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Original Article

The Impact of Crocin and Chronic Isolation Stress on Passive Avoidance Memory and Brain Electrical Activity in Male Rats

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ABSTRACT

Introduction: Crocin and stress affect different aspects of brain functions. Chronic isolation stress is prevalent in today's world. Therefore, this study investigated the impact of crocin and chronic isolation stress on learning, memory, and different brain waves in male rats.

Methods: Forty male Wistar rats were allocated to five groups: control, sham, chronic isolation stress (CIS), two stress groups receiving different doses of crocin (CIS-Cr30 and CIS-Cr60). Both chronic isolation stress (6h/day) and crocin administration were induced for 21 days. The passive avoidance test evaluated initial and step-through latencies (IL and STL, respectively), as well as total dark compartment, and stay time. Also, different brain waves were measured by EEG recording.

Results: The STL declined in the CIS and CIS-Cr30 groups while it significantly increased in only the CIS-Cr60 group. Also, the total dark compartment stay time increased in the CIS group, whereas it decreased by crocin (30 and 60 mg/kg) in the CIS group. The percentages of beta and alpha waves decreased whereas theta waves significantly increased in the CIS group. While the percentage of the beta and alpha waves increased as well as the percentage of the theta and delta waves decreased by crocin at a dose of 60 mg/kg in the CIS group.

Conclusion: Cronic isolation stress was so destructive and it impaired learning, memory as well as alpha, beta, and theta waves in the brain. Only a dose of 60 mg/Kg of crocin reversed memory deficit and affected all brain waves in subjects under chronic isolation stress. Therefore, the doses of 60 and 30 mg/kg of crocin had different effects on electrophysiological and behavioral brain functions under chronic isolation conditions.

Keywords:
Isolation stress
Crocin
Memory
EEG

Introduction

Herbal antioxidants with carotenoids play an important role in healthy humans (Maoka 2020). In this way, herbal plants such as saffron are usually used as a supplement due to their easy access and fewer side effects with respect to chemical medicines (Bandegi et al., 2014). Crocin is one of the most important herbal compounds of Saffron which clinically affects different functions of the central nervous system (Alavizadeh and Hosseinzadeh, 2014; Papandreou et al., 2011).

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Some previous studies have shown that saffron extract improves cognitive functions (Georgiadou et al., 2014; Ghotbeddin et al., 2021; Naghizadeh et al., 2013).

Memory also was defined as one of the important functions of the brain, which aims to classify, encode, store and retrieve a wide variety of subject-related information (Paul et al., 2009). There are different methods such as behavioral and electrophysiological methods for memory measurements. Electroencephalogram (EEG) is one type of electrophysiological monitoring technique that records the electrical activities in the brain cortex. Indeed, it is an efficient modality that helps to analyze the brain and its behaviors based on the respective frequency of the signal. Also, it helps to acquire the brain corresponding to various states (Kumar and Bhuvaneswari, 2012). Therefore, these waves help to study brain activities and the different types of memory such as working, spatial, recognition, and semantic memories (Kirk et al., 2020; Radahmadi et al., 2017). Also, the high-frequency brain waves make the connection between higher mental functions and the formation of memory and perception in different areas of the brain (Rajkishor et al., 2006). In fact, the mammalian brain is equipped with countless sensors to detect stress, as well as mechanisms to respond to stressful signals (Joëls and Baram, 2009; McEwen 2012). Some previous studies reported that stress influences some brain functions (Alfarez et al., 2003; Brunson et al., 2005; Kim and Diamond, 2002; McEwen et al., 2016). For instance, social isolation stress is associated with marked behavioral changes (Fone and Porkess, 2008). Therefore, investigating the effects of chronic isolation stress on some aspects of brain electrophysiological and behavioral functions is important.

Additionally, crocin probably improves chronic stress-induced learning and memory impairments through its antioxidant effect (Ghadrdoost et al., 2011; Hosseinzadeh et al., 2012) and exhibits radical scavenging effects (Hosseinzadeh et al., 2009; Khosravan, 2002). Also, crocin can improve ethanol-induced deficits of learning behavior in a dose-dependent manner (Soeda et al., 2007). Today, due to the widespread prevalence of reduced social interactions, the consequences of this type of chronic social stress may be nearly compensated by the use of herbal medicines. Hence, this study was designed to investigate the impact of crocin and chronic isolation stress on passive avoidance memory and brain electrical activity by EEG recordings in male rats.

