Kermanshah University of Medical Sciences

**Journal of Kermanshah University of Medical Science**

**Journal homepage: Htpp://journals.kums.ac.ir/ojs/index.php/jkums**

# The therapeutic effect of crocin on ketamine-induced retrograde amnesia in rats

**Original Article**

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## Article Info

**Keywords:** Ketamine, crocin, retrograde amnesia, shuttle box

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**Received:** 25 April, 2016
**Accepted:** 30 August, 2016

**J Kermanshah Univ Med Sci. 2016; 20(2): 68-73**

## Abstract

**Introduction:** The glutamatergic system plays an important role in learning and memory. Administration of crocus sativus (Saffron) or its constituent, crocin, facilitates the formation of memory. This research investigated the effect of crocin on antagonizing retrograde amnesia induced by ketamine, a glutamatergic receptor antagonist, in rats by shuttle box.

**Methods:** Male Wistar rats were tested to measure their learning behavior in the passive avoidance task. All animals were trained by a 1 mA shock. The drugs were injected immediately after the training was successfully performed. The animals were tested 24h after training to measure Step Through Latency (STL).

**Results:** On the test day, administration of ketamine (12 mg/kg, ip) impaired the memory after training. Different doses of crocin (2, 5 or 10 mg/kg, ip) were injected 30 min after ketamine, but only 2 mg/kg crocin could improve retrograde amnesia and 5 and 10 mg/kg doses did not have any significant effect on retrograde amnesia. Moreover, administration of crocin (2, 5 or 10 mg/kg, ip) after training had no significant impact on passive avoidance memory by itself.

**Conclusion:** Considering the therapeutic effect of post-training administration of crocin on ketamine-induced retrograde amnesia, it can be argued that crocin has an interaction with glutamatergic system in formation of passive avoidance memory in rats.

## Introduction

*N*-Methyl-D-aspartate (NMDA) receptors are a major class of glutamate receptors in the brain of mammals that are involved in the formation of learning and memory (1, 2). Inactivation of NMDA receptors in animals has been followed by cognitive disorder, which is derived in the memory of rodents and primates other than human through administration of NMDA antagonists (3, 4). Previous studies have shown that reduction of NMDA glutamate and ***alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid*** (AMPA) receptors along with the decreased activity of glutamate neurotransmitters occur in various areas of the brain of the patients with Alzheimer than in normal people (5).

Further, a study showed that long-term consumption of Phencyclidine (PCP), an antagonist of NMDA receptors, impaired the working memory of the consumers (6). Ketamine is a derivative of phencyclidine and an uncompetitive antagonist of NMDA receptors (4) and is used in human and animal medicine for inducing and maintaining general anesthesia in combination with a sedative (7). According to the animal studies conducted, administration of doses less than the dose of anesthetic ketamine impairs learning and memory in passive avoidance task (8). Another study indicated that administration of ketamine impaired the acquisition, learning and memory in Morris water maze (9).

Crocus sativus L. is planted in various parts of the world, including Iran, China, Spain, Italy and Greece. A chemical analysis of the pistils of crocus sativus has shown the presence of water-soluble carotenoids (crocin and crocetin), a little monoterpene aldehyde and glucoside (safranal and picrocrocin), and flavonoids (quercetin and ferrule) (10, 11). In traditional medicine, the pistil of crocus sativus has been used as an antispasmodic, a digestive substance, a nerve sedative, a carminative, an expectorant, an appetizer, a libido booster and menstruation inducer. It has also been proposed that the extract of crocus sativus has antitumor properties and its components are able to absorb the free radicals and reduce blood fat (12). The literature has shown that alcoholic extract of crocus sativus affects learning and memory (13). Moreover, the whole extract

of crocus has been indicated to neutralize the memory recovery failure and to improve the passive avoidance memory dysfunction due to scopolamine (a muscarinic cholinergic receptor antagonist) in rats (14).

