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# The *In Vivo* Antioxidant Action and the Reduction of Oxidative Stress by Boysenberry Extract Is Dependent on Base Diet Constituents in Rats

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ABSTRACT Dietary antioxidants are often defined by *in vitro* measures of antioxidant activity. Such measures are valid indicators of the antioxidant potential, but provide little evidence of activity as a dietary antioxidant. This study was undertaken to assess the *in vivo* antioxidant efficacy of a berry fruit extract by measuring biomarkers of oxidative damage to protein (carbonyls), lipids (malondialdehyde), and DNA (8-oxo-2′-deoxyguanosine urinary excretion) and plasma antioxidant status (antioxidant capacity, vitamin E) in rats when fed basal diets containing fish and soybean oils, which are likely to generate different levels of oxidative stress. Boysenberry (*Rubus loganbaccus* × *baileyanus* Britt) extract was used as the dietary antioxidant. The basal diets (chow, synthetic/soybean oil, or synthetic/fish oil) had significant effects on the biomarkers of oxidative damage and antioxidant status, with rats fed the synthetic/fish oil diet having the lowest levels of oxidative damage and the highest antioxidant status. When boysenberry extract was added to the diet, there was little change in 8-oxo-2′-deoxyguanosine excretion in urine, oxidative damage to proteins decreased, and plasma malondialdehyde either increased or decreased depending on the basal diet. This study showed that boysenberry extract functioned as an *in vivo* antioxidant and raised the antioxidant status of plasma while decreasing some biomarkers of oxidative damage, but the effect was highly modified by basal diet. Our results are further evidence of complex interactions among dietary antioxidants, background nutritional status as determined by diet, and the biochemical nature of the compartments in which antioxidants function.

**KEY WORDS:** • ***anthocyanins*** • ***berry fruit*** • ***boysenberry*** • ***free radicals*** • ***oxidative damage*** • ***oxidative stress***

## INTRODUCTION

NUMEROUS HEALTH BENEFITS are claimed for dietary antioxidants. These health benefits are often assumed to arise because of an ability of antioxidants to reduce oxidative damage.[1–5] Oxidative damage to cellular biomolecules such as proteins, lipids, and DNA is believed to result from an increased production of chemically reactive free radicals, which overwhelm the body's endogenous antioxidant defense system causing oxidative modification to cellular components. Even normal bodily functions such as respiration and exercise generate free radicals.[6] Free radicals attack healthy cells, reducing the body's resistance to some types of cancers,[3,7] and may potentially play a role in the development of disorders such as cardiovascular disease[1] and neurodegenerative conditions such as Alzheimer's disease.[8] Oxidative damage to DNA, proteins, and lipids is known to accumulate with age and has been postulated to be a major type of endogenous damage leading to aging and diseases associated with aging.[9–11]

The activities of dietary antioxidants are often determined by *in vitro* measurements, such as the oxygen radical absorbance capacity (ORAC)[12] and ferric-reducing antioxidant power assays.[13] While these measures are accepted as valid indicators of the antioxidant potential of a substance,[14] *in vitro* assays of this type do not provide evidence that a specific substance acts as an *in vivo* antioxidant when consumed. Specific *in vivo* experimental approaches are required to demonstrate the biological efficacy of a particular antioxidant.

The aim of this study was to determine if an extract of boysenberry, which has demonstrated antioxidant activity *in vitro*, also has *in vivo* antioxidant activity and is therefore able to reduce oxidative stress. To assess antioxidant activity *in vivo* we measured biomarkers of oxidative damage (1) to DNA as 8-oxo-2′-deoxyguanosine (8-oxodG) in the urine, as an indicator of the integrated rate of oxidative damage within the body,[15] (2) to proteins as protein carbonyl formation, as oxidative damage to proteins increases their carbonyl content and consequently carbonyl groups on amino acids are thought to indicate oxidative modification,[16] and (3) to lipids as malondialdehyde (MDA), a secondary prod-

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Table 1. Composition of the Boysenberry Extract Added to the Diets

| *Component* | *Composition* |
|---|---|
| Energy (kJ/100 g) | 1,580 |
| Protein (g/100 g) | 4 |
| Fat (g/100 g) | <0.1 |
| Saturated fat (g/100 g) | <0.1 |
| Moisture (g/100 g) | 3.6 |
| Ash (g/100 g) | 3.6 |
| Carbohydrate (by difference) (g/100 g) | 88.8 |
| Sugars (g/100 g) | 66.3 |
| Sodium (mg/100 g) | 2.8 |
| Potassium (mg/100 g) | 1,470 |
| Total phenolic (mg/g) | 262.67 |
| Total anthocyanin (mg/g) | 12.48 |

uct of lipid peroxidation that is relatively stable and readily diffusible within or outside of the cell.[17]

