Sains Malaysiana 48(10)(2019): 2221–2227
*http://dx.doi.org/10.17576/jsm-2019-4810-18*

# Effect of P-Hydroxycinnamic Acid in Mice Model of Cerebral Ischemia-Reperfusion Injury

(Kesan Asid P-Hidroksisinamik dalam Model Tikus dengan Kecederaan Serebrum Iskemia-Reperfusi)

ROMGASE SAKAMULA, CHANIKARN SAKDAPITAK & WACHIRYAH THONG-ASA*

ABSTRACT

*Multiple pathomechanisms of cerebral ischemia reperfusion (I/R) injuries can be ameliorated by certain high-potential pharmaceutical substances. In the present study, we investigated the acute effect of p-hydroxycinnamic (*p*HCA) acid against cerebral I/R injury in mice. Thirty male ICR mice were divided into Sham, Control-I/R, and* p*HCA-I/R groups. The* p*HCA 100 mg/kg and the vehicle were given 30 min before I/R induction. Thirty-minute bilateral common carotid artery occlusion followed by 45-min reperfusion was performed on the Control-I/R and* p*HCA-I/R groups. Brains were collected for biochemical analysis, infarction and histological study of the cerebral cortex and corpus callosum (CC). The results showed that I/R induction significantly induced biochemical changes ($p<0.05$) along with the increase of brain infarction ($p<0.05$), percentage of degeneration in cerebral cortex ($p<0.05$) and decrease of CC white matter density ($p<0.05$). Pretreatment with* p*HCA significantly reduced MDA ($p<0.05$), brain infarction ($p<0.05$), cerebral cortex neuronal degeneration ($p<0.05$) and prevented the reduction of white matter density in the CC ($p<0.05$). The present study concluded that pretreatment with* p*HCA helps prevent cerebral I/R injury by amelioration of lipid peroxidation, white matter damage and neuronal degeneration.*

*Keywords: Brain ischemia; infarction; oxidative stress; p-hydroxycinnamic acid; white matter*

ABSTRAK

*Pelbagai patologi mekanisme kecederaan serebrum iskemia-reperfusi (I/R) boleh diperbaikkan oleh beberapa bahan farmaseutik berpotensi tinggi. Dalam penyelidikan ini, kami mengkaji kesan akut p-hidroksisinamik (*p*HCA) terhadap kecederaan I/R serebrum pada tikus. Tiga puluh tikus ICR jantan dibahagikan kepada Sham, kawalan-I/R, dan kumpulan* p*HCA-I/R. PHCA 100 mg/kg dan pembawa diberikan 30 min sebelum induksi I/R. Oklusi arteri karotid selama 12 min diikuti oleh reperfusi 45 min dilakukan pada kumpulan Kawalan-I/R dan* p*HCA-I/R. Tisu otak dikumpulkan untuk analisis biokimia, infarksi dan kajian histologi korteks serebrum dan korpus kalosum (CC). Keputusan menunjukkan bahawa induksi I/R menunjukkan perubahan biokimia yang ketara ($p<0.05$) dengan peningkatan infarksi otak ($p<0.05$), peratusan degenerasi dalam korteks serebrum ($p<0.05$). Pra-rawatan dengan* p*HCA mengurangkan MDA ($p<0.05$), infarksi otak ($p<0.05$), degenerasi neuron korteks serebrum ($p<0.05$) dan menghalang pengurangan kepadatan bahan putih otak di CC ($p<0.05$). Kajian ini menyimpulkan bahawa prarawatan dengan* p*HCA membantu mencegah kecederaan otak I/R dengan memperbaik peroksidasi lipid, kerosakan bahan putih otak dan degenerasi neuron.*

*Kata kunci: Asid p-hidroksisinamik; bahan putih otak; infarksi; iskemia otak; tekanan oksidatif*

