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Original Article

Behavioral Effects of *Aronia Melanocarpa* Fruit Juice in a Rat Model of Ovariectomy-Induced Estrogen Deficit

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Abstract

Introduction: The ovariectomized rat is a model used to mimic the changes in female organism during menopause. *Aronia melanocarpa* fruit juice (AMFJ) is extremely rich in phenolic substances (procyanidins, flavonoids and phenolic acids).

Aim: The present study aimed to evaluate the effects of AMFJ on rat behavior in a model of ovariectomy-induced estrogen deficit.

Materials and methods: Four groups of female Wistar rats were used, each consisting of 14 animals – sham operated (SO), ovariectomized (OVX), OVX+$AMFJ_{5,}$ and OVX+$AMFJ_{10}$. After two-week recovery from the operation, three-month oral treatment was performed with distilled water for the SO and OVX groups, and AMFJ at doses of 5 ml/kg and 10 ml/kg for the OVX+$AMFJ_5$ and OVX+$AMFJ_{10}$ groups, respectively. Then, behavioral tests were conducted. Locomotor activity was assessed using the open field test (OFT). Anxiety was evaluated in the OFT, elevated plus-maze test and social interaction test. Depressive behavior was assessed in the forced swim test. Thermal pain sensitivity was measured in the hot plate test.

Results: OVX rats showed increased anxiety, depressive behavior and pain sensitivity in comparison with SO animals. Compared to OVX rats, anxiety, depressive behavior, and pain sensitivity of AMFJ-treated animals were decreased. Locomotor activity of AMFJ-treated rats was reduced in comparison with both SO and OVX animals, probably due to the sedative effect of the juice.

Conclusions: AMFJ was able to antagonize the negative impact of the estrogen deficit on rat behavior (anxiety, depression, pain sensitivity), probably due to the biological activity of its polyphenolic ingredients.

Keywords

anxiety, *Aronia melanocarpa*, depression, ovariectomized rats, pain

INTRODUCTION

The ovariectomized (OVX) rat is a model used to simulate the clinical findings in menopause.[1] Except for the metabolic consequences of the estrogen deficit, many behavioral changes are observed as well. Some longitudinal studies have shown that the decrease of estradiol (mainly) and the increase of FSH levels in postmenopausal women correlate with an increased risk for development of depressive symptoms.[2] Other authors consider it likely that the depressive state is related to menopausal symptoms rather than to decreased estrogen levels.[3] Anxiety is associated with both

natural and surgical menopause due to low plasma and brain estrogen levels.[4] The data regarding the association between human pain sensitivity and menopause have been inconsistent. In rodents, OVX-induced estrogen deficit is characterized by an increased mechanical and thermal pain sensitivity.[5]

Aronia melanocarpa (Michx) Elliot, named also black chokeberry, is a member of the *Rosaceae* family of plants. Aronia fruits are extremely rich in phenolic substances, especially flavonoids (proanthocyanidins, anthocyanins, quercetin glycosides) and phenolic acids (chlorogenic and neochlorogenic).[6,7] There are data that *Aronia melanocarpa* hot water extracts are able to antagonize the consequences of OVX-induced estrogen deficit on lipid profile and osteogenesis.[8] Previous experiments have demonstrated that *Aronia melanocarpa* fruit juice (AMFJ) exerts anxiolytic-like and antidepressant-like effects in healthy male rats.[9-11] There are no data regarding the effect of *Aronia melanocarpa* (or other berry fruits) on the behavioral consequences of estrogen deficit in OVX rats.

AIM

The aim of the present study was to investigate the effects of AMFJ, applied at two different doses, on locomotor activity, anxiety, depressive behavior and pain sensitivity threshold in OVX rats.

MATERIALS AND METHODS

Aronia melanocarpa fruit juice

AMFJ was produced from *Aronia melanocarpa* Elliot fruits grown in the Balkan Mountains, Bulgaria. The fruits were crushed and squeezed. The juice was filtered, preserved with potassium sorbate (1.0 g/l) and stored at 0°C until the experiment.[7] The contents of phenolic substances in AMFJ and the method of their determination are given in **Table 1**.

