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Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage

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Abstract

Free radicals are involved in neurodegenerative disorders, such as ischemia and aging. We have previously demonstrated that treatment with diets enriched with blueberry, spinach, or spirulina have been shown to reduce neurodegenerative changes in aged animals. The purpose of this study was to determine if these diets have neuroprotective effects in focal ischemic brain. Adult male Sprague–Dawley rats were fed with equal amounts of diets (blueberry, spinach, and spirulina) or with control diet. After 4 weeks of feeding, all animals were anesthetized with chloral hydrate. The right middle cerebral artery was ligated with a 10-O suture for 60 min. The ligature was later removed to allow reperfusional injury. Animals were sacrificed and brains were removed for caspase-3 enzymatic assays and triphenyltetrazolium chloride staining at 8 and 48 h after the onset of reperfusion. A subgroup of animals was used for locomotor behavior and biochemical assays. We found that animals which received blueberry, spinach, or spirulina enriched diets had a significant reduction in the volume of infarction in the cerebral cortex and an increase in post-stroke locomotor activity. There was no difference in blood biochemistry, blood CO_2, and electrolyte levels among all groups, suggesting that the protection was not indirectly mediated through the changes in physiological functions. Animals treated with blueberry, spinach, or spirulina had significantly lower caspase-3 activity in the ischemic hemisphere. In conclusion, our data suggest that chronic treatment with blueberry, spinach, or spirulina reduces ischemia/reperfusion-induced apoptosis and cerebral infarction.
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Introduction

Stroke is an acute and progressive neurodegenerative disorder. Besides the deprivation of oxygen and nutrients during the acute ischemic phase, generation of toxic compounds, such as free radicals, reactive oxygen or nitrogen species, and ONOO-, during the reperfusional stage can induce increased damage to the brain. It has been demonstrated that pharmacological agents that possess free radical scavenging or antioxidant properties reduce brain damage during stroke. Recently, several dietary supplements have been reported to have strong antioxidant effects and reduce neurological deficits in aged animals (Bickford et al., 2000; Gemma et al., 2002). Rats on blueberry-supplemented diets for 6 weeks developed less neuronal loss in the hippocampus after cerebral ischemia (Sweeney et al., 2002). These data suggest that these dietary supplements may have protective effect against neurodegeneration.

A recent epidemiological study has shown an inverse association between the incidence of cerebral infarction and intake of vegetables or β-carotene in male smokers (Hirvonen et al., 2000). The associations between incidence

of cerebral infarction and intake of fruits, berries, tea, or wine were not significant. These data suggest that some, but not all, diets may have protective effect against cerebral infarction.

Several ingredients in blueberries (Vaccinium spp.) have been shown to have strong antioxidant activity. These include the pigments flavonoids, anthocyanins, and others (Cao et al., 1999). Treatment with extracts from blueberries reduced oxidative stress and age-related declines in neuronal function in vitro and in vivo (Joseph et al., 1998). On the other hand, blueberry juice, taken acutely, did not enhance the antioxidant capacity to reduce potassium nitrosodisulfonate and Fe(III)-2,4,6,Tri(2-pyridyl)s-trizine in plasma (Pedersen et al., 2000). Similar to blueberry, spinach leaves also contain high levels of antioxidants flavonoids, p-coumaric acid (Bergman et al., 2001), 9-cis-β-carotene (Yan et al., 2001), and other water-soluble natural antioxidants (NAO). These ingredients can reduce LPS-induced inflammation and necrosis (Lomnitski et al., 2000) and doxorubicin-induced cardiac damage while increasing superoxide dismutase activities (Breitbart et al., 2001). Taken together, these data suggest that both blueberry and spinach have certain protective effects against oxidative stress and inflammation-induced damage.

Spirulina, a type of blue green algae that has been consumed for thousand of years as a primary food source for the Aztecs and Mayans, contains high levels of antioxidants, for example, carotenoids (Annapurna et al., 1991), especially β-carotene (Careri et al., 2001), and phycocyanin and phycocyanobilin (Reddy et al., 2000). In vivo studies also indicate that chronic treatment with spirulina diet for 6 weeks increased retinoid levels in liver (Mitchell et al., 1990). The extracts of spirulina have antioxidant activity both in vivo and in vitro (Miranda et al., 1998). Pretreatment with spirulina reduced oxidative stress and toxicity induced by cyclophosphamide and mitomycin in mice (Premkumar et al., 2001) and prevented lipid peroxidation induced by lead (Upasani et al., 2001). Phycocyanin and phycocyanobilin in the spirulina were also found to have strong anti-cyclooxygenase-2 and antioxidant activity (Bhat and Madyastha, 2001; Reddy et al., 2000) and to scavenger peroxidinitrite and reduce OONO- induced oxidative damage to DNA (Bhat and Madyastha, 2001). Besides the antioxidant effect, spirulina has other properties that can lower the risk of damage in various tissues. Pretreatment with spirulina maxima for 13 weeks elicited a dose-dependent decrease in serum cholesterol in male and female mice and increased the weight of seminal vesicles in male mice (Salazar et al., 1998). In a similar experiment, serum HDL level was increased in diabetic mice after receiving spirulina diet for 4 weeks. Such a response was not found in non-diabetic controls (Rodriguez-Hernandez et al., 2001). Taken together, these data suggest that chronic or subchronic treatment with spirulina can reduce lipid peroxidation, increase antioxi-

dants, reduce toxicity of OONO-, and furthermore, reduce cholesterol levels. All these factors have been previously reported to be involved in ischemic brain injury. The utility of spirulina diet in protection against stroke is still not known.

In this study, we study the protective effects of three diets: blueberry, spinach, and spirulina against stroke in rats. Our data indicate that these diets have differential effects to reduce ischemia-induced caspase-3 activity and cerebral infarction.

Materials and methods

Animals

Adult male Sprague–Dawley rats, purchased from the Charles River Laboratories, were used for this study. Animals were caged singly and separated into 4 groups according to the diets: (A) control NIH 07/31 diet, (B) blueberry diet (2%), (C) spinach diet (2%), and (D) spirulina diet (0.33%). All animals were fed with 5 food pellets (4–6 g per each pellet) per day for 4 weeks before stroke surgery.

