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Research Paper

Formulation and *In-Vitro* and *In-Vivo* Evaluation of a Mucoadhesive Gel Containing Freeze Dried Black Raspberries: Implications for Oral Cancer Chemoprevention

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Purpose. The purpose of these studies was to formulate mucoadhesive gels containing freeze dried black raspberries (FBR) and to determine optimum parameters for a subset of FBR bioactive compounds including anthocyanin stability, absorption and penetration *in-vitro* and *in-vivo*.
Materials and Methods. Berry gels were prepared having FBR at 5% and 10% w/w and final pHs ranging from 3.5 to 7.5. A HPLC assay was developed to quantify and determine the stability of the anthocyanins in the gels. A single time-point study was performed to determine anthocyanin uptake when the gels were applied to oral mucosa. Penetration of anthocyanins into human oral tissue explants was determined as a function of gel pH and FBR content. A HPLC-mass spectroscopy assay was utilized to quantify the anthocyanin levels in human oral tissue explants, saliva, and blood.
Results. The stability of anthocyanins in the gel was directly related to gel pH and storage temperature. Maximum stability of anthocyanins was found at lower pH (pH 3.5) and storage temperature (4°C). Anthocyanins contained in mucoadhesive berry gel formulations were readily absorbed into human oral mucosa tissue as evidenced by detectable blood levels within 5 min after gel application. There was a trend for greater penetration of anthocyanins into tissue explants for berry gels with a final pH of 6.5 versus pH 3.5.
Conclusions. Formulation and characterization of a novel gel formulation for local delivery of chemopreventive compounds to human oral mucosal tissues has been described. The results show anthocyanin stability was dependent upon gel pH and storage temperature and also demonstrate that the gel composition is well-suited for absorption and penetration into the target oral mucosal tissue site.

KEY WORDS: anthocyanins; black raspberries; chemoprevention; local delivery; oral cancer.

INTRODUCTION

Oropharyngeal cancer annually affects approximately 29,370 persons in the U.S., and more than 7,200 Americans die each year from this disease (1). Despite concerted efforts to improve treatments, 5 year survival rates for patients with advanced stage oral squamous cell carcinoma (oral SCC) remain among the lowest of solid cancers. Furthermore, even for those persons who are cured, morbidity remains high due to loss of tissues that are critical for esthetics and function (2). Another significant concern for persons diagnosed with oral SCC is the potential for tumor recurrence or the development of a second primary cancer (3–5). Clearly, prevention or early detection of precancerous oral lesions combined with strategies for local intervention to prevent progression to overt oral SCC could dramatically improve clinical outcomes.

Cancer chemoprevention, which represents one promising approach, is defined as the prevention, inhibition or reversal of carcinogenesis by intervention with chemically derived or naturally occurring dietary substances. While this concept is conceptually appealing, the results of previously conducted oral cavity chemoprevention trials have not been highly promising. Although pharyngeal premalignant lesions often respond to chemoprevention therapy, oral cavity lesions were frequently unresponsive, even to combination agents such as a cis-retinoic acid, IFN-α, and α-tocopherol cocktail (6). Some agents such as vitamin A derivatives were partially effective in managing oral premalignant lesions, but these formulations often caused toxicities such as severe mucositis

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(7). Recently, a protocol that used an attenuated adenovirus (ONYX-015) containing mouthwash to target p53 defective cells induced a 37% transient resolution of epithelial dysplasia (8). This protocol, however, was also associated with increases in circulating antiadenoviral antibody titers (8).

The majority of previously conducted oral chemoprevention trials employed systemic agent administration. Local delivery methods, however, have several advantages for site-specific, targeted disease management. Local therapies provide high, clinically effective concentrations directly to the treatment site, without causing deleterious systemic side effects. Local delivery is not always a panacea-as it is necessary to carefully select diseases amenable to such treatments. Oral epithelial dysplasia is an appropriate target as it can be directly visualized (facilitates both determination of efficacy and agent placement by patient) and compounds can be released into saliva to provide coverage throughout the mouth.

For persons with oral epithelial dysplasia, chemoprevention is likely to be necessary for the remainder of their lives. Consequently, identification of nontoxic, effective chemopreventive compounds is of paramount importance. Recent studies from our laboratories have demonstrated that black raspberries possess cancer preventing properties at both *in-vitro* and *in-vivo* levels (9–13). Dietary administration of freeze-dried black raspberries (FBR) successfully inhibited nitrosamine-induced esophageal tumorigenesis in rats (9) and also prevented dimethylbenz(a)anthracene (DMBA)-initiated oral carcinogenesis in the hamster cheek pouch (10). *In-vitro* studies, which showed freeze-dried black raspberries (FBR) prevent benzo(a)pyrene-induced transformation of Syrian hamster embryo cells (11), and inhibit activation of the redox responsive transcription activating factors NF-κB and AP-1 (12), demonstrated FBR's ability to scavenge reactive species and their cytoprotective properties. Further, treatment of oral SCC cell lines with an ethanol/water extract of FBR (RO-ET) elicited a wide array of chemopreventive effects that included reduction in pro-angiogenic cytokine release, inhibition of proliferation, induction of differentiation, and suppression of the "high output" nitric oxide synthase isoform (NOS-2) (13). Phase I human clinical trials conducted by our laboratories have confirmed that dietary administration of high dosages of freeze-dried black raspberries for periods up to 7 days is well tolerated (14). Finally, when contemplating development of a natural product based treatment, it is beneficial to first identify its bioactive constituents because preservation of these agents indicates compound stability in the final formulation. Recent characterization studies by Hecht *et al.*, which used spectral properties as well as comparison with anthocyanin standards, identified cyanidin glycosides as bioactive compounds in FBR extracts (15).

