Int J Clin Exp Med 2017;10(4):6728-6734
www.ijcem.com /ISSN:1940-5901/IJCEM0020759

Original Article

Eupatilin inhibits OGD/R-induced neuronal injury in PC12 cells

Jing Xu[1], Cong Hua[2], Xiaoqiang Pan[2], Xijia Fu[3], Wei Wu[2]

Departments of [1]Outpatient, [2]Neurosurgery, [3]Neurology, The First Hospital of Jilin University, Changchun 130021, China

Received November 28, 2015; Accepted February 15, 2016; Epub April 15, 2017; Published April 30, 2017

Abstract: Eupatilin, a pharmacologically active flavone derived from the Artemisia plant species, has been reported to have anti-oxidant, anti-inflammatory, and anti-tumor and neuroprotective activities. Hence, this study was to investigate the effects of eupatilin on oxygen and glucose deprivation/reperfusion (OGD/R) induced neuronal injury in differentiated PC12 cells. Our results showed that eupatilin pretreatment attenuated OGD/R-induced neuronal injury, with evidence of increased cell viability and decreased LDH leakage. It also inhibited OGD/R-induced intracellular ROS production in PC12 cells. In addition, the down-regulation of Bcl-2, up-regulation of Bax and the consequent activation of caspase-3 induced by OGD/R were reversed by eupatilin. Furthermore, eupatilin pretreatment inhibited OGD/R-induced p-JNK and p-p38 expression in PC12 cells. Taken together, these results suggested that eupatilin inhibits OGD/R-induced neuronal injury in PC12 cells through JNK and p38 signaling pathways.

Keywords: Eupatilin, oxygen and glucose deprivation/reperfusion (OGD/R), neuronal apoptosis

Introduction

Ischemic stroke is a life-threatening cerebrovascular disease with substantial morbidity and mortality worldwide, and constitutes approximately 80% of all strokes [1]. It is characterized by cerebral blood vessel occlusion, which results in an insufficient supply of glucose and oxygen to central nervous system tissue and leads to necrotic loss of neurons in center and a surrounding penumbra with a milder ischemic insult [2]. Despite advances in understanding of the molecular mechanisms responsible for stroke-associated neuronal damage [3, 4], it remains a significant problem that is the major cause of death and disability worldwide.

Cumulative evidence suggests that both oxidative stress and apoptosis play crucial roles in the pathogenesis of cerebral ischemia/reperfusion injury [5-7]. Under ischemic condition, intracellular reactive oxygen species (ROS) are rapidly increased in neurons and this increase induces the release of cytochrome c from mitochondria, which plays a critical role in the process of neuronal apoptosis [8]. Thus, inhibition of oxidative stress is a good therapeutic strategy for the treatment of ischemic stroke.

Eupatilin, a pharmacologically active flavone derived from the Artemisia plant species, has been reported to have anti-oxidant, anti-inflammatory, and anti-tumor activities [9-11]. For example, Choi *et al*. reported that eupatilin dramatically inhibited $FeSO_4$-induced ROS production in a dose-dependent manner, as well as reduces the expression of such oxidative-responsible genes in H_2O_2-treated gastric epithelial cells [12]. Moreover, eupatilin has been demonstrated to possess neuro-protective effect. Recently, one study reported that eupatilin can decrease apoptotic neuronal death and reduce infarct volume in a focal cerebral ischemia/reperfusion mouse model [13]. However, the effects of eupatilin on oxygen-glucose deprivation/reperfusion (OGD/R)-induced cell injury in the model of PC-12 cells and further, the potential mechanisms involved have not been explored. Hence, this study was to investigate

the effects of eupatilin on oxygen and glucose deprivation/reperfusion (OGD/R) induced neuronal injury in differentiated PC12 cells.

Materials and methods

Cell culture and OGD/R treatment

PC12 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured with high glucose DMEM medium supplemented with 5% (v/v) heat-inactivated FBS, 5% (v/v) horse serum, 100 IU/mL streptomycin, 100 IU/mL penicillin in a humidified atmosphere with 5% CO_2 at 37°C. Cells were passaged every 3-4 days with 0.25% trypsin. Moreover, before exposed to injury, PC12 cells were differentiated by treating with nerve growth factor (NGF) (50 ng/mL) for 6 days to generate nerve-like cells and the growth medium was changed every other day.

