

## Research paper

# Involvement of N-methyl-D-aspartate receptors and nitric oxide in the anticonvulsant effects of dantrolene against pentylenetetrazole-induced seizures in mice

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## ABSTRACT

**Objective:** N-methyl-D-aspartate (NMDA) receptors and nitric oxide (NO) have important roles in the pathology and treatment of pentylenetetrazole (PTZ)-induced seizures. We aimed to show the involvement of these two systems in the anticonvulsant effects of dantrolene against PTZ-induced seizures.

**Methods:** The male albino Swiss strain of mice (N = 56) randomly allocated to the seven separate groups and treated with dantrolene (40 mg/kg), dantrolene (40 mg/kg) + L-arginine (100 mg/kg, a NO donor), dantrolene (40 mg/kg) + N-Nitroarginine methyl ester (L-NAME) (100 mg/kg, a NO synthase inhibitor), dantrolene (40 mg/kg) + NMDA (50 mg/kg), dantrolene (40 mg/kg) + MK801 (1 mg/kg, a selective NMDA antagonist), Diazepam (5 mg/kg, the positive control) and saline (the negative control). Seizures were induced by intraperitoneal injection of PTZ (90 mg/kg). The onsets of clonic and tonic-clonic seizures, as well as the death of animals, were recorded.

**Results:** Dantrolene significantly increased the onset of clonic, tonic-clonic seizures and death of animals challenged with PTZ. The onset of tonic-clonic seizure in animals treated with dantrolene alone and dantrolene + L-NAME was higher than the control group. In contrast, the onset of tonic-clonic seizure in the animals treated with dantrolene + L-arginine was significantly lower than the dantrolene-treated group. The onset of clonic and tonic-clonic seizures in animals treated with dantrolene + MK801 were significantly higher than the control and dantrolene + NMDA groups.

**Conclusion:** Dantrolene protected animals against PTZ-induced seizures and mortality. The inhibition of NO synthase and NMDA receptors may contribute to the dantrolene anticonvulsant effects on the PTZ-induced seizure.

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## 1. Introduction

Dantrolene is a muscle relaxant which is mainly used for the treatment of patients with the lethal malignant hyperthermia.<sup>1</sup> This drug inhibits Ryanodine receptors (RyRs) and blocks calcium release from the intracellular store. RyRs are one of the most important modulators of intracellular calcium which are located in the sarcoplasmic reticulum membrane.<sup>2</sup> These receptors increase intracellular calcium via a mechanism called calcium-induced

calcium release system (CICR).<sup>3</sup> New evidence also shows that nitric oxide (NO)-induced calcium release is another mechanism for calcium modulation via RyRs in the neurons.<sup>4</sup>

A wide range of research findings has implied that calcium deregulation via RyRs has a plausible role in the generation and maintenance of epileptic seizures.<sup>5,6</sup> It has been shown that RyRs mutation may induce seizure in animals.<sup>7</sup> Moreover, therapeutic effects of some conventional antiepileptic drugs, at least in part, may be related to the modification of intracellular calcium via RyRs.<sup>8,9</sup> Caffeine, a ryanodine receptor agonist, particularly at the toxic doses lowers the threshold of seizure in animals and epileptic patients.<sup>10</sup> Furthermore, regulation of perturbed RyR-induced calcium homeostasis may suppress neuronal damage after status epilepticus.<sup>6,8</sup>

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New evidence also implies that dantrolene affects other processes like N-methyl-D-Aspartate (NMDA)-induced calcium release by mechanisms that are independent of RyR inhibition.<sup>11</sup> It has been also shown that dantrolene modulates NO production in the peripheral tissues.<sup>12</sup> Our previous study demonstrated dantrolene beneficial effects on the pentylentetrazole (PTZ)-induced seizures in mice.<sup>13</sup> However, the exact mechanism of action of dantrolene in the modulation of PTZ-induced seizures is not completely clear. By considering NMDA and NO roles in the pathology and treatment of PTZ-induced seizure,<sup>14–16</sup> we aimed to show the involvement of these two systems in the anticonvulsant effects of dantrolene against the PTZ-induced seizures.

