

Behavioral Effects of *Chaenomeles Maulei* Fruit Juice in Rats with Impaired Circadian Rhythm

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Abstract

Introduction: Impaired circadian rhythm (ICR) is a commonly used model of mild stress. The fruit juice of *Chaenomeles japonica* var. *maulei* (Mast.) Lavall, e (CMFJ) is rich in polyphenols known for their anti-inflammatory, antioxidant, and neuroprotective properties.

Aim: The aim of this study was to investigate the effects of CMFJ on the behavior of rats subjected to ICR.

Materials and methods: Male Wistar rats were divided into five groups of 10 animals each: control group (without ICR), the ICR, ICR+CMFJ_{2.5}, ICR+CMFJ₅, and ICR+CMFJ₁₀ groups. ICR was induced by exposing rats to 14 days of constant light. Over these days, oral treatment was administered with distilled water (the control and ICR groups) and CMFJ at doses 2.5, 5, and 10 ml/kg for the respective groups. Then we performed the open field test, the social interaction test (SIT), and the forced swim test (FST) to assess rats' locomotion, anxiety, and the depressive-like behavior, respectively.

Results: The ICR animals increased their horizontal and vertical locomotion when compared to the controls. The ICR rats did not change significantly the social interaction time in the SIT test and immobility time in the FST. The horizontal and vertical activity of the ICR+CMFJ₁₀ rats was reduced in comparison with ICR animals. Compared to ICR rats, the animals treated with CMFJ at doses of 2.5 and 10 ml/kg demonstrated an improved social interaction and decreased immobility time in the FST.

Conclusions: CMFJ prevented the development of ICR-induced hyperactivity and showed an anxiolytic-like and antidepressant-like effect, probably due to its high polyphenol content.

Keywords

anxiety, *Chaenomeles*, depression, mild stress, polyphenols

INTRODUCTION

The temporal pattern of animal behavior is regulated by the most significant environmental factor – the light. The environmental light controls the neuronal activity of the hypothalamic suprachiasmatic nucleus (SCN), which is involved in the regulation of the circadian rhythms of sleep^[1], the body temperature^[2], locomotion, and feeding.^[3] The circadian rhythms persist with slightly altered period lengths ranging between 20 and 28 hours. Circadian rhythms are

synchronized by the light-dark shifts and the length of the light-dark cycle within a day can affect the normal rhythmic patterns.^[4]

Disruption of the circadian rhythms is a commonly used model of mild stress induced by frequent shifts or altered periods of the light-dark cycle, by long periods of constant light or by forced activity during the normal sleep phase.^[5-7] Exposing rodents to such conditions can disrupt the circadian

an rhythmicity and the normal sleep-wake pattern.^[8] Artificial light for long intervals during the night promotes altered activity schedules, which can lead to conflicting signals to the biological clock.^[9] Altered circadian rhythms lead to anxiety, depressive-like symptoms, anhedonia, and elevated plasma levels of corticosterone.^[10] Constant light exposure is a useful model for studying learning/memory, anxiety-like behavior, and motor behavior of the same animals.

It has been proven that the circadian rhythm is involved in the regulation of the organisms' redox systems. There is a link between the redox state and the membrane excitability of the SCN neurons.^[11] Alterations in the circadian rhythms can influence the generation and scavenging of free radicals.^[12] Antioxidants can slow down the oxidation reactions and stimulate the production of endogenous antioxidants.^[13] Naturally occurring compounds like polyphenols can affect diverse functions in the body, possibly due to their antioxidant properties.^[14] Polyphenols can adjust or regulate also the circadian rhythm or modulate peripheral molecular clocks in rats.^[15] There is evidence that (-)-epigallocatechin-3-gallate can associate with the circadian clock and ameliorate diet-induced metabolic syndrome.^[16]

Chaenomeles japonica var. *maulei* (Mast.) Lavall, e is a polyphenol-rich plant belonging to the *Chaenomeles* genus. The plants from the *Chaenomeles* genus are widely used in the traditional Chinese medicine because of their anti-inflammatory, antioxidant, and neuroprotective properties. There is evidence that these effects may be attributed to the high concentrations of polyphenols such as flavonoids and phenolic acids in the fruits of the *Chaenomeles* species.^[17]

The effects of the *Chaenomeles* plants on the behavior of experimental animals are poorly studied.

