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Transitioning From Preclinical to Clinical Chemopreventive Assessments of Lyophilized Black Raspberries: Interim Results Show Berries Modulate Markers of Oxidative Stress in Barrett's Esophagus Patients

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Abstract: *Increased fruit and vegetable consumption is associated with decreased risk of a number of cancers of epithelial origin, including esophageal cancer. Dietary administration of lyophilized black raspberries (LBRs) has significantly inhibited chemically induced oral, esophageal, and colon carcinogenesis in animal models. Likewise, berry extracts added to cell cultures significantly inhibited cancer-associated processes. Positive results in preclinical studies have supported further investigation of berries and berry extracts in high-risk human cohorts, including patients with existing premalignancy or patients at risk for cancer recurrence. We are currently conducting a 6-mo chemopreventive pilot study administering 32 or 45 g (female and male, respectively) of LBRs to patients with Barrett's esophagus (BE), a premalignant esophageal condition in which the normal stratified squamous epithelium changes to a metaplastic columnar-lined epithelium. BE's importance lies in the fact that it confers a 30- to 40-fold increased risk for the development of esophageal adenocarcinoma, a rapidly increasing and extremely deadly malignancy. This is a report on interim findings from 10 patients. To date, the results support that daily consumption of LBRs promotes reductions in the urinary excretion of two markers of oxidative stress, 8-epi-prostaglandin F2α (8-Iso-PGF2) and, to a lesser more-variable extent, 8-hydroxy-2'-deoxyguanosine (8-OHdG), among patients with BE.*

Introduction

In recent years, a number of investigations have been conducted to evaluate the chemopreventive potential of black raspberry–derived extracts in vitro (1,2), and lyophilized black raspberries (LBRs) have been assessed as dietary inhibitors of chemically induced cancers in clinically relevant animal models (3–5). In vivo dietary administration of LBRs significantly inhibited chemically induced esophageal, colon, and oral cavity carcinogenesis (3–5). LBRs reduced measures of oxidative stress, decreased DNA damage, inhibited cellular proliferation rates, and reduced levels of esophageal and colon preneoplasia (3–5). Black raspberries are rich in a number of potential protective constituents (Table 1), including vitamins, minerals, phenolics, plant pigments, sterols, and fiber, which has been particularly protective against esophageal adenocarcinoma (EAC) development in human cohorts. These observations lead us to hypothesize that dietary administration of black raspberries may inhibit the progression of Barrett's esophagus (BE), a premalignant condition in which the normal stratified squamous epithelium lining the esophagus is replaced by metaplastic columnar epithelium containing goblet cells (6–8).

BE affects an estimated 700,000 U.S. adults and remains the only recognized precursor lesion to EAC, a cancer that has dramatically increased in incidence throughout most of the Western world over the last 3 decades (1–4). Rates of EAC tripled in the United States between 1976 and 1990, identifying EAC as the fastest increasing cancer type (9). The clinical importance of gastroesophageal reflux disease (GERD) and BE lies in the fact that these patients are at a 30- to 125-fold increased risk of developing EAC (10–13). Thus, the risk of malignancy associated with Barrett's metaplasia is similar in magnitude to the risk of heavy smokers developing lung cancer or carriers of chronic hepatitis B virus developing liver cancer, as pointed out in a review by Wild and Hardie (14).

A model of progression, similar to that developed for colon cancer, has been proposed for EAC (reviewed in Refs. 14–16). A number of markers associated with inflammatory

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responses, oxidative stress, and DNA damage are altered as the normal stratified squamous epithelium is replaced by columnar metaplastic Barrett's epithelium (17–24). A number of treatment options available to BE patients offer symptom relief, but none has proven curative or eliminated the risk of cancer progression. Furthermore, the prognosis for those diagnosed with esophageal cancer remains dismal with a 5-yr survival rate of only 14% (25). These statistics reflect the urgent need for improved treatment and preventive strategies. The precise reason for the rapid increase remains to be elucidated, but increasing rates have consistently been linked to chronic reflux (26–28) and obesity, particularly male pattern obesity (28–35). Other risk factors linked to the development of EAC include the presence of hiatal hernia, medication use, and multiple nutritional factors (14,36–38).