## MATERIALS AND METHODS

### *Animals*

Fifty-four 8-week-old male Sprague-Dawley rats weighing 220–250 g were individually housed in a controlled environment where air temperature and relative humidity were maintained at 22–24°C and 50–60% respectively, with a diurnal 12-hour light cycle. Rats were randomized into groups according to their body weight, so the mean weights of the groups were similar. The study reported here was approved and followed the procedures set out by the Massey University Animal Ethics Committee.[18]

### *Preparation of boysenberry extract*

Ten kilograms of frozen boysenberry fruit (*Rubus loganbaccus* × *baileyanus* Britt) was macerated in a food processor prior to the addition of 10 L of isopropyl alcohol. The mixture was left to stand overnight at ambient temperature. The next day the pulp was separated from the extract by filtration through stainless steel mesh, and the remaining pulp was extracted with a further 10 L of isopropyl alcohol for 24 hours. This second extract was recovered from the pulp, and the two extracts were combined before being concentrated by rotary evaporation at 50°C until all the isopropyl alcohol had been removed. The remaining aqueous extract was freeze-dried to yield a dark red powder (composition shown in Table 1).

### *Animal diets*

The compositions of the three diets used in this study are provided in Table 2. The chow diet (CD) was based on natural ingredients, whereas the soy oil diet (SOD) and fish oil diet (FOD) were based on semisynthetic components supplemented with soybean and fish oil, respectively. These diets were selected because in experiments prior to this study (authors' unpublished data) it was observed that the control levels and the response of markers of oxidative stress to antioxidants appeared to vary depending on the base diet. This study was undertaken to investigate the interactive effects of boysenberry extract and base diet on markers of oxidative stress.

All rats were raised on CD (Table 2). At the start of the experiment 36 rats were transferred to a soybean diet (SOD, containing 10% soybean oil). Fourteen days later 18 rats (half of the 36) were transferred to a diet where the soybean oil was replaced with equal amounts of fish oil (FOD, containing 10% fish oil). An outline of the study is shown

Table 2. Diet Components

| *CD* | *SOD* | *FOD* |
|---|---|---|
| 36.766% wheat | 67% wheat and corn flour | 67% wheat and corn flour |
| 27% barley | 12% lactic casein | 12% lactic casein |
| 6.3% fishmeal | 10% soybean oil | 10% fish oil |
| 5.4% meat and bone | 5% mineral mix[a] | 5% mineral mix[a] |
| 4.5% broll | 5% vitamin mix[b] | 5% vitamin mix[b] |
| 4.5% skim milk powder | 1% cellulose | 1% cellulose |
| 4.5% lucerne | | |
| 0.9% soybean oil | | |
| 5% mineral mix[a] | | |
| 5% mineral mix[b] | | |
| 0.09% methionine | | |
| 0.0046% NaCl | | |

[a]Mineral mix contains potassium phosphate, potassium sulfate, potassium citrate, NaCl, magnesium oxide, trace salt mix, and cellulose (as filler).

[b]Vitamin mix contains retinal acetate, ergocalciferol, menadione, biotin, folic acid, calcium panthothenate, riboflavin, thiamine HCl, pyridoxine HCl, inositol, choline chloride, sucrose, nicotinamide vitamin $B_{12}$, and vitamin E. The vitamin mix contributed 9 IU of vitamin E/kg to the final diet.

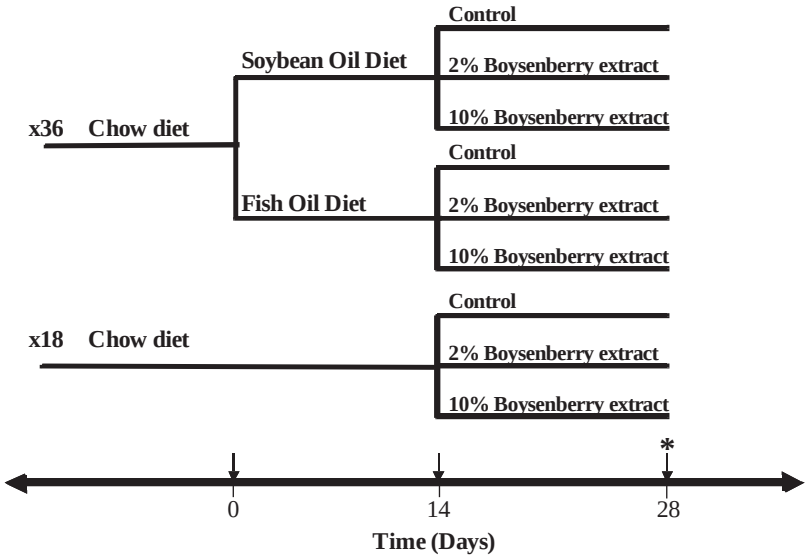


**FIG. 1.** Schematic of the study design. Fifty-four Sprague-Dawley rats were acclimatized for 2 weeks on CD, and then 18 animals were transferred to SOD and 18 animals to FOD. Following a further 2 weeks to acclimatize to the base diet the boysenberry extract was introduced at either 2% or 10% of diet. Data are mean ± SE values ($n = 6$). *Samples collected for analysis of biomarkers of oxidative damage and oxidative status.