Ischemia/reperfusion

Rats were anesthetized with chloral hydrate (0.4 g/kg, i.p). The ligation of the right MCA and bilateral common carotids (CCAs) was performed using methods previously described (Chen et al., 1986; Wang et al., 2003). The bilateral CCAs were identified and isolated through a ventral midline cervical incision. The CCAs were ligated with non-traumatic arterial clips. A craniotomy of about 4 mm^2 was made in the right squamous bone. The right MCA was ligated with 10-O suture for 60 min. The ligature was removed after 60-min ischemia to allow reperfusion. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during surgery. After surgery, the animals were kept in a temperature-controlled incubator to maintained body temperature at 37°C. After recovery from the anesthesia, the animals were returned to their home cages.

Caspase-3 enzymatic activity

Caspase-3 enzyme activity was measured using the ApoAlert kit (Clontech, Palo, Alto, CA. USA) as described previously (Wang et al., 2001a). Eight hours after the onset of reperfusion, animals were sacrificed. Brain tissue was removed, dissected, and homogenized in lysis buffer. Caspase-3 activity was determined fluorometrically by the formation of 7-amino-4-trifluromethyl coumarin (AFC) from Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC). The selective caspase-3 inhibitor DEVD-aldehyde (DEVD-CHO) was

included, at a concentration of 1 μM, in some assays to ensure that the enzymatic reaction was specific.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry

Animals were decapitated at 24 h after ischemia. The brains were taken out and cut into 30 μm sections in a cryostat. The sections were mounted onto Superfrost/Plus microscopy slides (Cat. 12-550-15; Fisher, PA) then air dried. A standard TUNEL procedure for frozen tissue sections with minor modifications was performed (Deng et al., 1999). Briefly, slide-mounted sections from rat brain were rinsed in 0.5% Triton X-100 in 0.01 M PBS for 20 min at 80°C to increase permeability of the cells.

To label damaged nuclei, 50 μL of the TUNEL reaction mixture was added onto each sample in a humidified chamber followed by a 60-min incubation at 37°C. Procedures for negative controls were carried out as described in the manufacturer's manual (Roche, IN) and consisted of not adding the label solution (terminal deoxynucleotidyl transferase) to the TUNEL reaction mixture. Material was examined under a fluorescent microscope.

Locomotor behavior measurements

Animals were placed in an Accuscan activity monitor (Columbus, OH) before and 2 days after ischemia for behavioral recording. The monitor contained 16 horizon-
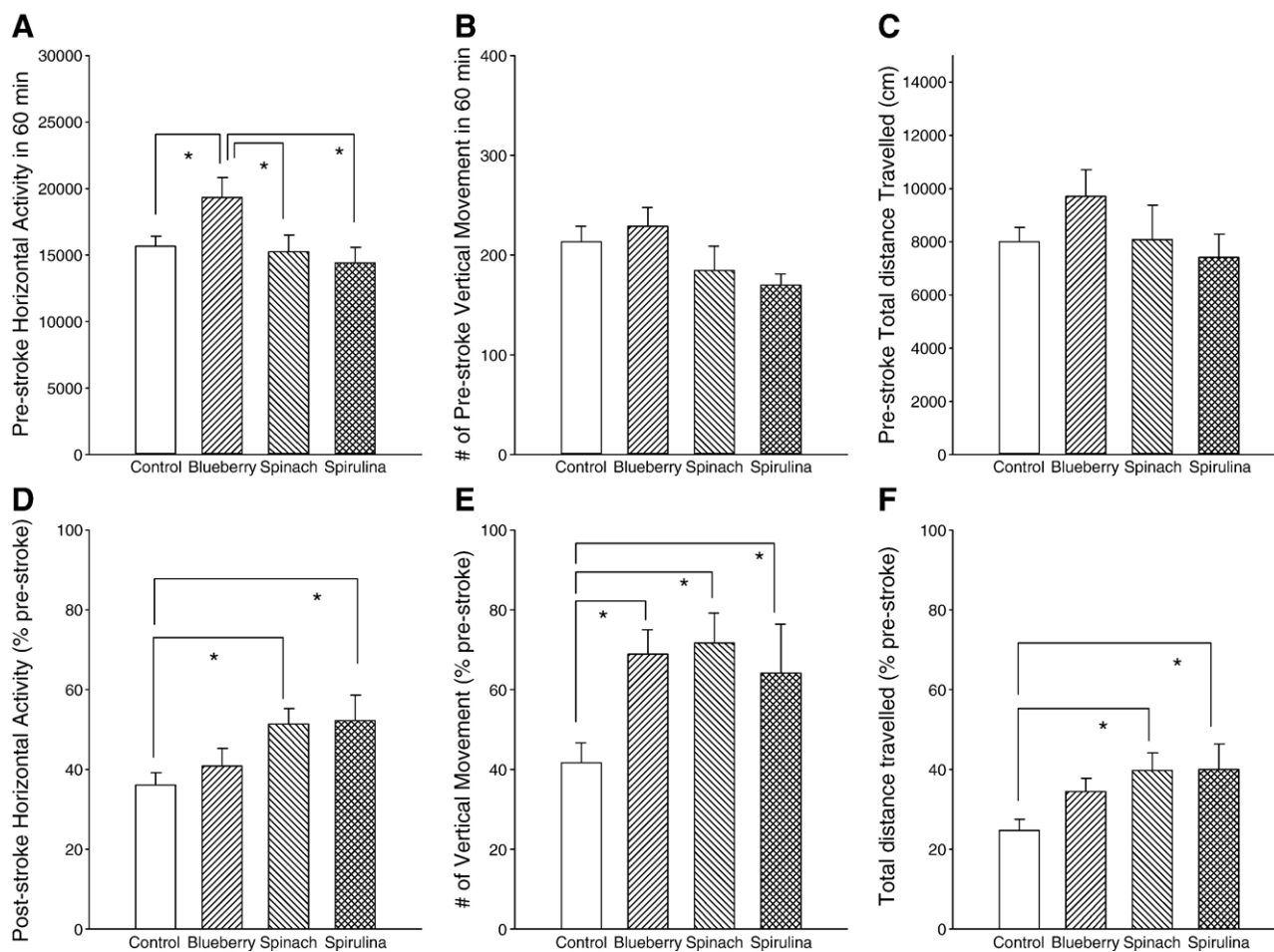