Plant-based diets have generally been associated with reduction of risk for EAC and those of animal origin with risk escalation (36–40). In terms of specific dietary components, fiber, β-carotene, folate, and vitamins C and B_6 are associated with decreased risk for EAC, whereas low consumption of fruits and vegetables, high dietary cholesterol, elevated intake of animal protein, and vitamin B_{12} are linked to increased risk (36–40).

Thus, we postulate that dietary administration of LBRs may reduce oxidative damage associated with GERD and slow the progression of BE. To explore the potential of LBRs to modulate markers of DNA or oxidative damage in patients at increased risk for cancer development, we are conducting a pilot dietary intervention in 20 patients with BE. The data presented in this article summarize findings on the modulation of two urinary markers of oxidative stress in 10 patients that have completed a 6-mo dietary intervention with LBRs. Additional tissue- and plasma-based biomarkers will be assessed in samples collected from patients at baseline and postintervention when all 20 patients have completed the 6-mo dietary intervention. This pilot study will also provide valuable information on the acceptability of a "food-based" chemopreventive approach in this cohort of patients.

Materials and Methods

Subjects

Twenty BE patients were recruited from The Ohio State University gastroenterology clinic. Results from 10 patients who have completed the intervention are included in this report. Eligibility assessments included 18 yr of age or older, complete medical history, a physical exam, completion of food-frequency questionnaire, positive endoscopy for BE (columnar lined esophagus, with specialized intestinal metaplasia/goblet cells) extending ≥1 cm above the gastroesophageal junction on the prescreening biopsy and on two previous biopsies, no history of invasive cancer within the past 5 yr, normal organ function, normal serum chemistries, and signed informed consent approved by the Institutional Investigational Review Board. Exclusion criteria included any severe chronic or life-threatening diseases (cardiac disease, pulmonary disease, malignancy within 5 yr, or severe neurological or rheumatologic disease), inability to return for scheduled follow-up visits, abnormal wound healing, esophageal varices or a history of varices or variceal bleeding, BE with high-grade dysplasia, coagulopathy that precedes taking esophageal biopsies safely, and excessive use of multivitamins or micronutrient supplements daily.

Ethics and Compliance With Good Clinical Practices

This study was conducted in compliance with the protocol, the Institutional Review Board (IRB), the Code of Federal Regulations, and International Conference on Harmonization/Good Clinical Practice Guidelines. Written IRB approval was obtained prior to initiating the study.

Chemopreventive Source and Berry Preparation

Fresh frozen black raspberries of the Jewel variety were supplied by the Stokes Fruit Farm (Wilmington, OH) and lyophilized by Van Drunen Farms (Momence, IL). Briefly, black raspberries were washed, drained of water, and passed through a Brown Pulper-Finisher (Brown International Corp., Covina, CA) equipped with a 0.02-inch screen to crush berries and remove cap-stems and seeds. Cap-stems were discarded. Black raspberry seeds were repulped to remove additional flesh and added back to the berry slurry prior to freeze-drying. Freeze-drying trays were lined one-half inch deep with the berry slurry and dried for 24 h in a Virtis freeze-drying unit (VirTis, Gardiner, NY). The powdered berries were then aliquotted and sealed into individual bags of 32 or 45 g and distributed to the patients on a rolling basis. Patients were instructed to store seven packets of berries in the refrigerator during the week they were scheduled to be consumed, and all other berries were stored in the freezer.

