Dantrolene: A Selective Ryanodine Receptor Antagonist, Protects Against Pentylenetetrazole-Induced Seizure in Mice

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Abstract- Ryanodine receptor abnormalities has implicated in the generation and maintenance of seizure. Dantrolene, a selective ryanodine receptor antagonist, may be a potential drug for the prevention of seizure. Therefore, we aimed to clarify the protective effects of dantrolene against pentylenetetrazole seizure in mice. Male albino mice were received an intra-peritoneal injection of pentylenetetrazole (80 mg/kg) in seven separate groups (n=8). We used dantrolene (10,20 and 40 mg/kg), caffeine (200 mg/kg), dantrolene (40 mg/kg) + caffeine (200 mg/kg), diazepam (5 mg/kg as a positive control) and vehicle 30 minutes before the injection of pentylenetetrazole. Then, we registered the latency time of the first seizure, the severity of seizures and the incidence of seizure and death. Kruskal-Wallis test followed by Mann-Whitney and Fisher's exact test were used to analyze the data. Dantrolene (10,20 and 40 mg/kg) significantly increased the latency time for the first seizure. Furthermore, dantrolene (20 and 40 mg/kg, but not 10 mg/kg) attenuated the severity of seizures in comparison to the vehicle group. Moreover, dantrolene only at the dose of 40 mg/kg prevented from tonic-clonic seizure and death in comparison to the vehicle group. In contrast, the addition of caffeine abolished the protective effects of dantrolene on the tonic-clonic seizure/death and inhibited the beneficial effects of dantrolene on the severity of pentylenetetrazol seizures. The acute dantrolene administration produced an anticonvulsant effect in the pentylenetetrazole-induced seizure. Moreover, caffeine prevented from dantrolene anticonvulsant effects. These results may imply about ryanodine receptors and intracellular calcium roles in the generation and control of pentylenetetrazole seizure.

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Keywords: Dantrolene; Ryanodine receptors; Caffeine; Pentylenetetrazole; Seizure

Introduction

Epilepsy is a common serious neurological disorder (1), which adversely affects the social, physical and neuropsychological well-being of sufferers (2). Currently, the great majority of patients benefit from drug therapy, albeit in about 30% epilepsy control is not satisfactory (3). Moreover, currently used antiepileptic drugs have important complications like serious side effects, drug interactions and limitations for use in especial populations (4). Therefore, it is an important necessity to find new drugs with enhanced efficacy and superior profile of safety (5). In line with this idea, new advances in the pathophysiology of epilepsy provide important clues about finding new anticonvulsant drugs.

It has been proposed that the intracellular calcium mobilization plays crucial roles in the pathophysiology and treatment of epilepsy (6). An important modulator of intracellular calcium is calcium-induced calcium release system (CICR) via ryanodine receptors (RyR) and inositol 1, 4, 5-trisphosphate (IP3) receptors (IP3Rs) (7). Recent evidence implies that functional abnormalities in RyR may be involved with the generation and maintenance of seizure (6,8). In this regard, caffeine, as a RyR agonist, lowers the threshold of convulsion in the patients and animal models of epilepsy (9). Moreover, it was suggested that some conventional antiepileptic drugs exert their effects, at least partly, via RyR associated CICR inhibition (10-11). Furthermore, inhibition of RyR-sensitive CICR activity may contribute to the suppression of neuronal detrimental events acutely after status epilepticus (8,11).

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Presently, dantrolene is the only approved drug that inhibits RyR on the endoplasmic reticulum in both neuronal and muscular cells (12). It blocks CICR from intracellular calcium stores (12). By considering RyR and calcium roles in the pathophysiology of seizure, dantrolene may be a potential drug to prevent seizure and its complications on the brain. In this regard, there are limited studies about anticonvulsant effects of dantrolene. However, in vitro and in vivo models imply about dantrolene ability to protect against seizure-induced neuronal death (13).

