was stored at −20 °C), lyophilized to dryness, resolubilized in 15 mL of 1:1 (v/v) methanol/water for quantification of mucin, and filtered (0.22 μm pore size) for HPLC analysis of ACNs.

ACN Extraction from Buccal Epithelial Cells. Preliminary studies were performed to determine the efficiency of extraction of ACNs from buccal cell pellets. Extraction solvents tested were methanol, acetronitrile, acetone, and 70% acetone. All solvents were acidified with formic acid (5% final concentration) to stabilize ACNs. ACN-rich extracts (15 nmol of Cy-3-glu equivalents in 25 μL of 0.01% HCl solution) from chokeberry and red grape were added to 0.25 mL suspensions of control washed buccal cells isolated from two subjects. Suspensions were sonicated (30 s, ice−water bath) before addition of 0.75 mL of test solvents and frozen (−20 °C) overnight. Samples were thawed, sonicated for 20 min in an ice−water bath, and centrifuged (20800g, 5 min). Supernatant was collected, and the pellet was re-extracted twice. Supernatants for the three extractions were transferred to a preweighed tube, concentrated by evaporation under N_2 (30 min, 37 °C), weighed, and filtered (0.22 μm pore size) prior to HPLC analysis. Recovery was calculated as (amount ACNs extracted/amount ACNs added) × 100%. Recovery using 70% acetone solvent exceeded 84% for all ACNs in chokeberry and red grape juices (data not shown), which was considerably greater than recovery after extraction into the other solvents tested (i.e., 36−51%). The extent of recovery of each ACN was not significantly affected by type of monosaccharide. Therefore, ACNs in all test samples were extracted into acidified 70% acetone for analysis.

HPLC Analysis. The content of ACNs in juices, retentate, mucus, and cell extracts was analyzed by HPLC-PDA-ESI-MS according to previously published methods[17] and reported as Cy-3-glu equivalents. Identification of ACNs was based on retention time (t_R) and $\lambda_{max\text{-}vis}$ and matched molecular weights of parent compounds and daughter fragments with those of available anthocyanin standards, isolated chokeberry anthocyanins, and reported elution profiles for chokeberry[23,24] and red grape.[25−27] The cellular content of ACNs was normalized by DNA content in isolated cell pellets (Quantification of DNA in Buccal Cells). The quantity of ACNs in the mucus layer was normalized per milligram of mucin (Quantification of Mucin).

Separation, identification, and quantification of reported phenolic metabolites were performed using a HPLC-PDA-ESI-MS system[17] with the mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient for the mobile phase was 0−30 min, 0−30% B; 30−31 min, 30−100% B; 31−32 min, 100−0% B; 32−37 min, 0% B. Partial flow from PDA detector was negatively ionized by the mass spectrometer with previously published parameters.[17] Mass spectra were obtained using scan mode (from m/z 50−800 with scan speed of 1000 amu/s) and selective ion monitoring (SIM) mode for PCA, PGA, gallic acid, syringic acid, vanillic acid, and 4-hydroxybenzoic acid at m/z 153, 153, 169, 197, 167, and 137, respectively.

Quantification of DNA in Buccal Cells. DNA in buccal cell aliquots was determined by diphenylamine (DPA) assay. The purpose of this test was to provide a basis for normalizing ACNs per unit buccal cells and allow for comparisons among the samples given that the number of cells collected for each buccal scrapping varies.

Briefly, 1 volume of cold acetaldehyde (0.16% in DI water) was mixed with 5 volumes of perchloric acid (20% v/v in DI water) to prepare the reagent. Cell suspensions were sonicated (20 min in ice bath) and aliquoted (40 μL) into a 96-well plate before addition of 60 μL of acetaldehyde solution. After mixing, 100 μL of DPA (4% in glacial acetic acid) was added to wells and the plate was incubated at 37 °C for 24 h. Absorbance was measured at 595 nm (corrected by subtraction of absorbance at a reference wavelength of 700 nm).

Corrected absorbance was compared to a five-point curve using DNA from herring sperm as standard.

