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Research report

Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity

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ABSTRACT

The aim of this study was to examine the effect of a polyphenol-rich extract (PrB) of *Vaccinium angustifolium* (wild blueberries) introduced intraperitonealy (i.p.) at 30 (PrB30) and 60 (PrB60) mg/kg body weight for 7 days, on cognitive performance, brain oxidative status and acetylcholinesterase activity in adult, male, 3–4-month-old *Balb-c* mice. Evaluation of rodent learning and memory was assessed by a step-through test on day 6 after a double training and an initial acquisition trial on day 5. Antioxidant status was determined by ferric reducing antioxidant power (FRAP), ascorbic acid concentration (FRASC), malondialdehyde and reduced glutathione levels in whole brain homogenates. Acetylcholinesterase (AChE) activity was determined by Ellman's colorimetric method. Results showed that the PrB60-treated mice exhibited a significant improvement in learning and memory (step-through latency time of 228 ± 38 s compared to 101 ± 32 s of the control group). PrB extract administration also resulted in reduced lipid peroxidation products (38 and 79%) and higher brain ascorbic acid levels (21 and 64%) in both PrB30 and PrB60-treated groups, respectively, and higher glutathione levels (28%) in the PrB60-treated group. Furthermore, salt- and detergent soluble AChE activity significantly decreased in both PrB-treated groups. Thus, the significant cognitive enhancement observed in adult mice after short-term i.p. supplementation with the blueberry extract concentrated in polyphenols, is closely related to higher brain antioxidant properties and inhibition of AChE activity. These findings stress the critical impact of wild blueberry bioactive components on brain function.

1. Introduction

It has been suggested that fruits and vegetables may play an important role in delaying the onset of Alzheimer's disease, particularly among those who are at high risk for the disease [15]. Furthermore, dietary intake of flavonoids has been inversely related to the risk of dementia [13,34]. High vegetable consumption may also be associated with slower rate of cognitive decline in older age [38]. The main mechanism proposed for the beneficial effect of fruits and vegetables is the potentiation of antioxidant defenses (including enzymatic and non-enzymatic antioxidants) by plant polyphenolic and other antioxidant nutrients. Oxidative stress is linked to neuronal protein misfolding, membrane dysfunction, cell death and glial cell activation that are associated with normal ageing or certain neurodegenerative diseases [1]. Although it is not yet clear whether oxidative stress is the primary cause, an epiphenomenon or a consequence, the brain is particularly vulnerable due to its high metabolic rate, specific features, like the abundant presence of polyunsaturated fatty acids, high levels of iron, and the reduced capacity for cellular regeneration [14].

Abbreviations: AChE, acetylcholinesterase; ao, ascorbic oxidase; ATCI, acetylthiocholine iodide; BDNF, brain-derived neurotrophic factor; BHT, butylated hydroxytoluene; BW, body weight; BSA, bovine serum albumin; BV2, immortalized mouse microglia; CA1, *Cornu Ammonis* area 1; COX, cyclooxygenase; CREB, cAMP-response element-binding protein; CNS, central nervous system; CSF, cerebrospinal fluid; DS, detergent soluble; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithiobis-2-nitrobenzoate ion; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-related kinase; FRAP, ferric reducing antioxidant power; FRASC, ferric reducing/antioxidant and ascorbic acid; G, globular; GABA, γ-aminobutyric acid; GAE, gallic acid equivalents; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione *S*-transferase; IC_{50}, median inhibition concentration; IL, initial latency; IGF, insulin-like growth factor; IGF-R, insulin-like growth factor receptor; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; ISO, isoproterenol; MDA, malondialdehyde; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PrB, polyphenol-rich blueberry extract; RT, room temperature; SS, salt soluble fraction; STL, step-through latency; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; TNFα, tumor necrosis factor-alpha; TPTZ, 2,4,6-Tris(2-pyridyl)-s-triazine; Tris–HCl, trisaminomethane hydrochloride.