## INTRODUCTION

Cerebral ischemia reperfusion (I/R) injury is the multiple pathomechanisms associated with neurodegeneration and disabilities. An ischemic event in the global brain activates multiple metabolic cascades that can lead to susceptibility and pathological occurrences in brain areas. Unlike focal cerebral ischemia, the brain area affected by I/R injury has been characterized into two categories of tissue; the ischemic core and penumbra, according to the severity of blood reduction. Initiation of neuronal damage is found beginning at ischemic onset and worsens during restoration of blood flow in the reperfusion period. Radical formation is believed to be one of the major deteriorative factors and results from multiple pathomechanisms that are activated during I/R. The death-promoting pathomechanisms of I/R injury are excitotoxicity, calcium overload, protein synthesis inhibition, inflammation, and free radical formation. These result in brain tissue edema due to blood-brain barrier failure, endothelial and mitochondrial dysfunctions, and activation of neuronal death via necrotic, apoptotic and necrotic-apoptotic continuums (Hou & MacManus 2002; Shuaib & Breker-Klassen 1997; White et al. 2000). Death- and survival-promoting mechanisms balancing during I/R will determine the affected neurons. The survival-promoting mechanisms are the increase of arterial pressure, activation of vasodilation to promote cerebral blood flow delivery, and the release of anti-inflammatory cytokines, anti-oxidant enzymes and anti-apoptotic proteins which help neurons survive during ischemic periods (Ladecola & Anrather 2011; White et

al. 2000). Severe stress of ischemia reinforces the death-promoting rather than survival-promoting mechanisms. When cerebral blood flow is returned, the death-promoting mechanisms are exacerbated. This is a result of high amounts of oxygen reacting with hypoxanthine to form toxic hydroxyl radicals and nitric oxide-derived peroxynitrite. These toxic radicals play pivotal roles in death-promoting mechanisms during the reperfusion period, damaging DNA and causing oxidation of proteins and cellular membrane damage by lipid peroxidation (Hou & MacManus 2002; Pan et al. 2007; Shuaib & Breker-Klassen 1997; White et al. 2000). As multiple pathomechanisms are present, treatment of I/R by using a single-substance therapy is challenging. Additionally, the therapeutic window of I/R injury is limited to a few hours after ischemic insult and affected by ischemic severity, neuronal strength, endurance and responsiveness to treatment (Kalogeris et al. 2012).

Preventive therapy is gaining attention for promoting cell viability with diet supplementation or nutraceutical products. Many active ingredients in foodstuffs act as natural antioxidants that help increase cell function and health and may reduce cell susceptibility to the causes of many diseases. P-hydroxycinnamic acid (*p*HCA), a phenolic that is synthesized from cinnamic acid by P450-dependent 4-cinnamic acid hydroxylase, has beneficial effects against degenerative diseases (Amalan et al. 2016). It can be found in fruits, vegetables and drinks-for example, apples, pears, beans, soybeans, tomatoes, potatoes, mushrooms, tea, coffee, beer and wine (Pei et al. 2015). It exerts a neuroprotective effect on embolic cerebral ischemia with antioxidant and anti-apoptotic features (Guven et al. 2015). There are varieties of beneficial effects of *p*HCA: anti-diabetic, anti-hyperlipidemic, anti-inflammation, anti-microbial, anti-cancer, antipyretic, analgesic, anti-arthritic, anti-ulcer, anxiolytic and antioxidant (Abdel-Wahab et al. 2003; Amalan et al. 2016; Guven et al. 2015; Yoon et al. 2014). It has low toxicity with a LD50 of about 2850 mg/kg and a half-life of 15.9 min to 1.3 h (Pei et al. 2015). In an earlier study, there is a report indicating that *p*HCA 100 mg/kg can increase nuclear respiratory factor-1 (NRF-1), which improve mitochondria dysfunction that is an important factor to induce neuronal death in I/R (Guven et al. 2015; Mehta et al. 2012) and *p*HCA in the same dose was found no toxicity and rapid absorption in the gastrointestinal tract after oral administration (Fentem & Fry 2009; Konishi et al. 2004; Lake 1997). Therefore, the present study aimed to investigate the *in vivo* benefit effects of *p*HCA against cerebral I/R injury in mice with biochemical and histological analysis.