To induce OGD/R injury, cell culture medium was removed and cells were washed twice with glucose-free DMEM (Invitrogen). Cells were then incubated in the glucose-free DMEM in an oxygen-free incubator (95% N_2 and 5% CO_2) for 4 h (OGD). Then, glucose was added back to the final concentration of 5 mg/ml and cells were incubated for additional 20 h (OGD/R) under normal conditions, in the absence or presence of eupatilin. Eupatilin was added to the cultures 30 min prior to the ischemic insult and was present during OGD and reoxygenation phases. Control groups were incubated in the glucose-free DMEM in a normoxic atmosphere for the same period.

Cell viability assay

Cell viability was determined by the CCK-8 assay (Beyotime, Shanghai, China). In brief, PC-12 cells at a density of 1×10^4 cells/well were seeded into 96-well plates and cultivated for 24 h to adhere. After treatment, 10 μL of kit reagent was added to the cells followed by incubation for 2 h. Absorbance was measured at 570 nm using an enzyme linked immunosorbent assay plate reader (Olympus, Tokyo, Japan).

LDH release assay

Cell membrane damage leading to cellular death was measured by the lactate dehydrogenase (LDH) assay [14]. In brief, PC-12 cells were treated with 0.5% Triton X-100, after being centrifuged; the supernatant was used for intracellular LDH level measurement by spectrophotometrical determination at 440 nm using a detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The equation LDH release (%) = (LDH level in the medium/total LDH level) × 100. Cultures under normal conditions (control group) represent basal LDH release.

Detection of ROS generation

Intracellular ROS was measured by flow cytometry using a cell-based ROS assay kit (Beyotime Biotechnology, Haimen, China). In brief, PC-12 cells were incubated with various concentrations of eupatilin for 4 h. Then, the cells were washed twice with PBS, and incubated with 10 μM dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C in the dark. ROS levels were measured as the fluorescence intensity of DCF by the FACSCaliber flow cytometer (BD Biosciences, CA, USA) with excitation and emission wavelengths of 488 and 525 nm. The measured fluorescence values were expressed as the fold changes relative to the control group.

Western blot

Proteins were extracted from PC-12 cells using RIPA lysis buffer. The concentration of protein was measured by BCA kit (Invitrogen, Carlsbad, CA, USA). A total of 30 μg of protein was fractionated by 12% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membranes were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline with tween (PBST), incubated with primary antibodies (anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-p-JNK, anti-JNK, anti-p-p38, anti-p38 and anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Subsequently, the membrane was incubated with secondary antibodies at room temperature for 2 h. An enhanced chemiluminescence system was applied according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

Data are expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA tests followed by Dunnett t test. A p

