



Berberine attenuates convulsing behavior and extracellular glutamate and aspartate changes in 4-aminopyridine treated rats

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ABSTRACT

Objective(s): K⁺ channel blocker 4-aminopyridine (4-AP) stimulates the release of glutamate from nerve terminals and induces seizures. Berberine as a potential herbal drug exerts several pharmacological actions on the central nervous system including anxiolytic, anticonvulsant, and neuroprotective properties. The present study aimed to investigate the effect of berberine on seizure onset and time course of the extracellular levels of excitatory amino acids (EAA), glutamate and aspartate, changes produced by 4-AP in rat hippocampus.

Materials and Methods: The rats were given either saline or berberine (50, 100 and 200 mg/kg, IP) 40 min before administration of 4-AP (15 mg/kg, IP) and the onset of seizure was recorded. A group of rats also received diazepam (DZP, 15 mg/kg, IP) 20 min prior to 4-AP administration. Hippocampal extracellular levels of EAA were also measured using microdialysis assay. Analysis of the dialysate samples was performed by reversed-phase high performance liquid chromatography (HPLC) with precolumn derivatization with o-phthalaldehyde and fluorescence detection.

Results: Our findings suggest that berberine significantly delayed the seizure onset following 4-AP injection. There was a considerable increase in the extracellular glutamate and aspartate levels in 4-AP treated rats and 4-AP-evoked release of EAA was sharply reduced (about 4-5 fold especially at 20 min after 4-AP administration) in berberine treatment groups.

Conclusion: The results of present study show that berberine attenuates 4-AP induced seizures by decreasing hippocampal aspartate and glutamate release in rats.

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Introduction

Excitotoxicity due to excessive glutamatergic neurotransmission is a well-studied phenomenon that has been associated with the mechanisms of neuronal death occurring in epilepsy and many other neurodegenerative disorders (1). 4-Aminopyridine (4-AP) as a voltage-sensitive K⁺-channel blocker, prolongs action potentials in neurons and causes high discharges of seizures in hippocampus and cortex which is related to its concentration. 4-AP extensively used in *in vitro* and *in vivo* experiments for evoking the release of glutamate and aspartate and propagation of epileptiform activity and neurodegeneration. Therefore, compounds which attenuate glutamate and aspartate release from nerve terminals, may have neuroprotective effects on the pathological conditions related to excessive excitatory amino acids (EAA) release (2-4).

Berberine is an isoquinoline alkaloid found in many plants especially *Berberidaceae* family. Berberine based formulations, are widely used in traditional systems of medicine including, Ayurveda and Traditional Chinese

Medicine (5). It has multiple pharmacological effects, including prevention of ventricular hypertrophy (6), reduction of apoptosis in myocytes exposed to ischemia/reperfusion (I/R) injury (7), positive inotropic, negative chronotropic, antiarrhythmic, and vasodilator properties (8), anticancer (9-11), anti-atherosclerosis (12), hepatoprotective (13), antidiabetic (14), antidepressant (15) and anti-inflammatory activities (16). Except for the above pharmacological effects, the neuroprotective properties of berberine in different neurotoxic conditions have been also documented (17). Berberine is shown to improve neural health and function and ameliorate memory deficits and cognitive impairments in animal models of Alzheimer's disease or ischemic stroke by improving redox status and synaptic plasticity and ameliorating neuroinflammation and apoptosis (18-23). Recent studies have also indicated that berberine exhibits an anticonvulsant effect in seizures induced by pilocarpine (24), kainate (25-27), pentylenetetrazole or maximal electroshock (28, 29). However, the exact mechanisms that underlie these

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neuroprotective effects remain to be elucidated. Therefore, the purpose of this research was to investigate the protective effects of berberine on seizure onset and time course of the EAA changes induced by 4-AP in rat hippocampus.

Materials and Methods

Chemicals and reagents

Berberine was purchased from Sigma-Aldrich Co (St. Louis, MO). 4-AP, urethane and liquid chromatographic grade solvents and reagents were obtained from Merck (Darmstadt, Germany).