## Materials and Methods

### ANIMALS

The Animal Ethics Committee, Kasetsart University Research and Development Institute (KURDI), Kasetsart University, approved the experimental protocol (ID#ACKU 02756). Thirty male ICR mice were delivered from the National Laboratory Animal Center (NLAC), Mahidol University, Salaya, Nakhon Pathom. We housed all animals in a well-controlled temperature and humidity room with 12:12 h light/dark exposure periods, and they received standard pellet food and RO water *ad libitum*.

### EXPERIMENTAL PROTOCOL

The mice were randomly divided into three groups of Sham (received vehicle without I/R induction), Control-I/R (received vehicle and I/R induction), and *p*HCA-I/R (received *p*HCA 100 mg/kg (Guven et al. 2015) and I/R induction). Treatments were orally administered for 30 min before I/R induction. Only Control-I/R and *p*HCA-I/R groups received I/R induction, which was induced by 30-min bilateral common carotid artery occlusion followed by 45 min reperfusion (Raghavendra et al. 2009). After I/R induction, the mice were killed and the brains were washed in a cold 0.9% normal saline solution (NSS) and homogenized in a 10% w/v 0.05 M phosphate buffer saline (PBS, pH7.4) preparation for further biochemical analysis; total protein, calcium, malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH). The brains were stained with 2, 3, 5-triphenyltetrazolium chloride (TTC) and kept in 10% formalin for infarction area analysis. Cerebral cortices were evaluated using 0.1% cresyl violet (CV) staining. White matter density evaluation in the corpus callosum (CC) was done by using 0.1% luxol fast blue (LFB) staining.

### TOTAL PROTEIN DETERMINATION

We mixed supernatant 0.2 mL with 2 mL of 2% w/v $Na_2CO_3$ in 0.1N NaOH: 0.5% w/v $CuSO_4$-$5H_2O$ in distilled water: 1% w/v $C_4H_4KNaO_6$-$4H_2O$ (48:1:1). The mixture was incubated for 10 min before 0.2 mL of 1N Folin-Ciocalteu reagent (1:1) was added. Samples were then incubated for 30 min and read at 600 nm. Protein concentration was calculated using the standard curve of bovine serum albumin (Lowry et al. 1951).

### CALCIUM DETERMINATION

We prepared test tubes of blank (distilled water 2.55 mL + working color reagent 1.5 mL), standard (distilled water 2.5 mL + working standard calcium solution 0.05 mL + working color reagent 1.5 mL), and test (distilled water 2.5 mL + sample 0.05 mL + working color reagent 1.5 mL) solutions. These were incubated at 25°C for 5 min before being read at 490 nm using the blank as reference and interpreted as mEq/L (Spare 1964).

### MDA DETERMINATION

We mixed homogenate 0.2 mL with 0.2 mL of 4% sodium dodecyl sulfate (SDS), 1.5 mL 20% acetic acid and 1.5 mL 0.5% thiobarbituric acid (TBA) and boiled the mixture for 60 min at 95°C. We centrifuged the mixture for 10 min

at 3,500 rpm, read supernatant at 532 nm and interpreted MDA in mM/mg of protein.

### SOD DETERMINATION

We mixed supernatant 0.1 mL with 0.1 mL of EDTA ($1\times10^{-4}$ M), 0.5 mL carbonate buffer (pH7.9) and 1 mL of epinephrine ($3\times10^{-3}$ M). This was read at 480 nm every 30 s for 3 min. Enzyme activity was determined in U/min/mg of protein using the standard SOD concentrations of 0, 0.0058, 0.0294, 0.117 and 0.294 µg/mg (standard SOD activity was 6150 U/mg, Merck, Germany).

### CAT DETERMINATION

Supernatant 50 µL was added up to a volume of 3 mL with 0.05 M PBS, pH7.4 containing 0.01 M $H_2O_2$ and read continuously at 240 nm every 30 s for 3 min. We calculated CAT activity with the extinction co-efficient of $H_2O_2$ and interpreted the results as U/mg of protein (Hadwan & Abed 2016).

