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# The involvement of neuronal nitric oxide synthase in the anti-epileptic action of curcumin on pentylenetetrazol-kindled rats

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**Abstract.** In this study, it was investigated whether a NO signaling pathway is involved in the anti-epileptic effect of curcumin on pentylenetetrazol (PTZ)-kindled rats. PTZ-kindled rats received different doses of curcumin that were administered intraperitoneally for 24 days. Either a non-selective inhibitor of nitric oxide synthase (NOS) (N-nitro-L-arginine methyl ester (L-NAME)), a selective inhibitor of neuronal NOS (7-Nitroindazole (7-NI)), a selective inhibitor of inducible NOS (aminoguanidine (AG)), or a NO precursor (L-arginine (L-ARG)) was administered chronically to evaluate the role of NO in curcumin's anti-seizure effect. A chronic administration of curcumin (200 mg/kg) was most effective for decreasing the mean frequency of epileptiform discharge. Furthermore, a pretreatment with L-NAME or 7-NI augmented the anti-epileptic effect of curcumin. In contrast, AG failed to significantly alter the anti-epileptic effect of curcumin. A pretreatment with L-ARG temporally reversed the anti-epileptic effect of curcumin in the early stage, but in the late stage, it potentiated curcumin's anti-epileptic effect. These findings suggest that the L-arginine–nitric oxide pathway may be involved in the anti-epileptic properties of curcumin, and that the role of nNOS (and not iNOS) is prominent in this neuroprotective feature.

Keywords: Curcumin, nitric oxide, nitric oxide synthase, epilepsy, pentylenetetrazol

## 1. Introduction

Curcumin is a biologically active phytochemical ingredient that is extracted from the dried rhizomes of Curcuma longa. For centuries, it has been used as a "folk" medicine in Asia to treat certain common ailments [1, 2]. Furthermore, curcumin has been reported to possess a variety of beneficial properties, such as anti-oxidative [3], anti-stress [4], and anti-amnesia [5] activities.

Recently, curcumin has been demonstrated to have yet another medical advantage as its seizure-suppressing efficacy has been demonstrated in several experimental models of

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chemically-induced epilepsy. More specifically, some scholars found curcumin to be effective against seizures induced by ferrous chloride [6] and kainic acid [7] as well as lithium pilocarpine-induced status epilepticus [8] in animals. Moreover, another study has demonstrated the anti-epileptic effect of curcumin via the activation of the adenosine-A1 receptor [9]. These findings imply that curcumin could have potent anti-epileptic efficacy; however, the mechanism of action of curcumin's anti-epileptic character has not yet been fully elucidated.

It has been suggested that nitric oxide (NO) plays a fundamental role in various physiologic and pathologic processes in the central nervous system (CNS) [10]. NO is synthesized from L-arginine (L-ARG) by activating nitric oxide synthase (NOS) [11], and NOS exists in three isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [12]. NO is considered to have a significant role in the genesis and spreading of epileptiform activity [13].

Several studies suggest NO mediates the effect of curcumin [6, 7]. It has been suggested that curcumin can alleviate the subacute stress response through the modulation of NO production within the hippocampus [6]. Other studies have indicated that the NO pathway is involved in the amelioration effects of curcumin in ethanol-induced memory deficits [7]. These findings suggest that curcumin may influence epileptiform activity through NO-dependent mechanisms.

In this study, the anti-epileptic effect of curcumin was investigated using a model of epileptiform activity in PTZ-kindled rats. To investigate the possible involvement of NO in the anti-epileptic effect of curcumin, the following were used: a non-specific inhibitor of NOS (N-nitro-L-arginine methyl ester (L-NAME)); a selective inhibitor of nNOS (7-Nitroindazole (7-NI)); a selective inhibitor of iNOS inhibitor (aminoguanidine (AG)); and a NO precursor L-ARG.

## 2. Material and methods

### *2.1. Animals*

The study protocol was approved by the Animal Ethics Committee of the University of Dalian (Dalian, China), and all experiments were carried out in accordance with the Guidelines for the Care and Use of Experimental Animals (National Institutes of Health, Bethesda, MD, USA).