Animals and operation

The sexually naïve female Wistar rats we used in the study were four-months old at the beginning of the experiment. They were housed in plastic cages, at a temperature of 20-25°C, under a 12-hour light/dark cycle, and had free access to food and drinking water. They were allocated to four groups consisting of 14 animals each: sham-operated (SO) and three groups of OVX rats: OVX, OVX+AMFJ$_5$, and OVX+AMFJ$_{10}$. On the day of the operation, rats were anesthetized with ketamine (30 mg/kg) and xylazine (30 mg/kg) and fixed. After removal of the abdominal hair and disinfection with iodine, a midline incision was performed. The abdominal cavity was sewed back immediately in the SO rats. Ovaries were isolated in the OVX rats, with clamping of uterine tubes and tying a thread around the oviduct and its blood vessels. The abdominal wall was closed. Antibacterial prophylaxis with cefazolin 200 mg/kg i.p. was performed for all rats. There was a recovery of two weeks after the operation. After that, in the course of three months, the animals were treated orally using an orogastric tube. SO and OVX rats received distilled water (10 ml/kg) while OVX+AMFJ$_5$ and OVX+AMFJ$_{10}$ were treated with AMFJ 5 ml/kg (diluted with distilled water to 10 ml/kg) and 10 ml/kg, respectively.

Animal care and all experiments were in conformity with national laws and policies as well as with the international guidelines (EU Directive, 2010/63/EU for animal experiments).

Three months after the operation, several behavioral tests were performed (on different days): open field test (OFT), elevated plus maze (EPM), social interaction test (SIT), forced swim test (FST), and hot plate test (HPT).

Open field test (OFT)

A wooden arena (100×100 cm) surrounded by walls 40 cm high was used. The floor was divided into 25 equal squares. Each rat was put in the center of the box and its behavior was observed for 5 minutes. The number of squares crossed (horizontal movements) and the number of rearings (verti-

Table 1. *Aronia melanocarpa* fruit juice (AMFJ) ingredients and methods of their determination

Ingredient	Content	Method of determination
Total phenols	5461 GAE/l	Folin-Ciocalteu procedure[12]
Total proanthocyanidins	3122.5 mg/l	Gravimetrically according to the procedure described by Howell et al.[13]
Cyanidin 3-galactoside	143.7 mg/l	HPLC
Cyanidin 3-arabinoside	61.7 mg/l	HPLC
Cyanidin 3-glucoside	4.4 mg/l	HPLC
Cyanidin 3-xyloside	11.6 mg/l	HPLC
Chlorogenic acid	585 mg/l	HPLC
Neochlorogenic acid	830 mg/l	HPLC

GAE: gallic acid equivalent; HPLC: high-performance liquid chromatography

cal movements) were recorded as measures of the locomotor activity. The time spent in the central squares (central time, CT) was registered as an index, inversely related to the level of anxiety.[14]

Elevated plus maze (EPM) test

An X-maze with two open and two closed arms elevated at 50 cm above the floor was used for this test. One rat was put in the center of the maze and in the course of 5 minutes, the following indices were recorded: number of entries into the open (OA) and closed arms (CA) as well as the time spent there. The index of open vs. total arm (TA) entries was calculated. Considering the fact that rodents naturally prefer to stay in the closed arms of the maze, it is postulated that the levels of anxiety are inversely proportional to the number of OA entries and the time spent there.[15]

Social interaction test (SIT)

In this test, two unfamiliar rats with similar weights were released in the opposite corners of the square arena used for the OFT. The index recorded during a 5-minute period was the time spent in social interaction (SI) which included sniffing, following, wrestling, crawling under or over the other rat, but not the passive contact such as lying or sitting over, under or next to the other animal. In this test, the level of anxiety is inversely proportional to the time spent in SI.[16]

Forced swim test (FST)

