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Original article

A potential mechanism for the ameliorative effect of thymoquinone on pentylenetetrazole-induced kindling and cognitive impairments in mice

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ABSTRACT

Cognitive dysfunction is commonly observed in epileptic patients. Pentylenetetrazole (PTZ) kindling is a well established animal model which simulates clinical epilepsy. This study evaluated the potential role of glutamate, oxidative stress and nitric oxide (NO) overproduction in pentylenetetrazole (PTZ)-induced kindling and associated cognitive impairments in mice and effect of thymoquinone on these parameters. Repeated treatment of mice with a subconvulsive dose of PTZ (35 mg/kg i.p.) once every alternate-day for 12 injections induced kindling. PTZ-kindled mice showed learning and memory impairments as assessed by acquisition and probe trials of Morris water maze and step-through latency of passive avoidance tests. Concurrently, the brain glutamate, malondialdehyde and nitrite levels were increased while the brain intracellular reduced glutathione level and glutathione peroxidase activity were decreased in PTZ-kindled mice. Also, the brain inducible but not neuronal NO synthase mRNA and protein expressions were increased in PTZ-kindled mice.

Treatment of mice with thymoquinonne (5, 10 and 20 mg/kg i.p.) along with alternate-day subconvulsive dose of PTZ produced dose-dependent protection against PTZ-induced kindling and learning and memory impairments. Moreover, treatment of mice with thymoquinonne (20 mg/kg) inhibited the biochemical alterations induced by PTZ in the brain except the elevation of brain glutamate level. The associated increase in brain inducible NO synthase mRNA and protein expressions were also inhibited. These results suggest that glutamate, and subsequent oxidative stress and NO overproduction, via inducible NO synthase, play an important role in the pathophysiology of PTZ-induced kindling and cognitive impairments in mice. Thymoquinone dose-dependently protects against PTZ-induced kindling and cognitive impairments. Inhibition of PTZ-induced brain oxidative stress and NO overproduction, via increase the expression and activity of inducible NO synthase, may play an important role in thymoquinone action.

1. Introduction

Cognitive impairments have long been known to accompany epilepsy [1]. PTZ-kindling is a well established animal model which simulates clinical epilepsy. In addition, induction of PTZ kindling was found to impair learning and memory in experimental animals [2,3]. Glutamate has been presumed to play a crucial role in the production of kindling seizures [4]. This effect is possibly mediated via the excessive stimulation of N-methyl-D-aspartate (NMDA) – receptor subtype [5]. Furthermore, Zaitsev et al. [5] found that blockade of NMDA glutamate receptors prevents PTZ-induced convulsions in rats.

Activation of the NMDA receptor by glutamate results in an increase in nitric oxide (NO) synthesis by NO synthase [6]. Many studies examining the role of NO in epileptogenesis provide controversial evidence with either anticonvulsant [7] or proconvulsant [8] effects in different seizure paradigms. Moreover, PTZ-induced kindling in experimental animals is associated with an increase in brain levels of NO [9,10]. The release of NO via inducible NO synthase or neuronal NO synthase plays an important role in PTZ-induced seizures in mice [11,12]. However, in PTZ-induced clonic seizures in mice, the role of neuronal NO synthase is

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prominent [13] but, inducible NO synthase/NO pathway was found to be involved in the hypersusceptibility to PTZ-induced seizures following hypoxia/ischemia in mice [14].

Overactivation of glutamatergic system is accompanied by increased formation of reactive oxygen species [15]. Several studies have reported that oxidative stress in different brain regions is implicated in experimental seizures and associated neuronal damage [16]. PTZ-kindled animals exhibited marked oxidative stress in the brain, leading to abnormal structural alterations of cellular proteins, membrane lipids, and genetic materials [10,17]. There is a correlation between increased brain glutamate level and cognitive impairments in experimental animals [4,18]. In addition, a number of observations support the hypothesis that elevation oxidative stress and NO overproduction in the brain might contribute to the development of cognitive dysfunction in experimental animals [19,20].

Thymoquinone, the major active component of the volatile oil of *Nigella sativa* seed, has been reported to afford neuroprotection against 6-hydroxydopamine [21] and acrylamide [22]–induced neurotoxicity in rats via its antioxidant activity and inhibition of NO production. Also, thymoquinone clearly protects brain tissue from radiation-induced nitrosative stress in rats [23]. Furthermore, thymoquinone attenuates cisplatin-induced hepatotoxicity [24] and methotrexate-induced hepatorenal damage [25] in rats via inhibiting oxidative stress and nitrosative stress manifested by increased NO with upregulation of inducible NO synthase

On the basis of these evidences, we studied the relation between PTZ-induced kindling and changes in cognitive functions in mice. In addition, the possible role of glutamate, oxidative stress, NO and NO synthase isoforms in these effects were determined. Moreover, the ameliorative effects of thymoquinone on these parameters were assessed.

