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ABSTRACT

Objective: Harmaline and other beta-carbolines act as an inverse agonist for GABA-A receptors and cause central nervous system stimulation and anxiety; thus, it may act hypothetically as a potential seizure augmenter. To examine the hypothesis, the effect of harmaline during the seizures induced by amygdala kindling is investigated here.

Methods: Seven groups of male rats were kindled by daily electrical stimulation of the amygdala. After being kindled, Groups I–III, respectively, received 5, 15 and 50 mg/kg harmaline through intraperitoneal injection. The rats in Groups IV and V received vehicle daily (1 ml/kg) and harmaline (5 mg/kg) daily through intraperitoneal injection. Groups VI and VII received artificial cerebrospinal fluid and harmaline (50 mM) through intraventricular injection, respectively.

Results: In addition to significant increase of some seizure parameters in the fully kindled groups, harmaline significantly increased cumulative afterdischarge duration ($P < 0.05$) and decreased stage 1 latency ($P < 0.01$) in the acquisition groups (Groups V and VII). In Group VII, seizure duration showed a significant increase ($P < 0.01$) while stage 1 latency and stage 4 latency decreased significantly ($P < 0.01$).

Discussion: According to the results, it is suggested that harmaline may increase neuronal activity and the production of high-frequency action potentials by stimulating NMDA receptors and inhibiting GABA receptors. Overall, drugs and plants containing harmaline may be harmful to epileptic-susceptible people during some traditionally and costume treatments, so these should be avoided.

1. Introduction

Some plant seeds bearing harmaline like those of Peganum harmala have been traditionally used as medications to cure some illness or as odorant steams during some religious rituals (customs) [1]. There are many reports indicating that harmaline can act as an inverse agonist for GABA-A receptors [2,3] and NMDA receptor stimulator [4], and facilitates some fear [5], anxiety [6] and central nervous system (CNS) stimulation [2,7]. It seems reasonable to assume that harmaline can act as a potential elevator for seizure susceptibility, acting as an epileptogenic factor.

With over 40 different types, epilepsy is considered to be one of the most common neurological disorders [8]. Thus, it is of paramount importance to develop different analytic tools and methods so that the possible causes and mechanisms involved can be understood, and more effective treatment options can be developed accordingly [9]. Since about 30% of patients with epilepsy are still resistant to currently used antiepileptic medications, a lot of research is underway to identify the possible underlying causes and mechanisms of epilepsy so that more effective treatment for these patients can be developed. To this end, various laboratory models such as kindling are used. Kindling is a suitable model for the study of temporal lobe epilepsy in that it is a useful technique to reveals the epileptogenic properties of non-well-known materials in this regards [10]. In this model, first proposed by Goddard [11], a certain region of the forebrain such as the amygdala is electrically stimulated at regular intervals.

However, there are some plants containing compounds that are not only ineffective in the treatment of epilepsy but can also cause seizure. Therefore, the possible side effects of medications derived from these plants need to be investigated. Currently, there are a host of studies performed to determine the pro/anticonvulsant of plants [12–14]. Apart from one single report introducing harmaline as a chemical inducing seizure, there is a lack of studies on the effects of harmaline [15]. Hence, the aim of the present study was to investigate the effect of harmaline, as one of the harmala alkaloids, on seizures induced by amygdala kindling.

2. Materials and methods

2.1. Animals

Fifty-two male SpragueDawley rats were individually housed under 12-h light/12-h dark conditions with ad libitum access to food and water. The rats weighed 280–320 g at the time of surgery. Procedures involving animals and their care were conducted in accordance with the 'Guide to the Care and Use of Experimental Animals.' [16]. All of the experiments were done during the same time of the day (8:00 a. m.–2:00 p.m.) in the morning to avoid the bias due to circadian rhythms [17].