Route and Schedule of Chemopreventive Administration

Eligible subjects who signed an informed consent were instructed to mix the LBRs (32 g for females and 45 g for males) with 170 ml of water and orally consume the LBRs each morning at a designated time of their choosing for 26 wk. This gram quantity of freeze-dried berries is equivalent to consuming approximately 5% berries in the diet based on average body weight figures and corresponding average caloric consumption requirements. This gram amount of lyophilized berries would approximate 1.5 cups and 2.0 cups of fresh berries for females and males, respectively. Patients recorded the date and time of daily berry consumption on a study calendar and returned all packaging materials including empty berry bags and any nonconsumed bags of berries.

Nutrient Analysis

Nutrient analysis of the LBRs was conducted as previously described (4) to determine the content of potential chemoprotective substances, including vitamins, minerals, and select phytochemicals. In addition, total phenolic content and the oxygen radical absorbance capacity (ORAC) were determined by Brunswick Laboratories (Wareham, ME) (41,42).

Urine Collection, Storage, and Analysis for 8-OHdG and 8-Iso-PGF2

Each subject collected urine for a 3-h period of time in the morning, at study baseline (pre–berry treatment), at Week 12 of study, and at the final time point of 26 wk. Urine was used to analyze biomarkers of oxidative damage and will be assayed for specific berry metabolites in the future. Urine was stored at –80°C without preservatives. Undiluted urine samples were shipped to Genox Corporation (Baltimore, MD) for analysis, in triplicate, for 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-epi-prostaglandin F2α (8-Iso-PGF2) using immunoaffinity chromatography-monoclonal antibody–based enzyme-linked immunosorbent assays (ELISAs) as previously described (43,44). ELISA kits utilized for detection of 8-OHdG and 8-Iso-PGF2 were catalog no. KOG-200SE (Genox) and catalog no. 900-010 (Cayman Chemical, Ann Arbor, MI), respectively. In addition, urinary creatinine levels were determined by using the Cayman Chemical creatinine assay (catalog no. 500701) according to manufacturer instructions. Levels of urinary 8-OHdG and 8-Iso-PGF2 were divided by levels of urinary creatinine to control for potential differences in urine volume between patients.

Data Analysis

Students'*t*-test (two-tailed) was used to evaluate the effect of LBRs treatment on urinary 8-OHdG and 8-Iso-PGF2 levels and to analyze changes in body mass index (BMI) measurements at Week 26 compared with baseline. Correlation analysis was conducted to derive a correlation coefficient value for evaluating the relationship between the two urinary markers of oxidative stress at 26 wk of study.

Toxicity and Compliance Evaluation

Subjects were contacted by telephone 2 wk after study initiation and queried regarding potential adverse events or toxicities associated with the intake of LBRs. Subjects were also queried at their Week 12 and Week 26 outpatient visits regarding any potential adverse events. In addition, at Weeks 2, 12, and 26 of study, the study nurse coordinator recorded information regarding berry consumption compliance for the previous week.

Results

Of 20 patients recruited for the 6-mo dietary intervention, data herein are presented on the first 10 Barrett's patients to complete the 6-mo intervention in which LBRs were consumed daily by study subjects. As shown in Table 1, LBRs are rich in a number of potential chemopreventive components. In addition, black raspberries possess strong antioxidant capacity compared with other fruits (45,46) as measured by their ORAC. The ORAC value determined for the black raspberries utilized in this study was 601.0 μmol of Trolex equivalents per gram of lyophilized berries.

Selected demographic characteristics of the patients are presented in Table 2. The average age at study enrollment was 59.1 yr with a range of 48–68 yr. All subjects were Caucasian and consisted of seven males and three females. Study subjects were well educated, with 30% holding college graduate degrees and 30% holding college undergraduate degrees. The average number of years subjects reported suffering from GERD was 26.7 yr, and the average age of GERD onset among study subjects was 22 yr; however, symptoms of GERD started in one subject at 13 yr of age. At baseline, the average length of the Barrett's tongue was 2.9 cm with a range of 2.0–8.0 cm. The length of the Barrett's lesion was unchanged following the 26-wk dietary intervention. Mean BMI at study baseline was 29.61 with a range of 24.84–40.97 kg/m^2. The average BMI of the study participants signifi-

