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Research article

Dietary supplementation with fruit polyphenolics ameliorates age-related deficits in behavior and neuronal markers of inflammation and oxidative stress

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Abstract

Dietary supplementation with fruit or vegetable extracts can ameliorate age-related declines in measures of learning, memory, motor performance, and neuronal signal transduction in a rat model. To date, blueberries have proved most effective at improving measures of motor performance, spatial learning and memory, and neuronal functioning in old rats. In an effort to further characterize the bioactive properties of fruits rich in color and correspondingly high in anthocyanins and other polyphenolics, 19-month-old male Fischer rats were fed a well-balanced control diet, or the diet supplemented with 2% extract from either blueberry, cranberry, blackcurrant, or Boysenberry fruit for eight weeks before testing began. The blackcurrant and cranberry diets enhanced neuronal signal transduction as measured by striatal dopamine release, while the blueberry and cranberry diets were effective in ameliorating deficits in motor performance and hippocampal HSP70 neuroprotection; these changes in HSP70 were positively correlated with performance on the inclined screen. It appears that the polyphenols in blueberries and cranberries have the ability to improve muscle tone, strength and balance in aging rats, whereas polyphenols in blueberries, cranberries and blackcurrants have the ability to enhance neuronal functioning and restore the brain's ability to generate a neuroprotective response to stress.

Introduction

Numerous studies suggest that one of the most important factors mediating the deleterious effects of aging on behavior and neuronal function are changes in the balance between antioxidant activity and reactive oxygen species (ROS) production. This imbalance in favor of ROS production can be defined as increased oxidative stress (OS) (see Floyd (1999) for review). The CNS appears to be especially vulnerable to ROS effects, partially as a result of additional factors such as increases in the ratio of oxidized to total glutathione (Olanow 1992), significant lipofuscin accumulation (Gilissen et al. 1999) with bcl-2 increases (Sadoul 1998), increases in membrane lipid peroxidation (Yu 1994), decreased glutamine synthetase (Carney et al. 1994), reductions in redox active iron (Gilissen et al. 1999; Savory et al. 1999) and alterations in membrane lipids (Denisova et al. 1998). Importantly, in addition to these considerations, it has been shown that not only is the CNS particularly vulnerable to OS but this vulnerability increases during aging (see Joseph et al. 1998a, b for review). With respect to aging we have shown that sene-

scent rats exhibited significantly greater motor behavioral deficits and decreased tyrosine hydroxylase immunoreactivity (in pars compacta) than young rats following intranigral applications of dopamine (DA), an ROS generator (Cantuti-Castelvetri et al. 2003).

Therefore, it might be suggested that enhancing defenses against these increases in vulnerability to OS through the administration of antioxidants would be useful in preventing or forestalling the deleterious effects of aging. However, the results of these studies have been mixed, especially when single antioxidants have been employed [e.g., vitamin E, see Cantuti-Castelvetri et al. (2000) for review]. For that reason, we have utilized dietary administration of fruit or vegetable extracts to determine if behavioral and neuronal deficits in aging could be moderated (Bickford et al. 2000; Joseph et al. 1998c, 1999; Youdim et al. 2000). Our research supports the hypothesis that the combinations of antioxidant/antiinflammatory agents found in whole fruits and vegetables can have significant antiaging effects.

Plants, including food plants (fruits and vegetables), synthesize a vast array of chemical compounds that are not involved in their primary metabolism. These "secondary compounds" instead serve a variety of ecological functions, ultimately to enhance the plant's survivability. Key among these components may be the polyphenolics, which provide protection against solar irradiation and oxidative damage. Interestingly, the polyphenolics may be responsible for the multitude of beneficial effects of fruits and vegetables on an array of health-related (e.g., anticancer and antiviral) bioactivities, two of the most important of which may be their antioxidant and antiinflammatory properties.

Among plant polyphenolics, which have attracted immense attention due to their potent antioxidant and antiinflammatory activities, are a subgroup of the flavonoids known as anthocyanins. These are natural pigments responsible for the orange, red and blue colors of fruits, flowers, vegetables and other storage tissues in plants (Wang et al. 1999; Seeram et al. 2001a, b). Anthocyanins have been reported to inhibit lipid peroxidation *in vitro* and also to inhibit the activity of cycloxygenase-1 and -2 (COX) enzymes (Seeram et al. 2002, 2003), and therefore possess both antiinflammatory and antioxidant activities.