The FST, known also as Porsolt test, is widely used to assess behavioral despair in rodents. The rat was put inside a water-filled glass cylinder (17 cm in diameter and 50 cm high) for 5 min. The cylinder was filled with water (~30°C) up to 30 cm to ensure that the animal could not touch the bottom of the pool with his hind paws or tail, and in this way, it was forced to swim. The test was conducted in two sessions separated by 24 hours. During the second session, immobility time was recorded as the marker of depressive behavior. After the test, rats were wiped and dried before returning to their home cages.[17]

Hot plate test (HPT)

This test reveals changes in rodent thermal nociception. HPT was performed on a heated (51±1°C) surface surrounded by a glass cylinder (24 cm in diameter) using the apparatus of Ugo Basile S. R. L., Italy. The time latency before shaking or licking the paw, or before jumping was recorded. The animal was removed from the plate after responding or after a cut-off time of 45 seconds to prevent tissue damage. This was repeated three consecutive times at two-hour intervals, the mean value was calculated for each animal and was regarded as an index for pain sensitivity.

Statistical analysis

The results from the experiments were analyzed by one-way ANOVA followed by Dunnett's multiple comparison post hoc test. Data are presented as mean ± SEM and considered significant at a value of $p<0.05$. GraphPad Prism 5.00 statistical software was used to perform the analyses.

RESULTS

Open-field test

The results from the OFT are presented in **Table 2**. One-way ANOVA revealed a decrease in the horizontal movements of AMFJ-treated rats in comparison with both SO rats ($p<0.01$ for the OVX+AMFJ$_5$ group and $p<0.001$ for the OVX+AMFJ$_{10}$ group) and OVX rats ($p<0.01$ for the OVX+AMFJ$_5$ and OVX+AMFJ$_{10}$ groups). Vertical movements of AMFJ-treated animals were significantly decreased in comparison with the SO rats ($p<0.05$ for the OVX+AMFJ$_5$ group and $p<0.01$ for the OVX+AMFJ$_{10}$ group) but were not significantly different compared to OVX rats. There were no significant differences between the groups regarding the time spent in the central squares of the open field.

Elevated plus-maze test

The results from the EPM test are presented in **Table 3**. There was no statistically significant difference between the groups concerning the number of OA entries. Compared to the SO group, the AMFJ-treated groups showed a decreased number of CA entries ($p<0.001$ for both OVX+AMFJ$_5$ and OVX+AMFJ$_{10}$ groups) as well as TA entries ($p<0.01$ for OVX+AMFJ$_5$ group and $p<0.001$ for OVX+AMFJ$_{10}$ group). The CA entries and TA entries of the OVX+AMFJ$_{10}$ rats were significantly lower ($p<0.05$) than these of OVX rats

Table 2. Number of crossings and rearings and time spent in the central squares (CT) in the OFT

Group	Crossings	Rearings	CT (sec)
SO	56.1±10.3	9.5±1.6	2.6±0.6
OVX	40.9±5.7	7.4±0.8	2.9±0.4
OVX+AMFJ$_5$	19.7±4.7$^{\&\&,**}$	5.2±1.5$^{\&}$	3.1±0.9
OVX+AMFJ$_{10}$	20.4±4.1$^{\&\&\&,**}$	4.2±0.8$^{\&\&}$	4.3±1.2

$^{\&}p<0.05$; $^{\&\&}p<0.01$; $^{\&\&\&}p<0.001$ vs. SO; $^{**}p<0.01$ vs. OVX

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Table 3. Number of open arm (OA), closed arm (CA) and total arm (TA) entries, index of open vs. total arm entries, time spent in the open and closed arms (s) in the elevated plus maze test

Group	OA entries	CA entries	TA entries	OA/TA entries	Time in OA (sec)	Time in CA (sec)
SO	2.1±0.5	4.7±0.6	6.8±0.9	0.3±0.1	30.3±6.7	269.7±6.6
OVX	2.1±0.4	3.1±0.7	5.2±1.0	0.4±0.1	35.6±8.4	264.4±8.4
OVX+AMFJ$_5$	1.5±0.4	1.7±0.4$^{\&\&\&}$	3.2±0.6$^{\&\&}$	0.4±0.1	28.2±7.5	271.8±7.5
OVX+AMFJ$_{10}$	1.1±0.3	1.6±0.7$^{\&\&\&,*}$	2.6±0.3$^{\&\&\&,*}$	0.4±0.1	21.6±6.0	278.4±6.0

$^{\&\&}p<0.01$; $^{\&\&\&}p<0.001$ vs. SO; $^{*}p<0.05$ vs. OVX

as well. The time spent in the open and closed arms of the maze, as well as the index of OA/TA entries did not differ significantly between groups.