in Figure 1. Diets were prepared at the Feed Processing Unit (Massey University, Palmerston North, NZ). The CD, SOD, and FOD diets contained minimal vitamin E levels (9 IU/kg) to ensure that the main source of dietary antioxidant was derived from the boysenberry extract. In the diets containing boysenberry extract, boysenberry extract replaced corn flour on an equal weight basis. In the CDs containing boysenberry extract, the equivalent percentage of diet was replaced with boysenberry extract. The antioxidant properties and phytochemical content of the diets are provided in Table 3.

All rats were provided with water *ad libitum* throughout the experiment. Feed intake of each rat was measured daily, and the rats were weighed every second day.

### *Sample collection*

On day 27 the rats were individually housed in metabolic cages (Nalgene® catalog number 650-0100, Nalge Nunc International, Rochester, NY), and an overnight urine sample was collected and frozen. At the end of the study (day 28), terminal exsanguination was performed by intracardiac puncture with an 18-gauge needle after administration of an isoflurane/oxygen mixture to anesthetize the rats. Approximately 10 mL of blood was obtained from the heart using EDTA as the anticoagulant. Plasma was recovered by centrifugation (2,000 *g* at 4°C for 15 minutes), and aliquots were distributed into tubes for each assay and placed immediately on dry ice before storage at −20°C until analysis. All samples were analyzed within 6 months of sample collection, for oxidized lipid, DNA, protein, and plasma antioxidant status [fluorescein ORAC ($ORAC_{FL}$) and vitamin E].

### *Determination of biomarkers of oxidative damage*

All solvents used were high performance liquid chromatography (HPLC) grade. Chemicals and reagents were analytical grade and were obtained from Merck New Zealand Ltd. (Palmerston North).

TABLE 3. CONCENTRATIONS OF ANTHOCYANINS, TOTAL PHENOLICS, AND ANTIOXIDANT CAPACITY (ORAC) IN THE DIETS

| *Diet* | *Total anthocyanin (mg/g of diet)* | *Total phenolics (mg/g of diet)* | *ORAC (μmol of TE/g of diet)* |
|---|---|---|---|
| SOD | | | |
| Control | 0.00 | 0.03 | 4.3 |
| 2% boysenberry extract | 0.18 | 1.29 | 19.3 |
| 10% boysenberry extract | 0.91 | 6.55 | 48.8 |
| FOD | | | |
| Control | 0.00 | 0.04 | 13.5 |
| 2% boysenberry extract | 0.10 | 0.90 | 17.5 |
| 10% boysenberry extract | 0.96 | 8.16 | 44.4 |
| CD | | | |
| Control | 0.00 | 0.88 | 33.6 |
| 2% boysenberry extract | 0.12 | 1.71 | 38.0 |
| 10% boysenberry extract | 0.75 | 5.99 | 69.6 |

*Urinary 8-oxodG excretion*

Strata X cartridges (part number 8B-S100-HCH-S, Phenomenex, Torrance, CA) were sequentially preconditioned with 10 mL of each of the following: methanol, water, and buffer A (50 mmol/L potassium dihydrogen phosphate, pH 7.5 with potassium hydroxide). One milliliter of urine sample was mixed with 1 mL of 1 mol/L NaCl solution and applied to the preconditioned cartridges, which were then sequentially washed with 4 mL of buffer A, 5 mL of buffer B (5% methanol in Buffer A), and 3 mL of ethyl acetate. Following this, the 8-oxodG was eluted with 2 mL of methanol. The final methanol eluant was concentrated to dryness under nitrogen and redissolved in 200 $\mu$L of 100 mmol/L sodium acetate buffer, pH 3.5.

Samples were analyzed by multidimension reverse-phase HPLC. The HPLC system consisted of a DG-980-50 three-line degasser, LG-980-02 ternary gradient unit, PU-980 Intelligent HPLC pump, AS-950 Intelligent autosampler, and UV-975 Intelligent ultraviolet/visible detector all from JASCO, Tokyo, Japan). 8-OxodG was detected electrochemically using an eight-channel CoulArray® electrochemical detector (ESA Inc., Chelmsford, MA), with the first channel set to 200 mV and each successive channel incremented by 45 mV. Samples (20 $\mu$L) were injected onto the first column (Adsorbosphere, C8 5 $\mu$m particle size, 150 $\times$ 4.6 mm, part number 287123, Alltech, Inc., Deerfield, IL) using an isocratic solvent program with a mobile phase consisting of 5% methanol in 100 m*M* sodium acetate buffer, pH 3.5 (mobile phase A). When 8-oxodG was eluted from the first column it was trapped in a 1-mL sample loop mounted on a switching valve (Rheodyne, Rohnert Park, CA) and transferred to the second column (Luna, C18 5 $\mu$m particle size, 250 $\times$ 4.6 mm, part number 00G-4252-EO, Phenomenex). The second column was eluted with mobile phase A and mobile phase B (20% methanol in mobile phase A) using a linear gradient program maintaining 100% A until 13 minutes, then 5% A/95% B at 30 minutes, and holding for 5 minutes before returning to the initial conditions. The concentration of 8-oxodG was determined using the peak height at 290 mV and an authentic standard (1 $\mu$g/mL) analyzed before and after each sample. The flow rate was 0.8 mL/minute for both columns.