Material and methods

Animals

Forty male Wistar rats (initial weight 250–300 g) were obtained from the Pasteur Institute of Tehran, Iran. The rats were housed under controlled humidity (50±5%) and light conditions (12h light/dark; lights on 07:00–19:00), in cages of similar sizes among all groups. The room temperature was set to 23±2°C and water was made available *ad libitum*. All behavioral experiments were performed from 14:00-16:00 .The Ethics Committee of Animal Use at the Isfahan University of Medical Sciences approved the study (IR.MUI.MED.REC.1398.036) and all experiments were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 2011). In the present study, the experimental period was 21 days to investigate the effect of chronic isolation stress and the protective roles of crocin on memory and electrical activity. Therefore, after 2 weeks of adaptation, the animals were divided equally into five groups (n=8): control (Co; rats were maintained in the cage with no special treatment for 21 days); sham (Sh; rats received saline as drug vehicle daily for 21 days); chronic isolation stress (CIS; rats maintained individual housing for 6h/day for 21days) and two chronic isolation stress- crocin groups receiving crocin (CIS-Cr30 and CIS-Cr60: the rats were under isolation stress (6 h/day for 21 days) and received crocin at doses of 30 mg/kg/day and 60 mg/kg/day respectively (Figure 1).

Experimental procedures

Drugs

Crocin (Sigma Aldrich Co., USA) was purchased in powder form and dissolved in saline. Crocin is injected intraperitoneally (i.p.) at doses of 30 and 60 mg/kg/day for 21 consecutive days. The doses of 30 and 60 mg/kg/day of crocin were indicated as the most common usable dose (Hosseinzadeh et al., 2010). Also, based on previous studies, these doses are the least effective injectable doses which show no biochemical, hematologic, or histopathological toxic effects in rodents' studies (Hosseinzadeh et al., 2010; Kianbakht and Hashem Dabaghian, 2015). It should be noted that there is a possibility of crocin toxicity at higher doses prescribed for

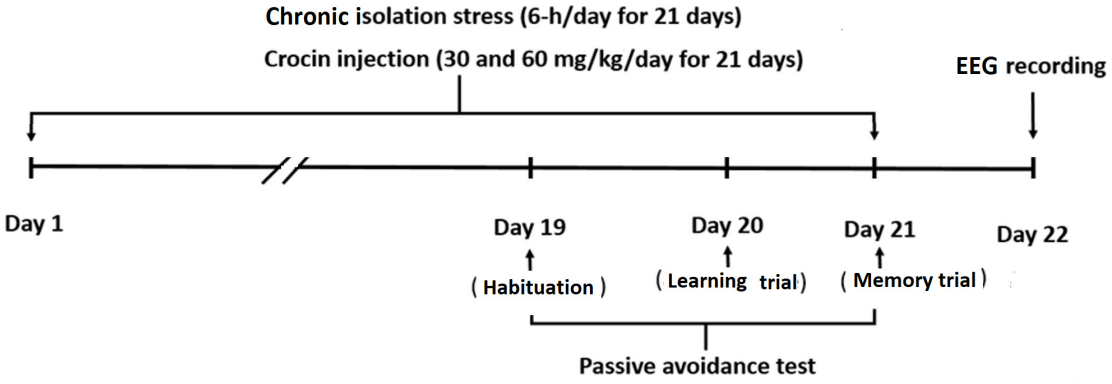

FIGURE 1. The study experimental design

a long time (21 days) (Khani et al., 2018). The sham group received only equal volumes of vehicle (Saline) (Khani et al., 2018)

Induction of chronic isolation stress

Isolation stress was induced 6h/day (from 8:00 to 14:00) for 21 consecutive days in the stressed groups (Khani et al., 2018). Therefore, each rat had individual housing (separated from their groupmates) before they were placed back in their communal home cage. Hence, they were deprived of social contact for 6h/day.