Fig. 1. Locomotor activity in animals treated with antioxidant-rich diets before (A–C) and 2 days after (D–F) MCAo. Animals received control or antioxidant food pellets daily for 4 weeks. The pre-stroke locomotor activity was examined 4 weeks after the start of dietary treatment. Animals were placed into infrared beam activity chamber for 60 min. (A) Horizontal activities were measured by the sum of the total number of beam interruptions that occurred in the horizontal sensors during the 60-min testing period. Animals receiving blueberry diet had significantly more horizontal activity than the other groups (*$P < 0.05$, one way ANOVA + Newman–Keuls test). There is no difference in (B) vertical movements, the frequency of beam interruptions that occurred in the vertical sensors, and (C) the total distance traveled, the distance, in centimeters, traveled by the animals in 1 h, among all groups. Post-stroke locomotor activity was examined 2 days after a 60-min MCA ligation. All post-stroke locomotor behaviors were normalized by comparison to the mean motor activity before MCAo. Animals receiving chronic spinach and spirulina diets showed increased horizontal motor activity (D and F; $P < 0.05$, one way ANOVA + Dunnett's test). All stroke animals that received antioxidant diets had a significant increase in vertical movement compared to the control animals (E, *$P < 0.05$).

tal and 8 vertical infrared sensors spaced 2.5 cm apart. The vertical sensors were situated 10 cm from the floor of the chamber. Each animal was placed in a 42 × 42 × 31 cm Plexiglas open box for 1 h. Motor activity was calculated using the number of beams broken by the animals. The following variables were measured: (A) horizontal activity (the total number of beam interruptions that occurred in the horizontal sensor in 1 h), (B) vertical movements (the frequency of beam interruptions that occurred in the vertical sensors), and (C) total distance traveled (the distance, in centimeter, traveled by the animals in 1 h).

Triphenyltetrazolium chloride (TTC) staining

Two days after MCA ligation, some animals were sacrificed and perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0 mm thick sections. The brain slices were incubated in 2% triphenyltetrazolium chloride (Sigma), dissolved in normal saline, for 10 min at room temperature, and then transferred into a 5% formaldehyde solution for fixation. The area of infarction on each brain slice was measured double blind using a digital scanner and the Image Tools program (University

Fig. 2. Pretreatment with antioxidant-rich diets for 4 weeks markedly reduced cortical infarction induced by middle cerebral arterial ligation and reperfusion. The right middle cerebral artery was ligated for 60 min after bilateral common carotid ligation. Animals were sacrificed for TTC staining 48 h after ischemia/reperfusion. Marked infarction (white areas) in the right cerebral cortex was found in animal receiving control diet. Pretreatment with antioxidant-rich diets reduced the infarction area in the other three groups.

of Texas Health Sciences Center, San Antonio). To minimize any artifacts induced by post-ischemic edema in the infarcted tissue, the infarction area in the right cortex was indirectly measured by subtracting the non-infarcted area in the right cortex from the total cortical area of the left hemisphere (Lin et al., 1993; Swanson et al., 1990). The total infarction volume in each animal was obtained from the product of average slice thickness

Fig. 4. Caspase-3 enzymatic activity was decreased in the ischemic brains of rats pretreated with antioxidant-rich diets for 4 weeks. All animals received 60-min ischemia and 8-h reperfusion. Caspase-3 activity in the ischemic hemisphere was normalized by comparison to the non-ischemic contralateral hemisphere. Animals receiving spinach or spirulina developed a significantly less increase in caspase-3 activity after ischemia and reperfusion (*P < 0.05).

(2 mm) and sum of the area of infarction in all brain slices.

Statistics

One way and two way ANOVA and post-hoc New-man–Keuls or Dunnett's test were used for statistical comparison. Data are presented as mean ± SEM.

Results

Locomotor activity

The rats were treated for 4 weeks with the various control or antioxidant-rich diets prior to the initial assessment of locomotor activity. The animals were individually placed into the activity chamber for 60 min to assess baseline locomotor activity before the ischemia/reperfusion injury (Figs. 1A–C). Animals that received the blueberry diet had increased horizontal activity (Fig. 1A, $P < 0.05$, $F_{(3,76)} = 3.323$). There was no difference in vertical movements (Fig. 1B, $P = 0.138$, $F_{(3,76)} = 1.894$) or total distance traveled (Fig. 1C, $P = 0.348$, $F_{(3,76)} = 1.116$) among all of the treatment groups.

