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Original Contribution

BEHAVIORAL EFFECTS OF ARONIA MELANOCARPA FRUIT JUICE IN EXPERIMENTAL ANIMALS WITH DIET-INDUCED METABOLIC SYNDROME

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ABSTRACT

PURPOSE: This study evaluated the effects of polyphenol-rich *Aronia melanocarpa* fruit juice (AMFJ) on the behavior of rats with metabolic syndrome (MS) induced by high-fat high-fructose diet. METHODS: Fifty rats were allocated into 5 groups (10 rats per group): control, MS, MS + $AMFJ_{2.5}$, MS + $AMFJ_5$ and MS + $AMFJ_{10}$. During the induction of MS in the course of 10 weeks, the last three groups were treated with AMFJ at doses 2,5 ml/kg, 5 ml/kg and 10 ml kg, respectively. The open field test was used to examine locomotion, the social interaction test was used to evaluate anxiety, the depression-like state was studied by the forced swimming test and spatial memory was evaluated by the place recognition test. RESULTS: No differences were found in the overall motor activity across the groups. Anxiety and memory impairment were established in MS rats. AMFJ counteracted the development of anxiety, and brought the memory function back to normal. CONCLUSIONS: The improvement of the MS-induced behavioral alterations might be attributed to the rich polyphenolic content of AMFJ, essential for its antioxidant, anti-inflammatory and metabolic actions, and probably underlie also the anxiolytic and memory-enhancing effects observed in the present study.

Key words: Aronia melanocarpa, behavior, metabolic syndrome, rats

INTRODUCTION

Aronia melanocarpa (Black chokeberry) originating from the eastern part of North America and eastern Canada is characterized by a very high content of polyphenols (PPs) in its fruits. Studies have shown that PPs exert antioxidant, anti-inflammatory, lipid-lowering and glucose-lowering effects and their benefits are documented in animal models of obesity, hypertension, dyslipidemia and diabetes (1).

Metabolic syndrome (MS) is a cluster of cardio-metabolic risk factors – central obesity, arterial hypertension, hyperglycemia, insulin resistance and atherogenic dyslipidemia (2). MS is one of the leading causes of disability worldwide. Patients with this condition have an increased risk of sudden cardiac death, as well as 1.5-fold higher total mortality risk (3). Due to the increasing rate and the poor prognosis, MS is one of the main problems of modern public health. This requires an active search and detection of this condition and timely measures. There is scientific evidence that MS is associated with anxiety and cognitive impairment in both clinical (4, 5) and experimental settings (6-8). The behavioral effects of *Aronia melanocarpa* fruit juice (AMFJ) have been studied on animals in different experimental models (9-17), but there are currently no published data about the effect of AMFJ on the behavior of animals with MS.

Therefore, the aim of this study was to evaluate the effects of polyphenol-rich AMFJ on the behavior of rats with diet-induced MS.

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MATERIALS AND METHODS

Aronia melanocarpa fruit juice preparation and determination of its polyphenolic content

AMFJ was prepared from fresh fruits grown in Troyan region of Bulgaria. It was filtered, canned with potassium sorbate (1.0 g/l) and stored in a refrigerator that reached no more than 5 °C. The polyphenol content of AMFJ, as determined by Valcheva-Kuzmanova *et al.* (18), is shown in **Table 1**.

Table 1. *Content of polyphenols in Aronia melanocarpa fruit juice; GAE – gallic acid equivalents [18]*

Ingredients	Content
Total phenols	5461 GAE/l
Total proanthocyanidins	3122.5 mg/l
Cyanidin 3-galactoside	143.7 mg/l
Cyanidin 3-arabinoside	61.7 mg/l
Cyanidin 3-glucoside	4.4 mg/l
Cyaniding 3-xyloside	11.6 mg/l
Chlorogenic acid	585 mg/l
Neochlorogenic acid	830 mg/l

The total content of phenolic ingredients in the juice was determined spectrophotometrically, the contents of cyanidin glycosides and phenolic acids were detected by high-performance liquid chromatography (HPLC) and the amount of total proanthocyanidins was measured gravimetrically (18).

Animals and experimental procedure

Fifty male Wistar rats (180-280 g) were included in the experiments. They were housed in plastic cages, at an ambient temperature 20-25 °C, under 12-hour light/dark cycle. They had free access to food and drinking water.

