Anticonvulsant effect of acute curcumin nanoparticle on pentylenetetrazole-induced seizures in mice: non-involvement of JNK restoration

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ABSTRACT

**Introduction**: Although several animal studies have indicated the antiepileptic effect for curcumin, there are reports stating the null antiepileptic effect of this substance. This inconsistency might be due to the low bioavailability of curcumin. Therefore, the current study aimed to assess the effect of oral bovine serum albumin (BSA)-based nanocurcumin on seizure caused by pentylenetetrazol (PTZ) in mice. Furthermore, due to the suggested involvement of JNK signaling in seizure pathology, the hippocampal pattern of JNK phosphorylation (activation) was evaluated.

**Methods**: BSA based nanocurcumin was administered at doses of 50 and 100mg/kg oral gavage to male NMRI mice, one hour before PTZ administration. Intravenous PTZ paradigm was used to determine the threshold dose of PTZ to induce clonic seizures, while the intraperitoneal PTZ paradigm was applied to evaluate the latency for appearance of generalized clonus. Upon completion of intraperitoneal PTZ paradigm experiments, the hippocampi were removed and Western blot analysis was performed to determine the phosphorylated and total forms of JNK.

**Results**: The results indicated that BSA-based nanocurcumin at the doses of 50 and 100mg/kg could significantly increase the threshold and latency of clonic seizure, which was a significant superior effect compared to natural curcumin. PTZ significantly increased the level of hippocampal JNK phosphorylation, but pretreatment of nanocurcumin did not modify this effect.

**Conclusion**: The present study shows that converting curcumin to BSA-based nanocurcumin can increase its antiepileptic effect. Furthermore, the antiepileptic effect of nanocurcumin was not associated with a modification in PTZ-induced hippocampal JNK hyper activation.

Keywords:
Curcumin
Nanoparticle
Seizure
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JNK
Pentylenetetrazol

Epilepsy, as one of the most prevalent and treatable neurologic diseases, is characterized by the abnormal activity of brain neural cells (Duncan et al., 2006). The available anti-epileptic medications can control epilepsy in about two thirds of the patients, but the remaining
suffer from the symptoms and regarded to be in the state of refractoriness (Tang et al., 2017). Additionally, the current anti-epileptic drugs have adverse effects, some of these anti-epileptic medications might even lead to hepatotoxicity (Vidaurre et al., 2017).

Herbal extracts are regarded as potential remedies in treating epilepsy. Curcumin is a polyphenolic phytochemical compound, extracted from Curcuma longa (turmeric) (Kocaadam and Sanlier, 2017). Curcumin is reported to have therapeutic effect on epilepsy. For example, it was shown to possess protective effect in iron-induced epilepsy model (Kumar et al., 2019), acute intraperitoneal pentylenetetrazol (PTZ) induced seizure (Akula and Kulkarni, 2014; Jahan et al., 2018), chronic intraperitoneal PTZ induced kindling model (Choudhary et al., 2013; Saha et al., 2016), kainate-induced epilepsy (Kiasalari et al., 2013) and pilocarpine-induced seizures (Peng et al., 2012). However, there are other studies reporting the null antiepileptic effect of curcumin in PTZ-induced seizures (Kaur et al., 2014), the seizures induced by kainic acid (Sumanont et al., 2007) and the seizure frequency in post-status epilepticus model of temporal lobe epilepsy (Drion et al., 2016). The absence of curcumin’s anti-epileptic effect in several studies might be due to its restricted accessibility to the brain since curcumin has low solubility in water (Tønnesen, 2002). Many animal and clinical studies in rodents and humans showed the low bioavailability of curcumin (Aggarwal et al., 2007; Goel et al., 2008). Subsequently, curcumin’s potential as a therapy might be restricted by its poor aqueous solubility and low oral bioavailability (Flora et al., 2013). Different strategies have been followed to improve curcumin absorption including the formulation of curcumin nanoparticles to enhance its water solubility and capability to cross the blood–brain barrier (Hashemian et al., 2017). In line, curcumin nanoparticles loaded on chitosan-alginate-sodium tripolyphosphate showed to reduce memory deficit and cell loss in PTZ kindling model of epilepsy (Hashemian et al., 2017). Additionally, it was shown that the liposomal formulations of curcumin attenuate seizures in different animal models of epilepsy, including PTZ-induced seizures, current electroshock seizures and status epilepticus in mice (Agarwal et al., 2013). Among the widespread biomolecules used for delivery of nanoparticles, albumin protein has some priority due to its nontoxicity and non-immunogenicity (Kratz, 2008). This protein is considered as a safe carrier for curcumin due to these properties and increases the bioavailability in comparison to native curcumin (Jithan et al., 2011; Delfiya et al., 2016). It was previously shown that albumin-based nanocurcumin has higher based nanocurcumin bioavailability in comparison to native curcumin both in vivo (SoukhakLari et al., 2018c; SoukhakLari and Moezi, 2019) and in vitro (Sookhaklari et al., 2019).