Male Wistar rats (180–250 g) were obtained from the Animals Experimental Research Centre of the Medical University of Dalian. The rats were housed under standard laboratory conditions. They were also allowed to acclimatize to the experimental room for at least one week before experimentation.

The kindling induction was conducted by modifying a previous method [14]. In brief, the rats were injected with a sub-convulsive dose of PTZ (35 mg/kg) once daily. After each PTZ administration, the rats were observed for 1h for any changes in convulsive behavior. The intensity of the seizure activity was estimated using the following 1-5 scale [15]: Stage 0: no seizure response; Stage 1: restlessness, eyelid, vibrissae, and ear twitching; Stage 2: frequently head nodding, clonic convulsions of head, and myoclonic jerk; Stage 3: partial rearing and unilateral forelimb clonic seizures; Stage 4: powerful bilateral forelimb clonus seizures with complete rearing or falling down; and finally Stage 5: generalized tonic–clonic seizure with hind limb extensor and failure of righting reflex. A rat was considered to be kindled when it experienced seizure activity at Stage 2 or above after five consecutive PTZ administrations. The animals that were not kindled by day 30 were excluded from the study.

After being kindled by PTZ, the rats were randomly divided into 17 different groups of six animals each. The groups were assigned as following: (1) PTZ (35 mg/kg); (2) Curcumin (50 mg/kg) + PTZ (35 mg/kg); (3) Curcumin (100 mg/kg) + PTZ (35 mg/kg); (4) Curcumin (200 mg/kg) + PTZ (35

mg/kg); (5) L-NAME (5 mg/kg) + PTZ (35 mg/kg); (6) 7-NI (15 mg/kg) + PTZ (35 mg/kg); (7) AG (50 mg/kg) + PTZ (35 mg/kg); (8) L-ARG (30 mg/kg) + PTZ (35 mg/kg); (9) L-NAME (5 mg/kg) + Curcumin (200 mg/kg) + PTZ (35 mg/kg); (10) 7-NI (15 mg/kg) + Curcumin (200 mg/kg) + PTZ (35 mg/kg); (11) AG (50 mg/kg) + Curcumin (200 mg/kg) + PTZ (35mg/kg); (12) L-ARG (30 mg/kg) + Curcumin (200 mg/kg) + PTZ (35 mg/kg); (13) Saline; (14) Polyethylene glycol 300 (PEG300)/saline; (15) PEG300/saline + PTZ (35 mg/kg); (16) Saline + PTZ (35 mg/kg); (17) Saline + PEG300/saline + PTZ (35 mg/kg).

The PTZ was administered every other day for 24 days. Curcumin, L-NAME, 7-NI, AG, and L-ARG were administered via the intraperitoneal route once daily for 24 days. Furthermore, the L-NAME, 7-NI, AG, or L-ARG was administered 15 min before curcumin was injected, and the curcumin was administered 20 min before PTZ was injected. The drug doses that were used in the present study were selected according to previous studies [16-18].

### *2.2. Identities and administration of drugs*

The following drugs were used throughout the study: curcumin, PTZ, L-ARG, L-NAME, 7-NI, AG, and PEG300. All of the drugs were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Curcumin and 7-NI were dissolved in PEG300/sterile physiological saline (2:3, v/v), respectively, whereas PTZ, L-ARG, L-NAME, and AG were dissolved in a sterile physiological saline solution. Solutions were administered via the intraperitoneal route at 3.5 ml/kg body weight and were freshly prepared prior to the experiments.

### *2.3. Electrocorticographic recordings*

The rats were anesthetized with urethane (1.25 g/kg, i.p.) and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). To monitor for electrocorticographic changes, two stainless-steel screw electrodes were placed over the bilateral primary motor cortex (electrode coordinates: 2 mm lateral to the sagittal suture and 1 mm anterior to the bregma). The two recording electrodes were stabilized firmly on the skull, and the scalp was stitched using surgical threads. The rats were recovered for seven days post-surgery.

The electrocorticographic activity was monitored continuously by a 32-channel electroencephalograph (Nicolet Biomedical, Middleton, WI, USA). The recording electrodes were connected to the electroencephalograph by two electrode holders. The reference electrode was fixed on the right pinna, and the other was placed in the rat's tail (ground electrode). Recordings were made under the conscious state and were stored on a personal computer.