The purpose of these studies was to formulate mucoadhesive gels containing freeze dried black raspberries (FBR) and evaluate these gels for anthocyanin stability, absorption and penetration into human oral mucosa both *in-vitro* and *in-vivo*.

MATERIALS AND METHODS

Materials

Noveon AA1 (NF) was obtained from Noveon, Inc. (Cleveland, OH). Carbopol 971P (NF) was a gift from BF Goodrich Specialty Chemicals (Cleveland, OH). Edetate disodium (EDTA; USP), 2-phenoxyethanol (BP), benzyl alcohol (USP), glycerin (USP), sodium hydroxide (NF), and formic acid (ACS reagent) were obtained from Spectrum Quality Products, Inc. (New Brunswick, NJ). Acetonitrile (HPLC grade) was from Fisher Scientific (Hampton, NH). Reagents used for the tissue explant analyses (sodium carboxymethylcellulose, sorbitol, NaCl, KCl, calcium chloride dehydrate, $MgCl_2$, Trizma hydrochloride) were purchased from Sigma Chemical Company (St. Louis, MO.).

All black raspberries used in these studies were of the Jewel variety (***Rubus occidentalis***) and were grown at the Stokes Raspberry Farm (Wilmington, OH.). All berries were grown in the same part of the field, and picked at about the same degree of ripeness (when the majority of the berries in a cluster have turned black—this occurs within a period of 1–2 weeks). The berries were harvested mechanically (with a picker) in a total period of 4 hr, washed, and frozen at −20°C within an hour of the time of harvest. The berries remain frozen until they are transported frozen to Van Drunen Farms in Momence, IL, where they are then freeze-dried and ground into a powder as described previously (9–14).

All berries picked during a single year were freeze-dried at the same time. The freeze drying process, which included the entire black raspberry fruit including seeds, resulted in very little destruction to any of the berry components measured which included minerals (calcium, copper, magnesium), vitamins and carotenoids (folate, α and β carotene), sterols (β-sitosterol, campesterol), phenolics (ellagic acid, ferulic acid, p-coumaric acid, and anthocyanins). The freeze-dried berries were shipped frozen from Van Drunen Farms to The Ohio State University and stored frozen before experimental use. Our analyses indicate that berries can remain frozen for at least 1 year with negligible loss of measured components. However, because not every component is measured, there is the possibility that some components may degrade during storage. Random samples of each batch of freeze-dried berries were shipped to Covance Laboratories for measurement of the components. On average, comparison of berries picked over a period of 5 years, indicated variation in content of most components is no more that 10–20%.

Preparation and Characterization of Prototype Berry Gels

Prototype mucoadhesive berry gels having different final pHs and either 5% or 10% w/w FBR were prepared for various analyses and *in-vivo* studies. The five studies were as follows: (1) pH 6.5 gels with 5% w/w FBR for a human pharmacokinetic study, (2) pH 6.5 gels with 5% and 10% w/w FBR for anthocyanin uptake studies in human mucosa explant tissue, (3) pH 4.5, 5.5, 6.5, and 7.5 gels with 10% w/w FBR to determine anthocyanin stability at 1 week at 4°C, 25°C, and 40°C, (4) pH 3.5 and pH 4.0 gels with 10% w/w FBR to determine anthocyanin stability over 1 month at 4°C and 25°C, and (5) pH 3.5 and pH 6.5 gels with 10% w/w FBR to compare difference in anthocyanin uptake into human mucosa explant tissue.

All gels (200 g batches) were prepared at room temperature in stainless steel vessels (350 mL) using a Caframo Stirrer Model BDC-1850 (Wiarton, Ontario, Canada) with attached metal blade. To the required amount of purified

water stirring in the vessel, Noveon AA1 and Carbopol 971P were added slowly and allowed to fully hydrate for at least 1 hr at 1,025 rpm. Next, glycerin, 2-phenoxyethanol, and benzyl alcohol were added followed by EDTA and the mixing speed was reduced to 875 rpm. The gel was allowed to mix for another 1 hr at room temperature at 875 rpm. Sodium hydroxide (2.5 N) was added to raise the pH of the gel to pH 7.5. Placebo gels were semi-transparent and homogenous in appearance. Finally, powdered FBR was added at room temperature while stirring at 875 rpm in different amounts to produce final concentrations of FBR of either 5% or 10% w/w. Either 2.5 N NaOH or 2.33 N HCl was added to adjust to the desired pH. Purified water was then added to q.s. the gels to weight. The berry gels were mixed for an additional 15 min to produce homogenous berry gels.

Gels having a final pH in the range of pH 4.5–7.5 had the following final concentration of excipients: Noveon AA1 (0.675% w/w), Carbopol 971P (0.788% w/w), glycerin (0.9% w/w), 2-phenoxyethanol (0.9% w/w), benzyl alcohol (0.9% w/w), and EDTA (0.09% w/w). Two adjustments were subsequently made for gels having a final pH of either pH 3.5 or 4.0. First, the amounts of Noveon AA1 and Carbopol 971P needed to produce a final berry gel having comparable viscosity at lower pH were significantly increased. Second, the concentration of all other excipients was increased by 10% to adjust for the dilutive effects of adding powdered FBR at the level of 10% w/w. Thus, gels having a final pH of either pH 3.5 or 4.0 had the following final concentration of excipients: Noveon AA1 (1.35% w/w), Carbopol 971P (1.575% w/w), glycerin (1.0% w/w), 2-phenoxyethanol (1.0% w/w), benzyl alcohol (1.0% w/w), and EDTA (0.1% w/w).