Table 1. Levels of Nutrients and Potential Chemopreventive Components in Lyophilized Black Raspberries[a]

Dietary Components	Lyophilized Black Raspberry Analysis	Units
Minerals		
Calcium	178.00	mg/100 g
Copper	0.86	mg/100 g
Iron	4.82	mg/100 g
Magnesium	184.00	mg/100 g
Manganese	3.68	mg/100 g
Phosphorus	220.00	mg/100 g
Potassium	1,350.00	mg/100 g
Zinc	2.57	mg/100 g
Selenium	<0.01	mg/100 g
Folate	0.08	mg/100 g
Vitamins		
Ascorbic acid	2.00	mg/100 g
β-Carotene	0.08	mg/100 g
α-Carotene	128.00	IU/100 g
E (natural)	19.30	IU/100 g
Sterols		
β-Sitosterol	97.00	mg/100 g
Campesterol	6.90	mg/100 g
Stigmasterol	<3.00	mg/100 g
Cholesterol	<1.00	mg/100 g
Total phenolics	5,938.00	mg/100 g
Ellagic acid	185.00	mg/100 g
ORAC	601.00	μmol TE/g

a: Abbreviations are as follows: ORAC, oxygen radical absorbance assay; TE, Trolex equivalent.

Table 2. Characteristics of Study Subjects[a]

Subject Characteristic	
Age at study enrollment (yr), mean (±SE), range	59.1 (1.91), 48–68
Race, Caucasian	100%
Education	
Graduate degree	30%
Some graduate-level work	10%
College graduate	20%
Some college	10%
High school graduate	30%
GERD	
Age of onset, mean years (±SE)	22 yr (5.79)
Age range of years of GERD onset	13–50
Number of years with GERD, mean (±SE)	26.7 (2.65)
Length of Barrett's tongue (cm)	
Baseline, mean (±SE), range	2.9 (0.59), 2.0–8.0
Week 26, mean (±SE), range	2.9 (0.60), 2.0–8.0
Body mass index (kg/m^2)	
Baseline, mean (±SE), range	29.61, 24.84–40.97
Week 26, mean (±SE), range	30.82, 26.75–44.75
Tobacco use	
Current smoker	20%
Past smoker	30%

a: Abbreviations is as follows: GERD, gastroesophageal reflux disease.

cantly increased to 30.82 ($P = 0.012$, paired *t*-test, two tailed) at 26 wk of study as graphically displayed in Fig. 1A. Individual changes in BMI are depicted in Fig. 1B. Patient 3 and Patient 7 experienced the greatest increases in body weight (15 and 14.5 lb, respectively), resulting in the largest changes in BMI over the course of the study. Overall, the average weight gain from baseline to 26 wk of study was 5.33 lb, a significant increase ($P = 0.023$). Only two patients were current smokers and three were past smokers. Approximately half of the subjects were current users of alcohol.

Mean concentrations of 8-Iso-PGF2 levels at baseline, Week 12, and Week 26 of study were 1.56E-10, 1.26E-10, and 1.15E-10 mg/ml of urine, respectively, as illustrated in Fig. 2. Levels of urinary 8-Iso-PGF2 were significantly reduced following 26 wk of daily LBRs administration ($P < 0.05$). As shown in Fig. 3, 60% of subjects experienced significant individual level decreases in 8-Iso-PGF2 levels following the 26-wk dietary intervention ($P < 0.05$) as depicted in Fig. 5.