The frequency and amplitude of the epileptiform discharge (clonic discharge and interictal discharge) were analyzed offline by our electroencephalographer (Dr. Zhu). She quantified the following every 10 min during the entire range: the electrocorticographic clonic/min frequency; the spike/min frequency; the highest microvolt voltage amplitude of clonic discharge and the highest microvolt voltage amplitude of interictal discharge. Our electroencephalographer also distinguished true epileptiform electrocorticographic discharge signals from any artifacts by monitoring the entire recording and marking for artifacts synchronously. The amplitude of the epileptiform discharge was measured through an automated capture monitoring software.

### *2.4. Statistical analyses*

Statistical procedures were conducted using SPSS v19.0 (IBM, Armonk, NY, USA). Statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test so as to correct for multiple comparisons of treatments. Data are represented as the mean ± SEM, and $p<0.05$ was considered significant.

## 3. Results

### *3.1. Effects of chronic administration of different doses of curcumin on PTZ-kindled rats*

The electrocorticogram of rats that were not given any substances was regarded as the blank control (Figure 1). Furthermore, an intraperitoneal injection of PEG300/saline and saline did not cause either the frequency or the amplitude of the electrocorticographic activity to change as compared to the blank control. The intraperitoneal administration of PTZ (35 mg/kg)-induced epileptiform activity was characterized by clonic activity and interictal discharge. In addition, an injection of PEG300/saline and/or saline + PTZ groups did not elicit significant differences as compared to the PTZ group alone.

The groups that were administered curcumin showed dose-dependent protection against seizures (Figures 1-4). An administration of a low curcumin dose (50 mg/kg) did not change the frequency or amplitude of the PTZ-induced epileptiform activity as compared to the PTZ group. However, a 100 mg/kg dose of curcumin significantly decreased the mean frequency of the interictal discharge and lasted for 40 min, but the mean frequency of the clonic discharge was not changed. Finally, a 200 mg/kg dose of curcumin significantly decreased the mean frequency of both the interictal discharge and clonic discharge, and these significant effects lasted for 60 min and 40 min, respectively. The 200 mg/kg curcumin dose most effectively decreased the mean frequency of the epileptiform discharge; however, administering curcumin did not influence the amplitude of the epileptiform discharge.

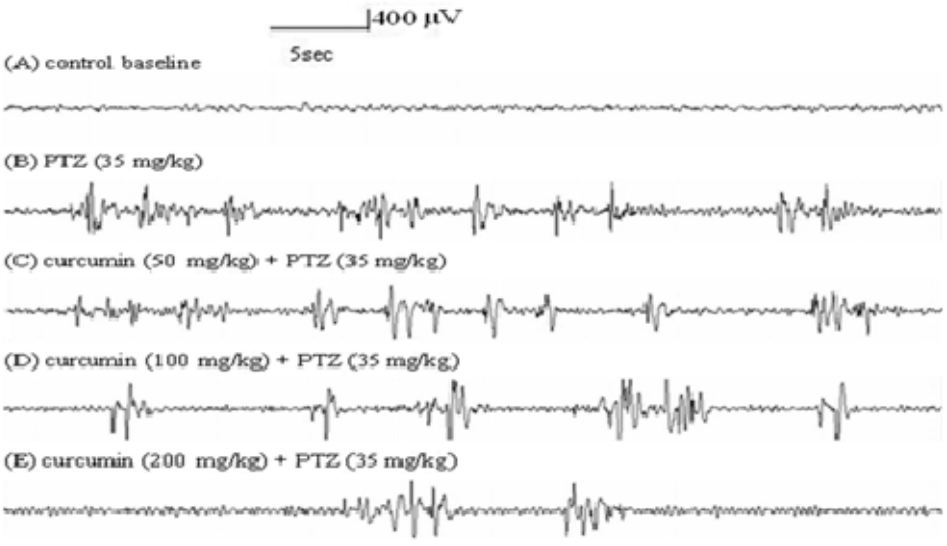


Fig. 1. (A) Baseline electrocorticographic activity; (B) PTZ (35 mg/kg)-induced clonic discharge on electrocorticography (ECoG); (C) Curcumin (50 mg/kg) did not influence PTZ-induced clonic discharge compared to the PTZ group alone; (D) Curcumin (100 mg/kg) did not influence PTZ-induced clonic discharge compared to the PTZ group alone; (E) Curcumin (200 mg/kg) significantly decreased PTZ-induced clonic discharge.

