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Chemoprevention of Oral Cancer by Topical Application of Black Raspberries on High At-Risk Mucosa

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Abstract

Objective—To evaluate the preclinical efficacy of topical administration of freeze-dried black raspberries (BRBs) to inhibit the progression of premalignant oral lesions and modulate biomarkers of cancer development in high at-risk mucosa (HARM).

Study Design—Hamster cheek pouches (HCPs) were treated with carcinogen for six weeks to initiate a HARM microenvironment. Subsequently, right HCPs were topically administered a BRB suspension in short-term or long-term studies. After 12 weeks, SCC multiplicity, SCC incidence, and cell proliferation rates were evaluated. mRNA expression was measured in short-term treated pouches for selected oral cancer biomarkers.

Results—SCC multiplicity (−41.3%), tumor incidence (−37.1%), and proliferation rate (−6.9%) were reduced in HCPs receiving BRBs. Topical BRBs correlated with an increase in *Rb1* expression in developing oral lesions.

Conclusion—Topical BRBs inhibit SCC development when targeted to HARM tissues. These results support the translational role of BRBs to prevent oral cancer development in humans.

Keywords

oral cancer; chemoprevention; black raspberries; oral premalignant lesions

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Introduction

In 2014, it is estimated that over 42,000 new cases of oral and oropharyngeal cancers will present in the United States¹ and in excess of 260,000 cases worldwide². Although localized tumor control is improving, the overall prognosis for these patients remains dismal, with 1-, 5- and 10-year relative survival rates of 84%, 62% and 51%, respectively^{1,3}. The majority of oral cancers (90%) are squamous cell carcinomas (SCC), etiologically linked independently to tobacco and alcohol exposure, with reported synergism between the two risk-enhancing behaviors^{4–11}. These exposures create an oral microenvironment that is high at-risk for premalignant initiation and malignant progression.

Epidemiologic data consistently associates increased fruit consumption with decreased oral cancer risk^{12–15}. Black raspberries (BRBs) are rich in bioactive phytochemicals, including four anthocyanins and additional bioactive plant polyphenols including: ellagic acid, ferulic acid, coumaric, quercetin, and phytosterols; and other micronutrients including: folic acid, selenium, and vitamin C, which that may act individually or in combination to inhibit various procarcinogenic processes^{16–20}. Dietary delivery of BRBs has demonstrated antitumor efficacy in numerous preclinical rodent models of epithelial cancer, including breast^{21,22} colon¹², esophagus^{23–26}, and the oral cavity²⁷. The hamster cheek pouch (HCP) model has been used extensively as a surrogate to study the role of dietary factors in the inhibition of oral cancer²⁸. In this context, we have previously shown that BRBs, when provided to hamsters in the diet before, during, and after treatment with the chemical carcinogen 7,12-dimethylbenz(a)anthracene, significantly inhibit oral tumor development by 44%²⁷. Many phytochemical chemopreventive agents have been shown to inhibit tumor cell growth *in vitro*. However, even less *in vivo* studies have addressed the effect of dietary whole foods on premalignant cells during oral cancer progression, and far fewer *in vivo* studies address the topical delivery of whole food-based entities. The current study was designed to test the ability of topically delivered BRBs to inhibit premalignant progression, inhibit development of oral SCCs, and modulate molecular biomarkers using an animal model with established premalignant lesions that mimics the high at-risk mucosal oral microenvironment of former smokers.

The need for complementary oral cancer prevention strategies, the robust anticarcinogenic bioactive profile of BRBs, and the direct accessibility of oral field-defective tissues led to the present investigation: to define the ability of topically applied BRBs to inhibit SCC progression in high at-risk mucosa (HARM) tissues.

Materials and methods

Chemicals

7,12-dimethylbenz(a)anthracene (DMBA), dimethylsulfoxide (DMSO) and 4000 cP methylcellulose were obtained from Sigma-Aldrich (St. Louis, MO). Saliva Substitute® was obtained from Roxane Laboratories (Columbus, OH). DMBA was dissolved at a concentration of 0.2% (w/v) in DMSO and stored in light-blocking containers at +4°C until each dosing.