Pitsikas et al. reported that the whole extract of crocus sativus improved the destructive effect of scopolamine on memory. They asserted that administration of crocus sativus extract had no impact on the spatial learning of the rats by itself but ameliorated their impaired spatial memory caused by scopolamine in the radial water maze (15). Another study evaluated the effect of crocin extracted from crocus sativus on prevention of learning impairment and spatial memory by scopolamine using Morris water maze. The results of this study showed that administration of scopolamine impaired the learning process and spatial memory in the animals but injection of crocin inhibited the effect of scopolamine on retrograde amnesia. Also, this study indicated that crocin had no impact on the learning process and spatial memory by itself (18). Abe et al. demonstrated that oral administration of saffron extract had no effect on the learning of the mice but could prevent the impaired memory due to ethanol consumption in the mice receiving ethanol. In addition, this extract could prevent the inhibition of long-term potentiation (LTP) at the CA1 region of hippocampus in rats. They also showed that crocin in the whole extract of crocus sativus might probably be responsible for the induced properties of crocus sativus in these studies (16, 17).

Based on the above discussion, most of the previous studies have been performed on the whole extract of crocus sativus not its active ingredients. On the other hand, the effect of crocin on other neurotransmitter systems involved in the learning process and memory, including glutamatergic system has not been assessed so far. Hence, the present study was carried out to specifically evaluate the role of crocin in the treatment of learning disorder due to inhibition of NMDA glutamate receptors in passive avoidance task in the rats.

## Materials and methods

This experimental study was performed on 60 male Wistar rats with the weight range of 200±20. The animals were kept in plexiglass chambers in a room with 12/12 light-dark cycle (light cycle from 8:00 am to 8:00 pm) at controlled ambient temperature of 23±2 ºC. The experiments were carried out in the 24-hour light cycle (between 9:00 and 11:00 am). The rats had free access to food and water except for the time of experiment. In all phases of the experiment, the ethical considerations of working with animals were taken into account. Meanwhile, each rat was tested only once and after experiment, deep anesthesia was applied until the animal died. The drugs used in this study were ketamine (Alfasan, The Netherlands) and crocin (Sigma, Germany). Each drug was prepared on the day of experiment and was used freshly. The solvent used for both drugs was normal saline. Memory was measured by step-through passive avoidance learning using a shuttle box (maze I) (19).

The shuttle box consisted of two separate chambers with 30×20 dimensions and 20 cm height, which were separated by a guillotine valve the animal could pass through when it was open. The walls and floor of one of the chambers were white (light chamber) and those of the other one were black (dark chamber). The floor of the dark chamber had transverse parallel metal bars with a distance of 1 cm from each other, through which electric shock with desired voltage and time could be transmitted to the animals' feet by a stimulator attached to them.

The experiment in this machine included three stages (19):

A) Adaptation: While the valve was open, the animal commuted between the light and dark chambers for three minutes and got familiar with them. The animal that did not enter the dark room during the three minutes was excluded from the study.

B) Training: Thirty minutes later, the animal was placed in the light room and while it entered the dark room instinctively, the guillotine valve was closed and after 20 seconds an electric shock (50 Hz, 1 mA) was applied to the animal's foot for 3 seconds. Therefore, the animal learned not to enter the dark room. After 20 seconds, the animal was taken out of the device and delivered to the cage. Then, the animal was placed in the device again after 2 minutes and the guillotine valve was opened. If the animal did not enter the dark chamber 120 seconds after opening the valve, the training was found to have been completed; otherwise, electric shock was applied again. The animal that entered the dark room after three electric shocks was excluded from the experiment. The drugs were administered during this day and after training.

C) Testing: This phase, in which the required data for statistical analysis were obtained, was performed 24 hours after training. While the guillotine valve was closed, the animal was placed in the light chamber. The valve was opened after 20 seconds and the animal's delay in stepping through the dark room was determined as the memory benchmark and recorded as Step Through Latency (STL). The final time limit was 600 seconds.

The rats were classified into four major groups via simple random sampling as follows:

A) Saline-saline (control): after raining, normal saline was administered to the rats twice with an interval of 30 minutes.

B) Ketamine-saline: the rats in this group were divided into three subgroups. After training, first, ketamine (3, 6 or 12 mg/kg) and 30 minutes later, normal saline were administered.

C) Saline-crocin: the rats in this group were divided into three subgroups, and with the sequence mentioned in A and B groups, normal saline and 30 minutes later crocin (2, 5 or 10 mg/kg) were administered.

D) Ketamine-crocin: the rats in this group were classified into three subgroups. After raining, they first received ketamine (12 mg/kg) and 30 minutes later they were administered saline or crocin (2, 5, or 10 mg/kg).

