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Original Article

Protective effect of catalpol on oxygen glucose deprivation neurons

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Abstract: Brain ischemia is one common cerebrovascular disease with unfavorable prognosis and higher morbidity and mortality rates. The re-perfusion injury after primary ischemia further aggravated the progression of cerebral ischemia. The development of effective drugs for protecting neurons during brain ischemia-reperfusion damage is thus of critical importance. With pluripotent bioactivities, the protective role of catalpol on oxygen glucose deprivation (OGD) neurons was studied. PC12 cells were prepared for OGD model, followed by the treatment using gradient concentrations of catalpol (0.1 mM, 1 mM and 2 mM). Cell proliferation was detected by MTT assay. The activity of lactate dehydrogenase (LDH), superoxide dismutase (SOD) and reactive oxygen species (ROS) was further tested. The level of caspase-3, tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β) was tested by enzyme linked immunosorbent assay (ELISA). Compared to control group, the survival of OGD model cells was decreased, accompanied with higher LDH and ROS levels and lower SOD level. The expression of inflammatory cytokine TNF-α and IL-1β were potentiated. Catalpol was found to facilitate cell survival, lower LDH, ROS, caspase-3, TNF-α and IL-1β expression while increase SOD activity ($P<0.05$) in a dose-dependent manner. Catalpol can inhibit apoptosis and inflammation via modulating oxidation homeostasis, and protect OGD injury neurons.

Keywords: Catalpol, oxygen glucose deprivation, apoptosis, brain ischemia

Introduction

Cerebrovascular disease is common worldwide [1] and is the most popular disease in China [2]. With the society advancement and life-style transition, both fast life rhythm and higher pressure lead to gradually increasing of cerebrovascular disease cases with younger ages [3, 4]. Cerebrovascular disease includes hemorrhage and ischemia types, the latter of which is more common in clinics [5]. Ischemic brain infarction is featured with higher incidence, worse prognosis and higher morbidity/mortality rates, thus severely affecting the life quality and causing major public health issues [6]. The clinical features of ischemia brain infarction mainly include lower cerebral circulation blood volume and commonly occur in those patients with coma and require cardio-pulmonary resuscitation. The consequent ischemia-reperfusion damage is critical for causing brain dysfunction [7]. Ischemia-reperfusion damage involves multiple steps, including energy depletion, excitatory amino acid toxicity, free radicals and inflammation [8, 9]. Previous studies have suggested the occurrence of body oxidation imbalance and production of reactive oxygen species (ROS) for leading to neuronal death during ischemia-reperfusion damage [10, 11]. Therefore the development of effective drugs for protecting neurons during brain ischemia-reperfusion damage is thus of critical importance.

Catalpol is one effective compound extracted from rehmanniae, and belongs to cyclic olefin glycosides compounds family [12]. Previous studies have suggested pluripotent bioactivities including anti-tumor, anti-fungal, anti-viral, treating Alzheimer's disease, inhibiting microvascular permeability and anti-inflammation [13]. Catalpol can also exert protective effects on H_2O_2-induced oxidative stress injury [14], but leaving its detailed mechanisms unclear. Therefore this study focused on the role of catalpol on oxygen glucose deprivation (OGD) treated neurons, along with the elucidation of molecular mechanisms, in an attempt to pro-

vide evidences for developing drugs for cerebral ischemia.

Materials and methods

Reagents

PC12 cell line was purchased from ATCC cell bank (US). Catalpol is obtained from China Pharmaceutical and Biological Products Identification Institute. DMEM medium, fetal bovine serum (FBS) and streptomycin-penicillin antibiotics were purchased from Hyclone (US). Polylysine, DMSO and MTT powders were provided by Gibco (US). Trypsin-EDTA lysis buffer was purchased from Sigma (US). Caspase-3 activity kit was produced from Pall Life Science (US). Enzyme linked immunosorbent assay (ELISA) kits for tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β) were purchased from R&D (US). RNA extraction kit and reverse transcription kit were produced by Axygen (US).