Animal models are useful tools in the epilepsy research for identification and screening of new antiepileptic drugs (14-15). In this line, a chemical convulsant agent like Pentylenetetrazole (PTZ) is widely used for the screening of new antiepileptic drugs (16). PTZ is generally considered as an accepted animal model for myoclonic seizure (17). Therefore, we aimed to clarify dantrolene effects, as a RyR antagonist and intracellular calcium modulator, in protecting against PTZ-seizures in mice.

Materials and Methods

Chemicals

Dantrolene, PTZ and Tween 80 were procured from Sigma, USA. We purchased diazepam and normal saline from Daru Pakhsh Pharmaceutical Co., Iran. Moreover, caffeine powder was a generous gift from Alhavi Pharmaceutical Co., Iran. Dantrolene was dissolved in saline and Tween 80 (1% V/V) while other substances dissolved in saline. Moreover, we used the solution of saline with tween (1% V/V) as the negative control. All compounds administered intraperitoneally (*i.p.*) 30 minutes before the PTZ injection. The solutions were prepared on a weight/volume basis on the day of use and administered with the volume of 0.1 ml/10 g of the animal body weight.

Animals

The experiment was carried out with the male albino Swiss strain of mice weighing between 25-40 g. Animals were housed in the Plexiglas cages (5 per cage), maintained in the controlled room (at 20-25° C and 12 h light/12 h dark cycles) with free access to food (standard rodent food) and water. We provided animals from the Bushehr University of Medical Science animal house, Bushehr, Iran. In total, 56 mice were randomly assigned into the seven separate groups (n=8). We used dantrolene (10, 20 and 40 mg/kg), caffeine (200 mg/kg), dantrolene (40 mg/kg)+caffeine (200 mg/kg) (dantrolene and caffeine were used at the same time but at the different injection sites), diazepam (5 mg/kg as a positive control) and vehicle 30 minutes before the injection of PTZ. The study was carried out under the approval of the Animal Ethics Committee of the Bushehr University of Medical Sciences, which is in accordance with the European Communities Council to minimize the quantity and suffering of animals.

PTZ-induced seizure

Mice were received i.p. injection of PTZ (80 mg/kg), moved into the separate cage and observed for 30 minutes. The seizure intensity was measured according to scale depicted from Ali *et al*., (18). The seizure scale has six stages as follow: (0) without any response, (1) twitches of mouth and facial, (2) myoclonic body twitching and nodding, (3) clonus of the forelimb, (4) dropping on the floor, rearing (uncoordinated movement), clonus locomotion of hindlimb and tonus of forelimb, (5) tonic-clonic seizure, status epilepticus and/or death. The maximal severity of convulsions was considered as the mouse seizure score. Moreover, we registered the latency time for the first seizure event and death, and the quantity of animals protected against PTZ-induced seizure and death.

Statistical analysis

We analyzed the severity of seizures, the latency of seizure onset and death via Kruskal-Wallis test followed by Mann-Whitney U-test as the post hoc paired comparisons. Fisher's exact test was used to assess the quantity of animals protected against PTZ-induced death. For the latency time of the first seizure, data were expressed as mean values±standard error of mean (SEM) while for the severity of seizures, median±upper, and lower quartiles was used. The significant level was considered as P.value of <0.05. Statistical analysis was carried out by the SPSS software version 18.

Results

Dantrolene effects on the PTZ-induced seizure

All animals experienced a seizure after treatment with PTZ (80 mg/kg, i.p.). Visual observation showed that dantrolene at doses of 20 and 40 mg/kg produced impaired locomotor activity. Acute administration of dantrolene (at doses of 10, 20 and 40 mg/kg) increased the latency time for the first seizure compared with the vehicle-treated group ($P<0.05$) (Figure 1). In addition, pretreatment with dantrolene (20 and 40 mg/kg but not 10 mg/kg) attenuated the severity of seizures ($P<0.05$)

(Figure 2) occurring after PTZ administration. Dantrolene only at the dose of 40 mg/kg prevented from PTZ-induced tonic-clonic seizure and death in comparison to the vehicle treated group (P<0.05) (Figure 3). Moreover, pretreatment of mice with diazepam (5 mg/kg i.p.), as a positive control, completely abolished seizures induced by PTZ.

