Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Penehyclidine hydrochloride improves cognitive function of rats with brain injury *via* CAMP/CREB signaling pathway

Yuting Wang[1,#], Wen Zhao[1,#], Shuhui Zhu[2], Liqun Wei[1,*]

[1] *Department of Anesthesiology, Huashan Hospital, Fudan University, Shanghai, China*
[2] *Shanghai Medical College, Fudan University, Shanghai, China*

Article Info

Article history:

Received: January 19, 2024
Accepted: April 12, 2024
Published: May 31, 2024

Use your device to scan and read the article online

Abstract

This study explored the impact of penehyclidine hydrochloride on cognitive function in rats with brain injury. Sprague-Dawley rats (n=36) were randomly assigned to sham-operation, model, and penehyclidine hydrochloride groups. Rats in the sham-operation group underwent craniotomy, while the model and penehyclidine hydrochloride groups received brain injury models and interventions with normal saline and penehyclidine hydrochloride, respectively. Specimens were obtained two weeks post-intervention. Neurological deficits were evaluated using Zea-Longa scores, and memory was assessed with the Morris water maze test. ELISA determined brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) content. mRNA expressions of BDNF and NGF were assessed via qPCR, and phosphorylated CREB (p-CREB) protein expression was measured by Western blotting. Compared to the sham-operation group, both model and penehyclidine hydrochloride groups showed increased Zea-Longa scores. Escape latencies were longer and platform crossings were fewer in model and penehyclidine hydrochloride groups compared to the sham-operation group, but penehyclidine hydrochloride demonstrated a shorter latency and more platform crossings than the model group. BDNF and NGF content decreased in model and penehyclidine hydrochloride groups compared to the sham-operation group, with an increase in the penehyclidine hydrochloride group compared to the model group. mRNA expression levels declined in model and penehyclidine hydrochloride groups but were higher in the latter. p-CREB protein expression was lower in model and penehyclidine hydrochloride groups compared to the sham-operation group but higher in the penehyclidine hydrochloride group than the model group. Penehyclidine hydrochloride exhibited neuroprotective effects by upregulating the cAMP/CREB signaling pathway, improving cognitive function in rats with brain injury.

Keywords: Brain injury, Penehyclidine hydrochloride, cAMP/CREB signaling pathway, Cognitive function

1. Introduction

Brain injury is a prevalent and debilitating condition, characterized by severe trauma to brain tissue, leading to cognitive impairment, memory deterioration, dementia, anxiety, and apathy in affected individuals [1-3]. The rising incidence of injuries from events such as traffic accidents and high-altitude falls has contributed to an increasing morbidity rate associated with brain injury, posing significant threats to patients' work capacity, quality of life, and overall safety.

The pathology of brain injury encompasses intricate mechanisms and responses that significantly impact the central nervous system's progression and recovery following injury [4,5]. Ischemic and hypoxic changes, along with ischemia/reperfusion injury in brain tissues, constitute critical elements of this complex process. In the quest for effective management, various treatments have been employed, each with its strengths and limitations. Common treatments for brain injury include pharmacological interventions, such as anti-inflammatory drugs and neuroprotective agents, aimed at mitigating the inflammatory response and reducing secondary damage. Surgical interventions, such as decompressive craniectomy, may be implemented to alleviate intracranial pressure. Rehabilitation therapies, including physical, occupational, and speech therapy, play pivotal roles in facilitating recovery and restoring functionality. Additionally, supportive measures like nutritional support and psychological counseling contribute to holistic patient care. While these treatments have demonstrated varying degrees of efficacy, they are not without shortcomings. Pharmacological interventions may have limited success in addressing the multifaceted nature of brain injury pathology. Surgical interventions, while relieving intracranial pressure, may pose risks and complications. Rehabilitation therapies often face challenges in achieving optimal patient participation and adherence. Furthermore, the individualized nature of brain injury responses necessitates a tailored and comprehensive approach, which may be challenging to implement consistently.