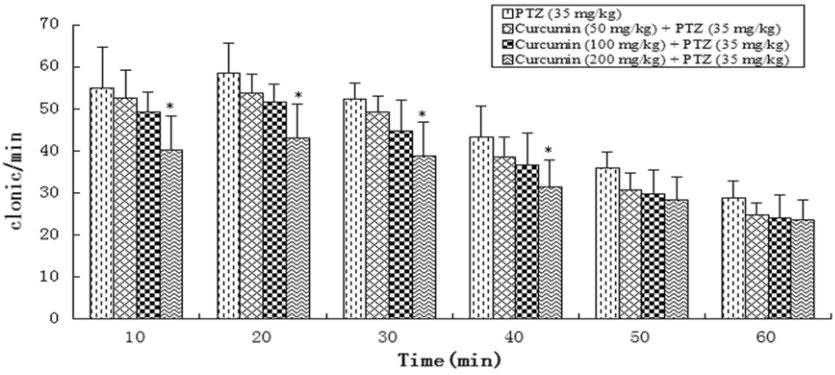


Fig. 2. Influence of chronic administration of different curcumin doses on the mean frequency of PTZ-induced clonic discharge. Data are the mean ± SEM from 6 animals. *p<0.05 compared to the PTZ (35 mg/kg) group.

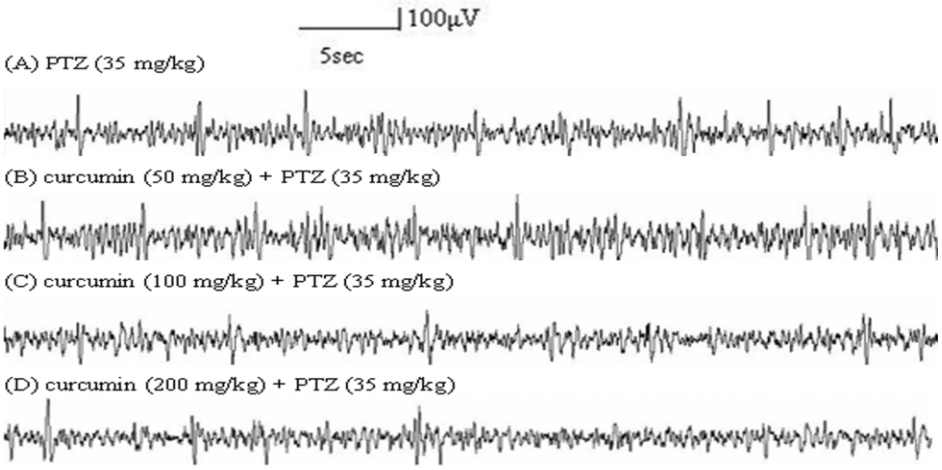


Fig. 3. (A) PTZ (35 mg/kg)-induced interictal discharge on ECoG. (B) Curcumin (50 mg/kg) did not influence PTZ-induced interictal discharge compared to the PTZ group alone. (C) Curcumin (100 mg/kg) decreased PTZ-induced interictal discharge. (D) Curcumin (200 mg/kg) significantly decreased PTZ-induced interictal discharge.