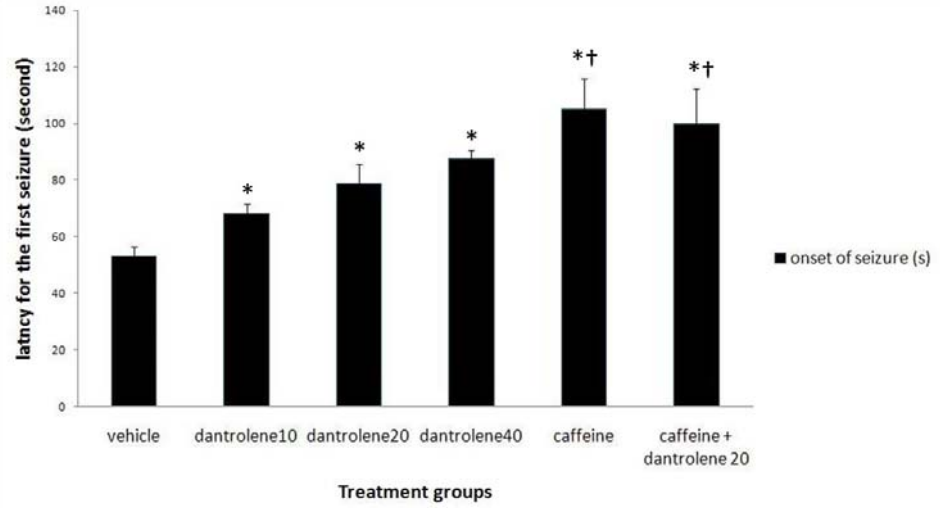

Figure 1. Dantrolene effects on the latency time for the first PTZ-induced seizures. All treatments were used 30 minutes before PTZ administration. The number of animals in each group was 8. Data was analyzed using Kruskal-Wallis rank followed by Mann-Whitney test. Data was presented as mean±standard error of means (SEM). P<0.05 was considered as significant value. * In comparison to the vehicle treated group and † in comparison to the dantrolene 20 mg/kg. (s:second).

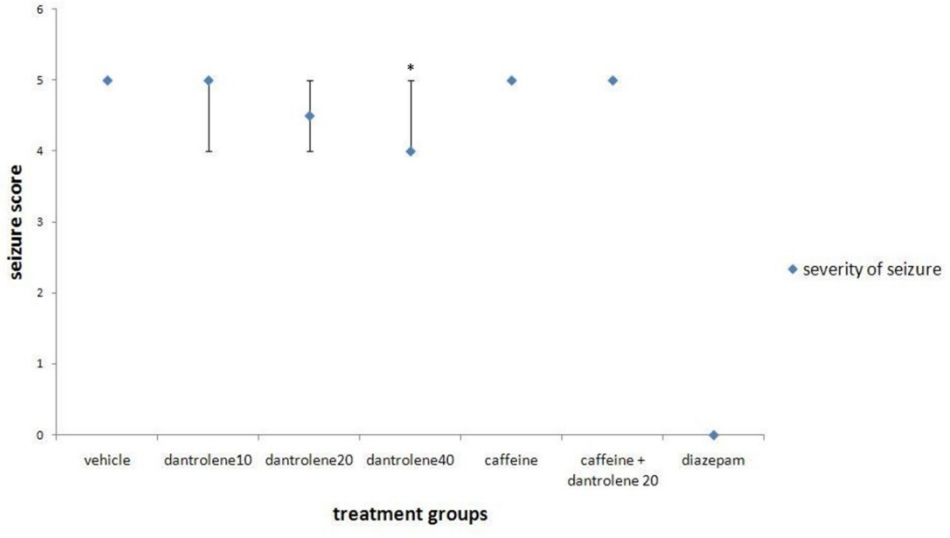

Figure 2. Dantrolene effects on the severity of PTZ-induced seizures. All treatments were used 30 minutes before PTZ administration. The number of animals in each group was 8. Data was analyzed using Kruskal-Wallis rank followed by Mann-Whitney test. Data was presented as median±upper and lower quartiles. * P<0.05 was considered as a significant value in comparison to the vehicle treated group.