To induce MS, a high-fat high-fructose (HFHF) diet was used (6). It consisted of regular rat chow enriched with 17% lard and 17% fructose, that was given together with a 10% fructose solution instead of drinking water. The calorie intake of this diet was 405 kcal/100 g, where lard provided 38% of the energy intake and fructose – 17%. The fructose solution accounted for additional 40 kcal/100 ml calorie intake. During the experimental period of 10 weeks, the animals were treated once daily with either distilled water or different doses of AMFJ administered by an orogastric tube.

There were 5 experimental groups (10 rats per group): control (on a regular diet and treated with 10 ml/kg distilled water); group MS (on HFHF diet and treated with 10 ml/kg distilled water), group MS + $AMFJ_{2.5}$ (on HFHF diet and treated with AMFJ 2.5 ml/kg diluted with distilled water to a volume of 10 ml/kg), group MS + $AMFJ_5$ (on HFHF diet and treated with AMFJ 5 ml/kg diluted with distilled water to a volume of 10 ml/kg) and group MS + $AMFJ_{10}$ (on HFHF-diet and treated with AMFJ 10 ml/kg).

All procedures concerning animal treatment and experimentation were conducted in conformity with the national and international laws and policies (EU Directive 2010/63/EU for animal experiments) and were approved by the Bulgarian Food Safety Agency (Document 177/07.07.2017).

Open field test (OFT)

Developed by Calvin Hall (19), the open field test gives a general idea about the overall locomotor activity. To carry out the test, a wooden field (100 cm x 100 cm) surrounded by 40 cm high walls was used. It was divided by blue lines into 25 squares measuring 20 cm x 20 cm. The test rat was placed in the center of the field and its behavior was studied by direct observation for 5 minutes. The total number of squares crossed by the four paws was counted as an indicator of horizontal motor activity. The vertical locomotor activity was evaluated by the number of the rearings.

Social interaction test (SIT)

The test, introduced by File and Hyde (1978) (20), serves as a valuable tool to determine the level of anxiety of experimental animals, as judged by the time of social interaction of a rat

with an unfamiliar test partner. To perform the test, two rats from different cages were simultaneously placed in the opposite corners of the open field arena. The animals had the same treatment and similar weights (with a difference of no more than 10%). The time spent in active social interaction (grooming, sniffing, following or crawling under/over the partner) was measured as an index inversely related to anxiety. Passive contact (e.g. lying or sitting with bodies in contact) was not considered as a social interaction.

Place recognition test (PRT)

The test is a modification of the novel object recognition test and is adapted to study the spatial memory of the tested animals (21). A walled field, measuring 60 cm x 40 cm and evenly lit by a source of artificial light, was used. The test was conducted in two sessions, the first being a training one. Two identical objects were located symmetrically and firmly attached to the floor, so that they could not be moved by the animal. The rat was placed in the center of the field and allowed to explore the objects for 3 min; 30 minutes later, a test session was performed, in which the location of one of the objects was changed. Again, within 3 minutes, the animal behavior was observed and the time spent in exploration of both objects was recorded. Exploration included approaching (to less than 1 cm), sniffing and climbing the objects. As an indicator of spatial memory, the discrimination index was calculated: B / (A + B), where: A was the time of exploring the object with the original location, and B – the time of exploring the object with the new location. The higher the discrimination index, the better the memory.

Forced swimming test (FST)

The method was described by Porsolt *et al.* (1977) (22). It is used to study the presence of depression-like behavior in rodents. When a rat is forced to swim with no possibility to escape, after an initial period of active struggling (swimming, climbing), the animal assumes an immobility posture with only minimal movements necessary to keep its head above the water. This condition of immobility is considered to reflect the animal's 'despair' as a result of a depression-like state. A glass cylinder (17 cm in diameter and 50 cm high) was used, filled with water (~30 ^{o}C) up to 30 cm to ensure that the animal could not touch the bottom of the pool with its hind paws or tail. The rat was placed in the cylinder for 5 minutes and the behavior was observed. The test was conducted in two identical sessions – a training and an experimental one, separated by 24 hours, with the immobility time during the second session only being recorded and analyzed.

Statistical analysis

The results from the experiments were analyzed by one-way ANOVA with Dunnet's multiple comparison test. GraphPad Prism 5.00 statistical software was used. The data are presented as means ± SEM and $p<0.05$ was considered to indicate statistical significance.

RESULTS

Open field test

The results from the OFT are presented in **Table 2**. No significant difference was seen in the horizontal and vertical movements across the groups.