Figure 4 presents the results for urinary 8-OHdG at baseline, Week 12, and Week 26 of study. There was no significant change in mean levels of urinary 8-OHdG following treatment with LBRs; however, at the individual level, five patients experienced significant declines in 8-OHdG. In addition, all five patients experiencing significant declines in 8-OHdG also had significant declines in 8-Iso-PGF2. Thus, a significant correlation ($r = 0.7$; $P = 0.024$) was noted between levels of these two markers of oxidative stress following berry administration as depicted in Fig. 6. However, in contrast to changes in urinary levels of 8-Iso-PGF2, four patients experienced significant increases in urinary 8-OHdG levels over the course of the intervention.

Discussion

Numerous preclinical investigations and observational epidemiology studies support that consumption of diets rich in fruits and vegetables decreases the risk of a number of cancers, including esophageal cancer (36–40). One mechanism by which plant-based diets may prevent cancer is through reducing oxidative stress and modulating damage to lipids, proteins, and nucleic acids (47,48). Oxidative DNA damage has been linked to a number of age-related degenerative diseases, including coronary heart disease, diabetes, and cancer. In this study, two potentially relevant markers of oxidative damage were utilized as indicators of intervention effect, 8-OHdG and 8-Iso-PGF2. In particular, 8-OHdG has been associated with cancer-related processes in preclinical models, and urinary elevations of this adduct have been reported in patients with bladder, breast, cervical, esophageal, and prostate cancers (45,49–51). Thus, these markers are postulated to provide a plausible and noninvasive measurement of oxidative stress.

These data are the first to report that daily administration of LBRs modulates urinary markers of oxidative stress in patients with BE. Urinary 8-OHdG is considered a marker of total DNA damage in humans, and increased levels of this adduct has been positively correlated with oxidative stress (46,52). Similarly, 8-Iso-PGF2, a prostaglandin-like compound (reviewed in Refs. 53 and 54) produced via cyclooxygenase-independent enzymes, is considered a reliable marker of lipid peroxidation and an indicator of oxidative status in vivo. Consumption of LBRs daily for 6 mo resulted in significant declines in urinary levels of isoprostane (8-Iso-PGF2) in 60% of BE patients, and 50% of patients had significant declines in urinary 8-OHdG compared with pre-intervention baseline levels. This is an important finding given that elevated DNA damage has been reported in Barrett's mucosa compared with normal squamous epithelium and gastric mucosa (18,23). Higher levels of DNA damage in Barrett's epithelium have been significantly linked to increased risk for progression to dysplasia and EAC (22). In addition, in vitro studies utilizing esophageal cell lines have shown that bile and acid refluxate induces DNA damage (24). However, post-intervention there appeared to be a differential response among patients with regard to levels of urinary 8-OHdG. Although 50% of patients experienced significant declines in 8-OHdG, 40% experienced significant increases in this marker for unknown reasons. Furthermore, posttreatment levels of 8-OHdG and 8-Iso-PGF2 were highly correlated, yet no patients experienced significant increases in urinary 8-Iso-PGF2 levels following the intervention, supporting that these two markers of oxidative stress may respond differently to environmental factors. This finding will be further explored when all patients have completed the intervention.

Identification of natural agents that inhibit DNA-damaging processes in high-risk cohorts, without imparting negative side effects, is a goal in cancer chemoprevention. Study findings support that daily consumption of LBRs at behaviorally achievable levels positively affects the in vivo