Animals and experimental procedure

Adult male Wistar rats weighing 300-350 g were obtained from the Animal Center of Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. The animals were housed in a pathogen-free facility on a 12 hr light/dark schedule and under constant temperature ($22 \pm 2^\circ\text{C}$) with free access to food pellets and tap water, available *ad libitum*. All animals were treated in accordance with the National Institutes of Health Guidance for the Care and Use of Laboratory Animals, and their use was approved by the Animal Ethics Committee of Mashhad University of Medical Sciences.

Convulsive behaviors

Rats (6-8 per group) were injected with berberine (50, 100 and 200 mg/kg) or saline (control) and 40 min later animals were injected with 4-AP (15 mg/kg, IP). A group of rats also received diazepam (DZP, 15 mg/kg, IP), 20 min prior to 4-AP administration. The animals were observed during 60 min after 4-AP administration and the seizures latencies were measured (30).

Based on behavioral alterations, doses of 50 and 200 mg/kg were selected for microdialysis assay.

Surgery and microdialysis procedure

Under urethane anesthesia (1.2 g/kg), a concentric microdialysis probe (CMA/11, 2 mm membrane length, 0.24 mm OD, cuprophane membrane, 6 kDa cut off) was stereotaxically inserted through the implanted guide cannula in the dorsal hippocampus (3.8 mm posterior to the bregma, 2.2 lateral to bregma and 4.1 mm below the skull surface). The dialysis probe was perfused with sterile artificial cerebrospinal fluid, aCSF (in mM: Na 150.0, K 3.0, Ca 1.4, Mg 0.8, P 1.0, Cl 155, glucose 10, pH 7.4) at a constant flow rate (2 $\mu\text{l}/\text{min}$) by a microinfusion pump. After 90 min stabilization period, three samples every 20 min (40 μl) were collected from each rat to assess the basal concentration of EAA (31).

The rats were then given either saline (10 ml/kg, IP, n=5) or berberine (50 mg/kg or 200 mg/kg, IP, n=5) and 40 min later, saline-treated and berberine-treated animals were administered with 4-AP (15

mg/kg, IP). In another experimental group, positive control, DZP (15 mg/kg, IP, n=5) was injected 20 min prior to 4-AP administration. Another group of rats (n=4) was given only berberine (200 mg/kg, IP) to investigate the effect of berberine on baseline hippocampal EAA concentration.

The probe placement was verified using 2% Coomassie blue solution at the end of each experiment. The probes were checked for *in vitro* recovery at 2.0 $\mu\text{l}/\text{min}$ and the values were 14% for aspartate and 13% for glutamate.

Amino acid analysis

Analysis of glutamate and aspartate in the dialysate was performed by reversed-phase high performance liquid chromatography (HPLC) with precolumn derivatization with o-phthalaldehyde (OPA) and fluorescence detection, as reported elsewhere with some modifications (31). Briefly, 15 μl of each dialysate sample were mixed with 15 μl OPA solution (40 mM, pH 9.3) and allowed to react for 1 min at room temperature. After the reaction was completed, 20 μl of the resulting mixture were injected into an HPLC system (Shimadzu Corp.) consisting of a solvent delivery system (LC-10 ADVP pump; Shimadzu Corp.), an octadecylsulfonate (ODS) C18 (25 \times 4.6 mm i.d., 5 μm particle size; Shimpak; Shimadzu Corp.) column and a fluorescent detector (RF-10 AXL; Shimadzu Corp.) with excitement (Ex) set at 350 λ and emission (Em) set at 470 λ . The mobile phase consisted of 92.5% sodium acetate buffer (0.1 M, pH 6.96), 5% methanol and 2.5% tetrahydrofuran (flow rate 1.2 ml/min).

Statistical analysis

The results were expressed as mean \pm SEM. The data of convulsive behavior were analyzed using one-way analysis of variance (ANOVA). In microdialysis experiment, the average of the four baseline samples was used to determine the basal EAA value. The results from microdialysis experiment were analyzed using ANOVA for repeated measurements following Bonferroni's *post hoc* test for multiple comparisons. Comparisons of basal values with subsequent values in each group were performed using the two-tailed student's t-test.

A $P < 0.05$ was considered to be statistically significant.