Behavioral Paradigms

In this study, learning and memory were measured via the shuttle box by the passive avoidance test. The protocol of the passive avoidance test was similar to a previous study (Radahmadi et al., 2015). The shuttle box apparatus had identical dark and light compartments with sliding guillotine gate and grid floors. This test was conducted with three phases (300 s) containing habituation (no electrical foot shock used on day 19), learning trial (electrical foot shocks used on day 20), and memory trial (no electrical foot shock given on day 21). An electrical shock was delivered to the animal's foot through the grid floor (0.5 mA, 50 v, and 2 s; once) in a learning trial. The initial latency (IL) of entry to the dark compartment was recorded before inducing the electrical foot shock. Also, the latency time of entry to the dark compartment (up to a maximum delay of 300 s) was measured after 1 day as the step-through latency (STL). The difference between the IL and STL was interpreted as the occurrence of learning in the animal experimental research. In addition, the total dark stay (DS) time was assigned as another variable of memory (Dastgerdi et al., 2018). The longer duration in the light compartment or absence of entry to the dark compartment indicated a positive response.

Surgical procedures for EEG recording

In the present study, an EEG recording was performed as an electrophysiological study. On day 22 of the experiment, rats were anesthetized with 1.5 g/kg urethane (Sigma-Aldrich Co. USA; i.p.) dissolved in sterile normal saline (Hosseini et al., 2017). Then, their heads were fixed in a stereotaxic apparatus (Stoelting Co., USA). The skull was exposed to assess the brain's electrical activity by EEG recording. Therefore, two extra small holes with a diameter of 0.5 mm were drilled 2 mm anterior to bregma and 1.5 mm lateral to the midline for the EEG recording. Since the EEG recording is an extracellular recording. It needs two electrodes (reference electrode and recording electrode) to establish an electrical current between them. Also, the frontal lobe was concerned with memory, controlling behavior, stress, and emotion (Kumar and Bhuvaneswari, 2012). Subsequently, the electrodes were placed sub-cranially at the level of the cortex for EEG recording. The electrodes were Teflon-coated stainless steel, with a diameter of 0.125 mm (Advent Co., UK). The brain- computer interface was connected to the rat and the device. In the electrophysiological study, to record signals properly, a rat was placed on a suitable pad (body temperature kept at 36.5±0.5 °C) and covered with a thin sheet during the experiment.

EEG recording

The EEG recording started as brain waves stabilized. The EEG waves were recorded for 20 min in the anes-

thetized rats. The waves were amplified (×1000) and bandpass filters (0.5–30 Hz) were applied (Radahmadi et al., 2017). These signals were monitored and collected by eProbe software and then were passed through an analog-to-digital interface (Data acquisition, Science Beam Co., MODEL D3111; Iran, Tehran) and transmitted to a computer. The obtained data were analyzed using the eTrace analysis software. The system processed the data to show the power of alpha, beta, delta, and theta waves. The waves with (1–4 Hz) counted as a delta, (4–8 Hz) were a theta wave, (8–13 Hz) were recorded as an alpha wave, and (13–30 Hz) were a beta wave. The total power of these four frequency bands was taken as the full percentage of 100%. Finally, the percentage for the ratio of each frequency band (quantity of alpha, beta, theta, and delta) to the total power was calculated for all examined groups (Miki Stein et al., 2017; Radahmadi et al., 2017; Rahimi et al., 2019). Since the alteration of memory and cognitive processing will also be revealed by changes in EEG waves (Ndaro and Wang 2018), the EEG recording was performed to assess brain electrical activity (Kafa et al., 2010).

Statistical analysis

All data of the behavioral and EEG waves were analyzed by One-way ANOVA followed by Tukey's post hoc test for multiple groups (between groups). Comparisons of IL and STL (within groups) were analyzed using the paired sample t-test. All the data were reported as means±standard error of the mean. P-values less than 0.05 were declared statistically significant. Finally, the calculations were performed using SPSS 24 (SPSS Inc. Chicago, IL, USA).

Results

None of the data showed significant differences between the Co and the Sh groups, indicating that the injection had no significant effects on these parameters. Therefore, all comparisons were performed with the control group.

Behavioral results

In the passive avoidance test, the ANOVA assigned different significant levels in IL, $F(4, 35) = 1.879$, $P=0.153$; in STL, $F(4, 35) = 15.366$, $P=0.000$; and in DS, $F(4, 35) = 9.746$, $P=0.000$.