* Corresponding author.
E-mail address: 1034710500@qq.com (L. Wei).
# These authors contributed equally
Doi: http://dx.doi.org/10.14715/cmb/2024.70.5.32

Penehyclidine hydrochloride, a commonly used selective anti-cholinergic drug in clinical practice, has demonstrated its potential to improve microcirculation, dilate capillaries, and protect brain tissues. However, the precise mechanisms underlying these effects remain unclear [6,7]. The cyclic adenosine monophosphate (cAMP)/cAMP-responsive element-binding protein (CREB) signaling pathway is intricately linked to brain tissue development, nervous system repair, and cognitive function. Therefore, this research aims to investigate the impact of penehyclidine hydrochloride on the cognitive function of rats with brain injury by modulating the cAMP/CREB signaling pathway.

Through this exploration, we seek to unravel the specific mechanisms by which penehyclidine hydrochloride influences cognitive function in the context of brain injury. The findings from this study may provide valuable insights into the development of therapeutic strategies aimed at enhancing cognitive recovery and quality of life for individuals affected by brain injury.

2. Materials and methods

2.1. Laboratory animals

A total of 36 SPF-grade laboratory Sprague-Dawley (SD) rats of either sex (1 month old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license No.: SCXK (Shanghai, China) 2014-0003]. Those rats were fed in the Laboratory Animal Center with normal diet and sterile filtered water every day under a 12/12 h light/dark cycle, conventional room temperature and humidity. This study was approved by the Animal Ethics Committee of Fudan University Animal Center.

2.2. Reagents and instruments

Penehyclidine hydrochloride was bought from Chengdu List Pharmaceutical Co., Ltd. (Chengdu, China), anti-phosphorylated (p)-CREB primary antibody and secondary antibody were provided by Abcam (Cambridge, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kit and quantitative polymerase chain reaction (qPCR)-related kits were offered by Vazyme (Nanjing, China). A light microscope was purchased from Leica (Wetzlar, Germany), a fluorescence qPCR instrument was bought from ABI (Applied Biosystems, Foster City, CA, USA), and a water maze was provided by the Chinese Academy of Sciences.

2.3. Grouping and intervention

The 36 SD rats of either sex were assigned to sham-operation group (n=12), model group (n=12) and penehyclidine hydrochloride group (n=12) using a random number table. The rat model of brain injury was established in penehyclidine hydrochloride group, and penehyclidine hydrochloride solution (0.01 mg/kg) was intraperitoneally injected daily after operation. The rats in model group were applied to prepare the model of brain injury and then intraperitoneally injected with an equal volume of normal saline every day after operation. In sham-operation group, however, only craniotomy was performed, and an equal volume of normal saline was injected intraperitoneally every day after operation. Specimens were obtained from each group at 2 weeks after operation.

2.4. Modeling methods

The rats were anesthetized with 3% pentobarbital sodium solution (5 mL/kg) injected intraperitoneally. After successful anesthesia, the head of the rat was depilated and disinfected, followed by skin preparation. Then the rats were fixed on an operation table, a longitudinal incision (about 3 cm) was made along the midline of scalp, and the periosteum was stripped. Next, a bone window was prepared at the middle of lambdoid suture and right anterior fontanelle, so as to expose the cerebral dura mater. Later, the cerebral dura mater in the bone window was hit by an impactor (height: 2 mm, velocity: 2 m/s, contact time: 80 s). Finally, the wound was sutured, and the rats were raised in separate cages.

2.5. Evaluation of neurological deficit of rats using Zea-Longa score

At 1 h after the last intervention, the neurological deficit of the rats was independently assessed by 2 researchers familiar with the Zea-Longa scoring rules according to the symptoms and manifestations of the rats.

2.6. Evaluation of rat memory through Morris water maze test

The rats were put into the water maze after the last intervention, where they were allowed to move freely. The escape latency and the times of crossing the platform in the water maze of the rats were recorded. After that, the platform in the water maze was withdrawn, and the rats were put into the water maze from any place. As the rats moved freely, the times of crossing the original platform and the residence time in the original quadrant were recorded within 70 s.