## 2. Materials and methods

### 2.1. Chemicals

We procured dantrolene, PTZ, L-arginine, N-Nitroarginine methyl ester (L-NAME), MK801, and NMDA from Sigma (USA). Diazepam and normal saline were purchased from Daru Pakhsh Pharmaceutical Co. (Iran). All the chemicals were dissolved in saline and used intraperitoneally (i.p.) 30 min before the injection of PTZ. Freshly prepared solutions were administered 0.1 ml/10 g of animal body weight.

### 2.2. Animals and treatment groups

The study was approved by the local animal study ethics committee and was in accordance with the European Communities Council to lessen the number and suffering of animals. The male albino Swiss strain of mice (25–35 g) were purchased from the animal lab of the Isfahan University of Medical Sciences and housed in Plexiglas cages (5 per cage). Animals were maintained at the controlled temperature of 20–22 °C and regular dark/light cycles with free access to standard rodent food and water. We randomly allocated 56 mice to the seven separate groups (N=8). The treatment groups were as follow: 1) dantrolene (40 mg/kg), 2) dantrolene (40 mg/kg) + L-arginine (100 mg/kg, a NO donor), 3) dantrolene (40 mg/kg) + L-NAME (100 mg/kg, a non-selective NO synthase inhibitor), 4) dantrolene (40 mg/kg) + NMDA (50 mg/kg), 5) dantrolene (40 mg/kg) + MK801 (1 mg/kg, a selective NMDA antagonist), 6) Diazepam (5 mg/kg, the positive control), and 7) saline (the negative control). The doses of administered drugs were selected according to the previous study and a pilot study.<sup>13,17,18</sup>

### 2.3. PTZ-induced seizure

PTZ (i.p.) at a dose of 90 mg/kg was used to induce the clonic and tonic-clonic seizures in mice. After the administration of PTZ, animals were monitored for 30 min in a separate cage. The latency of the clonic and tonic-clonic seizures, as well as the death of animals, was recorded. We considered clonic seizure as a clonus of the animal body for more than 3 s with losing righting reflex.<sup>19</sup> The tonic-clonic seizure was defined as a clonus of the animal whole body accompanied with forelimb and hindlimb extension.<sup>19</sup> The number of animals protected against PTZ-induced seizures and mortality was also recorded.

### 2.4. Statistical analysis

The Shapiro-Wilk Normality Test showed that the variables deviated from the normal distribution. Thus, the Kruskal-Wallis test followed by Dunn's test was used to analyze the onset of the clonic and tonic-clonic seizures as well as the latency for the death of animals. The seizure and death protection ratio were analyzed using the Fisher's exact test. Data were analyzed by SPSS software

version 23 and reported as the mean  $\pm$  SEM. We considered the p-value of lower than 0.05 as the significant level.

## 3. Results

### 3.1. Protective effects of different treatments on the PTZ-induced seizures and death

Diazepam protected all animals against PTZ-induced seizures and death. Further, dantrolene protected 37.5% of animals against PTZ-induced mortality and this was higher than the mortality in the control, dantrolene + L-arginine and dantrolene + L-NAME groups ( $X^2(3)=9.93$ ,  $p=0.019$ ). The combination of dantrolene + MK801 protected 37.5% of animals against tonic-clonic seizures and this was higher than those of the control, dantrolene alone and dantrolene + NMDA groups ( $X^2(3)=9.93$ ,  $p=0.019$ ). Further, all of the animals treated with the MK801 were protected against PTZ-induced mortality.

