RESEARCH PAPER

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Long-term effects of vitexin against development of pentylenetetrazole-induced kindling in rats

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Evidence is provided that the glycosylated flavonoid vitexin (apigenin-8-C-beta-D-glucopyranoside) attenuates pentylenetetrazole (PTZ)-induced acute tonic-clonic seizures in rats. However, the effects of chronic and systemic vitexin in PTZ-kindled rats remain unknown. The aim of this work was to investigate the effect of long-term treatment with vitexin in the PTZ-kindling model of epilepsy. Male Wistar rats received intraperitoneal injections of PTZ at a subconvulsive dose of 35 mg/kg every other day for 29 days. Either saline containing dimethyl sulfoxide – DMSO 1% (vehicle), diazepam (2 mg/kg; positive control) or vitexin (2.5 mg/kg) was administered intraperitoneally 30 min before each PTZ injection. The behavioral reactions were recorded by 30 min immediately after each PTZ injection. Furthermore, on the 31st day, that is, 48 h after the latter dose of PTZ, the animals were euthanized and renal and hepatic biochemical markers were evaluated in blood serum. Chronic treatment with either diazepam or vitexin attenuated the seizures provoked by PTZ injections. Neither diazepam nor vitexin caused changes in renal levels of creatinine and urea and in hepatic levels of aspartate aminotransferase and alanine aminotransferase. Our findings suggest that chronic administration of vitexin attenuates the progression of PTZ-induced kindling without causing side effects on kidneys and liver.

Key words: antiepileptic, epilepsy, flavonoid, kindling, pentylenetetrazole, seizures, vitexin

INTRODUCTION

Epilepsy is a neurological disorder characterized by a transient occurrence of abnormal and excessive synchronous neuronal firing in the brain, which results in irregular and unpredictable seizures (Engel, 2001; England et al., 2012). It has been reported that 30% of individuals with epilepsy are resistant to antiepileptic drugs because, even with treatment, they continue to experience seizures (WHO, 2019). Furthermore, the anticonvulsants drugs often cause side effects, which in several cases limit their clinical use (Wahad, 2010).

Bioactive phenolic compounds, a large family of natural compounds with phenolic hydroxyl groups, which are present in plants, fruits and vegetables, have been the target of many studies due to their diverse neuroprotective therapeutic properties (Youdim & Joseph, 2001; Luo et al., 2002; He et al., 2015; Costa et al., 2016). Polyphenols, including flavonoids, have been highlighted for producing relevant neuroprotective effects against conditions related to epilepsy (Medina et al., 1990; Diniz et al., 2015; Hossein & Marjan, 2017). Evidence from rodent studies demonstrates that the glycosylated flavonoid vitexin (apigenin-8-C-beta-D-glucopyranoside), one of the main active compounds in several medicinal plants with anticonvulsant properties, attenuates pharmacologically-induced acute and tonic-clonic seizures in rodents (Abbasi et al., 2012; Aseervatham et al., 2016; de Oliveira et al., 2020). In fact, intracerebroventricular microinjections of vitexin reduce acute seizures provoked by systemic injections of pentylenetetrazole (PTZ) (Abbasi et al.,

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2012), a selective blocker of the chloride channel coupled to the gamma-aminobutyric acid $(GABA)_A$ receptor complex, which has been used to provoke acute- or kindling-like seizures in rodents (De Deyn et al., 1992; Sejima et al., 1997; Hansen et al., 2004; Gelfuso et al., 2013; Mazzei-Silva et al., 2014; de Oliveira et al., 2016a, 2016b, 2018).

More recently, our team expanded the evaluation of the acute effects of vitexin on seizures induced by different drugs. We found that systemic administration of vitexin inhibits seizures induced by the GABAergic receptor antagonists PTZ and picrotoxin, but fails to reduce seizures provoked by the glutamate receptor agonists NMDA and kainic acid (de Oliveira et al., 2020). In fact, these findings indicate that the acute anticonvulsant effects of vitexin appear to be due to its action on GABAergic mechanisms. However, although vitexin has been tested in animal models of acute seizures, little is known about its effect in a chronic model of epilepsy.