All injections were performed intraperitoneally (1 mg/kg). The dose of the drugs used was chosen according to the pilot study. The sample size (n=60) was determined according to a similar study (18); a minimum of 6 rats in each subgroup. It should be noted

that ketamine administration thirty minutes after training in various behavioral models has been reported to impair learning in animal models (27).

The quantitative data showed the interval between the opening of guillotine valve and stepping through the dark chamber in the testing phase. Data were presented as Mean±SEM, and the results were analyzed by one-way ANOVA. In the case of significant differences, the follow-up Tukey test was run. P<0.05 was considered significant for all statistical analyses.

## Results

In this study, Step Through Latency (STL) was determined as a memory standard in the testing phase. Figure 1 indicates the effect of ketamine administration (3, 6, or 12 mg/kg, ip) on step-through latency in the testing phase. As shown, administration of ketamine (12 mg/kg, ip) reduced the remembering process compared to saline-saline group. The statistical analysis of this group showed a significant difference among the experimental groups in general ($F_{3, 20}$=54.62, P<0.001). The results of post-hoc test showed that ketamine (12 mg/kg, ip) could reduce the passive avoidance response in the rats so that the mean step-through latency was declined significantly in comparison with saline-saline group (p<0.001). According to figure 2, administration of different doses of crocin (2, 5, or 10 mg/kg, ip) did not significantly affect the mean memory and learning indices ($F_{3, 20}$=1.04, P=0.39).

Figure 3 illustrates the performance of the rats in control and ketamine (12 mg/kg) groups with and without treatment with crocin in the shuttle box. The results of statistical analysis showed a significant difference among the study groups in general ($F_{25, 4}$=8.78, P<0.001). Moreover, the findings of post-hoc test showed that administration of crocin after ketamine (12 mg/kg, ip) improved the performance of the rats in the shuttle box and the significant level was achieved with 2 mg/kg, ip crocin (P<0.001).

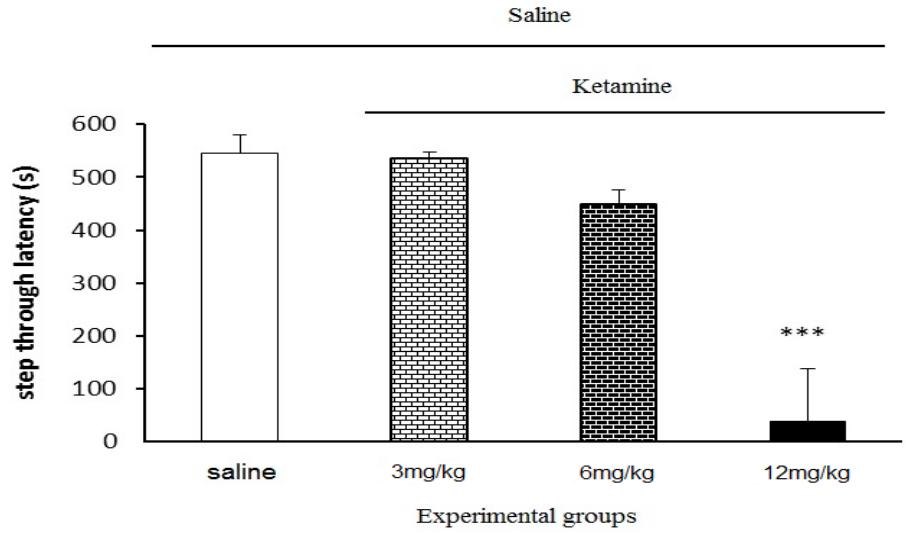


**Figure 1.** Effect of ketamine administration (3, 6, or 12 mg/kg) on the mean step-through latency in the testing phase compared to saline-saline group (n=6), ***P<0.001

