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ORIGINAL ARTICLE

Myricitrin Attenuates Hypoxic-Ischemia-Induced Brain Injury in Neonatal Rats by Mitigating Oxidative Stress and Nuclear Factor Erythroid 2-Related Factor 2/Hemeoxygenase-1/Antioxidant Response Element Signaling Pathway

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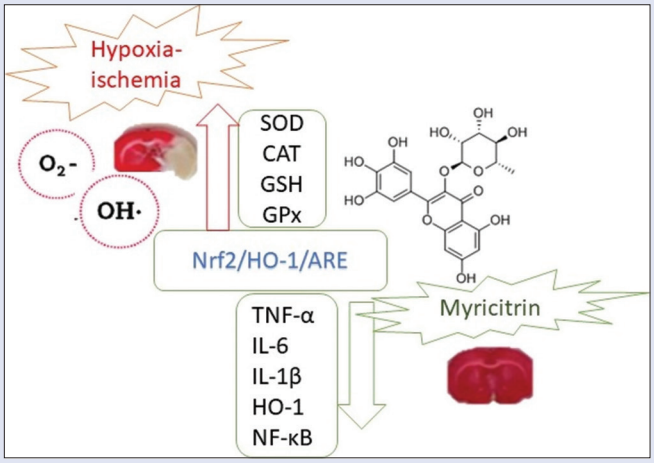
ABSTRACT

Background: Neonatal hypoxic ischemia (HI) is an overwhelming infantile neurological disability causing extreme morbidity and mortality with limited therapies available. **Objectives:** In this study, we aimed to explore the neuroprotective efficacy of myricitrin and elucidate the mechanism of action of myricitrin through the regulation of nuclear factor erythroid 2-related factor 2 (Nrf2)/hemeoxygenase-1 (HO-1)/antioxidant response element (ARE)-signaling pathways in neonatal rats. **Materials and Methods:** Seven-day-old neonatal rats were divided into four groups. Sham group served as the control group. After inducing HI through standard operation procedure, two groups of neonatal rats were intraperitoneally administered with 20 and 40 mg/kg bw of myricitrin twice a day for up to 1 week. At the end of the experiment, we assessed the brain infract area, edema, and motor coordination activity by using Rota Rod test. Furthermore, we measured the level of antioxidant enzymes and oxidative stress markers in the brain tissue samples. Real-time quantitative polymerase chain reaction was conducted to detect the inflammatory molecules expression including HO-1 being the activator of Nrf2 where further mediates the transcriptional activation of ARE. Subsequently, the hypothesis was further confirmed by the immunosorbent assay and Western blot analysis. **Results:** Myricitrin administration ameliorated HI-induced increase in infract volume and edema of the brain, as well as improved neurobehavioral impairments. Interestingly, myricitrin significantly reduced the level of nuclear factor kappa B (NF-κB) p65 and reduced the levels of nuclear fraction of Nrf2-ARE and cytosolic fraction of HO-1 enzyme activation. **Conclusion:** In summary, myricitrin improved the antioxidant defense mechanism and ameliorated the oxidative stress-associated neurological damage via modulating Nrf2/ARE-dependent HO-1 pathway in neonatal rats.

Key words: Antioxidant, hemeoxygenase-1, hypoxic-ischemic, myricitrin, neuroinflammation

SUMMARY

- Elevated reactive oxygen species (ROS) and decline in antioxidant status are signs of neurological inflammatory damage in HI rat brain
- Myricitrin is a well-known anti-inflammatory agent that restores the antioxidant levels and neutralizes the ROS during HI treatment
- Myricitrin attenuates hypoxia-induced ischemic brain damage in neonatal rats probably through nuclear factor erythroid 2-related factor 2/HO-1/antioxidant response element signaling pathway.



Abbreviations used: Nrf2: Nuclear factor erythroid 2-related factor 2; HO-1: Hemeoxygenase-1; ARE: Antioxidant response element; NF-κB: Nuclear factor kappa B; TNF-α: Tumor necrosis factor-alpha; IL-6: Interleukin-6; WHO: World Health Organization; ELISA: Enzyme-linked immunosorbent assay; TBARS: Thiobarbituric acid reactive substance

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INTRODUCTION

In general, neonates succumb to hypoxic-ischemic (HI) encephalopathy which causes neuronal damage during intrapartum asphyxia, and it accounts for about 25% of the infant mortality globally.[1] Perinatal HI encephalopathy remains a major cause of disability in infants, resulting in motor and learning impairment, epilepsy, seizures, and eventually cause death.[2] It is associated with an array of events contributing to neuronal damage, generation of free radicals and loss of ATP molecules, excitotoxicity, and failure of export/import of calcium ions.[3] Abnormal increase in the level of superoxide dismutase (SOD) and glutathione peroxidase (GPx)

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increases the formation of free radicals and lipid peroxidation products. Moreover, increased levels of iron (Fe^{2+}) in neonatal brain favor the formation of reactive oxygen species (ROS) through Fenton's reaction, which catalyzes the formation of hydroxyl radicals (OH). Hydroxyl radicals are highly oxidizing agents which cause severe brain damage.[4,5] Hemeoxygenase-1 (HO-1), one of the antioxidant enzymes, attenuates oxidative stress-mediated inflammation and alleviates neuronal loss with well-known cytoprotective effects.[6,7] However, the patients diagnosed with Parkinson disease (PD) has identified an elevated serum HO-1 which is also supports the hypothesis of oxidative stress mediated over expression of HO-1.[8]

In eukaryotic organisms, cellular redox balance is regulated by a transcription factor called nuclear factor erythroid 2-related factor 2 (Nrf2), which is encoded by *Nfe2l2*. Nrf2 binds with antioxidant response element (ARE) present in the HO-1 promoter region.[9] The abnormality in Nrf2-mediated defense in aging is thought to be one of the risk factors for PD, which is accompanied with diminished Nrf2/ARE-dependent cytoprotective enzyme activity.[10-13]