Results

Berberine significantly increased seizures onset induced by 4-AP

The onset of seizures in the control group was 3.46 ± 0.47 min. Administration of berberine at doses of 50 mg/kg, 100 mg/kg and 200 mg/kg, lead to significantly increase of seizure onsets to 7.22 ± 0.33 ($P < 0.001$), 7.78 ± 0.37 ($P < 0.001$) and 8.88 ± 0.32 ($P < 0.001$) min, respectively (Figure 1). DZP also significantly increase seizure onsets to 5.83 ± 0.29 min ($P < 0.001$) as compared with 4-AP treated animals.

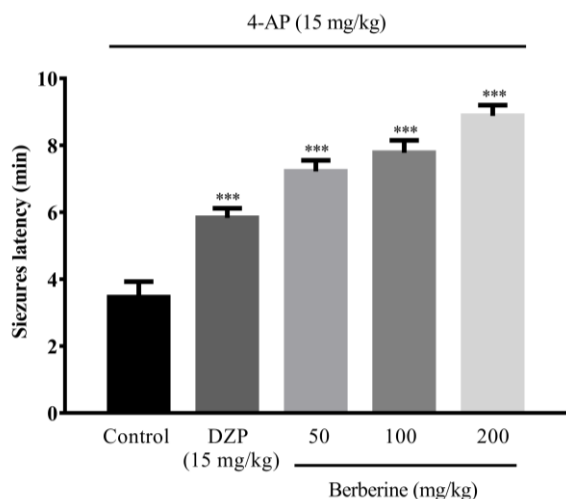


Figure 1. Anticonvulsant effects of berberine (50-200 mg/kg, IP) and diazepam (DZP, 15 mg/kg, IP) on 4-AP-induced seizures in rats. Graphs show latency to generalized convulsions in the five groups of rats. Values are mean±SEM (n=6-8). *** $P<0.001$ as compared to the 4-AP group

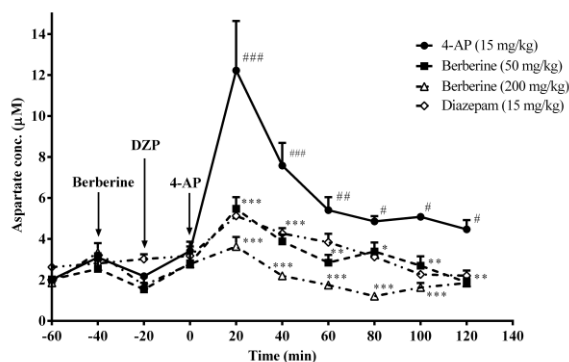


Figure 2. Time-course of extracellular aspartate concentrations (mean±SEM) in hippocampal dialysate samples of rats given either saline (10 ml/kg, IP, n=5), berberine (50, 200 mg/kg, IP, n=5) or diazepam (DZP, 15 mg/kg, IP, n=5), before 4-aminopyridine (4-AP, 15 mg/kg, ip) administration. The arrows represent the time of 4-AP, diazepam or berberine administration. ### $P<0.0001$, ## $P<0.01$, # $P<0.05$ as compared to the mean basal value. *** $P<0.001$, ** $P<0.01$, * $P<0.05$ as compared to the corresponding value in 4-AP group

According to the behavioral study, the doses of 50 and 200 mg/kg were chosen for microdialysis procedure.

Berberine inhibited 4-AP evoked aspartate release from rat hippocampal

As illustrated in Figure 2, the mean basal extracellular level of aspartate was not significantly different between groups ($P>0.05$). Treatment of the rats with berberine alone (200 mg/kg) did not significantly modify the extracellular hippocampal levels of aspartate throughout the experiments (data not shown).

