The neuroprotective effects of natural antioxidant against brain injury induced by paracetamol in a rat model of protein malnutrition

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Received: 25 October 2019
Revised: 30 November 2019
Accepted: 9 December 2019
Published: 24 March 2020

Egyptian Pharmaceutical Journal 2020, 19:55–61

Backgroundand objective
Apigenin (API), as flavonoid, is found in chamomile tea, parsley, celery, onions, lemon balm, and oranges. Therefore, in the present study, we investigated the neuroprotective role of API against oxidative stress, neuroinflammation, and neurotransmitter abnormality induced by cumulative dose of paracetamol (PA) in a rat model of protein malnutrition.
Materials and methods
A total of 30 male Wistar albino rats, weighing 150–200 g, were used in five groups. API (50 mg/kg, p.o., once daily for 1 week) was administered to low-protein-fed rats with PA (500 mg/kg, p.o, once)-induced brain injury.
Results and conclusion
API treatment obviously improved cerebral-reduced glutathione and malondialdehyde contents and also superoxide dismutase and myeloperoxidase activities. Additionally, it attenuated contents of serotonin, catecholamines, γ-aminobutyric acid, and cholinesterase activity. Moreover, API reduced the abnormal cerebral pathological lesions. Consequently, API has a protective effect on rat brain injury induced by both protein malnutrition and PA.

Keywords:
apigenin, brain injury, neurotransmitters, paracetamol, protein malnutrition

Egypt Pharmaceut J 19:55–61

1687-4315

Introduction

Malnutrition corresponds to severe health and socioeconomic problems worldwide. Nutritional inefficiency is a main nongenetic factor that leads to disturbances in neurogenesis, such as the inhibition in select γ-aminobutyric acid (GABA) interneuron systems [1]. Abundant studies have found biochemical changes in nervous systems in experimental models of malnutrition, especially those related to neurotransmitter systems [2,3].

The brain may be particularly vulnerable to free radicals owing to its high rate of oxidative metabolic activity, high content of polyunsaturated fatty acids, regions rich in iron concentration, and moderate levels of antioxidant [4]. We hypothesized that an increase in free radical content and/or its interaction with macromolecules, especially proteins, could be a potential mechanism for changes in brain development that are related to protein malnutrition (PM).

Paracetamol (PA) (Acetaminophen, *N*-(4-hydroxyphenyl)acetamide) is widely used as a prescription and over-the-counter analgesic and antipyretic agent [5]. PA is metabolized mainly in the liver via conjugation with glucuronic acid and sulfate, and finally excreted in urine. PA metabolism also yields a cytochrome P450-dependent highly reactive metabolite known as *N*-acetyl-p-benzoquinoneimine (NAPQI). This metabolite is able to react with glutathione (GSH) forming a nontoxic conjugate to be excreted via kidneys [6]. However, an overdose of PA results in formation of NAPQI that exceeds the capacity to detoxification. The excess NAPQI binds to cellular proteins, including mitochondrial proteins, leading to an oxidative stress-mediated damage [7]. The mechanism of action and safety of PA on the central nervous system still remains unclear. Probably, PA is assumed to be an additional factor in the development of neurobehavioral disturbances or neurodegenerative disorders [8]. According to literature data, PA can easily cross the blood–brain barrier, decrease GSH reactivity, and potentiate oxidative stress in neurons [9]. Recent data suggested a link between the common and the growing use of PA and increased incidence of autism among the youngest group of patients [10].

Diet is a major lifestyle factor that affects health; foods are not only a source of macronutrients (proteins, fats, and carbohydrates) and micronutrients (vitamins and minerals) but also contain various amounts of non-nutritional molecules called phytochemicals that have beneficial health effects [11]. Flavonoids are natural polyphenols produced by plants and have beneficial properties including antioxidant, anti-inflammatory, and anti-carcinogenic effects [12]. Naturally, apigenin (API) exists as apigenin-7-O-glucoside and various acylated derivatives. The best sources of API are parsley, chamomile, celery, onions, lemon balm, and oranges [13,14]. Dried parsley has been reported to have the maximum quantity of API [15]. The flavonoid API scavenges superoxide, singlet oxygen, and hydroxyl radicals *in vitro* and boosts up the cellular antioxidant defense system [16].