In previous studies we have found positive effects of blueberries (BBs) on age-related declines in motor, cognitive, and neuronal function (Bickford et al. 2000; Joseph et al. 1998c, 1999; Youdim et al. 2000). Among berryfruits, BBs contain high levels of a wide variety of anthocyanins including glycosides of four of the six major anthocyanidins: malvidin, petunidin, peonidin and cyanidin (Kalt et al. 1999). This abundance could explain the beneficial effects of BBs compared to other fruits and vegetables with different anthocyanin profiles. Of note are our recent findings that detectable amounts of several of these anthocyanins are present in the brains of rats fed a 2% blueberry-supplemented diet for 10 weeks (Andres-Lacueva et al., in press).

The present study was carried out to determine whether the beneficial effects of BBs would also be seen with other berryfruits [i.e., blackcurrants (BC), Boysenberries (BY), and cranberries (CB)] as compared with a control diet. In this effort, 19-month-old male Fischer 344 rats were maintained on a diet containing 2% BB, BC, BY, or CB extract or a control diet for eight weeks before performance on various motor and cognitive tasks was assessed. In addition we examined, *in vitro*, the activation of hippocampal heat shock protein 70 (HSP70) by lipopolysaccharide (LPS) and the levels of striatal DA release as indicators of antiinflammatory responsiveness and neuronal functioning, respectively.

HSP70 was chosen for study in the present experiments since previous studies have shown that increasing the levels of inducible HSP70 can protect cells from numerous insults ranging from ischemia, inflammatory agents and reactive oxygen species (Gordon et al. 1997; Guo and Mattson 2000; Lowenstein et al. 1991; Sharp et al. 1999). The loss of the ability of cells to respond to these insults by increasing HSP70 may contribute to the age-related declines in both neuronal and behavioral functioning. In the case of muscarinic activation of striatal DA release, numerous studies have indicated that this is a sensitive marker for assessing striatal muscarinic sensitivity in aging (Joseph et al. 1988a, b, 1990, 1996).

Materials and methods

Animals

Seventy 19-month-old male Fischer 344 rats were obtained from the NIA/Harlan colony. The rats were individually housed in stainless steel mesh cages,

provided food and water *ad libitum*, and maintained on a 12-h light/dark cycle. The rats were given two weeks to adjust to their new environment after which time they were weight matched and placed on five separate diets: 2% BB, BC, BY, or CB extract or a control diet for 13–16 weeks total. All animals were observed daily for clinical signs of disease. These animals were utilized in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, USPHS, Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Use and Care Committee of our Center.

Berryfruit extracts and diet

Berryfruit were obtained from different sources. Frozen blueberries were provided by the University of Georgia at Tifton, GA, frozen cranberries were obtained from the Cranberry Institute, East Wareham, MA, while frozen blackcurrants and Boysenberries were provided by Blackcurrants New Zealand Ltd. and Berryfruit Export Ltd., respectively. The berries [blueberries (*Vaccinium ashei* Reade), blackcurrants (*Ribes nigrum* L. cv. Ben Ard), Boysenberries (*Rubus loganbaccus* × *baileyanus* Britt cv. Riwaka Choice), or cranberries (*Vaccinium macrocarpon* Ait.)] were added to water in a 1:1 ratio, homogenized in a blender and centrifuged at 13,000 *g* for 15 min. The supernatant was frozen, crushed, and freeze-dried. The resulting freeze-dried fruit extract powders were shipped to Harlan Teklad (Madison, WI) where they were combined with the control diet, NIH-31 (20 g/kg). The amount of corn in the control diet was adjusted to compensate for the added volume (see Youdim et al., 2000 for more information on diet preparation and composition). The rats gained weight from 19 to 24 months, and there were no differences in weight between the groups over the course of the study ($P > 0.05$). There were also no differences in food intakes between the diet groups ($P > 0.05$).