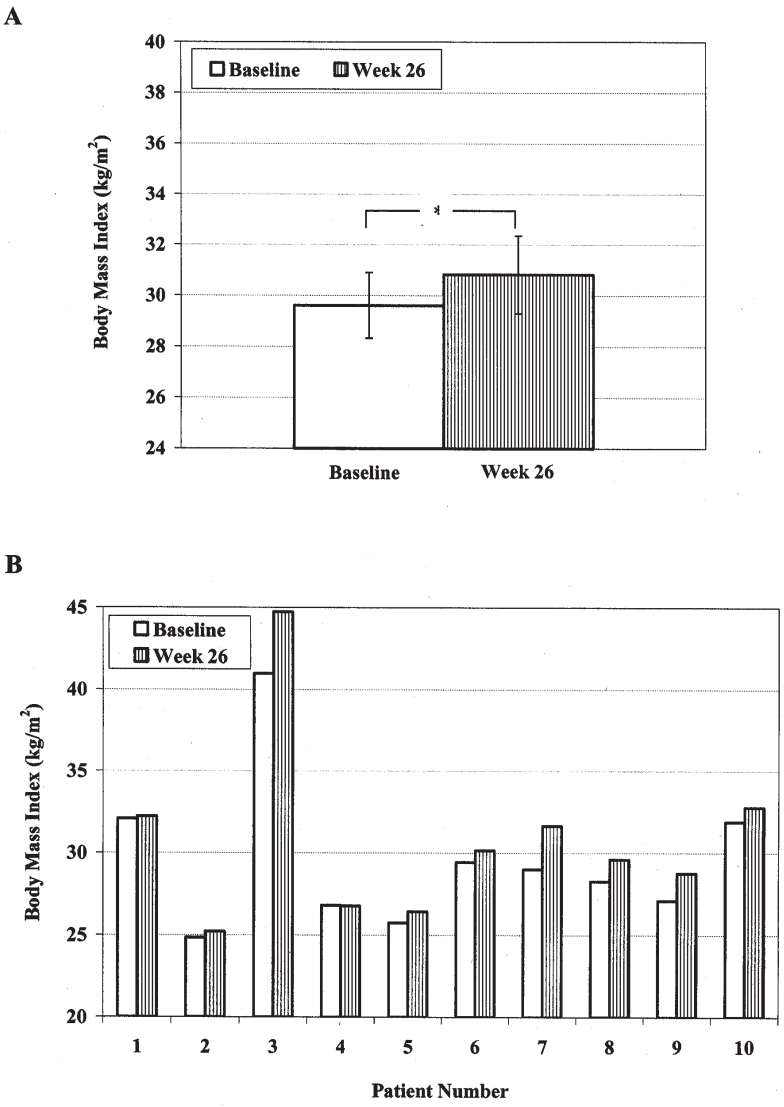

Figure 1. (A) Change in mean (±SE) body mass index (BMI) over 26 wk of study (*$P < 0.05$). Baseline values represent mean BMIs prior to the dietary intervention. Week 26 measurements were taken at study completion. (B) Changes in BMI per patient over 26 wk of study.

oxidative status of patients with BE. The precise mechanisms through which berries may decrease oxidative stress are under evaluation; however, LBRs are known to contain a number of potential chemopreventive components (Table 1) and to have high antioxidant capacity. A recent study by Clements et al. reported that BE patients were deficient in the plasma antioxidants selenium, vitamin C, β-cryptoxanthine, and xanthophylls compared with patients without BE (55). Similarly, Fountoulakis et al. reported that BE patients had significantly lower levels of vitamin C in their plasma compared with controls and that levels of vitamin C were also significantly lower in Barrett's mucosa specimens compared with levels found in normal squamous epithelium (24). Other studies have reported that phase II–detoxifying enzymes such as glutathione *S*-transferases, which function as antioxidants, are reduced in Barrett's epithelium compared with normal esophageal tissue (56,57), lending further support to the notion that oxidative stress is pivotal in the progression from Barrett's metaplasia to dysplasia and potentially EAC. Conversely, evidence supports that plant-based diets, which are rich in antioxidants, fiber, and other phytochemicals, decrease esophageal cancer risk. Thus, in BE patients, a food-based chemopreventive approach utilizing LBRs in conjunction with acid-suppressive therapy may better protect against oxidative damage and restore the balance between endogenous oxidants and antioxidants in susceptible esophageal tissues compared with traditional therapies alone. However, we caution that these data represent interim analysis in a small number of BE patients, and the statistical power of this study is insufficient to adequately assess the impact of potential confounders. Data analysis at study completion will further investigate the ability of LBRs to modulate urinary biomarkers, measure tissue-based markers in Barrett's epithelium, quantify plasma antioxidant levels, consider individual patient histories (diet, health, and risk factors), and assess potential linkages among these variables.