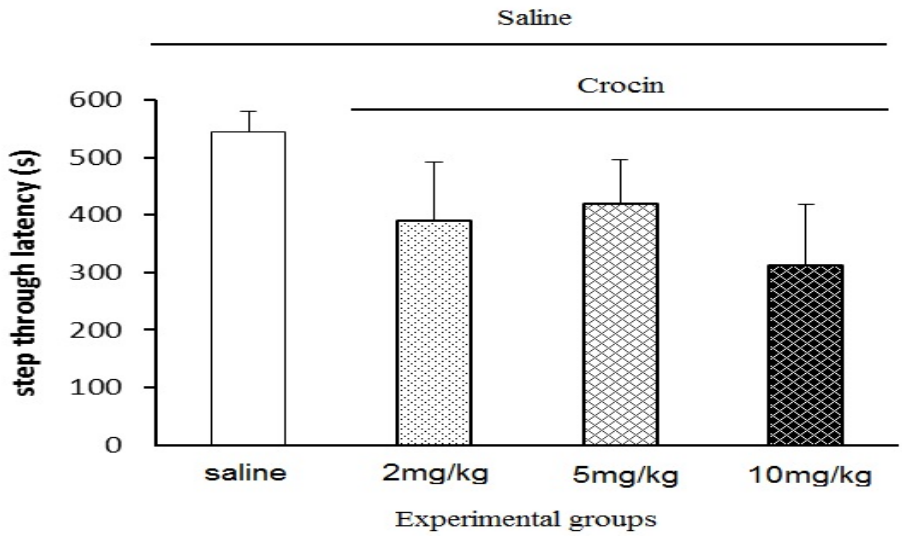


**Figure 2.** Effect of crocin administration (2, 5, or 10 mg/kg) on the mean step-through latency in testing phase compared to saline-saline group (n=6)

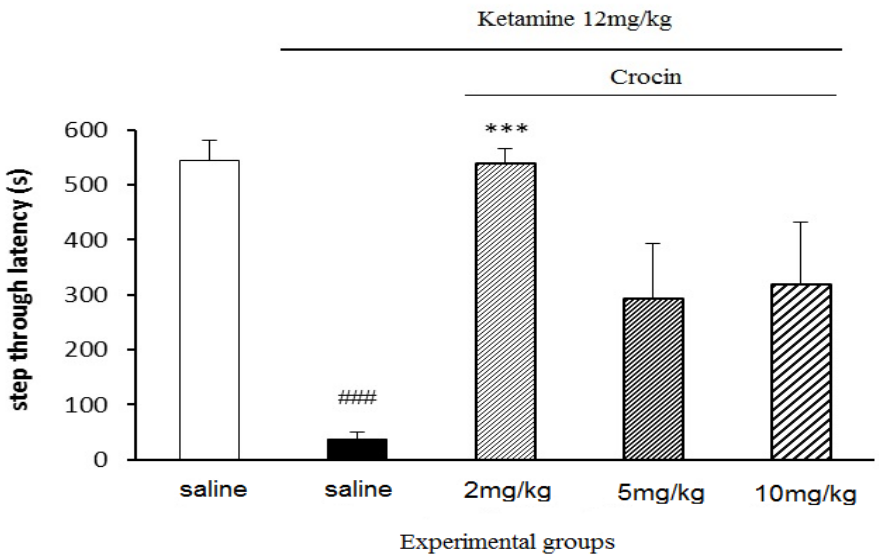


**Figure 3.** Effect of crocin administration (2, 5, or 10 mg/kg, ip) on ketamine-induced (12 mg/kg, ip) step-through latency in the testing phase compared to ketamine-saline group (n=6), ***P<0.001, and effect of ketamine administration (12 mg/kg, ip) on the mean step-through latency compared to saline-saline group (n=6), ###P<0.001

## Discussion

This study evaluated the effect of crocin on ketamine-induced amnesia in the male rats using a shuttle box. The same as previous studies (20), intraperitoneal administration of ketamine, an uncompetitive antagonist of NMDA receptors, impaired passive avoidance learning in the rats. Administration of ketamine (12 mg/kg, ip) reduced the step-through latency on the day of experiment, indicating the impairment of passive avoidance learning in the rats. Studies have shown that ketamine is a drug widely used for induction of anesthesia (21). It has also been reported that administration of doses lower than the anesthetic dose of ketamine impairs learning and memory in the passive avoidance task (8). On the other hand, injection of crocin (2, 5, or 10 mg/kg, ip) was shown not to affect the step-through latency by itself compared to the control group. Although crocin (10 mg/kg, ip) reduced the step-through latency in the rats but this reduction was not statistically significant.