The phytochemical composition of the four extracts was determined by HPLC according to the method of Connor and colleagues (Connor et al. in press). Briefly each extract was dissolved in methanol/water/formic acid (50:50:10 v/v/v) and analyzed by HPLC (Connor et al. in press). Total anthocyanins were measured by summing all the peaks observed at 520 nm and expressing as cyanidin 3-*O*-glucoside equivalents, total phenolics were measured by summing all the peaks observed at 325 nm and expressing as chlorogenic acid equivalents.

Procedures

Psychomotor testing

A battery of age- and diet-sensitive tests of psychomotor behavior (Joseph et al. 1983, 1998c, 1999; Youdim et al. 2000; Ingram et al. 1994; Shukitt-Hale et al. 1998) was administered in a randomized order to the animals at the end of the eighth week of dietary supplementation. Each test was performed once, separated by a break between tasks. Briefly, the tests included: (1) Rod Walk: The ability of rats to balance on a stationary, horizontal rod measures psychomotor coordination and the integrity of the vestibular system. Animals were placed in the center of a rod (100 cm long, 26 mm in diameter, positioned 23 cm above the table surface), and their latency to fall off the rod onto a cushion below was recorded (max score = 60 s). If the rats fell immediately after being placed on the rod, they were given another opportunity. (2) Wire Suspension: The prehensile reflex refers to an animal's ability to grasp a horizontal wire (12-gauge) with its forepaws and to remain suspended; it is a measure of muscle strength. Rats were raised to an elevated taut horizontal wire (55 cm above the tabletop) and the forepaws of each rat were placed on the wire. Each was given one trial, with the total hanging time in seconds recorded (max score = 60 s). (3) Plank Walk: Balance and coordination were measured by exposing the rats to one trial on each of three horizontal planks (wide = 38 mm; medium = 25 mm; narrow = 13 mm), each 100 cm long, placed 34 cm above the tabletop. The order of plank widths that the animal was given was randomized and counterbalanced across groups. Latency to fall (max score = 60 s), distance traveled (in cm), and number of turns on the planks were recorded and averaged for each trial. (4) Inclined Screen: This test measures muscle tone, strength and balance. Each rat was placed in one of six separate compartments of a wire mesh that was tilted 60° to the horizontal plane of the floor. Latency to fall from the screen (max score = 600 s) was recorded. (5) Accelerating Rotarod: Fine motor coordination, balance, and resistance to fatigue were quantitated by measuring the amount of time

that a rat could remain standing on a rotating, accelerating rod (Ugo Basile, Italy). The rod is a drum, 7 cm in diameter, and is machined to provide traction. Each rat was placed on the rod at 2 rpm until it maintained its grip and orientation without assistance. The rod then accelerated steadily for 5 min (by 2 rpm every 30 s) until it reached 20 rpm. Latency to fall (maximum = 300 s) was recorded. For a more detailed description of the tests, see (Shukitt-Hale et al. 1998).

Morris Water Maze

The Morris Water Maze (MWM), an accepted method of testing spatial learning and memory, is an age- and diet-sensitive (Joseph et al. 1998c, 1999; Youdim et al. 2000; Ingram et al. 1994; Shukitt-Hale et al. 1998; Brandeis et al. 1989) learning paradigm that requires rats to find the location of a hidden platform (10 cm in diameter) just below the surface (2 cm) of a circular pool of water (134 cm in diameter × 50 cm in height, maintained at 23 °C) based on distal cues in previous learning trials. Accurate navigation to the platform is rewarded by escape from the water. The pool was divided into four equal-size quadrants and the platform was submerged in the center of one of the quadrants; its location was changed to a different quadrant for each session of testing. The maze was placed in a room with the lights dimmed, and there were numerous extramaze cues on the walls.

