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ORIGINAL ARTICLE

Hesperidin attenuates oxidative and neuronal damage caused by global cerebral ischemia/reperfusion in a C57BL/J6 mouse model

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Abstract The aim of this study was to determine the effects of hesperidin (HP) on neuronal damage in brain tissue caused by global cerebral ischemia/reperfusion (I/R) in C57BL/J6 mice. For this purpose, a total of 40 mice were divided equally into four groups: (1) sham-operated (SH), (2) global cerebral I/R, (3) HP, and (4) HP+I/R. The SH group was used as a control. In the I/R group, the bilateral carotid arteries were clipped for 15 min, and the mice were treated with vehicle for 10 days. In the HP group, mice were administered HP (100 mg/kg) for 10 days without carotid occlusion. In the HP+I/R group, the I/R model was applied to the mice exactly as in the I/R group, and they were then treated with 100 mg/kg HP for 10 days. Cerebral I/R significantly induced oxidative stress via an increase in lipid peroxidation and a decrease in the components of the antioxidant defense system. Furthermore, cerebral I/R increased the incidence of histopathological damage and apoptosis in brain tissue. HP treatment significantly reversed the oxidative effects of I/R and inhibited the development of neurodegenerative histopathology. Therefore, the current study demonstrates that HP treatment effectively prevents oxidative and histological damage in the brain caused by global I/R. In this context, the beneficial effects of HP are likely a result of its strong antioxidant and free radical-scavenging properties. HP may be an useful treatment to attenuate the negative effects of global cerebral I/R.

Keywords Global cerebral I/R · Oxidative stress · Neuronal damage · Hesperidin · C57BL/J6 mice

Introduction

Stroke, the incidence of which has increased over the past four decades in developing countries, is among the most common causes of death and disability in humans [1]. Cerebral ischemic stroke typically originates from the initial interruption of blood flow and subsequent restoration of blood supply, which can result in the delivery of insufficient oxygen and glucose support cellular homeostasis [1, 2]. Global cerebral ischemia/reperfusion (I/R) can therefore lead to neurological damage and severe physical impairment or disability [3]. The interruption of blood flow leads to neuronal cell death in brain tissue via mechanisms including oxidative and nitrosative stress, inflammation and apoptosis [1, 2]. Although the exact mechanism of stroke is unclear, it is well known that oxidative stress caused by reactive oxygen species (ROS) is a critical component of cerebral ischemic injury [4, 5]. It is well recognized that oxidative damage can easily cause neuronal death and damage in brain tissue through the oxidization of intracellular molecules such as lipids, proteins, and DNA [4–6]. Because of this pathological process, it was thought that antioxidant agents, which have the ability to scavenge ROS, could attenuate the neurological damage caused by I/R. For example, Aras et al. [7] found that Ebselen, a synthetic seleno-organic compound, prevents

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neuronal I/R injuries in terms of oxidative and histological damage, via its ROS-scavenging properties. Therefore, antioxidant pharmacotherapy may be an important method to treat global cerebral I/R injury.

Flavonoids are a broad class of plant pigments and a structurally diverse class of polyphenolic compounds that are found ubiquitously in plants and produced as a result of plant secondary metabolism [8]. Hesperidin (HP) is a bioflavonoid found abundantly in *Citrus* species, such as lemon and orange, that has been reported to possess pharmacological activities including antioxidant, analgesic, anti-carcinogenic, anti-hypertensive, antiviral, and anti-inflammatory effects [9]. HP and its synthetic derivative diosmin, components of the drug daflon, can have a beneficial effect on the symptoms of varicose veins, through a mechanism involving increased blood flow. Furthermore, HP is also used effectively as a supplemental agent that helps to reduce edema or excess swelling in the legs [10]. It is believed that HP is a powerful radical scavenger that facilitates the cellular antioxidant defense mechanisms and can also traverse the blood brain barrier [11, 12]. Thus, HP can prevent the neurodegeneration caused by conditions such as I/R and can promote healthy brain functions [13].

The present study utilized a global cerebral I/R model in C57BL/6J mice to establish whether the antioxidative properties of HP would ameliorate the neurological damage (oxidative and histological) caused by I/R. We evaluated the changes in oxidative stress status and brain histopathology during I/R in C57BL/6J mice.