AIM

The aim of this study was to investigate the effects of the CMFJ on the behavior of rats subjected to impaired circadian rhythmicity (ICR) due to exposure to constant light.

MATERIALS AND METHODS

Experimental substances

Chaenomeles maulei fruit juice was produced from plants grown in the Balkan Mountains, Bulgaria, in the region of Troyan. The freshly handpicked fruits were grinded, crushed, and squeezed. The juice was filtered, preserved with potassium sorbate (1.0 g/l), and stored at 0°C until experiments. The total content of phenolic compounds in CMFJ proved to be very high - 8900.00 mg gallic acid equivalents per liter of juice. It was determined by spectrophotometric Folin-Ciocalteu assay.^[18] Absorbance was read at 760 nm. Gallic acid was used as a standard. The HPLC analysis confirmed the high content of polyphenols,

especially phenolic acids and flavonoids. The phenolic acids were presented in the highest concentration by vanillic acid (149.1 mg/l), caffeic acid (144.8 mg/l), and chlorogenic acid (110.0 mg/l).^[19] The most abundant flavonoids in the fruit juice were epicatechin (5.59 mg/l), catechin (52.5 mg/l), and (quercetin 35.8 mg/l).^[19]

Animals

We used healthy male Wistar rats with mean weight of 220±30 g, bred in the Animal Centre of Medical University of Varna. The animals were housed in plastic cages in a well-ventilated room maintained at 22±1°C and on a 12/12 light/dark cycle. They received standard rodent pelleted diet and water ad libitum. 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 Bulgarian Food Safety Agency (No. 141/23.06.2016).

Experimental design

In this study, 50 male Wistar rats were divided into five groups, each consisting of 10 animals. The groups were respectively the control, ICR, ICR+CMFJ_{2.5}, ICR+CMFJ₅, and ICR+CMFJ₁₀.

Treatment of the animals was carried out as follows: the animals from the control and ICR groups were treated once daily with distilled water (10 ml/kg) through an orogastric cannula. The animals from the ICR+CMFJ_{2.5} group received once daily 2.5 ml/kg of CMFJ diluted with distilled water to 10 ml/kg. The animals from the ICR+CMFJ₅ group were treated once daily with CMFJ at a dose of 5 ml/kg, diluted to 10 ml/kg with distilled water. The animals from the ICR+CMFJ₁₀ group received CMFJ at a dose of 10 ml/kg once a day.

The administration period was 14 days during which the animals from groups ICR, ICR+CMFJ_{2.5}, ICR+CMFJ₅ and ICR+CMFJ₁₀ were subjected to impaired circadian rhythmicity (ICR) through exposure to continuous light. The control animals had a normal lighting regime throughout the duration of the experiment. Fifteen days after the experiment started, one hour after the oral treatment, the open field test was performed. At 16 days, 1 hour after the oral treatment, the social interaction test was conducted. On the next two days, 1 hour after the oral treatment, a training and experimental session of the forced swim test were carried out.

Open field test (OFT)

The OFT is one of the experimental paradigms initially introduced to estimate the locomotor activity and willingness of animals to explore.^[20] The test allows measuring the behavior of an animal after it is released into an open, novel arena.^[21] The open field was a wooden arena

(100×100×40 cm) painted white. The floor was divided with blue paint into 25 equal-size squares. The duration of the test session for each animal was 5 min. The measure for the horizontal activity was the number of squares crossed with four paws (crossings). The vertical activity was measured by the times the animal stood on its hind limbs (rearings).

Social interaction test (SIT)

The test consists in placing unfamiliar pairs of animals under conditions of bright light and unfamiliar arena, according to the method described by File and Hyde.^[22] The square arena of the open field apparatus (100×100×40 cm) was used. The partner animals were matched by weight (difference of no more than 10 g was allowed). The two rats were gently placed at two opposite corners of the arena. The duration of the test period was 5 minutes during which the following behaviors were recorded: sniffing, nipping, grooming, following, mounting, kicking, jumping on, and crawling over or under the partner (active interaction). Passive interactions like lying or sitting next to each other was not considered a sign of social contact. The prolonged involvement in active interactions indicated reduced anxiety.