PC12 cell culture

PC12 cells were resuscitated in 37°C until complete thawing, and were centrifuged at 1 000 g for 3 min. Cells were then re-suspended in 1 mL fresh medium and were removed into 25-mL culture flask. Cells were cultured in a humidified chamber at 37°C with 5% CO_2 for 48 hours. Cells were then inoculated in polylysine-pretreated culture dish at 1×10^6 per cm^2 density. The culture medium consists of 90% high glucose DMEM (containing 100 U/mL penicillin and 100 μg/mL streptomycin) and 10% FBS. Cells at log-phase after 3~8 generations of passages were randomly divided into control, OGD model, low catalpol, moderate catalpol and high catalpol groups.

OGD model and drug treatment

Culture cells were changed for glucose-free DMEM medium and were incubated in anaerobic chamber (perfused with 95% N_2 and 5% CO_2) at 37°C for 12 hours. Then original high-glucose medium was applied along with oxygen supplied. In three drug treatment groups, catalpol (0.1 mM, 1 mM and 2 mM) was added 30 min before OGD treatment. After re-oxygenated, cells were also exposed at the same concentration of drugs for 24 hours.

MTT assay

PC12 cells at log-phase were seeded into 96-well plate at 3 000 cells per well. Different concentrations of catalpol (0.1 mM, 1 mM and 2 mM) were added into each well for 24-hour incubation. 20 μL MTT solution (5 g/L) was then added for further 4-hour incubation. After removing supernatants, 0.15 mL DMSO was added into each well for vortex until complete resolving of violet crystals. Absorbance (A) values at 570 nm was measured by a microplate reader to calculate cell proliferation rate. All experiments were performed in triplicates.

Lactate dehydrogenase (LDH) and superoxide dismutase (SOD) activity assay

Following instructions of SOD test kit, total cell proteins were firstly extracted and denatured at 95°C for 40 min. The lysate was then centrifuged at 4 000 g for 10 min and was eluted using ethanol-chloroform mixture (v/v 5:3) for testing LDH and total SOD activity.

ROS activity assay

To test the cellular level of ROS, cells after treatment were heated in 95°C for 40 min and cooled. Cells were then centrifuged at 4 000 g for 10 min. At 37°C, tissues were homogenized and incubated in 2',7'-dichlorofluorescein diacetate (DCF-DA) for 15 min. After centrifugation at 10 000 g for 15 min, supernatants were discarded to collect cell precipitation, which was re-suspended in sterilized PBS. After 37°C incubation for 60 min, spectrophotometer was used to quantify ROS levels, which were presented as ROS production percentage.

Caspase-3 activity assay

Using test kits, intracellular activity of caspase-3 was determined following manual instruction. In brief, cells were digested in trypsin, and were centrifuged at 600 g for 5 min. The supernatant was discarded followed by cell lysis buffer. The mixture was centrifuged at 20 000 g for 5 min. After adding 2 mM Ac-DECD-pNA, OD values at 405 nm was measured to reflect caspase-3 activity.

ELISA

Supernatants from all cells were collected to quantify the level of TNF-α and IL-1β using test kits following manual instruction. In brief, serially diluted standard samples were added into 96-well plate in triplicates. The plate was washed and removed for excess liquids. In

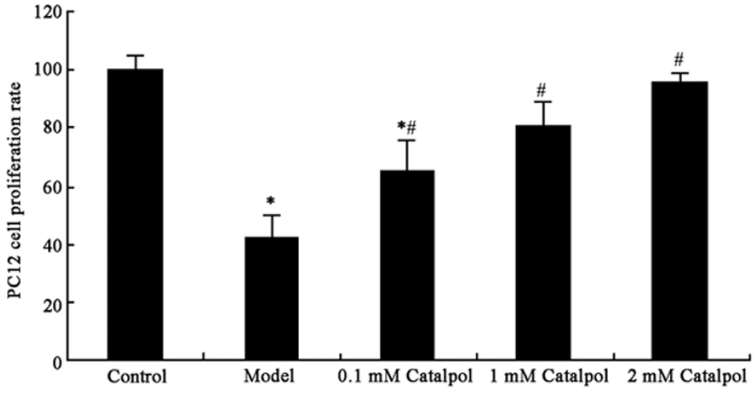

Figure 1. PC12 cell proliferation after catalpol treatment. *P<0.05 compared to control group; #P<0.05 compared to model group.

