Original Article

J Babol Univ Med Sci
Vol 20, Issu 11; Nov 2018. P:19-26

Anticonvulsant Effects of Hesperetin in Animal Model of Pentylenetetrazole-Induced Seizures

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J Babol Univ Med Sci; 20(11); Nov 2018; PP: 19-26

Received: Aug 5th 2018, Revised: Sep 17th 2018, Accepted: Sep 22nd 2018.

ABSTRACT

BACKGROUND AND OBJECTIVE: Hesperetin as the main flavonoid in citrus possesses various pharmacological properties including antioxidant and anti-inflammatory effects. In this study, the effects of hesperetin on seizures behavior and its function on total antioxidant capacity and lipid peroxidation has been investigated in pentylenetetrazol (PTZ)-induced seizures model.

METHODS: In this experimental study, thirty-five NMRI mice were divided into five experimental groups (n=7) as control, saline and hesperetin at doses of 10, 20 or 50 mg/kg. Animals received orally the related interventions for 7 days. On day 7, 30 minutes after oral gavage, convulsion was induced by single intraperitoneal (i.p.) injection of PTZ at dose of 60 mg/kg. After recording of convulsion behaviors including latency to myoclonic jerks, latency and duration of generalized tonic-clonic seizures, time to death, measuring of Ferric Reducing Antioxidant Power (FRAP) and Thiobarbituric acid reactive substances (TBARS) was carried out in hippocampus tissues.

FINDINGS: Pretreatment with hesperetin at dose of 50 mg/kg significantly increased the latency of myoclonic jerks (hesperetin 50: 22±3.35 s, p=0.032) and generalized tonic-clonic seizures (hesperetin 10: 1±21.48 s, p=0.0003, hesperetin 20: 35.2±83.6 s, 0.001, hesperetin 50: 34.5±2.30 s, p=0.004). The use of hespertin at dose of 10 mg/kg significantly reduced TBARS values compared to saline (p<0.003) and doses of 20 and 50 mg/kg hespertin (p<0.0001). Any significant difference in FRAP levels was not observed between different experimental groups.

CONCLUSION: The results of study indicate that hesperetin might be effective as supplementary treatment in epilepsy disorder.

KEY WORDS: *Hesperetin*, *Convulsion*, *Pentylenetetrazol*.

Please cite this article as follows:

Baradaran S, Ghasemi–Kasman M, Ebrahimpour A, Ahmadian SR, Pouramir M. Anticonvulsant Effects of Hesperetin in Animal Model of Pentylenetetrazole-Induced-Seizures. J Babol Univ Med Sci. 2018;20(11):19-26.

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[DOR: 20.1001.1.15614107.1397.20.11.3.1]
[DOI: 10.18869/acadpub.jbums.20.11.2]

Introduction

Epilepsy is the third most common neurological disorder with unpredictable seizures and cognitive decline. Earlier studies considered the release of inflammatory factors from activated glial cells as a major contributor to the onset of this disease (1,2). In addition, seizure activity during epilepsy decreases the antioxidant defense mechanism and increases the amount of free radicals, which in turn exacerbates oxidative stress in the brain. Free radicals play an important role in lipid peroxidation, cerebral edema and epilepsy (3).

Unlimited medications have been developed for the treatment of epilepsy, but about 20% to 30% of patients do not respond to common treatments for epilepsy. Also, continuous and prolonged treatment of antiepileptic drugs provides the basis for the side effects of multiple drugs (3). In recent years, the use of herbal medicines has increased due to its widespread dispersal, relative efficacy, less side effects and low prices compared to other drugs (4).

Previous reports have shown that natural antioxidants, like polyphenols, reduce cognitive functions and brain damage (5,6). Flavonoids are polyphenolic compounds found in many fruits, vegetables, and several reports confirm their antioxidant and anti-inflammatory effects (7-10). Hesperetin in citrus skin, which has broader healing properties than that of flavonoids, Hesperetin, has its own neuroprotective potential using radical decomposition properties and reduced levels of calcium and caspase-3 from PC-12 cells compared to the toxicity due to hydrogen peroxide (11, 12).