Forced swim test (FST)

The forced swim test, also known as the Porsolt test, was carried out in two sessions in two consecutive days.^[23] The training session took place 24 hours after the SIT and the test session took place exactly 24 hours after the training session. Because of its relative simplicity, the FST has become a widely used procedure in the screening of antidepressant drugs. FST measures coping behavior to an inescapable stress and allows the assessment of 'behavioral responses.'^[24] According to the protocol, the animals were tested one by one in a transparent glass cylinder (17 cm in

diameter and 60 cm in height). The cylinder was filled partially with water (22±1°C), so that there was some space left, but not allowing the animals to escape. Each animal was dropped down in water and its activity was documented for 5 min. The immobility time, which was the time during which the animal assumed an immobility posture with only minimal movements necessary to keep his head above the water, during the test session was recorded. The longer duration of the immobility time is related to increased behavioral despair while antidepressant drugs decrease the immobility time.^[25]

Statistical analysis

The results obtained were expressed as mean ± SEM. The data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison post hoc test. A level of $p < 0.05$ was considered significant. All analyses were performed using GraphPad Prism statistical software.

RESULTS

Open field test

The results from the OFT are presented in **Fig. 1**. The animals in the ICR group showed a statistically significant increase ($p < 0.05$) in the locomotion (crossings 63.4±6.8, rearings 22.4±2.3) in comparison with the control group (crossings 39.7±6.25, rearings 12.9±1.9). The number of crossings and rearings of the ICR+CMFJ_{2.5} and ICR+CMFJ₅ animals did not show a significant difference when compared to the controls or the ICR group. The crossings (29.8±5.2) and rearings (14.3± 2.2) of ICR+CMFJ₁₀ animals were significantly decreased ($p < 0.05$) in comparison with the ICR results.

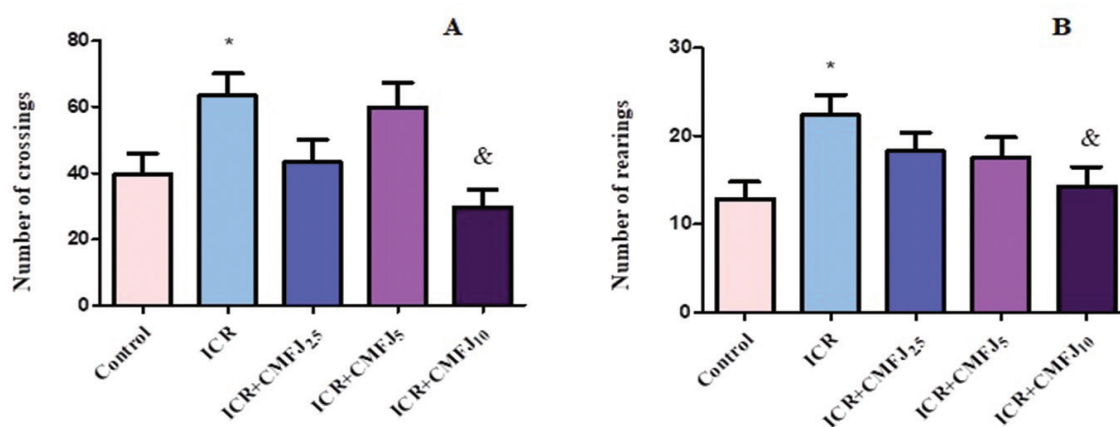


Figure 1. Effect of *Chaenomeles maulei* fruit juice (CMFJ) administration at doses of 2.5, 5, and 10 ml/kg on the number of crossings (panel A) and rearings (panel B) in the open field test in rats subjected to impaired circadian rhythm (ICR); * $p < 0.05$ vs. control; & $p < 0.05$ vs. ICR.

Social interaction test

The social interaction time of ICR rats (31.8 ± 4.0 sec) was slightly decreased compared to that of the control group (36.8 ± 10.3 sec). Administration of CMFJ_{2.5} produced an increase in the time spent in social interaction (52.98 ± 2.14) when compared to the ICR group ($p < 0.01$). The animals from the ICR+CMFJ₁₀ group also showed a significant increase ($p < 0.05$) in the social interaction time (51.6 ± 6.84) in comparison with the ICR animals. The social interaction time of the animals belonging to ICR+CMFJ₅ group was not significantly different from that of the control and ICR rats (Fig. 2).