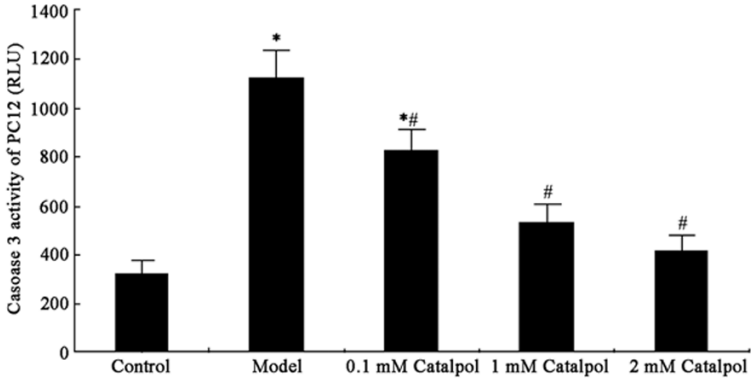

Figure 2. Caspase-3 activity in PC12 cells. *P<0.05 compared to control group; #P<0.05 compared to model group.

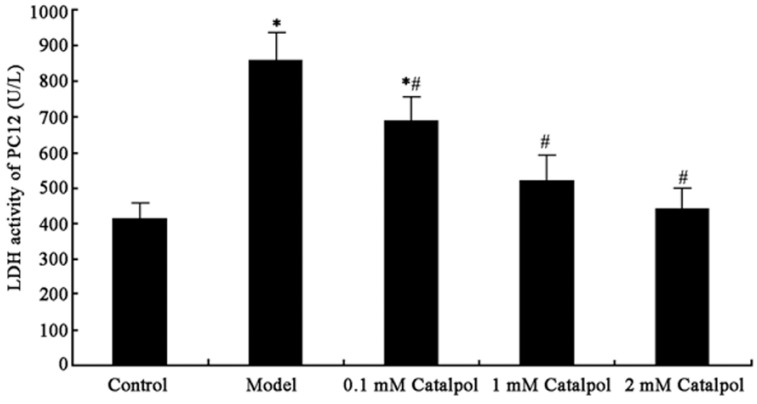

Figure 3. LDH activity in PC12 cells. *P<0.05 compared to control group; #P<0.05 compared to model group.

each well elution buffer was added to wash precipitations for five times. In each well, 50 μL enzyme labelled solutions were then added except blank control. The plate was then incubated at 37°C for 30 min. The plate was further washed and added with chromogenic substrate A and B (50 μL each). After development in dark at 37°C for 10 min, quenching buffer was added to stop the reaction. A microplate reader was used to measure OD values at 450 nm of each well within 15 min. A standard linear function is firstly plotted based on standard samples. The concentration of samples was then calculated from OD values.

Statistical analysis

SPSS 19.0 software was used to process all collected data, which were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare means across groups. A statistical significance was defined when P<0.05.

Results

Effect of catalpol on OGD neuron proliferation

MTT assay showed significantly depressed survival rate of neurons under OGD compared to control group (P<0.05, **Figure 1**). The pretreatment of catalpol on OGD neurons significantly facilitated the recovery of neuron proliferation in a dose-dependent manner (P<0.05, **Figure 1**). These results suggested certain protective effects of catalpol on OGD neurons by stimulating cell survival and proliferation.