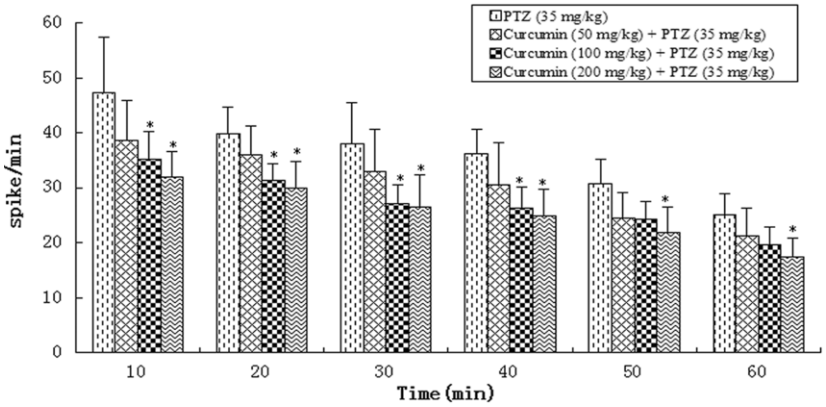


Fig. 4. Influence of chronic administration of different curcumin doses on the mean frequency of PTZ-induced interictal discharge. Data are the mean ± SEM from 6 animals. *p<0.05 compared to the PTZ (35 mg/kg) group.

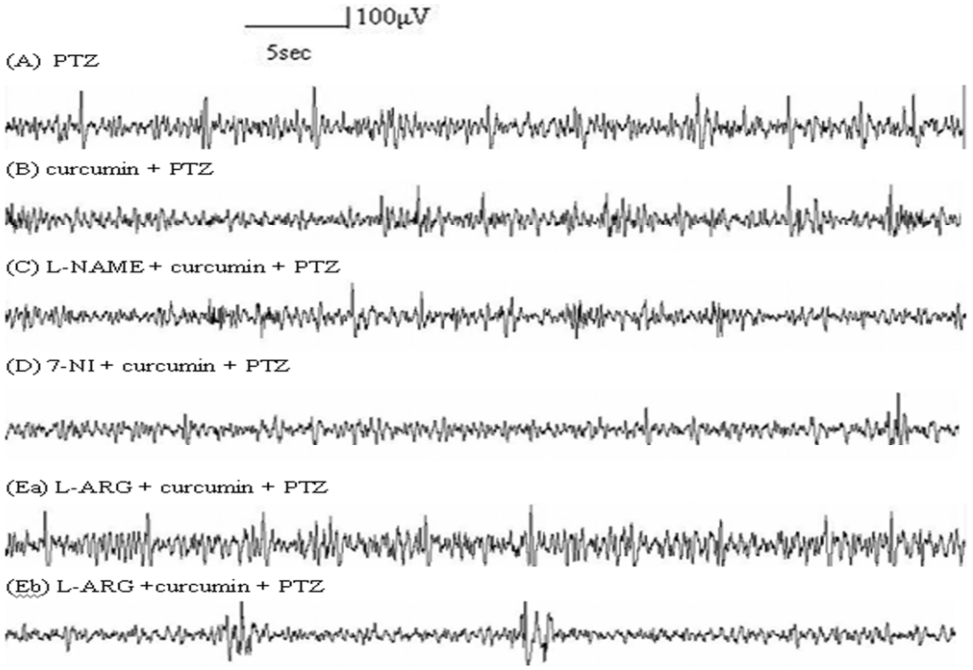


Fig. 5. (A) PTZ (35 mg/kg)-induced interictal discharge on ECoG. (B) Curcumin (200 mg/kg) significantly decreased PTZ-induced interictal discharge. (C) L-NAME augmented the anti-epileptic effect of curcumin. (D) 7-NI potentiated the anti-epileptic activity of curcumin. (Ea) L-ARG reversed the anti-epileptic effect of curcumin. (Eb) L-ARG potentiated the anti-epileptic effect of curcumin.