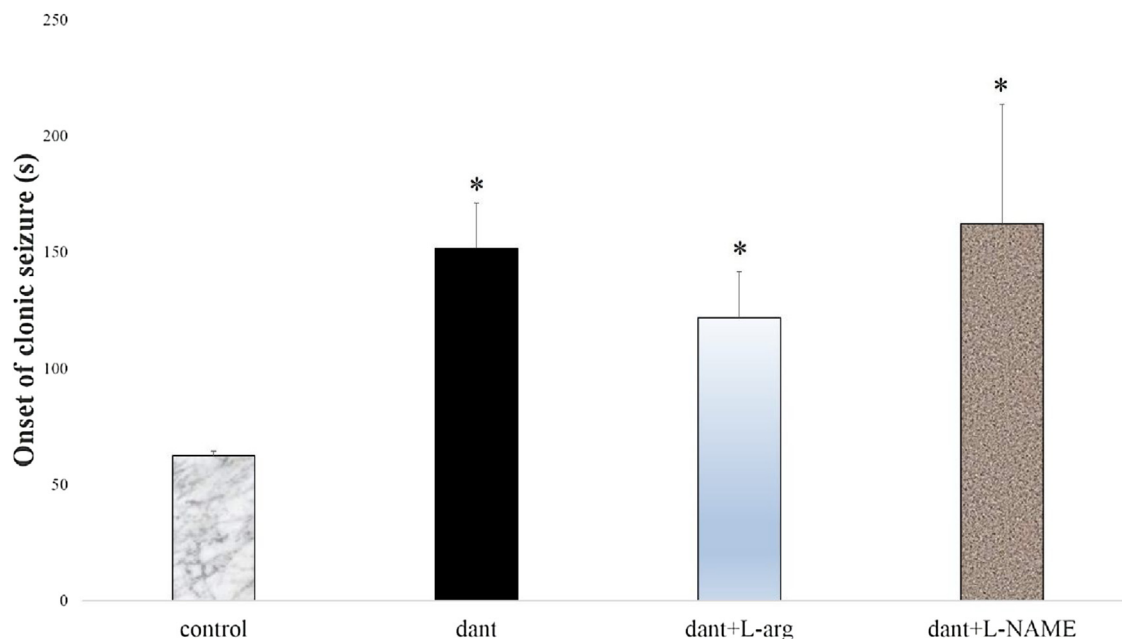
### 3.2. Contribution of NO modulators to the dantrolene anticonvulsant effects against PTZ

In the present study, the onset of clonic seizure ( $X^2(3)=18.42$ ,  $p=0.000$ ), tonic-clonic seizure ( $X^2(3)=19.15$ ,  $p=0.000$ ) and death ( $X^2(3)=16.98$ ,  $p=0.001$ ) were significantly different between animals treated with dantrolene with or without NO modulators and the vehicle-treated group. Pairwise comparison showed that the onset of the clonic seizure in the dantrolene ( $p=0.001$ ), dantrolene + L-arginine ( $p=0.024$ ) and dantrolene + L-NAME ( $p=0.002$ ) groups were significantly higher than the vehicle-treated group (Fig. 1). In contrast, there was no significant difference regarding clonic seizure between the dantrolene alone and dantrolene + L-arginine ( $p=1.000$ ) or dantrolene + L-NAME ( $p=1.000$ ) groups (Fig. 1).

The onset of the tonic-clonic seizure in animals treated with dantrolene alone ( $p=0.000$ ) and dantrolene + L-NAME ( $p=0.050$ ) was higher than the control group (Fig. 2). The onset of the tonic-clonic seizure in the animals treated with dantrolene + L-arginine was significantly lower than the dantrolene-treated group ( $p=0.043$ ) while was not significantly different from the vehicle-treated group ( $p=0.733$ ) (Fig. 2). Moreover, the time of the death of animals treated with dantrolene alone was significantly higher than the vehicle-treated group ( $p=0.000$ ) (Fig. 3). The latency for the death of animals treated with dantrolene + L-arginine ( $p=0.078$ ) or dantrolene + L-NAME ( $p=0.676$ ) was not significantly different from the control group (Fig. 3).

### 3.3. Contribution of NMDA modulators to the dantrolene anticonvulsant effects against PTZ

Our study showed that the onset of the clonic seizure ( $X^2(3)=24.51$ ,  $p=0.000$ ), tonic-clonic seizure ( $X^2(3)=21.95$ ,  $p=0.000$ ) and death ( $X^2(3)=12.77$ ,  $p=0.002$ ) were significantly different in animals treated with dantrolene, NMDA modulators, and the vehicle. Pairwise comparison showed that the onset of the clonic seizure in the animals treated with dantrolene + MK801 was significantly higher than the vehicle-treated group ( $p=0.005$ ) (Fig. 4). However, the onset of the clonic seizure in the animals treated with dantrolene + NMDA was not significantly different from the control group ( $p=0.624$ ) (Fig. 4). The onset of the tonic-clonic seizure in the animals treated with dantrolene + MK801 was significantly higher than the control group ( $p=0.000$ ) (Fig. 5). The onset of the tonic-clonic seizure in the dantrolene + NMDA group was significantly lower than the dantrolene alone group ( $p=0.013$ ) while was not significantly different from the control group ( $p=1.000$ ) (Fig. 5). Further, the time of death of animals treated



**Fig. 1.** Effects of dantrolene with and without nitric oxide modulators on the onset of pentylenetetrazole-induced clonic seizure.