*Plasma protein carbonyls*

Plasma protein carbonyl content was measured by an enzyme-linked immunosorbent assay supplied by Zenith Technology (Dunedin, NZ). The assay was performed according to the instructions of the manufacturer.

*Plasma MDA*

Plasma MDA was measured by a gas chromatography-mass spectrometry (GC-MS)-based method.[19] Briefly, MDA standards were prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma Inc., Sacramento, CA) at 40°C. Sample preparation involved treating 500 $\mu$L of plasma (or standard) with 350 $\mu$L of 10% trichloroacetic acid to precipitate the protein. Then 300 $\mu$L of 0.8% butylated hydroxytoluene in hexane was added, and the sample/standard was mixed using a vortex-mixer and centrifuged for 10 minutes at 12,000 *g*. After the upper hexane layer was discarded, 100 $\mu$L of 9 mmol/L phenylhydrazine (PH) (Sigma) was added, and the mixture was left for 1 hour at room temperature to convert MDA to the PH derivative. Derivatized MDA was extracted by adding 300 $\mu$L of hexane containing the internal standard (benzophenone, 100 ng/mL). Following centrifugation the hexane layer was removed, and the PH-MDA derivative was measured by GC-MS with selected ion monitoring (GCMS-QP5050A gas chromatograph/mass spectrometer, Shimadzu Corp., Kyoto, Japan) equipped with DB™–5MS 30-m, 0.25-mm inner diameter, 0.25-$\mu$m film thickness capillary GC column (part number 122-5532, J & W Scientific, Folsom, CA). The temperature gradient was as follows: 40°C for 1 minute, 10°C/minute to 300°C, and maintenance at 300°C for 10 minutes. The injection temperature was 250°C with a 2-$\mu$L splitless injection and a sampling time of 1 minute. The temperature of the GC-MS interface was 250°C, and the detector voltage was 1.8 kV. MDA and the internal standard were recorded at mass to charge ratios (*m*/*z*) of 76.90, 104.85, 143.90, and 182.05. The peak area of the PH-MDA derivative and benzophenone internal standard in the samples was calculated using Shimadzu GC-MS Solutions GC-MS software, quantified using a standard curve prepared with 0–450 ng/mL of derivatized MDA standards. The MDA concentration was calculated as ng/mL of plasma.

*Plasma antioxidant status*

*Antioxidant capacity (ORAC).* Plasma $ORAC_{FL}$ was measured using a method similar to that described by Ou *et al*.[20] Plasma samples were diluted 125-fold with 75 mmol/L potassium phosphate buffer, pH 7.4. For non-protein $ORAC_{FL}$ analysis plasma proteins were precipitated with an equal volume of 0.3% perchloric acid and centrifuged at 12,000 *g*, and the supernatant was diluted 6.67-fold with 75 mmol/L potassium phosphate buffer, pH 7.4. Standard curves were prepared using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E, at 50–200 $\mu$mol/L. Each sample or standard was analyzed in triplicate in a 96-well format, with one blank per sample and a total of 16 samples per plate. Plates were prepared by adding 10 $\mu$L of sample (or phosphate buffer for the blanks) and 160 $\mu$L of fluorescein ($4.8 \times 10^{-8}$ mol/L) to each well. Plates were preincubated in a fluorescence plate reader (Spectramax Gemini, Molecular Devices Corp., Sunnyvale, CA), to stabilize the temperature to 40°C. 2,2′-Azobis(2-amidinopropane) dihydrochloride (25 $\mu$L, 0.1 mol/L) was added to each well, and the fluorescence was measured every minute for 90 minutes. The excitation and emission wavelengths were 493 nm and 515 nm, respectively. The plate was shaken between each reading. The relative fluorescence for each standard was calcu-

lated, to give the area under the curve (AUC), and a standard curve was plotted. The antioxidant capacity [in mmol/L of Trolox equivalent (TE)] of the sample was calculated by $AUC_{sample} - AUC_{blank}$ and related to the results obtained from the standards.