Thus, considering the chronic nature of the development and progression of epilepsy, as well as the anticonvulsant action of vitexin against convulsive seizures elicited by GABAergic antagonists, the aim of this work was to examine the effects of chronic systemic administration of vitexin on PTZ-induced kindling in Wistar rats. Furthermore, using biochemical blood analysis screening, the toxic potential of chronic administration of vitexin was also evaluated.

METHODS

Male adult Wistar rats (weighing 150-180 g) from the Anilab company (Paulínia, SP, Brazil) were kept in groups of four animals per cage (41 × 34 × 17.8 cm), with water and food ad libitum, in a room with controlled lighting conditions (12 h dark/light cycle programming at 6 am / 6 pm), relative humidity (55 ± 10%) and standard temperature (20°C ± 2°C). All effort was made to minimize animal suffering. Housing and all experimental protocols adhered to the Guide for the Care and Use of Laboratory Animals ("Guide for the Care and Use of Laboratory" – Eighth Edition; Natural Research Council, 2014) and were performed in accordance with the recommendations of the Local Ethics Committee in Research at UNAERP (ECR-UNAERP, 11/2015) rules and legal approval.

Either the vitexin (apigenin-8-C-beta-D-glucopyranoside; CAS Number 3681-93-4, purity; Cayman Chemical Company, USA) at a dose of 2.5 mg/kg (de Oliveira et al., 2020), diazepam (GABA/benzodiazepine receptor complex agonist; União Química Farmacêutica Nacional S/A) at a dose of 2 mg/kg (Vieira et al., 2016) or physiological saline (0.9% NaCl) containing dimethyl sulfoxyde – DMSO 1% was intraperitoneally administrated before the ionophore GABAergic receptor antagonist PTZ (α,β-cyclopentamethylenetetrazole; CAS Number: 54-95-5; Sigma/Aldrich, St. Louis, MO, USA) at a dose of 35 mg/kg (Dhir, 2012; Ergul-Erkec et al., 2015).

PTZ-induced kindling

The kindling epilepsy induced by repeated administrations of subconvulsant doses of PTZ is a chronic animal model of epilepsy used for elucidating the epileptogenic mechanisms involved in the genesis of seizures and evaluate the effects of new antiepileptic compounds (Ergul-Erkec et al., 2015; Mason & Cooper, 1972). Here, animals were divided into 3 groups (n=6-7 per group). Either the vitexin at a dose of 2.5 mg/kg, diazepam at a dose of 2 mg/kg (positive control group) or vehicle (1% DMSO in 0.9% physiological saline) was administrated intraperitoneally 30 min before each systemic injection of PTZ. For the development of kindling, PTZ was administered intraperitoneally at a subconvulsant dose of 35 mg/kg every other day for 29 days (15 injections). Kindling was confirmed when the control group that receives PTZ exhibited convulsive episodes (myoclonus of the forelimb with or without loss of the righting reflex) in three consecutive administrations of PTZ (Dhir, 2012). Immediately after each PTZ injection, animals were placed in a circular arena with transparent acrylic walls (60 cm in diameter and 50 cm in height), with 350 lx fluorescent lighting, and their behaviors were recorded for 30 min with a video camera (Handycam, Sony Corporation, Osaki, Shinagawa-ku, Tokyo, Japan). At the end of each test/animal, the apparatus was cleaned with 70% alcohol. The PTZ-induced convulsive reactions were classified according to the Racine scale, modified by Pinel and Rovner (1978), as shown in Fig. 1.

After behavioral recording, all the animals were euthanized and blood was collected for biochemical analyses.