Quantification of Mucin. Mucin concentrations in buccal washings were determined by using a modification of the previously described[29] Alcian Blue assay.[30] Aliquots of supernatant were incubated for 30 min in a 1:1 (v/v) of 1% solution of Alcian Blue in 50 mM sodium acetate buffer containing 25 mM $MgCl_2$, pH 5.8, under constant agitation at room temperature. Samples were centrifuged (705*g*, 20 min), and supernatant was discarded. The pellets were washed twice with 1 mL of 40% (v/v) ethanol in 50 mM sodium acetate buffer containing 25 mM $MgCl_2$, pH 5.8, briefly vortexed, and incubated for 5 min before centrifugation (705*g*, 20 min); supernatant was discarded. Mucin−dye complexes were dissociated by the addition of 1 mL of dioctyl sulfosuccinate solution (1:90 w/v in DI water) followed by brief mixing and ultrasonication at 40 W for 10 s with a VibraCellTM (Sonics & Materials, Danbury, CT, USA). Samples were centrifuged (705*g*, 1 min) to eliminate the foam generated during sonication. The absorbance of the dye was determined spectrophotometrically at 605 nm and compared to a five-point standard curve using known concentrations of mucin from bovine submaxillary glands as standard. This method does not distinguish larger (MG1) and smaller (MG2) mucins.

Statistical Analysis. Each sample (retained juices, oral washes, and washed buccal cells) collected and prepared from each subject was aliquoted for a minimum of three independent analyses. Statistical analysis was performed using SPSS release 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Mixed-model analysis of ACN structure (fixed factor) and subject (random factor) with Bonferoni's adjustment of mean comparison was used. Pearson's product moment correlation analysis was used to determine correlation between salivary secretion and extent of degradation of ACNs. Repeated-measures ANOVA was used to evaluate the effect of inhibition of oral microbiota on reduction of ACN loss in the oral cavity. Independent-samples *t* test was used to compare (1) extent of ACN association with mucus and buccal cells between chokeberry and red grape juices and (2) abundance (%) of each ACN between samples, that is, juice, retained juice, mucus-bound, and buccal cell-associated fractions. Differences were considered significant at $P < 0.05$ unless indicated otherwise.

RESULTS

Characterization of Chokeberry and Red Grape Juices. Red grape and chokeberry were chosen because of their distinctive anthocyanin profiles, which were confirmed and monitored by HPLC-PDA-ESI-MS. Characteristics of each juice are listed in Table 1. Chokeberry juice contained four ACNs differing in the type of monosaccharides conjugated to the C3 position of the Cy aglycone. This material was chosen to allow for comparisons of anthocyanin structures with the same aglycone but different sugar substitutions. Red grape juice contained different anthocyanidins with each conjugated at the C3 position to glucose. This material was chosen to allow for evaluation of the impact of the type of aglycone, as it provides different aglycone structures, all with the same glycosylation pattern (just one glucose). ACN concentration in red grape juice was slightly less than that in chokeberry. Although the content of phenolic compounds and the pH for the two juices were comparable, red grape juice contained higher sugar content than chokeberry juice.

Metabolism of ACN in the Oral Cavity. Thirteen subjects (9 females, 4 males; ages 32.8 ± 9.5 years, range = 24−50 years; and body mass index (BMI) = 22.3 ± 3.1) were recruited for oral retention of chokeberry and red grape juices. Mean losses of total ACNs during the 5 min retention in the mouth were 12.5 ± 1.6% (range = 6.1−25.2%) and 15.3 ± 1.8% (range = 7.9−30.5%) for chokeberry and red grape juices, respectively. The loss of Cy-3-xyl was significantly ($P < 0.05$) greater than that of Cy-3-gal and Cy-3-arabinose in chokeberry juice (Figure 1A). Losses of Dp- and Pt-glucosides exceeded those of Cy-, Pn-, and Mv-glucosides during oral retention of red grape, although only the loss of Dp-3-glu was significantly greater than that of the other anthocyanidins ($P < 0.05$) (Figure 1B).