Materials and methods

Animals

Male Wistar albino rats, weighing 150–200 g, obtained from the animal house of the National Organization for Drug Control and Research (NODCAR, Cairo, Egypt) were used in the present study. Animals were housed for at least one week in the laboratory room before testing under controlled environmental conditions: constant temperature of 25±2°C, humidity of 60±10%, and alternating 12 h light/dark cycles. Standard pellet diet and water were allowed *ad libitum*. The present work was approved by the Ethics Committee of Animal Care and Use of Beni-Suef University (NUB-020-019) and complied with the Guide for Care and Use of Laboratory Animals published by means of the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Drugs and chemicals

API was purchased from Xi'an Lyphar Biotech Co. Ltd. (Xi'an, China). PA was obtained from Hebei Jiheng Group Pharmaceutical Co. Ltd. (Hengshui, China). Diet ingredients were obtained from Oxford Lab Chem (Vasai, Maharashtra, India). All chemicals used during the study were of standard analytical grade.

Experimental design

A total of 30 adult male rats were randomly allocated into five groups, with six rats each:

(1) Normal control group received normal diet during the 3 weeks, and received 1% Tween 80.
(2) PM control group received protein deficient diet during the 3 weeks [17,18].
(3) PA control group received normal diet during the 3 weeks, and received PA (500 mg/kg, p.o.; once) in 1% Tween 80 at the last day [19].
(4) PM+PA group received protein-deficient diet during the 3 weeks, and received PA (500 mg/kg, p.o.; once) in 1% Tween 80 at the last day.
(5) PM+PA+API group received protein-deficient diet during the 3 weeks, received API (50 mg/kg, p.o.) [20] in 1% Tween 80 daily during the last third week and received PA (500 mg/kg, p.o.; once) in 1% Tween 80 at the last day.

All animals were fasted 18 h before and 24 h after PA or vehicle administration with free access to water; after 3 weeks, all animals were decapitated.

Tissue sampling

Brain of each rat was immediately excised, and divided into two portions, one was kept in 10% formalin for histopathological examination, whereas the other was reserved for estimating the other biochemical parameters. As the cerebrum (cerebral cortex and hippocampus) were dissected, each of them was weighed and bisected. The first half of cerebrum was homogenized in ice cold saline to prepare 10% homogenate, which was used for the assessment of oxidative stress biomarkers, myeloperoxidase activity (MPO), as an inflammatory marker, as well as GABA and cholinesterase (ChE) cerebral contents. The other half of cerebrum was homogenized in ice cold solution of acidified n-butanol to obtain 10% homogenate for the determination of brain contents of serotonin (5HT), dopamine (DA) and norepinephrine (NE). Finally, the used animals were frozen till being incinerated.

Biochemical parameters

(1) Estimation of cerebral malondialdehyde (MDA) contents: determination of MDA was carried out according to the method of Buege and Aust [21] with a slight modification in the incubation period according to the method of Deniz *et al.* [22].
(2) Estimation of cerebral reduced GSH contents: the content of GSH was determined in homogenates according to the method described by Beutler *et al.* [23].
(3) Estimation of cerebral superoxide dismutase (SOD) activity: the activity of SOD was determined in the homogenate using Biodiagnostic kit (Cairo, Egypt) according to the method described by Kinouchi *et al.* [24].
(4) Estimation of cerebral proinflammatory activity of MPO: MPO enzyme was determined according to the method of Bradley *et al.* [25].

(5) Estimation of cerebral monoamine contents: serotonins as well as catecholamines (dopamine and norepinephrine) were determined according to the method of Ciarlone [26] using spectrophotoflourometer RF-5000 Shimadzu, Japan.
(6) Estimation of cerebral GABA: determination of GABA contents in brain was carried out according to the method described by Sutton and Simmonds [27].
(7) Estimation of cerebral ChE activity: ChE in cerebrum was determined according to the method of Ellman *et al.* [28] using DTNB-phosphate reagent after 10-min incubation of the brain homogenate with acetylthiocholine iodide.

Histopathological examination

Autopsy samples were taken from brain of rats in the different experimental groups then fixed in 10% formalin and prepared in saline for 12 h. Washing was done in tap water and then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene, and embedded in paraffin at 56°C degree in hot air oven for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4μm thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain according to the method of Bancroft *et al.* [29] for histopathological examination through the light microscope.