Table 2. *Number of squares crossed and number of rearings in the open field test*

Variable	Control	MS	MS + $AMFJ_{2.5}$	MS + $AMFJ_{5}$	MS + $AMFJ_{10}$
Number of squares crossed	60.7 ± 12.18	81.6 ± 13.15	64.7 ± 10.13	54.2 ± 10.37	43.8 ± 10.07
Number of rearings	20.7 ± 3.01	35.35 ± 3.17	22.9 ± 4.09	18.6 ± 2.09	17.5 ± 2.26

Social interaction test

One-way ANOVA showed statistically significant difference in the time spent in social interaction between the groups ($p=0.0218$). Dunnett's post-test revealed a significant decrease ($p<0.05$) in the social interaction time of the animals from the MS group (20.73 ± 1.86 sec) compared to the control group (28.96 ± 1.89 sec). Compared to the MS group, AMFJ-treated groups had a longer time for social interaction, the result being statistically significant ($p<0.05$) in MS+$AMFJ_{2.5}$ group (29.63 ± 2.21 sec) (**Figure 1**).

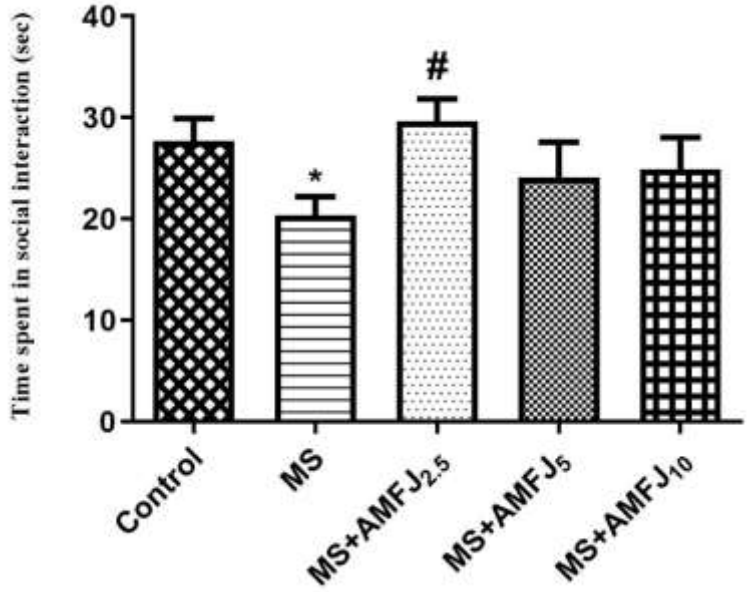

Figure 1. Time (sec) spent in social interaction in the social interaction test. *p<0.05 vs. control; #p<0.05 vs. MS

Place recognition test

The results are shown on **Figure 2**. One-way ANOVA revealed a significant difference across the groups (p=0.0076). Dunnett's multiple comparison test showed a deterioration of the spatial memory of rats from the MS group compared to the animals from the control group, as shown by the significant reduction of the discrimination index (0.44±0.04 vs. 0.65±0.07, p<0.01). There was a significant improvement in the spatial memory in all AMFJ-treated groups compared to the MS group (0.62±0.05 for MS+AMFJ$_{2.5}$, p<0.05; 0.68±0.04 for MS+AMFJ$_5$, p<0.01; 0.61±0.03 for MS+AMFJ$_{10}$, p<0.05).

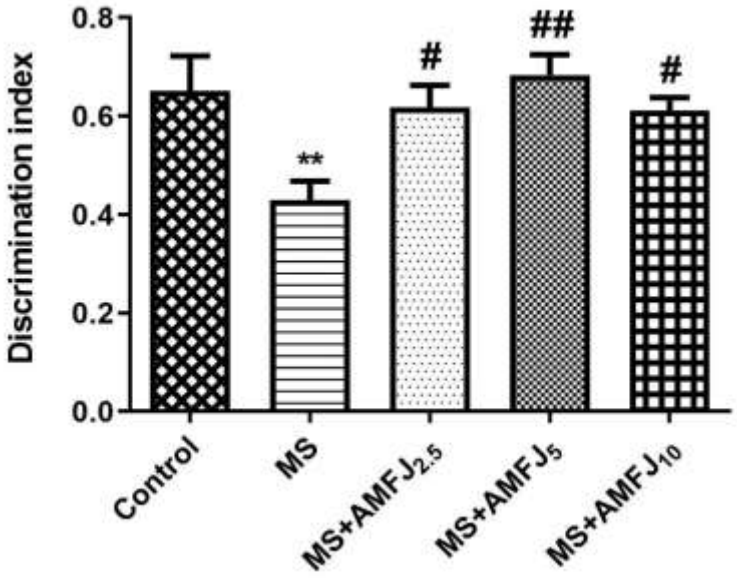

Figure 2. Discrimination index based on the results from the place recognition test. **p<0.01 vs. control, #p<0.05 vs. MS, ##p<0.01 vs. MS

Forced swimming test

There were no significant differences in the immobility times between the experimental groups (**Table 3**).