Table 1. Characteristics of Chokeberry and Red Grape Juices[a]

	chokeberry juice	red grape juice
monomeric ACN (nmol Cy-3-glu equiv/mL)	64.1 ± 1.4	52.9 ± 2.2
ACN composition (% of total ACN)	Cy-3-gal, 58.1 ± 1.1	Dp-3-glu, 9.3 ± 0.9
	Cy-3-glu, 3.8 ± 0.2	Cy-3-glu, 12.5 ± 0.1
	Cy-3-arab, 26.7 ± 0.2	Pt-3-glu, 8.0 ± 0.1
	Cy-3-xyl, 11.3 ± 1.0	Pn-3-glu, 42.2 ± 0.8
		Mv-3-glu, 28.1 ± 0.1
phenolic compounds (mg gallic acid equiv/mL)	272.4 ± 17.4	279.5 ± 7.8
pH	3.75	3.89
sugar (°Brix)	0.4 ± 0.2	19.6 ± 0.2

[a]Data are the mean ± SD of three independent analyses.

Microbial-Mediated Metabolism of ACN. Loss of ACNs during oral retention decreased in 8 of 10 individuals following treatment to reduce bacterial load (matched-pair analysis; Figure 2). The antimicrobial treatment also significantly decreased the mean loss of total ACNs in chokeberry juice from 9.57 ± 0.64 to 8.11 ± 0.77% ($P = 0.036$), supporting the likelihood that the observed loss was in part contributed by microbial activity during the 5 min retention. The extent of loss of ACNs was dependent on structure, with Cy-3-Glu and Cy-3-Gal demonstrating significantly greater loss in the presence of bacteria ($P < 0.01$, repeated-measured ANOVA, Figure 3).

Delivery of ACN to Mucus and Buccal Epithelium. Buccal scrapings were fractionated into mucus and epithelial cells to determine the relative distribution of ACNs delivered to the surface of this oral tissue. Greater than 97% of chokeberry ACNs in the buccal scraping were associated with mucus. The relative amounts of Cy-3-glu and Cy-3-arab in the mucus layer were significantly ($P < 0.01$) greater than that of Cy-3-xyl (Figure 4A). The relative amount of Cy-3-glu in washed buccal epithelial cells was significantly ($P < 0.05$) greater than those of the other Cy-3-monosaccharides (Figure 4B). The amount of red grape ACN associated with buccal mucus fraction was >98% of total scraping ACN after oral retention of the juice for 5 min. ACNs associated with mucus were significantly ($P < 0.001$) affected by anthocyanidin structure. Dp-3-glu was not detected in the mucus fraction, and the relative amount of Pt-3-glu was lower ($P < 0.05$) than those of Cy-, Pn-, and Mv-3-glucosides (Figure 5A). Dp- and Pt-3-glu were detected in the washed buccal cell fraction, although the relative amounts were lower than those of Cy-, Pn-, and Mv-3-glucosides ($P = 0.09$) (Figure 5B).

The amount of mucus-associated ACNs from red grape juice (Figure 5) was significantly less than that from chokeberry (Figure 4) (50.6 ± 11.1 vs 139.4 ± 18.5 μg ACN (mg mucin)$^{-1}$ (mg specific ACN intake)$^{-1}$, respectively; $P = 0.001$). Similarly, the total amount of ACNs from red grape juice that was present