Locomotor behavior was also examined on the 2nd day after MCAo. To eliminate the variability among animals before stroke, all post-stroke locomotor behaviors were normalized by comparing to the mean of activity in each group before MCAo (Figs. 1D–F). All animals receiving antioxidant enriched diets had significantly increased vertical movements compared with the control stroke animals (Fig. 1E, $P < 0.05$, $F_{(3,75)} = 3.946$). The stroke animals that received spinach or spirulina diets had
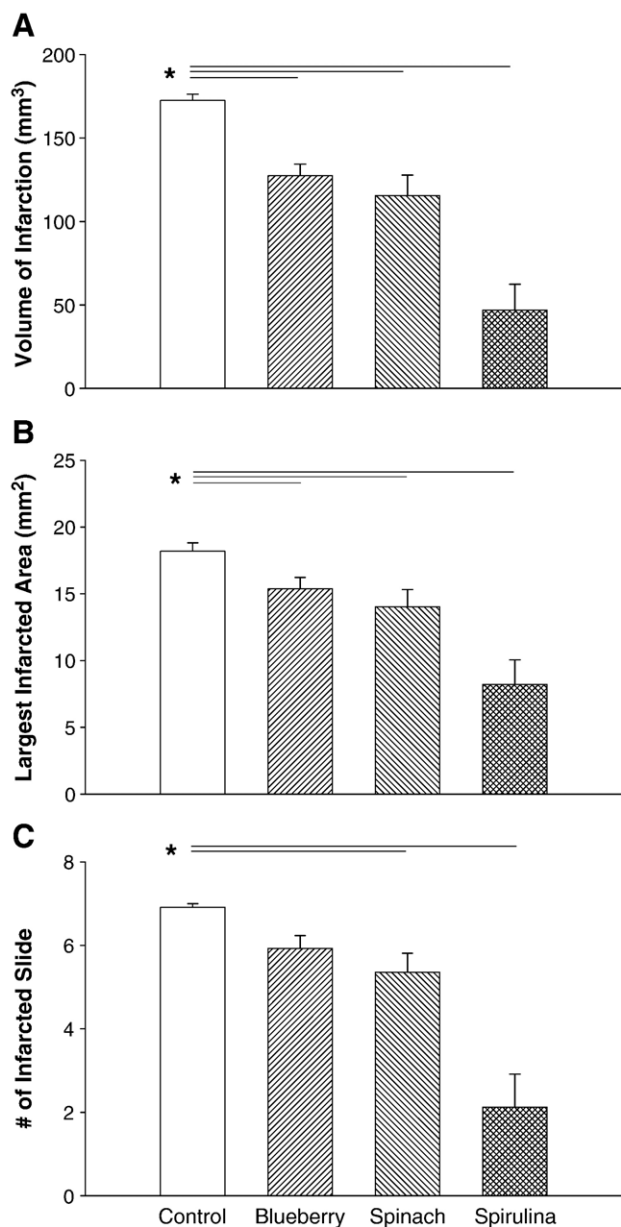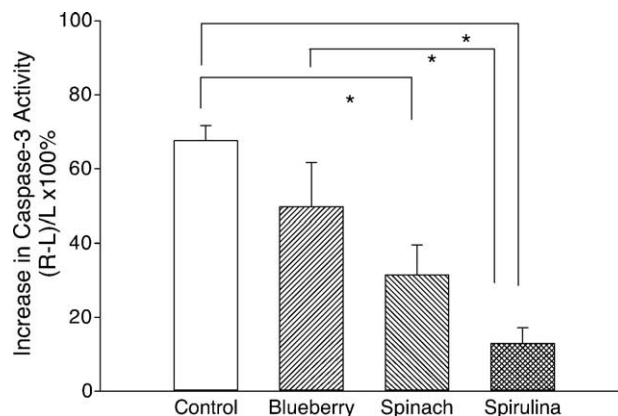

Fig. 3. Chronic antioxidant-rich dietary treatment reduced indices of cerebral infarction. (A) The volume of infarction was significantly decreased in the stroke animals pretreated with antioxidant enriched diets, as compared to those receiving control diet. The volume of infarction = [sum of the infarction area in 7 consecutive brain slices (mm²)] × [thickness of the slice, 2 mm]. (B) The area of the largest infarction in a slice and (C) the number of infarcted slices from each rat were also significantly diminished in animals receiving antioxidant enriched diets (*P < 0.05).

significantly increased horizontal activity (Fig. 1D, $P < 0.05$, $F_{(3,73)} = 3.435$) and traveled a greater distance than the control stroke rats (Fig. 1F, $P < 0.05$ $F_{(3,73)} = 3.299$).

Infarction

A total of 46 rats received a 60-min MCA occlusion 4 weeks after feeding with the special diets. Animals were sacrificed on the 2nd day after MCAo for TTC staining (Fig. 2). All animals receiving control diet ($n = 10$) developed prominent infarction on the ischemic cortex (Figs. 2 and 3). The volume of infarction in the control animals was 172.6 ± 6.6 mm³. Animals treated with blueberry ($n = 14$), spinach ($n = 14$), and spirulina ($n = 8$) enriched diets showed significantly decreased infarction (Figs. 2 and 3, $P < 0.001$, $F_{(3,42)} = 19.680$; $P < 0.05$). The largest infarction area in any slice and the total infarcted slice per animals were also significantly reduced in these animals (Fig. 3B, $P < 0.05$).

Caspase-3 enzymatic activity

A subgroup of animals were sacrificed at 8 h after MCA ligation. The brains were sliced coronally at 2-mm intervals. We have previously demonstrated that the largest lesion, as examined by TTC staining, occurred in the 4th slice from the rostral end, after 48-h reperfusion. We thus examined the caspase-3 enzymatic activity in the 4th slice from each animal (Fig. 4). Caspase-3 activity in the ischemic side, compared to the non-ischemic side, was significantly increased in the controls. The ischemia-induced increase in caspase-3 activity was significantly antagonized when Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), a selective caspase-3 inhibitor, was included in the incubation mixture as a control for specificity (data not shown). The increase in caspase-3 activity in the ischemic hemisphere (R) in each animal was normalized by comparison to activity in the non-ischemic (L) hemisphere using the formula: Δ caspase-3 activity = [(caspase activity in right hemisphere − caspase activity in left hemisphere) − caspase activity in left hemisphere] × 100% (Fig. 4). We found that animals that received spinach ($n = 12$), or spirulina diets ($n = 13$) for 4 weeks had a significant reduction in caspase-3 activity compared to the controls ($n = 26$, $P < 0.05$, $F_{(3,61)} = 12.557$). There is a trend in the animals that received blueberry diets ($n = 14$) for about a 30% reduction in caspase-3, although this difference did not reach significance.

TUNEL histochemistry

A total of 12 animals were used for TUNEL histochemical study. TUNEL (+) cells were found in the penumbra area in the control stroke animals (Figs. 5A1–A2). Animals pretreated with spirulina diet showed a remarkable reduction in the density of TUNEL-positive signals (Figs. 5D1–D2). The density of TUNEL (+) cells were quantified and averaged from two sections near bregma in all brains samples by blinded observers. There was a significant reduction in the density of TUNEL-positive cells in animals received antioxidant diet pretreatment (Fig. 5E, $P < 0.05$, $F_{(3,20)} = 13.829$).