Caspase-3 activity

Under OGD condition, caspase-3 activity in PC 12 cells was significantly enhanced compared to control cells (P<0.05, **Figure 2**). The introduction of catalpol significantly depressed cas-

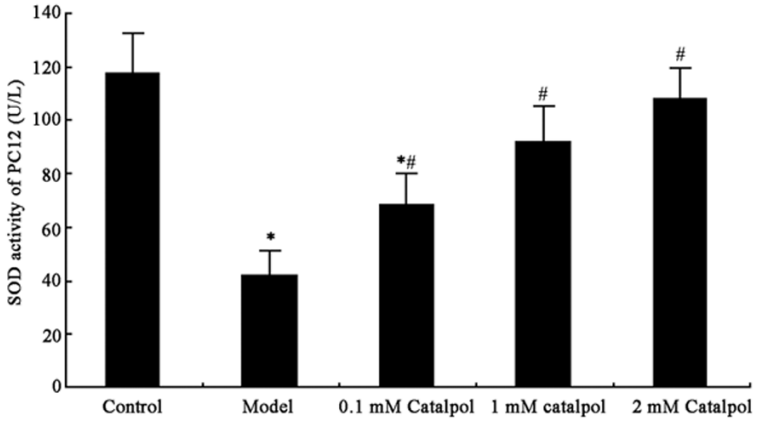

Figure 4. SOD activity in PC12 cells under OGD condition. *P<0.05 compared to control group; #P<0.05 compared to model group.

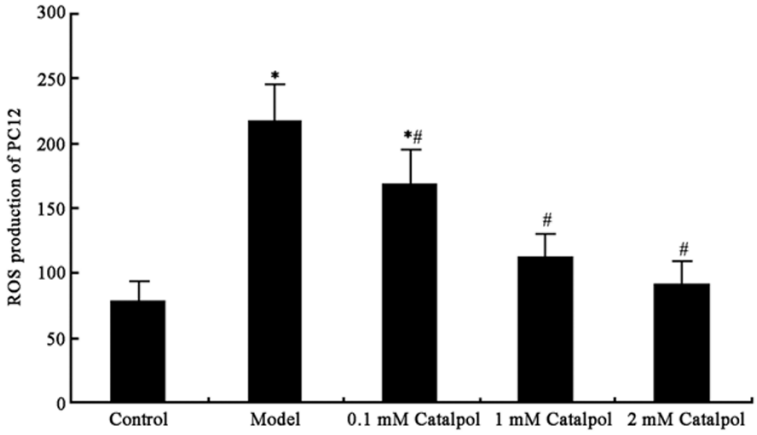

Figure 5. ROS production in PC12 cells under OGD condition. *P<0.05 compared to control group; #P<0.05 compared to model group.

pase-3 activity in OGD neurons compared to model cells in a dose-dependent manner (P<0.05, **Figure 2**). These results suggest the involvement of catalpol in regulating apoptotic proteins in protecting neurons from OGD injury.

LDH activity in OGD neurons

OGD condition caused significantly enhanced LDH activity in PC 12 cells compared to control cells (P<0.05, **Figure 3**). The pre-treatment of catalpol significantly depressed LDH activity in OGD neurons compared to model cells in a dose-dependent manner (P<0.05, **Figure 3**).

SOD activity in OGD neurons

As opposite to the condition of LDH, OGD treatment caused significantly depressed SOD activity in PC 12 cells compared to control cells (P<0.05, **Figure 4**). The pre-treatment of catalpol significantly enhanced SOD activity in OGD neurons compared to model cells in a dose-dependent manner (P<0.05, **Figure 4**).

Effect of catalpol on ROS productivity of OGD neurons

Under OGD condition, the productivity of ROS in PC 12 cells was significantly enhanced compared to control cells (P<0.05, **Figure 5**). The pre-treatment of catalpol significantly inhibited ROS level in OGD neurons compared to model cells in a dose-dependent manner (P<0.05, **Figure 5**).

Inflammatory cytokine level

Under OGD condition, the secretion of inflammatory cytokines including TNF-α and IL-1β in PC 12 cells was significantly increased compared to control cells (P<0.05, **Figure 5**). The intervention by catalpol significantly inhibited production level in OGD neurons compared to model cells in a dose-dependent manner (P<0.05, **Figures 6**, **7**).