Table 3*. Immobility time evaluated by the forced swimming test*

Variable	Control	MS	MS + $AMFJ_{2.5}$	MS + $AMFJ_{5}$	MS + $AMFJ_{10}$	One-way ANOVA (p)
Immobility time (sec)	122.1 ± 42.22	152.4 ± 48.30	128.3 ± 27.03	153.2 ± 34.54	123.1 ± 36.83	0.2276

DISCUSSION

An increasing body of evidence has demonstrated a connection between MS, obesity and/or diabetes and neurocognitive performance. Common features of these conditions include insulin resistance, mitochondrial dysfunction and oxidative stress (23).

It has been long believed that the liver, adipose tissue and skeletal muscle are the "classic targets" of insulin and the brain has been considered "insulin-insensitive". Emerging data show that insulin plays a significant role in regulating brain functions (24) as it affects food intake and energy homeostasis, odor sensitivity, neuronal survival and longevitiy, dendritic spine formation (25-27). "Brain insulin resistance" is considered as a modern concept of the pathogenesis of MS and related disorders (26). In insulin-resistant state, dysregulation of insulin signalling impaires the brain energy metabolism and the expression of insulin-responsive genes required for cognitive functions, and increases oxidative stress. Interestingly, peripheral insulin resistance indicates a state of cerebral insulin resistance. In support of this, there are data indicating that synaptic plasticity and cognitive performance negatively correlate with hyperglycemia and peripheral insulin resistance in animal models of obesity or diabetes (23). Especially, such correlation was documented in high fat diet (HFD)-induced obese insulin-resistant rats (28, 29). Clinically these relations have been demonstrated by a longitudinal study in patients with prediabetes, revealing that insulin resistance is the major predictor of memory decline (30).

Mitochondria are the main source of cell energy. Additionaly, they regulate thermogenesis, cell apoptosis, calcium homeostasis and reactive oxygen species (ROS) production and scavenging (31) Mitochondrial dysfunction is considered a common pathophysiological feature of MS (32) and neurocognitive disorders (33). As a consequence of impaired mitochondrial function, oxidative damage may amplifiy neuronal damage and induce neurodegeneration. In experimental models of obesity, diabetes or MS (conditions, characterized by oxidative stress) (31, 34, 35) it has been demonstrated that cognitive performance correlated negatively with the oxidative stress (36, 37).

Anxiety is a normal emotional response to real or potential threat, but when it is extreme and persistent, it is considered pathological. Anxiety disorders are the most common type of mental disorders. There is also a high risk of developing other psychiatric co-morbidities such as major depression (38). The association of MS with depression and anxiety has been a focus of considerable interest. While observational and cross-sectional studies generally confirm the connection of MS with depressive disorders (39), the link with anxiety has been more illusive. Despite the prevailing negative results, a recent meta-analysis reports a significant association between anxiety and MS (40). Interestingly, age-related differences have been found in one study, with anxiety being more common in adults with MS and depressive behaviour prevailing in children. The connections between MS and the anxious state appear to be bidirectional: anxiety increases the level of stress hormone cortisol and cortisol stimulates visceral adipogenesis (41). Additionally, it is supposed that interleukins, secreted by the abdominal adipose tissue, are able to stimulate cortisol release. Thus, interleukin antagonism in obese individuals has been associated with a decrease

in cortisol, systolic blood pressure and heart rate (42). Insulin resistance may be another link between mood dissorders and MS, as Grillo *et al*. found that down-regulation of hypothalamic insulin receptors is associated with a higher risk of depression and anxiety (43-45). In addition, a correlation has been established between lipid peroxidation and features of anxiety and depression in rats with diet-induced MS (6).

In the present study we aimed to test the effects of subchronic AMFJ treatment on the behavior of rats subjected to diet-induced MS.