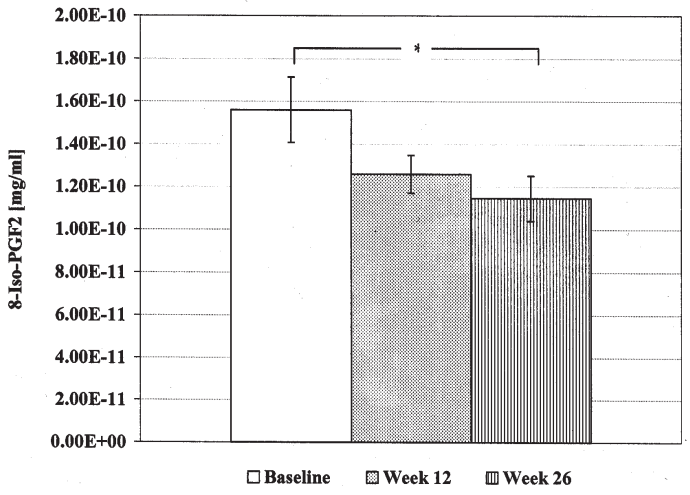

Figure 2. Comparison of mean (±SE) urinary 8-epi-prostaglandin F2α levels by week of study (*$P < 0.05$). Baseline values represent pre-intervention measurements. Week 12 measurements resulted from urine collected 2 h post–berry administration. Week 26 measurements were taken at study completion.

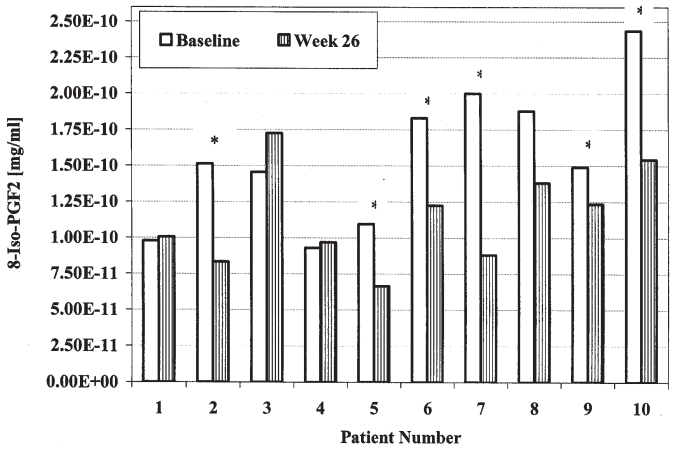

Figure 3. Changes of mean urinary 8-epi-prostaglandin F2α levels per patient over 26 wk of study (*$P < 0.05$).

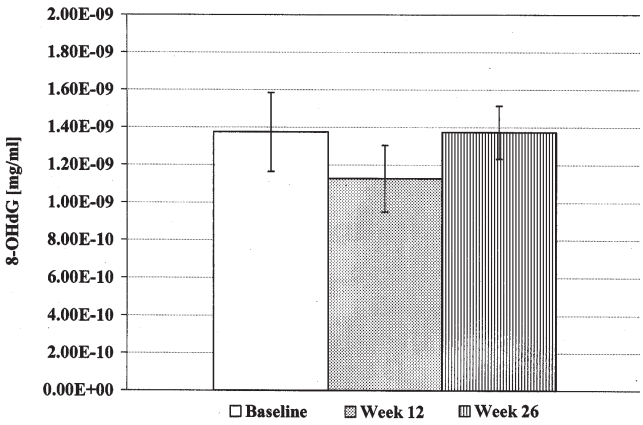

Figure 4. Comparison of mean (±SE) urinary 8-hydroxy-2′-deoxyguanosine levels by week of study. Baseline values represent pre-intervention measurements. Week 12 measurements resulted from urine collected 2 h post–berry administration. Week 26 measurements were taken at study completion.