2.2. Experimental groups

(A) *The effect of intraperitoneal injection (i.p) of harmaline on seizure stages (SSs) in the fully kindled rats*

(1) The rats were stimulated to be kindled. The stimulation was continued until the seizure parameters reached a constant value (with repeated stimulations from the first day onward, afterdischarge duration (ADD) and stage 5 duration (S_5D) increased while stage 1 latency (S_1L) and stage 4 latency (S_4L) decreased). After 24 h, sterile vehicle (10 ml/kg) was injected and kindling stimulation was applied after 30 min. Then, the seizure parameters were recorded. After 24 h, harmaline (5 mg/kg) was intraperitoneally injected. The kindling stimulation was applied after 30 min and then the seizure parameters were recorded.

(2) The procedure used for Group I was employed. In this case, 15 mg/kg harmaline was intraperitoneally injected. The kindling stimulation was applied after 30 min and then the seizure parameters were recorded.

(3) The method used for the first group was used except that harmaline was injected at 50 mg/kg intraperitoneally. Groups II and III are used to investigate the effect of harmaline doses on the seizure parameters.

(B) *The effect of intraperitoneal injection (i.p) of harmaline on the progress of seizures induced by electrical kindling of the amygdala*

(1) The rats were kindled twice a day. Sterile vehicle (10 ml/kg) was injected intraperitoneally 30 min before the first stimulation. Stimulation continued until the rats were fully kindled. The seizure parameters were recorded on a daily basis.

(2) The rats were kindled twice a day. Harmaline (5 mg/kg) was intraperitoneally injected 30 min before the first stimulation. Stimulation continued until the rats were fully kindled. The seizure parameters were recorded on a daily basis.

(C) *The effective administration of harmaline on the progress of seizures induced by kindling of the amygdala*

(1) Artificial cerebrospinal fluid (ACSF) was injected into the left lateral ventricle with a rate of 1 μl/2 min. The kindling stimuli were administrated after 30 min.

(2) The rats received 1 μl harmaline with a concentration of 50 mM (equivalent to 0.01 mg) through ICV injection on a daily basis. It should be injected into the left lateral ventricle within 2 min. The kindling stimuli began after 30 min. Harmaline dose was chosen based on a dose of 5 mg/kg, the molecular weight of harmaline (214.263).

2.3. Surgical and kindling procedure

The rats were anesthetized using Ketamine (100 mg/kg) and Xylazine (20 mg/kg) through the intraperitoneal injection [18]. For stereotaxic surgery, the animals were placed in a stereotaxic apparatus. According to the Paxinos Atlas [19], the coordinates of electrodes were determined unilaterally in the lateral nucleus of the left amygdala. The coordinates of the amygdala on the skull relative to bregma is as follows: AP = −2.5 mm, L = 4.8 mm and V = 7.5 mm (below dura). After precise determining the insertion point, a tripolar electrode was fixed in its own place by dental cement. Two monopolar electrodes were attached to the skull by screws.

For harmaline injection in the left lateral ventricle (Groups VI and VII), a 22-gauge guide cannula was also implanted in the left lateral ventricle (coordinates: AP = −0.8 mm; L = +4.1 and 2.6 mm below dura) in addition to insertion of electrodes in the amygdala.

At least 1 week after the surgery, the threshold intensity was used for stimulation. To obtain the threshold intensity, the animal was stimulated by a 10 μA electrical current. If the afterdischarge (AD) waves were recorded for at least 5 s, the current was known as the threshold intensity. Otherwise, the 10 μA current was increased with a time interval of 5 min until the AD waves were recorded. AD waves are 'after stimulus discharges' that evoked in stimulation point exactly after stimulus artifact and terminate after ending of discharges. We considered AD waves in which it was at least two times larger than baseline recording. It was recorded by computer after magnification and digitalization by the electromodule instrument (Electromodule D3111 and NeuroTrace provided by Science Beam Institute, Tehran, Iran). The animals were stimulated twice a day with the threshold intensity with an interval of at least 6 h to be kindled and show various SSs [18].

By gradual stimuli, the behavioral symptoms are also visible while of recording the AD waves (electrographic response). According to Racine's Scale (1972), the behavioral responses are divided into five distinct stages: Stage I: mouth and facial movements, Stage II: head nodding; Stage III: forelimb clonus on the opposite side of stimulated region; Stage IV: rearing with clonus; Stage V: rearing and falling with forelimb clonus [20].