As shown in Figure2 , no significant differences were observed in the IL values across the groups (Figure 2A). In the CIS and CIS-Cr30 groups, the STL values were significantly ($P<0.001$ and $P<0.01$, respectively) lower than the Co group, indicating the CIS and CIS-Cr30 severely impaired memory (Figure 2B). The STL had a significant ($P<0.001$) enhancement in the CIS-Cr60 group compared to the CIS group, whereas it was not

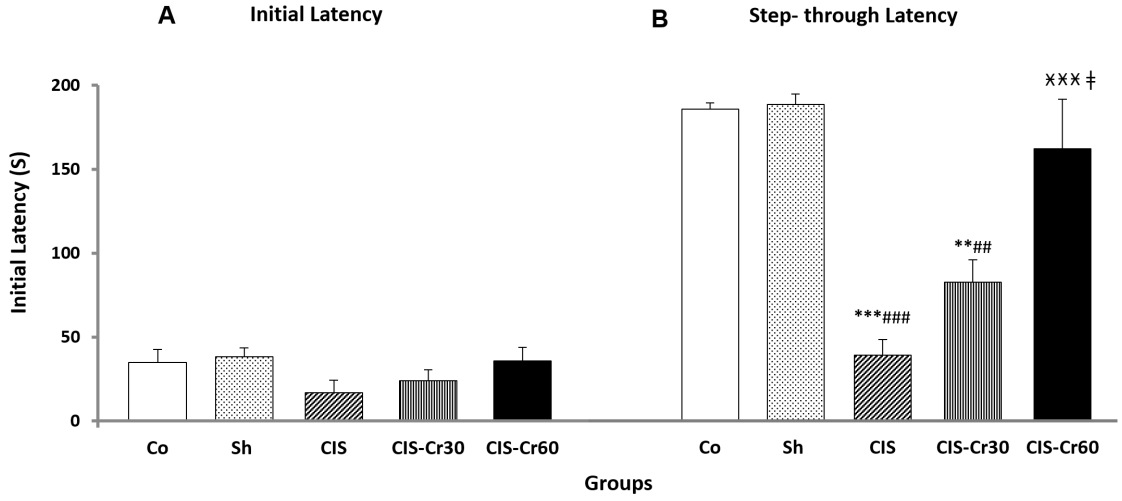

FIGURE 2. Initial and step-through latencies to enter the dark room of the passive avoidance apparatus for all groups before and after receiving foot shock (n =8). Results are expressed as means±SEM (One-way of ANOVA followed by Tukey's post hoc test). Crocin and CIS did not have significant effects on initial latency, whereas, CIS and CIS-Cr-30 impaired memory. While crocin at a dose of 60 mg/kg improved CIS-induced memory deficit. $^{***}P<0.001$ and $^{**}P<0.01$ compared to the Co group, $^{###}P<0.001$ and $^{##}P<0.01$ compared to the Sh group, $^{xxx}P<0.01$ compared to the CIS group, $^{ǂ}P<0.05$ compared to the CIS-Cr30 group.
Co: Control group, Sh: Sham group, CIS: Chronic isolation stress group, CIS-Cr30: Chronic isolation stress-Crocin 30 group, CIS-Cr60: Chronic isolation stress-Crocin 60 group.

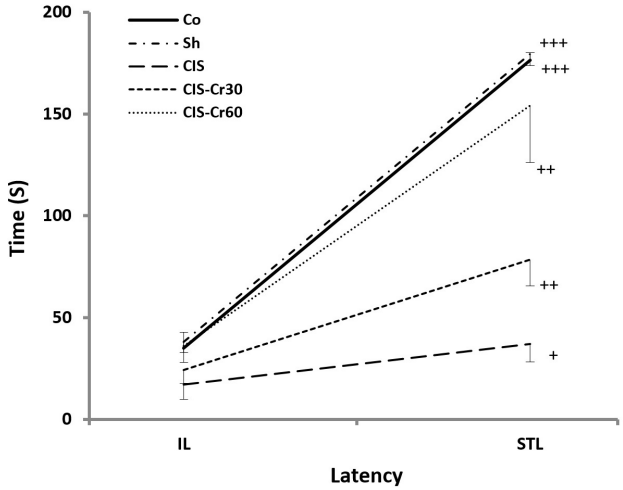

FIGURE 3. Initial latency (IL) and step-through latency (STL) after 1 day to enter the dark room of the passive avoidance apparatus before and after the foot shock (within groups) (n=8).). The difference between these delays was higher in the CIS-Cr60 group than CIS-Cr30 group. Also, it decreased in the CIS group. Results are expressed as means±SEM (paired sample t-test). +P<0.05, ++P<0.01, +++P<0.001 initial latency relative to the step-through latency.
Co: Control group, Sh: Sham group, CIS: Chronic isolation stress group, CIS-Cr30: Chronic isolation stress-Crocin 30 group, CIS-Cr60: Chronic isolation stress-Crocin 60 group.