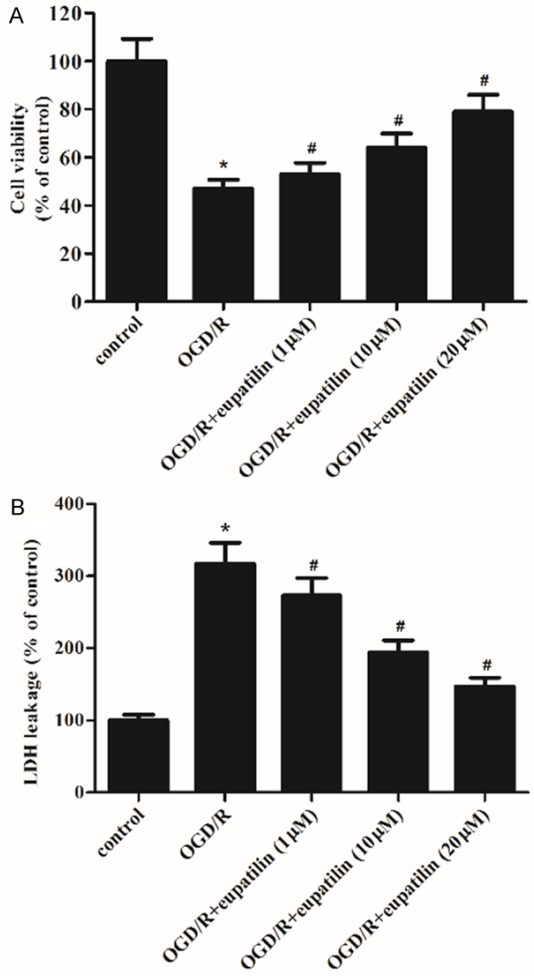

Figure 1. Eupatilin pretreatment inhibits OGD/R-induced cell viability loss in PC12 cells. PC12 cells were incubated for 4 h oxygen and glucose deprivation (OGD) followed by 20 h reperfusion with or without eupatilin. A. Cell viability was determined by CKK-8 assay. B. LDH leakage was detected with a LDH assay kit. Data are the mean ± SD from three independent experiments. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

value of less than 0.05 was considered to be significant.

Results

Eupatilin pretreatment inhibits OGD/R-induced cell viability loss in PC12 cells

To examine the neuroprotective effect of eupatilin against OGD/R induced injury, different concentrations of eupatilin were added to the culture medium 24 h during OGD/R. As shown in **Figure 1A**, the viability of the cells exposed to OGD/R was reduced significantly compared with the control. Treatment with eupatilin significantly attenuated OGD/R-induced cell death.

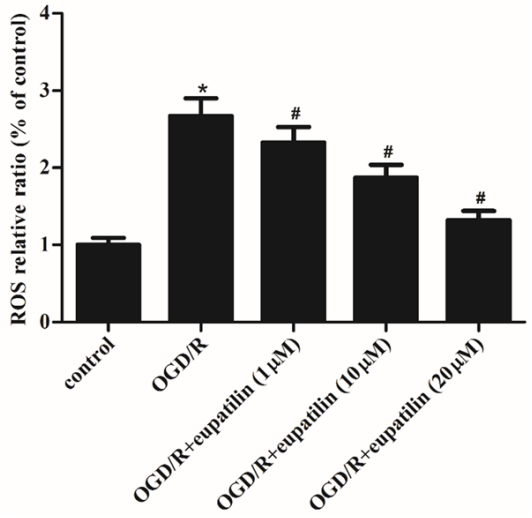

Figure 2. Eupatilin pretreatment inhibits OGD/R-induced intracellular ROS production in PC12 cells. PC12 cells were incubated for 4 h oxygen and glucose deprivation (OGD) followed by 20 h reperfusion with or without eupatilin. Intracellular ROS was detected using the 2',7'-dichlorofluorescein method. Data are the mean ± SD from three independent experiments. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

We also measured the effect of eupatilin on cell cytotoxicity caused by OGD/R by the LDH assay. As shown in **Figure 1B**, as compared with the control group, LDH release was significantly increased after OGD/R treatment. However, eupatilin attenuated OGD/R-induced LDH release in a dose-dependent manner.

Eupatilin pretreatment inhibits OGD/R-induced intracellular ROS production in PC12 cells

Then, we investigated the effects of eupatilin on the OGD/R-induced oxidative stress in PC-12 cells. As shown in **Figure 2**, OGD/R treatment significantly increased the production of intracellular ROS, while eupatilin (1, 10, and 20 μM) pretreatment decreased OGD/R-induced ROS production in a dose-dependent manner.

