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Potential Mechanisms Involved in the Anticonvulsant Effect of Walnut Extract on Pentylenetetrazole-Induced Seizure

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Key Words
Walnut kernel · Seizure · Rat · Anticonvulsant effect · Pentylenetetrazole

Abstract
Objective: It was the aim of this study to determine the potential effect of walnut kernel extract (WKE) on experimentally induced seizures in rats and to evaluate the role of benzodiazepines and ethosuximide (ESM) within these pathways. ***Materials and Methods:*** Male Wistar rats were selected and divided into eight groups. Seizures were evoked by intravenous infusion of pentylenetetrazole (PTZ; 2 mg/ml/min). In combination with PTZ, animals were treated with vehicle or WKE (100 mg/kg i.p.), with or without cotreatment with either flumazenil (FMZ; 5 mg/kg i.p.), ESM (150 mg/kg i.p.) or diazepam (DPZ; 0.5 mg/kg i.p.). ***Results:*** WKE administration significantly increased the PTZ dose needed to induce the first myoclonic jerk (13.09 ± 1.29 vs. 49.71 ± 12.03 mg/kg; $p < 0.001$), decreased the severity of seizure grades and reduced the mortality rate to 0%. FMZ did not significantly reduce the anticonvulsant effect of WKE. The combination of DPZ and WKE showed a synergic anticonvulsant effect, whereas ESM had no significant influence ($p > 0.05$) on the WKE effects. ***Conclusion:*** These findings indicated that WKE was effective at reducing seizure severity, at increasing the dose to the first myoclonic jerk and highly efficacious at preventing mortality, because 100% of animals were protected. It seems that this positive effect could apply through signaling pathways other than benzodiazepine-mediated γ-aminobutyric acid receptors and may at least in part be similar to ESM.

Introduction

Epilepsy is one of the most common central nervous system disorders, and uncontrolled seizures increase the comorbidities and the chance of mortality [1]. Antiepileptic drugs only provide symptomatic treatment as they suppress seizures but do not have the ability to cure the disease [2]. There is continual research focusing on new therapeutic approaches to prevent and treat epileptic seizures.

The walnut tree (*Juglans regia* L.) is cultivated throughout Eastern Asia, Southern Europe, Northern Africa, and the United States of America [3]. The Walnut kernel (WK) accounts for 40–60% of the nut weight. It has high levels of oil (52–70%) in which polyunsatu-

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rated fatty acids predominate [4]. WKs are enriched with many health-beneficial nutrients, especially Ω-3, Ω-6 and Ω-9 fatty acids that are essential for optimum health [4]. Previously, it was demonstrated that WK pretreatment has anticonvulsant and neuroprotective effects [5]. Other studies have also shown that WK consumption may delay the kindling procedure and attenuates the amygdala-kindled seizures in rats [6, 7]. Hence, the present study was conducted to evaluate the potential of WK extract (WKE) to suppress pentylenetetrazole (PTZ)-induced seizures in rats, focusing on the γ-aminobutyric acid (GABA)ergic system as the most important inhibitory pathway in the central nervous system as well as on the ethosuximide (ESM) performance pathway to clarify the probable mechanisms.

Materials and Methods

Drugs

The walnuts were collected from the Rabor area, Kerman Province, Iran, in September 2010. A voucher specimen was deposited at the herbarium of the Faculty of Pharmacy, Kerman University of Medical Sciences (No. 1401-1). PTZ hydrochloride, diazepam (DPZ), flumazenil (FMZ), ESM and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). PTZ, DPZ and FMZ were dissolved in saline (0.9% NaCl) immediately before use.

WK Extract

A 20-gram sample of Iranian walnuts *(J. regia)* was homogenized in 50 ml of methanol and centrifuged at 10,000 *g* for 5 min. After decanting the liquid, the remaining supernatant was dried under a nitrogen atmosphere to remove methanol, resuspended in 10 ml of 25 mM Tris-HCl, pH 7.5, and filtered through a Millipore 0.22-μm syringe-driven filter unit (Millipore, Billerica, Mass., USA). The prepared extract was stored in glass vials at –20°C prior to use.

Animals

Ninety-six male Wistar rats, 3 months old and weighing 250–300 g, were used in this study. The animals were housed in a temperature-controlled room (20 ± 2°C) in groups of 3 per cage and had free access to chow and water. The rats were divided into 8 groups (12 in each) and received drugs as follows: (1) PTZ (control group) 2 mg/ml intravenous infusion; (2) DMSO (the WKE vehicle, 0.1 ml i.p., 30 min before PTZ infusion) + saline (0.1 ml i.p., 10 min before DMSO administration) + PTZ; (3) WKE (100 mg/kg i.p., 30 min before PTZ infusion) + saline + PTZ; (4) WKE + FMZ (5 mg/kg i.p., 10 min before WKE administration) + PTZ [8]; (5) WKE + ESM (150 mg/kg i.p., 10 min before WKE administration) + PTZ [8]; (6) WKE + DPZ (0.5 mg/kg i.p., 10 min before WKE administration) + PTZ [9]; (7) ESM + PTZ, and (8) DPZ + PTZ.