*Vitamin E.* Vitamin E was measured in plasma using a method similar to that described by Gimeno *et al.*[21] Briefly, 500 $\mu$L of plasma was added to 100 $\mu$L of 50 $\mu$g/mL tocopherol acetate as the internal standard and vortex-mixed. Then 500 $\mu$L of absolute ethanol was added, and the sample was mixed for a further 10 seconds. Vitamin E was extracted by mixing the sample with 500 $\mu$L of hexane followed by centrifugation at 12,000 *g* for 15 minutes. The upper hexane layer was transferred to another tube, and the sample was extracted with a further 500 $\mu$L of hexane. Both hexane extractions were combined and concentrated to dryness under nitrogen at 30°C. The dried sample was redissolved in 200 $\mu$L of methanol, and then 40 $\mu$L was analyzed by reversed-phase HPLC (the same JASCO HPLC system was used as for measuring urinary 8-oxodG excretion) fitted with a Lichrocart C18 column (part number 1.50838.001, Merck, Darmstadt, Germany). The isocratic solvent program consisted of 100% methanol at 1 mL/minute with detection at 290 nm). A five-point standard curve was plotted using the peak area of $\alpha$-tocopherol related to the peak area of the tocopherol acetate internal standard. Peak areas from the samples were related to this curve, and the concentration was determined in $\mu$g/mL of plasma.

*Statistical analysis*

All statistical analyses were performed using GenStat for Windows version 7 (VSN International, UK, Hemel Hempstead, Herts, UK). To detect significant differences, parameters for oxidative stress were analyzed by a two-way analysis of variance. Differences between treatments were assessed using Fischer's protected least significant difference, and a value of $P < .05$ was considered significant.

## RESULTS

*Dietary intake and weight gain*

When the animals were transferred to the base diets, feed consumption changed depending on the diet. Consumption for the CD- and SOD-fed animals was similar, whereas consumption of the FOD-fed animals was approximately half. When animals were provided with diets containing boysenberry extract the difference in consumption between rats fed CD (mean 33 g/day) and SOD (mean 30 g/day) was not statistically different. Consumption for each treatment (control, 2% boysenberry, and 10% boysenberry) of FOD (mean 25 g/day) was lower than the mean consumption of animals fed the corresponding CD and SOD.

For the first 14 days of the study there was no difference in body weight gain of rats. With the introduction of the basal diets the body weight gain of the FOD-fed animals declined (Fig. 2), whereas the CD-fed rats gained significantly ($P < .05$) more weight than those fed either SOD or FOD. In addition, the rats on FOD had signifi-