Statistical analysis

All values were presented as means±SEM. Statistical analysis was performed using GraphPad Prism version 5 (Graph-Pad, San Diego, California, USA). A comparison between different groups was carried out using one-way analysis of variance, followed by a Tukey–Kramer's multiple comparisons test. A difference was considered significant when $P<0.05$.

Results

Oxidative stress

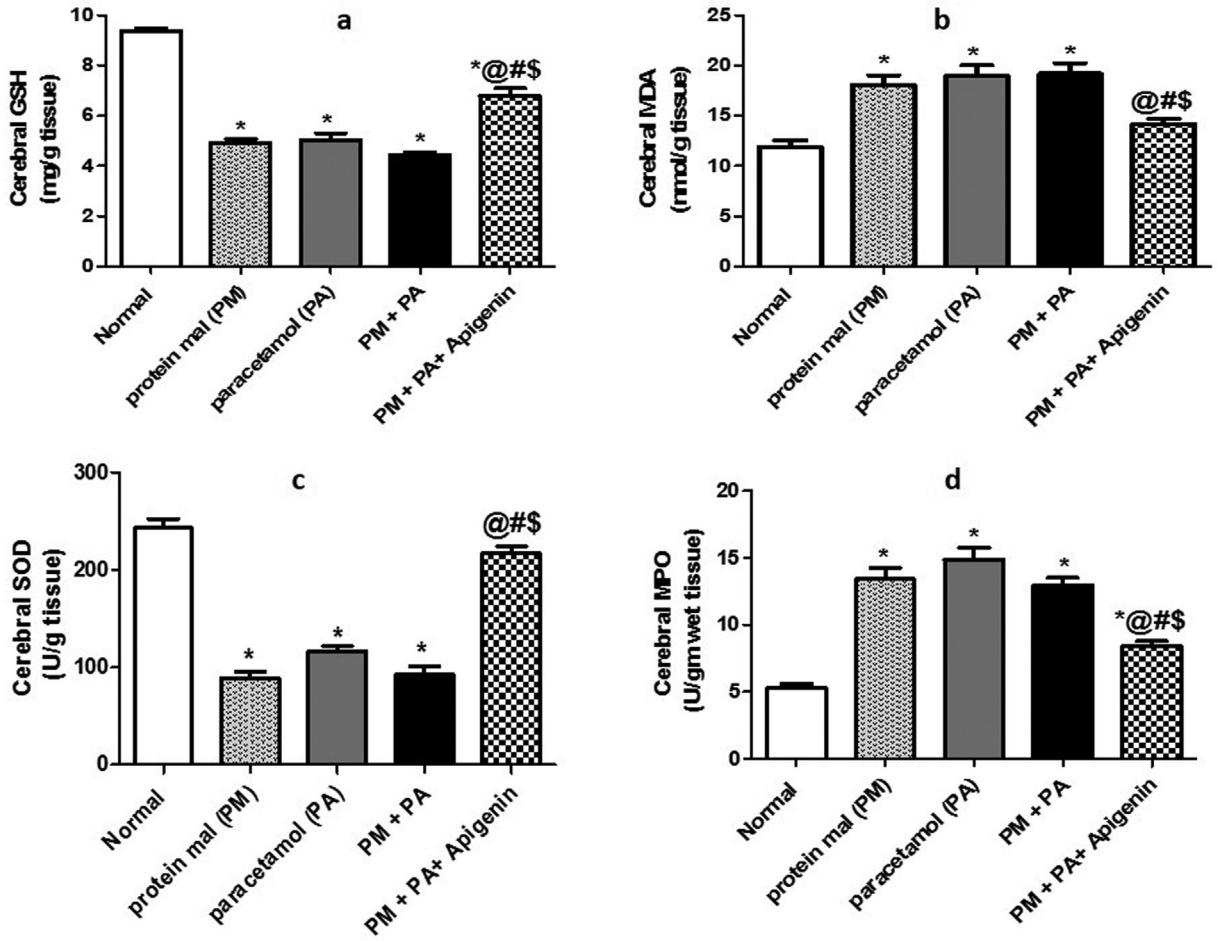
As illustrated in Fig. 1, the cerebral contents of GSH (a) and MDA (b) for normal rats were 9.37±0.11 (mg/g wet tissue) and 11.90±0.66 (nmol/g wet tissue), respectively, and the cerebral activities of SOD (c) and MPO (d) of normal rats were 243.10±9.43 and 5.28±0.31 (U/g wet tissue), respectively. We found that PM+PA induced oxidative stress in rats as evidenced by significant decrease in cerebral content of GSH by 53% and activity of SOD by 62%, whereas they caused a significant increment in MDA content by 61% and MPO activity by 146% compared with the normal control group. In addition, PM alone declined cerebral content of GSH significantly by 47% and activity of SOD by 64%, but they caused a significant increase in MDA content by 52% and MPO activity by 154% compared with the normal control group. Moreover, administration of PA alone caused significant decrease in cerebral content of GSH by 46% and activity of SOD by 52%; however, they caused a significant increase in MDA content by 60% and MPO activity by 182% compared with the normal control group.