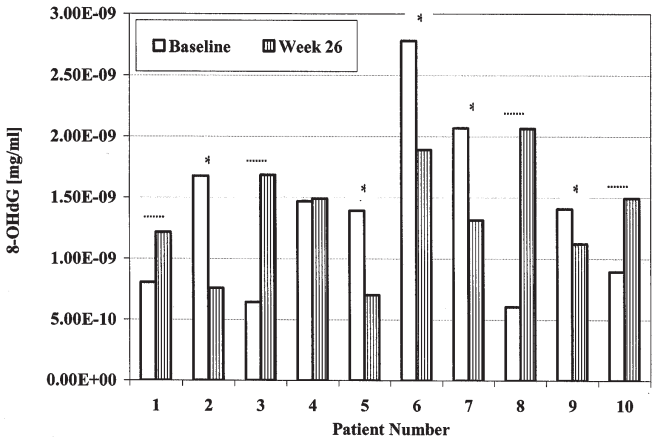

Figure 5. Changes of mean urinary 8-hydroxy-2′-deoxyguanosine levels per patient over 26 wk of study ($P < 0.05$; * indicates a significant decline and ···· indicates a significant increase).

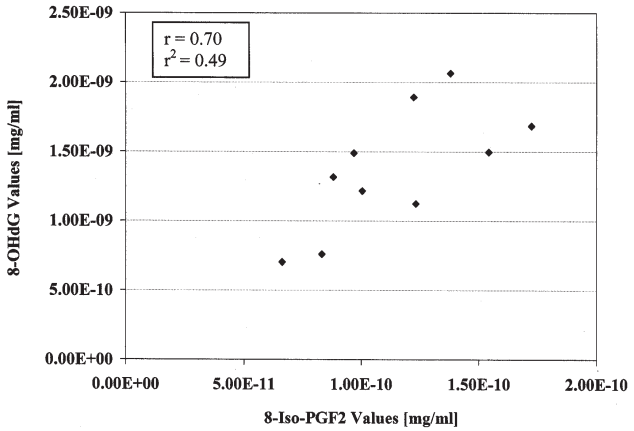

Figure 6. Correlation between urinary 8-epi-prostaglandin F2α levels and urinary 8-hydroxy-2′-deoxyguanosine levels per patient at 26 wk of study. At 26 wk of study, the two parameters show good correlation ($r = 0.70$; $P = 0.024$).

Overall, patient compliance has been extremely good, with subjects consuming the berries as scheduled over 90% of the time. This is particularly important for agents that must be present to exert protective effects. In addition, the berries were well tolerated in terms of potential side effects. Three patients reported symptoms of diarrhea, constipation, or epigastric pain, but symptoms were not severe, and all patients continued berry consumption throughout the 6-mo study. However, one unexpected study observation was that, on average, study patients gained 5.33 lb over the course of the study. Most patients experienced small weight gains; however, two patients gained over 14 lb, contributing to the significant change in mean weight gain across study subjects. Patients were not counseled to modify their normal diets in any way, and it is possible that the extra calories consumed as berries contributed to their excess caloric intake and mild weight gain. The intervention supplied an extra 115 kcal to female subjects and approximately an additional 162 kcal to male patients per day. This has implications for future interventions and should be a consideration especially when conducting interventions in BE patients where obesity is considered a risk factor for esophageal cancer progression.

In summary, this study provides support that daily consumption of LBRs promotes reductions in urinary excretion of 8-Iso-PGF2 and 8-OHdG among patients with BE. However, this is a small study with a number of limitations. Further assessments of these measurements as well as tissue-based markers of aberrant proliferation, apoptosis, differentiation, and DNA repair will occur in a total of 20 patients, and, if positive findings are confirmed, a larger randomized placebo-controlled trial will be planned to fully investigate the chemopreventive potential of LBRs in patients with BE.

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