Black Raspberries

BRBs (*Rubus occidentalis* 'Jewel' variety) were procured from the Stokes Berry Farm, LLC (Wilmington, OH). Ripened BRBs were mechanically harvested, immediately washed, quick frozen, and stored at -20°C . Frozen BRBs were shipped to Van Drunen Farms (Mokena, IL), freeze-dried, and pulverized into a powder. The freeze-dried BRB powder was stored at -20°C until it was suspended to a concentration of 10% (w/v) in a 1:1 mixture of Saliva Substitute[®] and sterile 2% methylcellulose. The phytochemical composition of this BRB powder has been previously reported elsewhere and is comparable to other reported lots of BRB used in chemoprevention studies²⁰.

Animal Care

Male Syrian Golden hamsters (*Mesocricetus auratus*), 5–6 weeks of age and weighing 50–60 grams, were obtained from Charles River Laboratories (Wilmington, MA). Three animals each were placed in plastic bottom cages with bedding and allowed to acclimate for one week. AIN-76A (Dyets, Inc.; Bethlehem, PA) pellets and water were given *ad libitum*. Temperatures were maintained at $21\text{--}24^{\circ}\text{C}$ and humidity was controlled to 40–60%. Hamsters were individually weighed prior to beginning the experiments, at bi-weekly intervals, and at the conclusion of the experiments. Body weights were averaged for animal groups at each interval. Food was exchanged every 5–7 days, using 120–150 grams of pelleted diet per cage, and the residual food was weighed to document the difference between beginning and ending values. Food consumption was recorded for each cage and averaged for the animal groups. All protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care.

Study Design

Two internally paired animal studies were conducted, such that HCPs were first treated bilaterally with chemical carcinogen for six weeks, followed by unilateral exposure to either topical BRBs or topical vehicle alone (Figure 1). In the first study, HCPs were exposed to DMBA for six weeks and then topically administered BRBs for an additional six weeks (Figure 1; Long-term Study, N=42) to determine the capacity of topical BRBs to inhibit tumor multiplicity and incidence. In the second study, HCPs were again exposed to DMBA for six weeks and then administered topical BRBs for two weeks to define the chemopreventive potential of topical BRBs on premalignant pathology and biomarkers of molecular efficacy (Figure 1; Short-term Study, N=6). One group of animals served as controls for food consumption and weight gain and was not treated with carcinogen or administered BRBs (Figure 1; Sentinel Group, N=6).

Chemical Carcinogenesis

The protocol for induction of lesions and administration of topical BRBs is presented in Figure 1. After acclimatization for one week, HCPs were bilaterally painted with DMBA as previously described by Casto *et al.*²⁷. For tumor initiation, the surfaces of both pouches were painted three times weekly for six weeks with a 0.2% solution of DMBA in DMSO using a No.4 sable hair brush. Six weeks of DMBA exposure initiates and promotes high at-

risk mucosa (HARM) fields of cellular deficiencies while fostering premalignant lesion development (Figure 2)²⁷. Our previously reported dietary BRB chemoprevention study²⁷ determined that after six weeks of DMBA treatment, HCPs are thicker (hyperplastic), less compliant, and grossly contain leukoplakias. Microscopically, these pouches harbor hyperplasia and mild (low-grade) epithelial dysplasia. After 10 weeks on study, some pouches begin to develop small exophytic tumors which are histopathologically classified as SCC. After 12 weeks, the incidence of SCC approaches 100%. These pouches additionally contain the whole range of discrete histopathological stages involved in the development of premalignant and malignant human oral cancers²⁹.