The seizure parameters after stimulation are as follows:

(1) Time delay between the onset of electrical stimulation to stage 1 seizure (S_1L: Stage 1 latency)
(2) Time delay between the onset of electrical stimulation to stage 2 seizures (S_2L: Stage 2 latency)
(3) Time delay between electrical stimulation to stage 4 seizure (S_4L: Stage 4 latency)
(4) Stage 5 duration (S_5D): the duration of stage 5
(5) Seizure duration (SD)
(6) Seizure stage (SS): This parameter represents the number of stimulations required to reach different stages of seizure.

2.4. Drug administration

After weighing the required amount of harmaline (Sigma-Aldrich, St. Louis, MO, USA), it was dissolved in ACSF. To prepare ACSF, the ingredients were weighed and then dissolved in a certain volume of distilled water. Ingredients include NaCl (114 mM), $MgSO_4$ (2 mM), KCl (3 mM), NaH_2PO_4 (1.25 mM), $CaCl_2$ (1 mM), $NaHCO_3$ (26 mM), Glucose (10 mM). The rats in Group VI received ACSF (1 μl/2 min) [21].

The rats in Group VII received 1 μl harmaline daily (with a concentration of 50 mM equivalent to 0.01 mg) through intra-cerebro-ventricular injection (i.c.v). For injection into the left lateral ventricle, a polyethylene tube (PE-20, Stoelting) was used where one end was attached to the needle G27. Before drug administration, the tube and needle were washed using distilled water and then rinsed with sterile drugs. After drug loading, the free end of the PE tube was connected to a Hamilton syringe (1 μl). Injection was performed by injection device (Microsyring pump) with a rate of 1 μl/2 min [21].

2.5. Histological confirmation

At the end of the experiment, rats were deeply anesthetized by ether and perfused with vehicle and 10% formalin. Their brains were detached and placed in 10% formalin for at least 24 h at room temperature. Then, slices (10 μm-thick) were prepared by a microtome (Leica Instruments, Germany) and processed with cresyl violet-based Nissl staining. The stained slices were qualitatively explored for recording electrode location using a light microscope (Olympus, Japan). The data of the animals with wrong placement of their electrode position or existence of any abnormality (such as lesion) were not included in the results.

2.6. Analysis methods

The mean data obtained from the experiments were reported as mean ± SEM with at least $n = 6$. The effects of different concentrations of treatments were evaluated using one-way ANOVA with the help of GraphPad Prism5. The differences between the means were evaluated by Tukey's post hoc test with a minimum significance level of $P < 0.05$. In Groups IV–VII, data were analyzed cumulatively. It means data are summed in each day with ones of its earlier days. SS was evaluated using Mann–Whitney test for non-parametric data. Since SS data are non-parametric, the Mann–Whitney test was used to analyze SS data.

3. Results

Histological assessment indicated that the recording electrodes were positioned in the amygdala. Twelve rats were eliminated from the study because of incorrect position of electrodes or disruption of their electrophysiological responses during freely moving records. There was no significant difference between threshold intensity of treatments and vehicle groups on the first day of stimulation (data not shown). It means that there was no difference in seizure susceptibility between all groups at the beginning of experiments. During experiments, animals were stimulated by the same threshold intensity which was measured on the first day.

3.1. The effect of intraperitoneal injection of harmaline on fully kindled seizures rats

In the first phase of the study, kindling stimulation was applied 30 min after i.p. injection of harmaline with a concentration of 5, 15 and 50 mg/kg. The seizure parameters were recorded and then compared with those received harmaline (vehicle) solution.

ADD: At all three concentrations (5, 15 and 50 mg/kg, i.p.), harmaline increased ADD as compared to vehicle. But the difference was only statistically significant at 15 mg/kg ($P < 0.05$) (Figure 1).