Social interaction test (SI)

The recorded times for SI were as follows: 30.4±4.6 s (SO group), 12.3±1.9 s (OVX group), 22.2±3.7 s (OVX+AMFJ$_5$ group) and 10.6±2.4 s (OVX+AMFJ$_{10}$ group). As seen in **Fig. 1**, the time spent in SI was significantly decreased ($p<0.001$) in the OVX group in comparison with the SO group. The time for SI of the OVX+AMFJ$_5$ rats was significantly prolonged ($p<0.01$) in comparison with that of OVX group and was not significantly different from that of the SO group. The SI time of OVX+AMFJ$_{10}$ group was significantly lower ($p<0.001$) than that of SO group and was comparable to that of OVX group.

Forced swim test

The IT of OVX rats (85.8±8.4 s) was significantly prolonged ($p<0.01$) compared to that of SO rats (54.7±6.8 s). The IT of OVX+AMFJ$_5$ group (87.3±9.0 s) was similar to that of OVX rats. The IT of OVX+AMFJ$_{10}$ group (59.9±6.9 s) was not significantly different from that of SO group and was significantly lower ($p<0.05$) than that of the OVX group **(Fig. 1)**.

Hot plate test

In the HPT, the time latency was significantly decreased ($p<0.05$) in the OVX group (28.3±1.2 s) compared to SO group (32.6±1.4 s). The time latency was 34.3±1.8 s and 35.5±1.0 s for groups OVX+AMFJ$_5$ and OVX+AMFJ$_{10}$, respectively. This values were not significantly different from the time latency of SO group and were significantly higher ($p<0.001$) than the respective value of OVX group **(Fig. 2)**.

DISCUSSION

The OVX rat model is often used as a standard in order to imitate the changes in the female organism during menopause.[1] The usual duration of this model is three months

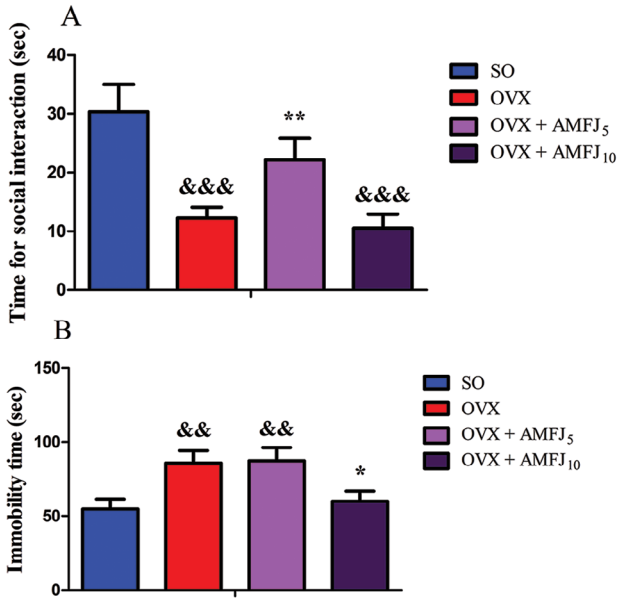

Figure 1. Time spent in social interaction (SI) in the SIT **(A)** and immobility time (IT) in the FST (s) **(B)** SO: sham-operated rats; OVX: ovariectomized rats treated with saline; OVX+AMFJ$_5$: ovariectomized rats treated with AMFJ 5 ml/kg; OVX+AMFJ$_{10}$: ovariectomized rats treated with AMFJ 10 ml/kg; $^{\&\&}p<0.01$ vs. SO; $^{\&\&\&}p<0.001$ vs. SO; $^{*}p<0.05$ vs. OVX; $^{**}p<0.01$ vs. OVX.