Materials and methods

Chemicals

HP was obtained from Accros Organics (Thermo Fisher Scientific; Geel, Belgium), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were either analytical grade or the highest grade available.

Animals and experimental protocol

The present study was approved by the Ethical Committee on Animal Research of Inonu University and carried out in accordance with the Guidelines for Animal Research of the National Institutes of Health (NIH). C57BL/6J male mice (clean grade), weighing 18–22 g, were supplied by the Inonu University Laboratory Animals Research Center (Malatya, Turkey), housed in sterilized polypropylene cages, and given an ad libitum diet of standard commercial food pellets and water. All mice were kept under a 12-h light/dark cycle at an ambient temperature of 21 ± 2 °C and a humidity level of 60 ± 5 %. A total of 40 animals were randomly divided into four groups ($n = 10$): (1) sham-operated (SH), (2) global cerebral I/R, (3) HP, and (4) HP+I/R.

This experimental design was described in our previous study [6]. HP (100 mg/kg) was dissolved in 0.1 % carboxymethyl cellulose (CMC) and administered intraperitoneally (i.p.) for 10 consecutive days. The dose of HP was based on preliminary dose-finding experiments in our lab, and HP treatment was initiated concomitant with the induction of global cerebral I/R. Mice in the SH group and the I/R group were treated with only the 0.1 % CMC solution as a vehicle. In the HP and HP+I/R groups, mice were treated with HP (100 mg/kg/day) for 10 days following the I/R procedure, after which all animals were killed under anesthesia, and tissue and blood samples were obtained for laboratory analyses.

Surgical procedure

This experimental design was described in our previous study [6]. For the induction of global cerebral ischemia, the mice were anesthetized with xylazine (5 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.), and the procedure was performed according to the methods of Yonekura et al. [14]. Briefly, after a midline cervical incision, the bilateral common carotid arteries of animals in the I/R and HP+I/R groups were isolated and occluded simultaneously for 15 min using two vascular miniclips. The same surgical procedure was applied to the SH and HP groups, except that the carotid arteries were not clipped. Following surgery, all mice were placed in a thermal room until they recovered from anesthesia.

Biochemical analyses

Tissue was homogenized as described previously [8]. The levels of thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH), together with the activities of catalase (CAT), CuZn-superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined using spectrophotometric methods as described previously [8, 9].

Histopathological examination

For light microscopic evaluation, brain samples were fixed in 10 % formalin and embedded in paraffin. Paraffin-embedded specimens were cut into 5-μm-thick sections, mounted on slides and stained with hematoxylin and eosin (H–E). Tissue samples were examined using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Table 1 Levels of SOD, CAT, GPx, GSH and TBARS in brain tissue of C57BL/J6 mice (mean ± SD)

	TBARS (nmol/g tissue)	Reduced GSH (nmol/ml)	CAT (k/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Sham	8.73 ± 0.81^{a}	188.1 ± 4.11^{a}	0.023 ± 0.0009^{a}	23.91 ± 2.02^{a}	275.6 ± 16.3^{a}
I/R	12.1 ± 0.93^{b}	143.9 ± 9.12^{b}	0.014 ± 0.0010^{b}	16.06 ± 1.82^{b}	198.4 ± 15.1^{b}
HP	6.53 ± 0.69^{c}	181.4 ± 7.9^{a}	0.022 ± 0.0014^{a}	24.14 ± 1.92^{a}	284.7 ± 21.4^{a}
I/R+HP	9.12 ± 1.20^{a}	191.8 ± 11.5^{a}	0.021 ± 0.0012^{a}	20.08 ± 2.13^{c}	249.9 ± 17.2^{c}

Mean values bearing different superscripts within the same column were significantly different ($p < 0.01$)

For immunohistochemical (IHC) analysis, sections were mounted on polylysine-coated slides. After rehydrating, the samples were transferred to citrate buffer (pH 7.6) and heated in a microwave oven for 20 min. After cooling for 20 min at room temperature, the sections were washed with phosphate-buffered saline (PBS). Sections were submerged in 0.3 % H_2O_2 for 7 min and washed with PBS. Sections were then incubated with a primary rabbit polyclonal antibody against caspase 3 (Boster, PA1302) for 2 h. After the primary incubation, sections were rinsed in PBS and incubated with biotinylated goat anti-polyvalent for 10 min and streptavidin peroxidase for 10 min at room temperature. Immunostaining was completed with a chromogen and substrate for 15 min, and slides were counterstained with Mayer's hematoxylin for 1 min, rinsed in tap water, and dehydrated. The caspase 3 antibody was used according to the manufacturer's instructions, and caspase 3 positive cells showed brown staining.

Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. For biochemical values, the statistical analyses were conducted using one-way analysis of variance (ANOVA) and post hoc Tukey's honestly significant differences test. The degree of significance was set at $p \leq 0.01$.

Results

Biochemical results

The levels of SOD, GPx, CAT, GSH and TBARS in brain tissue are presented in Table 1. Global cerebral I/R caused a significant increase in the levels of TBARS compared with the other groups. TBARS was significantly decreased in the HP treated group compared with the SH (control) group. Moreover, GSH, CAT, SOD and GPx levels were all significantly decreased in the I/R group compared with both the SH and HP groups. HP treatment also caused a significant attenuation of the I/R-induced increase in TBARS and increased GSH and CAT levels close to those of the SH group. In addition, the SOD and GPx levels of the I/R+HP group were significantly elevated compared with the I/R alone group.

Histopathological results

Morphological changes in brain tissue were detected by H–E staining. In the SH (Fig. 1a) and HP (Fig. 1b) groups, brain tissue was histologically normal. In the I/R group, extensive histological damage was detected compared with the SH group. In the I/R group, focal ischemic areas were visible in the cerebral cortex (Fig. 2a), in addition to vascular congestion (Fig. 2b), mononuclear cell infiltration (Fig. 2c) and the presence of cytoplasmic shrinkage and extensively dark picnotic neuronal nuclei (Fig. 2d). In the I/R+HP group (Fig. 3a, b), these histological changes were decreased, and an improvement in histological appearance was observed. HP had a significant protective effect against ischemia-induced histological changes.

Caspase 3 the activity of caspase 3 is shown in Fig. 4. Caspase 3 activity was significantly increased in the IR group compared with the IR+HP group. Immunohistochemically, caspase 3 positive cells were not observed in the SH (Fig. 4a) or HP (Fig. 4b) groups. The number of cells staining positive for caspase 3 was noticeably increased in the IR group (Fig. 4c). The proportion of immunohistochemically caspase 3 positive stained cells was decreased in the IR+HP group (Fig. 4d) relative to the IR group. HP treatment, therefore, significantly attenuated the IR-induced increase in caspase 3 positive cells.

Discussion

To our knowledge, this is the first report of the beneficial effects of HP following cerebral ischemia/reperfusion in a C57BL/J6 mouse model. The current study demonstrates that HP treatment (100 mg/kg) exerts a neuroprotective effects against I/R-induced damage. The mechanism of neuroprotection is associated with inhibition of neuronal apoptosis and suppression of oxidative stress via a reduction in lipid peroxidation and a significant induction of the enzymatic and non-enzymatic antioxidant defense systems.

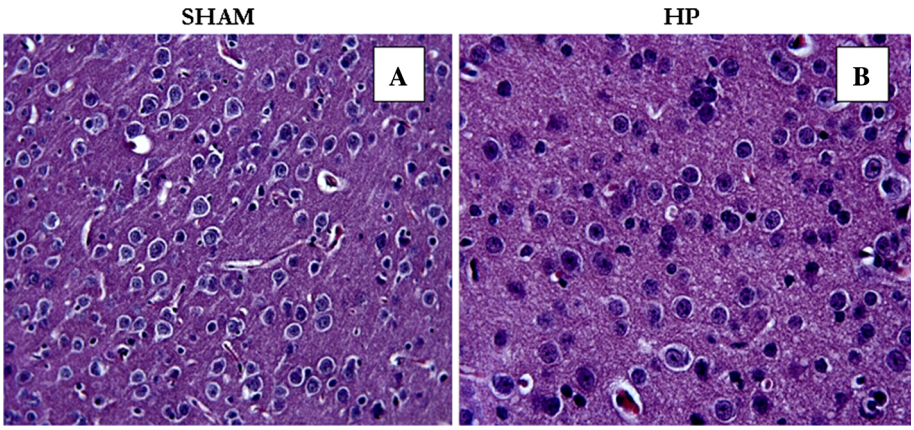

Fig. 1 Brain tissue from SH (**a**) and HP (**b**) groups displayed normal histological appearance. H–E; ×40