Eupatilin pretreatment inhibits OGD/R-induced cell apoptosis in PC12 cells

Thus, we measured the effect of eupatilin on the expression of Bcl-2 and Bax proteins in PC-12 cells induced OGD/R. As shown in **Figure 3A** and **3B**, OGD-R obviously reduced Bcl-2 expression and increased Bax expression com-

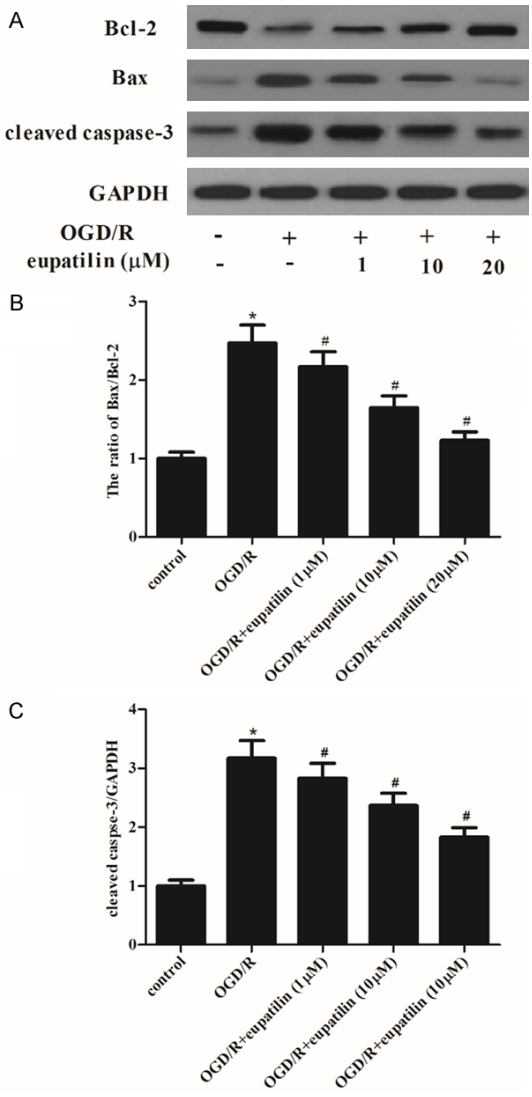

Figure 3. Eupatilin pretreatment inhibits OGD/R-induced cell apoptosis in PC12 cells. PC12 cells were incubated for 4 h oxygen and glucose deprivation (OGD) followed by 20 h reperfusion with or without eupatilin. A. The expression of Bcl-2, Bax, cleaved caspase-3, and GAPDH proteins was detected by western blot. B. The ratio of Bax/Bcl-2. C. Quantitative analysis of cleaved caspase-3 (fold of control). Data are the mean ± SD from three independent experiments. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

pared with the control cells. Treatment of PC12 with eupatilin was associated with greater Bcl-2 and attenuated Bax expression.

Next, we examined the effect of eupatilin on the OGD/R-induced activation of caspases. OGD/R treatment significantly increased the expression of cleaved caspase-3, however, in

