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Behavioral and genoprotective effects of *Vaccinium* berries intake in mice

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Abstract

Studies have shown that supplementation with berries rich in anthocyanins are effective in reducing oxidative stress associated with aging, and are beneficial in reversing age-related neuronal and behavioral changes. However, there are few reports on other biological activities of these polyphenols, such as genoprotective effects. The present experiments were performed to study the possible effects of 30-day administration of a lyophilized extract of *Vaccinium ashei* berries on cognitive performance using step-down inhibitory avoidance, open-field habituation and elevated plus-maze tasks, as well as on DNA damage in the hippocampus and cerebral cortex. The present study showed that the extract significantly enhanced long-term memory in the inhibitory avoidance task, induced an increase in the number of crossings during open-field habituation and had an anxiolytic effect in the elevated plus-maze task. Moreover, the extract reduced oxidative DNA damage in brain tissue in vitro. These results suggest that supplementation with *V. ashei* berries to mice improves performance on memory tasks and has a protective effect on DNA damage, possibly due to the antioxidant activity of polyphenols, including anthocyanins.

Keywords: *Vaccinium*; Anthocyanin; Memory; DNA damage

1. Introduction

Phenolic compounds are naturally occurring secondary metabolites from plants. They are present in fruits, vegetables, leaves, nuts, seeds, flowers and barks. These compounds are an integral part of the human diet and are also taken intentionally as medicinal preparations (Kuhnau, 1976; Kong et al., 2003). They act as inhibitors or activators for a large variety of mammalian enzyme systems, and as metal chelators and scavengers of free oxygen radicals (Sellappan et al., 2002; Ono et al., 2002). It has been suggested that free radical scavenging and antioxidant activities play an important role in the prevention of aging and disease-related free radical-induced damage (Joseph et al., 1998; Cantuti-Castelvetri et al., 2000; Youdim et al., 2000).

As with other fruits, blueberries and bilberries contain a range of micronutrients which are essential for health. In particular, many types of berries contain a high level of vitamin C (ascorbic acid), folic acid, resveratrol, pterostilbene and piceatannol (Rimando et al., 2004). However, berries may have additional health benefits as they are also rich in phytochemicals such as anthocyanins and flavonols (Prior et al., 1998; Sellappan et al., 2002; Beattie et al., 2005). It has been hypothesized that additive and synergistic effects of these complex mixtures of phytochemicals, instead of a single component, are responsible for the health benefits derived from fruits and vegetables (Aruoma et al., 2003).

The aim of this study was to carry out psychopharmacological screening to evaluate potential effects of a lyophilized extract of different cultivars from *Vaccinium ashei*, Reade (*Ericaceae*) berries, commonly known as rabbiteye blueberries, on memory, anxiety and locomotor performance, as well as on DNA damage in the hippocampus and cerebral cortex, which is

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thought to be a useful indicator of the bioavailability and efficacy of antioxidant supplements.

2. Materials and methods

2.1. Subjects

The subjects were 30 adult male Swiss mice (aged 3 months), weighing 35–45 g, obtained from our own breeding colony. They were caged in groups of five with free access to food (standard certified rodent diet) and water, and were maintained on a 12-h light/dark cycle (lights on at 07:00 h), at a temperature of 23 ± 1 °C. Animals were randomly assigned to each treatment group and all behavioural procedures took place between 8 am and 2 p.m.

The study was approved by the Animal Care and Use Committee of our center (Universidade Federal de Rio Grande Do Sul, Porto Alegre, Brazil), and all efforts were made to reduce the number of animals used and their suffering.

3. Preparation of lyophilized fruit extract and anthocyanin quantification

Representative samples of *V. ashei* Reade berries were collected at random from the following cultivars: *Woodard*, *Delite*, *Climax*, *Briteblue*, *Bluegen*, *Bluebelle*, *Aliceblue* and *Florida* (all originally American). Two selections (110 and 77) from these cultivars, both obtained from seedlings coming from open pollination of *Bonita* cultivars, were also used in the mixture. Plants were produced by EMBRAPA DE CLIMA TEMPERADO, Pelotas, RS, Brazil, and kept at −0.5 to 0 °C. Pesticide analysis was previously carried out and no sign of these substances was found, assuring no interference of pesticides on our results.