Pretreatment with API significantly increased the content of GSH by 38% and SOD activity by 144%, with consequent decrease of MDA content by 22% and MPO activity by 37% as compared with PM group. Moreover, API significantly increased GSH content by 35% and SOD activity to 87% with consequent decline in content of MDA by 25% and activity of MPO by 44% as compared with PA group. In addition, API increased the content of GSH by 53% and SOD activity by 135% significantly, with decrease of MDA content by 26% and MPO activity by 36% as compared with PM+PA group. Finally, pretreatment with API significantly decreased GSH content by 28% and increased MPO activity by 58% as compared with the normal group.

Neurotransmitters

As illustrated in Fig. 2, the cerebral contents of 5-HT (a), DA (b), NE (c), and GABA (d) for normal rats were 21.00±0.52, 23.00±1.40, 1.36±0.06, and 53.70 ±1.63 (μg/g wet tissue), respectively, and the cerebral activity of ChE (e) for normal rats was 75.01±2.75 (U/g wet tissue). The cerebrum content of 5-HT was significantly decreased in groups PM, PA, and PM+ PA by 90, 42, and 75%, respectively, as compared with the normal group. Similarly; the cerebrum activity of ChE was significantly decreased in groups PM, PA, and PM+ PA by 26, 12, and 33%, respectively, as compared with the normal group, accompanied with significant increase in DA, NE, and GABA by 27, 122, and 91% and by 29, 81, and 94% and by 90, 110, and 120%, respectively, as compared with normal group. Furthermore, as compared with combined group (PM +PA), treatment with API significant increased 5-HT and ChE by 58 and 36%, respectively, accompanied with significant decrease in the DA, NE, and GABA by 52, 54, and 41%, respectively. Similarly, as compared with cumulative dose of PA, treatment with API significant increased 5-HT by 28% accompanied with significant decrease in the DA, NE, and GABA by 60, 50, and 38%, respectively.

Figure 1



The neuroprotective effects of apigenin on cerebrum: reduced glutathione (a), malondialdhyde (b), superoxide dismutase (c) and Myeloperoxidase (d) against cumulative dose of paracetamol in a rat model of protein malnutrition. Each bar represents means (n=6)±SE. Statistical analysis was carried out by one-way analysis of variance followed by Tukey–Kramer multiple comparison test. *Significantly different from normal group at $P<0.05$. [@]Significantly different from PM control group at $P<0.05$. [#]Significantly different from PA control group at $P<0.05$. [$]Significantly different from combined group (PM+PA).

Finally, as compared with PM group, treatment with API significant increased 5-HT and ChE by 71 and 23%, respectively, accompanied with significant decrease in the DA and GABA by 27 and 31%, respectively.