Flavonoids are large groups of a polyphenolic compounds. Myricitrin is one among the polyphenols called hydroxy flavonoid and in terms of IUPAC, it is 3′, 4′, 5′, 5, 7-hexahydroxy flavone-3-*O*-α-L-rhamnoside [Figure 1] and is naturally present in various parts of bayberry including fruits, leaves, bark, and stem, as well as in the root bark of Myrica species of plants.[14-16] Myricitrin exhibits beneficial activities such as anticancer,[17,18] antimicrobial, antiviral,[19] and anti-nociceptive.[20] According to the literature, myricitrin can effectively scavenge free radical and shows better pro-oxidant properties than that of its counterparts such as quercetin and rhamnosides.[21]

Despite this, the number of therapeutic options available for HI-mediated abnormalities is low. However, pharmacological studies of isolated compounds from herbs have been evaluated for its neuroprotection under normal and hypoxic condition.[21] Interestingly, Nrf2/HO-1/ARE axis is the topic of research of many researchers. Therefore, in this study, we aimed to explore the role of myricitrin on the modulation of signaling pathways during HI-induced brain injury in neonatal rodents. To achieve this goal, hypoxia-induced ischemic brain injury was established in neonatal rats to evaluate the anti-inflammatory potential of myricitrin. Furthermore, to explore the molecular mechanisms, we studied the modulation of Nrf2/HO-1 via ARE and elucidated its critical neuroprotective effect.

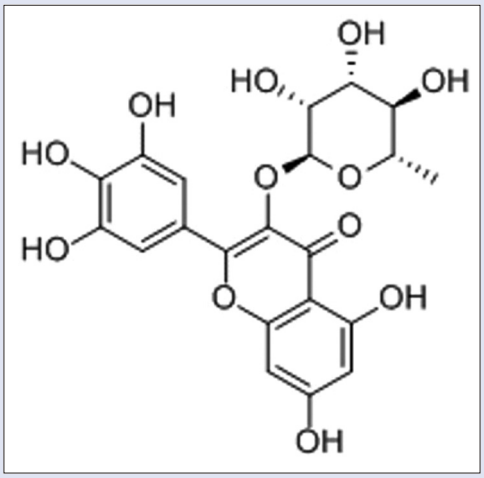

Figure 1: Molecular structure of myricitrin and molecular formula is $C_{21}H_{20}O_{12}$ and molecular weight = 464.3763

MATERIALS AND METHODS

Chemicals and reagents

Myricitrin was obtained from Selleckchem-S232701 (Shanghai, China). Phosphate buffer for lysis was obtained from Sigma-Aldrich (MO, USA). Total Nitric Oxide Assay Kit was purchased from Beyotime (Jiangsu, China). Pierce protein assay kit was obtained from Thermo Fisher Scientific Inc., (MA, USA). The tissue levels of malondialdehyde (MDA), which is a product of lipid peroxidation, SOD, catalase, and reduced glutathione (GSH)-GPx was measured using the following commercial micro test kits: MDA-A003, SOD-A001, CAT-A007, and GPx-A017, respectively, which were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) micro-test kits were obtained from Biovision Inc., (CA, USA). BCA protein assay kit was purchased from Beyotime (China). Poly-vinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA). The primary antibodies were obtained from Nrf2-Promega Corp., (Madison, WI, USA) and HO-1-CD143 Stressgen Biotechnologies, Victoria (BC, Canada). β-Actin was obtained from Beijing Zhongshan Goldenbridge Biotechnology, China. The horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Abcam, Cambridge, UK. All other chemicals used in this study were of analytical grade.

Experimental animals

In this study, we used 7-day-old female Sprague–Dawley rats (weighing 7 ± 0.8 g) free of pathogens that were procured from Animal Experimental Center of Ningxia Medical University (Yinchuan, China) (Permit number: SCXK Ningxia 2015-0001). Experiments involving neonatal rats were approved by the Taian City Central Hospital Institutional Animal Ethical Committee. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). All rats were housed in individual cages under constant regulated day/light cycles (6:00 AM to 6:00 PM), under controlled temperature (21°C). All animals were provided free access to water and food *ad libitum*. All the efforts were made to minimize the suffering of the animals. Pups from day 7 were chosen for the experiments as they are comparable to 32–34th-week neonates of humans.[22] Neonatal rats were randomly picked and divided into four different groups for experimental purposes.

Experimental groups

Animals were acclimated for about 1 week before the experiments. To investigate the neuroprotective efficiency of myricitrin during HI neuronal damage in perinatal infant rats, the animals were arbitrarily divided into four different groups.

Group I: Sham (n = 12)

Group II: HI induced + saline administered (n = 12)

Group III: HI induced + myricitrin administered (myricitrin was dissolved in saline contains 2% dimethyl sulfoxide (DMSO) and administered intraperitoneally (i.p.) at a dose of 20 mg/kg body weight until the end of the experiment (n = 12).

Group IV: HI induced + myricitrin (40 mg/kg body weight) administered as i.p. to pups until the end of the experiment (n = 12).

HI was induced as with modifications described previously.[22] Briefly, 7-day-old (P7) offspring pups were anesthetized with 2%–3% isoflurane through inhalation. Then, the carotid artery (CCA) at the right ventricle was surgically exposed and was double-ligated using 5.0 silk surgical sutures which was then cut open in between two ligation sites. After surgery, the animals were recuperated for 1.5 h and put into

hypoxic incubator (with 92% nitrogen balanced with 8% oxygen for 1.5 h at 37°C).[22,23] Then, the animals were allowed to recover after the hypoxic treatment. For sham treatment group, only CCA was exposed and the animals did not undergo ligation procedure and the hypoxic treatment.

Next, myricitrin was dissolved in sterile physiological saline containing 2% DMSO to obtain the desired concentration and were administered to the animals. It was administered after every 12 h for 7 consecutive days after induction of HI injury. Sham and HI pups were treated with saline with 2% DMSO as the vehicle control. At the end of day 7, all neonatal rats were sacrificed under pentobarbital sodium (50 mg/kg i.p.). The brain was harvested and fixed in 4% paraformaldehyde (w/v in phosphate-buffered saline [PBS]) for the histological analysis (six animals from each group). Phosphate buffer was used in the preparation of tissue homogenate for biochemical as well as for molecular biology analysis (n = 6 each group).