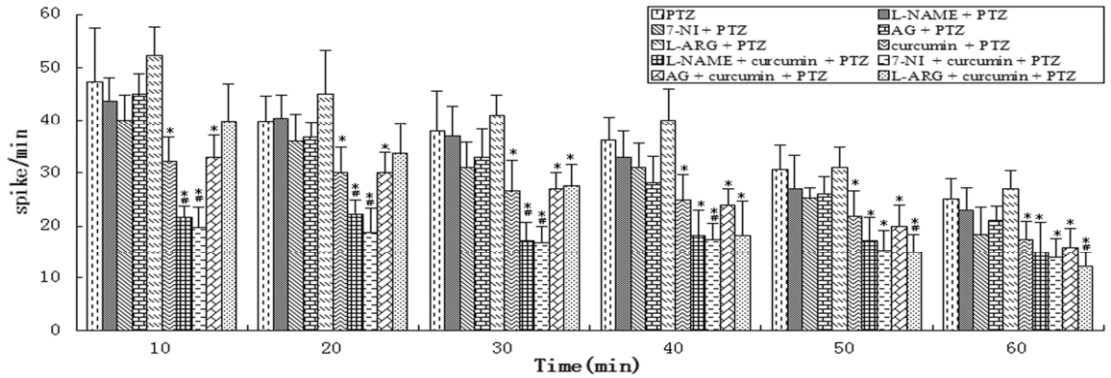


Fig. 6. Influence of NOS inhibitors and a NO precursor on the anti-epileptic activity of curcumin in PTZ-induced interictal discharge. *p<0.05 compared to the PTZ group. #p<0.05 compared to the curcumin + PTZ group.

### *3.2. Effects of NOS inhibitors and a NO precursor on the anti-epileptic activity of curcumin on PTZ-kindled rats*

A chronic administration of L-NAME, 7-NI, AG, or L-ARG did not affect the frequency or amplitude of PTZ-induced epileptiform activity as compared to the PTZ group (Figures 5 and 6).

In rats that were pretreated with L-NAME, the L-NAME improved the anti-epileptic activity of curcumin (Figures 5 and 6). In the L-NAME + curcumin + PTZ group, the mean frequency of the

interictal discharge decreased significantly after the PTZ injection, and the significant effect lasted for 30 min, but the amplitude of the interictal discharge was not influenced as compared to the curcumin + PTZ group. Administration of 7-NI also significantly potentiated the anti-epileptic action of curcumin, whereas an AG administration failed to influence curcumin's anti-epileptic activity (Figures 5 and 6). In the 7-NI + curcumin + PTZ group, the mean frequency of the interictal discharge decreased significantly after the PTZ injection, and the significant effect lasted for 40 min, but the amplitude of the interictal discharge was not influenced as compared to the curcumin + PTZ group. Furthermore, the AG administration did not affect either the frequency or the amplitude of the epileptiform electrocorticographic activity as compared to the curcumin + PTZ group (Figure 6). In the L-ARG + curcumin + PTZ group, contradictory results occurred (Figures 5 and 6). L-ARG temporally reversed the anti-epileptic effect of curcumin in the early stage, but in the late stage, it potentiated curcumin's anti-epileptic effect (Figures 5 and 6). Moreover, the mean frequency of the interictal discharge increased after the PTZ injection but then significantly decreased as compared to the curcumin + PTZ group, and this effect lasted for 20 min. L-ARG did not influence the amplitude of the interictal discharge as compared to the curcumin + PTZ group. All of the NOS inhibitors and the NO precursor did not influence either the amplitude or the frequency of the clonic discharge.

## 4. Discussion

It has been previously demonstrated that curcumin possesses neuroprotective activity [1]. Therefore, in the present study, we evaluated the effects of curcumin on PTZ-kindled rats. A dose–response curve was constructed for curcumin in order to determine the optimal dose for testing its anti-epileptic activity. Taking the results together, this study determined that a 200 mg/kg dose of curcumin was the most effective for decreasing the mean frequency of PTZ-induced epileptiform activity.

NO is considered to be involved in the pathophysiology of epilepsy [10], although the experimental results produced by several authors are inconsistent on this fact. In fact, some researchers have suggested that NO is an endogenous anti-convulsant [16], whereas other authors have suggested a pro-convulsant role for NO [17]. Nevertheless, a comprehensive understanding of the role of NO might aid in the development of effective anti-epileptic treatments [19].

In this study, the effect of a chronic administration of curcumin after pretreatment with NOS inhibitors (L-NAME, 7-NI, or AG) or a NO precursor (L-ARG) on PTZ-kindled rats was evaluated. A chronic administration of NOS inhibitors or a NO substrate alone did not affect the epileptiform activity, which is a finding that is similar to that produced by other scholars [16].