The working memory version of the MWM (Brandeis et al. 1989; Morris 1984) was performed daily for four consecutive days during week 9 or 10 of diet supplementation, with a morning and an afternoon session, two trials each session, with a 10-min intertrial interval between the two trials. At the beginning of each trial, the rat was gently immersed in the water at one of four randomized start locations. Each rat was allowed 120 s to escape onto the platform; if the rat failed to escape within this time, it was guided to the platform. Once the rat reached the platform, it remained there for 15 s (trial 1; reference memory or acquisition trial). The rat was returned to its home cage between trials (10 min). Trial 2 (the working memory or retrieval trial) used the same platform location and start position as trial 1. Performances were videotaped and analyzed with image tracking software (HVS Image, UK), which allows measurements of latency to find the platform (s), path length (cm), and swimming speed (cm/s). For a more detailed description of the maze and the paradigm used (see Shukitt-Hale et al. 1998).

In vitro *LPS treatment*

We recently showed that BB supplementation enhanced the brain's ability to generate an HSP70-mediated neuroprotective response to stress, since old rats fed the BB diet showed increased HSP70 levels against an *in vitro* stressor in hippocampal brain regions compared to old animals fed the control diet (Galli et al., in press). Therefore, in this study, brains were dissected on ice three to six weeks following behavioral testing and hippocampal regions were individually cross-cut (300 μm, McIlwain tissue chopper) and transferred into glass vials containing cold modified Krebs–Ringer buffer (see DA release section below) bubbled with 95% O_2/5% CO_2. Tissue samples were washed and aliquoted into fresh buffer with either 0 or 10 μg/ml LPS (Lipopolysaccharide, Sigma, *E. coli*, serotype 055:B5, TCA extraction), shaken in a 37 °C water bath for 90 min, with fresh buffer ± LPS replaced at 30-min intervals. Vials were then put in ice, and samples transferred to centrifuge tubes, spun at 8 K, 10 min, 4 °C, and dry tissue pellets frozen at −80 °C. For processing to total cytosolic lysates for Western blots, tissue pellets were resuspended in cold RIPA buffer (1× PBS, 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS, pH 7.4) plus protease inhibitors (40 μM PMSF, 2 μg/ml Leupeptin, 1 μg/ml Pepstatin, 0.1 nM Na Vanadate, 1 μM NaF, 5 nM DTT). Samples were vortexed, placed on ice for 30 min, centrifuged at 10,000 *g* at 4 °C for 10 min, and the supernatant was assayed for protein concentration. The resulting total cytosolic lysates were stored at −80 °C for Western immunoblots.

Western immunoblotting

For Western blots, samples (60 μg) were run on a 10% SDS-PAGE gel (Mendelson et al. 1996). The gel was soaked in protein transfer buffer (TB: 200 mM glycine, 25 mM Tris base, 20% methanol) for 10 min at room temperature, and then blotted onto nitrocellulose using a Hoeffer Transphor Unit. Blots were blocked with TBST (137 mM NaCl, 20 mM Tris, 0.1% Tween-20, pH 7.5) with 5% nonfat

Table 1. Anthocyanin and total phenolic concentration present in the extracts incorporated into the rat feed at the 2% level.

Berryfruit type	Total anthocyanin concentration (mg/g)	Total phenolic (325 nm) concentration (mg/g)
Blueberry	1.3	1.2
Blackcurrant	8.7	2.9
Boysenberry	9.2	2.0
Cranberry	3.3	3.6

dried milk and incubated in respective primary antibodies in TBST with 5% milk, followed by washing with TBST, and incubation with the appropriate horseradish peroxidase-conjugated secondary antibody in TBST with 5% milk. After final washing in TBST, signal was detected using the Renaissance Western Blot Chemiluminescence kit (DuPont NEN). Autogradiograms of the chemiluminescent immunoblots were quantified using a Molecular Dynamics Densitometer and expressed in units of optical density.