It has been previously established by our group that the HFHF-diet described here is able to induce symptoms of the MS such as visceral adiposity, glucose intolerance, insulin resistance, elevated triglycerides, oxidative stress in the experimental animals (6). These manifestations of MS have also been reproduced in the rats used in the present experiment (46). On the other hand, the effects of AMFJ on memory and mood disturbances have been tested previously in different experimental settings. The ability of AMFJ to improve learning and memory have been shown in young male rats subjected to subchronic treatment, using the two-way active (16) or passive avoidance test (17). Anxiolytic effects have also been demonstrated by using the SIT and the elevated plus maze test in young, healthy male rats, as well as an antidepressant activity in the FST after a single or prolonged administration of the AMFJ (11, 47). In addition, AMFJ has been found to exert anti-inflammatory and antioxidant effects, both in *in vivo* and *in vitro* conditions (18, 48, 49). Similar results have been reported for *Aronia* extracts and polyphenols by other authors in different experimental or clinical studies (50, 51).

In the present study, the most prominent behavioral alterations in the MS group were the increased anxiety-like state demonstrated in the SIT and the memory impairment observed in the PRT. The Porsolt test failed to demonstrate a significant diet-induced depression-like state despite the increase in the immobility time in the MS group.

At the background of increased anxiety caused by the consumption of HFHF diet, the administration of AMFJ effectively normalized the rat behavior in the lowest concentration used, as evaluated by the SIT. Given the role of oxidative stress (52) and possibly also inflammation (53) in the genesis of anxiety, the protective effect of AMFJ can be potentially related to attenuating of these pathogenic mechanisms. Moreover, it has been previously shown that the HFHF diet causes elevation of lipid peroxidation, which correlates with the anxiety measures in the rats (6).

The antioxidant and anti-inflammatory actions of AMFJ are even more plausible explanation of the results from the spatial memory test, where a significant improvement was reported at all doses used, antagonizing the deleterious effect of the diet. The spatial memory is known to be sensitive to manipulations with calorie-dense diets (54). Therefore, the antiamnestic effect in our study can be related to the antioxidant activity of AMFJ. In this line, *Aronia* juice has been demonstrated to counteract the reduction in the brain paraoxonase activity in rats fed HFHF diets (55). Neuroprotective effects of *Aronia* extract have been demonstrated also on mouse hippocampal cells *in vitro* (56), where the glutamate-induced cell death, ROS production and intracellular calcium levels were suppressed, accompanied by an increased activity of glutathione peroxidase and glutathione reductase. The role of anthocyanins for the cognitive-enhancing effect of *Aronia* fruits has been evaluated by Wen *et al*. (57) in an amyloid-beta (Aβ)-induced memory damage model in rats. It was observed that purified anthocyanin treatment was associated with improved spatial memory in Morris water maze test as well as hippocampal cell protection against Aβ-toxicity (57). Another study aimed to test the effect of *Aronia*-derived anthocyanins on age-related degenerative changes in rat brain induced by D-galactose injection. Anthocyanin supplementation prevented the age-related cognitive decline and improved the antioxidant protection of the neurons as shown by the decreased level of malondialdehyde (MDA) and increased activity of superoxide dismutase and glutathione peroxidase (58).

Given the role that insulin resistance plays in the development of cognitive impairment, it can be regarded as a target through which AMFJ could improve the neurobehavioral alterations induced by the HFHF diet. The effects of *Aronia melanocarpa* on insulin resistance have been studied in preclinical studies. In a HFD-induced and streptozotocin-induced type 2 diabetes in rats, 8-week *Aronia melanocarpa* berry extract supplementation significantly

decreased insulin levels and homeostatic model assessment for insulin resistance (HOMA-IR) score, improved glucose tolerance and increased glycogen content in the liver (59). Kim *et al.* demonstrated *Aronia melanocarpa* methanol extract modulated adipogenesis and improved insulin resistance in HFD -induced obese mice. These effects were accompanied by an improvement in the lipid profile with a decrease in serum triglycerides and low-density lipoproteins (60).

The results from this experiment, showing a prevention of the behavioral disturbances caused by the intake of HFHF diet in rats, are well in accordance with the known beneficial metabolic and behavioral effects of AMFJ.

CONCLUSION

This study showed that the administration of *Aronia melanocarpa* fruit juice to rats for 10 weeks, during the induction of metabolic syndrome, increased the social interaction between unfamiliar partners, indicative of an anxiolytic-like effect. The juice also prevented the diet-induced spatial memory impairment. We speculate that the anxiety-reducing and memory-enhancing effects observed in this study could be related to the antioxidant and insulin-sensitizing actions of *Aronia melanocarpa* fruit juice, dependent on its high polyphenol content.

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