Measurement of the brain infarct volume

For the measurement of the brain infarct volume, we followed previously described methods with some minor modifications. In this study, we used 2,3,5-triphenyltetrazoliumchloride (TTC) stain after 24 h and after 7 days of HI.[24] Briefly, the rat pups were anesthetized using 4% isoflurane and sacrificed. Then, the brain was harvested and kept frozen before staining. Then, the coronal sections (2 mm thick) of the brain tissue were obtained using microtome of Leica Microsystems (Wetzlar, Germany) and were incubated with 2% TTC at 37°C for 20 min. Then, the tissue samples were fixed in 4% formalin prepared using PBS and left overnight at 4°C. Subsequently, the samples were washed with PBS. The brain damage, in terms of percentage contralateral hemisphere damage, was quantified using the Image-Pro plus software version 7.0 (USA).

Measurement of brain cerebral edema (CO)

Wet (24 h after the HI and myricitrin administration) and dry weight of the brain (after drying at 105°C for 24 h) were measured.[24] CO was calculated as follows:

Evaluation of coordination deficits and motor activity

RotaRod experiment was conducted after 14 days of HI to measure the degree of coordination deficit and impairment in motor activity according to a previously described method.[25] Briefly, 2 days of training schedule was followed: On the 1st day, the animals were trained for 4 revolutions per minute (rpm) for 1 min, and on the 2nd day, they were trained for 4–40 rpm for 10 min for the maximum of the time. After the completion of training, 1 h rest was provided between each trial. The latency to fall in all rat pups was recorded in seconds.

Measurement of reactive oxygen species and lipid peroxidation and antioxidants

In this study, we used 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) dye to quantify the level of ROS.[26] In this assay, the oxidation product of DCFH to the fluorescent 2′,7′-dichlorofluorescein (DCF) formation is measured to quantify the level of ROS formed. DCF was measured by spectrofluorimetric method at an excitation wavelength of 504 nm and emission wavelength of 529 nm. Then, we measured the total nitric oxide (NO) levels by using Griess reagent, which allows spectrophotometric detection of the accumulation of nitrite and nitrate in the brain tissue homogenates. Next, pierce protein assay kit was used to measure the proteins present in the brain tissue homogenate.

Analysis of gene expression

In this study, we used RNA isolator reagent from Vazyme Biotech Co., Ltd., for the isolation of total RNA from the brain tissue homogenate and followed the manufacturer's instructions. HiScript® II 1st Strand cDNA Synthesis kit was used to perform reverse transcription reaction. AceQ qPCR SYBR Green Master Mix kit was used for the real-time polymerase chain reaction (RT-PCR) experiment by using the Bio-Rad CFX System and following the method described previously.[27] Table 1 shows the primers used for mice gene expression and were obtained from Vazyme Biotech Co., Ltd., (Nanjing, China).

Enzyme-linked immunosorbent assay

The expression of various proinflammatory cytokines was determined in cerebral tissue homogenate by using commercially available ELISA micro-test kits.

Western blot analysis of nuclear factor erythroid 2-related factor 2 and hemeoxygenase-1 expression

Nuclear fraction of Nrf2 and cytosolic fraction of HO-1 in the cerebral cortex of the brain was measured using respective nuclear and cytosolic kits, and the concentration of the extracted protein was estimated by using the BCA protein assay kit. Briefly, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used for the separation of the protein (40 μg/lane), and the separated proteins were electroblotted onto PVDF membranes. Tris-buffered saline with 1% Tween 20 solution containing 5% skimmed milk was used as the blocking solution to block the membrane from false signals. The membrane was subsequently incubated (4°C overnight) with primary antibodies of rabbit polyclonal anti-Nrf2 and HO-1 of 1:500 and 1:800 dilution, respectively, and anti-rat β-actin (polyclonal-rabbit) of 1:500 dilution. Subsequently, membranes were incubated with HRP-conjugated secondary antibody (1:10,000-Abcam, Cambridge, UK) diluted with TBS at the room temperature for 60 min. The band absorbance was quantified using DAB, and band intensity was measured by using ImageJ software developed by NIH (Bethesda, MA).

Hematoxylin and eosin staining

In this study, the brain tissue samples were fixed in 4% paraformaldehyde in PBS overnight at 4°C and embedded in paraffin wax. The 5 μm thick coronal sections of brain tissue were cut using a microtome. Finally, the microtome sections were stained with hematoxylin and eosin (H and E).[28]

Statistical significance

GraphPad Prism 8.0 developed by GraphPad Software Inc., (CA, USA) was used for the statistical analysis, and the results are presented as mean ± standard error of the mean. One-way analysis of variance followed by a Dunnett's t-test was conducted to calculate the significance of differences between the experimental groups for multiple data comparison. Statistical analysis of the neurological function was performed through nonparametric analysis. $P < 0.05$ value was considered to be significant.

RESULTS

Myricitrin administration reduces HI-induced cerebral infarct area in brain

Area of cerebral infarct (CI) in the rat brain was measured by using TTC stain [Figure 2]. The CI area which appears as white patches in the HI group. HI group shows significant ($P < 0.05$) increase in CI area (38.15% ± 2.11%) than that of the Sham control group. However, 20 and 40 mg/kg bw myricitrin reduced the CI area effectively: 25.21%

Table 1: Primes used in quantitative reverse transcription - polymerase chain reaction study listed

Gene name	Primer	
	Forward (5′-3′)	Reverse (5′-3′)
β-actin	GGAGATTACTGCCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG
TNF-α	CGGGCTCAGAATTTCCAACA	CGCAATCCAGGCCACTACTT
IL-6	GCCCTTCAGGAACAGCTATGA	TGTCAACAACATCAGTCCCAAGA
IL-1β	CAGGCTTCGAGATGAACAACAA	GTCCATTGAGGTGGAGAGCTTT
HO-1	TGCTCGCATGAACACTCTGGAGAT	ATGGCATAAATTCCCACTGCCACG
NFκB/P65	GAGACATCCTTCCGCAAACT	TCCTTCCTGCCCATAATCA

TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; HO-1: Hemeoxygenase-1; NFκB: Nuclear factor kappa B