Several studies indicate that epileptic seizures cause damage to hippocampal neurons, which in turn might be one of the most important factors in the reconstruction of loop during recurrent seizures (Buckmaster et al., 2014). The c-Jun N-terminal kinase (JNK) belongs to the mitogen activated protein kinases family and takes part in a wide range of biologic processes, such as inflammation and apoptosis (Yarza et al., 2016). Hence, it was proposed that JNK hyper activation might play a role in the pathogenesis of intractable epilepsy (Tai et al., 2017). The contribution of JNK has been shown in different chronic models of epilepsy, such as kainic acid (Mielke et al., 1999; Hsieh et al., 2007), electrical kindling models (Cole-Edwards et al., 2006), PTZ kindling model (Ben et al., 2014), as well as in acute model of PTZ-induced seizures (Morgan et al., 2006). The suppression of JNK pathway was previously reported to be involved in the therapeutic effects of curcumin (Collett and Campbell, 2004; Geng et al., 2018; Zhao et al., 2018). In the hippocampus, dietary curcumin administration reduced lipopolysaccharide induced hippocampal phosphorylated JNK elevation (Khan and Muhammad, 2019). Furthermore, the memory enhancing effect of nanocurcumin coincided with a diminished hippocampal JNK phosphorylation (SoukhakLari et al., 2018c).

In the current study, the effect of acute oral bovine serum albumin (BSA)-based nanocurcumin administration on the threshold and latency of clonic seizures induced by intravenous or intraperitoneal injection of PTZ in mice was assessed, and the results were compared with native form of curcumin. Moreover, considering the reported over activation of JNK in seizure, it was assessed to see if the potential antiepileptic effect of BSA based nanocurcumin coincides with JNK modulation.

**Materials and Methods**

**Materials**
PTZ was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Curcumin was bought from Exir GmbH, Austria. For western blot analysis, phosphorylated-JNK (p-JNK), total JNK (t-JNK), beta-actin and secondary HRP-conjugated antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). Amersham ECL select reagent kit was supplied from GE Healthcare Life Sciences, UK. Protease and phosphatase inhibitor was from Pierce, and PVDF membrane was purchased from Millipore. Other reagents were provided from the usual commercial sources.

BSA-based nanocurcumin preparation

In accordance with earlier studies (Delfiya et al., 2016; SoukhakLari et al., 2018c; Sookhaklari et al., 2019; SoukhakLari and Moezi, 2019), BSA was used as the polymer to prepare nanocurcumin. Briefly, 2.5% curcumin solution (in aceton) was added alternatingly to 3% BSA solution (in distilled water) on a magnetic stirrer. Afterward, 110µl of 8% glutaraldehyde solution (in distilled water) was added to the turbid solution to achieve a cross-linkage of the nanoparticles; a process performing on the magnetic stirrer overnight at 4°C. Differential centrifugation (3000rpm, 30min) was used to purify the resultant suspension (over 5 cycles). Lastly, the resulted pellet was dried by means of a freeze drier to achieve a powder. According to the previous publications, this desolvation technique led to the production of quite regular round nanoparticles with mean diameters of about 140nm (SoukhakLari et al., 2018c; Sookhaklari et al., 2019; SoukhakLari and Moezi, 2019).