Rotarod test

The rotarod test has been used to evaluate the balance and motor coordination of rats, enabling investigation of any possibly side-effects of drugs on locomotor function integrity (Dunham & Miya, 1957; Coimbra et al., 2001; Falconi-Sobrinho et al., 2017). The rotarod apparatus for rat used in our work (Acceler rotarod Ugo-Basile 7750, Comerio, Varese, Italy) con-

Score	Motor convulsive reactions
0	no seizure (exploratory behavior)
1	facial movements
2	facial movements and head nodding
3	facial movements, head nodding and forelimb clonus
4	facial movements, head nodding, forelimb clonus and rearing
5	facial movements, head nodding, forelimb clonus, rearing and falling
6	facial movements, head nodding, forelimb and hindlimb clonus, rearing and falling
7	Rotations, violent jumps and vocalization
8	All stage 7 behaviors followed by periods of hypertonia

Fig. 1. Scale of severity of convulsive reactions according to Racine's (1972), modified by Pinel and Rovner (1978).

sists of a revolving rod subdivided into four compartments (width 8.7 cm). 24 h after the end of the kindling (30^{th}), rats were subjected to three consecutive training sessions on the rotating bar for 5 min each. The following day (31^{st}), the animals were subjected to the rotarod test, which was conducted at a constant speed of 8 r.p.m. for 60 s. During the test, the number of falls from the rotarod was recorded.

Serum biochemical analysis

The rats were euthanized 24 h after behavioral experiments (30^{th} day). For biochemical analysis, blood samples were collected into tube without anticoagulant. Then, the serum was separated by centrifugation during 15 min for 2500 rpm and stored in a freezer at -20°C until use. Serum levels of creatinine, urea, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed on an autoanalyzer (Mindray BS-380) according to standard spectrophotometric methods using a reagent kit according to the manufacturer's instructions (Labtest, Brazil). The serum biochemical parameters were expressed as mean ± standard error of the mean (S.E.M).

Statistical analysis

Data from the convulsive seizures were submitted to a two-way repeated measures analysis of variance (two-way RM ANOVA) followed by Newman–Keuls *post-hoc* test, and reported as the means ± standard errors of the means (S.E.M.). Data from the rotarod test and biochemical measurements were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test, and reported as the standard deviation (S.D.). P values < 0.05 were considered to indicate statistical significance. The software used for statistical analysis and graph plotting was GraphPad Prism version 8.0 (San Diego, CA).

RESULTS

Effects of chronic treatment of either vitexin or diazepam on PTZ-induced kindling

According to the two-way ANOVA, there were significant effects of treatment ($F_{2,16}$=21.27; p<0.001) and days/time ($F_{14.224}$=9.403; p<0.01), as well as an interaction between them ($F_{28.224}$=2.319; p<0.001). Intraperitoneal injections of a sub-convulsive dose of PTZ every other day preceded by i.p. injections of the vehicle provoked a gradual increase in seizures severity, resulting kindling (p<0.05). In contrast, chronic treatment with either vitexin or diazepam suppressed the progression of kindling by reducing the severity of seizures from the 15^{th} day onwards (p<0.05, according to Newman–Keuls *post-hoc* test), as shown in Fig. 2. Additionally, data regarding kindling score for each animal in the experimental group, are shown in Table 1.

Effects of chronic treatment of either vitexin or diazepam on motor function integrity

According to one-way ANOVA, there were significant effects of pretreatment on the motor functions the animals kindled with PTZ ($F_{2,16}$=9.334; p<0.01). How-