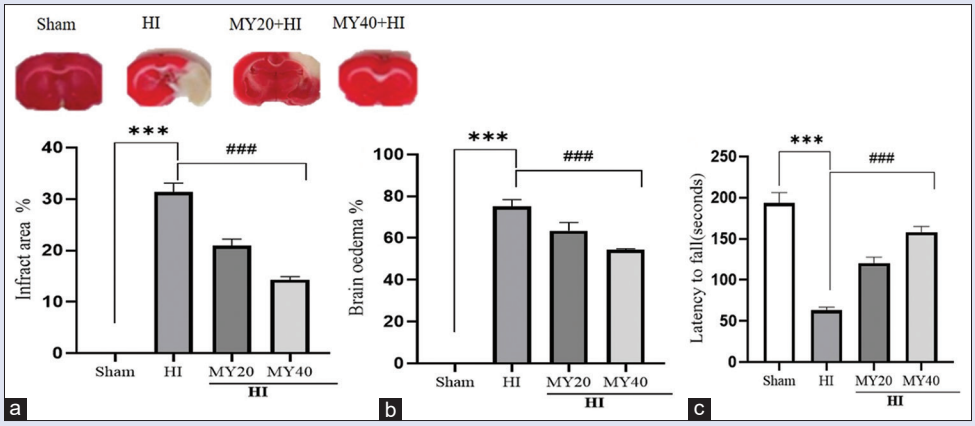

Figure 2: Myricitrin administration diminish infarct volume, edema (wet weight of the brain), improve neurological function studies in hypoxic-ischemic induced neonatal brain injury in a dose-dependent manner. (a) Coronal brain slices stained with TCC shows infarct area is smaller in myricitrin treated group than HI group and $n = 6$. (b) Effect of myricitrin on cerebral edema compared with HI-induced neonatal brain and $n = 6$. (c) RotaRod test was conducted and the expectancy to fall was expressed in seconds shown in C and $n = 10$. Statistical analysis data were expressed as the mean ± standard deviation. (*$P < 0.05$ compared with sham, #$P < 0.05$ compared with MY20 and 40)

±1.65% and 16.30% ± 2.13%, respectively. This indicates that myricitrin effectively controlled the spread of HI-induced CI area associated with brain damage.

Myricitrin reduces CO in HI-induced rat brain

CO of HI-induced rat brain tissue was drastically ($P < 0.05$) increased: 81.23% versus 0% in the Sham group. Myricitrin (20 and 40 mg/kg) reduced CO significantly ($P < 0.05$): 68.16% and 61.42%, respectively [Figure 2]. This demonstrates the neuroprotective effect of myricitrin in decreasing the level of water in the brain tissue when compared with the HI group.

Myricitrin improves coordination and motor activity in HI-induced rats

RotaRod test was conducted to identify the coordination and motor activity of rats. HI-induced rats showed poor activity with a very less time spent on the rod compared to the of animals in the Sham group [Figure 2]. Myricitrin improved the latency to fall when compared with Sham group [Figure 2]. High dose of myricitrin (40 mg/kg) administration increased the time of animals spent on the rod when compared with that of low dose (20 mg/kg).

Myricitrin improves antioxidant status in HI-induced rat brain

Product of lipid peroxidation (MDA), NO, and various antioxidant enzymes were analyzed to validate the effect of myricitrin on neuroprotection in HI-induced neonatal rat brain against ROS-induced oxidative stress [Figure 3]. The activity GSH, GPx, CAT, and SOD enzymes were significantly declined in HI-induced brain when compared to that of Sham group. Furthermore, the level of MDA was considerably increased in HI induced rat pups brain tissue ($P < 0.05$). Myricitrin treatment (20 and 40 mg/kg; $P < 0.05$) significantly restored the activities of antioxidant enzymes as well as decreased the formation of MDA and NO in brain tissues.

Myricitrin administration downregulated the inflammatory gene expression in HI-induced rat brain

Effect of myricitrin treatment on inflammatory marker genes in HI-induced brain tissue was determined by real-time PCR assay. The mRNA expression pattern of several proinflammatory cytokines was also measured to confirm the regulatory processes of inflammation. Figure 2 shows that HI resulted in the overexpression of mRNA of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, HO-1 and p65 NF-κB. Intraperitoneal administration of myricitrin to HI-induced rat pups controlled and downregulated the expression of proinflammatory cytokines and HO-1 [Figure 4]. High dose of myricitrin (40 mg/kg) significantly reduced the expression of inflammatory and oxidative stress markers than that of low dose myricitrin (20 mg/kg). These results show the positive effect of myricitrin in reducing inflammation after HI operation.

Myricitrin inhibits HI-induced inflammation in the brains of rat pups

The mRNA of inflammatory marker genes was analyzed by real-time PCR analysis. Next, the translational product was