BRB Chemoprevention

At 72 hours following the last DMBA treatment, the right HCP of each animal was administered 10 mg of BRBs suspended in 0.1 ml of 1:1 mixture of Saliva Substitute® (Roxane Laboratories, Columbus, OH) and 2% methylcellulose by direct injection onto the mucosal surface of the HCP using a feeding/oral gavage needle (Figure 3b). The contralateral (left) HCP was administered 0.1 ml of suspension vehicle only (1:1 mixture of Saliva Substitute® and 2% methylcellulose) (Figure 3b). Topical BRB administration occurred three times each week (M, W, F) for six weeks ("Long-Term" Study) or two weeks ("Short-Term" Study). Briefly, Saliva Substitute® is a commercially available saliva replacement solution and oral lubricant formulated for human use to coat the mucosal epithelia and provide relief to patients suffering dry mouth. Methylcellulose is a non-toxic, non-allergenic emulsifier and solidifier with a variety of commercial and clinical uses including: food preparation, treatment of constipation, personal lubricant, and saliva and tear replacement. An aqueous 1% solution of methylcellulose is liquid at 4C°, however, is semi-solid at 37C°, thus making an ideal vehicle to deliver and sustain water-soluble BRBs on the mucosal epithelium of the HCP.

Oral Lesion Incidence and Multiplicity (Long-term Study)

HCP tumors (5–8 mm) were observed following 10 weeks of DMBA exposure. Consequently, all HCPs were resected at 12 weeks following euthanasia of the hamsters by CO₂ asphyxiation and cervical dislocation. HCPs were everted and total lesions (leukoplakias, papillomas, and exophytic SCCs) were visually enumerated and recorded along with the size of each SCC (Figure 3c,d). All gross lesions were evaluated by at least two observers. SCCs were measured in two dimensions (W×L in mm), excised, cut into halves, and one half immediately placed in liquid nitrogen and the remaining half placed in 10% neutral buffered formalin (NBF) for 18–24h and processed for histology. The remaining HCP, after removal of the SCCs (denuded), was bisected longitudinally with one section placed in 10% NBF for histopathological and immunohistochemical staining and the other section frozen in liquid nitrogen and stored at –86°C. Tumors volumes were calculated by the formula $V = \pi/6 \times (\text{length}) \times (\text{width}) \times (\text{height})^3$.

Histologic Grading of Microscopic Lesions

Serial 5µm sections of each pouch were cut and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). A hematoxylin and eosin stained slide of each pouch was

prepared from each animal (left and right pouches) and scanned at 40X and 200X magnification. Each view in field was categorized into one of five histologic categories (Figure 2): normal epithelium, low-grade dysplasia, high-grade dysplasia, carcinoma in situ, or squamous cell carcinoma by an oral and maxillofacial pathologist (BTA). Low-grade (mild) dysplasia was characterized by changes in the epithelium such as basilar crowding and hyperplasia, cellular disorganization, and maturational disturbances not extending more than one-third of the epithelial thickness with little interruption of the keratin layer. High-grade (severe) dysplasia included the above parameters extending beyond one-half of the epithelial thickness but not affecting the entirety of the epithelium. Additional features included frequent mitotic figures, cellular pleomorphism, nuclear atypia, and some early disturbance of the keratin layer. *Carcinoma-in-situ* appeared as a full thickness epithelial change with the above features, an expansion of multiple layers of cells into the suprabasal and intermediate layers, and with disturbance of the keratin layer but without penetration of the basement membrane. Squamous cell carcinoma was defined as the above changes with invasion through the basement membrane.

Cell Proliferation (Long-term Study)

Immunohistochemical (IHC) staining was performed on SCC-denuded cheek pouches by The Ohio State University Comparative Pathology and Mouse Phenotyping Shared Resource on unstained, formalin-fixed, paraffin-embedded, 5 µm tissue sections. IHC was used to determine proliferation indices in dysplastic lesions in HCPs treated with BRBs or vehicle-alone for six weeks. Sections were pretreated with Target Retrieval Solution (Cytomation; Carpinteria, CA) under controlled temperature and pressure using a Decloaking Chamber (Biocare Medical; Concord, CA). Endogenous peroxidase activity was quenched by the pretreatment of the sections with hydrogen peroxide before incubation with a 1:200 dilution of Ki67 primary antibody (Novus Biologicals; Littleton, CO). The number of Ki-67 positive nuclei and total number of cells was counted in ten random fields containing full-thickness epithelium at 200X magnification and documented with digital photomicroscopy.