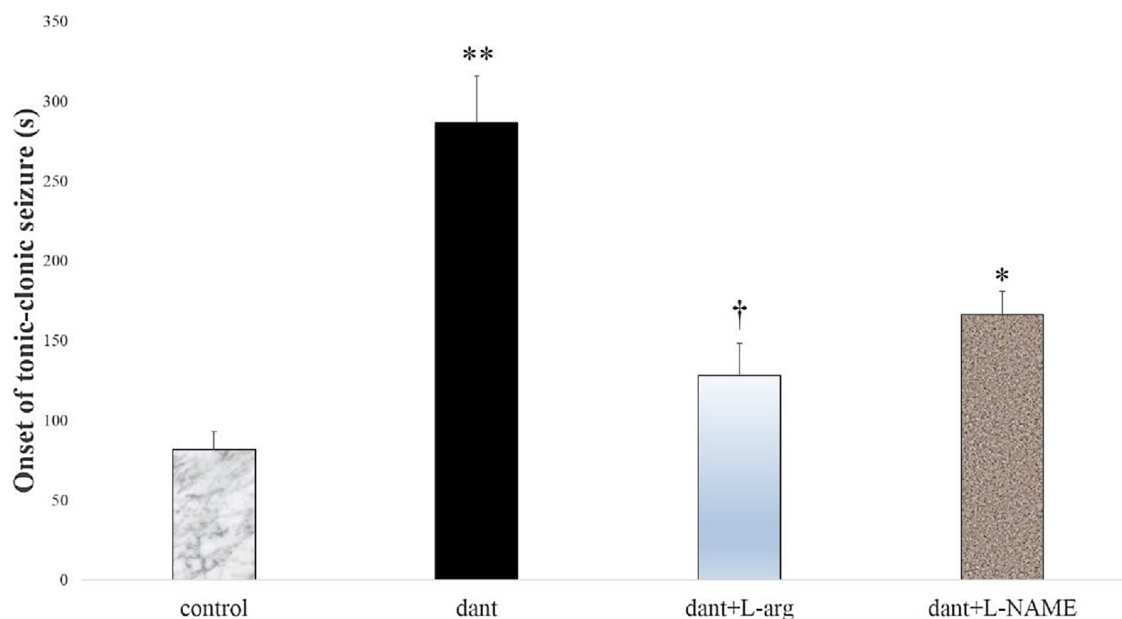
Animals treated with different agents 30 min before the injection of pentylenetetrazole (90 mg/kg) and monitored for the clonic seizure (a clonus of the animal body for more than 3 s with losing righting reflex). Data are presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed using Kruskal–Wallis test followed by Dunn's test. The p-value of  $<0.05$  was considered as the significant level. \* is  $p < 0.05$  compared with the control group. Dant: dantrolene, L-arg: L-arginine, L-NAME: N-Nitroarginine methyl ester, s: second.

with dantrolene + NMDA was not significantly different from the vehicle-treated group ( $p = 1.000$ ) (Fig. 6).