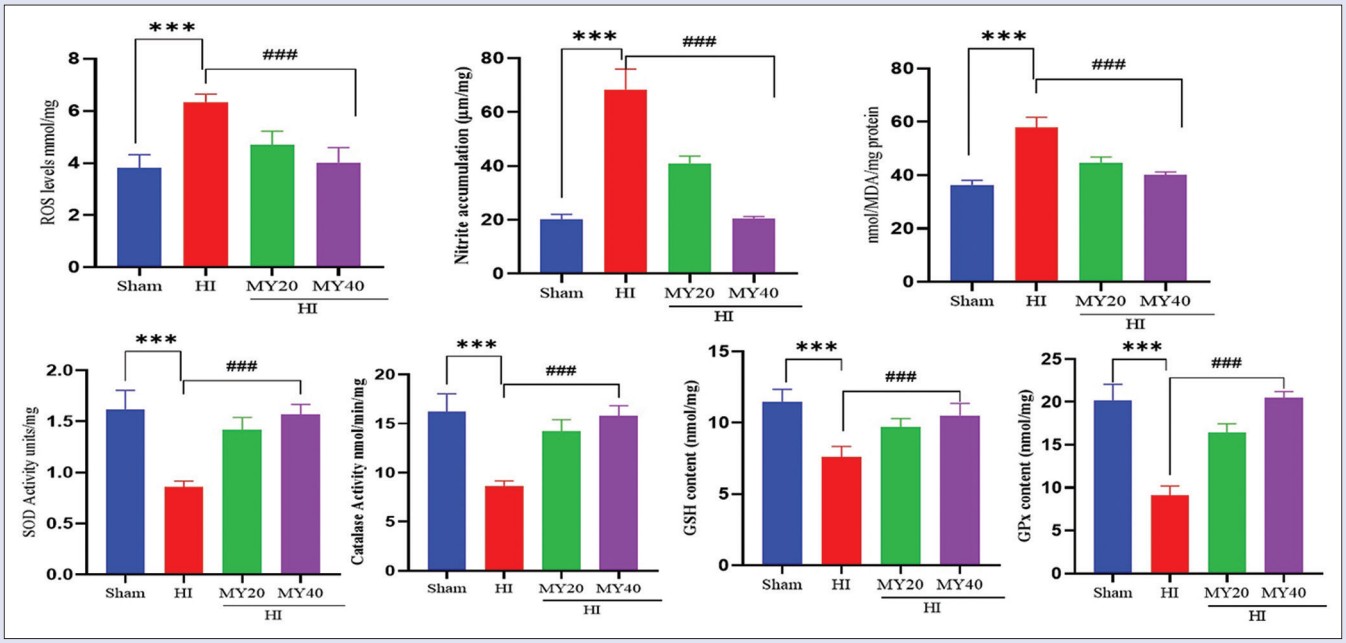

Figure 3: Shows the levels of total reactive oxygen species, malondialdehyde (lipid peroxidation), anti-oxidant enzymes (superoxide dismutase, catalase, reduced glutathione, and glutathione peroxidase) in the brain tissues of hypoxic ischemic hemispheres in brain tissues of hypoxic ischemic hemispheres. The results were expressed as mean ± standard error of the mean and statistically significant at $P < 0.05$. ### MY (Myricitrin) treated group compared to HI (hypoxic ischemic-induced group); ***HI group compared with Sham control

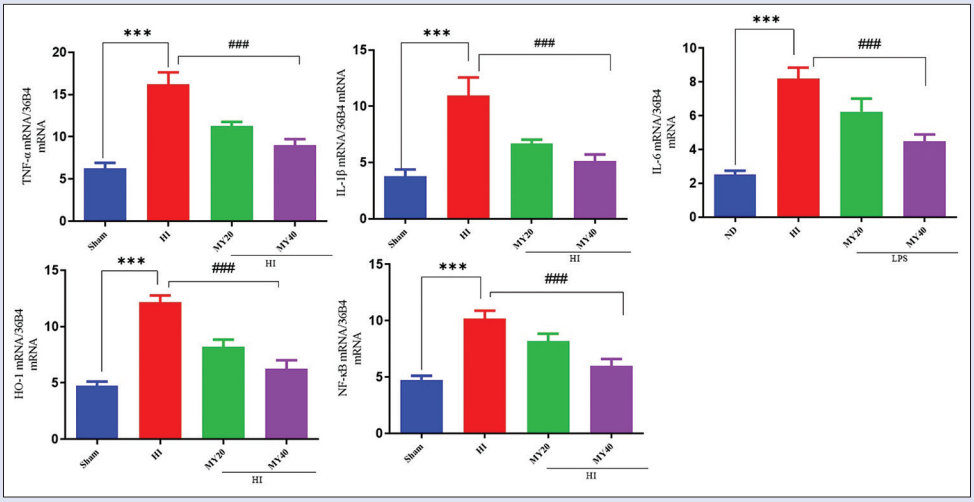

Figure 4: mRNA expression pattern of inflammatory mediators and the results were expressed as mean ± standard error of the mean and statistically significant at $P < 0.05$. ###MY (Myricitrin) treated group compared to HI (Hypoxic ischemic-induced group); ***HI group compared with Sham control

quantified by ELISA. The effect of myricitrin on the marker proteins after HI induction was assessed by evaluating the levels of oxidative stress-mediated ARE and inflammation-associated pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α as well as NF-κB free p65 subunit [Table 2]. HI-induced rat brain tissue showed statistically increased level of inflammatory marker protein molecules ($P < 0.05$) (HO-1, IL-6, IL-1β, TNF-α, and NF-κB free p65) than that of Sham control group. Myricitrin (40 mg/kg) significantly reduced the HI-induced abnormal increase in the level of inflammatory marker proteins ($P < 0.05$).

Myricitrin augmented protein expression of nuclear nuclear factor erythroid 2-related factor 2 and cytosolic hemeoxygenase-1 in the cerebral cortex of rats

Western blot analyses were performed to confirm the effects of myricitrin on the protein expression pattern of HO-1 of cytosolic and Nrf2 of nuclear fractions [Figure 5]. The protein expression of Nrf2 and HO-1 in the cerebral cortex of HI-induced rat brain was unusually upregulated ($P < 0.05$) which was similar to the Sham control group. Of note, myricitrin treatment (20 and 40 mg/kg) reversed ($P < 0.05$) the upregulated expression of HO-1 and Nrf2 after HI insult.

HI-induced brain damage normalized by myricitrin

The neuroprotective effect of myricitrin on HI-induced brain damage was observed in H and E-stained brain tissue. Sham group tissue sections showed normal intact round-shaped cell bodies, whereas HI group tissue sections revealed atrophic cell bodies in the intensely stained sections. HI-induced myricitrin treated neonatal brain tissue showed reduced cell atrophy which resembled the normal morphology [Figure 6]. Furthermore, 40 mg/kg myricitrin showed normal cellular morphology, which indicates the protective effect of myricitrin.