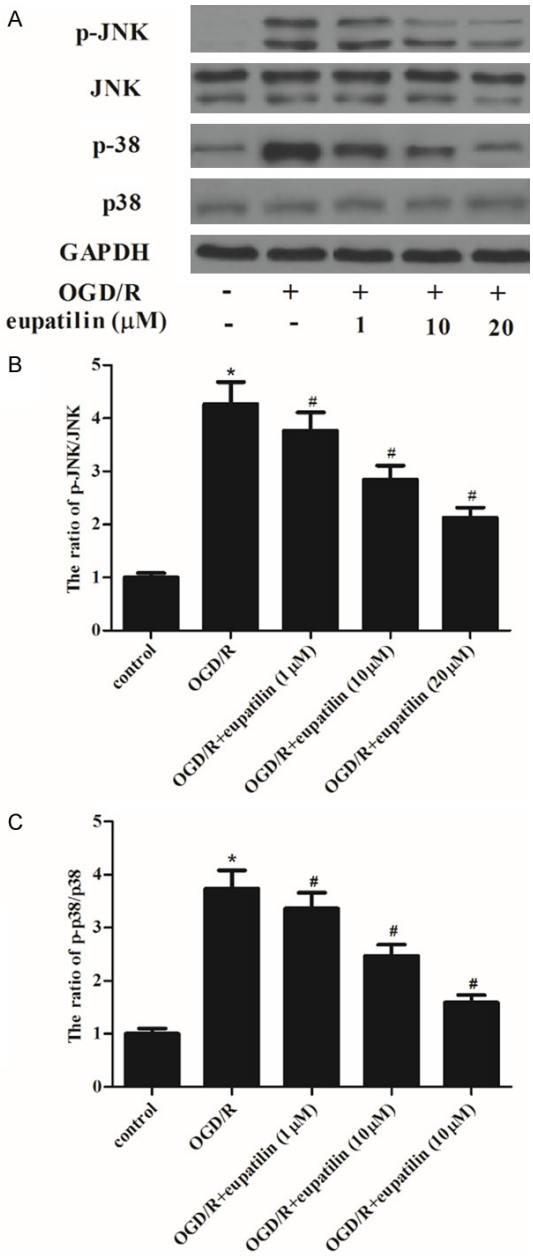

Figure 4. Eupatilin pretreatment inhibits OGD/R-induced activation of JNK and p38 signaling pathways in PC12 cells. PC12 cells were incubated for 4 h oxygen and glucose deprivation (OGD) followed by 20 h reperfusion with or without eupatilin. A. The expression of p-JNK, JNK, p-p38 and p38 proteins was detected by western blot. B. Quantitative analysis of p-JNK (fold of JNK). C. Quantitative analysis of p-p38 (fold of p38). Data are the mean ± SD from three independent experiments. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

the eupatilin-pretreated group; the expression of cleaved caspase-3 was down-regulated in a dose-dependent manner (**Figure 3A** and **3C**).

Eupatilin pretreatment inhibits OGD/R-induced activation of JNK and p38 signaling pathways in PC12 cells

JNK and p38 signaling pathways play important roles in regulation of apoptosis. Thus, we explored the molecular mechanism by which eupatilin rescued the PC12 cells from OGD/R induced neuronal apoptosis and oxidative stress, and measured the expression phospho-JNK and phospho-p38. As shown in **Figure 4**, the levels of phospho-JNK and phospho-p38 expression were enhanced by OGD/R pretreatment; however, eupatilin obviously inhibited OGD/R-induced activation of JNK and p38 in PC-12 cells.

Discussion

In the current study, we found that eupatilin pretreatment inhibited OGD/R-induced cell viability loss in PC12 cells, as well as the production of intracellular ROS. In addition, the down-regulation of Bcl-2, up-regulation of Bax and the consequent activation of caspase-3 induced by OGD/R were reversed by eupatilin. Furthermore, eupatilin obviously inhibited OGD/R-induced activation of JNK and p38 in PC-12 cells.

Previous studies demonstrated that eupatilin confer neuroprotective effects in transient middle cerebral artery occlusion mice model [13]. Consistent with the prior study, in the present study, we found that eupatilin pretreatment inhibits OGD/R-induced cell viability loss in PC12 cells.

Numerous studies have indicated that ROS plays a critical role in the early events of ischemia reperfusion injury [15-17]. Moreover, eupatilin has been demonstrated to possess antioxidant effect. One study showed that eupatilin inhibited tumor necrosis factor (TNF)-α-induced intracellular ROS accumulation in human breast epithelial (MCF-10A) cells [18]. In the present experiment, we found that OGD/R treatment significantly increased the production of intracellular ROS, while eupatilin pretreatment decreased OGD/R-induced ROS production in a dose-dependent manner. These results suggest that eupatilin protected PC12 cells against OGD/R by reducing the production of intracellular ROS.

Apoptosis is a physiological process which is regulated by a number of well-characterized genes and is important for during neuronal development and under pathological conditions including cerebral ischemia [19]. Bcl-2 protein plays an essential role to control the process of cell death through preventing various apoptosis signaling pathways. The Bax protein has a role in the release of a factor that stimulates apoptosis into the cytoplasm. Thus, the balance of the expressions of these proteins is important in the process of cell death [20]. In the present experiment, we observed that OGD-R obviously reduced Bcl-2 expression and increased Bax expression compared with the control cells. Treatment of PC12 with eupatilin was associated with greater Bcl-2 and attenuated Bax expression. In addition, caspase-3 also plays an important role in apoptotic mechanisms after ischemia [21-23]. Similarly, in our experiments, the results showed that eupatilin pretreatment inhibits OGD/R-induced the expression of cleaved caspase-3 in PC12 cells. These results suggest that eupatilin protected against OGD/R induced neurotoxicity, is probably associated with the inhibition of neuronal cell death.