All prepared gels were stored at 2–8°C (unless stated otherwise), sealed and protected from light in 10 g white Aluminum Tubes, sealed neck with Morton PE 1090 liner (Montebello Packaging; Ontario, Canada). Gels were characterized for pH, viscosity, osmolality, and anthocyanin content. The pH of gels was measured using an Orion pH meter Model 520A. Gel viscosity was determined using a Brookfield Cone & Plate Rheometer Model RVDV III+ (Brookfield Engineering, Middleboro, MA) at 25°C for 1 min at an RPM of 4 using Spindle CPE-52. The osmolality was measured using a FISKE 110 Freezing-Point Depression Osmometer.

The Microbial Limits Test <61> in USP 29 was used to determine the total microbial count and for the presence of yeasts and molds for only the final prototype 10% mucoadhesive berry gel (pH 3.5) shown in Table I.

Table I. Composition of Final Prototype Mucoadhesive Berry Gel (pH 3.5) for Mucosal Application

Ingredient (Grade)	Function	% w/w
Purified Water	Base vehicle	Qs to 100%
Noveon AA1 (NF)	Mucoadhesive	1.35
Carbopol 971P (NF)	Mucoadhesive/ Polymer thickening	1.575
Glycerin (USP)	Emollient	1.0
Edetate Disodium (EDTA) (USP)	Metal chelator (anti-oxidant)	0.1
2-Phenoxyethanol (BP)	Anti-microbial preservative	1.0
Benzyl alcohol (USP)	Anti-fungal preservative	1.0
2.5 N NaOH or 2.33 N HCl	Adjust to final pH of 3.5	as needed
Freeze-dried black raspberries (FBR)	Active (5% or 10% w/w)	5.0 or 10

HPLC Assay for Quantification and Stability Determination of Berry Anthocyanins

A stability-indicating reversed-phase HPLC assay was developed and partially validated to quantify anthocyanins in FBR. The HPLC assay was developed based on a previously described assay by Tian *et al.* (16). For the assay, a Thermoquest HPLC system with a UV6000LP photodiode array detector, a Water's Symmetry C18 column (3.9 × 150 mm, 5 μm), and a Phenomenex Security Guard with C18 cartridge were employed. FBR standards (0–2 mg/mL) were prepared by dissolving FBR in aqueous 1% formic acid (mobile Phase A) and applying some vortexing to completely dissolve the powder. The slightly cloudy standards were filtered through a 0.45 μm hydrophilic PTFE syringe filter into a HPLC vial. A linear gradient (flow rate of 1.0 mL/min) of mobile Phase A (1% formic acid) and mobile phase B (100% acetonitrile) was utilized at a column temperature of 40°C as follows: 93% A (isocratic) for 0–2 min, 93% A to 89% A (linear gradient) for 2–18 min, 89% A to 0% A (linear gradient) for 18–19 min, 0% A (isocratic) for 19–25 min, 0% A to 93% A (linear gradient) for 25–26 min, and 93% A (isocratic) for 26–35 min. The total run time for the assay was 35 min; 25 min to allow for all potential degradation components to elute, and the remaining 10 min to sufficiently re-equilibrate the column to the initial conditions. The injection volume was 10 μL and the detection wavelength for the anthocyanins was 520 nm. For berry gels, 100 mg of gel was added to a 10 mL volumetric flask with aqueous 1% formic acid followed by the subsequent steps as described above.

Pharmacokinetic Analyses to Evaluate Anthocyanin Uptake from Human Oral Mucosa

Participation of human subjects in these studies was conducted in accordance with an IRB approved protocol (The Ohio State University IRB protocol #2003C0050). None of the human subjects had a diet rich in anthocyanin compounds prior to participation in either the pharmacokinetic or tissue explant studies. For the pharmacokinetic analyses, nine consented adult volunteers dried the anterior floor of their mouth, placed 1.0 g of 5% berry gel (pH 6.5), and massaged the gel in place for 30 s to facilitate uptake. Two minutes after gel application saliva was collected for the next 3 min and peripheral blood was drawn 5 min after gel application. Following clotting, sera samples were collected, and both the sera and saliva samples stored at −80°C until LC-MS analysis as described below.

Tissue Explant Studies to Assess the Effects of Berry Gel FBR Content and pH on Uptake of Anthocyanins by Human Oral Mucosal Explants

Human oral tissues for the tissue explant studies were obtained from consented volunteers who were undergoing

elective oral surgery procedures. To confirm "no pathologic diagnosis," a portion of each of these tissues was submitted for light microscopic evaluation. All tissue donors were systemically healthy and did not use tobacco products. Tissues for explant experiments were immediately placed in an artificial saliva transport medium, which also served as the incubation medium for the gel absorption studies. The composition of the artificial saliva (pH 7.4) used for tissue transport and incubation was (reagents listed in final concentrations): sodium carboxymethylcellulose (0.5%), sorbitol (165 mM), NaCl (14 mM), KCl (16 mM), calcium chloride dehydrate (1.0 mM), $MgCl_2$ (0.63 mM), and Trizma hydrochloride (2.0 mM).

Tissues from eight donors were used for preliminary studies to compare the penetration of anthocyanins from the 5% berry gel and the 10% berry gel, prepared at a final pH of 6.5. In order to account for heterogeneity among human donors, tissue samples were hemisected and one tissue portion treated with 5% and the other with 10% berry gels. One gram of berry gel (containing either 5% or 10% w/w FBR) was placed on the epithelial surface of the explant, followed by gently rubbing of the site for 30 s to facilitate uptake. Explants were then placed into artificial saliva, incubated for 30 min (37°C, 5% CO_2), and treated tissues then frozen at −80°C until HPLC-mass spectroscopy analyses, as reported in our recent publication (13). Tissue preparation for HPLC analyses consisted of homogenization of the entire oral mucosal specimen, removal of an aliquot for sample protein level determination, followed by the addition of collagenase type II (0.1 mg/mL, GIBCO, Grand Island, NY) and 1% formic acid. Samples were then placed on a Sep Pack (Millipore, Billerica, MA.), eluted with methanol, and dried under argon gas.