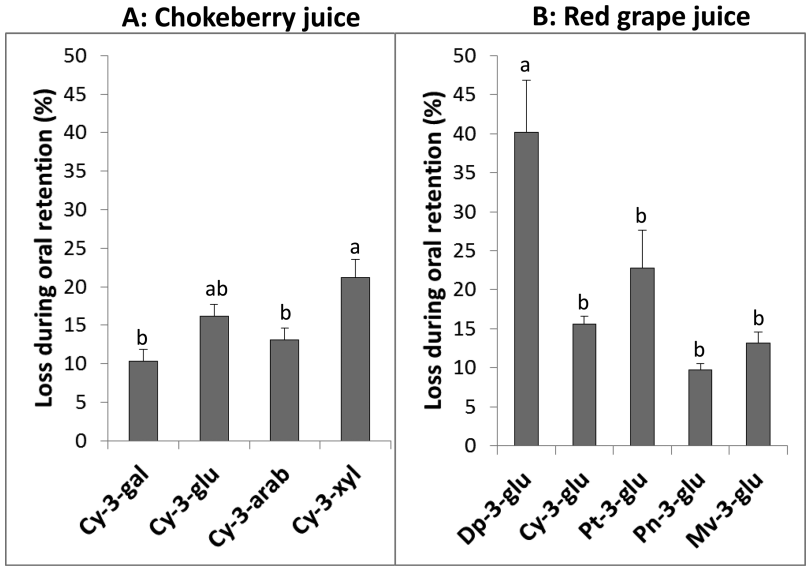

Figure 1. Loss of ACN during 5 min of retention of chokeberry (A) and red grape (B) juices in the oral cavity. Data are the mean ± SEM for 12 and 13 participants for chokeberry juice and red grape juice, respectively. Columns with different letters are significantly different ($P < 0.05$, two-sample t test).

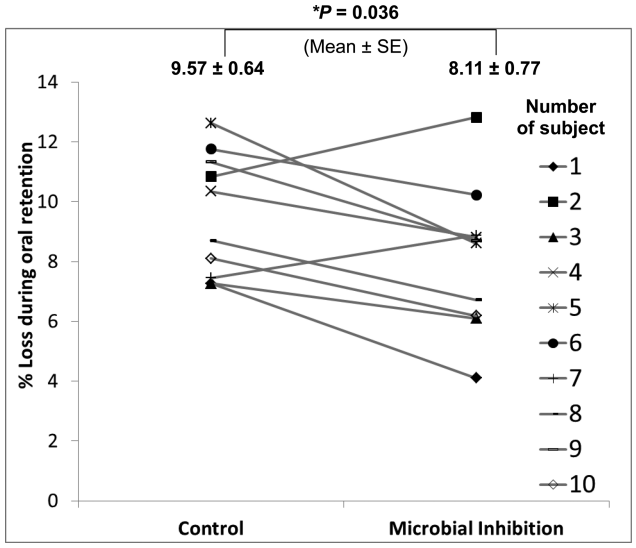

Figure 2. Extent of loss of total ACN in chokeberry juice during retention in the oral cavity was significantly decreased after removal of plaque and rinsing with antibacterial mouthwash. Matched-pair analysis and lines represent change in total chokeberry ACN in the oral cavity before and after removal of bacterial biofilms. The asterisk (∗) denotes significant ($P < 0.05$) difference between microbe-rich and microbe-depleted environments of 10 subjects.

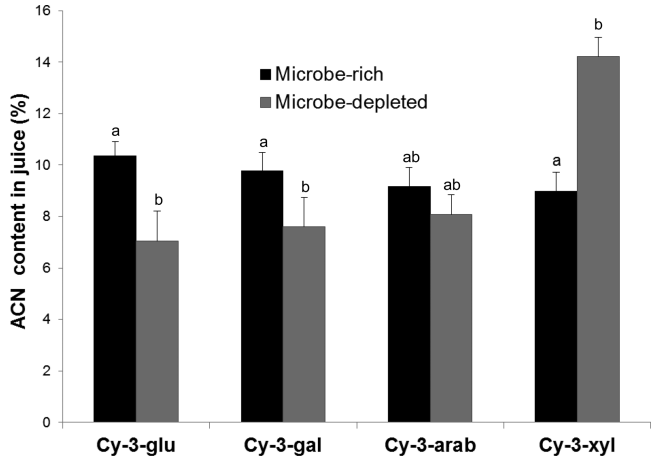

Figure 3. Degradation of ACN following treatment to reduce oral bacterial load. Data are the mean ± SEM for 10 subjects. Different letters above bars indicate that means differ significantly ($P < 0.05$).

in washed buccal cells was less than that after retention of chokeberry juice in the mouth for 5 min (2.16 ± 0.64 vs 13.5 ± 2.3 μg ACN (mg DNA)$^{-1}$ (mg specific ACN intake)$^{-1}$, respectively ($P = 0.001$). Moreover, comparison within the same subjects showed lesser relative amounts of red grape ACN in both mucus-associated and cellular fractions ($n = 5$ and 9, respectively).