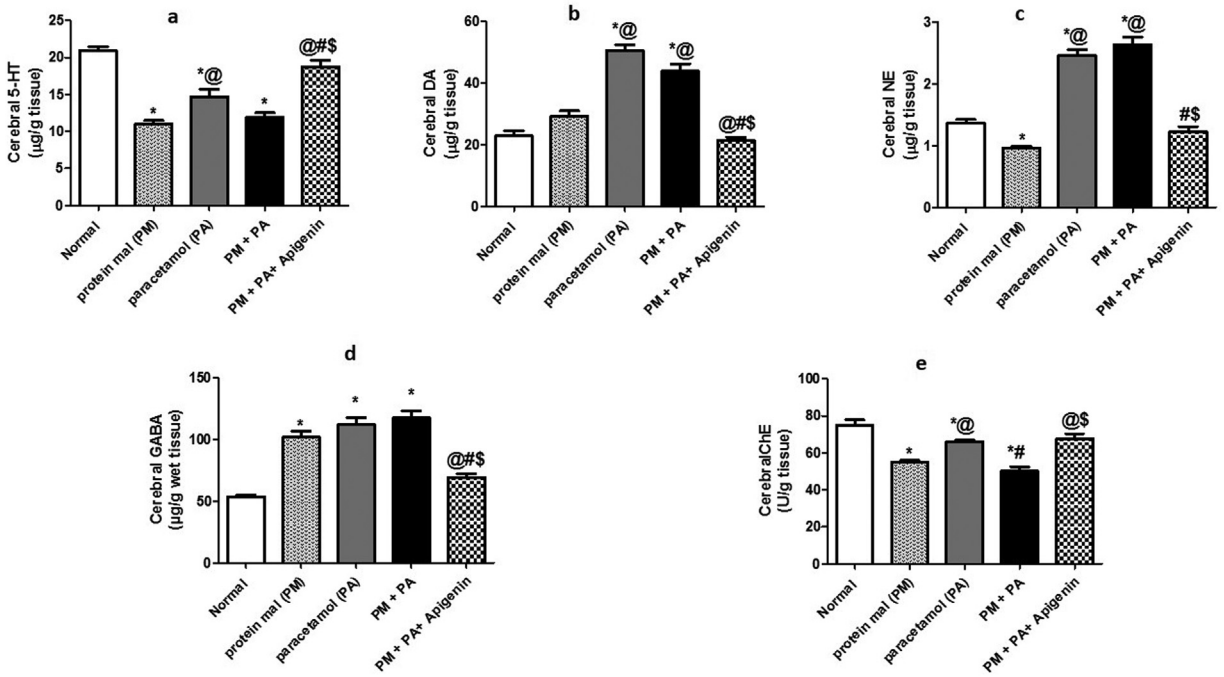
Histopathological examinations (hematoxylin and eosin)

As illustrated in Fig. 3, photomicrographs of rat's brain sections stained with hematoxylin and eosin of normal group (A) showed normal histopathological appearance. Sections of rat's brain from PM group showed necrosis of neurons and neuronophagia (B1) and focal gliosis (B2). Sections of rat's brain from group PA showed meningitis and congestion of meningeal blood vessel (C1) and necrosis of neurons and neuronophagia (C2). Sections of rat's brain from combined group PM+PA showed necrosis and pyknosis of neurons (D1) and hemorrhage, and congestion in the meninges (D2). API-treated group (E) showed no histopathological changes (H&E ×400).

Discussion

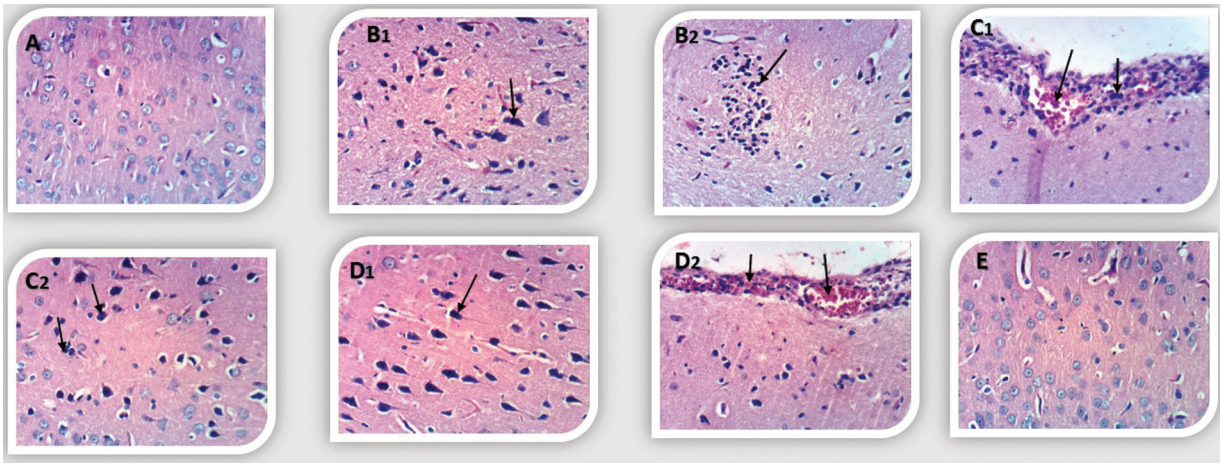
Previous studies carried out over the years have indicated that API has many interesting pharmacological activities [30]. However, API is reported here for the first time to cure the cerebral adverse effects of combination of PM and PA. In this model, PM and PA were combined to induce cerebral disorders, which are represented by reduction in antioxidant biomarkers, GSH and SOD, plus increment in oxidative biomarker, MDA.In contrast, API improved this situation, as it has antioxidant property according to Valdameri *et al.* [31], who found that API is able to quench the lipid peroxidation chain and is capable of shielding the membrane from free radicals which cause injuries. In agreement with our hypothesis, Patel *et al.* [32]