c-Jun N-terminal kinase (JNK) is an important stress-responsive kinase that is activated by various forms of brain insults [24-26]. It was reported that JNK activity was obviously up-regulated in the brain after ischemia, and inhibition of JNK prevented ischemia-induced mitochondrial translocation of Bax and Bim, release of cytochrome c and Smac, and activation of caspase-9 and caspase-3 [27]. In addition, p38 was also involved in cerebral ischemia/reperfusion injury, and that inhibition of p38 activation could alter the outcome of ischemic brain injury in vitro and in vivo experimental models [28-30]. Similarly, in our experiments, the results demonstrated that the levels of phospho-JNK and phospho-p38 expression were increased significantly after OGD/R treatment; however, eupatilin obviously prevented this increase in phospho-JNK and phospho-p38 expression induced by OGD/R. On the basis of these data, we suggest that eupatilin inhibits OGD/R-induced neuronal injury via suppressing the JNK and p38 MAPK signaling pathways in PC12 cells.

In summary, the present work shows that eupatilin inhibits OGD/R-induced neuronal injury via suppressing the JNK and p38 MAPK signaling pathways in PC12 cells. These data suggested

that eupatilin treatment is a potential therapeutic approach for protecting against ischemia/reperfusion injury.

Disclosure of conflict of interest

None.

Address correspondence to: Wei Wu, Department of Neurosurgery, The First Hospital of Jilin University, Xinmin Street No. 71, Changchun 130021, Jilin, China. Tel: +86-0431-88782222; Fax: +86-0431-88782222; E-mail: wuwei_neuro@163.com

References

[1] Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. Lancet 2008; 371: 1612-1623.

[2] Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 2003; 4: 399-414.

[3] Lipton P. Ischemic cell death in brain neurons. Physiol Rev 1999; 79: 1431-1568.

[4] Nishizawa Y. Glutamate release and neuronal damage in ischemia. Life Sci 2001; 69: 369-381.

[5] Saito A, Maier CM, Narasimhan P, Nishi T, Song YS, Yu F, Liu J, Lee YS, Nito C, Kamada H. Oxidative stress and neuronal death/survival signaling in cerebral ischemia. Mol Neurobiol 2005; 31: 105-116.

[6] Rajesh M, Pan H, Mukhopadhyay P, Bátkai S, Osei-Hyiaman D, Haskó G, Liaudet L, Gao B, Pacher P. Pivotal Advance: Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis. J Leukocyte Biol 2007; 82: 1382-1389.

[7] Crack PJ, Taylor JM, Flentjar NJ, De Haan J, Hertzog P, Iannello RC, Kola I. Increased infarct size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to ischemia/reperfusion injury. J Neurochem 2001; 78: 1389-1399.

[8] Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cerebr Blood F Met 2001; 21: 2-14.

[9] Choi EJ, Lee S, Chae JR, Lee HS, Jun CD, Kim SH. Eupatilin inhibits lipopolysaccharide-induced expression of inflammatory mediators in macrophages. Life Sci 2011; 88: 1121-1126.

[10] Kim MJ, Kim DH, Na HK, Oh TY, Shin CY, Surh YJ. Eupatilin, a pharmacologically active flavone derived from Artemisia plants, induces apoptosis in human gastric cancer (AGS) cells. J Environ Pathol Tox 2005; 24: 261-9.

[11] Hwang KE, Choi YS, Choi JH, Kim HY, Kim HW, Lee MA, Chung HK, Kim CJ. The antioxidative properties of Ganghwayakssuk (Artemisia princeps Pamp.) extracts added to refrigerated raw chicken nugget batter against lipid oxidation. Korean J Food Sci An Re 2011; 31: 166-175.