Dopamine release

We have previously shown that the muscarinic enhancement of dopamine release from superfused striatal slices is an indicator of receptor sensitivity and is sensitive to both aging, oxidative stress, and dietary supplementation (Joseph et al. 1988a–c, 1990, 1996, 1999). DA release was conducted on freshly dissected and cross-cut (300 μm) striatal slices from the brains of animals maintained on the various diets. The slices were placed in small glass vials containing modified Krebs–Ringer basal release medium (BRM) that had been bubbled for 30 min with 95% O2/5% CO_2 and which contained (in mM): $NaHCO_3$ 21, glucose 3.4, NaH_2PO_4 1.3, EGTA 1, $MgCl_2$ 0.93, NaCl 127 and KCl 2.5 (low KCl) (pH 7.4). They were then placed in the perfusion chambers where they were maintained at 37 °C and perfused with the BRM for 30 min. Following this equilibration period, the medium was switched to one containing (in mM): KCl 30, $CaCl_2{\cdot}2H_2O$ 1.26 (in place of EGTA) and NaCl 57, and 0 or 500 μM oxotremorine, and then the enhancement of potassium-evoked dopamine release was assessed. DA release was then quantitated by HPLC coupled to electrochemical detection. Data were expressed as pmol/mg protein as determined by the Lowry and colleagues procedure (Lowry et al. 1951).

Statistical analyses

For each dependent variable, a one-way analysis of variance (ANOVA) was performed using Systat (SPSS, Chicago, IL) to compare the five diet groups at the $P < 0.05$ level of statistical significance. Post-hoc comparisons, to determine differences between the control group and the diet groups, were performed using Fisher's LSD post-hoc analysis. Correlations between behavior and brain measures were carried out using a Pearson's *r* correlation.

Results

The concentrations of total anthocyanins and total phenolics for each fruit extract are provided in Table 1. Both blackcurrant and Boysenberry extracts contained greater anthocyanin concentrations than either blueberry or cranberry, but all berryfruit extracts contained similar amounts of phenolic compounds measured as chlorogenic acid equivalents. The relative concentrations of phytochemicals present in the extracts are consistent with concentrations expected in the berryfruit.

Of the motor tasks, only the inclined screen yielded significant results between diet groups. ANOVA

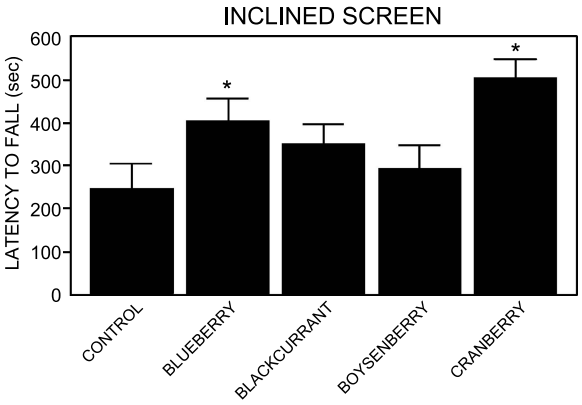

Figure 1. Latency to fall (mean ± SEM, s) on the inclined screen test for the control, blueberry, blackcurrant, Boysenberry, and cranberry diet groups. *Significant difference from the control group ($P < 0.05$; Fisher's LSD).

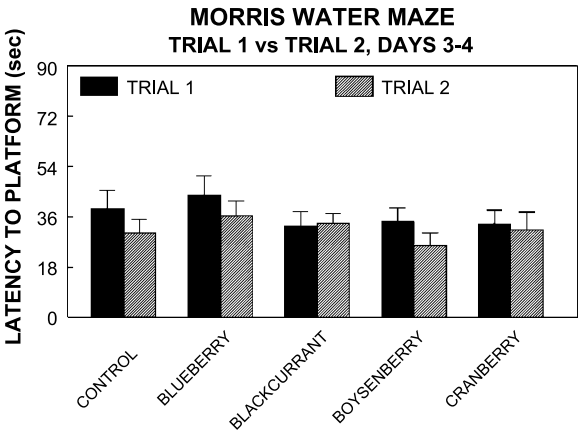

Figure 2. Morris water maze performance in the control, blueberry, blackcurrant, Boysenberry, and cranberry diet groups. Performance was assessed over four days (2 sessions/day, 2 trials/session). Results are given as latencies (mean ± SEM) to find the hidden platform for the first and second trials on days 3 and 4.

showed overall significance ($F_{4, 64} = 3.76$, $P < 0.01$); post-hoc testing revealed that both the blueberry ($P = 0.038$) and cranberry ($P = 0.001$) groups performed better than the control group (Figure 1). Morris Water Maze performance showed no differences among the diet groups when trial 1 or trial 2 latencies were analyzed on days 3 and 4 (Figure 2). Furthermore, when trial 1 latency was compared to trial 2 latency within each group, no significant differences were detected (all $P > 0.05$).