To assess changes in the ACN profile of chokeberry and red grape juices during oral retention and delivery to the buccal surface, relative abundances of each ACN (i.e., percent area under curve at 520 nm) in juice, after retention in the mouth, and associated with buccal mucus and epithelium are compared in Figure 6. Notably, increases of relative abundance of Cy-3-arab (by 70%, $P < 0.001$) and Cy-3-glu (by 330%, $P < 0.001$), as well as decreases in Cy-3-gal (by 30%, $P < 0.001$) and Cy-3-xyl (by50%, $P < 0.005$), were observed in buccal cells compared to intact chokeberry juice (Figure 6A). Decreased relative amounts of Dp-3-glu (by 80%, $P < 0.001$) and Pt-3-glu (by 63%, $P < 0.005$) were coupled with increased relative amounts of Pn-3-glu (by 30%, $P < 0.001$) in buccal cells compared to red grape juice (Figure 6B).

Because protocatechuic acid (PCA) and cyanidin aglycone were recently reported to be present in the saliva of several subjects after oral retention of black raspberry juice for 3 min,[31] we examined the juice and cell extracts for the presence of possible metabolites before and after oral retention. HPLC chromatograms (250−600 nm) of juices and cell extract before and after retention were compared. Phloroglucinal aldehyde (PGA) was not found in any samples, which may be due to instability of aldehyde. PCA could not be quantified in retained chokeberry juice due to coelution with other compounds.

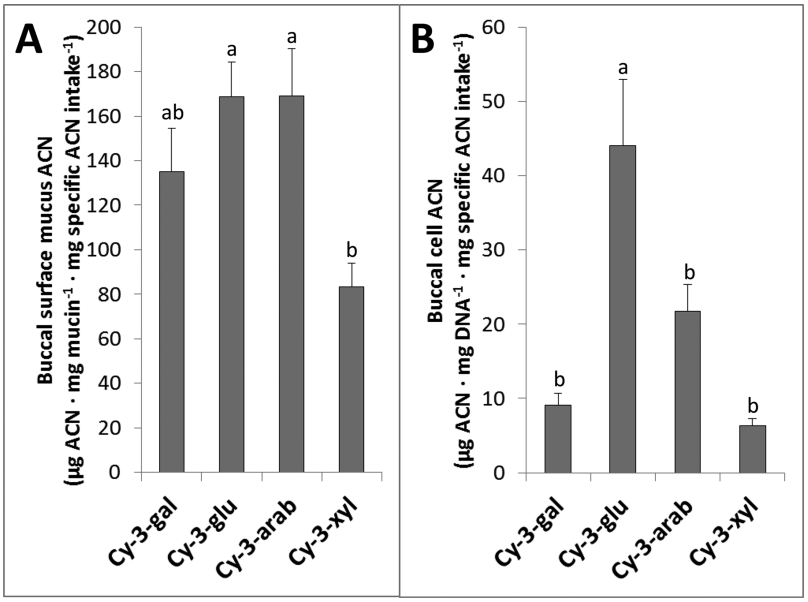

Figure 4. Adherence of chokeberry cyanidins to buccal surface mucus (A) and delivery to buccal epithelial cells (B). Data are the mean ± SEM for 8 subjects (A) and 12 subjects (B), respectively. Different letters above bars indicate that means differ significantly ($P < 0.05$).