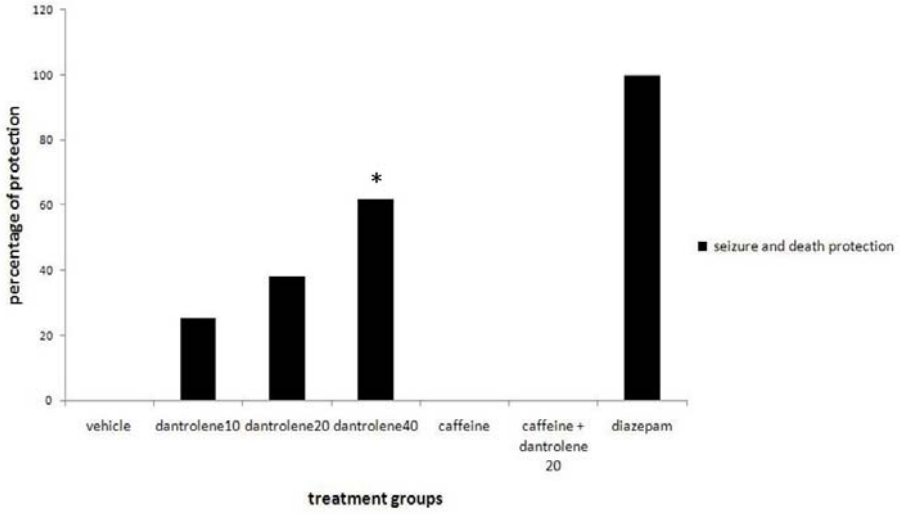

Figure 3. Protective effects of dantrolene against PTZ-induced tonic-clonic seizure and death. All treatments were used 30 minutes before PTZ administration. The number of animals in each group was 8. Data was analyzed using Fisher's exact test. Data was presented as a percentage of animals protected against tonic-clonic seizure and death. **P*<0.05 was considered as a significant value in comparison to the vehicle treated group.

Caffeine effects on the PTZ-induced seizure

All animals treated with caffeine experienced tonic-clonic seizure and death after PTZ administration. There was no difference between caffeine and vehicle groups with respect to the seizure severity (Figure 2). Moreover, the addition of caffeine reversed the protective effects of dantrolene on the tonic-clonic seizure and death (Figure 3). In addition, caffeine inhibited the beneficial effects of dantrolene on the severity of PTZ-seizure ($P<0.05$) (Figure 2). On the other hand, caffeine increased the latency time of the first clonic seizure induced by the PTZ in comparison to the vehicle and dantrolene-treated groups ($P<0.05$) (Figure 1).

Discussion

In our experiment, we used dantrolene, as a specific antagonist of RyRs and intracellular calcium modulator (13), to inhibit seizures produced by the PTZ. Dantrolene prolonged the latency time for the first clonic seizure and attenuated the severity of seizure in the mice. Moreover, dantrolene protected against PTZ-induced tonic-clonic seizure and death. There are some discrepancies between studies about anticonvulsant effects of dantrolene. In agreement with our results, dantrolene suppressed seizure in an animal model of complex partial seizure (19). Another study revealed that higher doses of dantrolene (62.5 to 500 mg/kg, i.p.) inhibited limbic seizures induced by a selective metabotropic glutamate receptor agonist (20). Furthermore, dantrolene prevented from kindling development in the rodents (21). On the contrary, it was revealed that dantrolene, at lower doses (5 to 20 mg/kg) did not alter the electroconvulsive threshold (22). This inconsistency between the studies may be attributed to the utilization of different animal models and dantrolene doses. Accordingly, it may be proposed that dantrolene is more effective against myoclonic and complex partial seizure models.