2.7. Specimen acquisition

The rats were anesthetized with 3% pentobarbital sodium solution (5 mL/kg) via intraperitoneal injection. Then the brain tissues were directly obtained from 6 randomly selected rats in each group, flushed with normal saline, put into Eppendorf (EP) tubes and stored at -80°C for later use. As for the remaining 6 rats in each group, the thoracic cavity was cut open to expose the heart, and 400 mL of 4% paraformaldehyde was perfused from the left atrial appendage. Subsequently, the brain tissues were taken out and immediately immersed with 4% paraformaldehyde solution, followed by fixation for 48 h.

2.8. Detection of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) content in brain tissues via ELISA

The freshly obtained brain tissues were mashed. Based on the instructions of the ELISA kit, samples were loaded, and standard substance, biotinylated antibody working solution and enzyme conjugate working solution were added. Finally, the plate was washed, and the tissues were detected using a microplate reader at 450 nm.

2.9. QPCR assay

The fresh brain tissues were added with ribonucleic acid (RNA) extraction reagent to extract the total RNA in the specimens. Next, the total RNA obtained was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit and a designed reaction system (20 μL) under the following conditions: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s and annealing at 62°C

Table 1. Primer sequences.

Name	Primer sequence
NGF	Forward: 5'TGTTTAAAAAGCGGCGACTCC3' Reverse: 5'TCCTTGCCCTTGATGTCTGTG3'
BDNF	Forward: 5'CTGTATCAAAAGGCCAACTGAA3' Reverse: 5'GTGTCTATCCTTATGAATCGCCA3'
GAPDH	Forward: 5'ACGGCAAGTTCAACGGCACAG3' Reverse: 5'GAAGACGCCAGTAGACTCCACGAC3'

for 30 s for 35 cycles. △Ct value was calculated first, and then the expression difference of target genes was calculated. The detailed primer sequences are shown in Table 1.

2.10. Western blotting assay

The lysis buffer was added into the cryopreserved brain tissues for 1 h of ice bath, then the tissues were centrifuged at 14,000 g for 10 min in a centrifuge, and the proteins were quantified using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Next, the absorbance and standard curve of the proteins were obtained through the microplate reader, based on which the protein concentration in tissues was calculated. Subsequently, the proteins in tissue specimens were subjected to denaturation and separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The position of the Marker proteins was observed, and the electrophoresis was stopped when the Marker proteins reached the bottom of glass plate in a straight line. Later, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and reacted with blocking buffer for 1.5 h. After that, anti-p-CREB primary antibody (1:1000) and secondary antibody (1:1000) were added in sequence. Finally, the image was fully developed with chemiluminescent reagent in the dark for 1 min after rinsing.

2.11. Statistical analysis

In this research, Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was adopted for statistical analysis. The measurement data were expressed as mean ± standard deviation. t-test was performed for the data meeting normal distribution and homogeneity of variance, corrected t-test was used for the data meeting normal distribution and heterogeneity of variance, and non-parametric test was conducted for the data not meeting normal distribution and homogeneity of variance. The rank sum test was utilized for the ranked data, and the enumeration data were examined by chi-square test.

3. Results

3.1. Zea-Longa score in each group

Compared with sham-operation group, model group and penehyclidine hydrochloride group had notably increased Zea-Longa scores, with statistically significant differences ($P<0.05$), while penehyclidine hydrochloride group exhibited a remarkably decreased Zea-Longa score in comparison with model group, and the difference was statistically significant ($P<0.05$) (Figure 1).

3.2. Water maze test results in each group

The model group and penehyclidine hydrochloride group displayed obviously longer escape latencies and markedly fewer times of crossing the original platform than sham-operation group, showing statistically significant differences ($P<0.05$). However, the escape latency was evidently shortened, but the times of crossing the original platform were increased distinctly in penehyclidine hydrochloride group in contrast model group, and there were statistically significant differences ($P<0.05$) (Figure 2).