The delay between onset of seizure to Stages 2 and 4 (S_2L and S_4L): At all concentrations (5, 10 and
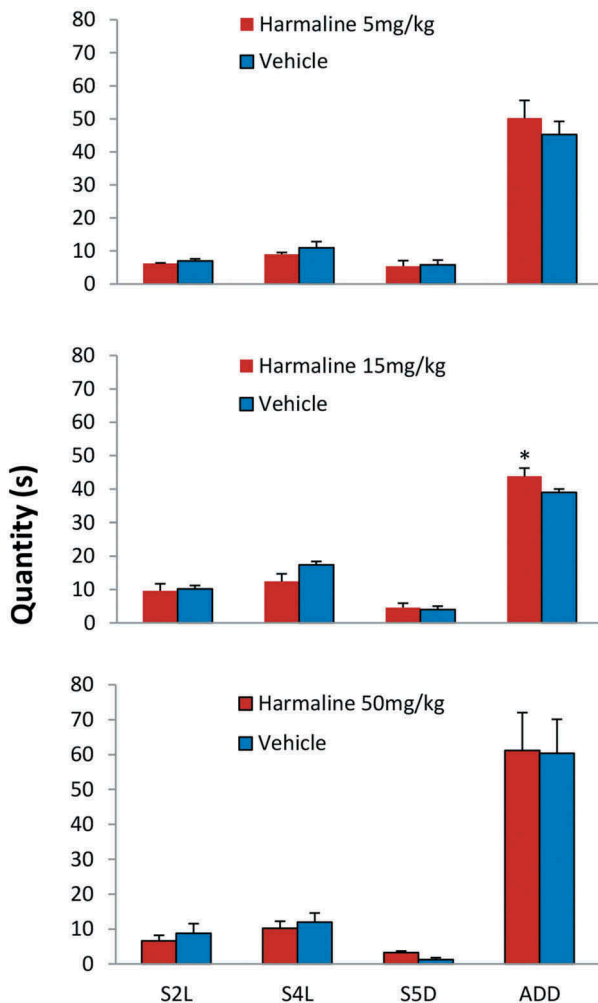
Figure 1. The effect of i.p injection of harmaline (15 mg/kg) on seizure parameters in fully kindled rats. At all three concentrations (5, 15 and 50 mg/kg), harmaline increased ADD as compared to vehicle. But the difference was only statistically significant at 15 mg/kg ($P < 0.05$).

50 mg/kg, i.p.), harmaline did not decrease S_2L and S_4L as compared to vehicle.

S_5D: At 15 and 50 mg/kg, harmaline did not increase S_5D as compared to vehicle.

3.2. The effect of i.p. injection of harmaline on seizures during kindling acquisition

In the second phase of the study, kindling stimulations were applied 30 min after i.p. injection of harmaline. The seizure parameters were recorded and compared statistically.

ADD: The repeated measures ANOVA revealed that harmaline (5 mg/kg, i.p.) significantly increased ADD as compared to those receiving vehicle. Figure 2 shows cumulative ADD. Cumulative ADD (s) is added to the values obtained in previous days. Statistical comparisons were performed and displayed for the cumulative values [$F_{(14, 112)} = 1.8$; $P = 0.04$] (Figure 2(a)).

S_1L: The repeated measures ANOVA revealed that harmaline (5 mg/kg, i.p.) (30 min before the kindling stimulation) significantly reduced S_1L [$F_{(15, 120)} = 17.7$; $P = 0.00$] (Figure 2(b)).

3.3. The effect of i.c.v. injection of harmaline on seizures during kindling acquisition

S_1L: The repeated measures ANOVA revealed that i.c.v. injection of harmaline (50 mM) (30 min before the kindling stimulation) significantly reduced S_1L [$F_{(14, 112)} = 14.9$; $P = 0.00$] (Figure 3(a)).

S_4L: The repeated measures ANOVA revealed that i.c.v. injection harmaline (50 mM) (30 min before the kindling stimulation) significantly reduced Stage 4 Latency [$F_{(11, 77)} = 16.6$; $P = 0.00$] (Figure 3(b)).

SD: The repeated measures ANOVA revealed that i.c.v. injection of harmaline (50 mM) (30 min before the kindling stimulation) significantly changed the SD [$F_{(14, 98)} = 12.9$; $P = 0.00$] (Figure 3(c)).