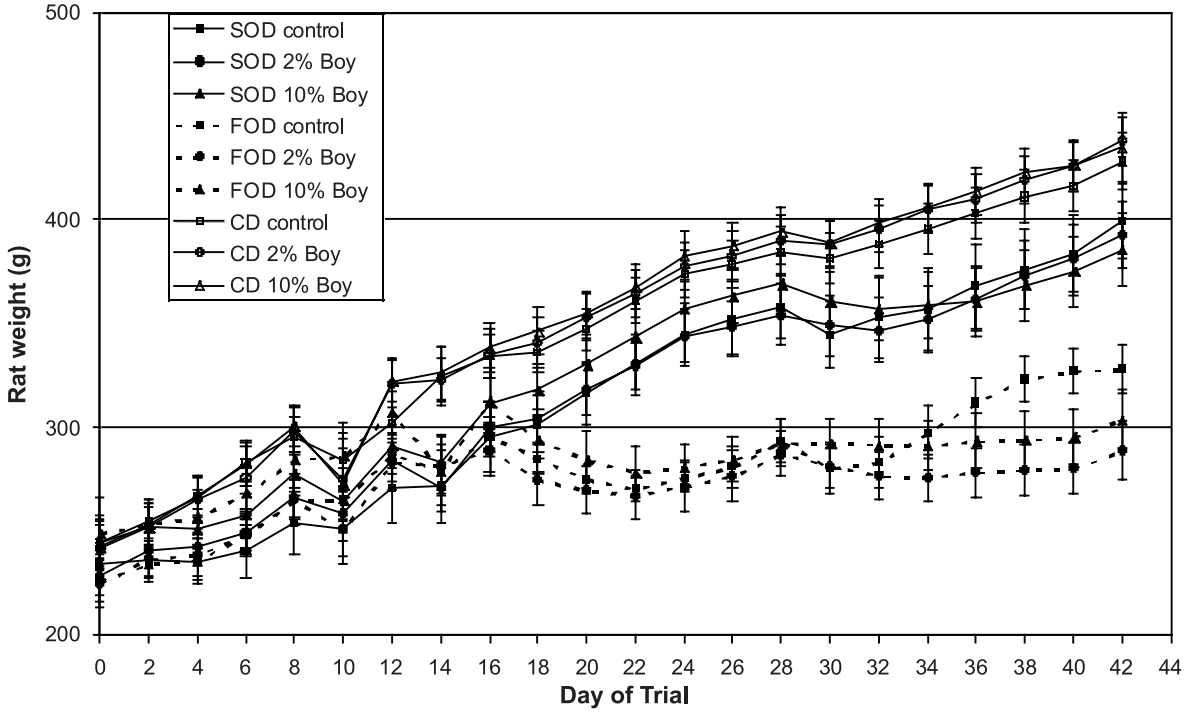


**FIG. 2.** Body weight gain for animals in the study. On days 1–14 animals were fed CD, on days 15–28 animals were divided into base diet, and on days 29–42 boysenberry (Boy) extract was introduced into the diet. Data are mean ± SEM values ($n = 6$).

cantly ($P < .05$) lower weights than rats in all other groups. When the boysenberry extract was added to FOD, the rats gained weight at a lower rate than their respective control groups.

### *Biomarkers of oxidative damage*

The oxidative stress present in the animals on the different dietary regimes was determined by measuring the concentrations of specific biomarkers of oxidative damage (8-oxodG, protein carbonyls, and MDA) together with measures of plasma oxidative status ($ORAC_{FL}$ and vitamin E). The results are shown in Table 4.

CD-fed rats excreted greater amounts of 8-oxodG than rats on either FOD or SOD. When boysenberry extract was added to the diet, 8-oxodG excretion increased significantly for the CD/2% boysenberry-fed rats compared with the CD/control-fed rats. There were no other significant differences in the excretion of 8-oxodG between any of the boysenberry extract treatment groups and their respective controls. Plasma protein carbonyl concentrations were significantly less for the CD/control-fed rats compared to the FOD and SOD control-fed rats. When boysenberry extract was added to the diet the plasma protein carbonyl concentrations were reduced for CD-fed rats at both concentrations of boysenberry extract but were reduced for only the 10% boysenberry concentration in the SOD-fed rats. In the FOD-fed rats, the addition of boysenberry extract did not change plasma protein carbonyl concentrations. There were large differences between the control treatments for each of the basal diets for plasma MDA concentration. When boysenberry extract was added to each of the diet groups, the response differed. For the SOD-fed rats MDA concentration was significantly reduced compared to the control when 2% and 10% boysenberry extract was added. For CD there was no significant difference. However, for the FOD-fed rats, plasma MDA increased for rats fed 2% boysenberry extract compared with the FOD controls, suggesting a pro-oxidant effect.

### *Plasma antioxidant status ($ORAC_{FL}$ and vitamin E)*

The antioxidant capacity ($ORAC_{FL}$) of the total plasma and non-protein plasma was significantly greater ($P \leq .05$) for the FOD control rats than both the SOD and CD control rats, suggesting a higher antioxidant capacity for rats fed the FOD diets. When the boysenberry extract (2% and 10%) was incorporated into the FOD there was no significant change relative to the FOD control. When both SOD and CD were supplemented with 2% boysenberry extract the non-protein and total $ORAC_{FL}$ values increased significantly ($P \leq .05$). Plasma vitamin E concentration was highest in rats fed FOD and lowest for the SOD control diets. Boysenberry extract (10%) treatment significantly ($P \leq .05$) decreased the concentration of plasma vitamin E concentration in rats fed FOD.

## DISCUSSION

This study was initiated to determine if a boysenberry extract with *in vitro* antioxidant activity was able to affect the level of oxidative stress as determined by biomarkers of oxidative damage and antioxidant status. In preliminary experiments, we observed apparent differences in the response to added boysenberry extract depending on the type of basal diet used. Therefore, to investigate the effect of the addition of boysenberry extract and the effect of basal diet on the response, this study was designed with three basal diets (CD, SOD, and FOD) each with a control group and two concentrations of added boysenberry extract. The results showed that boysenberry extract did function as an *in vivo* dietary antioxidant as a number of measures of oxidative damage were decreased. However, the *in vivo* antioxidant response observed appeared to be modulated by basal diet.

### *Effect of diet on basal levels of oxidative damage*

Although there were differences in food intake and body weight gain for the control diets, there were minimal dif-

Table 4. Measures of Oxidative Stress and Antioxidant Capacity After 2 Weeks on Each Diet