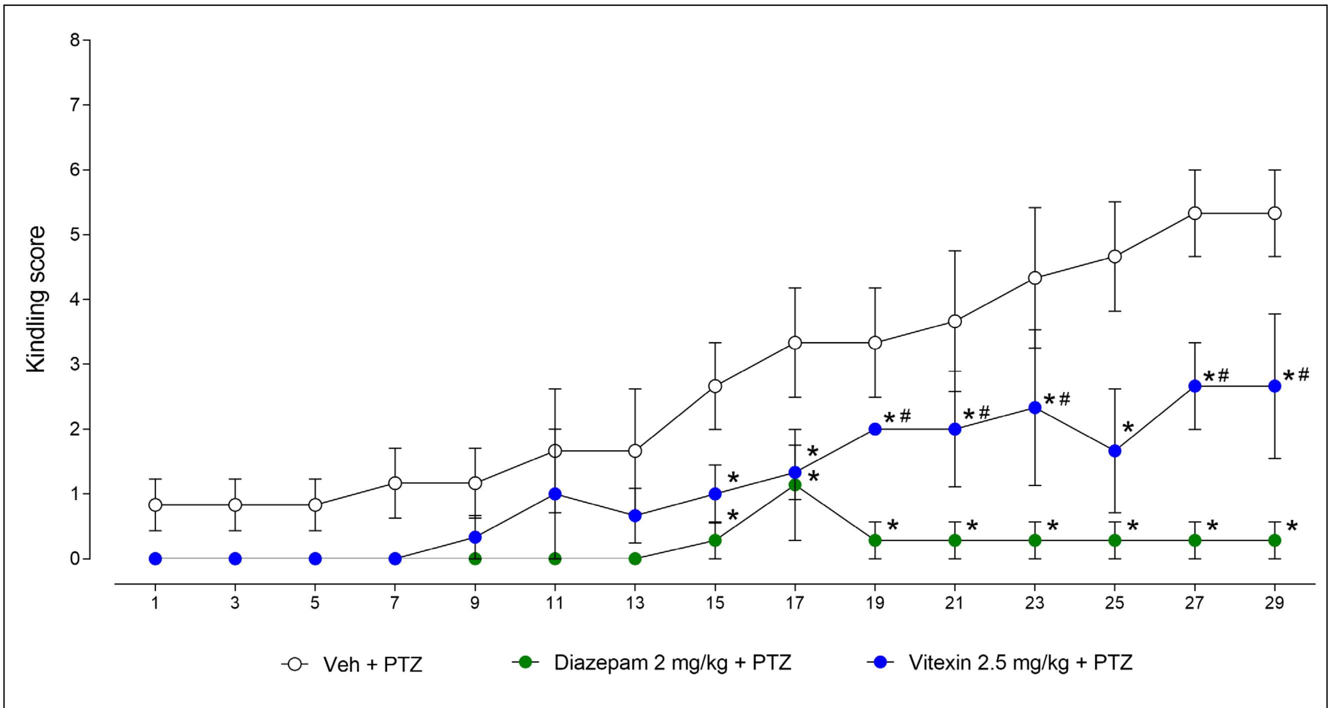

Fig. 2. Effect of chronic administration of either vitexin (2,5 mg/kg) or diazepam-DZP (2 mg/kg) on PTZ-induced kindling. Data are presented as the mean ± standard error of the mean (S.E.M.); **p*<0.05 compared with control group (Vehicle + PTZ); #*p*<0.05 compared with positive control group (Diazepam + PTZ), according to repeated measures two-way ANOVA followed by Neuman–Keuls *post-hoc* test.

ever, although pretreatment with diazepam caused more rotarod falls compared to the vehicle control group ($p<0.01$), vitexin did not provoke significant differences in the number of falls from the rotarod compared to the same vehicle control group ($p>0.05$), as shown in Fig. 3.