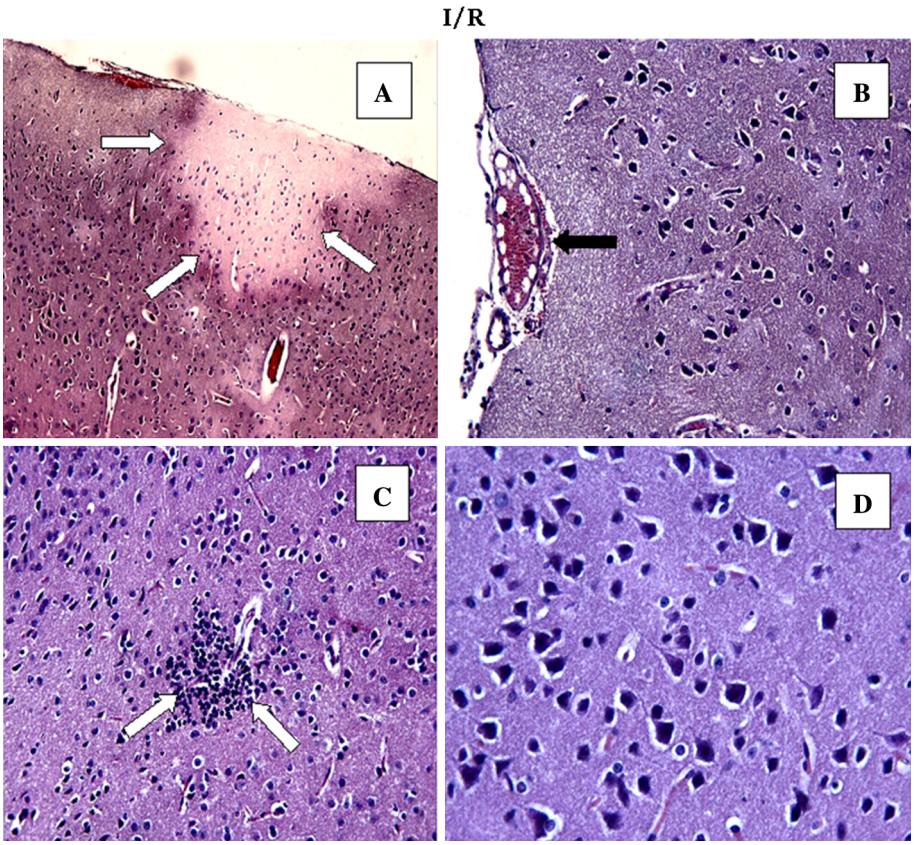

Fig. 2 Areas of focal ischemia (*white arrows*) (**a**), vascular congestion (*black arrow*) (**b**), mononuclear cell infiltration (*white arrows*) (**c**), cytoplasmic shrinkage and extensively dark picnotic neuronal nuclei (**d**) were detected in the I/R group. **a**, **b**, **c** H–E; ×20. **d** H–E; ×40

Oxidative stress, which is caused by an imbalance between TBARS and the antioxidant defense systems (SOD, CAT, GPx, and GSH), is induced by the I/R process and may play an important role in ischemic stroke injury [2, 15]. Lipid peroxidation following I/R is one of the primary pathological hallmarks of irreversible cell death in neurons, together with induction of ROS and down regulation of elements of the antioxidant defense systems [2, 16]. In this study, we observed that global cerebral I/R induced lipid peroxidation as a result of elevated TBARS levels, leading to neuronal damage in the brain. Furthermore, the antioxidant defense system was suppressed by I/R via decreases in the enzymatic activities of SOD, CAT, and GPx and in the expression levels of GSH, a specific ROS scavenger. We established that HP treatment in control mice (SH group) exhibits strong antioxidant activity, associated with an increase in SOD, CAT, GSH, and GPx levels. Importantly, HP treatment in ischemic mice caused a significant attenuation of IR-induced TBARS elevation and significant increases in SOD, CAT, and GPx activities and GSH levels. This suggests that HP treatment reduces oxidative stress and