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Blueberries (fruits of various *Vaccinium* species) are among the fruits with high antioxidant power and rich in anthocyanins. In pigs fed wild blueberries for 4 weeks, anthocyanins were detected in the liver, eye, cortex, and cerebellum [28]. Furthermore, in aged rats fed blueberries for 8 weeks, anthocyanins were found in the cerebellum, cortex, hippocampus or striatum in their unmetabolized forms [2]. Interestingly, Williams et al. [51] reported that flavanol levels were higher than anthocyanin levels in both plasma and brain tissue of aged rats supplemented with blueberries for 12 weeks.

Studies have shown that short-term dietary supplementation (8 weeks) of aged rats (19 months old) was effective in reversing age-related deficits in cognitive and motor function [26]. Reactive oxygen species (assayed as 2′,7′-dichlorofluorescein diacetate) in the striata from all treated animals were lower than in the control group [26]. It is clear, however, that the significant effects of blueberries on both motor and cognitive behavior (motor behavioral performance on the rod walking and accelorod tasks, learning and memory in the Morris water maze, object recognition memory on the visual paired comparison task) involve a multiplicity of actions, including neuronal signalling and anti-inflammatory effects [42,48]. In particular, short-term dietary supplementation with blueberries of aged rats improved striatal dopamine release and GTPase activity, synaptosomal Ca^{2+} recovery after H_2O_2 challenge [26,52], cerebellar ISO potentiation of GABAergic inhibition [11], hippocampal plasticity parameters, i.e., higher hippocampal neurogenesis, extracellular receptor kinase activation, and resulted in higher IGF-1 and IGF-1R levels [12]. Additionally, short-term dietary supplementation activated the ERK-CREB-BDNF pathway [51], increased hippocampal heat shock protein 70-mediated neuroprotective response to inflammatory challenge [20], and resulted in lower brain NF-κB levels [23]. Furthermore, blueberry supplementation inhibited the production of the inflammatory mediator, nitric oxide (NO), as well as interleukin-1β and tumor necrosis factor-alpha (TNFα) in cell conditioned media from lipopolysaccharide-activated BV2 microglia and reduced mRNA and protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) [33].

Only a few studies have been performed in young/adult rodents. A 2-month dietary supplementation of rats with blueberries prevented deficits in learning performance induced by bilateral hippocampal injections of kainic acid and prevented the loss of CA1 pyramidal neurons [17] and deficits in cognitive performance, and dopamine release, induced by ^{56}Fe particle irradiation [47]. Furthermore, adult rats (aged 12 months), that consumed lyophilized berries for 30 days (approximately 3.2 mg/kg day of anthocyanins), had significantly enhanced short-term but not long-term memory in the inhibitory avoidance task and improved working memory in the radial maze [43]. A similar dietary intervention in adult mice (aged 3 months) improved performance in memory tasks and had a protective effect on DNA damage in the hippocampus and cerebral cortex [7].

The central cholinergic system is essential for the regulation of cognitive functions, as evidenced by the extensive loss of cholinergic neurons observed in the forebrain of Alzheimer's disease patients and the learning and memory deficits of anti-cholinergic drugs, such as scopolamine, in a variety of cognitive animal models [45,49,53]. Agonists of cholinergic receptors and inhibitors of acetylcholinesterase (AChE) have been extensively used in order to increase endogenous acetylcholine levels and thus overcome cognitive deficits. Acetylcholinesterase metabolizes acetylcholine to choline and acetyl-CoA. AChE exists into different molecular forms, which can be distinguished on the basis of their shapes, e.g., collagen-tailed asymmetric forms and globular (G) forms [32]. The latter is present in the mammalian brain in different multiples of the monomer subunit, e.g., monomer, dimer and tetramer. It has also been found that these isomeric forms are differentially localized in the neuron. The G1 form is cytosolic and the G4 form is membrane bound by hydrophobic amino acid sequences or glycophospholipids. The detergent soluble (DS) and salt soluble (SS) fraction of AChE contain predominantly the G4 and G1 forms, respectively. Recent studies have shown that dietary supplementation of mice with green tea polyphenols for 7 weeks improved cognitive performance and inhibited AChE activity in scopolamine-induced amnesic mice [31]; however, the effect of blueberries on the above has not been studied.