| *Diet treatment* | *8-OxodG (nmol/kg of body weight/24 hours)* | *Protein carbonyl (nmol/mg of protein)* | *MDA (ng/mL)* | *ORAC (mmolL)* | *Non-protein ORAC (mmolL)* | *Vitamin E (μg/mL)* |
|---|---|---|---|---|---|---|
| SOD | | | | | | |
| Control | 1.49 ± 0.26 | 0.38 ± 0.07 | 182 ± 28.6[a,b] | 7.32 ± 0.39 | 0.99 ± 0.04[a] | 2.54 ± 0.26[b] |
| 2% boysenberry extract | 1.41 ± 0.36 | 0.33 ± 0.04 | 23.0 ± 4.31[c] | 9.04 ± 0.74[c] | 1.53 ± 0.13[c] | 2.90 ± 0.18 |
| 10% boysenberry extract | 0.92 ± 0.17 | 0.18 ± 0.05[c] | 64.9 ± 28.3[c] | 8.92 ± 0.98[c] | 1.51 ± 0.05[c] | 2.81 ± 0.16 |
| FOD | | | | | | |
| Control | 2.10 ± 0.63 | 0.30 ± 0.09 | 44.8 ± 18.0[c] | 9.21 ± 0.92[c] | 1.52 ± 0.08[b,c] | 3.77 ± 0.37[c] |
| 2% boysenberry extract | 2.36 ± 0.79 | 0.37 ± 0.11 | 189 ± 57[a] | 8.10 ± 1.11 | 1.44 ± 0.11 | 2.58 ± 0.28[a] |
| 10% boysenberry extract | 2.81 ± 0.73 | 0.23 ± 0.05 | 115 ± 27.2[a] | 9.76 ± 1.04 | 1.52 ± 0.15 | 2.84 ± 0.11[a] |
| CD | | | | | | |
| Control | 3.40 ± 1.04[a,c] | 0.21 ± 0.07[c] | 83.9 ± 16.5[c] | 8.52 ± 1.04 | 1.06 ± 0.13[a] | 3.26 ± 0.52 |
| 2% boysenberry extract | 4.98 ± 1.03[b] | 0.09 ± 0.02[b] | 47.3 ± 18.7 | 11.1 ± 1.00[b] | 1.42 ± 0.06[b] | 2.60 ± 0.10[b] |
| 10% boysenberry extract | 3.57 ± 1.21 | 0.07 ± 0.03[b] | 50.6 ± 23.4 | 8.88 ± 0.56 | 1.38 ± 0.05[b] | 2.60 ± 0.13[b] |

Data are mean ± SEM values ($n = 6$).
Mean values statistically different ($P > .05$) from the [a]FOD-control, [b]CD-control, or [c]SOD-control.

ferences in 8-oxodG excretion and plasma protein carbonyl content between the rats fed the three different basal diets. In contrast, there were significant differences in plasma MDA, plasma non-protein $ORAC_{FL}$, and plasma vitamin E. For example, rats consuming the FOD-control diet had the highest plasma $ORAC_{FL}$, non-protein $ORAC_{FL}$, and vitamin E and the corresponding lowest plasma MDA. In contrast, the SOD-fed rats had the lowest plasma $ORAC_{FL}$, non-protein $ORAC_{FL}$, and vitamin E and the corresponding highest plasma MDA. This suggested that the higher antioxidant plasma status of the FOD-fed animals resulted in lower lipid peroxidation. However, this increased antioxidant status did not result in reduced oxidative damage to DNA (8-oxodG) and protein (protein carbonyls) in these animals, suggesting that there are distinctly separate mechanisms for the control of oxidative damage for DNA, proteins, and lipids in plasma.

These results, showing differences in oxidative damage in the control animals, suggests that the SOD diet produced more oxidative stress than the FOD diet, with the CD diet being intermediate in its capacity to induce oxidative stress. This is in contrast to other studies that have shown that increased consumption of fish oil containing polyunsaturated fatty acids leads to higher levels of oxidative stress. The suggested mechanism for this is that consumption of fish oil results in a greater incorporation of polyunsaturated fats into membrane systems of the animal, which promotes increased lipid oxidation. In contrast, diets high in monounsaturated fats, such as those in soybean oil, have been thought to generate less oxidative stress.

### *Effect of adding additional antioxidant (boysenberry extract) to the diet*

The addition of either 2% or 10% boysenberry extract resulted in little change in food intake or body weight gain compared to the control for each of the basal diets. However, the introduction of boysenberry extract produced changes in the concentrations of some indicators of oxidative damage and provided evidence that oxidative stress can be modulated by dietary antioxidants such as boysenberry extract.

When boysenberry extract was introduced to animals fed the basal CD, the indicator of oxidative damage to protein was significantly reduced, but the indicators for DNA and lipid damage remained unchanged. Concurrently the indicators for antioxidant status increased for $ORAC_{FL}$ (2% only) and non-protein $ORAC_{FL}$, but plasma vitamin E concentration decreased. In contrast, when boysenberry extract was added to the SOD-fed animals, all indicators of oxidative damage to DNA, protein, and lipid decreased. At the same time the indicators for antioxidant status all increased significantly with the exception of plasma vitamin E. Therefore in animals fed the SOD diet, boysenberry extract functioned as an effective dietary antioxidant according to most of the parameters measured. When animals fed FOD were also fed boysenberry extract, the indicators of oxidative damage to DNA and protein remained unchanged, whereas plasma MDA concentration increased, indicating more lipid oxidation in the boysenberry-fed animals. The corresponding measures of antioxidant status were unchanged for both ORAC and non-protein ORAC, but the plasma concentrations of vitamin E decreased. Together, the increase in plasma MDA and the decrease in vitamin E concentration with the introduction of boysenberry extract suggest that in these FOD-fed animals boysenberry extract induced oxidative damage to lipids and increased the level of oxidative stress.