A mixture of fresh berries from the cultivars and selections described above were triturated mechanically and later lyophilized and kept sheltered from light. Anthocyanins were isolated following the procedure described in the European Pharmacopeia (2002).

For the experiments, lyophilized berries were homogenized in 96% ethanol, agitated for 30 min and centrifuged at 3000 rpm for 15 min. The supernatants were filtered through filter paper and concentrated by rotary evaporation at 30 °C. After relyophilization, extracts were stored until the time of administration to the animals, when they were redissolved in distilled water. These ethanol extracts contained 999 mg of anthocyanins per 100 g, and this value was used for dosage calculation. The daily quantity of extract offered to the animals was calculated to provide either 0.6–1.0 or 2.6–3.2 mg/kg/day of anthocyanins. The volume of juice provided was 10 ml/mice/day in all cases. During the whole procedure, including at the time of administration, the extract was kept sheltered from light.

4. Experimental design

Animals were acclimatized to the laboratory environment and to the investigator who handled them. They underwent training in an inhibitory avoidance task (described below) and were subsequently divided into a control group which drank water ad libitum and two groups which drank water supplemented with different concentrations of lyophilized *Vaccinium* berries extract for 30 days ($n = 10$ animals/group), with group 1 receiving 0.6–1.0 mg/kg/day and group 2 receiving 2.6–3.2 mg/kg/day of anthocyanins. Food was available ad libitum. Juice intake and weight were recorded daily.

During the 30-day period, behavioral procedures (inhibitory avoidance testing, open-field habituation and elevated plus-maze) were performed as described below. At the end of this period, animals in the control group and in group 2 were sacrificed. Hippocampi and cerebral cortices were dissected and used to test DNA damage through an alkaline single cell electrophoresis (comet) assay.

5. Behavioral procedures

Animals were subjected to the following behavioral tasks: (a) step-down inhibitory avoidance, (b) open-field habituation and (c) elevated plus-maze.

5.1. Inhibitory avoidance

The inhibitory avoidance apparatus was a 50 × 25 × 25-cm acrylic box, whose floor consisted of parallel 1.0 mm diameter stainless steel bars spaced 1.0 cm apart. A 10-cm^2, 2-cm high, platform occupied the center or the floor. In the training session, immediately after stepping down and placing their four paws on the grid, animals received a 0.4 mA, 2.0 s scrambled foot shock. In test sessions, no foot shock was used and step-down latency (with a ceiling of 180 s) was used as a measure of memory retention. Test sessions were carried out 24 h, 7 days and 30 days after training (Barros et al., 2000, 2001, 2002).

5.2. Open-field habituation

The open-field apparatus consisted of a 40 × 50-cm wide arena whose brown linoleum floor was divided into 12 equal squares by white lines. In the first exploration session, animals were placed in the rear left square and left to explore the arena freely for a 5-min period (Barros et al., 2000), during which the number of line crossings and rearings were counted and used to measure locomotion and exploratory activity. Twenty-four hours later, animals were left to explore the apparatus again for another 5 min and the same measures were recorded to evaluate habituation to the task.

5.3. Elevated plus-maze

The elevated plus-maze consisted of a central platform (10 × 10 cm) with four 45 × 10-cm arms, of which two were open and two were closed. Arms were arranged in such a way that the two arms of each type were opposite to each other. The maze was kept 88 cm above floor level and tests were carried out under dim red light. Animals were placed individually on

the central platform of the plus-maze facing an open arm. Two observers recorded the number of rearings, the time spent in the open and enclosed arms and the number of entries in each arm during 5 min. The percentage of time spent in the enclosed arms and the number of entries in these arms were used as a measure of anxiety (Barros et al., 2000; Izquierdo et al., 2002; Pellow et al., 1985).