Animal experiments were performed in accordance with the Ethics Committee Guidelines of Kerman University of Medical Sciences (No. 89/123KA).

WKE Dose Selection

A pilot study with 4 different doses of WKE (50, 100, 200 and 500 mg/kg) was done for dose selection. Despite the dose-dependent anticonvulsant effect of WKE, the dose of 100 mg/kg, the minimum dose which showed a significant anticonvulsant effect, was used for the experiment.

Convulsion Test

To induce convulsion, 30 min after injection of 100 mg/kg of WKE and other drugs based on a designed protocol for each group, PTZ was infused using a method described in a previous study [10]. Briefly, the rat was restrained for the insertion of a 22-gauge angiocath, connected by an appropriate tube to a syringe containing the drug, into the lateral vein of the tail. Then, the rat was released to a plexiglass cage and allowed free movement. The PTZ solution, containing 2 mg/ml of PTZ, was infused at a constant rate of 1 ml/min using a syringe pump, and the rat was observed during the infusion period [10]. Convulsion severity was scored as follows: stage 0, no change in behavior; stage 1, ear and facial twitching; stage 2, isolated myoclonic jerks; stage 3, clonus of the forelimbs, neck and/or head; stage 4, clonus of the forelimbs, neck and/or head with rearing and falling; stage 5, generalized clonic seizures (GCS; without the tonic phase) beginning with running and followed by loss of righting reflex [11]. The onset of the first myoclonic jerk was considered as seizure threshold, and with the onset of generalized tonic-clonic convulsions, the infusion was ended [10]. The durations of the convulsant infusion necessary to observe a seizure threshold and generalized tonic-clonic convulsion were measured. The amount of convulsant agent required for induction of the threshold or clonic convulsions was calculated by the following parameters: concentration of the convulsant in the injected liquid, infusion duration, infusion rate, and animal weight [10]. If animals did not show a seizure and/or clonic convulsion after receiving the 80 mg/kg of PTZ [9], the PTZ injection was stopped and the threshold and/or clonic dose of PTZ was considered as 100 mg/kg. In addition, animals were excluded from the study if the tail vein angiocath insertion was not successful for intravenous infusion of PTZ, or if leakage of injected fluid occurred under the skin of the animal.

Statistical Analysis

Threshold values and clonic doses of PTZ are presented as means ± SEM. Comparisons of threshold and clonic doses of PTZ among different groups were performed using the Kruskal-Wallis test followed by the post hoc Bonferroni test. Severity scores are presented as medians. Differences in the severity of convulsions were determined by the Kruskal-Wallis and the Mann-Whitney test, and in mortality by Fisher's exact test. A p value <0.05 was considered statistically significant.

Results

Threshold doses, i.e. the required values of PTZ for the induction of a first myoclonic jerk, in the different groups are shown in figure 1. DMSO and saline did not have a significant effect on the threshold dose of PTZ ($p > 0.05$). Pretreatment with WKE significantly increased the threshold dose compared with the PTZ group and the

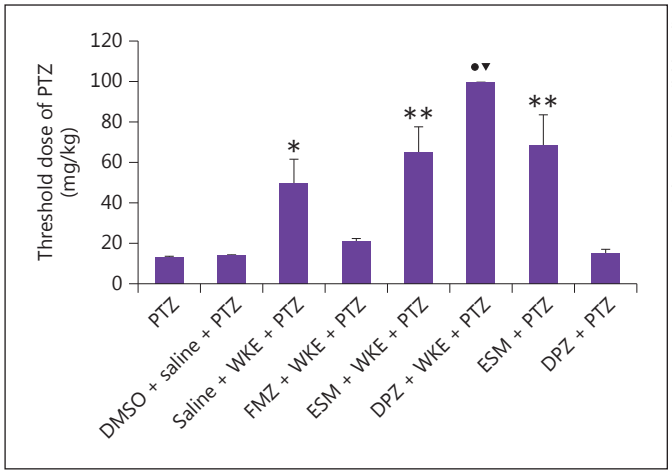

Fig. 1. Required doses of PTZ for the induction of threshold convulsions in the different animal groups. Data are presented as the mean ± SEM (n = 8–11). * $p < 0.05$ versus the PTZ and DMSO + saline + PTZ groups. ** $p < 0.01$ versus the PTZ, DMSO + saline + PTZ and DPZ + PTZ groups. ● $p < 0.001$ versus the PTZ, DMSO + saline + PTZ, DPZ + PTZ and FMZ + walnut + PTZ groups. ▼ $p < 0.01$ versus the saline + walnut + PTZ group.