2. Materials and methods

2.1. Animals

Male adult Swiss–Webster mice weighing 22–28 g from the animal house of Assiut University were used in all experiments. The animals were housed in stainless steel cages under a 12 h light/dark cycle at 25 °C and allowed water and food (laboratory chow) *ad libitum*. They were divided into groups, 16 animals each. The research was conducted in accordance with the internationally accepted principles for Guide for the Care and Use of Laboratory Animals. The experiments reported here were approved by our institutional ethics committee.

2.2. Chemicals

Pentylenetetrazole, thymoquinone, L-glutamic acid, ß-nicotinamide adenine dinucleotide and Ellman's reagent were obtained from Sigma-Aldrich Co. (USA). Malondialdehyde-bis (dimethylacetal) was purchased from Merk (Germany). All other chemicals were of analytical grade.

2.3. Induction of kindling

Kindling was elicited in all mice by repeated intraperitoneal (i.p.) injections of a subconvulsive dose of PTZ (35 mg/kg, 1% solution in saline) once every 48 h. Mice were observed for 30 min after each PTZ injection. Seizure intensity was evaluated using the following modified scale [26]: Stage 0 (no response); Stage 1 (hyperactivity and ear and facial twitching); Stage2 (head nodding and myoclonic body jerks); Stage 3 (forelimb clonic seizures); Stage 4 (generalized clonic seizures with falling); Stage 5 (generalized clonic – tonic seizures). The animals were considered to be kindled after having reached at least three consecutive stage 4 or stage 5 seizures. Kindled mice retain their convulsive behavior for a long time (21 days after withdrawal of PTZ injections). Later a single subconvulsive dose of PTZ (35 mg/kg) can provoke clonic-tonic convulsion within this period.

2.4. Treatment of animals

Animals of group-I were kindled mice (treated with PTZ only). Group-II, III and IV mice were treated with thymoquinone [1% solution in normal saline in the presence of 0.1% (v/v) tween 80] intraperitoneally (i.p.), at dose levels of 5, 10 and 20 mg/kg, respectively, along with alternate-day PTZ. Control groups of animals were treated likewise with the pure vehicle. Behavioral parameters were evaluated after 24 h of the last PTZ injection. The animals of group-I (treated with PTZ only), IV (treated with 20 mg/kg thymoquinone + PTZ) and control group were divided into 2 equal subgroups. One subgroup of animals from each group was used for each behavioral test. The animals utilized in a certain test were not evaluated in any other one to avoid any contribution of the animal's experience or training in the results of other tests.

2.5. Behavioral tests

2.5.1. Morris water maze test

The water maze was a circular stainless steel tank (85 cm in diameter and 25 cm in height). The tank was divided into 4 equal quadrants with four starting locations called north, east, south and west at equal distance on the rim. The tank was located in an experimental room containing several extramaze cues (e.g. pictures, lamps, etc., which could be used by the mice for spatial orientation) and filled with water (25± 2 °C). An escape platform (10 × 10 × 10) was inserted and placed in a constant quadrant of the pool throughout the trials at a level of 1.5 cm below the water surface. Powdered milk, or non-toxic white paint was used to make the water opaque. Immediately prior to behavior testing, the mice were allowed an adaptation period of 10–15 min in the pool to adapt white light. Before removing from the pool, each mouse was then given 1 min upon the platform. Each mouse was then placed into the water at a start point in the middle of a quadrant not containing the escape area with its head pointed to the side of the pool. Each mouse was given daily four trials from each 3 starting positions with 5 min intertrial intervals for 6 successive days (acquisition trials). For each trial, the latency to reach the platform, the distance traveled and swimming speed were evaluated. If the mouse failed to find the escape platform within 90 s, it was manually guided to the platform by the experimenter and allowed to stay on it for 30 s. Twenty-four h after the previous trials, the probe trials were carried out. In these trials the water pool was set without the escape platform. Each mouse was released with its head pointed towards the side of the water pool and allowed it to swim freely for 90 s before the end of the session. The latency to reach the target quadrant (that previously contained the platform) and the time each mouse spent in it were calculated in these probe trials. During the different versions of the test, the animals were monitored by a digital camera fixed above the maze.

2.5.2. Passive avoidance test

The step-through cage consisted of 2 compartments (22 × 22 × 25 cm each) separated by a partition containing a communicating hole (8 cm in diameter). The start compartment was kept illuminated by a light fixture fastened to the cage lid. The escape compartment was dark and its floor bars, wired to a constant current scrambler circuit. The test consisted of an acquisition and retention trials and was performed in 2 consecutive days. In acquisition trials, each mouse was placed individually in the