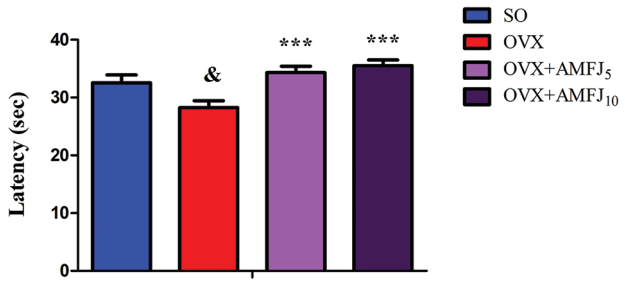

Figure 2. Time latency in HPT (s).
SO: sham-operated rats; OVX: ovariectomized rats treated with saline; OVX+AMFJ$_5$: ovariectomized rats treated with AMFJ 5 ml/kg; OVX+AMFJ$_{10}$: ovariectomized rats treated with AMFJ 10 ml/kg; $^{\&}p<0.05$ vs. SO; $^{***}p<0.001$ vs. OVX.

or 100 days. There are some data concerning the beneficial effects of *Aronia melanocarpa* extracts on bone mineral density and lipid profile in rats with OVX-induced estrogen deficit.[8] Grape powder, which is also rich in anthocyanins and quercetin, was able to improve the anxiety behavior in OVX rats.[18] In this experiment, we aimed to highlight the effects of AMFJ, applied at two different doses, on rat behavior in a model of ovariectomy-induced estrogen deficit.

AMFJ-treated OVX rats showed a decreased locomotor activity which was manifested by the decreased number of crossings and rearings in the OFT as well as decreased TA entries in the EPM. This might be due to a sedative effect of AMFJ. Such an effect was demonstrated by Valcheva-Kuzmanova et al. in healthy animals, treated with AMFJ 10 ml/kg for more than three weeks.[19] The mechanism of this sedative action is probably linked to GABA-receptor activation by the polyphenolic compounds in the juice.[20]

The time spent in the central squares in OFT is used to assess anxiety behavior in rodents. In the OFT, there was a slight but not significant increase of the CT in AMFJ-treated groups. The EPM test is a classical paradigm for evaluation of rodent anxiety. In the EPM test, animals treated with the two doses of AMFJ, showed a significantly decreased number of CA entries and a slight decrease in the OA entries. The decrease in CA entries against the background of unchanged TA entries is an indicator of anxiolytic effect. In this experiment, the TA entries were significantly decreased indicating a decreased locomotor activity probably due to a sedative effect as demonstrated in the OFT as well. However, as the number of TA entries was decreased mainly due to the decreased number of CA entries, we might suppose that AMFJ exerted an anxiolytic effect that was affected by its sedative action.

The effect of AMFJ on anxiety was manifested also in the SIT test. The decreased time for SI of OVX rats demonstrated an increased anxiety which is consistent with other experiments in the OVX estrogen-deficit model.[21,22] AMFJ was able to prevent the OVX-induced anxiety, as rats treated with AMFJ 5 ml/kg showed a SI time that was not significantly different from that of the SO rats and was significantly higher than that of the OVX rats. The higher AMFJ dose of 10 ml/kg did not demonstrate such an effect and the reason for that might be the higher sedative action of this dose resulting in a decreased locomotor activity which interfered with the results in the SIT. A previous study demonstrated that AMFJ administered subchronically to rats caused a dose-dependent decrease in locomotion, probably due a sedative effect.[19] The anxiolytic effect of AMFJ is probably due to $GABA_A$ receptor activation by the phenolic constituents of the juice[20], probably at the benzodiazepine-binding site of the receptor[23].