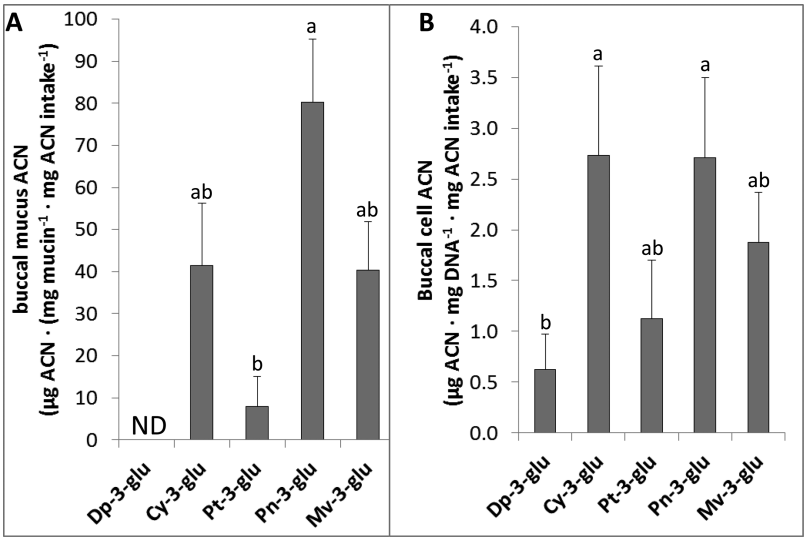

Figure 5. Adherence of ACN to buccal mucus (A) and delivery to epithelium (B) during retention of red grape juice in the oral cavity. Data are the means ± SEM for 8 subjects (A) and 12 subjects (B). Different letters above error bars indicate that means are significantly different ($P < 0.05$, A; $P = 0.09$, B). ND, not detected.

Cyanidin aglycone and PCA also were not detected in buccal cell extracts collected from 11 subjects after retention of chokeberry juice rinse (limits of detection for cyanidin aglycone and PCA are 2.0 and 3.6 ng/mL, respectively). Similarly, there was no detectable gallic acid, PCA, vanillic acid, or syringic acid, compounds that have been reported as products of cleaved B-ring of Dp, Cy, Pn, and Mv, respectively,[28,32−34,35] in either postrinse red grape juice or buccal cell extracts (limits of detection for gallic acid, vanillic acid, and syringic acid were 2.0, 2.5, and 3.3 ng/mL, respectively).

DISCUSSION

ACN-rich fruits and extracts have been shown to possess chemopreventive and chemotherapeutic activities in oral cell lines,[11,32] hamster buccal tissue,[10] rat esophagous,[36] and the human oral cavity.[13,14] This efficacy of ACN has been reported to be dependent on chemical structure.[37,38] It is also known that ACNs are degraded in the gastrointestinal (GI) tract[32,39,40] and the oral cavity.[17,31] An understanding on the effect of ACN structure on its relative stability, types of metabolites produced in the oral cavity, and availability to oral tissues is expected to provide insights for dietary recommendations and the strategic formulation of ACN-containing foods, beverages, confectionaries, and muco-adhesive gels for the promotion of oral health. We recently showed that the extent of biological and chemical degradation of ACNs in saliva is dependent in part on their chemical structure.[17] More specifically, Dp- and Pt-glycosides were degraded to a greater extent than Cy-, Pn-, and Mv-glycosides during ex vivo incubation in saliva. In the present study, we investigated the in vivo stability of ACN in the oral cavity and the levels of these compounds in buccal mucus and epithelial cells in the same human subjects who participated in

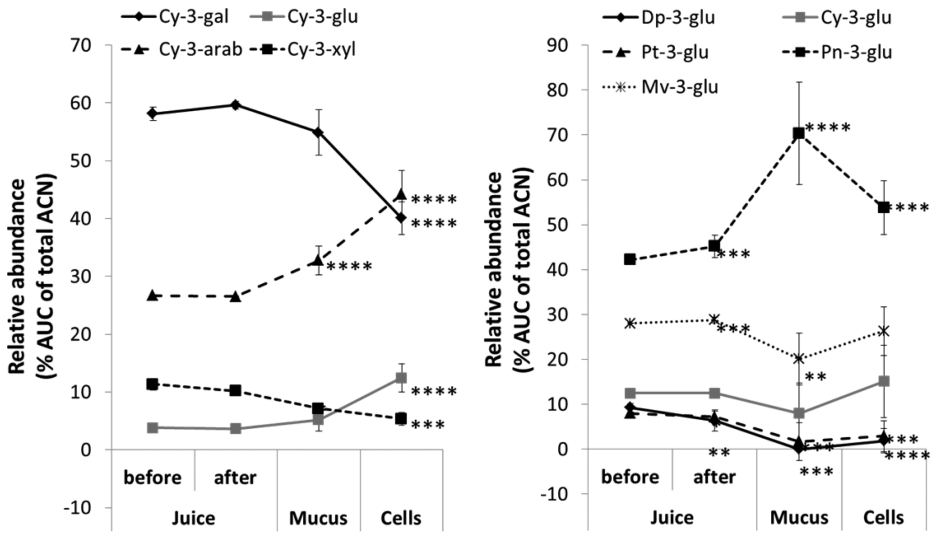

Figure 6. Change of ACN profile of ACN in chokeberry (A) and red grape (B) juices during oral retention and delivery to the buccal surface. Data are the mean ± SD for ≥8 subjects. Asterisks denote statistical difference compared to juice before retention at $P < 0.05$ (*), < 0.01 (**), < 0.005 (***), and <0.001 (****).

