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Original Article

EFFECT OF *ARONIA MELANOCARPA* FRUIT JUICE ON LEARNING AND MEMORY IN THE TWO-WAY ACTIVE AVOIDANCE TASK IN RATS

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Summary

Aronia melanocarpa fruits are one of the richest natural sources of phenolic substances, among them flavonoids, mainly from the subclass of anthocyanins. Flavonoids, which are constituents of *Aronia melanocarpa* fruit juice, have been found to localize in various brain regions that are important for memory. The aim of the present study was to investigate the learning and memory effects of *Aronia melanocarpa* fruit juice (AMFJ) in young/healthy male Wistar rats. AMFJ was applied orally for 21 and 30 days at a dose of 10 ml/kg b.w., and comparisons were made with saline-treated (10 ml/kg b.w.) controls. Learning and memory functions were evaluated, using the two-way active avoidance task (shuttle box). Administered for the two treatment periods, the juice significantly increased the number of avoidances on training days 1 and 2, as well as at the retention test (24 h after the 2nd training session). These effects were more pronounced in rats treated with AMFJ for 30 days. The learning and memory effects of AMFJ are probably due to its polyphenolic constituents. The findings from the present study suggest that AMFJ applied subchronically improved learning and memory in young/healthy rats.

Key words: *Aronia melanocarpa*, learning, memory, active avoidance

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Introduction

Aronia melanocarpa [Michx.] Elliot (black chokeberry) originates from the eastern parts of North America and East Canada. Around 1900 it migrated to Europe and Russia. *Aronia* fruits are commonly used to produce juice, syrup, jellies, tea and wine. Chokeberry fruits are extremely rich in plant secondary metabolites, known as polyphenols [1, 2]. These polyphenols are flavonoids (mainly from the subclass of anthocyanins), procyanidins and phenolic acids.

Indeed, there has been intense interest in the neuroprotective effects of polyphenols, which are

powerful antioxidants in vitro [3]. A prospective study has provided strong evidence that dietary flavonoid intake is associated with the preservation of cognitive performance with ageing [4]. Furthermore, there is much evidence to suggest that flavonoids found in fruits and fruit juices (most notably flavanols, flavanones and anthocyanins) have the capacity to improve memory [5-8].

In pigs, anthocyanins have been detected in the cortex and cerebellum [9]. In aged rats, anthocyanins have been found in the cerebellum, cortex, hippocampus or striatum in their unmetabolized forms [10]. Interestingly, Williams et al. [11] reported that flavanol levels were higher than anthocyanin levels in brain tissue of aged rats supplemented with blueberries.

Rodent models have been used as models of human declarative memory to predict potential effects of flavonoids on human cognitive performance [12]. A number of animal studies, using diets containing freeze-dried fruit/fruit juice, have demonstrated that grape, pomegranate, and blueberry, as well as pure flavonoids (epicatechin, quercetin), are capable of affecting several aspects of memory and learning [8, 11, 13-16].

Most studies, investigating the effects of flavonoid-rich foods on cognition, have been carried out on aged animals and in experimental models of memory impairment. To date, there has been only a limited number of studies in young/healthy animals [7].

The aim of the present study was to investigate the effect of *Aronia melanocarpa* fruit juice (AMFJ) on learning and memory in male young/healthy male rats, using the two-way active avoidance task (shuttle box).

Materials and Methods

Aronia melanocarpa fruit juice (AMFJ) preparation

AMFJ was produced from *Aronia melanocarpa* (Michx.) Elliot fruits grown in the Balkan Mountains, Bulgaria. They were handpicked in September, crushed and squeezed. The juice was filtered, pasteurized at 80°C for 10 min and stored at 0°C till the experiment. The contents of phenolic substances in 100 ml AMFJ were: total phenolics, 709.3±28.1 mg as gallic acid equivalents, determined spectrophotometrically according to the Folin-Ciocalteu procedure [17]; total flavonoids, 189.4±8.6 mg as catechin equivalents, measured by a colorimetric assay developed by Zhisten et al. [18]; total anthocyanins, 106.8±6.2 mg as cyanidin-3-glucoside equivalents, determined by a pH-differential spectrophotometry at pH 1.0 and pH 4.5 [19]; quercetin, 11.8±0.8 mg, measured by a high-performance liquid chromatography method [20]. The values were the mean of duplicate determinations of three samples.

Animals and treatment

Male Wistar rats (200-240 g at the beginning of the experiments) were housed in polypropylene boxes with free access to food and water. The experiments were carried out according to the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences, in compliance with the national policies and the EEC Directive of 1986 (86/609/EEC).