5.4. Alkaline single cell electrophoresis (comet) assay

DNA damage was evaluated through the alkaline single cell electrophoresis (comet) assay, performed as described by Singh et al. (1988) and Tice et al. (2000), with some modifications. Hippocampi and cerebral cortices were dissected for five animals from the control group and five animals from group 2. Tissue was homogenated in 500 µl of cold (4 °C) phosphate-buffered saline solution (PBS). For each sample, an aliquot (30 µl) was diluted in PBS to a final volume of 100 µl and another aliquot (30 µl) was diluted in PBS plus H_2O_2 (1 mM) to a final volume of 100 µl. The in vitro assay with H_2O_2 was performed on ice for 5 min (Psimadas et al., 2004). Finally, 10 µl of these cellular suspensions were diluted in 80 µl of low melting point agarose (0.65%) and added to fully frozen slides, which had been covered with a layer of 0.65% normal melting point agarose. Following layer solidification, cells in slides were lysed (2.5 M NaOH, 0.1 M EDTA, 0.01 M Tris, 1% sodium sarcocinate, 1% Triton X-100 and 10% dimethyl sulfoxide, pH 10) overnight at 4 °C. Subsequently, samples were placed in the electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH 13) for 30 min to allow DNA unwinding. Electrophoresis was then performed during 35 min at 25 V and 280 mA. Finally, slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with 50 µl of ethidium bromide (20 µg/ml) and analyzed using a Zeiss-Axioplan epifluorescence microscope (400× magnification). In 100 randomly selected cells in duplicated slides, DNA damage was classified as undamaged (class 0) or as presenting short migration of DNA (class 1), medium migration (class 2), long migration (class 3) and complete migration (no nucleus remaining, class 4). The final score was calculated by adding the scores for each cell in the slide, resulting in a final score of 0 for no DNA damage and 400 for maximum damage.

5.5. Statistical analysis

Data for the inhibitory avoidance task are expressed as median and interquartile intervals for test session latencies. To evaluate differences among groups, a Kruskal–Wallis test was used, followed by Mann–Whitney's *U*-test when appropriate.

For the plus-maze and open-field habituation, data are expressed as mean and standard errors of mean, and groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test against the control group when appropriate. Intragroup comparisons among the two test sessions were performed using a paired samples *t*-test.

For alkaline single cell electrophoresis (comet) assay, data are expressed as mean and standard errors and analyzed with one-way ANOVA. In all comparisons, $p<0.05$ was considered to indicate statistical significance.

6. Results

There were no significant differences in weight between the groups over time ($p>0.05$) (data not shown). There were also no differences in water/juice intake between the groups over the course of the study ($p>0.05$).

6.1. Inhibitory avoidance

Results for the inhibitory avoidance task are displayed in Fig. 1. Groups treated with lyophilized fruit extract after training had higher memory retention in all test sessions, an effect which was statistically significant at 7 ($U=23.5$, $p<0.05$) and 30 days of supplementation ($U=0.0$, $p<0.05$) for group 1, and at 24 h ($U=7$, $p<0.05$), 7 days ($U=4.0$, $p<0.05$) and 30 days ($U=3$, $p<0.05$) for group 2. Training session step-down latency differences among groups were not significantly different among groups.

6.2. Open-field habituation

Results for open-field habituation are presented in Table 1. There was no significant difference in locomotor activity among the three groups in the first session ($p<0.05$). In the second session, there was a decrease in exploration in the control group and in group 1 indicating habituation to the open-field environment ($F=5.638$, $t=2.34$, $p<0.05$). This effect was not as clearly observed in group 2, in which there was no significant difference in the number of crossings among sessions. Moreover, this group had an increased number of crossings in the second session when compared to the control group and group 1 ($t=3.61$, $p<0.05$), an effect which could be due to increased locomotion or impaired habituation. There was also a