SS: The statistical analysis by Mann–Whitney test revealed that the number of stimulations to reach Stage 2 ($P = 0.04$), Stage 3 ($P = 0.03$) and Stages 4 and 5 ($P = 0.009$) in the group receiving harmaline was significantly lower than those receiving vehicle (Figure 4).

4. Discussion

The results obtained in the present study suggested that harmaline had proconvulsant effect in the fully kindled animals. Moreover, the injection of harmaline (5 mg/kg) during kindling led to a significant increase in ADD. According to the results obtained from fully kindled and kindling acquisition groups, harmaline is able to significantly increase the ADD. The increase in the neuronal activity, and production of high-frequency action potentials [2] and consequently the increase in ADD induced by harmaline can be due to that fact that the presence of harmaline strengths the stimulatory mechanisms while weakening inhibitory mechanisms in the brain. ADD is an indicator of the activity of local circuits in the stimulating area (amygdala) which is dependent on focal excitability. It is also presumed that harmaline increases this parameter during the kindling process by stimulating the neuronal circuits of the amygdala area.

In the present study, harmaline was injected intra-cerebro-ventriculary so that the direct effect of harmaline on the brain could be investigated. It was shown that the i.c.v. injection of 50 mM harmaline was associated with a significant increase in SD (Figure 3(b)). However, the presence of harmaline led to a significant reduction of S_1L and S_4L (Figure. 3(a) and 3(c)). The results showed that compared to those receiving vehicle, those receiving harmaline needed significantly fewer number of stimulations to reach SS 2–5 (Figure 4). S_4L parameter indicates the development of seizure
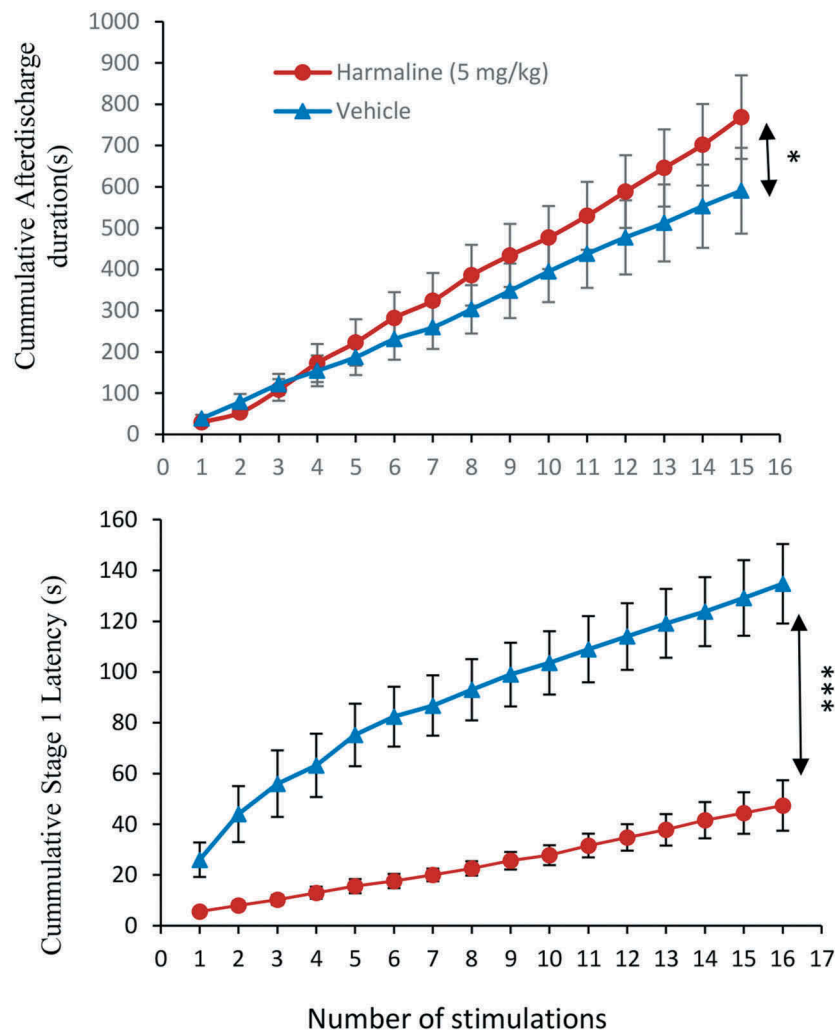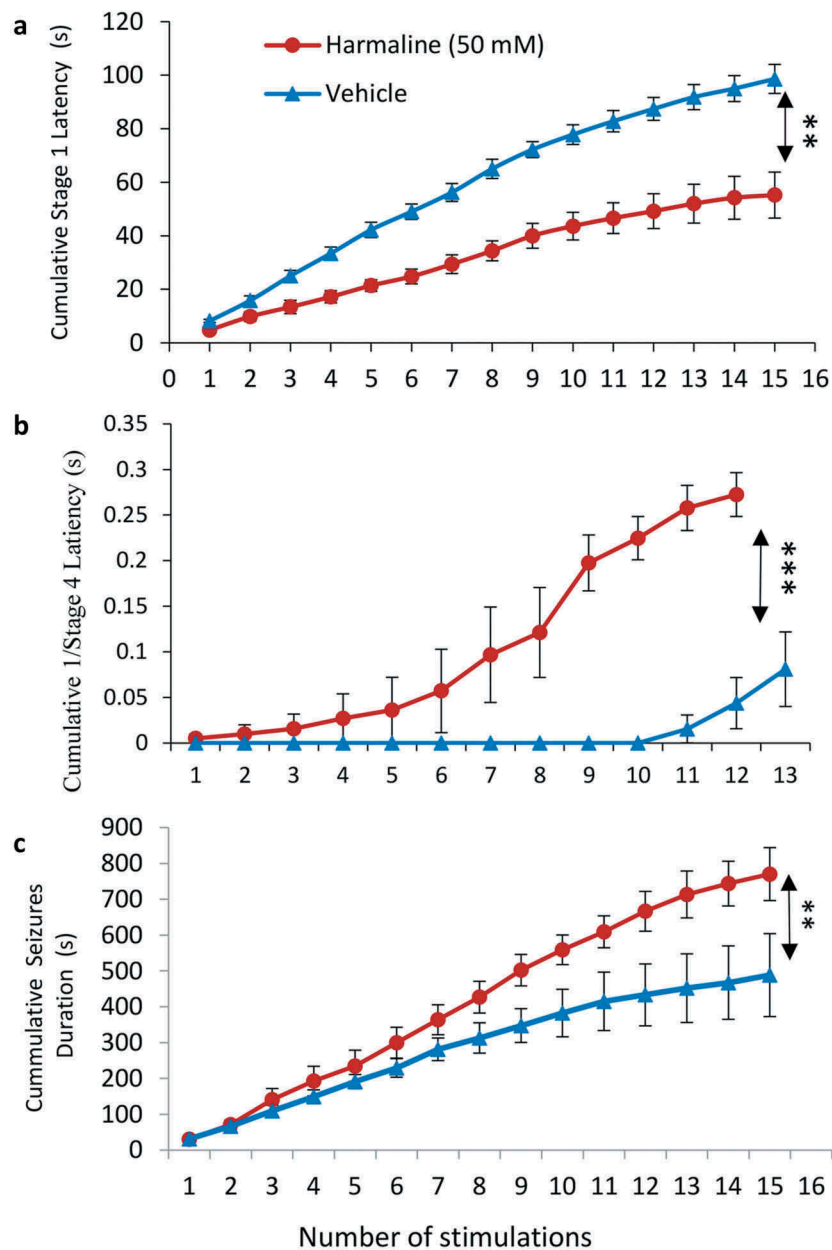
Figure 2. The effect of i.p. injection of harmaline (5 mg/kg) on (a) the cumulative after discharge duration (add) during amygdala kindling. Harmaline injection increases the cumulative add as compared to the vehicle group; (b) the stage 1 latency (s_1l) during the amygdala kindling. Harmaline (5 mg/kg) injection reduces the cumulative stage 1 latency compared to the vehicle group (vehicle injection). The cumulative data are shown as Mean ± SEM. *indicates $P < 0.05$, **indicates $P < 0.01$ when compared with vehicle injection ($n = 6$).

and the involvement of brainstem circuits leading to the generalized seizure. In other words, harmaline shortens the development phase of the seizure in the amygdala kindling. On the other hand, harmaline injection increased the SD, an observation which can be explained by the pro-convulsive properties of this chemical compound.