[12] Choi EJ, Oh HM, Na BR, Ramesh T, Lee HJ, Choi CS, Choi SC, Oh TY, Choi SJ, Chae JR. Eupatilin protects gastric epithelial cells from oxidative damage and down-regulates genes responsible for the cellular oxidative stress. Pharm Res Dordr 2008; 25: 1355-1364.

[13] Cai M, Phan PT, Hong JG, Kim DH, Kim JM, Park SJ, Liu X, Han JE, Park H, Choi JW. The neuroprotective effect of eupatilin against ischemia/reperfusion-induced delayed neuronal damage in mice. Eur J Pharmacol 2012; 689: 104-110.

[14] Tabakman R, Jiang H, Levine RA, Kohen R, Lazarovici P. Apoptotic characteristics of cell death and the neuroprotective effect of homocarnosine on pheochromocytoma PC12 cells exposed to ischemia. J Neurosci Res 2004; 75: 499-507.

[15] Honda HM, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. Ann NY Acad Sci 2005; 1047: 248-258.

[16] Tompkins AJ, Burwell LS, Digerness SB, Zaragoza C, Holman WL, Brookes PS. Mitochondrial dysfunction in cardiac ischemia-reperfusion injury: ROS from complex I, without inhibition. BBA Mol Basis Dis 2006; 1762: 223-231.

[17] Kaminski KA, Bonda TA, Korecki J, Musial WJ. Oxidative stress and neutrophil activation-the two keystones of ischemia/reperfusion injury. Int J Cardiol 2002; 86: 41-59.

[18] Kim MJ, Kim DH, Na HK, Surh YJ. TNF-α induces expression of urokinase-type plasminogen activator and β-catenin activation through generation of ROS in human breast epithelial cells. Biocheml Pharmacol 2010; 80: 2092-2100.

[19] Nicholson D. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell death Differ 1999; 6: 1028-1042.

[20] Rossé T, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B, Borner C. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature 1998; 391: 496-499.

[21] Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. Stroke 2009; 40: e331-e339.

[22] Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 1998; 18: 3659-3668.

[23] Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA, Moskowitz MA. Caspase activation and neuroprotection in caspase-3-deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. Proc Natl Acad Sci U S A 2002; 99: 15188-15193.

[24] Yatsushige H, Ostrowski RP, Tsubokawa T, Colohan A, Zhang JH. Role of c-Jun N-terminal kinase in early brain injury after subarachnoid hemorrhage. J Neurosci Res 2007; 85: 1436-1448.
[25] Savage MJ, Lin YG, Ciallella JR, Flood DG, Scott RW. Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition. J Neurosci 2002; 22: 3376-3385.
[26] Gu Z, Jiang Q, Zhang G. Extracellular signal-regulated kinase and c-Jun N-terminal protein kinase in ischemic tolerance. Neuroreport 2001; 12: 3487-3491.
[27] Gao Y, Signore AP, Yin W, Cao G, Yin XM, Sun F, Luo Y, Graham SH, Chen J. Neuroprotection against focal ischemic brain injury by inhibition of c-Jun N-terminal kinase and attenuation of the mitochondrial apoptosis-signaling pathway. J Cerebr Blood F Met 2005; 25: 694-712.
[28] Qi LL, Fang SH, Shi WZ, Huang XQ, Zhang XY, Lu YB, Zhang WP, Wei EQ. CysLT 2 receptor-mediated AQP4 up-regulation is involved in ischemic-like injury through activation of ERK and p38 MAPK in rat astrocytes. Life Sci 2011; 88: 50-56.
[29] Hwang SG, Shim J, Choi EJ. CIIA negatively regulates neuronal cell death induced by oxygen-glucose deprivation and reoxygenation. Mol Cell Biochem 2014; 397: 139-146.
[30] Jiang M, Li J, Peng Q, Liu Y, Liu W, Luo C, Peng J, Li J, Yung KKL, Mo Z. Neuroprotective effects of bilobalide on cerebral ischemia and reperfusion injury are associated with inhibition of pro-inflammatory mediator production and down-regulation of JNK1/2 and p38 MAPK activation. J Neuroinflamm 2014; 11: 1-17.