Nucleic Acid Isolation and Quantitative Gene Expression Analysis (Short-term Study)

HCP tissues were lysed in RLT Buffer (Qiagen; Valencia, CA), homogenized using a BioSpec Mini-Beadbeater-16 (Bartlesville, OK), and RNA isolated using the Qiagen AllPrep DNA/RNA Mini Kit according to the manufacturer's protocol. RNA was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE). RNA quality and size distribution was qualified using an Agilent Bioanalyzer 2100 (Agilent Technologies; Santa Clara, CA) and RNA integrity was determined using the RNA Integrity Number (RIN) calculated by 2100 Expert Software (Agilent Technologies). RNA with high RIN values (>9.0) were used for relative real-time Reverse Transcription PCR (RT-PCR). First-stand (cDNA) synthesis was performed on 400ng of total RNA using the High Capacity cDNA RT Kit (Life Technologies, Carlsbad, CA). Relative changes in molecular biomarkers were assessed using quantitative real-time PCR (RT-qPCR) with custom hamster TaqMan Assays (Life Technologies) designed from available *Mesocricetus auratus* GenBank sequences using Primer Express Software (Life Technologies). RT-qPCR was performed using Fast Universal 2× Master Mix (Life Technologies) and an Applied

Biosystems 7900HT Fast Real-Time PCR System (Life Technologies). Fold expression changes were calculated using the C_q method using the geometric mean of the C_q of *Actb* and *Gapdh*. The “ C_q analysis” method and the 2^{-C_q} method were used to determine relative fold change in gene expression^{4,6,8,10}.

General Statistical Methods

Body weights and food consumption were compared using analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test ($p < 0.05$). Sample size for SCC evaluation was estimated from the results of previously completed hamster studies at an effect size of 40% reduction in mean numbers of SCCs. To achieve 80% power at $\alpha = 0.05$, this study required 36 pairs of pouches (paired t -test for mean differences). Sample size was adjusted to 42 animals to account for mortality. Each animal served as a pair of observations (*i.e.*, topical BRB pouch versus vehicle control pouch). Incidence was tested using McNemar's two-sample test for binomial proportions for matched data. Multiplicity was tested using a two-sided paired t -test at $\alpha = 0.05$, or alternatively, Wilcoxon signed-rank test at $\alpha = 0.05$, where the assumptions of the t -test were not met. Secondary endpoint measures, such as papillomas, leukoplakias, microscopic lesions, and Ki67 proliferation indices were analyzed using the same statistical procedures. Statistical differences in relative mRNA between treated and untreated tissues were compared using a two-sided t -test ($p < 0.05$), multiple testing was adjusted for using a Holm Procedure for Multiple Comparisons.

Results

Long-term topical BRB administration does not significantly alter food consumption or animal weight

There were no adverse effects of topical BRB administration on either food consumption or weight change. The amount of food consumed by animals receiving topical BRBs was 5.90 grams/day/hamster compared to 5.97 grams/day/hamster in DMBA-only control animals. Individual hamsters were weighed every two weeks and the weights averaged first for hamsters in each cage, and then for all cages in each respective group. There were no significant differences in the final average weights of the topical BRB group animals compared to DMBA-only controls (140.8 grams/animal vs 133.9 grams/animal).

Long-term topical application of BRBs inhibits oral SCC multiplicity, oral SCC incidence, and oral dysplasia cell proliferation

Exposure of the HCP mucosal epithelium to DMBA for six weeks established a pre-initiated high at-risk oral mucosa (HARM) microenvironment with low-grade dysplastic change. Subsequently, topical application of BRBs for six weeks to HCPs with HARM resulted in no significant differences in the numbers or appearance of gross premalignant lesions (*e.g.*, leukoplakias) nor were there differences in the numbers or appearance of gross benign lesions (*e.g.*, papillomas) (Table 1). There were no differences in the histopathological designation or appearance between topical BRB-treated and vehicle control treated SCC-denuded HCPs. However, there was a significant 37.1% ($p < 0.05$) inhibition of SCC incidence (Table 1, Figure 4a) and a significant 41.3% ($p < 0.05$) decrease in SCC multiplicity (Table 1, Figure 4b) following long-term topical BRB administration. Figure 5