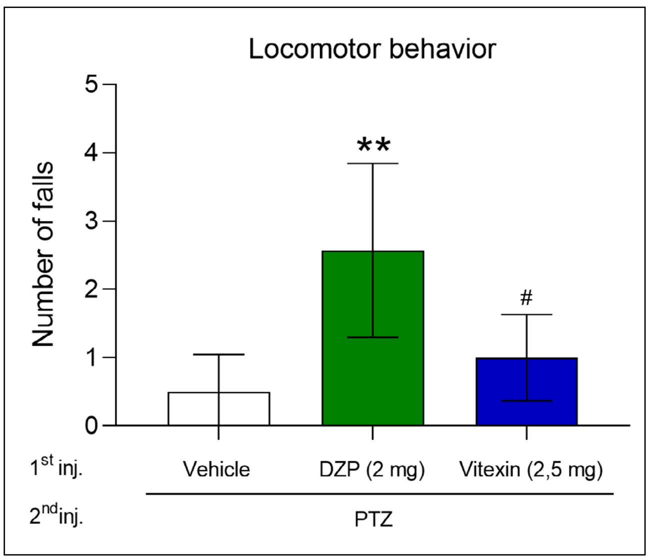

Fig. 3. Effect of chronic administration of either vitexin (2.5 mg/kg) or diazepam-DZP (2 mg/kg) on the locomotor integrity, expressed by number of falls from the revolving rod during the rotarod test, in animals kindled with PTZ. Data are presented as the standard deviation (S.D.); ***p*<0.01 compared with control group (Vehicle + PTZ), #*p*<0.05 compared with positive control group (Diazepam + PTZ), according to one-way ANOVA followed by Tukey's *post-hoc* test.

Effects of chronic treatment with either vitexin or diazepam on plasma levels in the kidney and liver biomarkers of kindled rats

According to one-way ANOVA, there were no significant differences in the levels of creatinine ($F_{2,16}$=0.6892; $p>0.05$) urea ($F_{2,16}$=1.321; $p>0.05$), AST ($F_{2,16}$=0.6934; $p>0.05$) and ALT ($F_{2,16}$=1.278; $p>0.05$) from the different animal groups kindled with PTZ. Indeed, neither vitexin nor diazepam treatments produced statistically significant differences in kidney creatinine and urea levels, and liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels when compared with the control group ($p>0.05$, according to Tukey's *post-hoc* test), as shown in Fig. 4A-4D.

Table 1. Data regarding the kindling score of animals per group.

Kindling score	Experimental groups																		
	Veh + PTZ						Diazepam + PTZ							Vitexin + PTZ					
Animals / Days	1	2	3	4	5	6	1	2	3	4	5	6	7	1	2	3	4	5	6
1st	0	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3rd	0	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5th	0	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7th	0	2	2	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9th	0	2	2	0	3	0	0	0	0	0	0	0	0	0	0	2	0	0	0
11th	0	2	6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0
13th	0	2	6	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	2
15th	2	2	6	2	2	2	0	0	0	0	2	0	0	0	0	2	0	2	2
17th	2	2	6	6	2	2	0	6	0	0	2	0	0	0	2	2	2	0	2
19th	2	2	6	6	2	2	0	0	0	0	2	0	0	2	2	2	2	2	2
21st	2	2	6	6	0	6	0	0	0	0	2	0	0	2	2	6	0	2	0
23rd	2	6	6	6	0	6	0	0	0	0	2	0	0	0	0	6	0	6	2
25th	2	6	6	6	6	2	0	0	0	0	2	0	0	0	2	2	6	0	0
27th	6	6	2	6	6	6	0	0	0	0	2	0	0	2	2	2	2	2	6
29th	2	6	6	6	6	6	0	0	0	0	2	0	0	0	6	6	2	2	0

Behavioral index	Veh no threat	Veh threatened	NMDA threatened	F value
Defensive attention	1.24 ± 0.17	1.53 ± 0.24	2.16 ± 0.52	$F_{2,15}$=1,81
FBA	0.17 ± 0.17	0.30 ± 0.21	0.41 ± 0.14	$F_{2,15}$=0,50
SAP	0.88 ± 0.44	2.30 ± 0.30*	1.93 ± 0.32	$F_{2,15}$=4,13
Total risk assessment	1.90 ± 0.23	4.05 ± 0.52*	4.14 ± 0.85*	$F_{2,15}$=4,59
Defensive immobility	0 ± 0	0 ± 0	0.91 ± 0.29*#	$F_{2,15}$=9,87
Escape	0 ± 0	1.99 ± 0.23*	2.30 ± 0.39*	$F_{2,15}$=22,9