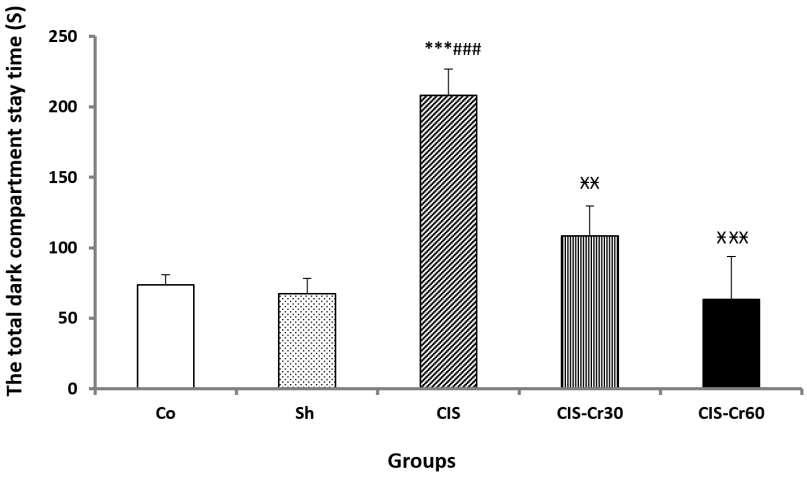

FIGURE 4. Total stay time in dark room of the passive avoidance apparatus for all groups 1 day after receiving the foot shock (n = 8). Results are expressed as means±SEM (One-way of ANOVA followed by Tukey's post hoc test). CIS significantly increased total dark compartment stay time, while both doses of crocin decreased it. $^{***}P<0.001$ compared to the control group, $^{\#\#\#}P<0.001$ compared to the sham group, $^{xx}P<0.01$ and $^{xxx}P<0.001$ compared to the CIS group.
Co: Control group, Sh: Sham group, CIS: Chronic isolation stress group, CIS-Cr30: Chronic isolation stress-Crocin 30 group, CIS-Cr60: Chronic isolation stress-Crocin 60 group.

significant in the CIS-Cr30 group (Figure 2B). It is indicated that daily administration of crocin at a dose of 60 mg/kg had more beneficial effects than 30 mg/kg on memory improvement in the chronic isolation stress condition.

The results of initial and step-through latencies (IL and STL respectively) were analyzed using the paired sample t-test within the groups. As shown in Figure 3, significant differences were detected between IL and STL in all experimental groups (in Co group, t(7)=-15.468 *P*=0.000; in Sh group, t(7)= -8.911 *P*=0.000; in CIS group, t(7)= -2.606 *P*=0.35; in the CIS-Cr30, t(7)= -5.427 *P*=0.01; and in CIS-Cr60 groups t(7)= -3.886 *P*=0.06). Therefore, learning happened in all experimental groups.

As shown in Figure 4, a significant (*P*<0.001) enhancement was observed in total dark compartment stay time (DS) in the CIS group in comparison with the Co group. Also, the DS significantly decreased in the CIS-