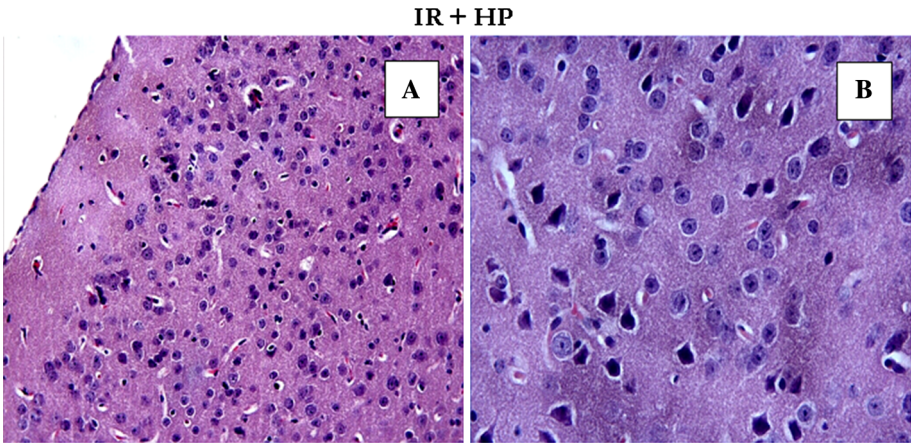

Fig. 3 Decreased areas of focal ischemia, vascular congestion, mononuclear cell infiltration, cytoplasmic shrinkage and extensively dark picnotic neurons nuclei in the I/R+HP group. **a** H–E; ×20. **b** H–E; ×40