In the FST, the prolonged IT of OVX rats in comparison with SO animals indicated the development of depression which was demonstrated in other estrogen-deficit-model experiments.[21,22] AMFJ was able to prevent the OVX-induced depression, as rats treated with AMFJ 10 ml/kg showed an IT time that was not significantly different from that of SO rats and was significantly decreased in comparison with that of OVX animals. This is consistent with the experiments performed with healthy male rats by Valcheva-Kuzmanova et al. and Eftimov et al.[9-11] The antidepressant effect of AMFJ in OVX rats may be attributed to the polyphenolic ingredients of the juice such as anthocyanins[24] and chlorogenic acid (unpublished data from our experiments). It is probably due to increased brain concentrations of serotonin and noradrenalin[25] due to MAO-inhibition[26]. The anxiolytic and antidepressant effects of AMFJ might also be linked to the neurotrophic properties of its constituents. Such effects have been demonstrated for chlorogenic acid[27] and quercetin[28].

OVX animals demonstrated thermal hyperalgesia in HPT, which is in agreement with other investigations.[5,29] AMFJ reversed thermal hyperalgesia in OVX rats. Authors report antioxidant and anti-inflammatory actions of AMFJ which probably correlate with the antinociceptive effect.[30,31] The anti-inflammatory actions of the juice might be attributed to its influence on inflammatory and anti-inflammatory cytokines. The capacity of AMFJ to reduce the levels of the inflammatory IL-6 has been demonstrated in other experiments with the juice.[32,33]

The beneficial effects of AMFJ on behavior of OVX rats can be attributed, at least in part, to the phytoestrogenic properties of some anthocyanins (especially cyanidin) and quercetin, both present at high concentrations in *Aronia melanocarpa* fruits.[34,35]

CONCLUSIONS

Aronia melanocarpa fruit juice was able to antagonize the negative impact of the estrogen deficit on rat behavior (depression, anxiety, and increased pain sensitivity). The measured effects are probably due to its polyphenolic ingredients possessing important biological activities, including phytoestrogenic properties of anthocyanins and quercetin, present at high concentrations in the juice.

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Поведенческие эффекты сока плодов аронии черноплодной (*Aronia melanocarpa*) в экспериментальной модели дефицита эстрогена, вызванного овариэктомией, у крыс

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Резюме

Введение: Крыса с овариэктомией представляет собой модель, используемую для имитации изменений в женском организме во время менопаузы. Сок плодов аронии черноплодной (*Aronia melanocarpa*) (СПАЧ – AMFJ) чрезвычайно богат фенольными веществами (процианидины, флавоноиды и фенольные кислоты).

Цель: Настоящее исследование было направлено на оценку влияния AMFJ на поведение крыс в модели дефицита эстрогена, вызванного овариэктомией.

Материалы и методы: Были использованы четыре группы самок крыс Wistar, каждая из которых состояла из 14 животных – ложнооперированных (SO), овариэктомированных (OVX), OVX+ $AMFJ_5$ и OVX+ $AMFJ_{10}$. После двухнедельного восстановления после операции проводилось трёхмесячное пероральное лечение дистиллированной водой для групп SO и OVX и AMFJ в дозах 5 мл/кг и 10 мл/кг для групп OVX+ $AMFJ_5$ и OVX+ $AMFJ_{10}$., соответственно. Затем были проведены поведенческие тесты. Двигательную активность оценивали с помощью теста «открытое поле» (open field test – OFT). Тревожность оценивали в тесте OFT приподнятого крестообразного лабиринта (elevated plus maze – EPM) и тесте социального взаимодействия. Депрессивное поведение оценивали в тесте принудительного плавания. Термическую болевую чувствительность измеряли в тесте с горячей пластиной.

Результаты: Крысы OVX проявляли повышенную тревожность, депрессивное поведение и болевую чувствительность по сравнению с животными SO. По сравнению с крысами OVX тревожность, депрессивное поведение и болевая чувствительность у животных, получавших AMFJ, были снижены. Двигательная активность крыс, получавших AMFJ, была снижена по сравнению с животными SO и OVX, вероятно, из-за седативного действия сока.

Заключение: AMFJ смог противодействовать негативному влиянию дефицита эстрогенов на поведение крыс (беспокойство, депрессия, болевая чувствительность), вероятно, благодаря биологической активности его полифенольных ингредиентов.

Ключевые слова

тревога, *Aronia melanocarpa*, депрессия, овариэктомированные крысы, боль