Discussion

Under normal physiological condition, the production and clearance of free radicals are at homeostatic status. The pathological condition, however, may produce abundant free radicals. On the other hand, the lowering anti-oxidation ability may also induce the occurrence of oxidative stress. The cerebral ischemia-reperfusion damage is one important factor inducing the aggravation of brain ischemia infarction. Under the ischemia-reperfusion injury, body may have oxidative stress, which can break the oxidation/anti-oxidation system, leading to the production of various oxidative free radicals, such as ROS, superoxide anion, and hydroxyl

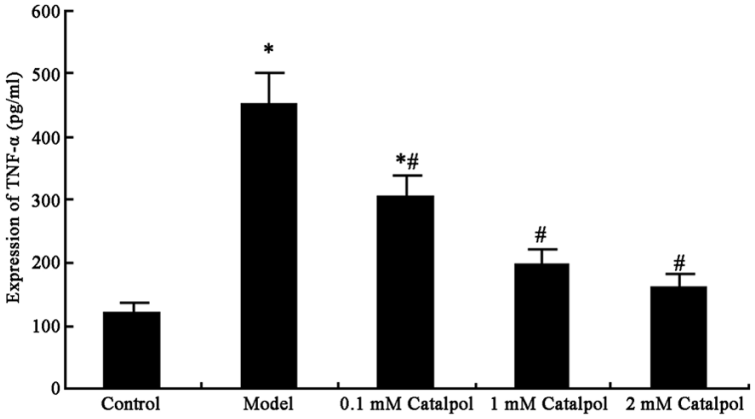

Figure 6. TNF-α level in PC12 cells under OGD condition. *P<0.05 compared to control group; #P<0.05 compared to model group.

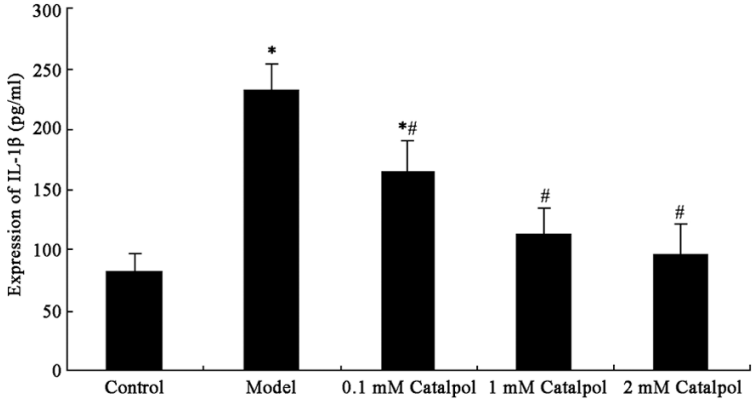

Figure 7. IL-1β levels in PC12 cells under OGD condition. *P<0.05 compared to control group; #P<0.05 compared to model group.

radicals. Those factors may cause the peroxidation of lipid membrane of neurons, leading to structural and functional deficits of DNA, further causing neuronal death [15, 16]. Such injured cells further release large amounts of inflammatory cytokines, further interrupting body's inflammation/anti-inflammation balance, causing a positive feedback loop for aggravation of ischemia-reperfusion damage [17, 18].