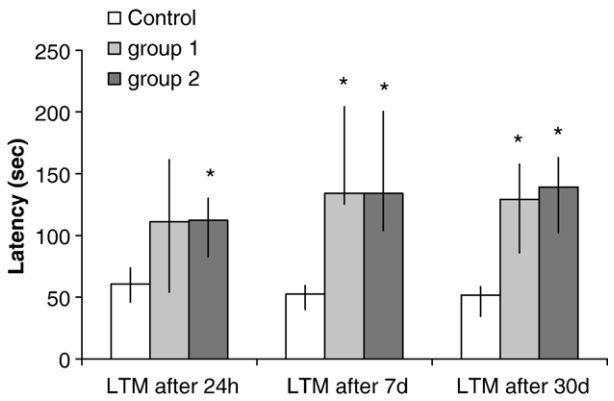

Fig. 1. Test latencies for the inhibitory avoidance task measured 24 h, 7 days and 30 days after training in controls, group 1 (0.6–1.0 mg/kg/day of anthocyanins) and group 2 (2.6–3.2 mg/kg/day). Data express median (interquartile range) test session latencies in seconds. Asterisks indicate significant differences in retention test performance from respective control groups (Mann–Whitney *U*-test, two-tailed, $p<0.05$), $n=10$ per group.

Table 1
Open-field habituation

	Crossings		Rearings	
	1st session	2nd session	1st session	2nd session
Controls	63.10±6.62	46.30±2.77 [a]	19.30±2.17	23.50±1.58
Group 1	61.70±3.81	49.40±3.30 [a]	17.40±2.04	20.30±1.97
Group 2	70.40±3.13	63.10±1.85 [b]	23.60±1.77	27.80±1.83

Effect of treatment with both dosages of *Vaccinium* lyophilized extract in the open-field habituation task. Data are expressed as mean±S.E.M.
$n=10$ per group.
[a] Indicates a significant difference between the first and second sessions.
[b] Indicates a significant difference from the control group in the same session.

trend toward an increase in rearings in group 2, but this was not statistically significant.

6.3. Elevated plus-maze

Results are presented in Table 2. In this test, group 2 spent significantly more time in the open arms of the maze, suggesting an anxiolytic effect of the treatment. Group 2 also presented statistical differences in the number of entries in the open arms when compared to the control group ($F=5.638$, $t=2.53$, $p<0.05$).

6.4. Alkaline single cell electrophoresis (comet) assay

Treatment with lyophilized fruit extract significantly decreased the DNA damage in hippocampal tissues in comparison to the respective control groups as evaluated by the single cell electrophoresis (comet) assay (Fig. 2) ($p<0.05$). In the assay after in vitro treatment with H_2O_2, similar scores were found among treated animals and controls. In the cerebral cortex (Fig. 3), similar results were found: DNA damage was decreased in the treated group ($p<0.05$) when compared to controls, whereas after H_2O_2 treatment no differences were found (data not shown).

7. Discussion

Recent studies have demonstrated that dietary supplementation with blueberry polyphenolics may have beneficial actions on motor and cognitive function, and that they may improve antioxidant status (Joseph et al., 2005). Berry extracts have high

Table 2
Elevated plus-maze

	Total entries	Entries open arms	%Time open arms	Rearings
Controls	11.60±1.55	8.10±1.50	20.0±0.25	15.1±1.57
Group 1	9.05±1.50	7.30±1.34	23.33±0.32	12.50±2.20
Group 2	14.60±1.51	14.10±2.10 *	36.03±0.30 *	15.10±2.51

Total number of entries, number of entries in open arms, percentage of time spent in open arms and rearings for the three study groups in the elevated plus-maze task. Values are expressed as mean±S.E.M.
$n=10$ per group.
* Difference in relation to control group ($p<0.05$).