Our findings also indicated that the i.c.v. injection of harmaline significantly reduced the average number of stimulations required to reach stages 2–5 (Figure 4). The five stages of kindling seizures can be divided into two periods: focal seizure including stages 1–3 and the generalization of seizures including stages 4 and 5. As the findings of the present study revealed, harmaline had a significantly greater effect on the reduction of stimulations needed for the fourth and fifth stages of seizure to be reached than it did for the second and third stages (Figure 4). Therefore, harmaline plays an important facilitating role in the transition of the focal seizure to the generalized one. In other words, if any subjects would be susceptible to seizure, harmaline may start convulsions, and even make it worse.

Harmaline is a beta-carboline found in *Peganum harmala* with important effects on the body, especially the nervous system [1]. The first systematic studies on the effect of harmaline and other beta-carbolines on the laboratory animals (rats and rabbits) were conducted by Gunn [2]. Their results showed that harmaline causes tremor and provocative behavior [1,2]. There is some evidence indicating the relationship between the mechanisms involved in tremors induced by harmaline and the mechanisms involved in seizure. For example, anticonvulsant compounds such as valproate and carbamazepine suppressed harmaline-induced tremor [22]. Moreover, as seizure induced elevation of Ro 15–4513 binding sites in the dentate gyrus, harmaline led to an increase in the number of $GABA_A$ receptor $\alpha 4$ subunit [7]. Thus, the examination of

Figure 3. The effect of intraventricular injection of harmaline (50 mM) on (a) the stage 1 latency (S_1L) during the amygdala kindling. Harmaline injection reduced the cumulative stage 1 latency compared to the vehicle group (ACSF injection); (b) the cumulative stage 4 latency (S_4L) during the amygdala kindling. Harmaline injection significantly decreased the cumulative S_4L compared to the vehicle group; (c) the cumulative seizure duration during the amygdala kindling. Harmaline injection significantly increased the cumulative seizure duration compared to the vehicle group. The cumulative data are shown as Mean ± SEM. **indicates $P < 0.01$ when compared with ACSF injection ($n = 6$).

the mechanisms involved in the tremors induced by harmaline is likely to reveal the mechanisms through which harmaline causes seizure. It has been shown that the increased activity of NMDA receptor plays an important role in the pathophysiology of some neurological diseases such as Parkinson's disease [23] and epilepsy [24]. Harmaline has stimulatory effects (rhythmic activity) on neurons through increasing the formation of cGMP and through opening the calcium channels of NMDA receptor [4]. As a result, it is likely that harmaline results in an increased ADD level (rate), thus increasing the likelihood of the occurrence of