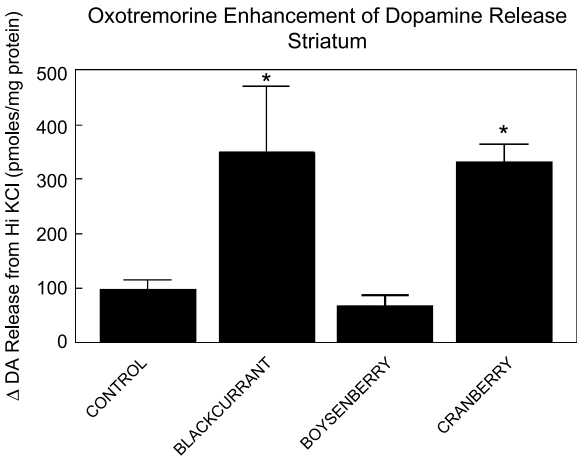

Figure 3. Oxotremorine enhancement of DA release from striatal slices obtained and prepared from animals maintained on the control, blackcurrant, Boysenberry, or cranberry diets (mean ± SEM). *Significant difference from the control group ($P < 0.05$; Fisher's LSD).

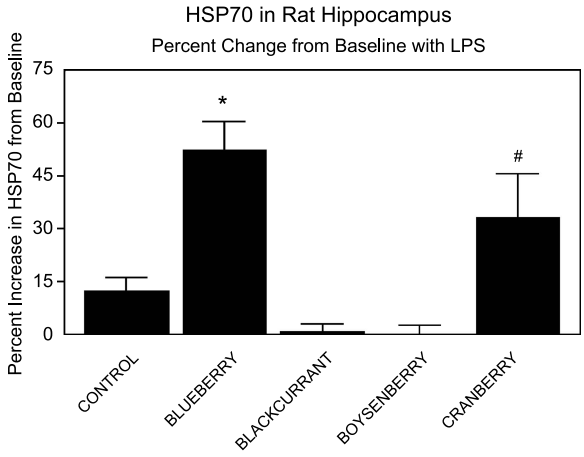

Figure 4. Percent increase in hippocampal HSP70 levels from baseline in response to an *in vitro* LPS challenge of animals maintained on the control, blueberry, blackcurrant, Boysenberry, or cranberry diets (mean ± SEM). *Difference of $P < 0.05$ (Fisher's LSD), # $P = 0.06$ (Fisher's LSD) from the control group.

Dopamine release was different among the groups ($F_{3, 19} = 7.09$, $P < 0.01$]; post-hoc testing found that the blackcurrant ($P = 0.004$) and cranberry ($P = 0.007$) groups were significantly better than the control and Boysenberry groups (Figure 3). The effects of BB supplementation on striatal dopamine were not examined in this study, since it had been replicated in previous studies (Joseph et al. 1999; Youdim et al. 2000).

Percent change in HSP70 also showed differences among the groups ($F_{4, 21} = 10.50$, $P < 0.001$), with post-hoc analyses showing that HSP70 responsiveness in the blueberry group was significantly higher than the control group ($P = 0.001$), while the cranberry group showed a trend toward being higher than the control group ($P = 0.060$) (Figure 4). Furthermore, HSP70 levels positively correlated with inclined screen performance; i.e., latency to fall from the inclined screen increased as the percent change in HSP70 increased ($r = 0.39$, $P = 0.048$).

Discussion

There were significant effects of the diets found on several of the measures in this study. The inclined screen, dopamine release and HSP70 results all suggest that there is a range of effectiveness associated with the different berryfruit diets. The blackcurrant

and cranberry diets enhanced neuronal signal transduction, while the blueberry and cranberry diets were effective in ameliorating deficits in motor performance (inclined screen) and hippocampal HSP70 neuroprotection; these changes in HSP70 were positively correlated with performance on the inclined screen. Previous studies have shown that polyphenols such as anthocyanins, present in blueberries and cranberries, may have the ability to improve muscle tone, strength and balance in aging rats (Joseph et al. 1999; Youdim et al. 2000). This possibility is further supported by the results of this study as reflected in the benefits of these berryfruits on inclined screen performance. Additionally, the effects on inclined screen performance are correlated with the ability of blueberries and cranberries to restore the increases in LPS-induced HSP70 levels in the hippocampal tissue obtained from these animals. However, it does not appear that the anthocyanin component is the one solely responsible for these improvements, as the blackcurrant and Boysenberry fruits are higher in anthocyanin level, but not as effective in improving motor performance. Future studies should examine other components, such as the proanthocyanidins and flavans, in addition to the anthocyanins.