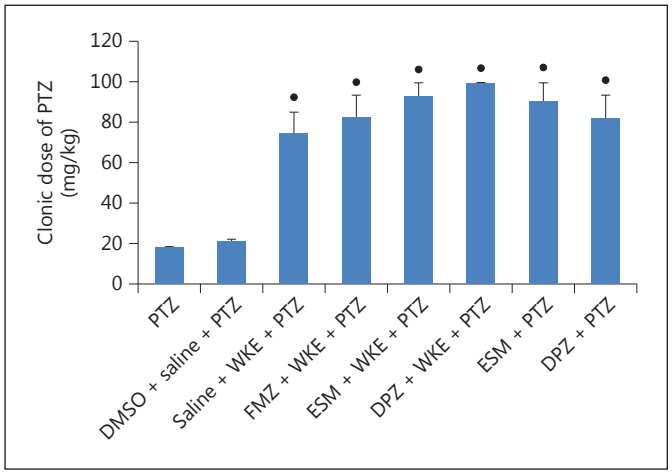

Fig. 2. Required doses of PTZ for the induction of GCS in the different animal groups. Data are presented as the mean ± SEM (n = 8–11). ● $p < 0.001$ versus the PTZ and DMSO + saline + PTZ groups.

PTZ + DMSO + saline group ($p < 0.05$). Coadministration of WKE with FMZ did not change the threshold dose. However, injection of WKE + DPZ amplified the anticonvulsant effect of WKE and increased the threshold dose ($p < 0.001$ compared to the PTZ, WKE + PTZ, WKE + FMZ + PTZ, and PTZ + DZP groups). ESM administration also enhanced the effect of WKE but there was no significant difference between the threshold doses of ESM alone or in combination with WKE (fig. 1).

Color version available online

Table 1. Convulsion severity and mortality rate in the different animals groups (n = 8–11)

Groups	Convulsion severity	Mortality
PTZ (n = 9)	5 (5–5)	9 (100%)
DMSO-saline-PTZ (n = 9)	5 (5–5)	9 (100%)
Saline + WKE + PTZ (n = 11)	4 (0–5)[a, b]	0 (0%)
FMZ + WKE + PTZ (n = 8)	3.5 (1–5)[c, d]	0 (0%)
ESM + WKE + PTZ (n = 11)	0 (0–5)[e]	0 (0%)
DPZ + WKE + PTZ (n = 8)	0 (0–0)[f]	0 (0%)
ESM + PTZ (n = 8)	0 (0–5)[g]	1 (12.5%)
DPZ + PTZ (n = 8)	4 (1–5)[h]	2 (25%)

Seizure severity is expressed as the median, with ranges in parentheses. [a] $p < 0.05$ versus the PTZ group. [b] $p < 0.05$ versus the DMSO + saline + PTZ group. [c] $p < 0.05$ versus the DMSO + saline + PTZ and ESM + walnut + PTZ groups. [d] $p < 0.05$ versus the DMSO + saline + PTZ group. [e] $p < 0.01$ versus the DMSO + saline + PTZ, FMZ + walnut + PTZ and DPZ + PTZ groups. [f] $p < 0.001$ versus the DMSO + saline + PTZ, saline + walnut + PTZ, FMZ + walnut + PTZ and DPZ + PTZ groups. [g] $p < 0.01$ versus the DMSO + saline + PTZ, FMZ + walnut + PTZ and DPZ + PTZ groups. [h] $p < 0.01$ versus the DMSO + saline + PTZ and DPZ + walnut + PTZ groups.

Administration of WKE significantly increased the PTZ dose for GCS induction ($p < 0.001$ vs. the PTZ group and the PTZ + DMSO + saline group). Moreover, there was no significant difference between the doses of PTZ for GCS in the presence of WKE alone or combined with FMZ or ESM or DZP. In addition, the PTZ dose of GCS in the PTZ + ESM and PTZ + DZP groups in the presence or absence of WKE did not show a significant difference (fig. 2).

The severity of convulsions was significantly reduced by WKE ($p < 0.05$ vs. the PTZ group and the PTZ + DMSO + saline group). This effect of WKE was not affected by FMZ. Furthermore, jerky movements were not seen in the WKE + DZP group at all (table 1).