Furthermore, it was demonstrated that a co-administration of L-NAME and curcumin augmented the anti-epileptic activity of curcumin. This result supports findings that L-NAME can improve the protective actions of some chemicals with anti-epileptic properties [17]. This study's results also showed that an administration of a selective inhibitor of nNOS, 7-NI, also significantly potentiated the anti-epileptic action of curcumin, whereas administering a selective iNOS inhibitor, AG, failed to influence curcumin's anti-epileptic activity. These findings imply that nNOS participates in the anti-epileptic activity of curcumin, whereas iNOS activity is not involved in the protective effect of curcumin against PTZ-kindled rats. Moreover, these findings are in accordance with other studies that emphasize the role of the nNOS pathway in the anti-epileptic response to different antiepileptic agents in addition to studies that demonstrate that 7-NI increases the efficacy of anti-epileptic therapy [17]. Studies have shown that there is increased NO production in several parts of the brain during experimentally-induced seizures [20]. Moreover, it has been reported that a type of nNOS can be

detected in several brain regions [21] and that PTZ-kindling is associated with an increased amount of nNOS [22]. In addition, a local depletion of the nNOS substrate reduced NO production, thereby suggesting that NO is predominantly synthesized by nNOS in the brain [23]. One interpretation of the present experiments is that PTZ-induced seizures may be associated with a overproduction of NO, which would make curcumin beneficial because it may restore moderate concentrations of NO by inactivating NOS and thus ameliorating convulsive seizures. A selective inhibitor of nNOS, 7-NI, significantly potentiated the inhibition effect of curcumin on the increased nNOS activity and NO concentration. These findings suggested that a potential site-specific neurobiological mechanism is associated with the anti-epileptic effect of curcumin.

Several investigations have revealed that directly injecting NO into the brain cortices of rats results in convulsive attacks [24]. Furthermore, some reports have suggested that a NO donator, L-ARG, aggravates the severity of experimental epileptic attacks, and NO has been speculated to have a pro-convulsive effect [25]. However, some examples are in disagreement. For example, L-ARG effectively decreased the mean frequency of epileptiform discharge in the penicillin-kindling rats [26], and it was reported that L-ARG in combination with non-effective doses of melatonin showed a significant anti-epileptic effect in comparison to either L-ARG or non-effective doses of melatonin alone [27]. However, in this study, L-ARG played a dual role. The anti-epileptic effect of curcumin was reversed in the early stage, but by the late stage, it was augmented, and L-ARG aggravated the interictal activity in the early stage but relieved it in late stage. These results suggest that NO might have a dual effect during the progression of PTZ-induced epilepsy. This result supports findings that NO has acted differently, either as a convulsant or an anticonvulsant, in the same experiment [28]. In addition, it has been reported that the application of L-ARG induces an excessive release of NO and may lead to a hyperexcitable state via the production of cyclic guanosine monophosphate [10]. Some studies have shown that endogenously-released NO participates in excitatory transmission through NMDA receptors [29] and reduces the activity of the inhibitory γ-aminobutyric acid (GABA) ergic receptors [30]. However, NO was suggested to have neuroprotective actions for it can also suppress overactive NMDA receptors [31]. This may explain the conflicting role of L-ARG in this study. Some studies have also shown that NO is a multi-faceted molecule that can exert dichotomous regulatory roles depending on the exposure dose and the specific cell type [32]. Perhaps, the application of L-ARG interfered with the balance of NO, and excessive NO induced abnormal cortical excitability, which through some feedback mechanism, triggered a subsequent inhibitory effect. Furthermore, when the substances used in this study were added, the resulting effects were not exactly the same for the two kinds of aberrant epileptiform activities, which may suggest that there are differences in the formation mechanism between the two types of epileptiform discharge (clonic activity and interictal discharge).

In this study, it was demonstrated that a chronic administration of curcumin exerts dose-dependent protection against seizures. It was revealed that the anti-epileptic property of curcumin is mediated (at least in part) through the L-arginine–NO pathway, and it was concluded that nNOS (and not iNOS) is likely involved in this phenomenon. Lastly, NO might play a biphasic role in the PTZ-kindled model.

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