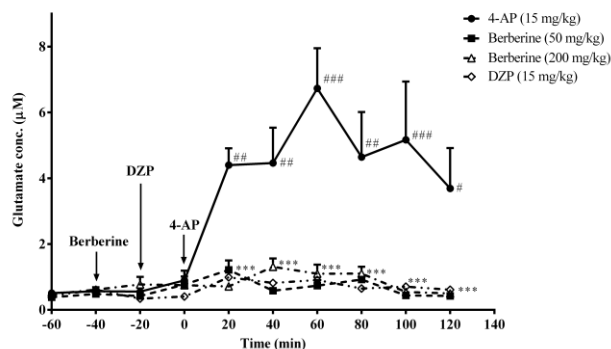


Figure 3. Time-course of extracellular glutamate concentrations (mean±SEM) in hippocampal dialysate samples of rats given either saline (10 ml/kg, IP, n=5), berberine (50, 200 mg/kg, IP, n=5) or diazepam (DZP, 15 mg/kg, IP, n=5), before 4-aminopyridine (15 mg/kg, IP) administration. The arrows represent the time of 4-AP, diazepam or berberine administration. ### $P<0.001$, ## $P<0.01$, # $P<0.05$ as compared to the mean basal value. *** $P<0.001$, ** $P<0.01$ as compared to the corresponding value in 4-AP group

After 4-AP injection in saline treated rats, the extracellular concentration of aspartate significantly increased in comparison to mean basal value; about 5 ($P<0.001$), 3 ($P<0.001$), 2 ($P<0.001$), 2 ($P<0.01$) and 2 ($P<0.05$) fold at 20, 40, 60, 80-100 and 120 min, respectively.

DZP significantly reduced the 4-AP-evoked release of aspartate in rat hippocampus about 2.5 fold ($P<0.001$) at 20 min after 4-AP administration.

Berberine significantly reduced the 4-AP-evoked release of aspartate in rat hippocampus; about 2 fold (50 mg/kg, $P<0.001$) or 3.5 fold (200 mg/kg, $P<0.001$) at 20 min after 4-AP administration, respectively.

Berberine inhibits 4-AP-evoked glutamate release from rat hippocampal

As shown in Figure 3, the mean basal extracellular level of glutamate was not significantly different between groups ($P>0.05$). Treatment of the rats with berberine alone (200 mg/kg) did not significantly modify the extracellular hippocampal levels of glutamate throughout the experiments (data not shown).

After 4-AP injection in saline treated rats, the extracellular concentrations of glutamate significantly increased as compared to mean basal value; about 7 ($P<0.001$), 11 ($P<0.001$), 7 ($P<0.001$), 8 ($P<0.001$) and 6 ($P<0.001$) fold at 20-40, 60, 80, 100 and 120 min, respectively. DZP significantly decreased the release of glutamate in rat hippocampus about 3 fold ($P<0.001$) at 20 min after 4-AP administration.

Berberine administrating substantially reduced 4-AP-evoked hippocampal glutamate release; about 2 fold (50 mg/kg, $P<0.001$) or 4 fold (200 mg/kg, $P<0.001$) at 20 min after 4-AP administration.

Discussion

This study reveals that berberine significantly delayed the seizure onset and decreases extracellular hippocampal EAA release induced by 4-AP.

Previous studies showed that berberine pretreatment (25, 50 and 100 mg/kg) could attenuate spontaneous recurrent seizures in an intrahippocampal kainate model of temporal lobe epilepsy in the rat (27). Also berberine (25, 50, and 100 mg/kg) delayed the onset of first seizure in a pilocarpine induced epilepsy model in rats (32). Sadeghnia *et al* showed that berberine (400 mg/kg) increases both minimal clonic seizures and generalized tonic-clonic seizures latencies in pentylenetetrazol-induced epileptic seizures in rats (29). Daily administration of berberine at a dose of 50 mg/kg also ameliorates intrahippocampal kainate-induced status epilepticus in rats (26). With regards to these results, in our behavioral study, berberine (50, 100, 200 mg/kg) delayed the onset of seizure induced by 4-AP.

We used 4-AP, which is a convulsant that enhances neurotransmitter release by blocking A-type and delayed rectifier K⁺ channels (33). It has been proved experimentally that 4-AP stimulates the release of glutamate, norepinephrine, serotonin and dopamine (34). In the hippocampus, 4-AP enhances the release of glutamate, aspartate and GABA dose-dependently, and causes typical convulsive electroencephalographic (EEG) changes (34).