Many studies have also shown that hesperetin can increase the antioxidant defense capacity by increasing the enzymes catalase, superoxide dismutase and glutathione peroxidase (13,14). Kumar et al. indicated that hesperedin, a flavanone glycoside, has significant anticonvulsant properties in the pentylenetetrazole-induced chemical kindling model (PTZ). Also, the results of this study showed that hesperedin with increasing glutamate, superoxide dismutase, catalase and mitochondrial complexes, as well as reduction of malondialdehyde and nitrate levels exerts its anticonvulsant effects in the groups receiving pentylene tetrazole (15). In addition, the *Citrus aurantium aqueous* extract has been shown to increase the seizure threshold in the gutter fish (16). Empirical models for inducing epilepsy provide the possibility of analyzing the mechanisms and predisposing factors of epilepsy and assessing anticonvulsants and new therapies (17). One of the common animal models for epilepsy is the induction of acute seizure with PTZ injection. PTZ as an antagonist of GABAA receptors causes seizures (18). According to available evidence, a study has not been done to evaluate the effects of hesperetin pre-treatment on seizure parameters, total antioxidant capacity and lipid peroxidation index. Therefore, this study was designed to evaluate the anticonvulsant effects of hesperetin on mice in the acute seizure model induced by pentylenetetrazol. Additionally, the level of lipid peroxidation and antioxidant index were measured by measuring the Thiobarbituric acid reactive substances (TBARS) and total antioxidant power (FRAP).

Methods

Medications: Pentylenetetrazole and hesperetin (95% purity) were prepared from Sigma-Aldrich (Germany) and dissolved in normal saline.

Animals: In this experimental study, after approval in the Ethics Committee of the Babol University of Medical Sciences (ethical code: MUBABOL.HRI.REC.1396.98), NMRI male mice in the range of 20-25 g from (animal room of Babol University of Medical Sciences) were kept in standard conditions and had free access to water and food. Before the experiments, the animals were weighed and labeled and transferred to the lab and remained in the laboratory for 30 minutes and adapt to laboratory conditions.

Experimental group: 35 mice were randomly divided into 5 groups (n=7) as controls, saline and doses of 10, 20 and 50 mg/kg hesperetin.

Control group: Animals did not receive any intervention in this group;

[DOR: 20.1001.1.15614107.1397.20.11.3.1]
[DOI: 10.18869/acadpub.jbums.20.11.2]

Saline+Pentylenetetrazole Group: In this group, animals received saline pre-treatment (gavage) as a hesperetin solvent for one week and half an hour after the last saline gavage, received PTZ 60 mg/kg intraperitoneally.

Hesperetin+Pentylenetetrazole group: In this group, hesperetin was administered orally at 10, 20, or 50 mg/kg for one week (14) and half an hour after the last hesperetin gavage, pentylenetetrazole was injected intraperitoneally.

The animals were monitored for recording the symptoms of seizure for 20 minutes after PTZ injection. Since the hippocampal tissue during epileptic injections is one of the most important areas that causes neuronal damage (19), the tissue of the hippocampus was extracted and examined for biochemical studies. Total antioxidant capacity and lipid peroxidation were evaluated using FRAP and TBARS methods.

Seizure model: In order to develop acute seizure model, intraperitoneal injection of PTZ (60 mg/kg) was used (20). After PTZ injection, the animals were monitored for 20 minutes and seizure symptoms were examined. Behavioral Symptoms were classified in Stage 0= No response, Stage 1= Contraction of the facial and ears muscles, Stage 2= Radiation of the contraction in the body and movement of the head up and down, Step 3= Myoclonic jerks, Step 4= Standing on two-foot and clonus of anterior organs, and stage 5= Generalized tonic-colonic attacks and loss of standing reflexes (19).

Total antioxidant capacity measurement: After recording the behavioral symptoms, animals were first anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the hippocampus tissue was rapidly removed and transferred to a microtube in -80 ºC until biochemical measurements.

Each tissue of the hippocampus was equally well-balanced in 0.5 µL of normal saline and after centrifugation for 5 minutes at 1000 g, the soup was analyzed for biochemical studies. 1.5 mL of the FRAP ready-to-use reagent (Buraşatat, TPTZ reagent and chloro-ferric solution, 11: 1: 1, respectively) were added to all test tubes and incubated for 37 minutes at 5 °C. In the next step, 51 µL of the sample (tissue extracts or different standards) was added to the appropriate tubes and mixed well and re-incubated for 15 minutes at 37 °C. After this time, the intensity of the color at 593 nm in front of the blank (1.5 mL of FRAP solution and 50 µL of distilled water) was measured by spectrophotometer. The adsorption rates were plotted using a standard curve drawn using iron sulfate solution at concentrations of 125-450-500 - 1000 µM as a standard (21). Tubes in triplicate were used for biochemical assessments.