3.3. Content of BDNF and NGF in brain tissues in each group detected via ELISA

As shown in Figure 3, the content of BDNF and NGF in brain tissues was reduced distinctly in model group and

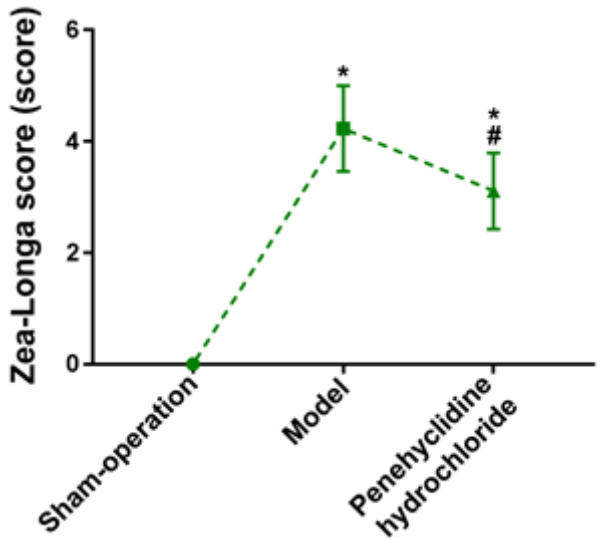

Fig. 1. Comparison of Zea-Longa score among groups Note: *P<0.05 vs. sham-operation group, #P<0.05 vs. model group.

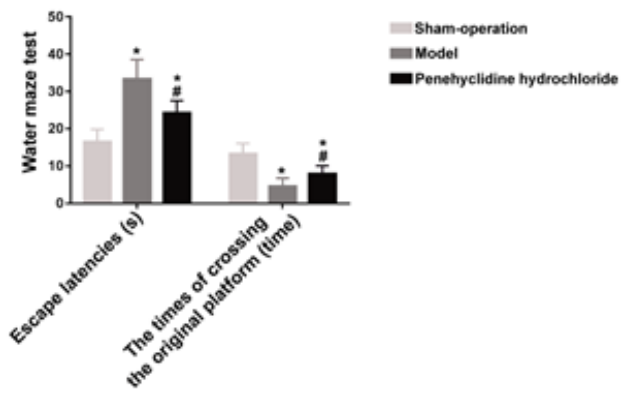

Fig. 2. Comparison of water maze test results among groups. Note: *P<0.05 vs. sham-operation group, #P<0.05 vs. model group.

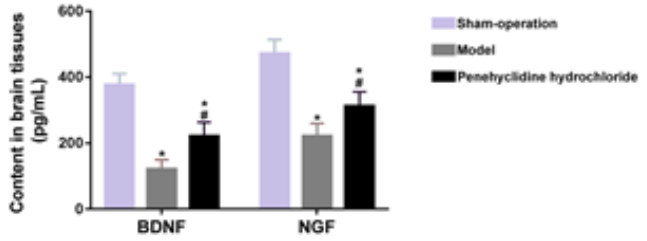

Fig. 3. Comparison of content of BDNF and NGF in brain tissues among groups. Note: *P<0.05 vs. sham-operation group, #P<0.05 vs. model group.

penehyclidine hydrochloride group compared with that in sham-operation group, and it was raised clearly in penehyclidine hydrochloride group in comparison with that in model group. All the differences were statistically significant (P<0.05).

3.4. Messenger RNA (mRNA) expressions of BDNF and NGF in brain tissues in each group determined through qPCR

The relative mRNA expression levels of BDNF and NGF in brain tissues declined prominently in model group and penehyclidine hydrochloride group in contrast with those in sham-operation group, and the differences were statistically significant (P<0.05). Furthermore, the opposite results were observed between penehyclidine hydrochloride group and model group, with statistically significant differences (P<0.05) (Figure 4).

3.5. Protein expression of p-CREB in brain tissues in each group measured by Western blotting

The protein expression of p-CREB was high in sham-operation group but low in model group (Figure 5A). According to the statistical results (Figure 5B), the relative protein expression level of p-CREB in brain tissues was apparently lower in model group and penehyclidine hydrochloride group than that in sham-operation group, displaying statistically significant differences (P<0.05), whereas it was remarkably higher in penehyclidine hydrochloride group than that in model group, and the difference was statistically significant (P<0.05).