In this study, all doses of crocin increased the step-through latency in the rats receiving ketamine (12 mg/kg, ip), but only 2 mg/kg crocin could significantly improve ketamine-induced learning disorder in comparison with ketamine-saline group. The results of this study are in line with those of previous studies such as the study of Abe et al. in which they reported administration of whole extract of saffron could inhibit the destructive effect of ethanol on memory and could induce the hippocampal long-term potentiation (LTP), but had no impact on the learning behaviors in the mice by itself. They found these effects were induced by crocin in crocus sativus extract (17).

Another study showed that crocin could prevent the effect of ethanol-induced inhibition on neuronal responses mediated by NMDA receptors in the hippocampus of the rats (16). Also, the effect of crocin on impairment of learning and spatial memory induced by scopolamine (a cholinergic muscarinic receptor antagonist) in Morris water maze was assessed by another study. The findings indicated that administration of scopolamine impaired the spatial learning process in the animals so that crocin inhibited the effect of scopolamine in induction of amnesia in a dose-dependent manner but crocin alone had no effect on the formation of learning and spatial memory (18).

The whole extract of saffron and safranal (another constituent of crocus sativus) has been reported to reduce the memory damage resulting from hyoscine in the rats in Morris water maze. This study showed that crocus extract improved the memory only in lower doses while it had no impact on memory in higher doses. On the other hand, safranal impaired the memory by itself; therefore, the researchers suggested that the effects of whole extract of crocus were probably generated by crocin (22). However, no definite conclusion can be made regarding the activity of crocin against learning and memory disorders. The glutamatergic system along with acetylcholine system constitute glutamate/ aspartate-acetylcholine cycle which is associated not only with memory and learning but also with perception and cognition.

Perception disorders induced by Alzheimer may be caused by the loss of neurons of glutamate system and acetylcholine. Moreover, amyloid-beta peptide, which causes synaptic dysfunctions in Alzheimer, inhibits the glutamatergic system and reduces the synaptic flexibility (23, 24). Possibly, one of the important mechanisms involved is the induction of hippocampal long-term potentiation, a kind of activity-dependent synaptic plasticity, which may be the basis of learning and memory. This mechanism has been discussed in some previous studies. Given the protective effects of crocus extract and crocin on ethanol-induced suppression of

hippocampal LTP, it can be suggested that the activity of N-Methyl-D-aspartate and Calcium influx into postsynaptic cells through NMDA channels induce the given activity (17). It is not quite clear whether crocin directly affects NMDA receptors or indirectly influences the activity of NMDA receptors. However, since crocin has been able to improved the ethanol-inhibited LTP, which has an inhibitory effect on NMDA receptors, it seems that crocin clearly affects the activity of this channel directly or indirectly (17). On the other hand, it has been reported that crocin does not change LTP in hippocampus by itself and has no impact on the synaptic potential of non-NMDA glutamate receptors (25, 26).

NMDA receptors, in addition to a location for glutamate, have other sites for $mg^{2+}$ ion and glycine molecule (16). Since ketamine has also a specific site for inhibition of NMDA receptors (27) and that crocin has no effect on learning and memory by itself, crocin may inhibit the effect of ketamine on the activity of NMDA receptors by antagonizing its binding site. However, further research is needed to prove this assumption. Furthermore, various regions of central nervous system may be involved in the therapeutic effects of crocin on improving the dysfunction induced by ketamine in NMDA receptors. On the other hand, because administration of different doses of crocin alone had no effect on the learning behavior of the rats, it can be proposed that crocin exerts its effects on the learning process by interfering with the performance of ketamine. Based on the results of the current and previous studies, further studies are suggested to evaluate the role of crocin in interaction with other neurotransmitter systems involved in the memory. Moreover, central administration of crocin into different nuclei of the brain and use of microanalysis techniques can be helpful to determine the mechanisms of crocin.

## Conclusion

In general, the findings showed that crocin, as a constituent of crocus sativus extract, could treat ketamine-induced retrograde amnesia in passive avoidance task in rats.

## Acknowledgements

This article was taken from a research proposal (grant no: 93395) approved by deputy of research at Kermanshah University of Medical Sciences. The authors would like to extend their gratitude to all the concerned authorities for their sincere support.

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