The aim of this study was to investigate the effect of a 7-day intraperitoneal (i.p.) administration of a polyphenol-rich wild blueberry (*Vaccinium angustifolium*) extract (PrB) on the cognitive performance of adult mice as assessed by a passive avoidance test, brain oxidative status (total antioxidant capacity, ascorbic acid, malondialdehyde (MDA) levels and reduced glutathione (GSH) content) and AChE activity. Results showed that short-term intraperitoneal administration of blueberry polyphenols to healthy, adult mice significantly enhanced cognitive performance in the passive avoidance test and attenuated brain oxidative stress markers. We have documented for the first time that the nootropic action of blueberry polyphenols is related to decreases in brain AChE activity and lipid peroxidation.

2. Materials and methods

2.1. Plant material and extraction

Wild blueberries were purchased as a composite from Wyman's (Cherryfield, ME), freeze-dried with standard procedures by American Lyophilizer Inc. (Bridgeport, PA, USA) and powdered. Polyphenols were extracted from the above as previously described [21]. In brief, 2 g of blueberry powder was extracted in the dark, under magnetic stirring with 15 mL/g of methanol, acetic acid, and distilled water at a ratio of 25:1:24, respectively, for 2 h. The extract was centrifuged at 1200 × *g* for 5 min at room temperature (RT), filtered through a 0.2 μm filter and evaporated to dryness in a Speed Vac system (Freeze dryer 4.5; Labconco Corp., Kansas City, MO, USA). The dry residue was stored at −20 °C until further use.

The dry residue was re-dissolved with 1 mL of 3% formic acid in water (w/v), centrifuged and the supernatant was absorbed on a C18 Sep-Pak cartridge. The cartridge was washed with methanol, equilibrated with 5 mL of 3% formic acid in water (w/v) and eluted with 5 mL 3% formic acid in 50% methanol (w/v). The polyphenols eluted from the cartridge were evaporated under vacuum until dryness and kept at −20 °C until use.

2.2. Determination of total phenolics and anthocyanins

Total phenolics were measured with the Folin-Ciocalteau reagent method [50]. The total polyphenolic content was expressed as gallic acid equivalents (GAE), using a standard curve with 50–600 mg/L gallic acid. Absorbance was measured at 765 nm with a UV-spectrophotometer (Pharmacia LKB-Biochrom4060). Additionally, total anthocyanin content was estimated according to Giusti and Wrolstad [22] by UV–vis spectroscopy at 538 nm after dissolution in a mixture of methanol and 0.1 M HCl at a ratio of 85:15. Total anthocyanin content was expressed as cyanidin 3-rutinoside equivalents.

2.3. In vitro acetylcholinesterase inhibition assay

The assay for AChE activity was performed with the colorimetric method of Ellman et al. [19], utilizing acetylthiocholine iodide (ATCI) as a substrate. The rate of production of thiocholine is determined by the continuous reaction of the thiol with 5,5′-dithiobis-2-nitrobenzoate (DTNB) ion to produce the yellow anion of 5-thio-2-nitro-benzoic acid. Briefly, in the 96 well plates, 25 μL of 15 mM ATCI, 75 μL of 3 mM DTNB and 50 μL of 50 mM Tris–HCl, pH 8.0, containing 0.1% bovine serum albumin (BSA), and 25 μL of the tested phytochemicals were added and the absorbance was measured at 405 nm after 5 min of incubation at RT. After 25 μL of 0.22 U/mL of AChE from electric eel (Sigma–Aldrich Corporation, St. Louis, MO, USA) was added, the absorbance was measured again after 5 min of incubation at RT. Percentage of inhibition was calculated by comparing the rate of enzymic hydrolysis of ATCI for the samples to that of the blank (50% aqueous methanol in buffer). Galanthamine (1–32 μM) was used as a reference standard and was supplied by Sigma–Aldrich. IC_{50} values were determined by GraphPad Prism4.0 (GraphPad Software Inc., USA). All determinations were carried out at least five times, and in triplicate, at each concentration of the standard and samples.

2.4. Animals

Male, *Balb-c* mice (34–38 g, BW), 3–4 month-old, were kept in polyacrylic cages (38 cm × 23 cm × 10 cm) with nine animals per cage and housed in a room under