Blood biochemistry, electrolytes, and CO₂ contents

A total of 35 animals were sacrificed for blood biochemistry, serum electrolytes, and CO_2 content. About 1 mL whole blood was taken directly from the heart on the 2nd day after MCAo. There was no difference in most indices of liver, kidney, and pancreatic function, as well as blood electrolytes and triglyceride levels (Table 1). There was a slight but significant decrease in cholesterol in animals receiving spinach diets ($P < 0.05$).

Body weight

The body weight of animals in each group did not show any differences either before or 1 month after treatment ($P > 0.05$, one way ANOVA). The means of body weight in each group was 257–265 g before and 392–396 g after the special diet.

Discussion

Reactive oxygen species, generated during cerebral ischemia and reperfusion, can induce further neurodegeneration. It has been reported that low antioxidant activity in plasma is associated with higher lesion volumes and neurological impairments in stroke patients (Leinonen et al., 2000). Pretreatment with antioxidant chemicals reduced ischemic brain injury (Clark et al., 2001; Fujimura et al., 2000; Yang et al., 2001). Similarly, dietary intake of vegetables or β-carotene, antioxidants, are associated with lower risk for cerebral infarction in male patients (Hirvonen et al., 2000). In this study, we examined the neuroprotective effect of various diets against ischemia. We found that

Fig. 5. Density of TUNEL (+) cells is decreased in stroke animals receiving antioxidant diet pretreatment. Animals were pretreated with (A) control, (B) blueberry, (C) spinach, or (D) spirulina diets for 4 weeks before MCAo. Abundant TUNEL-positive signals were found in the control brain slice section near bregma at low (A1) and high (A2) magnification 1 day after the MCAo. Marked reduction in TUNEL-positive signals was observed in animals pretreated with antioxidant diets (B–D). Scale bar A1–D1: 100 μm. A2–D2: 12.5 μm. (E) The density of TUNEL (+) cells was quantified and averaged from three sections near bregma in each animal. There is a significant reduction in the density of TUNEL-positive cells in animals receiving antioxidant diet pretreatment (*$P < 0.05$).
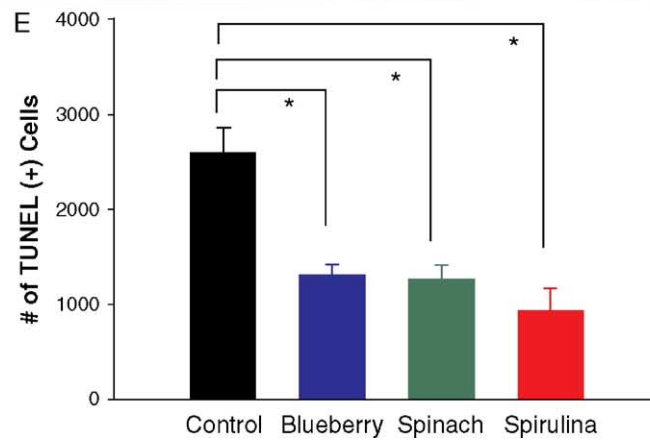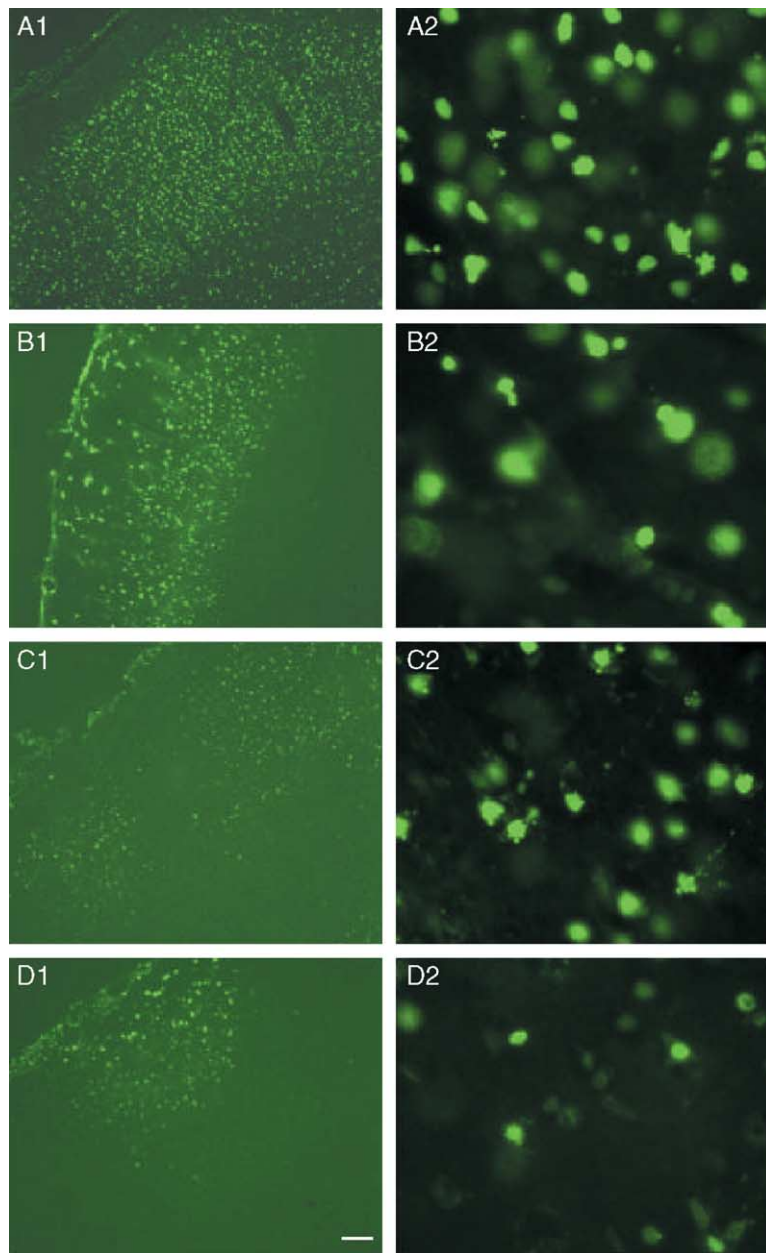
Table 1
Blood biochemistry, CO_2, and electrolytes from the stroke animals that received antioxidant diets