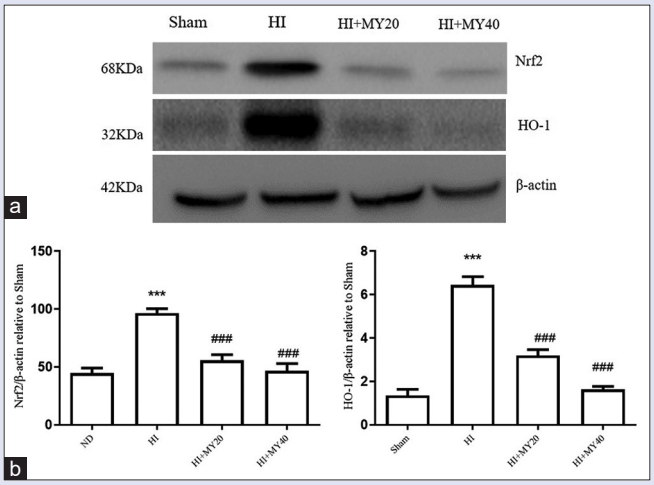

Figure 5: Myricitrin mediates the overexpression of nuclear factor erythroid 2-related factor 2 (a) and cytosolic hemeoxygenase-1 (b) protein in HI-induced cerebral cortex of experimental neonatal rats. Results were expressed as mean ± standard error of the mean and statistically significant at $P < 0.05$. *Induced group compared to control; ###MY (Myricitrin) treated group compared to HI (Hypoxic ischemic induced group); ***HI group compared with Sham control

DISCUSSION

Myricitrin is a flavone class of bioactive compounds occurring naturally in various herbs. The majority of the studies have explored its efficiency both under *in vivo* and *in vitro* conditions. Studies have confirmed that myricitrin might activate Nrf2-mediated HO-1 and NQO-1, also improve the level of anti-oxidant enzymes this would further attenuates high glucose induced cell apoptosis in H9c2 cells via activation of Akt signaling. Previous studies have clearly demonstrated that nuclear translocation of Nrf2 can be induced by phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway. Thus, activation of PI3K/Akt pathway can subsequently modulate the Nrf2-mediated HO-1 expression, which has been considered as a possible approach to reduce HI-induced neuronal damage.[29] Interestingly, it has been reported that myricitrin shows remarkable anti-inflammatory and antifibrotic properties.[30] Nonetheless, the effects of myricitrin on HI-induced brain damage in the newborn and its protective mechanism has not yet been studied. To the best of our knowledge, this is the first report on myricitrin that demonstrates the role of Nrf2/HO-1/ARE signaling pathway in attenuating the HI-induced brain injury. ROS-induced oxidative stress and the subsequent inflammatory responses after HI induction are the key mediators of pathological change associated with HI.[31,32]

In general, brain cerebral ischemic condition experiment with rodents, the degree of damage in neuronal part is mainly assessed by brain infarct volume measurement assay.[33] In our experimental measurement of brain infarct volume and edema, the HI-induced pups developed a more infarct area and a higher rate of edema and inflammatory neuronal damage and response. Coordination and motor activity observation study exhibits poor time spent on RotaRod test by HI-induced pups

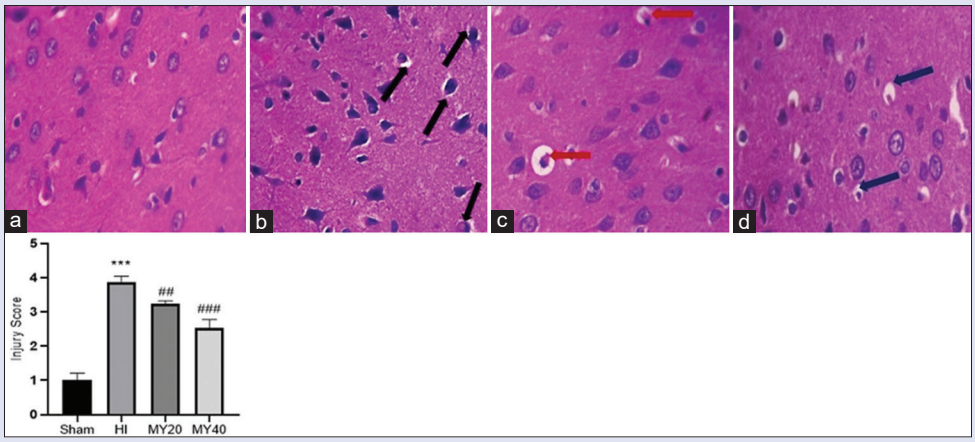

Figure 6: Neuroprotective effect of myricitrin observed in HE staining of neonatal cerebral cortex of brain tissue with × 200 (a), Sham; (b), HI; (c), MY20 + HI; (d), MY40 + HI. The bar graph shows cell damage scores. Data are expressed as mean ± standard deviation ($n = 6$). ###$P < 0.05$ versus HI group. ***$P < 0.05$ versus Sham group

Table 2: Significant effect of myricitrin on proinflammatory cytokines and hemeoxygenase-1 in of hypoxic ischemic induced neonatal rats

Group	TNF-α (pg/mg)	IL-1β (pg/mg)	IL-6 (pg/mg)	HO-1 (pg/mg)
Sham	60.31±3.22	47.69±2.11	91.40±1.20	48.16±1.32
HI	128.42±4.32***	144.54±8.20***	162.74±3.13***	112.41±2.21***
HI + MY20	96.13±6.28###	117.49±3.97###	141.43±4.64##	81.13±5.54##
HI + MY40	82.69±7.54###	93.22±6.84###	128.25±7.43###	68.23±6.13###

###MY treated group compared to HI-induced group, ***HI group compared with Sham control. Results were expressed as mean±SD and statistically significant at $P<0.05$. MY: Myricitrin; HI: Hypoxic ischemic; SD: Standard deviation; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; HO-1: Hemeoxygenase-1

indicates impairment in coordination and motor activity. In contrast, myricitrin-treated pups demonstrated improved coordination and the latency to fall when compared with pups in HI and Sham groups. Our results with regard to infarct volume area agree with those of Karalis *et al.*[28] The authors noted that resveratrol treatment in HI-induced rat pups significantly decreased the infarct area of the brain by preventing the demyelination.[34] This ameliorative effect would highly correlate with the observed pathological changes made by myricitrin.