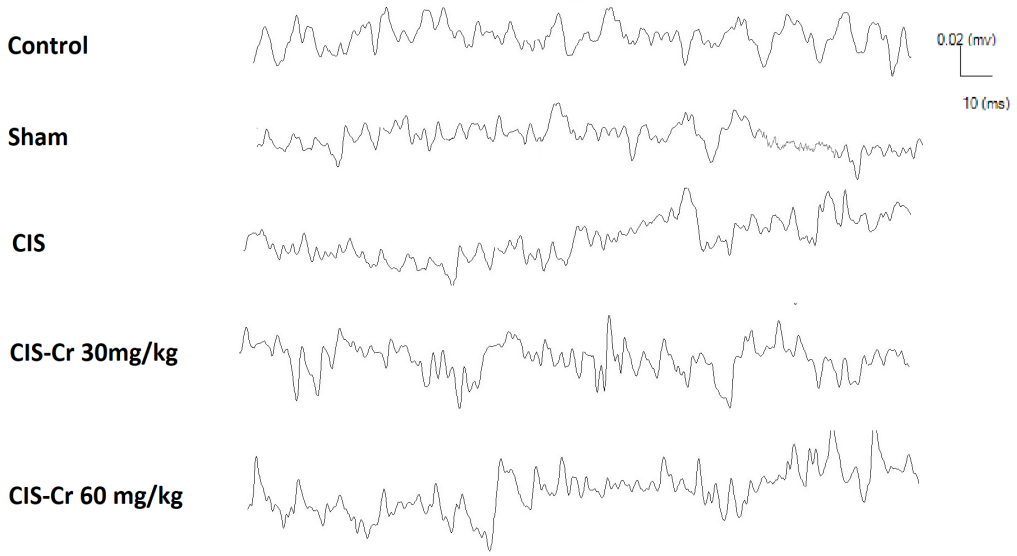

FIGURE 5. Representative EEG trace for all experimental groups.

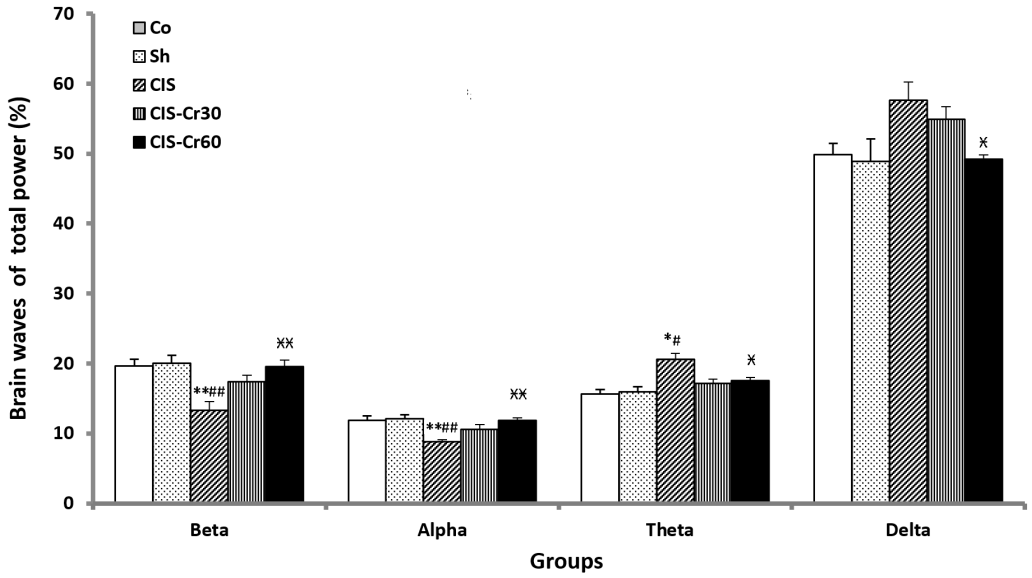

FIGURE 6. Comparison of percentages of different waves (Beta, Alpha, Theta and Delta) of total power (%) in all groups' waves of total power (%). Data represent means±SEM (One-way of ANOVA followed by Tukey's post hoc test). The percentages of beta and alpha waves of total power were decreased in the CIS group. Whereas, the theta wave of total power increased in this group. There were significant increases in the percentage of beta and alpha waves in the CIS-Cr60 group in comparison with the CIS group. While the theta and delta waves of total power had significant decreases in the CIS-Cr60 group compared to the CIS group. $^{**}P<0.01$ and $^{*}P<0.05$ compared to the control group; $^{\#\#}P<0.01$ compared to Sham group; $^{x}P<0.05$ and $^{xx}P<0.01$ compared to CIS group.
Co: Control group, Sh: Sham group, CIS: Chronic isolation stress group, CIS-Cr30: Chronic isolation stress-Crocin 30 group, CIS-Cr60: Chronic isolation stress-Crocin 60 group.

Cr30 and CIS-Cr60 groups compared to the CIS group ($P<0.01$ and $P<0.001$; respectively).

EEG results

The EEG traces were represented for all experimental groups (Figure 5).

In the EEG recording, the ANOVA assigned different significant levels in beta waves, $F(4, 35)=7.459$, $P=0.000$; in alpha waves, $F(4, 35)=5.983$, $P=0.001$; in theta waves $F(4, 35)=8.711$, $P=0.000$; and in delta waves $F(4, 35)=4.009$, $P=0.009$. Also, Figure 6. indicated the responses of the different EEG brain waves in the cortex of experimental groups.