TBARS Assay: To measure TBARS, one mL of tissue extract was mixed with 2 mL of TBARS reagent and placed in a boiling water bath for 15 minutes. The resulting mixture was centrifuged at 2500 rpm for 10 minutes and the optical absorption was read by a spectrophotometer at 535 nm. (22).

Statistical analysis: Data were analyzed by one-way ANOVA and Tukey's post-test. $P<0.05$ was considered significant.

Results

Intravenous injection of pentylenetetrazole resulted in severe seizure attacks in the 9.41±51.6 s in the saline group. Although pre-treatment with hesperetin at doses of 10 and 20 mg/kg delayed the onset of seizure attacks (10 mg/kg: 16.17±4.12 s, dose 20 mg/kg: 15.17 ±2.96 s), but this difference was not significant (in the hesperetin group 10 mg/kg: p=0.3703, in the hesperetin group 20 mg/kg: p=0.4977). Compared with the saline group, the use of hesperetin at a dose of 50 mg/kg significantly increased the onset of seizure attacks (22±3.25 s, p=0.032). In addition, our results showed no significant difference between the groups receiving hesperetin (Fig 1A). Investigating the delay time in generalized tonic-clonic seizures showed that applying hesperetin 10 mg/kg (39.3±20.35s) effectively increased the time of this parameter compared to the saline group (1±21.48 s) (p=0.0003). In addition, compared to saline recipients, pre-treatment with doses of 20 mg/kg (35.2±83.6 s) and 50 mg/kg (34.2±5.30 s) also significantly increased the time of onset of

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generalized tonic-clonic seizures (hesperetin 20 mg/kg: p=0.001, hesperetin 50mg/kg: p= 0.004) (Fig. 1B). Measurement of generalized tonic-clonic seizure duration showed that, although compared with saline (10.1±33.308 s), pre-treatment with hesperetin at doses of 20 and 50 mg/kg reduced the duration of generalized tonic clonic seizures, this difference was not significant (dose 20 mg/kg: 7.6±1.16 s, p=0.43, dose 50 mg/kg: 8±1.065 s, p=0.52). Furthermore, hesperetin 10 mg/kg showed a more effective reduction compared to other recommended doses, but this difference was not significant (6.33±1.30 s, p=0.11, Fig. 1C). Additionally, the measurement of the death time (time betweenPTZ injection and death of the animals) in the per-treated groups with different doses of hesperetin (dose 10 mg/kg: 426/2±133/1 s, p= 0.36; dose 20 mg/kg: 621.8±197.3, p= 0.087; dose 50 mg/kg: 589.3± 154.5, p=0.095) indicated an increase compared to the control group (85.6±20 s). However, this increase was not significant in comparison with the control group (Fig. 1D). Therefore, the results suggest that use of hesperetin in some doses reduces the manifestation of behavioral seizures. The results of this study showed that although the use of pentylenetetrazol reduced the total antioxidant capacity in saline groups (360.1±32.08 µM, p= 9989), hesperetin 20 mg/kg (297.3±54.90 µM, p=0.8564) and 50 mg/kg (243.4±43.35 µM, p= 0.009) compared to the control group (382.38±9.59 µM), but this differences were not statistically significant. However, the use of hesperetin at a dose of 10 mg/kg (457.105± 1.8 µmol/L) resulted in an increase in antioxidant activity compared to the saline recipient group, but this difference was not significant (Fig. 2A).

In addition, the study of lipid oxidation index using TBARS showed that lipid peroxidation significantly increased in saline recipients compared to control group (p<0.0001). Also, in comparison with the control group, the lipid peroxidation index increased significantly in the groups receiving 10 mg/kg (p<0.009), 20 mg/kg (p<0.0001) and 50 mg/kg (p <0.0001), respectively. Applying a low dose of hesperetin (10 mg/kg) significantly decreased TBARS in comparison with the saline group (p<0.003). In addition, our results indicated that there was a significant difference in lipid peroxidation index between dose of 10 mg/kg hesperetin with doses of 20 mg/kg (p<0.0001) and 50 mg/kg (p<0.0001). Pre-treatment with hesperetin, especially in low dose, reduced the level of lipid peroxidation (Fig. 2B).