	Control	Blueberry	Spinach	Spirulina	F	P^a
Glucose mg/dL	225.00 ± 29.76	175.83 ± 7.26	180.88 ± 14.44	199.00 ± 30.67	$F_{(3,30)} = 0.746$	0.533
Cholesterol mg/dL	86.75 ± 3.14	78.80 ± 7.55	66.25 ± 4.44[a]	82.00 ± 5.08	$F_{(3,30)} = 4.082$	0.015[a]
Triglycerides mg/dL	112.00 ± 11.92	123.80 ± 10.29	122.88 ± 24.21	90.17 ± 16.59	$F_{(3,31)} = 0.601$	0.619
Na^+ mM	143.69 ± 1.24	145.80 ± 0.66	146.25 ± 0.62	145.17 ± 0.75	$F_{(3,31)} = 1.052$	0.384
K^+ mM	4.79 ± 0.11	4.70 ± 0.21	4.72 ± 0.13	4.97 ± 0.24	$F_{(3,18)} = 0.401$	0.754
Cl^- mM	101.50 ± 0.56	102.60 ± 0.81	101.38 ± 0.73	101.67 ± 0.33	$F_{(3,31)} = 0.462$	0.711
CO_2, total mM	25.57 ± 0.53	28.20 ± 1.50	26.00 ± 0.85	25.00 ± 0.78	$F_{(3,29)} = 2.052$	0.128
Creatinine mg/dL	0.49 ± 0.04	0.37 ± 0.03	0.41 ± 0.04	0.35 ± 0.02	$F_{(3,30)} = 2.810$	0.056
BUN mg/dL	23.21 ± 1.55	20.40 ± 1.63	20.63 ± 1.39	18.17 ± 2.36	$F_{(3,29)} = 1.490$	0.238
Albumin g/dL	3.53 ± 0.06	3.46 ± 0.07	3.48 ± 0.08	3.48 ± 0.11	$F_{(3,29)} = 0.175$	0.912
Ca^{++} mM	2.37 ± 0.04	2.39 ± 0.04	2.34 ± 0.04	2.40 ± 0.04	$F_{(3,29)} = 0.301$	0.824
Mg^{++} mM	1.14 ± 0.05	1.00 ± 0.04	1.21 ± 0.08	0.98 ± 0.03	$F_{(3,29)} = 2.669$	0.066
Phosporus, inorganic mg/dL	7.81 ± 0.34	7.48 ± 0.36	8.56 ± 0.68	7.27 ± 0.36	$F_{(3,30)} = 1.266$	0.304
Alkaline phosphatase U/L	176.71 ± 11.79	190.00 ± 11.32	189.38 ± 7.57	205.83 ± 19.61	$F_{(3,29)} = 0.839$	0.483
ALT/GPT U/L	90.54 ± 8.77	69.20 ± 8.89	92.75 ± 9.18	81.67 ± 6.74	$F_{(3,28)} = 1.023$	0.397
AST/GOT U/L	373.89 ± 90.45	244.33 ± 33.91	378.57 ± 55.18	303.80 ± 36.06	$F_{(3,20)} = 0.483$	0.698
Bilirubin, total mg/dL	0.39 ± 0.15	0.23 ± 0.03	0.20 ± 0.02	0.22 ± 0.03	$F_{(3,25)} = 0.645$	0.593
Bilirubin, direct mg/dL	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	$F_{(3,28)} = 1.008$	0.404
Amylase U/L	2841.36 ± 113.20	2883.25 ± 72.90	2640.71 ± 179.18	2647.33 ± 219.94	$F_{(3,24)} = 0.578$	0.635
Lipase U/L	28.77 ± 5.81	17.00 ± 4.18	21.63 ± 7.31	32.00 ± 14.01	$F_{(3,28)} = 0.561$	0.645
Protein, total g/dL	5.92 ± 0.12	5.76 ± 0.18	4.98 ± 0.73	5.87 ± 0.17	$F_{(3,29)} = 1.447$	0.249
Uric acid mg/dL	1.44 ± 0.25	1.04 ± 0.20	1.45 ± 0.38	1.30 ± 0.28	$F_{(3,29)} = 0.287$	0.835

[a] Significantly different from the control; one way ANOVA + Newman–Keuls test.

animals pretreated with blueberry, spinach, and spirulina diets for 4 weeks had reduced cerebral infarction after ischemia and reperfusion. These data suggest that diet pretreatment reduces ischemic brain injury in rats. These diets have effects in addition to the antioxidant effects that we describe, thus it is likely that a combination of antioxidant and anti-inflammatory effects as well as other possible actions are acting in combination to produce the observed results.

Increasing evidence indicates that cytosolic cytochrome C activates caspase-3 and apoptosis. The translocation of cytochrome C from mitochondria to cytosol during ischemia/reperfusion and subsequent DNA fragmentation are suppressed in animals with overexpression of superoxide dismutases (Fujimura et al., 2000; Wang et al., 2003), suggesting that prevention of apoptosis is involved in antioxidant-induced protection against ischemia/reperfusion. In this study, we also found that animals that received antioxidant diets demonstrated decreased TUNEL (+) cells and caspase-3 activity in the ischemic hemisphere when compared with controls. Furthermore, there was a correlation between the reduction of caspase-3 activity and decrease in cerebral infarction among various diets [volume of infarction = $-6.903 + (3.295 \times \Delta$ caspase-3 activity), $r = 0.964$, $P = 0.037$]. Animals pretreated with spirulina had both the least cerebral infarction and lowest activated caspase-3 activity after ischemia and reperfusion. There was a smaller reduction in cerebral infarction and caspase-3 activity in animals pretreated with the blueberry diet. Taken together, these data suggest that antioxidant enriched dietary pretreatment may induce protection through an anti-apoptotic mechanism.