Figure 2



The neuroprotective effect of apigenin on cerebrum Serotonin (a), Dopamine (b), Norepinephrine (c), γ-aminobutyric acid (d) and cholinesterase (e) against cumulative dose of paracetamol (PA) in a rat model of protein malnutrition (PM). Each bar represents means (n=6)±SE. Statistical analysis was carried out by one-way analysis of variance followed by Tukey–Kramer multiple comparison test. *Significantly different from normal group at $P<0.05$. [@]Significantly different from PM control group at $P<0.05$. [#]Significantly different from PA control group at $P<0.05$. [$]Significantly different from combined group (PM+PA).

Figure 3



Photomicrographs of rat's brain sections stained with hematoxylin and eosin of normal group (A) showed normal histopathological appearance. Sections of rat's brain from PM group showed necrosis of neurons, neuronophagia (B1), and focal gliosis (B2). Sections of rat's brain from group paracetamol showed meningitis and congestion of meningeal blood vessel (C1) and necrosis of neurons, and neuronophagia (C2). Sections of rat's brain from combined group protein malnutrition+paracetamol showed necrosis, pyknosis of neurons (D1), and hemorrhage, and congestion in the meninges (D2). API-treated group (E) showed no histopathological changes (hematoxylin and eosin ×400).

confirmed that API could effectively remove a variety of ROS.

Furthermore, PM, PA, or their combination resulted in a significant increment in MPO content. In fact, MPO acts as a detector, and its discharge can catalyze hydrogen peroxide that induced the formation of TNF-α, IL-6, and IL-1β. On the contrary, treatment with API produced a significant decline in MPO content. According to Li *et al.* [33], API significantly inhibited nitric oxide production in macrophages, which may be associated with the attenuation of activation for MPO.

According to the results obtained from our research, we realized that combination of PM and PA decreased 5-HT and ChE contents plus increased DA, NE, and GABA contents significantly as compared with normal group. In contrast, API nearly restored the previous parameters to normal. It was previously reported that API inhibited monoamine oxidase activity [34] and so increased the levels of brain monoamines, such as 5-HT, which have been related to the alleviation of depression [35]. Moreover, API inhibited GABA receptor function and so accounted for API's antidepressant activity [36]. In addition, Yi *et al.* [37] reported that the antidepressant-like effects of API might be related to an upregulation in cAMP signaling associated with elevation in platelet adenylyl cyclase activity.

The histopathological examination performed on the brain tissues is in complete harmony with our biochemical results, as it showed necrosis of neurons, neuronophagia, gliosis, meningitis, and congestion in brain tissues exposed to PM and PA. However, administration of API renovated these damage almost to normal.

Conclusion

Our study demonstrated that API dramatically alleviated oxidative stress, inflammation, and neurotransmitters disturbance induced by cumulative dose of PA in a rat model of PM. We speculated that API may also attenuate neurotoxicity by its antioxidative effects. Moreover, the underlying mechanism of the neuroprotective effect induced by API also needs to be clarified.

Acknowledgements

The authors would like to thank Professor Dr Kawkab A. Ahmed (Pathology Department, Faculty of Veterinary Medicine, Cairo University, Egypt) for her assistance in the histopathological examinations.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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