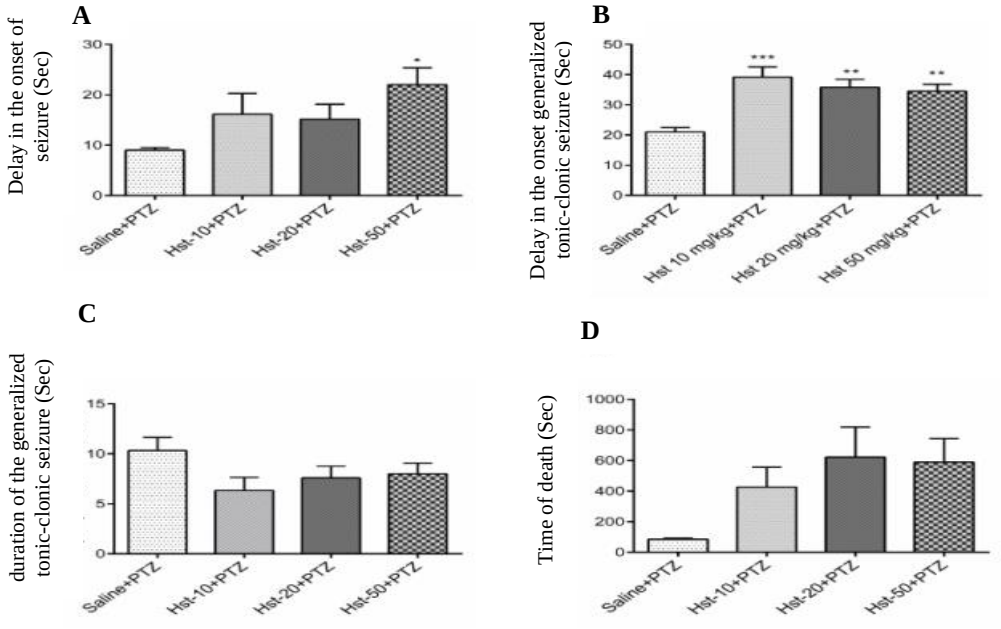

Figure 1. The effect of hesperetin on the delay of the onset of myoclonic contractions (A), latency of generalized tonic-clonic seizure (B), the duration of the generalized tonic-clonic seizure (C), and the time of death (D). *p<0.05, **p<0.01 and ***p<0.001 compared with the saline group. Number of animals per group (n=7).

[DOR: 20.1001.1.15614107.1397.20.11.3.1]
[DOI: 10.18869/acadpub.jbums.20.11.2]

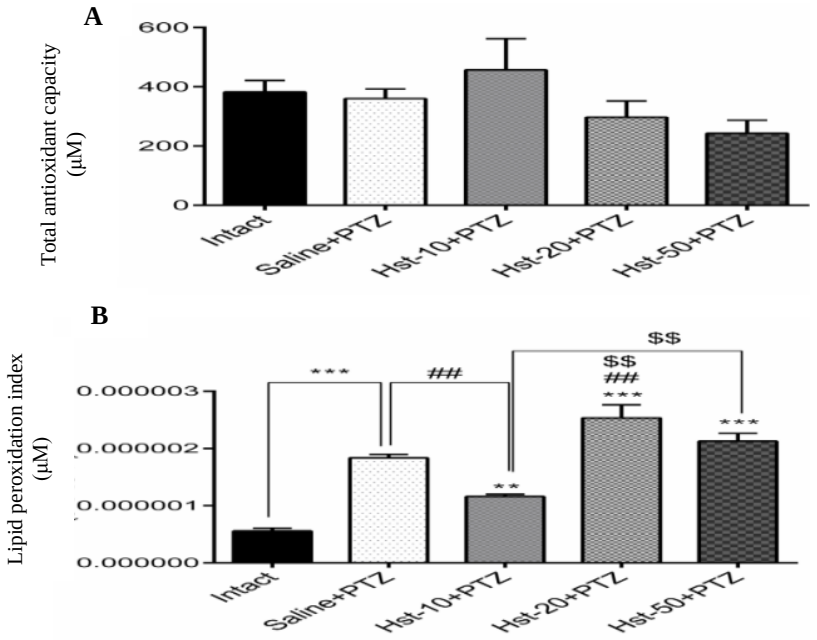

Figure 2. Effect of hesperetin pre-treatment on total antioxidant capacity (A) and lipid peroxidation index (B). *p<0.01 and **p<0.001 compared with the intact group; $^{\#\#}$p<0.01 compared with the saline group and $^{\$\$}$ p<0.01 compared with the hesperetin group at dose of 10 mg/kg. Number of animals per group (n=7).