A second series of oral mucosal explant studies were conducted to compare the effect of berry gel pH on absorption into oral mucosal explants. Tissues from eleven donors were used for these studies, and handling was as described above with the exception that all gels contained 10% w/w FBR and were formulated at a final pH of 3.5 or 6.5.

HPLC-MS Assay to Determine Anthocyanin Content in Human Sera, Saliva and Oral Mucosal Tissue Explants

The respective samples (sera, saliva or tissue homogenates) were dissolved in 90% solvent A (water plus 1% formic acid) and 10% solvent B (acetonitrile plus 1% formic acid) and then separated on a Symmetry C_{18} reversed-phase column and analyzed using a Waters 2695 gradient HPLC separation module equipped with a 996 photodiode-array (PDA) UV/visible absorbance detector (Milford, MA). The solvent system consisted of a step gradient from 90% A to 50% B over 15 min, and the absorption spectra were recorded from 200–800 nm with the inline PDA detector. Mass spectrometry was conducted on a quadrupole ion tunnel mass spectrometer equipped with a Z-spray ESI source. Quantification of anthocyanins and their metabolites was conducted using a standard curve generated from standard cyanidin 3-glucoside; range, 0–255 pmol; R^2 0.985. The method detection limit (MDL) was determined to be 0.31 pmol according to the U.S. Environmental Protection Agency approach that the MDL is the concentration corresponding to 3ó of seven measurements of the analyte at very low concentrations. The lower limit of quantification is 0.98 pmol according to an EPA approach representing the concentration corresponding to 3.18 time of MDL. All anthocyanins are expressed as cyanidin 3-glucoside equivalents.

Determination of Explant Tissue Protein Levels

Explant protein levels were determined by the Lowry method, using bovine γ-globulin as the standard protein (17).

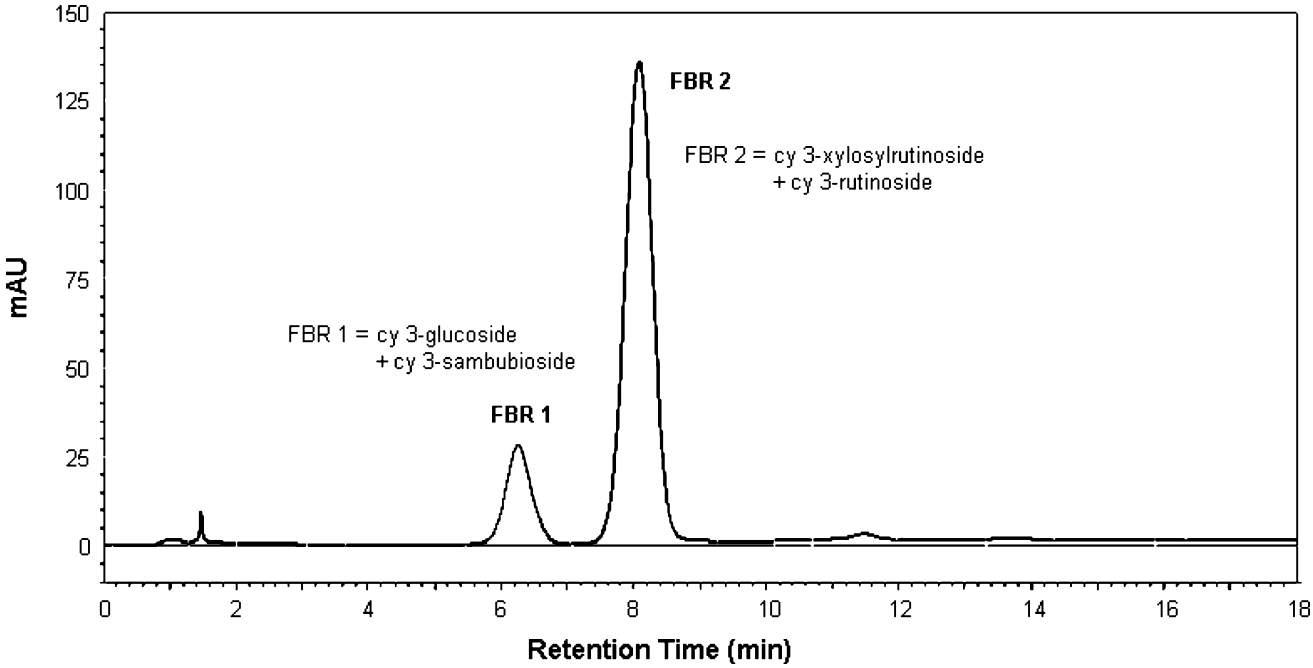

Fig. 1. Standard HPLC chromatogram of anthocyanins in freeze-dried black raspberries. FBR 1 corresponds to cy 3-glucoside and cy 3-sambubioside and FBR 2 corresponds to cy 3-xylosylrutinoside and cy 3-rutinoside. The 1% formic acid used in this HPLC assay was not able to separate into the two components of FBR 1 and FBR 2.

Statistical analyses. A two-tailed Mann Whitney *U*-test was used to determine whether or not gel pH (pH 3.5 relative to 6.5) significantly affected anthocyanin uptake by oral mucosa.