Behavioral Duration (s)	Veh no threat	Veh threatened	NMDA threatened	F value
Defensive attention	2.69 ± 0.44	2.60 ± 0.25	4.90 ± 0.66*#	$F_{2,15}$=7,31
FBA	0.69 ± 0.52	0.59 ± 0.43	1.45 ± 0.64	$F_{2,15}$=1,92
SAP	0.99 ± 0.47	4.69 ± 0.52*	5.11 ± 0.71*	$F_{2,15}$=15,3
Total risk assessment	3.89 ± 0.61	7.88 ± 0.72*	11.5 ± 1.20*#	$F_{2,15}$=18,4
Defensive immobility	0 ± 0	0 ± 0	12.1 ± 3.69*#	$F_{2,15}$=10,7
Escape	0 ± 0	4.91 ± 0.81*	14.6 ± 1.75*#	$F_{2,15}$=44,6

*p<0.05 compared to the group (*Veh_no threat*); #p<0.05 compared to the group (*Veh_threatened*).

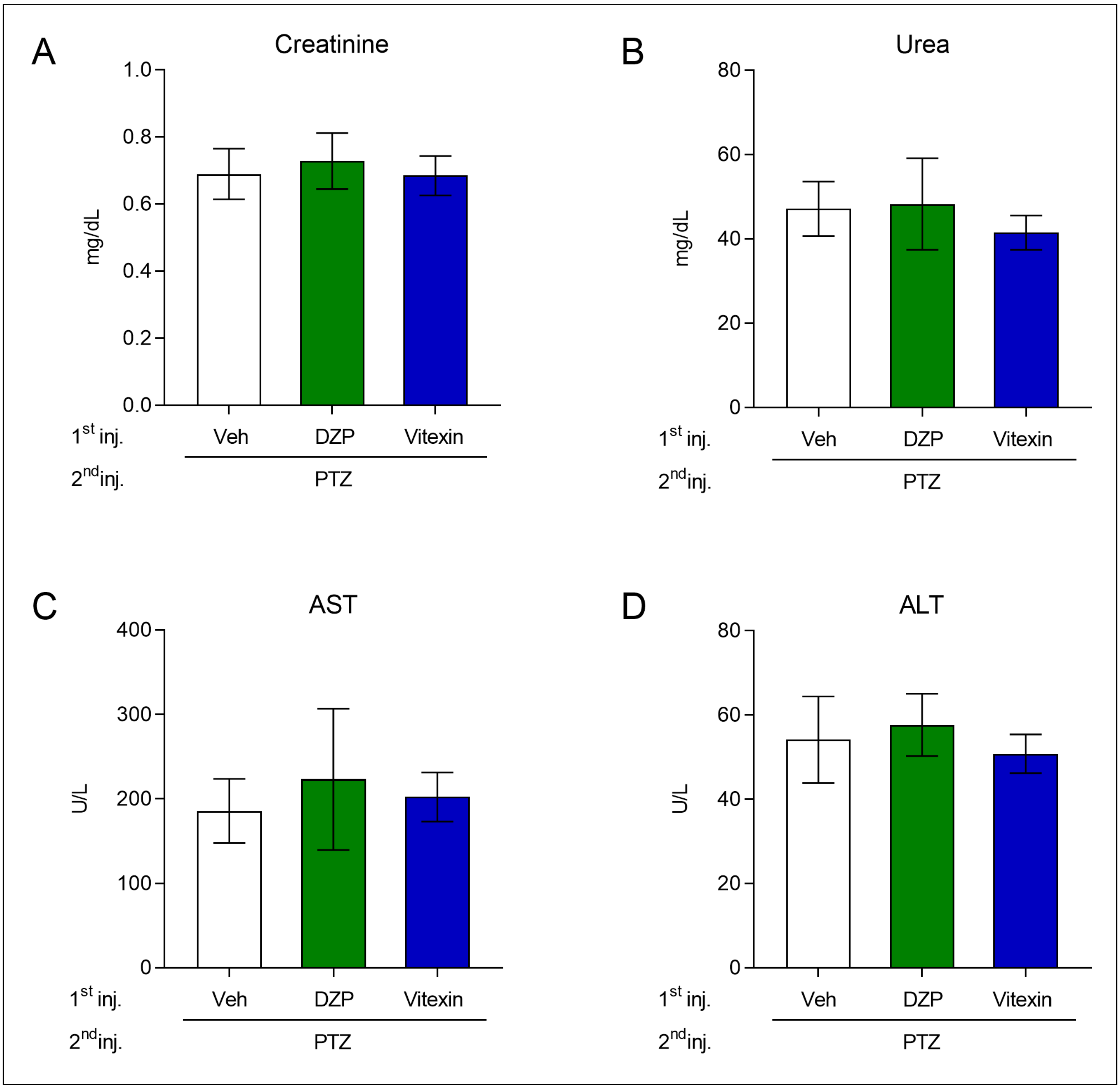

Fig. 4. Lack of effect of chronic administration of either vitexin (2.5 mg/kg) or diazepam (2 mg/kg) on renal levels of creatinine (A) and urea (B) and on hepatic levels of hepatic aspartate aminotransferase-AST (C) and alanine aminotransferase-ALT (D) in PTZ-kindled rats. Data are presented as the standard deviation (S.D.). Biochemical data were submitted to one-way ANOVA followed by Tukey's *post-hoc* test.

DISCUSSION

The present study sought to investigate the anticonvulsant potential of vitexin in a PTZ-induced chronic animal model of epilepsy. When it was preceded by intraperitoneal injections of vehicle, disinhibition of GABAergic neurotransmission by systemic injections of PTZ, a noncompetitive $GABA_A$ receptor antagonist, induced kindling epilepsy. PTZ has been widely used to induce acute seizures (De Deyn et al., 1992; Mazzei-Silva et al., 2014; de Oliveira 2016a, 2016b, 2018) and when administered at a subconvulsive dose over a longer period, it can cause progressive and permanent convulsive reactions (Ergul-Erkec et al., 2015; Singh et al., 2021; Thapliyal et al., 2023). Here, PTZ-kindled rats exhibited progressive clonic seizures, which were characterized by myoclonic movements of the paws and the head, with loss of postural control. In turn, long-term pretreatment with the flavonoid vitexin reduced the severity of seizures displayed by the PTZ-kindled rats. In fact, our data showed that chronic treatment with vitexin appears to be able to prevent the progression of kindling by decreasing severity of seizures from the second week onwards.