The experiments were performed on 40 rats. The animals were treated orally through an orogastric cannula for 21 days (three weeks) or for 30 days (one month). There were two types of treatment: the control groups were treated with saline (10 ml/kg) and AMFJ groups were treated with AMFJ at a dose of 10 ml/kg. Thus, there were 4 groups of 10 animals: $Control_{21}$, $AMFJ_{21}$, $Control_{30}$ and $AMFJ_{30}$ (the index indicates the duration of treatment). The dose and the treatment periods were chosen on the basis of previous investigations (unpublished data), which have shown that AMFJ at that dose and treatment durations had significant effects on the central nervous functions in rats.

Two-way active avoidance task – shuttle box

In the shuttle box task, in order to avoid a mild foot shock, the rat must learn to shuttle from one end of the box to the other every time a warning signal (conditioned stimulus) is presented. The two-way active avoidance task was carried out after the method of Buresova and Bures [21], modified by Petkov et al. [22]. The shuttle box apparatus (50 x 29 x 21 cm) was divided in two equal compartments provided with a round opening in the centre. Light (20 W switched on alternately in the two compartments) was used as a conditioned stimulus. The conditioned stimulus was switched on in the part of the cage opposite to the part, in which the rat was located at the end of the inter-trial period. The unconditioned stimulus was an electric shock (0.5 mA, 50 Hz) applied to

the grid floor for 12 sec. The conditioned stimulus preceded the onset of the unconditioned stimulus by 9 sec and continued during the action of the unconditioned stimulus. An avoidance response (correct response) was recorded when the animal avoided the unconditioned stimulus within 9 sec after the onset of the conditioned stimulus. The inter-trial interval was 9 sec. There were two learning sessions (on two consecutive days), each consisting of 50 trials for each rat. A retention test was carried out 24 h after the second training session: the light stimulus was applied for 9 sec and was followed by 2 sec electric shock. The inter-trial interval was 9 sec. AMFJ was applied 60 min before the two learning sessions and was not given to the animals before the retention test. The number of avoidances was recorded in the shuttle-box learning tests (on the first and second training days) and the retention test (24 hours after the second training day). Before each test, the apparatus was wiped clean and dried. The experiments were performed between 9.00 h and 13.00 h.

Statistical analysis

Results are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to analyze the data. Findings from the ANOVA were post-hoc analyzed using the Student-Newman-Keuls (SNK) test. A level of $p < 0.05$ was considered significant.

Results

After the treatment period of 21 days, ANOVA demonstrated significant effects of AMFJ on the number of avoidances on the first training day ($F_{1,19}$=6,639; p⩽ 001), on the second training day ($F_{1,19}$ =4.042, p ⩽ 0.05) and at the retention test ($F_{1,19}$=4.158; p⩽ 0.05). The post-hoc comparisons showed that AMFJ significantly increased the number of avoidances on the first training day (t=2.577, p⩽ 0.01), on the second training day (t=2.01, p⩽ 0.03) and at the retention test (t=2.039, p⩽ 0.02), as compared with the saline-treated controls (Figure 1A).

After the 30-day treatment period, ANOVA showed significant effects of AMFJ on the first training day ($F_{1,19}$ =13.784, p⩽ 0.001), on the second training day ($F_{1,19}$=6.691, p⩽ 0.01) and at the retention test ($F_{1,19}$=13.8, p⩽ 0.001). The post-hoc comparisons showed that AMFJ significantly increased the number of avoidances on the first training day (t=3.713, p⩽ 0.001), on the second training day (t=2.587, p⩽ 0.009) and at the retention test (t=3.715, p⩽ 0.001) as compared to the saline-treated controls (Figure 1B).

Discussion

In this study, the learning and memory processes of rats tested by the active avoidance task were markedly improved by AMFJ applied to the animals for 21 and 30 days. This effect was probably due to the activity of the juice polyphenolic ingredients, predominantly flavonoids. There are literature data that polyphenols from berries do accumulate in the brain following long-term consumption [16]. Most of the biological actions of flavonoids on the brain have been attributed to their antioxidant actions through their ability to scavenge reactive species or to improve the cellular antioxidant status [23, 24]. Indeed, AMFJ possesses pronounced antioxidant and radical scavenging effects, which have been demonstrated by many authors [25-28] and reviewed by Kokotkiewicz et al. [29] and Denev et al. [2].

However, this classical antioxidant activity probably does not account for all biological actions of flavonoids *in vivo*, particularly in the brain, where they are found in only very low concentrations [30]. Instead, it has been postulated that their effects on the brain are mediated by an ability to protect vulnerable neurons and enhance existing neuronal function. Flavonoids are potentially capable of inducing vascular effects and increasing peripheral blood flow. These vascular effects are potentially significant because increased cerebrovascular function is known to facilitate adult neurogenesis [30, 31].