Animals that received blueberry diet had increased horizontal motor activity before stroke. Previous experiments from our laboratory have demonstrated that the aged animals receiving blueberry diets showed improved performance on rotorod and other indices of balance and coordination (Joseph et al., 1999). There is also an increase in electrophysiological neuromodulation, induced by β-adrenergic agonists, in these animals (Bickford et al., 2000). As we have observed in the effects of these diets on both markers of oxidative damage such as malondialdehyde and markers of inflammation such as the pro-inflammatory cytokines TNFα (Gemma et al., 2002), it is possible that mechanisms besides anti-oxidation are involved in such enhanced locomotor activity in the non-stroke animals.

We found that, 2 days after stroke, animals that received all diets developed enhanced vertical movement. However, there is less correlation between the improvement in vertical movement and caspase-3 activity ($r = 0.661$, $P = 0.339$) or volume of infarction ($r = 0.693$, $P = 0.307$) among these animals. Animals treated with spirulina diet had the least infarction and caspase-3 activity, but did not show greater improvement in vertical movement, as compared to the stroke animals that received other diets. In no case did rats perform equivalent to what would be expected in a non-lesioned animal, thus the functional recovery of movement is significant, but some degree of deficiency is still observed at this time point. To further examine if such discrepancies in anatomical recovery of stroke volume versus recovery of motor function may relate to changes in physiological parameters, we examined the blood biochemistry, electrolytes, as well as CO_2 content in a subset of rats. We found that there was no difference in any of these physiological

parameters among the animal groups. Although there was a slight decrease in cholesterol level in animals that receive spinach diet, the cholesterol levels in the other groups were all within normal limits. Our data also indicate that cerebral blood flow, measured by an ultra fine (0.45 mm in diameter) laser Doppler flowmeter (PF-5010, Periflux system, SE) (Wang et al., 2001a,b), did not differ among all animal groups (unpublished observation), indicating that these physiological parameters are not involved in the improvement in motor behavior. Further experiments are required to investigate the involvement of other factors, such as cognitive changes and neuroregeneration, on locomotor activity in these animals after stroke. In addition, it would be of interest to examine if combinations of the diets would have an additive effect.

Previous studies have indicated that treatment with blueberry, spinach, and/or spirulina enriched diets increases cerebellar glutathione levels, reduces malondialdehyde levels, decreases pro-inflammatory cytokines, and improves both spatial and motor learning in aged rats (Bickford et al., 2000; Gemma et al., 2002; Joseph et al., 1999). These diets were given after the onset of neurodegeneration in aged rats. In this study, we pretreated the animals with the diets for 4 weeks before ischemia. It is possible that differential neuroprotective and neuroregenerative mechanisms are involved in these two animal models of neurological dysfunction. Furthermore, as mentioned above, there are many possible mechanisms by which these diets could be having an effect. The antioxidant mechanism is one possibility. An action as anti-inflammatory mechanisms is also possible as all of these diets have an effect on pro-inflammatory cytokines.

In conclusion, our data indicate that the blueberry, spinach, and spirulina diets have neuroprotective effects against transient focal ischemia. The mechanism of protection may involve anti-apoptosis. These data may have clinical implications insofar as administration of diets enriched in certain fruits and vegetables could improve behavioral outcome and reduce neuronal damage in stroke patients.

Acknowledgments

References

Annapurna, V.V., Deosthale, Y.G., Bamji, M.S., 1991. Spirulina as a source of vitamin A. Plant Foods Hum. Nutr. 41, 125–134.

Bergman, M., Varshavsky, L., Gottlieb, H.E., Grossman, S., 2001. The antioxidant activity of aqueous spinach extract: chemical identification of active fractions. Phytochemistry 58, 143–152.

Bhat, V.B., Madyastha, K.M., 2001. Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from Spirulina platensis: protection against oxidative damage to DNA. Biochem. Biophys. Res. Commun. 285, 262–266.

Bickford, P.C., Gould, T., Briederick, L., Chadman, K., Pollock, A., Young, D., Shukitt-Hale, B., Joseph, J., 2000. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Res. 866, 211–217.

Breitbart, E., Lomnitski, L., Nyska, A., Malik, Z., Bergman, M., Sofer, Y., Haseman, J.K., Grossman, S., 2001. Effects of water-soluble antioxidant from spinach, NAO, on doxorubicin-induced heart injury. Hum. Exp. Toxicol. 20, 337–345.

Cao, G., Shukitt-Hale, B., Bickford, P.C., Joseph, J.A., McEwen, J., Prior, R.L., 1999. Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants. J. Appl. Physiol. 86, 1817–1822.

Careri, M., Furlattini, L., Mangia, A., Musc, M., Anklam, E., Theobald, A., von Holst, C., 2001. Supercritical fluid extraction for liquid chromatographic determination of carotenoids in Spirulina Pacifica algae: a chemometric approach. J. Chromatogr. A. 912, 61–71.

Chen, S.T., Hsu, C.Y., Hogan, E.L., Maricq, H., Balentine, J.D., 1986. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. Stroke 17, 738–743.

Clark, W.M., Rinker, L.G., Lessov, N.S., Lowery, S.L., Cipolla, M.J., 2001. Efficacy of antioxidant therapies in transient focal ischemia in mice. Stroke 32, 1000–1004.

Deng, X., Ladenheim, B., Tsao, L.I., Cadet, J.L., 1999. Null mutation of c-fos causes exacerbation of methamphetamine-induced neurotoxicity. J. Neurosci. 19, 10107–10115.