### GSH DETERMINATION

We mixed homogenate 1 mL with 1 mL 10% tricarboxylic acid (TCA), centrifuged (10,000 g) 10 min before collecting 0.5 mL of the supernatant and mixing it with 2 mL 5, 5'-dithios 2-nitro benzoic acid (DTNB) up to a volume of 3 mL with PBS and read at 412 nm. We prepared standard glutathione from 0, 0.065, 0.163, 0.326, 0.490 and 0.653 mM and interpreted the results as mM/mg of protein.

### INFARCTION ANALYSIS

Brains were removed from skulls, washed in cold 0.9% NSS, and cut with a surgical blade to yield 2 mm of thickness, then stained with 2% TTC at 37°C for 10 min and kept in a 10% neutral buffer formalin for 24 h. The lethally damaged areas did not retain TTC reactions and appeared pale, while viable areas were red. We differentiated pale areas relative to red ones using NIH ImageJ and represented this total area as the percentage of infarction (Fishbein et al. 1981).

### HISTOLOGICAL ANALYSIS

Paraffin-embedded brains were cut to 5 µm of thickness and five brain slides from each animal at -1.98 from the bregma (Paxinos & Franklin 2008) with 100-µm intervals were used. Overnight incubation was conducted in a hot-air oven at 60°C. The brain slides were deparaffinized and rehydrated via serially changing xylene, 100% Ethanol (EtOH) and 95% EtOH, respectively. Slides were soaked in 70% EtOH distilled water and stained with 0.1 % CV for 30 s. After washing in distilled water, brain slides were dehydrated *via* serially changing of 95%, 100% EtOH, xylene, then mounted with mounting media and covered with glass.

Photomicrographs of the cerebral cortex were captured in three non-overlapping images for each hemisphere at 400×. Neuronal cells were counted as viable and degenerating cells in the cerebral cortex. Viable cells were characterized by light purple CV cytoplasm staining of a visible nucleus and nucleolus (Wachiryah & Kanokwan 2014; Wachiryah et al. 2015). Degenerating cell characterizations were a dark purple stained, shrinking pyknotic/fragmented nucleus surrounded with a vacuole (apoptotic-like neurons) and karyolysis, pyknosis, karyorrhexis with a swollen cell (necrotic-like neurons) (Chaitanya & Babu 2008; Elmore et al. 2016). We used NIH ImageJ for cell counting and expressed data as the percentage of neuronal degeneration.

Corpus callosum white matter density analysis, in brief, brain sections were deparaffinized, rehydrated, and stained with 0.1% LFB in 95% EtOH and incubated overnight at 56°C. Slides were processed further in 95% EtOH, distilled water, 0.05% lithium carbonate solution, 70% EtOH, distilled water, and dehydrated and finished with cover glass. Photomicrographs of corpus callosum were captured in three non-overlapping images in each hemisphere at 200× of magnification and analyzed for myelinated fiber density using NIH ImageJ (Wachiryah et al. 2017). We interpreted data as the percentage of white matter density.

### STATISTICAL ANALYSIS

We analyzed all data through a one-way analysis of variance followed by Fisher' PLSD post hoc test, represented data as mean ± standard error of mean (S.E.M.), and indicated statistical significance by p-value < 0.05.

## RESULTS AND DISCUSSION

Ischemia-reperfusion injury induced by 30 min bilateral common carotid artery occlusion followed by 45 min of reperfusion significantly increased calcium ($p = 0.0012$) and MDA ($p = 0.0003$) levels with significantly reduced CAT ($p = 0.0434$) and SOD ($p = 0.0079$) activities. Pretreatment with *p*HCA ameliorated only MDA ($p = 0.0003$) but not calcium levels. PC increased CAT, SOD and GSH but not by a significant difference (Table 1). Biochemical assessment implies that cerebral I/R models in mice significantly induce lipid peroxidation and pretreatment with *p*HCA 100 mg/kg can ameliorate only lipid peroxidation.

Brain infarction (Figure 1) was significantly increased in the Control-I/R group compared to the Sham group ($p < 0.0001$). Pretreatment with 100 mg/kg of PC acid significantly reduced the infarction area in the *p*HCA-I/R group ($p = 0.0004$). Photomicrographs of the cerebral cortex with 0.1% CV staining are shown in Figure 1(a)-1(c). Histological study of the cerebral cortex indicated that I/R significantly increased the percentage of degeneration ($p = 0.0010$), and pretreatment with *p*HCA significantly reduced the percentage of degeneration in the *p*HCA-I/R group ($p = 0.0282$).