Although increasing evidence demonstrates the neuroprotective effects of flavonoids in animal models of epilepsy (Abbasi et al., 2012; Hossein & Marjan, 2017), to our knowledge, the present study is the first demonstration long-term neuroprotective effects of systemic administration of vitexin in PTZ-kindled rodents.

Previously, a recent study by our team demonstrated that systemic vitexin inhibits acute seizures induced by GABAergic receptor antagonists PTZ and picrotoxin, but fails to reduce seizures provoked by the glutamate receptor agonists NMDA and kainic acid (de Oliveira et al., 2020). Taken together, the findings of our present and previous studies suggest that the anticonvulsant effect of vitexin is due to its selective action on GABAergic system. Furthermore, although other systems such as acetylcholine (Serra et al., 1997), dopamine, and norepinephrine (Szot et al., 1997) have been shown to be affected by PTZ administration, the fact that the epileptogenic effect of PTZ is believed to occur mainly due to its action on $GABA_A$ receptors (Hansen et al., 2004), reinforce our argument that the anticonvulsant action of vitexin is mediated by GABAergic mechanisms. Most notably, Abbasi et al. (2012) presented evidence that PTZ-induced acute seizures rats are attenuated by intracerebroventricular microinjections of vitexin. These latter authors linked the anticonvulsant effects provoked by vitexin to the blockade at the benzodiazepine site of the γ-aminobutyric acid type A receptor complex, because pretreatment with the benzodiazepine antagonist flumazenil suppressed the anticonvulsivant effects of vitexin. These findings indicate that vitexin promotes anticonvulsant action by modulating the $GABA_A$-mediated Cl^- channel complex. However, a previous study performed by Aseervatham et al. (2016) suggested that the anticonvulsant effects of vitexin could also be related to the modulation of glutamatergic activity. They demonstrated that chronic vitexin (10 mg/kg, oral), reduced the severity of pilocarpine-induced seizures while inhibiting the mRNA expression of metabotropic glutamate receptors 1 (mGluR1) and 5 (mGluR5), as well as ionotropic N-methyl-D-aspartate receptor (NMDAr).