In this study, PTZ and PTZ + DMSO-induced convulsions were associated with a high mortality rate (100% of animals). However, pretreatment with WKE alone or along with FMZ, DPZ and ESM decreased the mortality rate to 0%. In addition, the mortality rate in the ESM + PTZ group and the DPZ + PTZ group was 12.5 and 25%, respectively. All groups showed significant differences whenever compared with the PTZ and PTZ + DMSO groups ($p < 0.001$; table 1).

Discussion

The results showed that WKE increased the required doses of PTZ for the induction of threshold convulsions and GCS. In addition, WKE significantly reduced the severity of convulsions and completely prevented the deaths caused by seizures. The combined use of WKE and DPZ showed a stronger anticonvulsant effect and increased the dose of PTZ for both threshold convulsions and GCS when compared with WKE or DPZ alone. In addition, the animal group that received a combination of WKE and DPZ did not show jerky movements and all animals survived after seizure induction. Administration of FMZ did not significantly decrease the enhancing effect of WKE on the PTZ threshold dose but did not have a prominent effect on the PTZ GCS dose for seizure induction, seizure severity and mortality rate. On the other hand, similar to DPZ, ESM with or without WKE increased the threshold convulsions and GCS and decreased the convulsion severity and mortality; however, there was no significant difference between the effect of ESM alone or along with walnuts.

The GABAergic system is the most important inhibitory system in the central nervous system, but its function may be disturbed in different conditions [12]; GABA is an inhibitory neurotransmitter that can affect $GABA_A$ and $GABA_B$ receptors [12]. $GABA_A$ are voltage-gated receptors that act with increasing chloride intracellular influx [13].

PTZ is a noncompetitive antagonist of $GABA_A$ receptors that acts through the t-butyl-bicyclo-phosphorothionate site of the receptor and decreases its activity [14]. Another possibility of PTZ action is to change the potassium and calcium channel conductance [15]. DPZ, as a benzodiazepine receptor agonist [16], can increase the conduction of chloride ion through $GABA_A$ receptors and induce the anticonvulsant effect [17]. ESM can decrease the conduction of calcium ion from T-type calcium channels and thereby show its anticonvulsant effect [18]. According to the low impact of FMZ as a benzodiazepine receptor antagonist [16] on WKE effects and the synergistic effect of DPZ and walnuts in this study, it is possible that the major anticonvulsant effects of WKE applies through receptors other than the benzodiazepine pathway. On the other hand, the pattern of interaction between the effects of ESM and WKE on the control of seizures raises the possibility that at least part of the anticonvulsant mechanisms of these agents may be similar.

Other pathways may contribute to the anticonvulsant effects of WKE. There is an increasing number of different studies regarding the role of nitric oxide (NO) in the pathophysiology of disorders such as stroke, trauma and seizure disorders [19, 20] that could help to explain the anticonvulsant effect of WKE. In epilepsy, NO is also considered an essential pathogenic factor and may have a function in the mechanisms underlying seizure induction and progression [21]. Consistent with this, impressive (five-fold) elevations in NO production were found for the duration of the seizures induced by PTZ. The levels of secondary products resulting from lipid peroxidation have also been shown to be significantly increased in the cerebral cortex of rats with PTZ-induced seizures [22]. A recent study confirmed that walnut extracts diminished the production of NO, tumor necrosis factor-α as well as the expression of inducible NO synthase in BV-2 microglial cells activated by lipopolysaccharide [23]. In another study, primary fatty acids were found to be able to suppress NO production in macrophages [24]. In addition, a recent study has revealed that fatty acids decrease NO production in macrophages stimulated by lipopolysaccharide through iNOS protein expression [25]. WKE and its component ellagic acid have also been shown to possess anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in KS483 cell lines [26]. In addition, it is reported that excessive production of free radicals has been implicated in the pathogenesis of some neurological disorders, including epilepsy, and it has been suggested that antioxidants as adjuncts to antiepileptic drugs may be helpful for better seizure control [27]. Walnuts contain the highest total level of antioxidants, including both free antioxidants and antioxidants bound to fiber [28]. Therefore, part of the anticonvulsant effect of WKE observed in the present study may mediate through mechanisms that modulate NO production, impede the proinflammatory process and also inhibit the redox imbalance.

Conclusion

Our findings show the anticonvulsant effect of WKE and suggest that this effect could be exerted through routes other than the involvement of the benzodiazepine action pathway. Activation of the ESM function pathway, reduction in brain NO production, activation of anti-inflammatory mechanisms and reinforcement of the antioxidant system are possible paths of action for WKE in the control of seizures. However, further studies are needed to elucidate the exact mechanisms in which WKE attenuates the PTZ-induced seizures.

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Disclosure Statement

The authors declare that they have no conflicts of interests.

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