the earlier ex vivo study.[17] Loss of endogenous ACN from juice during brief retention in the oral cavity was used as a surrogate for metabolism in the present study. Because this loss may be attributable to spontaneous degradation, metabolism by oral microbiota,[17] adsorption to teeth and mucus,[41] and uptake by epithelial tissues,[31] the effect of each of these parameters was measured.

The extent of in vivo loss of chokeberry and red grape ACNs was affected by their structures (Figure 1B). The relative amount of Cy-3-xyl in chokeberry juice decreased during oral retention (Figure 1A), and this loss could not be attributable to selective binding to mucus (Figure 4A), metabolism by bacteria (Figure 3), or uptake by buccal epithelium (Figure 4B). Bermudez-Soto et al. reported that Cy-3-xyloside was less stable than the other Cy-3-glycosides during in vitro digestion of chokeberry juice.[42] Loss of Cy-3-gal, on the other hand, appeared to be due to microbial degradation and binding to mucus.

The preferential loss of Dp-3-glu from red grape juice during oral retention was similar to that during ex vivo incubation in saliva.[17] Our results are also in agreement with the report that glycosides of Dp, but not those of Pt, Cy, Pn, or Mv, in Concord grape juice were preferentially lost from the mouth to the ileum of ileostomy subjects.[43] However, the extent of loss could not be attributable to mucus binding (Figure 5A) or epithelial cell uptake (Figure 5B), leading us to speculate that microbial degradation or other causes may be responsible for this observation. The amount of saliva secreted during oral retention of red grape juice significantly exceeded that from subjects during retention of chokeberry juice (data not shown). We suspect that this was due to the higher concentration of simple sugars in the red grape juice (Table 1). Losses of Dp-3-glu and Pt-3-glu during 5 min of retention of red grape juice in the oral cavity were significantly ($P < 0.05$) correlated with amount of secreted saliva (data not shown). Enhanced salivary flow is associated with increased concentrations of sodium and bicarbonate ions in saliva.[44] We have shown that the presence of inorganic constituents, including bicarbonate, in artificial saliva resulted in the preferential degradation of Dp and Pt.[17]

We recently reported that microbial activity was a major contributor of ex vivo degradation of ACNs in saliva at 37 °C.[17] To evaluate whether oral microbiota contribute to the in vivo metabolism of ACNs in the oral cavity during a 5 min oral retention of chokeberry juice, the extent of loss of ACNs was compared in individual subjects before and after extensive oral disinfection (dental prophylaxis and antibacterial mouth rinse). This method has been shown to reduce the total bacterial load in the oral cavity significantly.[3] Because oral bacteria exhibit significant interindividual variability, a crossover study design was used to examine the effects of bacteria on loss of ACN during retention of juice in the oral cavity. The intervention resulted in a notable inhibition (ca. 15%) of ACN loss during the brief period of retention of juice in the oral cavity (Figure 3), suggesting that oral bacteria play a vital role in the metabolism of ACNs and may be a source of increasing availability of bioactive compounds to the host tissues.

We investigated whether there was preferential delivery of specific ACNs to buccal cells, because squamous cell carcinomas originate in this anatomical location[45] and because ACNs from black raspberries have been shown to inhibit oral cancer in hamsters[12] and human buccal lesions.[13,14] Chokeberry juice was used to investigate the effect of the type of conjugated monosaccharide on delivery of ACNs to the buccal epithelium. Strikingly, Cy-3-glu was preferentially enriched in the buccal epithelium (Figures 4B and 6A), suggesting preferential binding or uptake. When suspensions of buccal epithelial cells from two subjects were spiked with ACN-rich extract from chokeberry and incubated at 0 °C for 5 min, the relative amounts of the four Cy-glycosides were not significantly altered. This suggests that the increased bioavailability of Cy-3-glu compared to that of the other Cy-3-glycosides in chokeberry juice was temperature dependent and likely to be enzyme mediated. It has been reported that transport of Cy-3-glu by Caco-2 human intestinal cells exceeds that of Cy-3-gal.[46] The authors suggested that this difference was mediated by the specificity of the sodium-dependent glucose cotransporter-1 (SGLT1).[31] SGLT1 has also been detected in primary human buccal epithelial cells.[31,47] The possibility that Cy-3-glu is preferentially bound or taken up into