demonstrates that the major inhibitory effect was on smaller volume, early developing SCCs, whereas larger SCCs were generally unaffected by topical BRB application. When SCC-denuded HCPs were examined for cell proliferation in dysplastic HARM tissues (N=10 pairs, Ki67 staining, Figure 6a), exposure to DMBA alone was shown to increase the proliferation index in dysplastic lesions 13–18% above that observed in adjoining normal tissues (Figure 6b). After topical delivery of BRBs, there was a 6.9% ($p<0.01$) relative reduction in the proliferation index when compared to the DMBA-initiated dysplastic lesions. In this context, 34% of the mean increased proliferation rate in dysplastic lesions was mitigated by topical BRB application. Among selected lesions in which the largest increases in proliferation index were observed, there was a 9.65% average decrease in proliferation index between topical BRB-administered and DMBA-initiated dysplasia with a range of 7.19–13.53% reduction in proliferation index (Figure 6b).

Short-term topical application of BRBs does not significantly alter premalignant lesion multiplicity or histopathological designation

Topical application of BRBs for 14 days following 6 weeks of DMBA exposure had no significant effect on the gross or microscopic incidence, multiplicity, or types of existing preneoplastic oral lesions when compared to those identified in DMBA-only treated HCPs. Grossly, there were 13 and 14 small (<1 mm) leukoplakias in BRB and DMBA-only treated HCPs treated for 14 days. In general, the histopathological designation of these pouches was low-grade dysplasia. The lack of redistribution in lesion histopathology (gross or microscopic) is not unexpected following such an abbreviated exposure to topical BRBs after dysplasia had already been established.

Short-term topical delivery of BRBs alters the expression of molecular biomarkers of oral carcinogenesis

HCPs were resected and denuded of SCC, if present, and the effects of short-term topical BRB application on gene expression levels were evaluated using 13 molecular biomarkers associated with cell proliferation and oral carcinogenesis. Only the retinoblastoma gene (*Rb1*) was significantly changed by short-term application of topical BRBs in the high at-risk mucosa (HARM) of the HCP (Table 2, $p<0.05$).

Discussion

Black raspberries, their phytochemical components, and their derived bioactive metabolites have demonstrated remarkable efficacy as chemopreventive interventions across diverse epithelial malignant phenotypes. Pioneering studies by Stoner and collaborators^{12,14} established a role for dietary BRB phytochemicals in animal models of colon and esophageal cancer prevention. However, much less is known for BRB-mediated risk-reduction approaches for oral cancer. Our studies were designed to test if a low-dose topical application of BRBs would inhibit premalignant progression and SCC formation in a carcinogen-initiated high at-risk oral microenvironment.

First, we wanted to demonstrate that a targeted topical approach to reducing oral cancer risk is a practical alternative to systemic dietary interventions. This approach would allow a

decreased absolute amount of BRBs to be administered by concentrating their phytochemical bioactivities directly on the high at-risk tissues of a pre-initiated oral microenvironment. Instead of relying on the abbreviated oral contact that occurs during food consumption, or waiting for systemic trafficking back to the oral cavity, we intended to increase physicochemical contact time between the HARM tissue and BRB components by using an artificial saliva delivery model. The BRBs were incorporated into a topical application containing a commercial product (Saliva Substitute[®]) and a standard pharmaceutical compounding agent (methylcellulose) to increase residence time in oral cavity cheek pouches, while still being available (via swallowing) for further systemic processing. Briefly, in a 1% solution, methylcellulose at 4°C will form a viscous liquid, however as the temperature rises to that of the oral cavity of hamsters (~36°C), the solution will gel. Thus, the gel coats the pouch and remains in contact with the HCP increasing the residence time of suspended chemopreventive agents (i.e., BRB).