This study generated a cell model of OGD for mimicking cerebral ischemia-reperfusion damage, confirming the elevated secretion of ROS and inflammatory cytokines, along with depressed neuronal activity during the process of neuronal damage. In the oxidation/anti-oxidation system, SOD is one important anti-oxidation enzyme in body defense system, and plays a crucial role in the oxidation/anti-oxidation homeostasis. The activity of SOD directly reflects the ability of the body to clear oxygen free radicals [11]. Mainly existed in cytoplasm, LDH has a relatively stable expression level and cannot penetrate membrane under normal condition. When cells are damaged or undergone death process, however, LDH may be abundantly released through membrane [19]. Therefore LDH can work as one index for cell activity. This study illustrated the significantly lowered SOD activity in ischemia-reperfusion damage, which can also increase LDH activity, suggesting the disruption of oxidation/anti-oxidation balance, which leads to enhanced caspase-3 activity and lower survival rate of neurons. Extracted from traditional Chinese medicine, catalpol has multiple pharmaceutical functions including anti-inflammation, modulating oxidative-reductive homeostasis, and neural protection in disease such as Alzheimer's and Parkinson's [20]. The protective role and mechanism of catalpol in cerebral ischemia injury and ischemia-reperfusion damage, however, remains unclear. This study investigated the effect of catalpol regarding homeostatic control of oxidation/anti-oxidation balance, anti-inflammation, and anti-apoptosis on an OGD neuron cell model. Our results demonstrated the decrease of LDH and ROS contents, along with higher SOD activity by catalpol in a dose-dependent manner. Catalpol thus can facilitate the recovery of oxidation/anti-oxidation balance, inhibiting apoptosis via decreasing caspase-3 expression, and facilitating recovery of neurons by depressing inflammatory cytokines TNF-α and IL-1β, all of which can benefit the survival of neurons.

In summary, this study suggested the protective effect of catalpol on OGD neurons via mod-

ulating oxidation/anti-oxidation balance, inhibiting cell apoptosis and inflammation. This study provides further evidences and new insights for developing novel treatment plants for clinical management of brain ischemia infarction.

Disclosure of conflict of interest

None.

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References

[1] Bao C, Wang Y, Min H, Zhang M, Du X, Han R, Liu X. Combination of Ginsenoside Rg1 and Bone Marrow Mesenchymal Stem Cell Transplantation in the Treatment of Cerebral Ischemia Reperfusion Injury in Rats. Cell Physiol Biochem 2015; 37: 901-10.

[2] Cao Y, Wang F, Cheng Q, Jiao X, Lv X. Fasting blood glucose levels affect hospitalization time and relapse and mortality rates of cerebral infarction patients. Int J Clin Exp Med 2015; 8: 11508-13.

[3] Yang PS, Lin PY, Chang CC, Yu MC, Yen TL, Lan CC, Jayakumar T, Yang CH. Antrodia camphorata Potentiates Neuroprotection against Cerebral Ischemia in Rats via Downregulation of iNOS/HO-1/Bax and Activated Caspase-3 and Inhibition of Hydroxyl Radical Formation. Evid Based Complement Alternat Med 2015; 2015: 232789.

[4] Merante A, Gareri P, Castagna A, Marigliano NM, Candigliota M, Ferraro A, Ruotolo G. Transient cerebral ischemia in an elderly patient with patent foramen ovale and atrial septal aneurysm. Clin Interv Aging 2015; 10: 1445-9.

[5] Chen H, Li Z, Hong H, Xing S, Liu G, Zhang A, Tan S, Zhang J, Zeng J. Relationship between visible branch arteries distal to the stenosis on magnetic resonance angiography and stroke recurrence in patients with severe middle cerebral artery trunk stenosis: a one-year follow up study. BMC Neurol 2015; 15: 167.

[6] Li G, Tian Y, Zhang Y, Hong Y, Hao Y, Chen C, Wang P, Lei H. A Novel Ligustrazine Derivative T-VA Prevents Neurotoxicity in Differentiated PC12 Cells and Protects the Brain against Ischemia Injury in MCAO Rats. Int J Mol Sci 2015; 16: 21759-74.

[7] Ichijo M, Ishibashi S, Li F, Yui D, Miki K, Mizusawa H, Yokota T. Sphingosine-1-Phosphate Receptor-1 Selective Agonist Enhances Collateral Growth and Protects against Subsequent Stroke. PLoS One 2015; 10: e0138029.

[8] Kang BK, Kim MK, Kim SY, Lee SJ, Choi YW, Choi BT, Shin HK. Anti-Neuroinflammatory Effects of Uncaria sinensis in LPS-Stimulated BV2 Microglia Cells and Focal Cerebral Ischemic Mice. Am J Chin Med 2015; 43: 1099-115.