The neonatal brain is more susceptible to ROS-induced oxidative stress than that of adult counterpart and neonatal brain has contain high polyunsaturated fatty acids this could utilize more oxygen resulted subsequent antioxidants deficiency. Therefore, neonatal brain is more susceptible to pathological increase in the amount of free radicals generated, and subsequently, it causes neuronal damage in the brain tissue of neonatal rat.[34,35]

In order to identify the role of myricitrin in antioxidant profile and oxidant defense, we measured the level of cellular antioxidant enzymes. Myricitrin administration significantly improved the level of antioxidant enzymes and reduced the rate of lipid peroxidation and formation of NO in the brain tissue. This data strongly supports the reported antioxidant role of myricitrin in various mechanistic studies. Pinocembrin is flavonoid class of compound exhibits neuroprotection via the oxidative stress improvement in ROS or 6-OHDA (6-hydroxydopamine) and (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mediated neuro inflammation to establish PD. This data correlates our results on myricitrin, which mediates antioxidant defense in HI-induced brain damage. In this study, the mechanism of neuroprotection by myricitrin was achieved via the antioxidant defense improvement which subsequently induced the HO-1 enzyme activity and activation of Nrf2. Meanwhile, irrespective to neuroprotection pinocembrin could mitigate the oxidative stress through Nrf2-HO-1 signaling in carbon tetrachloride (CCl_4)-induced liver fibrosis and nephrotoxicity.[28]

Number of studies explored that flavonoid group of compounds showed neuroprotection has been attributed via regulation of Nrf2 and HO-1.[34] Moreover, previous studies have revealed that flavonoids could also inhibit the expression of NF-κB, inducible nitric oxide synthase, cyclooxygenase-2, and proinflammatory cytokines such ass TNF-α, IL-1β, and IL-6. According to our results, the mRNA expression of inflammatory molecules in rat pups treated with myricitrin shows limited expression of inflammatory marker genes and the quantification data of inflammatory cytokines also correlated with the mRNA expression pattern of inflammatory markers.

Neurotoxicity induced by various neurotoxic agents such as H_2O_2, paraquat, methylglyoxal, and 6-OHDA might ameliorate efficiently by well-known flavonoid compound pinocembrin. The experimental neuroprotection via the constructive role of Nrf2-HO-1 axis was evidenced by the Nrf2 silencing study or HO-1 inhibition by ZnPP IX (0.5 μM). In addition, it has been shown that flavonoids exert anti-inflammatory effect against lipopolysaccharide and H_2O_2-induced inflammatory damage in BV-2 microglial cells and SH-SY5Y cells respectively through the Nrf2-HO-1 axis.[35] In order to identify the role of myricitrin on Nrf2-HO-1-ARE axis in HI-induced neuronal damage, we measured the protein expression of the nuclear fraction of Nrf2 and cytosolic fraction of HO-1 in the brain cerebral cortex. Our results showed that myricitrin significantly suppressed the Nrf2 and HO-1 expression that indicates translocation of Nrf2-mediated activation of ARE-HO-1 also inhibited.

This study has certain limitations and yet to identify the interlink between Nrf2/HO-1 associated other signaling cascade mechanism include contributors of HIE such as apoptosis, mitochondrial dysfunction as well as its relationship with bioenergetics. In future, we plan to substantiate the mechanism. In this study, we focused the neuroprotective effects of myricitrin on rat pups subjected to HI insult accompanying ROS-induced oxidative stress, antioxidant status, and inflammatory signaling response.

CONCLUSION

In summary, myricitrin can be used in the treatment of inflammatory neurological abnormalities and the regulation of Nrf2/HO-1/ARE is possible signaling mechanism via myricitrin attenuates hypoxia-induced ischemic brain damage in neonatal rats. We recommend further research on humans for a better understanding of the mechanism of action of myricitrin.

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Ethical approval statement

Animal experiments were approved by the ethical committee of Taian City Central Hospital, (No: 2019-20/ML 2231) Taian, 271000, Shandong, China.

REFERENCES

1. Varghese B, Xavier R, Manoj VC, Aneesh MK, Priya PS, Kumar A, *et al.* Magnetic resonance imaging spectrum of perinatal hypoxic-ischemic brain injury. Indian J Radiol Imaging 2016;26:316-27.
2. Hossain MA. Hypoxic-ischemic injury in neonatal brain: Involvement of a novel neuronal molecule in neuronal cell death and potential target for neuroprotection. Int J Dev Neurosci 2008;26:93-101.
3. Qin X, Cheng J, Zhong Y, Mahgoub OK, Akter F, Fan Y, *et al.* Mechanism and treatment related to oxidative stress in neonatal hypoxic-ischemic encephalopathy. Front Mol Neurosci 2019;12:88.
4. Aridas JD, Yawno T, Sutherland AE, Nitsos I, Ditchfield M, Wong FY, *et al.* Detecting brain injury in neonatal hypoxic ischemic encephalopathy: Closing the gap between experimental and clinical research. Exp Neurol 2014;261:281-90.
5. Ten VS, Starkov A. Hypoxic-ischemic injury in the developing brain: The role of reactive oxygen species originating in mitochondria. Neurol Res Int 2012;2012:542976.
6. Chao XJ, Chen ZW, Liu AM, He XX, Wang SG, Wang YT, *et al.* Effect of tacrine-3-caffeic acid, a novel multifunctional anti-Alzheimer's dimer, against oxidative-stress-induced cell death in HT22 hippocampal neurons: Involvement of Nrf2/HO-1 pathway. CNS Neurosci Ther 2014;20:840-50.
7. Piras S, Furfaro AL, Brondolo L, Passalacqua M, Marinari UM, Pronzato MA, *et al.* Differentiation impairs Bach1 dependent HO-1 activation and increases sensitivity to oxidative stress in SH-SY5Y neuroblastoma cells. Sci Rep 2017;7:7568.
8. Mateo I, Infante J, Sánchez-Juan P, Gorostiaga G, Rodríguez RE, Higuera JL, *et al.* Serum hemeoxygenase-1 levels are increased in Parkinson's disease but not in Alzheimer's disease. Acta Neurol Scand 2010;121:136-8.
9. Park EJ, Jang HJ, Tsoyi K, Kim YM, Park SW, Kim HJ, *et al.* The heme oxygenase-1 inducer THI-56 negatively regulates iNOS expression and HMGB1 release in LPS-activated RAW 264.7 cells and CLP-induced septic mice. PLoS One 2013;8:e76293.
10. Francisqueti-Ferron FV, Ferron AJ, Garcia JL, Silva CC, Costa MR, Gregolin CS,*et al.* Basic concepts on the role of nuclear factor erythroid-derived 2-like 2 (Nrf2) in age-related diseases. Int J Mol Sci 2019;20:E3208.
11. Jiang S, Deng C, Lv J, Fan C, Hu W, Di S, *et al.* Nrf2 weaves an elaborate network of neuroprotection against stroke. Mol Neurobiol 2017;54:1440-55.
12. Kumar H, Koppula S, Kim IS, More SV, Kim BW, Choi DK. Nuclear factor erythroid 2-related factor 2 signaling in Parkinson disease: A promising multi therapeutic target against oxidative stress, neuroinflammation and cell death. CNS Neurol Disord Drug Targets 2012;11:1015-29.
13. Zhang M, An C, Gao Y, Leak RK, Chen J, Zhang F. Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. Prog Neurobiol 2013;100:30-47.