Blockade of calcium release by the mediation of RyRs is the main possible mechanism of action of dantrolene against the PTZ-induced seizure. As mentioned above, RyRs and IP3 receptors are important mediators for the regulation of intracellular calcium (7). It was suggested that abnormalities in the regulation of intracellular calcium by the mediation of RyRs may be connected with the induction and maintenance of seizure (23). In line with this idea, some convulsant agents like kainic acid have up-regulated RyR subtypes in the central nervous system of the rodents (8). Furthermore, it was proposed that RyRs may have contributed to the antiepileptic mechanism of action of sodium valproate and carbamazepine, two agents that are commonly used for the treatment of epilepsy (10-11,24). In parallel, it was documented that the PTZ-induced seizures may be related to the augmentation of intracellular calcium release from endoplasmic reticulum (25). Therefore, it

can be postulated that dantrolene attenuated PTZ-induced seizure by the inhibition of RyR-induced calcium release from the endoplasmic reticulum. Moreover, our study provided other support for the involvement of intracellular calcium in the development of PTZ-induced seizure.

In order to confirm this hypothesis, we used caffeine as an activator of RyR-mediated calcium release. In agreement with our hypothesis, caffeine suppressed dantrolene favorable effects in the PTZ-induced seizure in mice. In this regard, ithas been shown that caffeine abolished protective effects of some antiepileptic drugs in the PTZ model (26). Furthermore, it was shown that caffeine has reversed or reduced the protective effects of some antiepileptic drugs that their mechanism of action was very relevant to the RyRs (27). Although caffeine is not a selective RyR agonist (28), dantrolene produces selective effects on the RyRs. Therefore, it may be proposed that RyRs are the most probable mediator for the alteration of dantrolene anticonvulsant effects by the caffeine in the PTZ-induced seizure. Moreover, the results of our study provided further support for the pro-convulsive effects of caffeine.

There are three different isoforms of RyRs. RyR-1 is predominantly expressed in the skeletal muscle and in the cerebellar Purkinje neurons (29-30). RyR-2 is the most important isoform in the cardiac muscle while its expression in the brain is significant (30-31). Moreover, RyR-3 is expressed in the different tissues including mammalian brain (12,30). According to our experiment, it is not possible to show which RyR isoform(s) is (are) responsible for the dantrolene effects in the PTZ-seizure. However, it can be proposed that RyR-3 may be the most important mediator for the dantrolene effects against the PTZ-induced seizure. It was revealed that seizure provocation had increased RyR-3 isoform expression in the rodent brain (8). In parallel, in vivo studies suppose that dantrolene only inhibits RyR-1 and 3, while has no affinity for the type 2 of RyR (12). Therefore, it is tentative to assume that dantrolene exerts its antiepileptic effects by the inhibition of RyR-3.

Our experiment showed that caffeine delayed the latency time for the first PTZ-induced seizure. The exact mechanism for an explanation of this caffeine effect is not completely clear. However, it was shown that caffeine has an affinity to bind to the $GABA_A$ receptors (32). Simultaneously, it was proposed that the PTZ interaction with GABA receptors, at least partly, was responsible for the convulsive effects of this substance (33). Thus, it can be assumed that caffeine and PTZ, at least immediately after PTZ exposure, compete for the binding to the $GABA_A$ receptor and this might delay the convulsant effects of PTZ. In this regard, some evidence has shown that caffeine decreased susceptibility of rodents to the convulsant agents like PTZ and bicuculline (34). However, the majority of studies provides support for the proconvulsant effects of caffeine (35). Elucidation of this hypothesis needs more researches about caffeine mechanism of action in the PTZ seizure.

Dantrolene had protective effects against PTZ-induced seizures. Moreover, caffeine abolished beneficial effects of dantrolene against seizures produced by PTZ. These results suggest that RyRs and intracellular calcium may have an important contribution to the development and control of PTZ-induced seizure. Moreover, dantrolene may be a potential drug that can be considered for the control of myoclonic seizure.

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