The percentage of beta and alpha waves of total power

had significant (in both $P<0.01$) decreases in the CIS group compared with the Co group. Whereas, the theta wave of total power was significantly ($P<0.05$) higher than the Co group (Figure 6).

As shown in figure 6, there were significant increases in the percentage of beta and alpha waves (in both $P<0.01$) in the CIS-Cr60 group in comparison with the CIS group. While the theta and delta waves of total power had significant (in both $P<0.05$) decreases in the CIS-Cr60 group compared to the CIS group.

Discussion

This study investigated the impact of crocin and chronic isolation stress on learning, memory, and different brain waves in male rats.

The main findings of the present study showed that chronic isolation stress greatly impairs passive avoidance learning and memory. Some previous studies reported chronic stress impairs brain function in both humans and experimental animals (McEwen and Gianaros, 2011; Schwabe et al., 2012) in different behavior tasks such as radial-arm water maze (McLaughlin et al., 2007) and Y-maze (Kim and Diamond, 2002). More specifically, in a previous study, it was observed that chronic stress has no effect on memory which was evaluated by the radial arm maze (Alavizadeh and Hosseinzadeh, 2014). These conflicting recommendations could be related to duration and type of stress, age, nature of the subject, and type of used memory task for experiences (Ranjbar et al., 2016).

Based on the current electrophysiological results, it was found that chronic isolation stress decreased alpha and beta waves, whereas it increased theta waves. However, the delta waves did not show differences in the EEG recordings. Knyazev et al. (2006), also reported the enhancement of alpha waves in anxious individuals (Knyazev et al., 2006). A human study indicated beta and alpha waves of EEG recordings changed during mild and moderate stress when exposed to examination stress (Jena, 2015). Another study offered that noise stress did not induce any marked differences in the EEG recorded of the prefrontal region (Loganathan and Rathinasamy, 2016). In addition, during stress and strong exciting emotions, brain electrical activity had a desynchronized pattern (Aftanas et al., 2004). However, the reduction of the beta and alpha waves may indicate decreased attention, lower cognitive performance, a stressful and non-relaxed condition (Murao et al., 2013). In contrast, Mrdalj et al. (2013) reported no clear EEG activities during stress (Mrdalj et al., 2013). It is noticeable that brain waves confirmed the results of passive avoidance in chronic isolation stress in the present study. Different mechanisms were proposed for brain waves in stress conditions. For example, some reports indicated that the alterations of acetylcholine and serotonin secretion in the neocortex increased cortical activity (Dringenberg et al., 2002; Lagopoulos et al., 2009). Unfortunately, acetylcholine and serotonin secretion was not measured in the present study. The synchronized firing of brain neurons is another mechanism involved in the high power of EEG waves at specific frequencies (Brunson et al., 2005). The EEG (as a biological marker with different representations in the power of its waves) can be a moderator in the emotional processes of life (Azimi et al., 2012). Moreover, it seems that this difference may be due to the different methods used in EEG analysis (Mrdalj et al., 2013; Radahmadi et al., 2017).

According to other present findings, only the dose of 60 mg/Kg of crocin improved memory and brain electrical activity in EEG recording. Therefore, it is suggested that crocin has potential protective applications against the detrimental effects of chronic isolation stress on some brain functions. According to our previous study, the results of two behavioral tests (the object location and novel object recognition tests) that were used to evaluate spatial and cognitive memories, showed that the dose of 60 mg/Kg of crocin has superior effects in reversing memory impairment due to chronic isolation stress (Khani et al., 2018). Other studies also reported that crocin protects brain functions by behavioral assessments such as the passive avoidance and morris water maze tests (Dastgerdi et al., 2018; Hosseinzadeh et al., 2012). In addition, it was reported that the saffron extract improved memory impairments and stress (Abe and Saito, 2000; Dastgerdi et al., 2018; Hosseinzadeh and Ziaei, 2006; Khani et al., 2018 ; Roustazade et al., 2021; Sugiura et al., 1994).