Restorative effects of blueberry supplementation in senescent rats, as measured by the brain's ability to generate an HSP70-mediated neuroprotective response to stress, were shown in a previous study (Galli et al., in press). The results presented here show that the blackcurrant and cranberry diets increased dopamine release to an extent similar to that seen previously in blueberry-treated animals (Joseph et al. 1999), but in the case of blackcurrants this increase in dopamine release did not translate into enhanced motor performance on the inclined screen. These findings support the hypothesis that antioxidants from berryfruit diets help improve neuronal functioning, and as shown by HSP70 and dopamine release measures, restore the protective ability of the brain against oxidative or inflammatory stressors. However, the effects of each berryfruit are modulated differently and this is presumably a result of the variety of phytochemicals in each berryfruit.

We were somewhat puzzled about the failure to find any effect of diet on cognitive performance in the MWM and one of the psychomotor tests (the accelerating rotarod), in contrast to findings from many earlier studies. However, an examination of the data from the control group from this study and the control groups from previous supplementation studies revealed that the old rat control group averaged about 39 ± 6 s latency on trial 1 for days 3 and 4 in this study on the MWM, and 30 ± 5 for trial 2 (Figure 3), while in previous studies (Joseph et al. 1999; Youdim et al. 2000; Shukitt-Hale et al. 1998), the senescent control animals averaged 59 ± 5 s latency on trial 1 and 44 ± 4 on trial 2. Likewise for the accelerating rotarod, the average latency to fall for the control in this study was 26.7 ± 5 s compared with 8.5 ± 1 s for the control animals in previous studies (Joseph et al. 1999; Youdim et al. 2000; Shukitt-Hale et al. 1998). Thus, since the controls performed significantly better in these experiments, there could be a floor effect which lowered the signal to noise ratio and prevented any cognitive effects of the berryfruit on these tasks from being observed. This did not seem to be the case on the other motor tasks, where the controls in the current study had performances that were not different than those of previous supplementation studies, thereby contributing to the detection of a positive effect of berryfruit supplementation. We are currently employing more challenging cognitive tests in our ongoing studies and are also beginning to test higher concentrations of the berryfruits supplemented in the diet to offset the increased performance of the control groups that we have been observing.

The findings in this study, and those from previous experiments using these and various other fruit extracts in a cell model (Joseph et al. 2004), showed differential effects of the fruits in their protective ability. In the cell study, we assessed the effectiveness of fruit extracts in countering the toxic effects of Aβ 25–35 and DA on calcium buffering (Recovery) following oxotremorine-induced depolarization in M_1AChR-transfected COS-7 cells, and on cell viability following DA exposure (Joseph et al. 2004). The extracts showed varying degrees of protection against the deleterious effects of DA or Aβ on Recovery and viability in comparison to the nonsupplemented controls, with the BB pretreatment being the most effective. The results for the other extracts tested were dependent upon which stressor was used as the pretreatment, indicating that it is possible to reduce both the deleterious effects of DA and the putative toxic effects of Aβ via fruits high in antioxidant activity.

In summary, these results suggest that the anthocyanins and other polyphenolic phytochemicals contained in these berryfruit extracts have, in addition

to antioxidant effects, a multiplicity of actions that might be altering the course of age-related deficits in motor and cognitive function. Indeed, studies have shown decreased development of Alzheimer's Disease in individuals who have a high intake of fruits and vegetables (Commenges et al. 2000; Deschamps et al. 2001; Youdim and Joseph 2001), suggesting that fruit supplementation may indeed provide multiple levels of protection against the course of this devastating disease.

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