buccal epithelium during the brief retention of chokeberry juice in the oral cavity by SGLT1 and/or other unidentified transporters warrants further investigation.

Red grape juice allowed us to assess the effect of anthocyanidin structure on the bioavailability of ACNs to the buccal epithelium as it contains five distinct anthocyanidin-3-glucose compounds. Preferential loss of Dp-3-glu and Pt-3-glu in expectorated juice (Figure 1B) was coupled with lesser amounts of these ACN associated with both buccal mucus and buccal cells (Figure 5). These data suggest that the instability of Dp and Pt in the oral cavity lessens their accessibility to the buccal epithelial cells. Similarly, Stalmach et al. reported that loss of Dp-glycosides in Concord grape juice was greater than that of Pt-, Cy-, Pn-, and Mv-glycosides during passage from the mouth to the ileum of human ileostomists.[43] Delivery of ACNs to the washed buccal cell fraction during retention of red grape juice in the oral cavity was less than that for chokeberry juice (Figures 4 and 5). This difference appears to be due to the lower amount of ACN in the red grape juice (Table 1) and the greater instability of Dp-3-glu and Pt-3-glu.

Genistin (genistein-7-*O*-glucoside) is taken up by the SSC-9 human oral epithelial cell line and hydrolyzed to genistein aglycone.[48] It is possible that once ACNs are taken up by buccal epithelial cells, they are metabolized to anthocyanin aglycones that spontaneously degrade to phenolic compounds. Other investigators have reported that the degradation product PCA was detected in saliva from 2 of 10 subjects 4 h after an oral rinse with black raspberry for 3 min.[31] We did not detect ACN aglycone, PCA, PGA, or other phenolic products of ACN B-ring in the buccal cell extracts from 11 subjects immediately after the 5 min retention of chokeberry or red grape juices in the oral cavity. It appears that the generation of bioactive metabolites of ACNs may require increased duration of ACNs in the oral cavity. The possibility that there is time-dependent release of ACNs from mucin with some of the compounds becoming available to the buccal epithelium for uptake and metabolism merits investigation.

In summary, the present study provides novel insights on the effect of ACN structure on oral stability and delivery to the buccal mucosa. The results demonstrate that ACNs can be degraded in the human oral cavity and that the loss is partially due to microbial activity. These results also suggest that the structure of ACNs can affect oral stability and delivery to the buccal surface. Cy-3-glu appeared to be preferentially transferred to the buccal epithelium compared to other Cy-3-glycosides. Instability of Dp-3-glu and Pt-3-glu in the oral cavity resulted in association of lesser amounts of the ACNs with the buccal epithelium. The instability appeared to be associated with the amount of salivary secretion. These in vivo results with ACNs in a food matrix generally largely agree with the ex vivo stability and metabolism of ACNs in saliva.[17] The source of ACNs merits consideration for development of ACN-containing products for promoting oral health. The relationship between oral metabolism of ACNs and efficacy, for example, anti-inflammatory activity, as well as the role of metabolites of ACNs, warrants further investigation.

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ABBREVIATIONS USED

ACN, anthocyanin/anthocyanidin; CDC, Centers for Disease Control and Prevention; Cy, cyanidin; DMF, decayed, missing, filled teeth; Dp, delphinidin; DPA, diphenylamine assay; GI, gastrointestinal; Mv, malvidin; PCA, protocatechuic acid; PGA, phloroglucinal acid; Pn, peonidin; Pt, petunidin; SGLT1, sodium-dependent glucose cotransporter-1; WHO, World Health Organization

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