RESULTS

Preparation and Characterization of Mucoadhesive Berry Gels

Gels were prepared by adding FBR to semi-transparent and homogenous placebo gels to achieve a final FBR concentration of up to 10% w/w. For unbuffered placebo gels, the addition of FBR resulted in a lowering of the gel pH in proportion to the amount of FBR added. For example, the addition of 10% FBR to a placebo gel with a pH of 6.9 resulted in a final pH of the 10% w/w gel of 5.9. However, the pH of the 10% w/w berry gel could easily be adjusted with NaOH or HCl to achieve a gel with the desired pH. The drop of pH after the addition of FBR was due to the naturally low pH of the FBR since a 10% w/w slurry of the FBR in water had a pH of 4.3. The addition of FBR to placebo gels resulted in a red-to-purple colored gel, and the color intensity increased with increased FBR content. All berry gels were homogenous in texture and appearance with no visible suspended particulates. The berry gels had viscosities in the range of 16,000 to 20,000 cP using the viscosity method and conditions described. Gel osmolality increased with increasing FBR content; with the highest osmolality determined in the pH 3.5 10% mucoadhesive berry gel (811 ± 9 mOsm/kg H_20).

For the final prototype 10% mucoadhesive berry gel (pH 3.5) shown in Table I, the total aerobic microbial count was <300 colony forming units (cfu)/g and the gel was found to be free of *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Pseudomonas aeruginosa.* No mold was found in the gel after storage for 6 months at 2–8°C; however, yeast was present in the gel at 300 cfu/g and was identified as *Candida colliculosa*. All microbial counts were determined to be within the predetermined GMP acceptable bioburden range.

HPLC Assay for Quantification and Stability Determination of Berry Anthocyanins

An HPLC assay was developed to quantify the anthocyanin content and stability in the berry gels. As shown in Fig. 1, the standard chromatogram gives two peaks, termed FBR 1 and FBR 2. These two peaks correspond to the four peaks shown in Tian *et al*. (16). It was subsequently determined using LC-MS (see below) that FBR 1 correspond to cyanidin 3-glucoside and cyanidin 3-sambubioside and FBR 2 correspond to cyanidin 3-xylosylrutinoside and cyanidin 3-rutinoside. For initial development of the HPLC assay, a 1% formic acid mobile phase was used instead of 10% formic acid as previously described by Tian *et al*. to conserve the stability and integrity of the C18 column. The standard curve for both FBR 1 and FBR 2 showed excellent linearity over the range of 50 μg/mL to 2,000 μg/mL. System suitability for the assay calculated by averaging the peak areas of all standard injections showed that the relative standard deviation (RSD) of the assay was less than 1%. In addition, the recovery of anthocyanins (FBR 1 and FBR 2) from the gels was greater than 99%.

The Effect of Gel pH and Storage Temperature on Anthocyanin Stability

Initial studies indicated that lower pH and lower storage temperatures were more favorable for FBR1 and FBR2 peak stability. Figure 2A shows the percent detected for 10% berry gels at various pHs after 24 hr storage at 4, 25, and 40°C. For both FBR1 and FRB2, there was a very strong correlation between pH storage temperatures in terms of peak retention. It is well known that anthocyanins exist at low pH as the flavylium cation, which is their naturally occurring form. The flavylium cation is highly electron deficient and intensely colored (red or orange) at a pH below about pH 4.5. At higher pHs anthocyanins exist as either a quinoidal base, carbinol pseudobase, or the chalcone pseudobase. Thus, in addition to being less stable at higher pH, the flavylium cation is known to be less stable at increased temperatures (18,19).

As a result of this initial study, a second stability study was performed using four gels formulated at 3.5 and 4.0 with and without 0.1% w/w ascorbic acid as an additional anti-

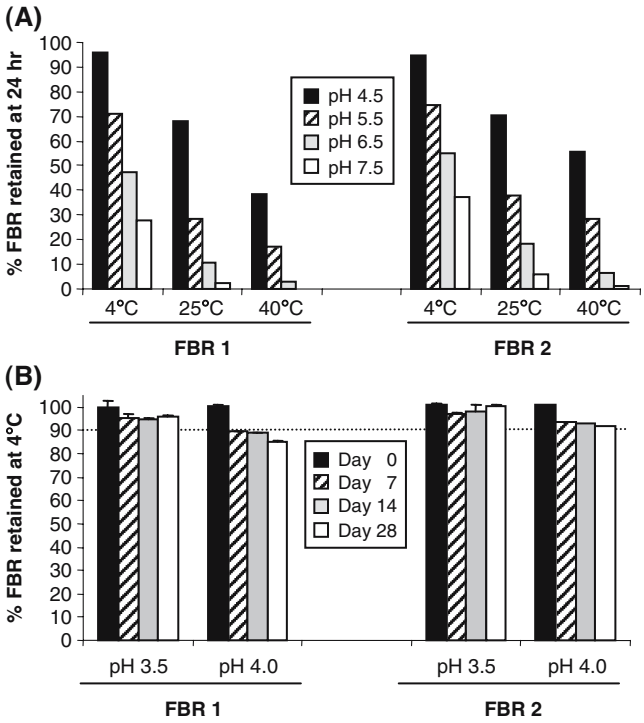

Fig. 2. Anthocyanin stability in the berry gels as measured by retention of FBR 1 and FBR 2. (A) Anthocyanin stability in the berry gels at 24 hr as a function of final gel pH (pH 4.5, 5.5, 6.5, and 7.5) and storage temperature (4, 25, and 40°). Storage conditions were: (1) 4°C (controlled 2–8°C refrigerator), (2) 25°C (controlled stability chamber at 25°C/60% relative humidity, and (3) 40°C (controlled stability chamber at 40°C/75% relative humidity. The results showed a strong and direct effect of both pH and temperature, with lower pH and temperature resulting in the greatest retention of FBR 1 and FBR 2 peak area. (B) Anthocyanin stability in the berry gels over 28 days as a function of final gel pH (pH 3.5 and 4.0). The results showed nearly quantitative retention of FBR 1 and FBR 2 over 28 days at a pH of 3.5.

oxidant. The results as shown in Fig. 2B demonstrate that the pH 3.5 gel provided excellent stability for FBR1 (96.0 ± 0.8%) and FBR 2 (100.2 ± 0.4%) over 1 month at 4°C. Gels at pH 4, gels stored at 25°C, and gels with 0.1% ascorbic acid (not shown) demonstrated less stability for both FBR1 and FBR2. The results of the second stability study were very encouraging and demonstrated that pH 3.5 provided for the greatest stability of FBR 1 and FBR 2 when 10% FBR was formulated in the gel and stored at 4°C. With regard to bioactive compound stability, a 10% w/w berry gel, formulated at pH 3.5 and stored at 4°C was therefore identified as a promising clinical formulation.