#### 4. Discussion

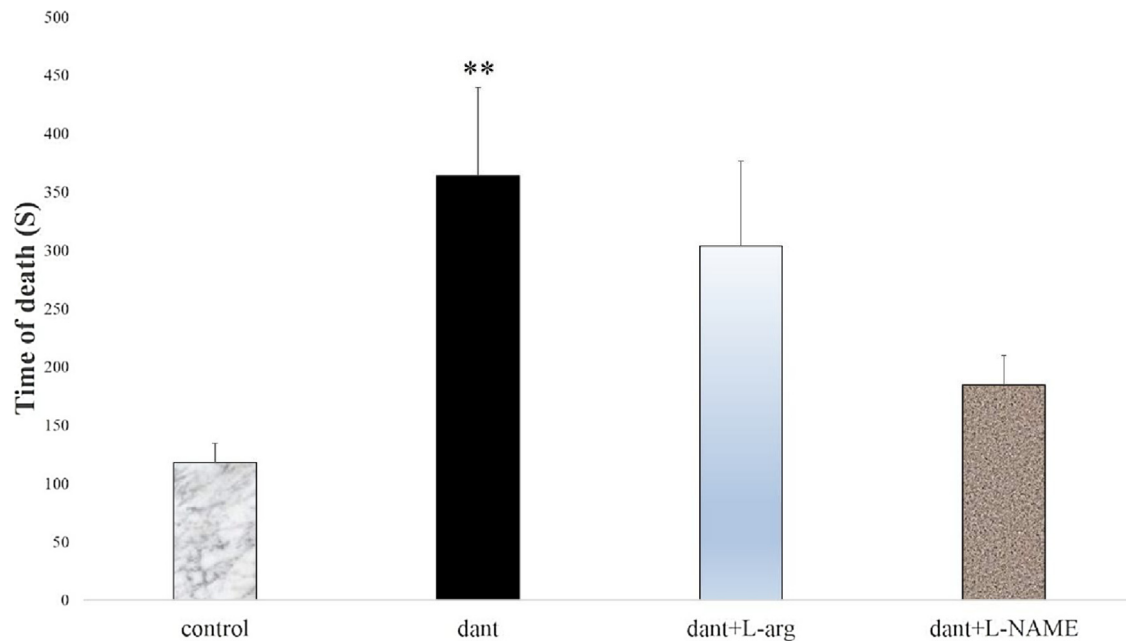
We previously showed that dantrolene increased the latency of PTZ-induced seizures in mice.<sup>13</sup> The present study confirmed the anticonvulsant effects of dantrolene against PTZ-induced seizures

and mortality. There are some controversies in the literature about the dantrolene anticonvulsant effects. These controversies may be related to the differences in seizure models and the doses of the administered drug. In line with our study, it has been shown that dantrolene inhibited seizure in a seizure susceptible EL mice.<sup>20</sup> Moreover, high doses of dantrolene suppressed seizures induced by intracerebral injection of a selective glutamate agonist.<sup>21</sup> In contrast, some reports have shown neuroprotective effects but not



**Fig. 2.** Effects of dantrolene with and without nitric oxide modulators on the onset of pentylenetetrazole-induced tonic-clonic seizure.

Animals treated with different agents 30 min before the injection of pentylenetetrazole (90mg/kg) and monitored for the tonic-clonic seizure (a clonus of the animal whole body accompanied with forelimb and hindlimb extension). Data are presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed using Kruskal–Wallis test followed by Dunn's test. The p-value of  $<0.05$  was considered as the significant level. \* is the  $p < 0.05$  compared with the control group and † is the  $p < 0.05$  compared with the dantrolene-treated group. Dant: dantrolene, L-arg: L-arginine, L-NAME: N-Nitroarginine methyl ester, s: second.