14. Aloud AA, Veeramani C, Govindasamy C, Alsaif MA, El Newehy AS, Al-Numair KS. Galangin, a dietary flavonoid, improves antioxidant status and reduces hyperglycemia-mediated oxidative stress in streptozotocin-induced diabetic rats. Redox Rep 2017;22:290-300.
15. Liao HW, Liu EG, Wang DY. Chemical constituents of the bark of *Myrica ruba*. Cent South Pharm 2006;4:196-9.
16. Liu ZG, Zhang YC, Shuang JI, Meng DL, Xian LI. Isolation and identification of constituents from bark of *Myrica ruba* (Lour.) Sieb.et Zucc. J Shenyang Pharm Univ 2010;27:34-6.
17. Lee ER, Kang GH, Cho SG. Effect of flavonoids on human health: Old subjects but new challenges. Recent Pat Biotechnol 2007;1:139-50.
18. Kale A, Gawande S, Kotwal S. Cancer phytotherapeutics: Role for flavonoids at the cellular level. Phytother Res 2008;22:567-77.
19. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicro Agents 2005;26:343-56.
20. Meotti FC, Luiz AP, Pizzolatti MG, Kassuya CA, Calixto JB, Santos AR. Analysis of the antinociceptive effect of the flavonoid myricitrin: Evidence for a role of the L-arginine-nitric oxide and protein kinase C pathways. J Pharmacol Exp Ther 2006;316:789-96.
21. Wu JH, Huang CY, Tung YT, Chang ST. Online RP-HPLC-DPPH screening method for detection of radical-scavenging phytochemicals from flowers of Acacia confusa. J Agric Food Chem 2008;56:328-32.
22. Vannucci SJ, Hagberg H. Hypoxia-ischemia in the immature brain. J Exp Biol 2004;207:3149-54.
23. Wang L, Ke J, Li Y, Ma Q, Dasgupta C, Huang X, *et al.* Inhibition of miRNA-210 reverses nicotine-induced brain hypoxic-ischemic injury in neonatal rats. Int J Biol Sci 2017;13:76-84.
24. Jin Z, Liang J, Wang J, Kolattukudy PE. MCP-induced protein 1 mediates the minocycline-induced neuroprotection against cerebral ischemia/reperfusion injury *in vitro* and *in vivo*. J Neuroinflammation 2015;12:39.
25. Pazos MR, Cinquina V, Gómez A, Layunta R, Santos M, Fernández-Ruiz J,*et al.* Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. Neuropharmacology 2012;63:776-83.
26. Rodrigues Siqueira I, Fochesatto C, da Silva Torres IL, Dalmaz C, Alexandre Netto C. Aging affects oxidative state in hippocampus, hypothalamus and adrenal glands of Wistar rats. Life Sci 2005;78:271-8.
27. Guruvaiah P, Guo H, Li D, Xie Z. Preventive effect of flavonol derivatives abundant sanglan tea on long-term high-fat-diet-induced obesity complications in C57BL/6 mice. Nutrients 2018;10:E1276.
28. Karalis F, Soubasi V, Georgiou T, Nakas CT, Simeonidou C, Guiba-Tziampiri O, *et al.* Resveratrol ameliorates hypoxia/ischemia-induced behavioral deficits and brain injury in the neonatal rat brain. Brain Res 2011;1425:98-110.
29. Zhang B, Chen Y, Shen Q, Liu G, Ye J, Sun G, *et al.* Myricitrin attenuates high glucose-induced apoptosis through activating Akt-Nrf2 signaling in H9c2 cardiomyocytes. Molecules 2016;21:E880.
30. Domitrović R, Rashed K, Cvijanović O, Vladimir-Knežević S, Škoda M, Višnić A. Myricitrin exhibits antioxidant, anti-inflammatory and antifibrotic activity in carbon tetrachloride-intoxicated mice. Chem Biol Interact 2015;230:21-9.
31. Lu Q, Wainwright MS, Harris VA, Aggarwal S, Hou Y, Rau T, *et al.* Increased NADPH oxidase-derived superoxide is involved in the neuronal cell death induced by hypoxia-ischemia in neonatal hippocampal slice cultures. Free Radic Biol Med 2012;53:1139-51.
32. Arteaga O, Revuelta M, Montalvo H, Cañavate ML, Alonso-Alconada D, Martinez-Ibargüen A, *et al.* Neuroprotective Effect of Antioxidants in Neonatal Rat Brain after Hypoxia-Ischemia: Microscopy: Advances in Scientific Research and Education. Badajoz, Spain: Formatex Research Center; 2014. p. 335-43.
33. Fukui K, Omoi NO, Hayasaka T, Shinnkai T, Suzuki S, Abe K, *et al.* Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. Ann N Y Acad Sci 2002;959:275-84.
34. Yousuf S, Atif F, Ahmad M, Hoda N, Ishrat T, Khan B, *et al.* Resveratrol exerts its neuroprotective effect by modulating mitochondrial dysfunctions and associated cell death during cerebral ischemia. Brain Res 2009;1250:242-53.
35. Wang Y, Miao Y, Mir AZ, Cheng L, Wang L, Zhao L, *et al.* Inhibition of beta-amyloid-induced neurotoxicity by pinocembrin through Nrf2/HO-1 pathway in SH-SY5Y cells. J Neurol Sci 2016;368:223-30.