Anthocyanins are Readily Detected in Berry Gel Treated Oral Mucosal Explants

Preliminary studies, which compared the ability of 5% relative to 10% berry gels (both adjusted to a pH of 6.5) to absorb into human oral mucosa explants, revealed that the 10% berry gel delivered detectable levels of all four anthocyanin compounds (as shown in Fig. 3) into all eight of the tissue explants evaluated. In contrast, only two anthocyanins (cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside) were identified in tissues treated with the 5% gel formulations.

Bioadhesive Berry Gels are Readily Absorbed into Human Oral Mucosa

Concurrently conducted human pharmacokinetic studies which used the 5% berry gel (pH 6.5) demonstrated that bioadhesive berry gels are readily absorbed into human oral mucosa (Table II). All nine participants had detectable anthocyanin levels of three anthocyanins (cyanidin 3-rutinoside, cyanidin 3-xylosylrutinoside, cyanidin 3-glucoside) in either their saliva or blood, and 4 donors had detectable levels of two anthocyanins (cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside) in their peripheral blood. Saliva from eight of the nine donors contained detectable levels of either two (cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside-four donors) or three anthocyanins (four donors).

Higher Berry Gel pH Values are Associated with Trends Towards Greater Penetration into Human Oral Tissue Explants

In contrast to the human pharmacokinetic evaluations, tissue explant studies showed the presence of all four anthocyanins contained in FBR, inclusive of cyanidin 3-sambubioside. Further, these data, demonstrated approximately 2–3 fold higher levels of the respective anthocyanin in tissue explants relative to levels determined in either saliva or blood. As shown in Table III, studies to compare the effect of gel pH on tissue anthocyanin uptake reveal trends that show greater penetration with the pH 6.5 gels. Four donors' tissues showed increased penetration of all four anthocyanin compounds with the pH 6.5 relative to the pH 3.5 berry gels. Notably, there were large variations that reflected both the anthocyanin under evaluation as well as the penetrability of the individual donors' tissues. Further, depending upon the anthocyanin compound under analysis, there were between four to six tissue donors who consistently showed greater penetration with the pH 3.5 gel. Large differences (up to 15-fold) in detectable anthocyanin levels were found in the gel treated donor tissues; findings that likely reflect the extensive heterogeneity within human tissues that affects tissue penetrative abilities. Finally, although these data demonstrate

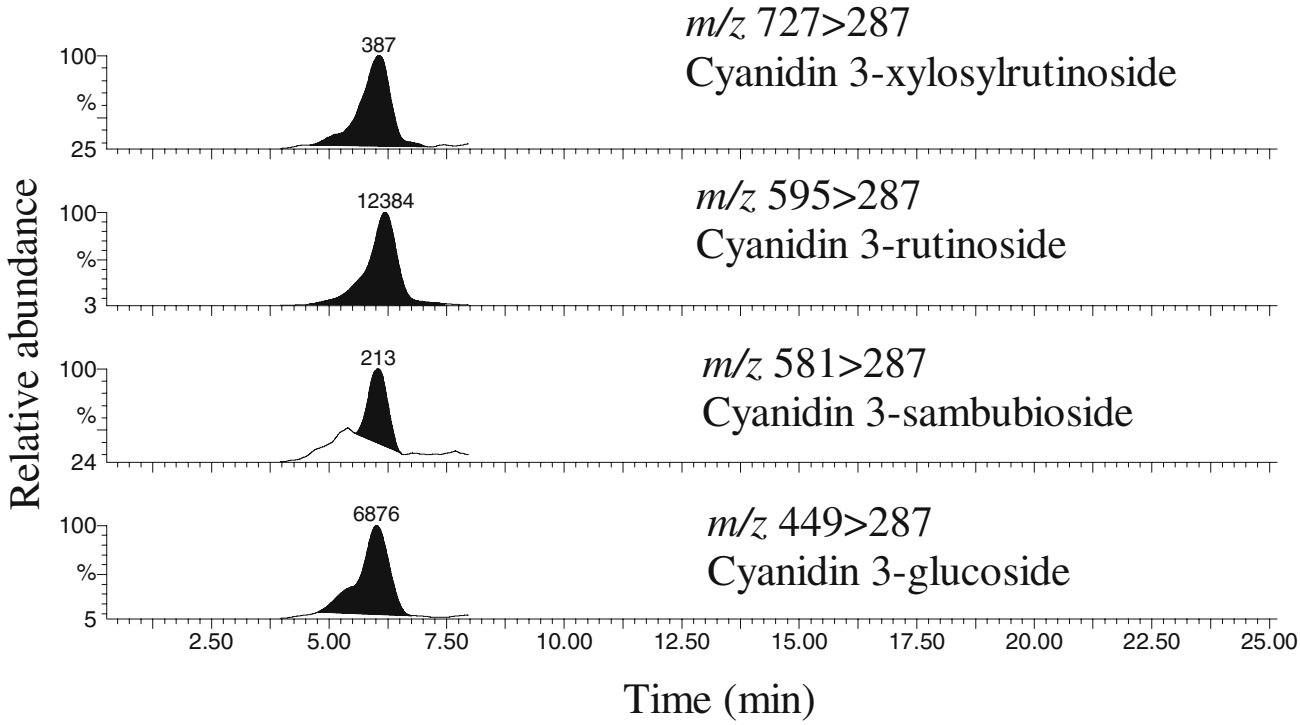

Fig. 3. Representative mass spectroscopy HPLC chromatogram, which shows the presence of peaks corresponding to the four anthocyanins present in black raspberries, of an oral mucosal tissue explant following topical application of berry gel containing 10% w/w FBR.