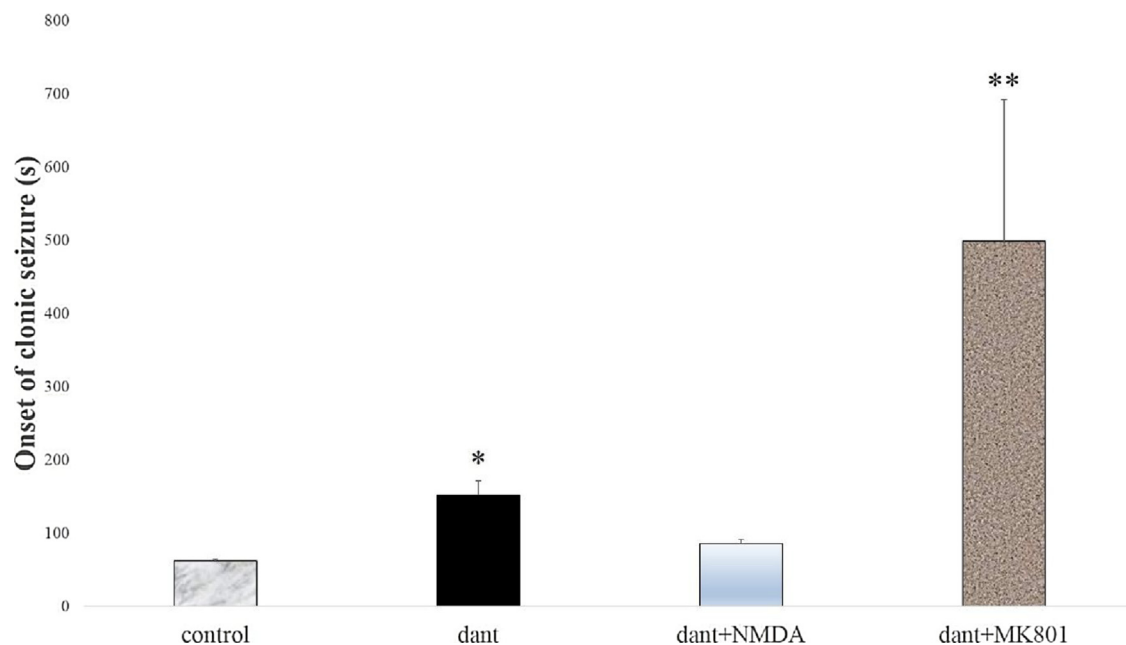


**Fig. 3.** Effects of dantrolene with and without nitric oxide modulators on the time of death of animals challenged with pentylenetetrazole. Animals treated with different agents 30 min before the injection of pentylenetetrazole (90 mg/kg). Data are presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed using Kruskal–Wallis test followed by Dunn's test. The p-value of  $<0.05$  was considered as the significant level. \* is  $p < 0.05$  compared with the control group. Dant: dantrolene, L-arg: L-arginine, L-NAME: N-Nitroarginine methyl ester, s: second.

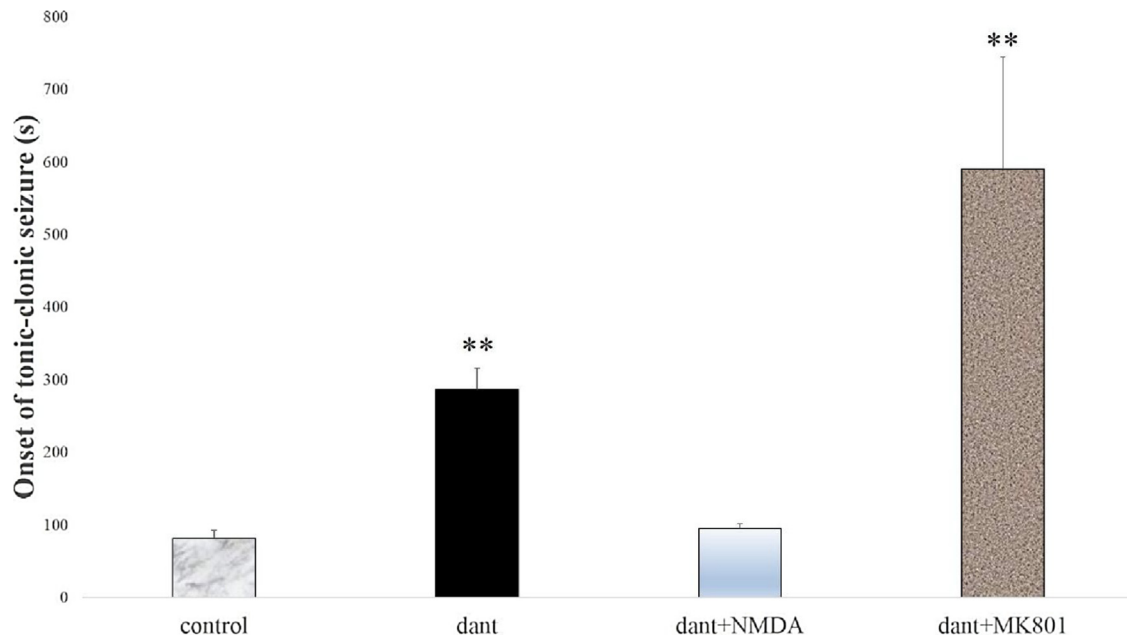
anticonvulsant effects of dantrolene in an animal model of seizure.<sup>22</sup> Our study may prove the anticonvulsant effects of dantrolene and added the contribution of NO and NMDA systems to its anticonvulsant effects.