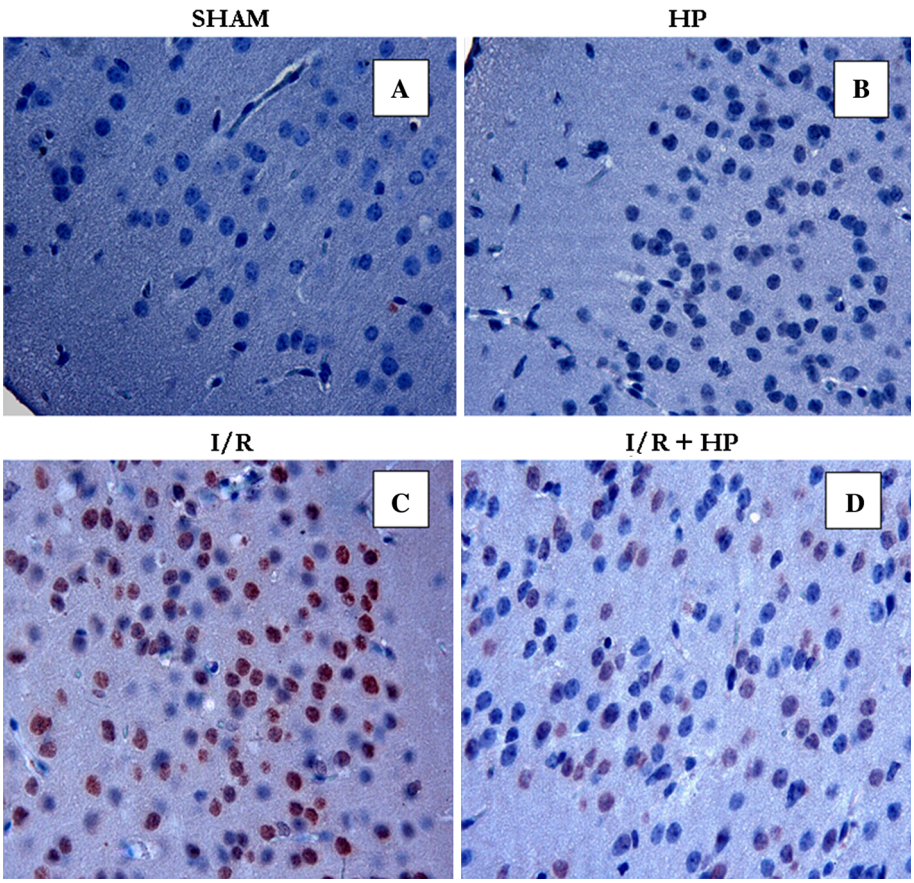

Fig. 4 Caspase 3 staining of tissue sections. There are no caspase 3 positive cells in the SH (**a**) and HP (**b**) groups. Caspase 3 positive staining is greater in the I/R group (**c**) compared with the I/R+HP group (**d**). Caspase 3; × 40

thereby reverses the neuronal damage caused by I/R. Recent studies clearly show that global cerebral I/R causes significant neuronal damage and oxidative stress in the brain tissue of both mice and rats experimentally [17–19]. Furthermore, our previous study showed that cerebral I/R generates a significant increase in TBARS levels and a significant inhibition of the antioxidant defense system in C57BL/6J mice [6]. These findings are consistent with the results of the present study.

As far as we are aware, little research has been conducted concerning the relationship between HP treatment and neuronal damage in cerebral I/R. The study by Gaur and Kumar [10] showed that 7 days of HP treatment in rats with cerebral I/R significantly improved oxidative defense via altered levels of lipid peroxidation, nitrite concentration, GSH, and SOD and CAT activities. In addition, Gandhi et al. [20] determined that HP protects the heart tissue of rats against focal myocardial I/R via an increase in

antioxidant status. Such findings are in agreement with the present study and confirm our results. The signaling pathways underlying I/R remain largely unknown, but our results and those of previous studies suggest that oxidative stress plays an important role [2, 6, 21]. It is believed that elevated oxidative stress leads to significant cell death and neuronal damage in brain tissue. Therefore, we hypothesized that a strong antioxidant agent, such as HP, may protect brain tissue against oxidative stress. We observed that HP treatment does indeed protect against I/R, owing to its strong antioxidant properties, and may prevent damage and lipid degeneration in neurons.

Histopathological and immunohistopathological analyses revealed that significant structural changes occurred in the brain tissue of the I/R group, compared with the SH and other experimental groups. The primary damage included diffuse focal ischemic areas in the cerebral cortex as well as secondary issues, such as cell infiltration, vascular congestion, cytoplasmic shrinkage, and the presence of extensively dark pyknotic nuclei in cortical neurons. Additionally, I/R caused an increase in the number of caspase 3 stained cells, which are indicative of the apoptotic state of neurons. In our previous study, similar lesions were detected following a similar experimental procedure, consistent with the current study [6]. Moreover, Yonekura et al. [14] also determined that 14 min of global cerebral ischemia leads to injury in all brain regions of C57BL/6J mice. We observed that HP treatment attenuates the negative histological changes caused by I/R, including a notably significant decrease in the number of caspase 3 stained cells. This demonstrates that HP protects neurons against cell death. Gaur and Kumar [10] confirm our results by reporting that a 7-day treatment of HP at a dose of 50 and 100 mg/kg attenuates the histopathological changes caused by I/R in animals. Moreover, Wang and Cui [22] demonstrated that neohesperidin, a HP analogue, prevents cerebral I/R via inhibition of neuronal apoptosis. In this context, it can be assumed that there is a correlation between the oxidative status of the brain and I/R-induced histopathological changes. For this reason, we conclude that HP may provide protective effects in ischemia by inhibiting the elevation of oxidative stress status and preventing histological damage in brain tissue.

Conclusion

In the present study, it was demonstrated that HP treatment at a dose of 100 mg/kg/day attenuates the neurodegenerative effects caused by global cerebral I/R in a C57BL/J6 mouse model. Global cerebral I/R caused increased oxidative stress and resulted in histopathological changes in brain tissue. HP treatment for 10 consecutive days reversed the negative effects of I/R. This effect of HP is likely a result of its strong antioxidant and radical-scavenging properties. Therefore, we conclude that HP attenuates the neuronal damage caused by global cerebral I/R in the brain.

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Conflict of interest The authors have declared no conflict of interest.

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