The results, taken in their entirety, suggests that the addition of boysenberry extract to the diet at either the 2% or 10% level reduced oxidative stress by reducing the amount of oxidative damage to key biomolecules, but that the degree of antioxidant activity appears to be dependent on the basal diet. For example, plasma protein carbonyl content was unchanged in the FOD-fed animals, but there was a decrease in the CD-fed animals when boysenberry extract was introduced. This suggests that the antioxidant power of the boysenberry extract with respect to plasma carbonyl proteins was only effective in particular nutritional circumstances as determined by the basal diet. As the concentrations of carbonyl proteins were similar in the controls for all the diets, the FOD diet did not appear to induce additional oxidative damage to proteins, but it appeared to inhibit the antioxidant effect of the boysenberry extract on plasma proteins. Clearly the nutritional status of the animals, determined by the basal diet, had a large effect on the *in vivo* antioxidant function of boysenberry extract in these animals. Compared with protein oxidation, an opposite effect was observed for the biomarker of lipid oxidation, MDA. The concentrations of MDA in plasma from the animals fed the control diets for FOD- and CD-fed animals were similar. However, when boysenberry extract was added, MDA increased for the FOD-fed animals, indicating a pro-oxidant effect, whereas for the CD-fed animals the MDA concentration was not significantly different. Thus the biomarkers for oxidative damage to DNA, protein, and lipid behaved differently when boysenberry extract was added to the diet.

Vitamin E is an important lipid-soluble antioxidant and may be important for reducing lipid peroxidation,[22,23] whereas protein oxidation is more likely to involve aqueous environments. The correlation coefficients between plasma vitamin E concentrations and 8-oxodG, protein carbonyl, and MDA were $-0.0567$, 0.3306, and $-0.4141$, respectively. As anticipated there appears to be an inverse relationship between plasma vitamin E concentration and MDA; however, there was also a substantial positive correlation between vitamin E concentration and protein carbonyls. Although it is recognized that there appears to be a relationship between water-soluble antioxidants (vitamin C) and lipid-soluble antioxidants (vitamin E),[24] the results presented here suggest that the *in vivo* mechanisms for restricting oxidative damage in the lipophilic and hydrophilic compartments may be independent of each other.

The boysenberry extract was prepared by simple water–alcohol extraction followed by freeze-drying and had the

composition described in Table 2. Like other fruit of the *Rubus* genus, boysenberries contain relatively high concentrations of anthocyanins composed of the aglycone cyanidin to which different types of sugars are conjugated.[25] Anthocyanins are considered to benefit health and well-being because of their high antioxidant activity,[26] which can protect low-density lipoproteins from oxidation,[27] and have been reported to have anti-inflammatory and anticancer activity.[28–30] A number of reports have established that anthocyanins are bioavailable, but at an apparently low level.[31–34] Anthocyanins were one of the major antioxidant components of the boysenberry extract used in this study. Previous studies have shown that anthocyanins or anthocyanin-rich extracts can provide some resistance to oxidative damage. For example, Ramirez-Tortosa *et al.*[35] demonstrated that a highly enriched anthocyanin extract of *Abies koreana* improved plasma antioxidant capacity and, in vitamin-E deficient rats, reduced the concentration of hydroperoxides and 8-oxodG in the liver. When oxidative stress was induced in rats by treatment with paraquat, anthocyanins isolated from eggplant and red cabbage were able to provide some protection and reduced the oxidative damage as measured by several indices.[36] Furthermore, anthocyanin-containing berry fruit incorporated into juice mixtures increased both antioxidant status (plasma Trolox equivalence antioxidant capacity) and reduced oxidative damage (plasma MDA) in human studies.[37,38] In contrast, the study by Young *et al*.[39] in which human subjects consumed 750, 1,000, or 1,500 mL of a blackcurrant/apple juice combination observed no change in plasma antioxidant status, a decrease in plasma MDA, and an increase in plasma protein oxidation. However, these previous studies taken together, and combined with the current study, suggest that anthocyanin-rich foods may function as *in vivo* antioxidants but that the effect is modulated by the basal diet and by the type of *in vivo* antioxidant effect investigated. The current study suggests that *in vivo* antioxidants may only be active against the oxidation of particular biomolecules, and under specific conditions. In this respect boysenberry extract appeared to be an effective *in vivo* antioxidant against protein oxidation in the CD-fed rats and lipid peroxidation in the SOD-fed rats.

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