Fujimura, M., Morita-Fujimura, Y., Noshita, N., Sugawara, T., Kawase, M., Chan, P.H., 2000. The cytosolic antioxidant copper/zinc-superoxide dismutase prevents the early release of mitochondrial cytochrome c in ischemic brain after transient focal cerebral ischemia in mice. J. Neurosci. 20, 2817–2824.

Gemma, C., Mesches, M.H., Sepesi, B., Choo, K., Holmes, D.B., Bickford, P.C., 2002. Diets enriched in foods with high antioxidant activity reverse age-induced decreases in cerebellar-adrenergic function and increases in proinflammatory cytokines. J. Neurosci. 22, 6114–6120.

Hirvonen, T., Virtamo, J., Korhonen, P., Albanes, D., Pietinen, P., 2000. Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers. Stroke 31, 2301–2306.

Joseph, J.A., Denisova, N., Fisher, D., Shukitt-Hale, B., Bickford, P., Prior, R., Cao, G., 1998. Membrane and receptor modifications of oxidative stress vulnerability in aging. Nutritional considerations. Ann. N.Y. Acad. Sci. 854, 268–276.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., Bickford, P.C., 1999. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J. Neurosci. 19, 8114–8121.

Leinonen, J.S., Ahonen, J.P., Lonnrot, K., Jehkonen, M., Dastidar, P., Molnar, G., Alho, H., 2000. Low plasma antioxidant activity is associated with high lesion volume and neurological impairment in stroke. Stroke 31, 33–39.

Lin, T.N., He, Y.Y., Wu, G., Khan, M., Hsu, C.Y., 1993. Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. Stroke 24, 117–121.

Lomnitski, L., Carbonatto, M., Ben Shaul, V., Peano, S., Conz, A., Corradin, L., Maronpot, R.R., Grossman, S., Nyska, A., 2000. The prophylactic effects of natural water-soluble antioxidant from spinach and apocynin in a rabbit model of lipopolysaccharide-induced endotoxemia. Toxicol. Pathol. 28, 588–600.

Miranda, M.S., Cintra, R.G., Barros, S.B., Mancini, F.J., 1998. Antioxidant activity of the microalga Spirulina maxima. Braz. J. Med. Biol. Res. 31, 1075–1079.

Mitchell, G.V., Grundel, E., Jenkins, M., Blakely, S.R., 1990. Effects of graded dietary levels of Spirulina maxima on vitamins A and E in male rats. J. Nutr. 120, 1235–1240.

Pedersen, C.B., Kyle, J., Jenkinson, A.M., Gardner, P.T., McPhail, D.B., Duthie, G.G., 2000. Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. Eur. J. Clin. Nutr. 54, 405–408.

Premkumar, K., Pachiappan, A., Abraham, S.K., Santhiya, S.T., Gopinath, P.M., Ramesh, A., 2001. Effect of *Spirulina* fusiformis on cyclophosphamide and mitomycin-C induced genotoxicity and oxidative stress in mice. Fitoterapia 72, 906–911.

Reddy, C.M., Bhat, V.B., Kiranmai, G., Reddy, M.N., Reddanna, P., Madyastha, K.M., 2000. Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from *Spirulina platensis*. Biochem. Biophys. Res. Commun. 277, 599–603.

Rodriguez-Hernandez, A., Ble-Castillo, J.L., Juarez-Oropeza, M.A., Diaz-Zagoya, J.C., 2001. Spirulina maxima prevents fatty liver formation in CD-1 male and female mice with experimental diabetes. Life Sci. 69, 1029–1037.

Salazar, M., Martinez, E., Madrigal, E., Ruiz, L.E., Chamorro, G.A., 1998. Subchronic toxicity study in mice fed *Spirulina* maxima. J. Ethnopharmacol. 62, 235–241.

Swanson, R.A., Morton, M.T., Tsao-Wu, G., Savalos, R.A., Davidson, C., Sharp, F.R., 1990. A semiautomated method for measuring brain infarct volume. J. Cereb. Blood Flow Metab. 10, 290–293.

Sweeney, M.I., Kalt, W., MacKinnon, S.L., Ashby, J., Gottschall-Pass, K.T., 2002. Feeding rats diets enriched in lowbush blueberries for six weeks decreases ischemia-induced brain damage. Nutr. Neurosci. 5, 427–431.

Upasani, C.D., Khera, A., Balaraman, R., 2001. Effect of lead with vitamin E, C, or *Spirulina* on malondialdehyde, conjugated dienes and hydroperoxides in rats. Indian J. Exp. Biol. 39, 70–74.

Wang, Y., Chang, C.F., Morales, M., Chou, J., Chen, H.L., Chiang, Y.H., Lin, S.Z., Cadet, J., Deng, X., Wang, J.Y., Chen, S.Y., Kaplan, P.L., Hoffer, B.J., 2001a. Bone morphorgenetic protein-6 reduces ischemia-induced brain damage in rats. Stroke 32, 2170–2178.

Wang, Y., Hayashi, T., Chang, C.F., Chiang, Y.H., Tsao, L.I., Su, T.P., Borlongan, C.V., Lin, S.Z., 2001b. Methamphetamine potentiates ischemia/reperfusion insults after transient middle cerebral artery ligation in mice. Stroke 32, 775–782.

Wang, Y., Chang, C.F., Morales, M., Chiang, Y.H., Su, T.P., Tsao, L.I., Thiemermann, C., 2003. Neuroprotective effects of diadenosine teraphosphate in animal models of stroke and Parkinson's disease. J. Neurosci. 23, 7958–7965.

Yan, J., Liu, Y., Mao, D., Li, L., Kuang, T., 2001. The presence of 9-*cis*-beta-carotene in cytochrome b(6)f complex from spinach. Biochim. Biophys. Acta 1506, 182–188.

Yang, G.Y., Pang, L., Ge, H.L., Tan, M., Ye, W., Liu, X.H., Huang, F.P., Wu, D.C., Che, X.M., Song, Y., Wen, R., Sun, Y., 2001. Attenuation of ischemia-induced mouse brain injury by SAG, a redox-inducible antioxidant protein. J. Cereb. Blood Flow Metab. 21, 722–733.