[9] Lin L, Chen H, Zhang Y, Lin W, Liu Y, Li T, Zeng Y, Chen J, Du H, Chen R, Tan Y, Liu N. IL-10 Protects Neurites in Oxygen-Glucose-Deprived Cortical Neurons through the PI3K/Akt Pathway. PLoS One 2015; 10: e0136959.

[10] Hedna VS, Ansari S, Shahjouei S, Cai PY, Ahmad AS, Mocco J, Qureshi AI. Validity of Laser Doppler Flowmetry in Predicting Outcome in Murine Intraluminal Middle Cerebral Artery Occlusion Stroke. J Vasc Interv Neurol 2015; 8: 74-82.

[11] Zhou M, Ren H, Han J, Wang W, Zheng Q, Wang D. Protective Effects of Kaempferol against Myocardial Ischemia/Reperfusion Injury in Isolated Rat Heart via Antioxidant Activity and Inhibition of Glycogen Synthase Kinase-3beta. Oxid Med Cell Longev 2015; 2015: 481405.

[12] Xue B, Ma B, Zhang Q, Li X, Zhu J, Liu M, Wu X, Wang C, Wu Z. Pharmacokinetics and tissue distribution of Aucubin, Ajugol and Catalpol in rats using a validated simultaneous LC-ESI-MS/MS assay. J Chromatogr B Analyt Technol Biomed Life Sci 2015; 1002: 245-53.

[13] Wang JM, Yang LH, Zhang YY, Niu CL, Cui Y, Feng WS, Wang GF. BDNF and COX-2 participate in anti-depressive mechanisms of catalpol in rats undergoing chronic unpredictable mild stress. Physiol Behav 2015; 151: 360-8.

[14] Xu Z, Zhang L, Li X, Jiang Z, Sun L, Zhao G, Zhou G, Zhang H, Shang J, Wang T. Mitochondrial fusion/fission process involved in the improvement of catalpol on high glucose-induced hepatic mitochondrial dysfunction. Acta Biochim Biophys Sin (Shanghai) 2015; 47: 730-40.

[15] Kim DW, Cho JH, Cho GS, Kim IH, Park JH, Ahn JH, Chen BH, Shin BN, Tae HJ, Hong S, Cho JH, Kim YM, Won MH, Lee JC. Hyperthermic preconditioning severely accelerates neuronal damage in the gerbil ischemic hippocampal dentate gyrus via decreasing SODs expressions. J Neurol Sci 2015; 358: 266-75.

[16] Silachev DN, Plotnikov EY, Zorova LD, Pevzner IB, Sumbatyan NV, Korshunova GA, Gulyaev MV, Pirogov YA, Skulachev VP, Zorov DB. Neuroprotective Effects of Mitochondria-Targeted Plastoquinone and Thymoquinone in a Rat Model of Brain Ischemia/Reperfusion Injury. Molecules 2015; 20: 14487-503.

[17] El-Sahar AE, Safar MM, Zaki HF, Attia AS, Ain-Shoka AA. Neuroprotective effects of pioglitazone against transient cerebral ischemic reperfusion injury in diabetic rats: Modulation of antioxidant, anti-inflammatory, and anti-apoptotic biomarkers. Pharmacol Rep 2015; 67: 901-6.

[18] Zhang W, Xing B, Yang L, Shi J, Zhou X. Icaritin Attenuates Myocardial Ischemia and Reperfusion Injury Via Anti-Inflammatory and Anti-Oxidative Stress Effects in Rats. Am J Chin Med 2015; 43: 1083-97.

[19] Yang SM, Liu J and Li CX. Intermedin protects against myocardial ischemia-reperfusion injury in hyperlipidemia rats. Genet Mol Res 2014; 13: 8309-19.

[20] Zhu J, Chen X, Wang H, Yan Q. Catalpol protects mice against renal ischemia/reperfusion injury via suppressing PI3K/Akt-eNOS signaling and inflammation. Int J Clin Exp Med 2015; 8: 2038-44.