Currently, dantrolene is the only agent for the treatment of malignant hyperthermia. The established mechanism of action of this agent is to inhibit RyRs and regulate intracellular calcium in the skeletal muscles.<sup>23</sup> Dantrolene also modulates intracellular

calcium release in neurons.<sup>24,25</sup> However, the function of RyRs and mechanism of dantrolene in the CNS should be further clarified. Independent studies from different groups have shown that neuroprotective effects of dantrolene may be related, at least in part, to its inhibitory effects on the NO and glutamate systems.<sup>26</sup> However, very limited studies are available regarding the interaction of these two systems with the dantrolene anticonvulsant effects in the animal models of seizure.



**Fig. 4.** Effects of dantrolene with and without N-Methyl-D-Aspartate modulators on the onset of pentylenetetrazole-induced clonic seizure. Animals treated with different agents 30 min before the injection of pentylenetetrazole (90 mg/kg) and monitored for the clonic seizure (a clonus of the animal body for more than 3 s with losing righting reflex). Data are presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed using Kruskal–Wallis test followed by Dunn's test. The p-value of  $<0.05$  was considered as the significant level. \* is  $p < 0.05$  and \*\* is  $p < 0.001$  compared with the control group. Dant: dantrolene, NMDA: N-Methyl-D-Aspartate, s: second.

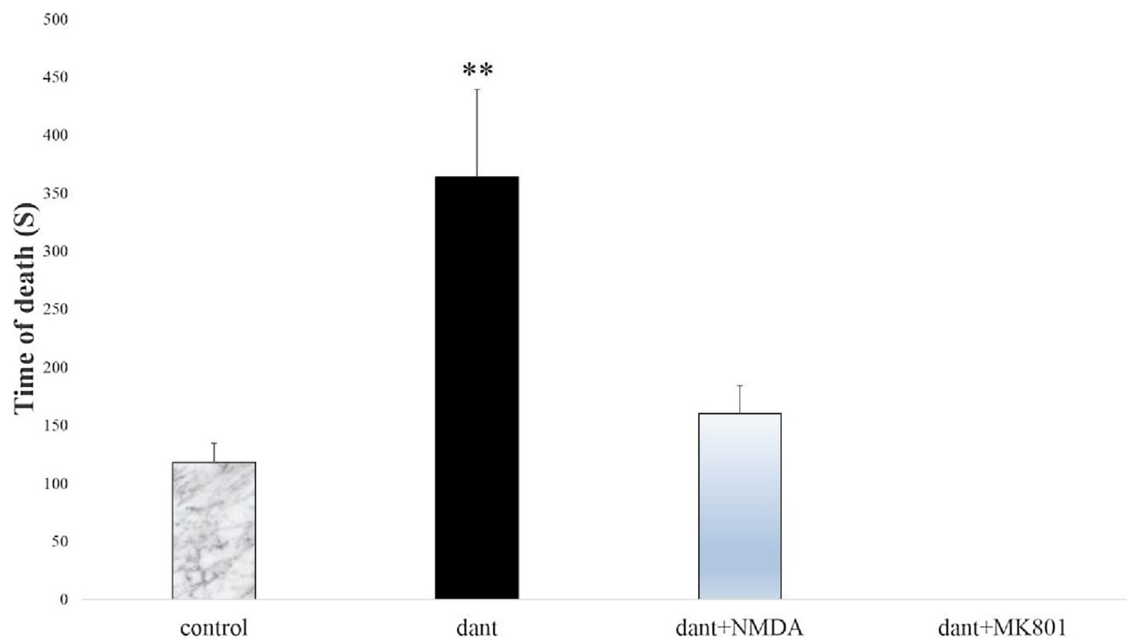


**Fig. 5.** Effects of dantrolene with and without N-Methyl-D-Aspartate modulators on the onset of pentylenetetrazole-induced tonic-clonic seizure.

Animals treated with different agents 30 min before the injection of pentylenetetrazole (90mg/kg) and monitored for the tonic-clonic seizure (a clonus of the animal whole body accompanied with forelimb and hindlimb extension). Data are presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed using Kruskal–Wallis test followed by Dunn's test. The p-value of  $<0.05$  was considered as the significant level. \* is  $p < 0.05$  and \*\* is  $p < 0.001$  compared with the control group. Dant: dantrolene, NMDA: N-Methyl-D-Aspartate, s: second.