In the current study, the dose of 30 mg/Kg of crocin somewhat ameliorated the destructive effects of chronic isolation stress only on dark compartment stay time as another variable of memory assessment. A study reported that low doses of crocin have no effect on animal behavior in the object recognition test (Radahmadi et al., 2020). In contrast, the results of some studies have

shown that low doses of crocin improve ethanol and scopolamine-induced memory impairment (Ghadami and Pourmotabbed, 2009; Ghadrdoost et al., 2011; Pitsikas et al., 2007). Also, the dose of 30 mg/Kg of crocin was more useful for the improvement of brain function in chronic restraint stress (as one of the models of emotional stress) (Dastgerdi et al., 2017). Therefore, it seems that the brain response can be related to both doses of crocin and stress type. However, the mechanisms of action underlying the role of crocin on brain functions such as memory are completely unclear. Different mechanisms may be involved in the positive effect of crocin on cognitive functions containing anti-oxidant effect, anti-inflammatory properties (Bathaie and Mousavi, 2010; Papandreou et al., 2011), changes in some neurotransmitters (Ettehadi et al., 2013), increases of CREB and BDNF levels in the brain (Behravanfar et al., 2017), inhibiting of the synaptic transmission in the glutamatergic system (Abe and Saito, 2000; Georgiadou et al., 2014), glutamate NMDA receptors (Dharmshaktu et al., 2012), cholinergic system (Ghadami and Pourmotabbed, 2009; Pitsikas and Sakellaridis, 2006) and corticosterone hormone (Khani et al., 2018). Also, it was reported that crocin could reduce some pro-inflammatory mediators (e,g., TNF-α, iNOS, COX-2, and IL-1β) in a dose-dependent manner (Lv et al., 2016), increasing the latency to start the first spike-wave as well as reducing of both frequency and amplitude of spike-waves in the brain (Tamaddonfard et al., 2012).

According to the current EEG results, only the dose of 60 mg/Kg of crocin increased percentages of alpha and beta waves as well as decreased theta and delta waves in chronic isolation stress conditions. Therefore, the dose of 30 mg/kg of crocin did not improve CIS-induced memory deficit and all brain waves. It seems that chronic isolation stress was so destructive that crocin at a lower dose did not improve both behavioral and electrical brain activity. Therefore, it seems that the brain response to crocin depends on not only the dose of crocin but also the type of stress. In addition, behavior is the result of functional connectivity between different brain regions, therefore perhaps the lower dose of crocin may have been able to make only serum biochemical changes such as corticosterone (Khani et al., 2018), but was not able to improve the electrical brain activity with this usage duration. It was proposed that crocin has different functions in a dose-dependent manner (Ahmadi et al., 2017; Khalili and Hamzeh, 2010; Roustazade et al., 2021). Since all brain waves changed only with the dose of 60 mg/Kg of crocin, so it can be concluded that crocin affects electrical brain activity in a dose-dependent manner. It was reported that a high dose of crocin improved consciousness and information processing in a stress condition (Schacter, 1977). In contrast to the present findings, the dose of 80 mg/kg of crocin did not affect the EEG power (Masaki et al., 2012). Also, the chronic isolation stress did not significantly affect delta waves, whereas crocin decreased delta waves in the chronic isolation stress animals. It seems that this result was due to the role of crocin alone. However, similar to our finding, a previous study reported that the isolated animals did not show an increase in brain delta power (Ramesh and Gozal, 2009).

It should be considered that not much is known regarding the neuroprotective mechanism of crocin. Also, no research has been conducted to examine the effect of crocin in various rhythmical EEG activities. As above mentioned, it seems that the various mechanisms are complicated as various EEG waves produce changes in the secretion of neurotransmitters and hormones (Heim and Nemeroff, 2002; Seo et al., 2010). Also, the previous study with the same experimental protocol reported that change in glucocorticoid production is possibly the reason for the different effect of the higher dose of crocin (60 mg/kg) compared to the lower one (30 mg/kg) under chronic stress conditions (Khani et al., 2018).

It was concluded that different doses of saffron and its components had different effects on different brain functions under stressed and non-stressed conditions (Roustazade et al., 2022). However, further studies on other molecular, cellular, structural, and biochemical mechanisms are needed to assess the effects of different doses of crocin on improving brain dysfunctions in chronic stress conditions.

Conclusion

It was concluded that chronic isolation stress was so destructive that it impaired learning, memory as well as alpha, beta, and theta waves in the brain. Also, only a dose of 60 mg/Kg of crocin reversed memory deficit and affected all brain waves in subjects under chronic isolation stress. Therefore, the doses of 60 and 30 mg/kg of crocin had different effects on electrophysiological and behavioral brain functions under chronic isolation

conditions.

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Conflict of interests

The authors declare that they have no conflict of interest.

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