Whereas excessive and abnormal hyper-synchrony of electrical discharges involving hyperexcitable neurons in the pathogenesis of epilepsy is linked to an imbalance between inhibitory (mediated by GABA) and excitatory (mediated by glutamate) neurotransmission (Dalby & Mody, 2001; Engel, 1996; Naylor, 2010), the pharmacological manipulation of these neurotransmitters through vitexin injections, in addition to providing new insights into the long-term neuroprotective effects of this flavonoid against seizures, may provide a better understanding of the neural mechanisms involved in seizure modulation.

The present work also evaluated the liver and kidney function of PTZ-kindled rats that were pre-treated with vitexin (2.5 mg/kg, i.p). Our biochemical data showed that systemic administration of vitexin every other day for 29 days caused no significant changes in AST, ALT, creatinine and urea levels. In addition, vitexin also did not cause locomotor impairment in animals subjected to the rotarod. These data indicate that chronic vitexin produced neither nephrotoxic, hepatotoxic effects, nor motor impairment in PTZ-kindled rats.

CONCLUSION

In summary, our findings provide original insights on antiepileptic effects the vitexin on a chronic animal model of epilepsy. Long-term administration of vitexin reduces the severity of epileptic seizures without causing motor impairment and excitotoxic effects on renal and hepatic functions in PTZ-kindled rats.

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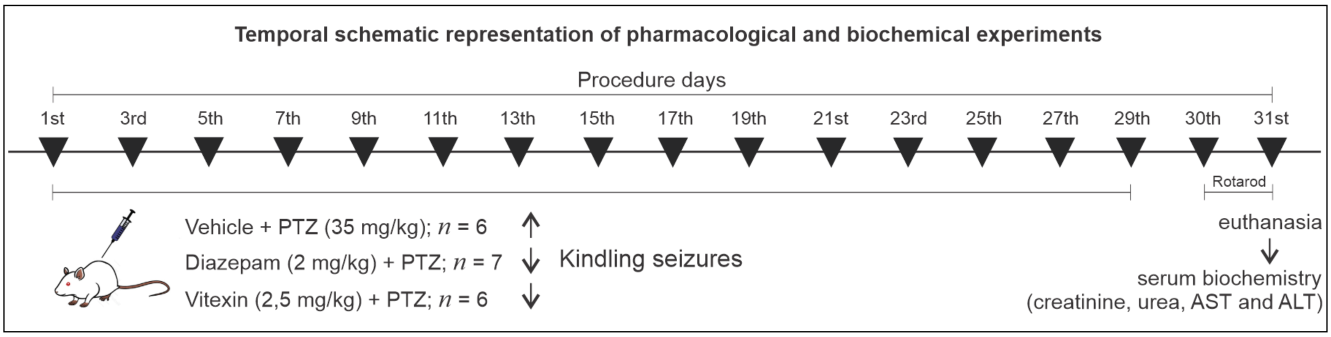
SUPPLEMENTARY MATERIALS



Graphical abstract. Schematic representation of the experimental design and summary of the findings obtained in our work.