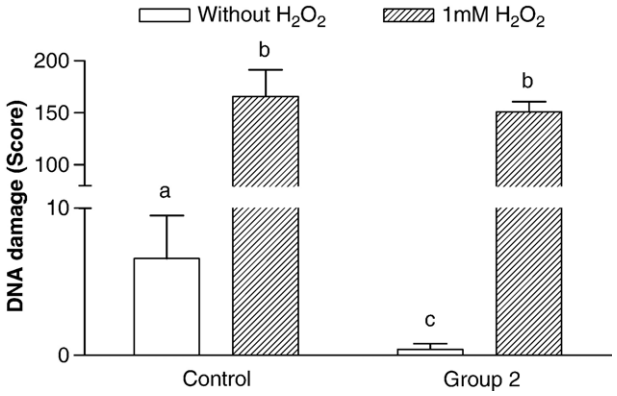

Fig. 2. Effect of the higher dose (2.6–3.2 mg/kg) of *Vaccinium* berries lyophilized extract on DNA damage evaluated by alkaline single cell electrophoresis (comet) assay in hippocampal tissue with (white) and without (striped) treatment with 1 mM of H_2O_2. Values are mean±S.E.M. ($n=5$). Similar letters mean absence of statistical differences ($p>0.05$) between groups, while different letters indicate significant differences ($p<0.05$).

antioxidant activity, and this activity correlates with their content of anthocyanins and total phenolic compounds (Prior et al., 1998; Sellappan et al., 2002; Ono et al., 2002; McAnulty et al., 2004).

A number of investigators have found that flavonoids, including some anthocyanins, possess oral bioavailability in rats (Tsuda et al., 1999; Miyazawa et al., 1999; Matsumoto et al., 2001; McGhie et al., 2003) and that they are able to cross the rat blood–brain barrier after blueberry (Andrés-Lacueva et al., 2005) and blackberry (Talavera et al., 2005) supplementation, as well as after a single administration (Youdim et al., 2003; Passamonti et al., 2005) suggesting that these compounds can feasibly have a direct effect on brain processes. Dietary consumption in some individuals has been estimated to be up to 200 mg/day of anthocyanins, which is higher than that of other flavonoids (23 mg/day) such as quercetin (Scalbert and Williamson, 2000; Frank et al., 2002; McGhie et al., 2003). During our experiment, animals ingested approximately 0.3–

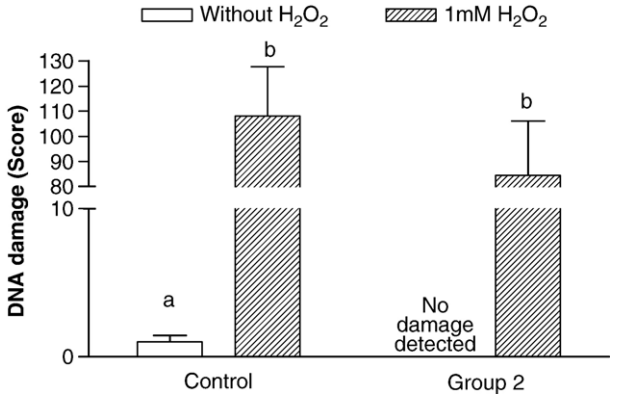

Fig. 3. Effect of the higher dose (2.6–3.2 mg/kg) of *Vaccinium* berries lyophilized extract on DNA damage evaluated by alkaline single cell electrophoresis (comet) assay in cortex tissue with (white) and without (striped) treatment with 1 mM of H_2O_2. Values are mean±S.E.M. ($n=5$). Similar letters mean absence of statistical differences ($p>0.05$) between groups, while different letters indicate significant differences ($p<0.05$).

3.2 mg/kg/day of the anthocyanins; thus, their dietary intake was approximately of the same order of magnitude as that which occurs in humans.