Photomicrographs of corpus callosum with 0.1% LFB are shown in Figure 1(d)-1(f). Ischemia reperfusion

TABLE 1. Biochemical analysis of brain tissue; total protein, calcium, malondialdehyde (MDA), catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD)

| Groups | Total protein (mg/g) | Calcium (mEq/L) | MDA (mM/ml/mg protein) | CAT (U/ml/mg protein) | GSH (mM/mg protein) | SOD (U/µl/mg protein) |
|---|---|---|---|---|---|---|
| Sham | 173.85 ± 9.59 | 0.29 ± 0.06 | 101.74 ± 9.86 | 2.73 ± 0.99 | 0.45 ± 0.19 | 2.00 ± 0.16 |
| Control-I/R | 178.61 ± 11.79 | 0.80 ± 0.12* | 176.17 ± 15.08* | 0.69 ± 0.08* | 0.20 ± 0.05 | 0.16 ± 0.0.06* |
| *p*HCA-I/R | 172.52 ± 9.47 | 0.73 ± 0.07* | 103.10 ± 7.02# | 1.89 ± 0.37 | 0.23 ± 0.04 | 1.00 ± 0.47* |

Data are expressed as mean ± S.E.M.* indicates a significant difference from the Sham group at $p < 0.05$, # indicates a significant difference from the Control-I/R group at $p < 0.05$