Forced swim test

The immobility time of the ICR animals (115.6 ± 11.4 sec) was slightly but not significantly increased when compared with the control (105.8 ± 15.1 sec). The administration of CMFJ at doses of 2.5 and 10 ml/kg produced a significant reduction of the immobility time in comparison with the ICR group. The results obtained were 84.9 ± 7.7 sec for the ICR+CMFJ_{2.5} group ($p < 0.01$ vs. ICR) and 73.8 ± 11.7 SEC for the ICR+CMFJ₁₀ group, respectively ($p < 0.05$). The administration of CMFJ at the dose of 5 ml/kg resulted in an immobility time of 100.3 ± 18.9 sec which was not significantly different from that of the control and ICR rats (Fig. 3).

DISCUSSION

Literature data show that the short-term exposure of animals to impaired circadian rhythmicity for up to 2 weeks could significantly increase the levels of corticosterone and could activate the monoamine system.^[26] The elevated levels of monoamines and corticosterone are associated with behav-

ioral stress and motor hyperactivity.^[27] In this experiment, we can explain the increased motor activity of the ICR group in the open field test with the increased levels of monoamines and the activation of the HPA axis. Administration of CMFJ antagonized the increased motor activity of ICR rats.

The social interaction test mimics natural interactions by allowing the free contact between the rats. A decreased interaction between the animals indicates social avoidance, which reflects a stress-induced anxiety.^[28] The chronic mild stress has been known to increase the social avoidance in male rats resulting in a decreased social interaction.^[29] In the present experiment, the disturbed light-dark cycle reduced the time spent in social interactions, although this effect was not statistically significant. The social interaction time was increased by administration of CMFJ at doses of 2.5 and 10 ml/kg. These results demonstrated the anxiolytic-like effect of the treatment. This effect might be due to the polyphenols found in CMFJ exerting an anxiolytic effect probably by decreasing the HPA activity, the oxidative stress levels or by activation of the dopaminergic system in the frontal cortex, as demonstrated for other polyphenols.^[30]

Apart from anxiety, the constant light exposure leads to depressive-like behavior in animals.^[31] In the forced swim test, the immobility time was slightly elevated, though falling short of statistical significance, for the ICR group when compared to the control animals. The administration of CMFJ at doses of 2.5 ml/kg and 10 ml/kg reduced significantly the immobility time of ICR rats. CMFJ at a dose of 2.5 ml/kg had the most pronounced effect on the immobility time when compared to the ICR group. In the open field test, the doses of 2.5 ml/kg and 10 ml/kg were associated with the most evident decrease in the motor activity. So, the decrease of the immobility time could not be attributed to a decreased motor activity. Thus, the combination of results of the both tests suggested that CMFJ at the doses of 2.5 and 10 ml/kg exerted an antidepressant-like effect in the

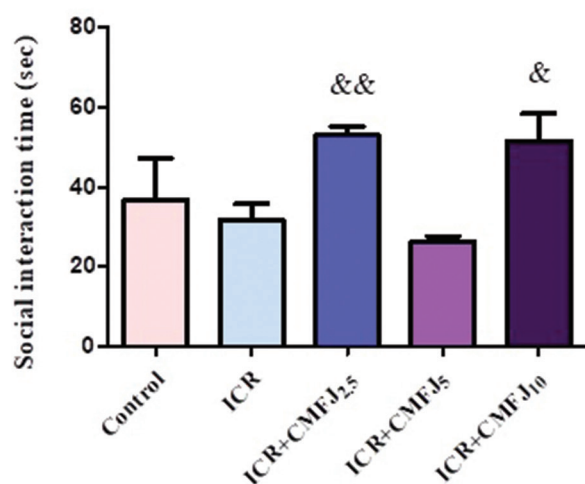


Figure 2. Effect of *Chaenomeles maulei* fruit juice (CMFJ) administration at doses of 2.5, 5, and 10 ml/kg on the social interaction time in rats subjected to impaired circadian rhythm (ICR); & $p < 0.05$ vs. ICR, && $p < 0.01$ vs. ICR.