seizures as the neuronal activity increases. On the other hand, it has been reported that harmaline is involved in neuroprotective process, because of its interaction with a large number of neurotransmitter receptors and ion exchangers. For example, it has been reported that harmaline inhibits Ca^{2+} currents [25–27]. In fact, there are some reports that Ca^{2+} channels are necessary to induce tremor by harmaline [28]. The fact that part of our results, especially those observed for the 50 mg/kg i.p. dosage, are not compatible with the nature of proconvulsive effect can be accounted for by the possible inhibition of Ca^{2+} channels caused by the presence of Harmaline.
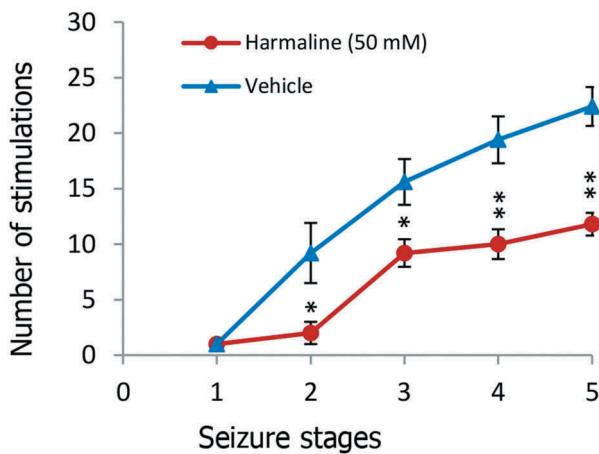
Figure 4. The effect of intraventricular injection of harmaline (50 mM) on the seizure stages during the amygdala kindling, the number of stimulations to reach stages 2–5 in the rats receiving harmaline showed a significant decrease as compared to those receiving ACSF. The cumulative data are shown as Mean ± SEM. *indicates $P < 0.05$ and **indicates $P < 0.01$ when compared with ACSF injection ($n = 6$).

Harmaline (0.5 mM) inhibits more than 90% of the GABA receptors in that it occupies GABA binding sites, preventing GABA reactions [29]. It has been observed that harmaline acts as an inverse agonist binding site of benzodiazepines (BZs) on GABA receptors. Because harmaline connects to the BZ binding site in GABA-BZ receptor complex, it has adverse effects on BZs including induced anxiety, CNS stimulation and seizures [7]. On the other hand, it has been shown that kindling results from increased GABAA receptor ligand binding between 4 and 8 h after electroconvulsive shock; therefore, it may be part of proconvulsant effects of harmaline due to GABAA receptors occupation. As it has been reported, BZ receptors are one of the action sites for harmaline [29].

Moreover, the proconvulsant effect of harmaline can be due to the inhibition of MAO-A and MAO-B [30] activity and the consequent increase in dopaminergic transmission [31], and in serotonin level [32] in nerve synapses. In fact, dopamine is associated with both proconvulsant as well as anticonvulsant effects [33]. Moreover, although serotonin is believed to have anticonvulsant effects, a wide range of pharmacological and genetic manipulation studies clearly demonstrate that some subtypes of dopamine and serotonin receptors have a proconvulsant effect while others have an anticonvulsant action [34].

5. Conclusion

The results of the present study suggested that harmaline may increase neuronal activity and the production of high-frequency action potentials through stimulating NMDA receptors and inhibiting GABA receptors. In general, it seems that the medications and plants containing harmaline are harmful for epileptic-susceptible people although they are traditionally given to patients as part of their treatment. The review of the studies on the effect of harmaline on the seizures induced by experimental epilepsy models showed that there is a dearth of studies in this area. Therefore, more research studies are needed to broaden and deepen our understanding of the potential effect and mechanisms of such medications.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Azam Alenajaf is graduated in Animal Physiology from Ferdowsi University of Mashhad. She holds a bachelor of biology and a diploma in experimental sciences. Now he is the teacher of biology. He was interested in studying the environmental factors on epilepsy.

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Masoud Fereidoni has focused on nervous system function specially in the field of pain during his PhD when he did his PhD at Tarbiat Modares University of Tehran, Iran. Afterward, he became a professor of neuroscience and a research group leader as head of Behavior, neuro-degenerative/inflammation laboratory at the department of biology, Faculty of Science, Ferdowsi University of Mashhad, Iran. Research in his group is more concentrated in neuroinflammation and neurodegeneration using some cognitive and behavioral, cellular and molecular examinations both in vivo and in vitro, thereby connect his experience in the fields of neurobiology to the areas of neurodegenerative disease.

Mohammad Mohammad-Zadeh has focused on nervous system function specially in the field of Kindling and Field potential recording during his PhD when he did his PhD at Tarbiat Modares University of Tehran, Iran. Now he is Associated professor of Physiology and have a laboratory at the department of Physiology, Faculty of Medicine, Sabzevar University of Medical Sciences, Iran. Research in his Lab. is more concentrated in epilepsy field using some behavioral, electrophysiological, cellular and molecular examinations.

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