Animals

Male NMRI mice (age 8-10 weeks, weight 25–30g) were obtained from the animal lab of Shiraz University of Medical Sciences. The animals had access to water and food ad libitum and were kept under controlled lighting (07:00 to 19:00) and temperature (20±2°C) status. All experimental protocols were approved by the Shiraz University of Medical Sciences Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines (approval number: IR.SUMS.REC.1398.674). Each mouse was used once and each group consisted of 6-8 mice.

Behavioral seizure evaluation

Experiment 1: Assessment of the effect of nanocurcumin on seizure threshold

An intravenous (i.v.) PTZ infusion with a continuous flow rate provokes seizure in a reproducible, reliable and rapid manner (Mandhane et al., 2007). This test determines the threshold dose of PTZ to prompt seizures contrary to fixed dose PTZ paradigms that detect latency time to induce seizures (Mandhane et al., 2007). The screening of potential anti-epileptic effect of a substance through the intravenous PTZ test, usually provides better insight about its modulatory effects on seizure susceptibility (Loscher et al., 1991). A dental needle (30-gauge) was placed in the lateral tail vein of the animal. The precision of needle location in the vein was confirmed by arrival of blood in the cannula. An adhesive tape was used to secure the needle to the tail. While the animal was moving freely, the 0.5% PTZ solution (diluted in saline) was infused, using an infusion pump (Harvard, USA) at a continual rate of 0.5ml/min. The time latency till the appearance of first clonus subsequent to full clonus of the body was recorded (Shafaroodi et al., 2015; Moezi et al., 2018; Inaloo et al., 2019). The threshold dose of PTZ for the appearance of clonic seizure was calculated using the following formula:

$$\text{PTZ} \left[ \frac{mg}{kg} \right] = \frac{\text{infusion duration (s)} \times \text{infusion rate (ml/s)} \times \text{PTZ concentration (mg/ml)}}{\text{weight (kg)}}$$

To determine the potential effective doses of nanocurcumin, a preliminary study was performed during which nanocurcumin at the doses of 25, 50 and 100mg/kg oral gavage (SoukhakLari et al., 2018b; SoukhakLari et al., 2018a) was dissolved in distilled water and then administered 60min before PTZ infusion (data not shown). Accordingly, the doses 50 and 100mg were chosen as effective doses for further investigations. Moreover, two other separate groups received natural curcumin at the equal doses of 50 and 100mg/kg. Mice were divided into 5 groups (n=6-8/each group) as below:

1- i.v.PTZ group (n=8): administered distilled water/oral gavage (vehicle for nanocurcumin) 1h before the venous infusion of threshold dose of PTZ (dissolved in saline); 2- i.v.PTZ+ nanocurcumin 50mg/kg (n=8): received nanocurcumin 50mg/kg oral gavage 1h before the venous infusion of threshold dose of PTZ;
3- i.v.PTZ+ nanocurcumin 100mg/kg (n=7): received nanocurcumin 100mg/kg oral gavage 1h before the venous infusion of threshold dose of PTZ; 4- i.v.PTZ+ curcumin 50mg/kg (n=6): received curcumin 50mg/kg oral gavage 1h before the venous infusion of threshold dose of PTZ; 5- i.v.PTZ+ curcumin 100mg/kg (n=8): administered curcumin 100mg/kg oral gavage 1h before the venous infusion of threshold dose of PTZ.

Experiment 2: Assessment of the effect of nanocurcumin on seizure latency

In this experiment, intraperitoneal PTZ (85mg/kg, dissolved in saline) was injected 60min following nanocurcumin, curcumin or vehicle administration. This dose induces a generalized tonic–clonic seizure, which resembles tonic-clonic seizures in human (Loscher et al., 1991; Kupferberg, 2001). Immediately following i.p.PTZ administration, animals were observed for the appearance of generalized clonus and the latency to this phase was measured (Loscher and Lehmann, 1996; Shafaroodi et al., 2012). Latency was assumed as the time interval between i.p.PTZ injections and the initiation of seizure. The animals were divided into 5 groups (n=7-8/ each group) as below:

1- i.p.PTZ group (n=7): received distilled water, oral gavage (vehicle for nanocurcumin) 1h before the intraperitoneal PTZ at the dose of 85mg/kg (dissolved in saline); 2- i.p.PTZ+ nanocurcumin 50mg/kg (n=7): received nanocurcumin 50mg/kg oral gavage 1h before the intraperitoneal PTZ at the dose of 85mg/kg; 3- i.p.PTZ+ nanocurcumin 100mg/kg (n=8): received nanocurcumin 100mg/kg oral gavage 1h before the intraperitoneal PTZ at the dose of 85mg/kg; 4- i.p.PTZ+ curcumin 50mg/kg (n=7): received curcumin 50mg/kg oral gavage 1h before the intraperitoneal PTZ at the dose of 85mg/kg; 5- i.p.PTZ+ curcumin 100mg/kg (n=7): received curcumin 100mg/kg oral gavage 1h before the intraperitoneal PTZ at the dose of 85mg/kg.

Western blot analysis

The Western blot technique was performed as formerly described (Mooeavi, Abbasi et al. 2014, Amiri, Ghasemi et al. 2016). Equal amount of protein sample (40μg) was loaded on a 10% SDS–PAGE and then transferred onto PVDF membranes (Millipore, Burlington, MA, United States), using Bio-Rad transfer system (Bio-Rad, USA). The 5% BSA in TBST (100mM Tris, 2.0% NaCl pH 7.6, 1% Tween-20) was used as the blocking agent. The primary antibodies, p-JNK (1:2000), t-JNK (1/3000) and beta-actin (1/2000) were exposed to PVDF membranes overnight at 4°C. After washing with TBST, the blots were incubated in horseradish-peroxidase-conjugated secondary anti-rabbit antibody (1/5000) for 1h. The blot was then washed three times with TBST. Enhanced chemiluminescence (ECL select; GE Healthcare) was used to illuminate the bands. The blots were then exposed to photographic film in a dark room. ImageJ software from NIH (Bethesda, MD, USA) was used to quantify the intensities of protein bands.

Statistical analysis

Prism 6 (GraphPad Software, San Diego, CA) was used for one-way ANOVA (analysis of variance) followed by Tukey’s post hoc test. Differences between groups were considered significant at \( P<0.05 \). The data are expressed as mean±SEM.

Results

Experiment 1: Nanocurcumin increased the threshold dose of i.v.PTZ induced seizure

Figure 1 shows the effect of nanocurcumin or natural curcumin at the doses of 50 and 100mg/kg on i.v.PTZ-induced clonic seizure threshold. One-way ANOVA revealed a significant difference between groups (F(4,32)= 48.42, \( P<0.0001 \)). Post hoc Tukey’s test indicated that a single oral gavage of nanocurcumin at the doses of 50 and 100mg/kg, 1h before PTZ administration, significantly increased the threshold of
seizure in comparison with the PTZ group, demonstrating its antiepileptic effect ($P<0.0001$). As it is depicted in Figure 1, the antiepileptic effect of nanocurcumin appeared at the dose of 50mg/kg, the dose at which natural curcumin had no significant antiepileptic effect. The antiepileptic effect of natural curcumin appeared at the dose of 100mg/kg upon which nanocurcumin exerted a significant superior effect ($P<0.0001$) compared to natural curcumin.

**Experiment 2: Nanocurcumin increased the latency of generalized clonus in i.p.PTZ induced seizure paradigm**

The effect of nanocurcumin on i.p.PTZ induced generalized tonic–clonic seizure was assessed in i.p.PTZ induced seizure paradigm. Figure 2 shows the effect of acute administration of nanocurcumin or curcumin at the doses of 50 and 100mg/kg on the onset of clonic seizure induced by i.p.PTZ. One-way ANOVA revealed a significant difference between groups ($F(4,31)= 49.08$, $P<0.0001$). Acute gavage of nanocurcumin significantly increased the clonic seizure latency at both doses of 50 ($P<0.01$) and 100mg/kg ($P<0.0001$). Natural curcumin increased clonic seizure latency at the dose of 100mg/kg ($P<0.0001$), but not at 50mg/kg.