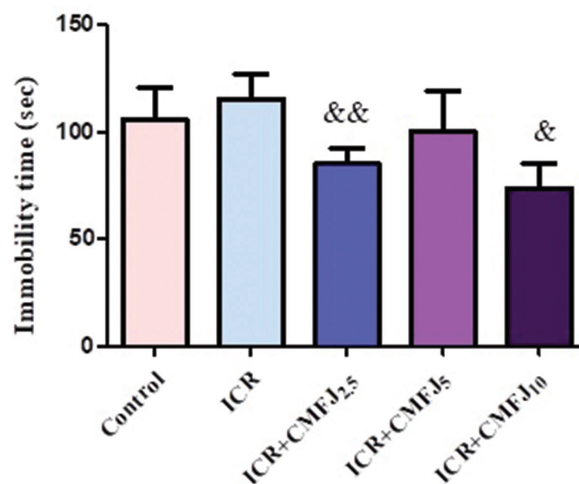


Figure 3. Effect of *Chaenomeles maulei* fruit juice (CMFJ) administration at doses of 2.5, 5, and 10 ml/kg on the immobility time in the forced swim test in rats subjected to impaired circadian rhythm (ICR); & $p < 0.05$ vs. ICR, && $p < 0.01$ vs. ICR.

presence of mild stress.

The full development of depressive-like behavior requires exposure to chronic stress lasting at least 4 weeks. During this time, the typical biochemical and behavioral indicators of depressive behavior can develop in the animals. Since the exposure of animals to impaired 24-hour light rhythm did not produce a depressive-like behavior, we assumed that the 14-day presence of constant light was not enough for the full development of depression. Nevertheless, the administration of CMFJ showed a potential to protect against stress-induced behavioral changes. The reduced immobility time in the forced swim test can be explained by the biological activity of the polyphenols found in CMFJ. For example, the flavonoid quercetin reduced the motor activity and the immobility time in a model of chronic mild stress in rats. The administration of quercetin restored the levels of oxidative stress, the MAO activity, and the levels of serotonin in the CNS. These mechanisms of action might explain the observed effects in the behavioral tests in animals.^[32]

CONCLUSIONS

Administration of *Chaenomeles maulei* fruit juice could ameliorate anxiety-like and depression-like behavior of animals subjected to impaired circadian rhythm. These effects of the juice might be attributed to the biological activity of its polyphenolic ingredients.

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Competing Interests

The authors have declared that no competing interests exist.

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Поведенческие эффекты фруктового сока *Chaenomeles Maulei* у крыс с нарушением циркадного ритма

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Резюме

Введение: Нарушение циркадного ритма (ICR) является широко используемой моделью лёгкого стресса. Сок плодов *Chaenomeles japonica* var. *maulei* (Mast.) Lavalle (CMFJ) богат полифенолами, известными своими противовоспалительными, антиоксидантными и нейропротекторными свойствами.

Цель: Целью данного исследования было изучение влияния CMFJ на поведение крыс, подвергнутых ICR.

Материалы и методы: Самцов крыс линии Вистар разделили на пять групп по 10 животных в каждой: контрольная группа (без ICR), группы ICR, ICR+CMFJ2.5, ICR+CMFJ5 и ICR+CMFJ10. ICR индуцировали, подвергая крыс воздействию постоянного света в течение 14 дней. В эти дни перорально применяли дистиллированную воду (контрольная и ICR -группы) и CMFJ в дозах 2.5, 5 и 10 ml/ kg для соответствующих групп. Затем мы провели тест «открытое поле», тест социального взаимодействия (SIT) и тест принудительного плавания (FST) для оценки локомоции, тревожности и депрессивного поведения крыс соответственно.

Результаты: Животные группы ICR увеличили свою горизонтальную и вертикальную локомоцию по сравнению с контрольной группой. Крысы ICR существенно не изменили время социального взаимодействия в тесте SIT и время неподвижности в тесте FST. Горизонтальная и вертикальная активность крыс ICR+CMFJ10 была снижена по сравнению с животными ICR. По сравнению с крысами ICR у животных, получавших CMFJ в дозах 2.5 и 10 ml/ kg, наблюдалось улучшение социального взаимодействия и уменьшение времени неподвижности в FST.

Заключение: CMFJ предотвращал развитие индуцированной ICR гиперактивности и проявлял анксиолитический и антидепрессантный эффект, вероятно, из-за высокого содержания полифенолов.

Ключевые слова

тревога, *Chaenomeles*, депрессия, лёгкий стресс, полифенолы