Table II. Anthocyanin Concentration (pmol/ml) in Saliva and Peripheral Blood 5 Min After Topical Application of a 5% Berry Gel, pH 6.5 to the Floor of the Mouth

Patient		SML 101	SML 102	SML 103	SML 104	SML 105	SML 106	SML 107	SML 108	SML 109
Gender		F	M	M	F	M	M	M	F	M
Age		50	62	29	48	42	59	33	56	48
Current Smoking Status		Non-Smoker	Non-Smoker	Non-Smoker	Non-Smoker	Non-Smoker	Non-Smoker	Non-Smoker	Non-Smoker	Non-Smoker
Saliva (pmol/ml)	cyanidin 3- rutinoside	3.5	1.7	ND	4.6	8.3	6.1	9.3	4.2	2.2
	cyanidin 3- xylosylrutinoside	2.0	0.5	ND	1.5	2.3	1.9	1.7	1.0	1.2
	cyanidin 3- glucoside	ND	ND	ND	11.2	9.7	ND	19.7	ND	22.8
Blood (pmol/ml)	cyanidin 3- rutinoside	ND	ND	2.3	ND	7.4	5.6	5.8	ND	ND
	cyanidin 3-xylosyl rutinoside	ND	ND	0.6	ND	1.1	0.8	1.3	ND	ND
	cyanidin 3- glucoside	ND	ND	ND	ND	ND	ND	ND	ND	ND

*ND = not detectable

Table III. Effect of Final Berry Gel pH on the Anthocyanin Uptake into Human Oral Mucosa Explant Tissues

Tissue Explant Sample No.	Cyanidin 3-glucoside (pmol per mg tissue)			Cyanidin 3-sambubioside (pmol per mg tissue)			Cyanidin 3-rutinoside (pmol per mg tissue)			Cyanidin 3-xylosylrutinoside (pmol per mg tissue)		
	Gel pH 3.5	Gel pH 6.5	% change 3.5–6.5	Gel pH 3.5	Gel pH 6.5	% change 3.5–6.5	Gel pH 3.5	Gel pH 6.5	% change 3.5–6.5	Gel pH 3.5	Gel pH 6.5	% change 3.5–6.5
001	128.1	296.6	131.6	7.4	30.9	319.4	116.4	668.3	474.1	14.2	88.1	522.5
002	13.6	18.5	36.2	0.6	2.9	427.3	9.3	67.8	627.2	1.2	10.3	747.1
003	14.4	12.0	−16.6	1.8	1.4	−19.7	34.3	29.2	−14.8	5.7	6.4	12.1
004	1.6	12.0	653.5	1.3	0.4	−66.4	26.3	9.4	−64.4	3.6	1.3	−65.1
005	32.0	52.9	65.3	0.3	0.1	−61.8	3.5	2.8	−20.0	0.9	0.5	−45.9
006	31.5	60.3	91.3	2.3	10.1	330.8	45.7	211.1	362.1	9.9	35.5	258.5
007	17.0	22.8	34.5	0.4	0.4	−14.6	5.6	8.1	45.1	1.1	1.0	−9.4
008	33.2	19.7	−40.7	0.0	1.9	–	0.4	35.1	8905.1	0.0	6.3	–
009	21.2	45.3	113.9	2.9	10.3	256.9	43.9	207.4	395.1	9.9	41.3	318.4
010	13.9	4.6	−66.9	2.0	0.1	−92.9	37.1	3.2	−91.4	6.7	0.5	−91.9
011	18.9	7.5	−60.1	2.1	0.0	−100	28.7	0.4	−98.7	6.0	0.00	−100
Avg. ± SEM	29.6 ± 10.3	50.2 ± 25.3	85.6 ± 60.4	1.9 ± 0.6	5.3 ± 2.8	89.0 ± 60.3	31.9 ± 9.8	113.0 ± 60.3	956.3 ± 797.9	5.4 ± 1.4	17.4 ± 8.3	140.6 ± 85.6

trends towards enhanced tissue penetration with the higher pH 6.5 berry gel, due to large inter and intra donor standard deviations, these differences were not statistically significant.

DISCUSSION

The majority of oral cavity chemoprevention trials conducted to date have employed systemic administration of synthetic retinoids. Many of these studies resulted in some degree of clinical success (6,7,20–24). Critical evaluation of the clinical trials data, however, provides a strong rationale to evaluate other chemopreventive compounds. Retinoid use was associated with significant toxicities that included mucositis, conjunctivitis, dry skin and hypertriglyceridemia (20–24). Also apparent from the synthetic retinoid trials was the transient nature of any clinical response (23,24). The majority of patients relapsed within several months after discontinuation of therapy, emphasizing the need to identify effective, well-tolerated agents for long term applications. In addition to vitamin A derivatives, there are many additional chemopreventive compounds that have elicited a variety of cancer preventing effects including induction of apoptosis, stimulation of differentiation and suppression of cell proliferation in both *in-vitro* and *in-vivo* models (25–28). Relevant to this current study, compounds which provide natural colors to fruits and vegetables such as the anthocyanins, have been shown to demonstrate a wealth of chemopreventive properties (15,27–30). While anthocyanins represent the primary flavinoids in FBR, black raspberries also contain additional chemopreventive compounds including ferulic acid, coumaric acid, quercetin, and phtyosterols. The freeze drying process concentrates the berries bioactive constituents approximately 10-fold, introducing a greater chemopreventive impact within a 10% berry gel formulation. On the basis of their chemical composition as well as strongly supportive preclinical *in-vitro* and *in-vivo* data generated by our laboratories, FBR was selected as the bioactive constituents to incorporate into these gels (9–13). Essential for human applications, our Phase I clinical trial demonstrated that consumption of large quantities of FBR are well tolerated in humans (14). A final consideration is the pharmacologic benefit derived from local delivery formulations; specifically the ability to obtain therapeutically relevant local levels without development of systemic complications. Collectively, these data imply that a topically applied FBR bioadhesive gel represents a promising strategy for human oral cancer chemoprevention.