**Effect of nanocurcumin on the phosphorylation levels of hippocampal JNK in i.p.PTZ induced seizure paradigm**

Figure 3A shows the demonstrative bands of p-JNK, JNK and beta actin (as an internal control), determined via Western blot technique in control, i.p.PTZ with/without nanocurcumin at the doses of 50 and 100mg/kg. The ratio of p-JNK/JNK is shown in Figure 3B. One-way ANOVA showed a significant difference between groups ($F(3,14)= 8.47$, $P=0.0019$). Post hoc Tukey test indicated that PTZ significantly increased the level of hippocampal p-JNK/JNK ($P<0.05$). Pretreatment of nanocurcumin did not affect PTZ induced elevation of hippocampal p-JNK/JNK.

**Discussion**

Oral administration is considered as the most comfortable way to deliver a drug (Scheepens et al., 2010; Rein et al., 2013). However, due to low solubility of curcumin following its oral administration, more than 75% of it, excretes in feces (Wahlstrom and Blennow, 1978). It was suggested that BSA based nanocurcumin represents a superior therapeutic effect over natural curcumin (Soukhaklari et al., 2018c; Sookhaklari et al., 2019; Soukhaklari and Moezi, 2019). Considering the non-antigenic and non-toxic properties of BSA (Jithan et al., 2011; Delfiya et al., 2016), the present study aimed to evaluate whether oral gavage of BSA based nanoparticles increases its efficacy in i.v.PTZ and/or i.p.PTZ seizure paradigms.

Animal seizure models are extensively used in
FIGURE 3. The effect of nanocurcumin or curcumin at the doses of 50 and 100mg/kg on latency time to clonic seizure induced by intraperitoneal PTZ administration. Data are presented as mean±SEM. **P<0.01 show the statistical difference between groups. NC represents nanocurcumin and C represents curcumin.

epilepsy research to identify and characterize the new antiepileptic agents (Rogawski, 2006). PTZ blocks GABAA receptor by interacting with its picrotoxin site (Huang et al., 2001). In this study, a single intraperitoneal fixed dose of PTZ was used to measure the latency of seizure response (Mandhan et al., 2007). However, since the calculation of PTZ threshold dose to induce seizure is impossible via the fixed dose method, intravenous PTZ infusion (Nutt et al., 1986) was also utilized to assess PTZ dose threshold and seizure susceptibility (Mandhan et al., 2007).

In the present study, using both i.v.PTZ and i.p.PTZ paradigms, it was shown that a single oral gavage of nanocurcumin increased both the seizure threshold and latency, indicating its potential antiepileptic effect. This protective effect appeared at the dose of 50mg/kg while the same dose of natural curcumin did not have any significant effect on seizure. Although, natural curcumin showed an antiepileptic effect at the dose of 100mg/kg, its equivalent dose of nanocurcumin showed a significant stronger antiepileptic effect on seizure threshold in i.v.PTZ paradigm. This higher efficacy of nanocurcumin can be attributed to its greater bioavailability, since the smaller diameter of curcumin nanoparticles, evidently affects its solubility (Jithan et al., 2011; Kim et al., 2011). Previously, intraperitoneal injection of curcumin C3 complex nanoparticles (50 mg/kg/day) for 4 consecutive days was shown to attenuate pilocarpine rat model of status epilepticus (Khadrawy et al., 2018). In PTZ-induced kindling model of status epilepticus in mice, intraperitoneal injection of curcumin-loaded chitosanalginate-STPP nanoparticles for 20 days exhibited an anticonvulsant activity (Hashemian et al., 2017). A single liposomal formulation of curcumin was shown to attenuate seizure latency induced by intraperitoneal PTZ (Agarwal et al., 2013).