Previously, we showed that a 5% BRB dietary intervention significantly inhibited DMBA-induced oral SCCs. The average amount of freeze-dried BRBs consumed by each animal daily was approximately 400 mg. Since HCPs are contiguous evaginations of the oral vestibule and because hamsters store their food in these epithelial cups, we hypothesized that the storage of BRB diet was a *de facto* sustained contact microenvironment for BRB phytochemical delivery. Dietary BRB intervention studies in preclinical animals models of colon, esophagus, and oral cavity cancers have established the chemopreventive potential of such a whole-food approach on epithelial malignancies when incorporated as 5% or 10% freeze-dried BRBs into the diet. Early phase human clinical trials have recapitulated these dosing regimens, requiring participants to consume 32–60 grams of freeze-dried BRBs daily, or the equivalent of more than 4 cups of fresh BRBs every day³⁰. A pilot human clinical trial conducted by Mallery and coworkers using a bioadhesive gel has shown that that a 6-week topical application of 10% w/v freeze-dried BRBs significantly reduced the instances of LOH in clinical premalignant lesions, and exhibited varied abilities to inhibit size and malignant progression/regression^{16,18}. Very recently, Mallery and coworkers have extended the results of their pilot study in a multicenter clinical trial demonstrating reductions in lesion sizes, histologic grades, and LOH events in lesions treated with a 10% freeze-dried BRB mucoadhesive gel and identified a cohort of highly BRB-responsive individuals³¹. While there was appreciable inter-patient variation, and premalignant lesion phenotypes are subject to dynamic changes and spontaneous regression, these studies clearly support the value of a targeted topical BRB delivery strategy by decreasing the daily and cumulative BRB levels required for efficacy. We demonstrate that a 6-week low-dose (10 mg/day, 3×/week) topical administration of freeze-dried BRBs significantly reduced SCC multiplicity and incidence as well as reducing the proliferation index in initiated premalignant tissues in an animal model recapitulating the essential elements of these early clinical studies. Although there were no significant changes in the distribution of premalignant lesions in either the Short-term Study or the Long-term Study, cumulative effects reflected a decrease in squamous cell carcinomas (Long-term Study). Consistent with a preventive, but not therapeutic efficacy, topical BRBs were most effective in reducing the number of smaller oral lesions. These studies support our hypothesis that a directed topical application of BRBs, in contrast to a generalized dietary intervention, would require a lesser

amount of whole-food agent (30 mg/week topical BRB vs 2,800 mg/week dietary BRB) to demonstrate preventive efficacy in the oral HARM tissues. It is reasonable that comparable human studies would also benefit from a topical delivery system (troche, mouth wash, gel) and the decreased absolute and cumulative amounts of BRB necessary for efficacy.