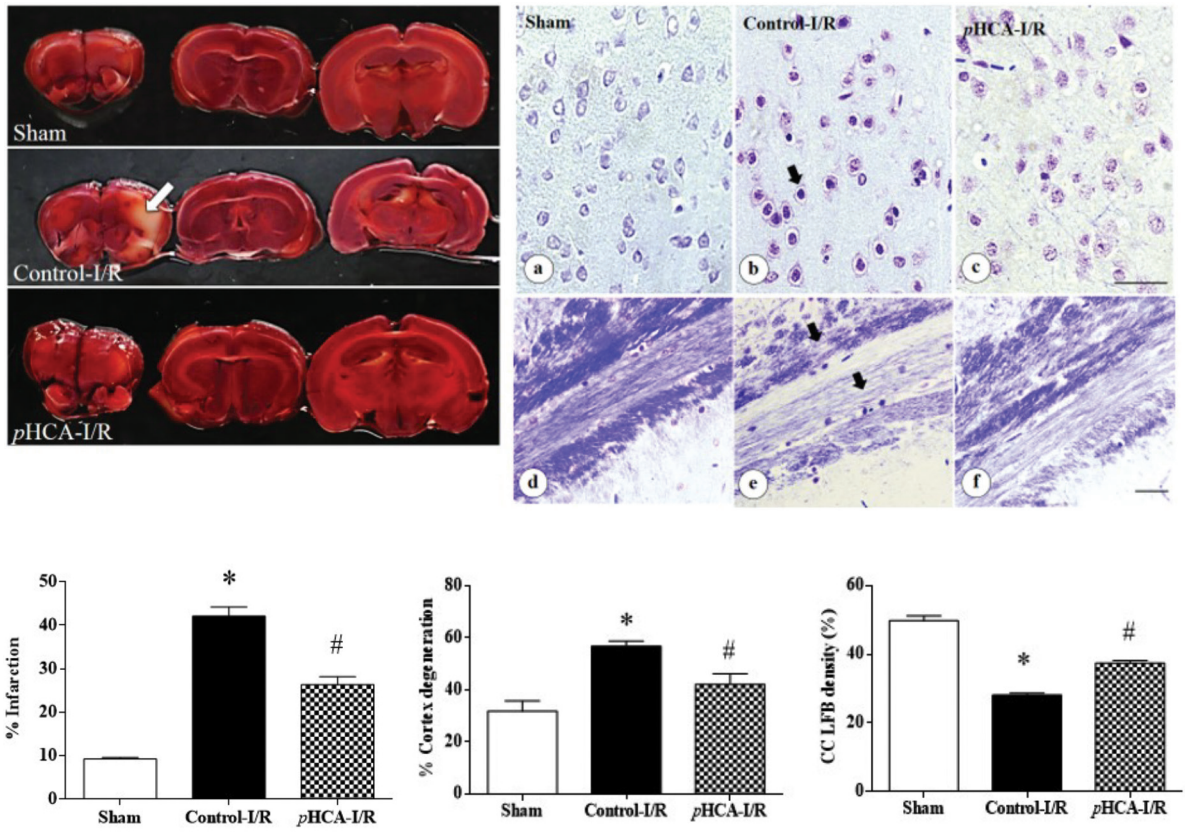


FIGURE 1. Brain with TTC staining for analysis of infarction area (left panel). White arrows indicate brain infarction (pale area). Histogram shows percentage of brain infarction (%). * indicates a significant difference compared to Sham. # indicates a significant difference compared to Control-I/R. Photomicrographs of cerebral cortex (a-c) with 0.1% CV staining captured at 400× of magnification (right panel). Black arrow (b) indicates dead cell with dark purple stained, pyknotic nucleus shrinkage and appearance of a vacuole around the cell. Photomicrographs of corpus callosum with 0.1% LFB staining captured at 200× of magnification. Black arrows (e) indicate axonal fragmentation with reduced LFB myelination density in I/R. Bars represent 50 µm. Histograms represent the percentage of degeneration in the cerebral cortex (g) and the corpus callosum (CC) LFB myelination staining density. * indicates a significant difference compared to Sham. # indicates a significant difference compared to Control-I/R

induced white matter changes with weaker LFB staining and axonal fragmentation in the remaining portion. The density was significantly reduced in Control-I/R ($p < 0.0001$), and pretreatment with *p*HCA can prevent the reduction of white matter density in *p*HCA-I/R ($p < 0.0001$).

The present study showed the beneficial effects of *p*HCA in mice with cerebral I/R injury. An ischemia reperfusion injury that was induced by a 30 min bilateral common carotid artery occlusion followed by 45 min of reperfusion significantly induced biochemical changes in the brain tissue such as an increase of calcium and MDA levels and reduced CAT and SOD levels in the present study. The results of the I/R induced oxidative stress were similar to the previous report (Raghavendra et al. 2009). Brain infarction was significantly increase together with cerebral cortex and CC white matter degeneration. The ischemic

vulnerable brain area such as the cerebral cortex showed a significant increase in the percentage of degenerating cells after the I/R induction had been described, resulting from energy failure, calcium overload, excitotoxicity, free radical formation, necrotic and apoptotic cell death (Hou & MacManus 2002; Mehta et al. 2007; Phillis et al. 2002; White et al. 2000).

Brain infarction was determined by using TTC staining, the presence of NADH depicts aerobic respiration adequacy of tissue; it acts as the reducing agent and reacts with TTC to turn healthy tissue a red color. We found I/R significantly increases brain infarction in the Control-I/R group and brain infarction was lower in the *p*HCA-I/R group which indicates *p*HCA can prevent brain infarction after I/R induction. Focusing on a particular brain susceptibility to global I/R injury such as the cerebral cortex, the neuronal degeneration promptly appeared (Martin et al. 1998; White et al. 2000). The protective effect of *p*HCA on the cerebral cortex together with the biochemical assessment, brain susceptibility and multiple pathomechanisms of I/R injury in the present study indicated that *p*HCA can counteract pathomechanisms of global cerebral I/R injury. We found a single dose pretreatment with *p*HCA significantly reduced MAD but not calcium levels. However, *p*HCA 100 mg/kg was previously used in embolic cerebral ischemia (Guven et al. 2015) and it can ameliorate lipid peroxidation which was similar to our study. The neuroprotective effects of *p*HCA arise from ERK and Akt signaling pathways that are the modulation of the apoptotic mechanism (Guven et al. 2015).