In vitro work has indicated that flavonoids and their physiological metabolites are capable of activating signaling pathways, critical in controlling synaptic plasticity [32], but only at low nanomolar concentrations [33], similar to those reported in the brain. Such signaling pathways are the extracellular receptor kinase (ERK) and protein kinase B/Akt pathways [30, 34]. These pathways are known to be critical in controlling the morphological mechanisms behind memory storage in the hippocampus and cortex of the brain. Flavonoids have the potential to enhance memory and learning by activating

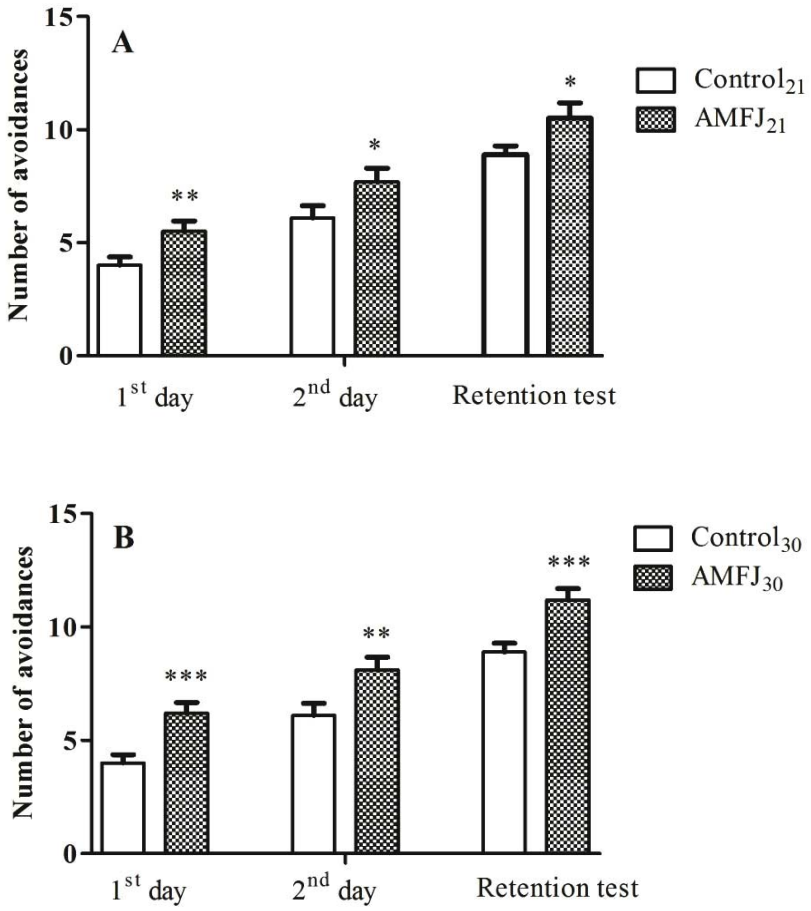

Figure 1. Number of avoidances of rats during the training sessions (1st day and 2nd day) and during the retention test in the active avoidance task (shuttle box) after treatment with *Aronia melanocarpa* fruit juice (AMFJ) for 21 days (**A**) and 30 days (**B**). Results are presented as mean ± S.E.M.; n = 10; *p<0.05 vs. Control; **p<0.01 vs. Control; ***p<0.001 vs. Control

kinases within these pathways. They act by regulating proteins such as the cAMP response element-binding protein (CREB), which is involved in the expression of important genes linked to memory. For example, CREB is crucial for the production of neurotrophins such as brain-derived neurotrophic factor (BDNF), which are known to be required during memory acquisition and consolidation [3]. The study of Rendeiro et al. [7] suggests that consumption of flavonoid-rich blueberries has a positive impact on spatial learning performance in young healthy animals, and these improvements are linked to the activation of ERK-CREB-BDNF pathway in the hippocampus.

The central cholinergic system is essential for the regulation of cognitive functions. There are data that flavonoids and procyanidins are able to inhibit acetylcholineesterase activity [23] and to restore acetylcholine brain contents in cognitively impaired rats [35].

From the review of literature, it is clear that plant polyphenolic substances might improve memory by several mechanisms: antioxidant activity, vascular effects, activation of signaling pathways, and inhibition of acetylcholinesterase activity. The exact mechanism of AMFJ-induced improvement of memory and learning performance in rats remains to be elucidated.

Conclusions

The results from the the two-way active avoidance task (shuttle box) showed that AMFJ,

administered to young/healthy male rats for periods of 21 and 30 days, improved learning and memory. This effect was more pronounced after 30 days treatment, i.e. it was time-dependent. The effect was probably due to the polyphenolic ingredients of the juice. However, further research is required to find out the exact mechanisms of these results.

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