Based on the inhibition of SCC in HCPs and reduction in cell proliferation in dysplastic tissues at 12 weeks, 13 proliferation-associated genes were evaluated for topical BRB molecular efficacy in HARM tissues. Since these cells are carcinogen-initiated and early in the multistep progression of oral carcinogenesis, we anticipated that diverse growth promoting mechanisms would be deregulated, and that significant growth inhibitory controls would be activated in the high at-risk epithelium following topical BRB application. Freeze-dried BRBs have been shown to modulate cell cycle associated biomarkers, including *Mki67*²³, *Pcna*^{2,32,33}, *Vegfa*^{1,24}, *Nfkb1*^{5,7,9,11,23}, *Cdc42*^{13,15,34}, *Ccnc*^{17,19,34}, *Cdkn3*^{24–26,35}, and *Tp53*^{27,36} in other preclinical animal model systems, and is a hallmark feature *in vitro*^{28,37–40}. Because the hamster is not available as a curated genome, selection of pertinent cell cycle biomarkers is restricted to prior relevant publications and partial GenBank sequences. The retinoblastoma gene (*Rb1*) is a canonical tumor suppressor gene that is inactivated during the course of oral SCC genetic progression^{27,41}. After short-term topical BRB delivery, *Rb1* was significantly up-regulated in the HARM tissues of the DMBA-initiated HCPs. Although the mechanism of *Rb1* up-regulation is not known in our current pre-clinical model, it is worth noting that BRBs have demonstrated epigenetic activities^{24,42}. To this end, BRB phytochemicals enhance tumor suppressor gene expression through promoter demethylation via inhibition of DNMT1 and DNMT3B in colon cancer *in vitro*, and an early phase human study^{42,43}. It is likely that BRBs elicit their effects similarly in premalignant oral epithelium presenting the possibility of reversible methylation may be involved in the selective induction of *Rb1* following topical BRB delivery. This molecular efficacy following topical BRB application is consistent with prior evidence supporting a role for BRB phytochemicals in growth control, inhibition of cell proliferation, induction of apoptosis, and restoration of normal homeostatic measures. Other potential mechanisms of BRB-mediated chemoprevention were reviewed by Stoner and Casto et al., (2008)²⁶. These include protection against oxidative DNA damage via scavenging of reactive oxygen species (ROS), reducing the formation of carcinogen-induced DNA adducts, promoting DNA repair, and modulation of signaling pathways involved with proliferation, inflammation, apoptosis, and cell cycle arrest²⁶. The dominant bioactive phytochemicals in BRBs are the polyphenolic compounds such as the anthocyanins⁴⁴. Anthocyanins are redox active phytochemicals that elicit both redox scavenging and redox generating effects⁴⁵. Therefore, scavenging aberrant ROS reduces DNA damage and subsequent DNA adduct formation in affected cells. This is supported by our previous research demonstrating that 5% BRB administered in the diet reduced DNA adduct formation in the HCP²⁷. Further, by scavenging ROS, these compounds inhibit ROS-mediated cellular signaling and in turn reduce unremitting proliferation³¹. In the present study, we demonstrate reduced proliferation rates in dysplasia after treatment with BRB and it can be postulated that this is due, in part, to the antioxidant effects of BRBs.

The application of topical BRBs to pre-initiated high at-risk oral mucosa demonstrates a significant preclinical efficacy by reducing SCC incidence, multiplicity, and proliferation index, while providing molecular efficacy in the up-regulation of the *Rb1* tumor suppressor. These low-dose topical prevention studies provide the evidence and support for a food-based oral cancer risk-reduction strategy using freeze-dried BRBs. The carcinogen-initiated oral microenvironment presents as HARM tissue strewn with field-defect cells awaiting the necessary promoting and progression signals. Such a microenvironment can be envisioned for oral cavities exposed to tobacco smoke carcinogens. Following removal of the oncogenic insult (DMBA in HCP, tobacco smoke in human oral cavity) topical BRB phytochemicals demonstrate the potential to inhibit tumorigenesis and suppress oral carcinogenic progression.

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Statement of Clinical Relevance

This preclinical study supports the hypothesis that black raspberries (BRBs), when applied directly to high at-risk oral mucosa, reduces the progression to squamous cell carcinoma. These results support the translational role of BRBs in modifying oral cancer risk in humans.

Figure 1.

Preclinical paired study design for exposure to DMBA and topical delivery of 10% BRBs. All carcinogen-exposed animals were painted with DMBA three times per week for six weeks to the entire mucosal surface of the bilateral HCP mucosa. Animals on the "Long-term" protocol were administered either delivery vehicle alone (left HCP, L) or topical 10% BRBs (right HCP, R) for an additional six weeks and were evaluated for SCC incidence and multiplicity, and proliferation indices in normal and dysplastic epithelia in the HCP. Animals on the "Short-term" protocol were administered topical 10% BRBs for two weeks following DMBA-initiated carcinogenesis and evaluated for molecular efficacy in preneoplastic tissues. Sentinel animals received neither DMBA nor 10% BRBs.

Figure 2. Continuum of epithelial dysplasia in the HCP model of oral SCC. Photomicrographs from H&E stained HCP sections at 200X were collected from areas of normal, mild (low-grade) dysplasia, severe (high-grade) dysplasia, *carcinoma in-situ*, and squamous cell carcinoma. Lesion grades were classified as described in the Methods.

Figure 3.