As it was mentioned before, among the widespread biomolecules used for delivery of nanoparticles, albumin
protein has some priority due to its nontoxicity and non-immunogenicity (Kratz, 2008). BSA is broadly used for drug delivery due to its nontoxicity, biocompatibility, biodegradability, abundance and non-immunogenicity. Additionally, BSA improves the water-solubility of prodrugs (Karimi et al., 2016). Based on these properties of BSA and the previous research work on BSA based nanocurcumin (SoukhakLari et al., 2018c; Sookhaklari et al., 2019; SoukhakLari and Moezi, 2019), the present study evaluated the effect of this type of nanocurcumin on both seizure threshold and latency in PTZ model of animal seizure. The threshold dose of PTZ for provoking seizure cannot be calculated, using fixed dose PTZ administration, which detect the time latency to induce seizures (Mandhane et al., 2007). Therefore, i.v.PTZ method was applied to determine seizure threshold, which is an implicational characteristic in screening the potential anti-epileptic effect of a substance on seizure susceptibility (Loscher et al., 1991). Moreover, in the present study, the effect of nanocurcumin was compared with native curcumin, showing a significant superior effect in i.v.PTZ model.

Previous studies have shown that epileptic seizures cause damage to hippocampus and this damage might be one of the most important factors in the reconstruction of loop during recurrent seizures (Buckmaster et al., 2014). JNK becomes activated in response to numerous stress signals and has been shown to be involved in various cellular events, including excitotoxic hippocampal injury following seizures (Spigolon et al., 2010; Zhao et al., 2012). Therefore, the additional aim of this study was to assess the hippocampal JNK activation (phosphorylation). The animals received a fix dose of PTZ in i.p.PTZ model were selected for JNK signaling assay. A control group receiving the vehicles of PTZ and nanocurcumin was included in Western blot study to assess PTZ effect on hippocampal JNK phosphorylation. The results indicated that a single administration of i.p.PTZ at the dose of 85mg/kg induced a significant increase in p-JNK/t-JNK ratio, indicating potent JNK activation. This finding is in line with previous studies, showing an increase in JNK activation following kainic acid or pilocarpine model of epilepsy in the brain or hippocampal area (Mielke et al., 1999; Tai et al., 2017). The pharmacological inhibition of JNK activity or its genetic deletion was shown to protect against neuronal loss induced by kainate or kindling (Yang et al., 1997; Cole-Edwards et al., 2006; Wang et al., 2015). It was suggested that the inhibition of JNK phosphorylation might serve as a possible target for antiepileptic drugs development. In this line, the antiepileptic effects of agents such as ketogenic diet, acylated ghrelin as well as baclofen and muscimol co-application were shown to be associated with JNK signaling inhibition (Zhang et al., 2018).

Previous works showed that curcumin acts as an inhibitor of JNK. For instance, curcumin inhibited JNK in a rat model of LPS-induced neurotoxicity (Khan and Muhammad, 2019) and treatment of mice with curcumin alone led to a significant reduction in hippocampal JNK (SoukhakLari et al., 2018c). Therefore, in the present study, it was assessed to see if the attenuating effect of nanocurcumin on chemical seizure was associated with JNK deactivation. However, the results of Western blot analysis revealed that curcumin could not reduce PTZ induced hippocampal JNK activation. Therefore, it seems that JNK modulation is not involved in the attenuating effect of a single treatment of curcumin on PTZ induced seizure. If JNK deactivation does not play a role in the anti-seizure effect of curcumin nanoparticle, what other mechanisms might be involved? Curcumin was suggested to act as an effective anti-inflammatory and antioxidant molecule (Dhir, 2018). The oxidative stress and neuroinflammation are known as the two major pathological events in seizure and epilepsy (Koblarek et al., 2019). Hence, the anti-oxidative and anti-inflammatory properties of curcumin nanoparticle might serve as the potential mechanisms of the anti-seizure effect of this molecule.

**Conclusion**

In brief, the results of the current study indicated that a single oral dose of BSA-based nanocurcumin, attenuates the severity of seizure both in i.v.PTZ and i.p.PTZ paradigms of status epilepticus in the mice. Additionally, it was shown that PTZ over activates hippocampal JNK signaling and the antiepileptic effect of nanocurcumin is not associated with the modulation of PTZ induced JNK over activation.

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Conflict of interest
The authors declare that they have no conflict of interests.

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