NO is a very important intracellular messenger with an essential function in the brain.<sup>27</sup> New evidence also shows that NO affects RyRs and enhances intracellular calcium release in neurons.<sup>4</sup> Various reports have shown NO effects as a proconvulsant or anticonvulsant in the PTZ-induced seizures.<sup>28</sup> Our study showed that L-arginine, a NO donor, diminished inhibitory effects of dantrolene against PTZ-induced tonic-clonic seizure in mice. This may imply that dantrolene anticonvulsant effect, at least in

part, may be related to the inhibition of NO synthesis in neurons. In vitro studies have demonstrated that NO enhances the probability of RyR1 opening,<sup>29</sup> and increase the intracellular calcium leak from skeletal muscle in the pathological conditions.<sup>30,31</sup> Further studies have shown that NO mediates intracellular calcium release from neurons.<sup>4</sup> Thus, it is possible to assume that NO donors may counteract dantrolene inhibitory effects on the calcium release in neurons in the PTZ-induced tonic-clonic seizure. In this regard, it



**Fig. 6.** Effects of dantrolene with and without N-Methyl-D-Aspartate modulators on the time of death of animals challenged with pentylenetetrazole.

Animals treated with different agents 30 min before the injection of pentylenetetrazole (90mg/kg). Data are presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed using Kruskal–Wallis test followed by Dunn's test. The p-value of  $<0.05$  was considered as the significant level. \* is  $p < 0.05$  and \*\* is  $p < 0.001$  compared with the control group. Dant: dantrolene, NMDA: N-Methyl-D-Aspartate, s: second.



has been shown that dantrolene counteracts endotoxin-induced NO rising in the peripheral tissues.<sup>12</sup> However, Nagatomo et al.<sup>20</sup> have shown that dantrolene had no effect on the NO production in the seizure susceptible mice. The inconsistency between our study and Nagatomo investigation may be related to the model differences. However, the effects of dantrolene on the NO in neurons needs to be further elucidated.

Some reports have implied that RyRs interaction with NMDA receptors. This interaction may contribute to the calcium deregulation induced by different neurotoxic insults and may lead to the neuronal injury.<sup>25,32,33</sup> It has been shown that dantrolene blocks the intracellular calcium elevation and neurotoxicity induced by NMDA or glutamate.<sup>25,32,33</sup> However, there is very limited information about the interaction of dantrolene with NMDA receptors in the epileptic seizure. Our study showed that NMDA inhibited dantrolene effects against PTZ-induced seizure. Some evidence has implied that dantrolene may reduce intracellular calcium by inhibiting NMDA receptors rather than the blockade of RyRs.<sup>11</sup> In this line, dantrolene suppressed NMDA-induced intracellular calcium rise in the cultured hippocampal neurons.<sup>33</sup> However, receptor binding and patch-clamp studies did not show any direct interaction of dantrolene with NMDA receptors.<sup>25,32</sup> Therefore, dantrolene interaction with NMDA receptors in the PTZ-seizure may result from the opposite effects of these two agents on the intracellular calcium.

The main limitation of this study may be the use of a non-selective inhibitor of NO synthase (NOS). However, we wanted to screen dantrolene effects on the NO in the PTZ-induced seizures. Thus, we propose to use selective inhibitors of NOS isoforms in the future studies. Further, measuring brain NO level may help to understand dantrolene direct effects on the NOS. It is also proposed to explore the contribution of NO/NMDA-induced calcium release to the seizures induced by PTZ in the future studies.

## 5. Conclusion

Taken together, dantrolene protected against PTZ-induced seizures and mortality. The inhibition of NOS and NMDA receptors may contribute to the dantrolene anticonvulsant effects on the PTZ-induced seizure. However, it is unclear that the observed effects were produced from the direct interaction of dantrolene with NMDA receptor and NOS or may result from the RyR interaction with these two systems.

## Author's contribution

M. Keshavarz: conception and design, analysis data, drafting of the manuscript, critical revision of manuscript, final approval of manuscript.

Fatemeh Heidary: conception and design, collecting data, drafting of manuscript, critical revision of manuscript, final approval of manuscript.

Samad Akbarzadeh: conception and design, collecting data, drafting of manuscript, critical revision of manuscript, final approval of manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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