Evaluation and chemoprevention in the HCP model of oral SCC. (a) Sentinel animal demonstrating an everted, thin, flexible HCP free of macroscopic lesions. (b) Carcinogen-treated animals receiving 0.1 mL of 10% BRB gel into the HCP using a blunt, rigid gavage needle. The 10% BRB gel was expressed onto the entire mucosal surface of the HCP and would form a persistent coating delivering BRB phytochemicals directly to affected mucosal surface. *, 10% BRB gel, 10% BRB w/v in 1:1 Saliva Substitute® and 2% methylcellulose; **, vehicle, 1:1 Saliva Substitute® and 2% methylcellulose (c) HCP demonstrating a number of small- and medium-sized exophytic carcinomas (arrows). (d) HCP demonstrating a large-sized carcinoma.

Figure 4.

Topical administration of 10% BRBs significantly reduced the incidence and multiplicity of tumors in the HCP. (a) The number of HCPs containing measureable oral SCC (≥ 1 mm) was significantly reduced (-37.1%) in pouches receiving topical 10% BRBs for six weeks following DMBA-initiated oral carcinogenesis. (b) The number of SCCs per HCP was significantly reduced (-41.3%) in pouches receiving 10% BRBs for six weeks following DMBA exposure. *, p -value < 0.05 .

Figure 5.

Topical delivery of 10% BRBs preferentially reduces the mean number of small SCCs relative to intermediate or large SCCs. HCP SCCs were categorized as small (<10 mm³), intermediate (10–100 mm³) or large (>100 mm³) based on volume estimates. While each distributions shows a reduction in SCC multiplicity, the most significant BRB effect is demonstrated in tumors <10 mm³. *p-value*<0.05 following Bonferroni adjustment.

Figure 6.

Topical administration of 10% BRBs reduces the proliferation index (PI) in dysplastic HCP tissues relative to matched normal tissues. Box plots are transected to indicate the median value (50% percentile) with the 25% and 75% quartiles. The outermost lines indicate the 0% quantile (minimum, lower whisker) and 100% quantile (maximum, upper whisker). 10% BRBs reduce the mean relative Ki67 PI by 6.9% (0.597 vs 0.556; $p < 0.01$; two-sided paired t test) in dysplasia. This reduction accounts for 34% of the total increased PI between normal and dysplastic tissues.

Table 1

Inhibition of DMBA-induced oral lesions by topical 10% BRBs

Long-Term Study (6-week Topical BRBs)	Leukoplakia Multiplicity	Papilloma Multiplicity	Tumor Multiplicity	Tumor Incidence
Vehicle control Left HCP	52	7	63	81%
+ Topical BRBs Right HCP	61	6	37	51%
% Inhibition	0%	14.3%	41.3% *	37.1% *

* significant when compared to DMBA controls (p -value <0.05).

Table 2

Molecular efficacy in cell proliferation biomarkers following topical BRB administration in DMBA-initiated HCPs

Transcriptional regulation of cell proliferation biomarkers following 10% topical BRB administration in DMBA-initiated HCPs. mRNA expression of biomarkers were estimated using custom *TaqMan* expression assays. Relative mRNA expression fold-changes in BRB-treated pouches were calculated relative to vehicle-only treated pouches in a paired study. BRBs increased the expression of pRb1 after 14 days of treatment ($p=0.05$, two-sided paired t test).

Gene	<i>Bax</i>	<i>Bcl2</i>	<i>Ccnb2</i>	<i>Cdk2</i>	<i>Cdk4</i>	<i>Mdm2</i>	<i>Myc</i>	<i>Cdkn2a p13^{Arf}</i>	<i>Cdkn2a p16</i>	<i>Rb1</i>	<i>Tp53</i>	<i>Vegfa</i>
Fold-change	-1.24	-1.07	+1.09	+1.04	-1.04	+1.04	-1.11	-1.08	-1.16	+1.30	-1.38	+1.06
<i>s.d.</i>	0.22	0.21	0.41	0.15	0.05	0.21	0.22	0.312	0.49	0.21	0.27	0.30
<i>p</i> -value	0.56	0.25	0.52	0.23	0.18	0.27	0.55	0.69	0.69	0.05*	0.29	0.10

* significant when compared to DMBA controls (p -value <0.05).