Necrotic, apoptotic and necrotic-apoptotic cell death had been reported in cerebral I/R injury. During ischemic periods, neuronal cell death is mainly a result of excitotoxicity, an energy failure that leads to excessive neurotransmitter release with over activation of glutamate receptors and further leads to calcium overload. Free radical formation, inhibition of protein synthesis, lipid peroxidation, protein and DNA and RNA oxidations lead to prompt necrotic cell death or necrotic-apoptotic continuum (Hou & MacManus 2002; Mehta et al. 2007; Phillis et al. 2002; White et al. 2000). In the present study, we counted degenerating cells in both forms of apoptotic- and necrotic-like cells that are presented after I/R induction. The form of cell death in Control-I/R was necrotic-like rather than apoptotic-like cell while *p*HCA-I/R were mostly the apoptotic form. This may involve the cell death mode switching as has been described of self-protective mechanisms for ameliorating the spread of cell death in the ischemic brain (Ueda & Fujita 2004). The *p*HCA fails to inhibit the augmentation of calcium levels in the present study, it may imply excitotoxicity persistency. Although a previous study indicated that *p*HCA is able to increase the levels of GSH, SOD and CAT in doxiorubicin-induced cardiotoxicity (Abdel-Wahab et al. 2003), the present study showed a trend that only CAT and SOD were increased in the *p*HCA-I/R group but there was not a significant difference when compared to the Control-I/R group. Comparing the *p*HCA effect on CAT and SOD levels in the present and previous study at the same given dose of 100 mg/kg, we found an increase of these enzymatic activities, but it was not statistically significant. In the previous study, *p*HCA was given by intraperitoneal injection, and in our study, it was given by oral administration. The latter given route results in a $t_{max}$ range from 3.72 to 10 min and 15.9 min to 1.3 h for $t_{1/2}$ which may lower *p*HCA blood level more than the beginning dose and may not be high enough to activate anti-oxidant enzymatic activity during the I/R period (Pei et al. 2015).

Brain is vulnerable to ischemia because brain contains large amounts of polyunsaturated fatty acid which are most susceptible macromolecules to oxidative stress and free radical attract. Several layers of cerebral cortex, hippocampus, basal ganglia, thalamus, deep white matter areas and the Purkinje cell of the cerebellum are vulnerable regions (Lee at al. 2000; Mendelow et al. 1984; Small et al. 1999; Wang et al. 2016). Cerebral cortex is one of vulnerable zone to global cerebral ischemia, which a lot of glutamate NMDA receptor (N-methyl-D-aspartate receptors) and easily activate excitotoxicity and induced neuronal death after I/R (Luo et al. 2018). In the present study, we found the treatment with *p*HCA can protect neuronal death in cerebral cortex. White matter was also vulnerable to I/R and more susceptible than the gray matter (Wakita et al. 2002). We found the reduction of myelination with axonal fragmentation after I/R induction and pretreatment with *p*HCA can reduce the reduction of white matter density in the CC. Vulnerability of white matter to cerebral ischemia is due to the lower blood supply, especially the deep white matter area. Oligodendrocytes, myelin producing cells in brain, are sensitive to ischemia-induced oxidative stress, excitotoxicity, and inflammation, which contribute to oligodendrocyte death and white matter damage after ischemia (Wang et al. 2016). Oligodendrocytes have a lower susceptibility and responsiveness to ischemia than neurons, therefore, the treatment with *p*HCA in the present study can rescue the white matter. However, this study has a limitation because further study for the precise molecular action of *p*HCA of indeed mechanisms are needed.

## Conclusion

In summary, *p*HCA has an acute benefit effect on cerebral I/R in mice by decreasing lipid peroxidation and percentage of degeneration in cerebral cortex and prevent the decrease of CC white matter density.

## Acknowledgements

This work was funded by the Graduate and Project in Zoology grants of Department of Zoology and Faculty of Science, Kasetsart University.

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Physiology division, Animal Toxicology and Physiology
Specialty Research Unit (ATPSRU)
Department of Zoology, Faculty of Science
Kasetsart University
Bangkok 10900
Thailand

*Corresponding author; email: fsciwyth@ku.ac.th

Received: 3 April 2019
Accepted: 8 August 2019