In this work, the effects of extract from *V. ashei* were studied in several behavioral animal models. In the step-down inhibitory avoidance task, a classic model to evaluate memory with a strong aversive component (Cahill et al., 1986), the extract exerted significant effects in long-term retention of the task. Although our protocol does not allow us to tell if these effects occurred on memory consolidation or retrieval, a clear enhancement of retention was seen 7 and 30 days after training. Twenty-four hours after training, this was also observed for the higher dose (2.6–3.2 mg/kg), whereas a visible, albeit nonsignificant, trend towards enhancement was seen with the lower dose (0.3–0.6 mg/kg). This seems to indicate that extract is effective in a broad spectrum of dosage, confirming our expectation that the composition of the juice and the time of administration are more significant than the dosage used.

In the open-field test, which allows us to measure general locomotor activity as well as habituation to a new environment (Novas et al., 1988), animals treated with the higher dose of the extract showed an increase in the number of crossings in the second session of habituation. This could be due to an effect of the blueberry extract on motor behaviour, as has been found on other tasks such as the rod walking and accelerating rota rod tests (Joseph et al., 1998, 1999), or eventually to interference with habituation to the task, although the memory-enhancing effects observed in the inhibitory avoidance task argues against the latter possibility (Ramirez et al., 2005). Other factors such as volition and motivation for exploring the arena should also be considered.

Several flavonoids have been reported to exhibit anxiolytic action (Marder et al., 1995; Salgueiro et al., 1997). This activity is usually demonstrated using a popular test, the elevated plus-maze task (Pellow et al., 1985; Rodgers et al., 1997).

Our results are in agreement with these findings (Table 2), as group 2 showed an increase in time spent in the open arms, suggesting an anxiolytic effect of *V. ashei* extract.

Finally, the potential benefits of the *V. ashei* extract were also observed in the comet assay, a genotoxicity test which has been widely used in recent years to analyze protective effect on DNA damage. In our study, the higher (3.2 mg anthocyanins/kg) dose of the extract decreased DNA damage in both hippocampal and cortical tissues in basal conditions. This effect was not sufficient to significantly decrease DNA damage after H_2O_2 treatment, although it is possible that a protective effect could be observed after lower-intensity oxidative stress (such as lower concentrations of H_2O_2 or shorter treatment times). It is also possible that the antioxidant properties of compounds found in the extract are dependent on mechanisms which are functioning in vivo but are no longer working after tissue dissection and homogenizing. Results similar to ours have been reported for other phenolic compounds found in fruit, including berries containing quercetin, ellagic acid and some anthocyanins. Possible mechanisms for these genoprotective effects include protection of DNA from alkylation or formation of anthocyanin-DNA complexes, which stabilize the molecule against oxidative attack (Ramirez-Tortosa et al., 2001; Beattie et al., 2005).

It is unclear, however, if the behavioral effects of *V. ashei* extract in our study can be explained by the same mechanisms. As the effect of the extract on memory was already seen 24 h after the beginning of treatment, it seems more likely that other mechanisms could account for the behavioral findings. Recent studies suggest that blueberry supplementation enhance several signaling pathways which have been widely shown to be important in memory formation, including hippocampal protein kinase Cα (PKCα) and extracellular-regulated kinase (ERK) (Joseph et al., 2003; Youdim et al., 2004). Interestingly, Andrés-Lacueva et al. (2005) reported that blueberry supplementation and cognition are positively correlated with the total number of anthocyanin compounds found in the different brain regions. Alterations in these signaling pathways, therefore, as well as other factors such as regulation of neurotransmission, should be studied as possible mechanisms for the effects we have found. This does not exclude, however, that the long-term effects of supplementation could also be partly explained by neuronal preservation due to the extract's antioxidant activity (Joseph et al., 2005).

In conclusion, a lyophilized extract of *V. ashei* berries was shown to have memory-enhancing, anxiolytic and locomotion-increasing effects in mice, as well as protective effects against free radical-induced DNA damage in the brain. These results are consistent with the hypothesis that flavonoids (including anthocyanins) and other polyphenols can have effects in cell signaling and decrease oxidative damage, and also suggest that they might act directly on cognition. These effects may contribute to the prevention of age-related and pathological degenerative processes in the brain. The effects of these compounds in these pathological conditions remain to be tested.

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