Our data suggested that berberine at doses of 50 and 200 mg/kg decreased hippocampal glutamate and aspartate concentrations in 4-AP-induced seizure model in rats. Consistent with our data, Lin *et al*, using cerebrocortical synaptosomes preparations, showed that berberine inhibits 4-AP-evoked glutamate release by reduction of Ca²⁺ influx through Ca_{v2.1} channels, and extracellular signal-regulated kinase (ERK)/synapsin I signaling cascade, in the absence of any effect on nerve terminal excitability (35). This event was prevented by the chelating extracellular Ca²⁺ ions and the vesicular transporter inhibitor bafilomycin A1, but was insensitive to the glutamate transporter inhibitor DL-threo-beta-benzyl-oxyaspartate. Inhibition of glutamate release by berberine was not due to diminishing synaptosomal excitability, for the reason that berberine did not alter 4-AP-mediated depolarization (35). In the same way, we found that berberine does not affect basal EAA release.

In this work, reducing glutamate release may explain, in part, the neuroprotective mechanism of berberine. An excessive release of glutamate is generally considered to be one of the molecular mechanisms of neuronal damage in several neurodegenerative disorders (36). Moreover, some neuroprotective agents have been revealed to diminish glutamate release in brain tissues (37, 38). In synaptic terminals, activation of Na⁺ channels or inhibition of K⁺ channels is known to regulate

membrane excitability (35). Excessive glutamate release and activation of glutamate receptors induces an increased oxygen free radicals production, disturbed mitochondrial function, and protease activation, that finally kill the neurons and involved in acute insults for instance epileptic seizures, traumatic brain, stroke and spinal cord injury as well as, chronic neurodegenerative disorders such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (35, 39).

It has been confirmed that berberine penetrates through the blood-brain barrier. Wang *et al* showed that berberine rapidly increased in rat hippocampus with slow elimination rate, suggesting that berberine could accumulate in the hippocampus and have a direct action on neurons (40). It was shown that berberine lacks any cytotoxic, genotoxic or mutagenic activities at doses usually used in clinical situations (41).

It has neuroprotective effects in both *in vivo* and *in vitro* models of neurotoxicity such as ischemia and Alzheimer's disease (20, 42, 43). Berberine has been shown to suppress oxygen and glucose-deprivation- or NMDA-induced neurotoxicity (18). By increasing nuclear factor-like (Nrf)2, berberine also mediates neuroprotection and decreases ROS damages in NSC34 motor neuron-like cells (44).

It has been shown that berberine blocks transient outward potassium channels and delayed rectifier potassium current of hippocampal CA1 neurons and has benefit effect on cation balance of neurons under anoxic/ischemic injury which results in increased cell survival and suppression of apoptosis (45).

Previous researches have demonstrated that these beneficial effects mediated through antioxidant effect, scavenging oxygen-free radicals, decreasing intracellular calcium concentration, inhibiting N-methyl-D-aspartate (NMDA) receptor activity, and anti-inflammatory effects (5, 46, 47).

In a research berberine exerts dose-dependent anticonvulsive activity in pilocarpin-induced epilepsy model in rats. Berberine improved neuronal degeneration and memory impairments and decreased the degree of oxidative stress in the hippocampal CA1 region (32). Pretreatment with berberine also attenuated spontaneous recurrent seizures in kainate-induced temporal lobe epilepsy in rats. This protective effect is due to its effectiveness in lessening of oxidative stress (27).

Berberine is also known to promote the survival and differentiation of hippocampal precursor cells. In the rat peripheral nervous system, berberine also showed the neuroregenerative effect and improved axonal regeneration of injured nerves (19).

Kalalian-Moghaddam *et al* showed that treatment with berberine ameliorates streptozotocin-induced learning and memory impairments and improves hippocampal synaptic plasticity through the down regulation of CA1 pyramidal neurons apoptosis (48).

There are some evidence that berberine can modulate nitric oxide synthesis (15). It has been suggested that activation of excitatory amino acid receptors leads to NO release and contributes to the genesis of seizure activity (27, 49, 50).

Conclusion

Our results suggest that berberine could prevent 4-AP-induced seizures partially via inhibition of aspartate and glutamate release. This study may provide a further understanding of the mode of berberine action in the brain, emphasizing the therapeutic potential of this compound to treat neurological and neurodegenerative disorders.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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