Discussion

The results of this study indicate that pretreatment with hesperetin reduces some behavioral symptoms of seizure. In addition, the amount of lipid peroxidation in animals receiving low-dose hesperetin decreased. The previous study suggests that hesperedin and its aglycone hesperetin reduce the electrical activity of the hippocampal pyramidal neurons *in vitro* (23). The study by Rosa-Falero et al. indicated that the use of aqueous extract of *Citrus aurantium* increased the duration of seizure attacks, and this was modulated by NMDA receptors, mGluR's I and II (16). Also, the study by Kumar et al. showed that Hesperdin through the effect on inhibitory receptor of GABA and benzodiazepine, exerts its anticonvulsant effects in the pentylenetrazole-induced seizure model (24).

Similar to these studies, our study also found that pretreatment with hesperetin reduced seizure manifestations in the phenylenetetrazole-induced seizure model. In order to determine the possible anticonvulsant mechanism/s of hesperetin, total antioxidant capacity and lipid oxidation index in the hippocampus were evaluated. Our results indicated that low dose of hesperetin reduced the amount of lipid oxidation in the hippocampal tissue. In agreement with our results, a previous study by Kumar et al., demonstrated that hesperedin treatments reduces oxidative stress, mitochondrial dysfunction and cognitive impairment in the pentylenetetrazole-induced kindling model. The results of this study indicate that a part of the neuroprotective effects of hesperetin may be obtained by modifying the nitric oxide pathway (15). Frequent studies have shown that hesperetin is effective in reducing oxidative stress (25-27).

Previous studies have shown that hesperetin and its nano-crystal form can effectively reduces the oxidative stress and increases the antioxidant capacity of the brain tissue in the animal models of Alzheimer's disease and autism (28, 14). Also, the results of Baradaran et al. study showed that hesperetin effectively reduced the activity of glial cells and the extent of the demyelination region in local demyelination model of optic chiasma (29). Hesperetin has been demonstrated to play its antioxidant role in two ways, one with direct inhibition of radicals and the other increasing the antioxidant defense of the cell. Thus, protects DNA, proteins and tissues from damage to inherent factors, such as tumors and external agents such as radiation, inflammation and toxins. Hesperetin may inhibit ROS production and prevent the formation

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of apoptosis cascades that lead to cell death (30). This compound increases the antioxidant defense capacity by inducing hemoxygenase-1 through ERK/Nrf2 signaling. This induction can also increase the expression of the gene and the level of antioxidant enzymes such as CAT, SOD, and glutathione (GST).

In addition, hesperetin has several mechanisms for protecting neurons, including the prevention of ROS formation and caspase 3 activity, decreased membrane damage and DNA, increased activity of antioxidant enzymes, maintenance of calcium homeostasis, mitochondrial potential and modulating signaling of cell survival (31, 9). In spite of the mentioned evidence regarding the effects of hesperetin on increasing the antioxidant capacity, we did not find any significant difference between the studied groups. However, a low dose of hesperetin could increase total antioxidant capacity, compared with other hesperetin groups, but this difference was not significant. The interesting point in our study was that while dose of 10 mg/kg of hesperetin reduces TBARS levels, its administration at high doses (20 and 50 mg/kg) increased the lipid peroxidation index. According to our results, Bouayed et al., study showed that although natural compounds with antioxidant properties can be effective in reducing the oxidative stress level, high doses of them may have toxic effects and increase the level of oxidative stress in the body (32).

In addition, Khadir et al. showed that arbutin, the hydroquinone glycosylated form in the wild pear tree at a dose of 50 mg/kg increased total antioxidant capacity, while its high dose (100 mg/kg) increased TBARS (33).

Overall, the results of this study indicate that pretreatment with hesperetin reduces seizure symptoms. Although a part of the anticonvulsion effect of hesperetin seems to be modulated by its effects on reduction of lipid peroxidation index, but further studies are needed to identify the exact mechanism of hesperetin anticonvulsant effects.

Acknowledgment

Hereby, we would like to thank deputy of Research and Technology of Babol University of Medical Sciences for Supporting this research project.

[DOR: 20.1001.1.15614107.1397.20.11.3.1]
[DOI: 10.18869/acadpub.jbums.20.11.2]

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[DOR: 20.1001.1.15614107.1397.20.11.3.1]
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