The human pharmacokinetic studies generated both predicted and less anticipated results. As expected, following sublingual berry gel application, anthocyanins were detected in the saliva in eight out of nine participants. The saliva analyses also demonstrated appreciable inter-donor variations in anthocyanin levels; findings that likely reflect differences in both saliva production and anthocyanin metabolism. High saliva production would dilute the anthocyanins, facilitate local clearance and/or increase anthocyanin sequestration. Also, as the HPLC-MS assay detected the flavylium cation form of anthocyanins, individuals with high capacity for anthocyanin metabolism may generate undetectable forms. Anthocyanin detection in the saliva following topical berry gel application supports the premise that bioadhesive gels can provide field coverage throughout the mouth.

In contrast to the saliva analyses, the peripheral blood results were more surprising. Although sublingual drug administration is established as a delivery site for rapid systemic drug uptake, such rapid absorption kinetics for the anthocyanins was not anticipated. The detection of anthocyanins in peripheral blood within 5 min of berry gel application provides compelling evidence that the gel formulation efficiently delivers bioactive anthocyanin compounds to the target tissue site. For the pharmacokinetic study, 1 g of a 5% berry gel was placed on the anterior floor of the mouth. Thus, the 1 g dose of berry gel corresponded to 50 mg FBR (5% w/w) or approximately a 885 μg dose of total anthocyanins. Blood levels of the anthocyanins at only 5 min were in the 1–20 pmol/mL range. Although it is difficult to compare these results to several human studies investigating the oral absorption of much larger quantities of anthocyanins in food or beverages (31–33) it is fair to conclude that the transmucosal absorption of anthocyanins from the berry gel was relatively rapid and potentially greater from topical application to the mouth compared to levels obtained by ingestion. The data also showed that not every donor's sera contained detectable levels of anthocyanins; results that may be attributable to both anatomic and metabolic differences such as the proximity of vascularity to the surface, the relative thickness of the surface epithelium, the retention of anthocyanins within the surface epithelium, and anthocyanin metabolism in the oral cavity.

The initial tissue explant evaluations, which showed increased tissue uptake with the 10% berry gel relative to the 5% berry gel formulations, may reflect both increased FBR content as well as differences in the gels' osmotic pressures. Hyperosmotic gels would induce cell membrane contraction and facilitate uptake, as the cells in contact with the gel release water in an attempt to attain an iso-osmotic state. Evaluations to assess the effects of berry gel pH on tissue capacity to absorb and retain anthocyanins showed trends implying better uptake with higher 6.5 pH gel. Notably, at pH 6.5, anthocyanins are predominantly in the hemiketal form. As the HPLC-MS assay used only detects the flavylium cation form, our data probably underestimated the tissue absorption with regard to the pH 6.5 gel. While the explant studies suggested that berry gel formulations have the potential to provide a pharmacologic advantage to the target tissue site (higher anthocyanin levels in explants relative to either saliva or blood), these *in vitro* assays did not include either the dynamics of salivary flow, salivary digestive enzymes or blood perfusion. Accordingly, additional human pharmacokinetic analyses are ongoing to assess the effects of berry gel pH (pH 3.5 versus 5.5) on targeted tissue absorption, as well as penetration and uptake of the berry gel compounds into both saliva and peripheral blood. For these analyses, we will develop a refined HPLC-MS assay that will detect both the hemiketal and flavylium cation anthocyanin forms, and we will determine anthocyanin levels in tissue, saliva and peripheral blood. Findings from these studies will provide essential data for future product development. While we have established that the anthocyanins are more stable at lower pH values, in the event that superior tissue uptake is obtained with the higher pH berry gel formulation, then considerations such as a two vial system for product storage will be entertained.

On-going formal stability studies of the cGMP prepared clinical batch in use in our oral dysplasia clinical trial (10% berry gel, pH 3.5) through 6 months controlled storage at 2–8°C have shown retention of gel pH (pH 3.4–3.5), viscosity (12,000–18,000 cP), and both FBR 1 content (>76% of original) and FBR 2 content (>87% of original). These recent physical/chemical data on the cGMP batch support and extend the initial 28 day data for the pH 3.5 gel shown in Fig. 2B. Notably, none of the ten persons enrolled as normal control participants nor any of the 16 persons with epithelial dysplasia who completed the study, developed any adverse effects such as contact mucositis from sustained gel applications (four times daily × 6 weeks). Data analyses to determine therapeutic outcomes are in progress.

A primary goal of oral cavity chemoprevention is to redirect growth and facilitate orderly maturation of the surface epithelium. Accordingly, chemopreventive compounds need to be delivered to cells responsible for regeneration of the surface epithelium i.e., basal layer keratinocytes and their respective transient amplifying cell pools. Formulation of a delivery vehicle that provides both stability of bioactive compounds and good absorptive capacity is therefore essential. The prototype bioadhesive berry gels described in this study represent a potential strategy to deliver effective, yet well-tolerated chemopreventive compounds to lesional tissues without eliciting systemic complications.

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