4. Discussions

Brain injury is a common outcome of traumatic craniocerebral injury, in which trauma and violence usually lead to a series of pathological lesions in brain tissues, including hemorrhage, ischemia, hypoxia and ischemia/reperfusion injury, resulting in apoptosis, necrosis, autophagy and other pathological responses of brain neurons and damaging the normal physiological functions of the central nervous system. Studies have demonstrated that the greatest impact of brain injury on the physiological functions of the brain is to affect the cognitive function of patients so that the patients manifest memory deterioration, cognitive impairment, anxiety and apathy, which severely influence patients' work capacity and quality of life and even threaten their lives [8-10]. Therefore, it is essential to deeply investigate the related pathological mechanisms and responses after brain injury as well as therapeutic methods for the disease. It has been discovered through studies that neuronal damage (apoptosis, necrosis, autophagy, etc.) in the brain induced by brain injury is not only an important cause of affected brain functions but also a crucial pathological factor for cognitive impairment in patients [11,12]. Hence, how to protect the brain neurons after brain injury is one of the vital strategies to repair the nervous system and ameliorate cognitive impairment following brain injury. Both BDNF and NGF are pivotal neurotrophic factors in organisms, which exert important nutritional effects on the brain neurons. Specifically, they can promote the development and growth of the brain neurons, facilitate the synapse formation in the brain and maintain the normal physiological functions of neurons. According to research [13,14], BDNF and NGF are able to stimulate the repair and remyelination of neurons after brain injury, thus playing a vital neuroprotective role. As one of the crucial signaling pathways in the body, the cAMP/CREB signaling pathway have important functions in the development and generation of neurons, formation of synapses and maintenance of neurological functions. Studies have revealed that the high expression of cAMP in the signaling pathway can prominently promote the phosphorylation of its downstream CREB and play an essential regulatory role by increasing p-CREB [15-17]. Based on further research, BDNF and NGF are downstream effectors of the cAMP/CREB signaling pathway, and the activation of this signaling pathway exerts pivotal neuroprotective effects through enhancing the expressions of BDNF and NGF. It was found in this research that the protein expression of p-CREB, a key molecule of the cAMP/CREB signaling pathway, was decreased clearly in the brain tissues of rats with brain injury, suggesting that the phosphorylation level of CREB is lowered, and the cAMP/CREB signaling pathway is repressed. Meanwhile, the content of BDNF and NGF was reduced. Therefore, it is inferred that the cAMP/CREB signaling pathway is inhibited in the pathological process of brain injury, thereby affecting the expressions of BDNF and NGF, which may be an important pathologi-

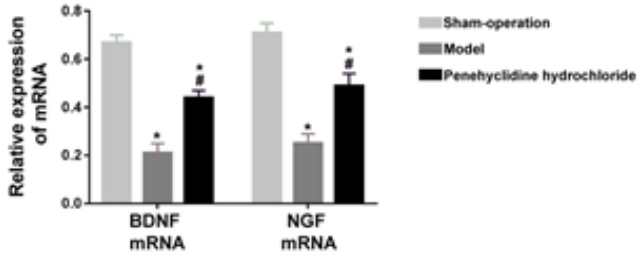

Fig. 4. Comparisons of mRNA expressions of BDNF and NGF in brain tissues among groups. Note: *P<0.05 vs. sham-operation group, #P<0.05 vs. model group.

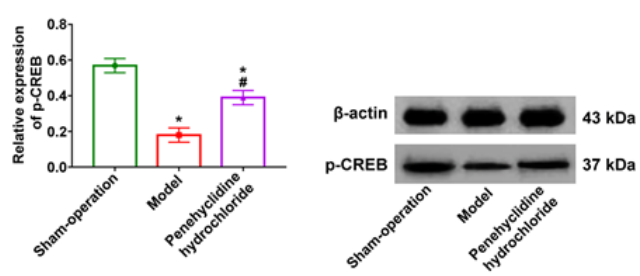

Fig. 5. Comparison of expressions of p-CREB in brain tissues among groups. Note: (A) statistical results of relative expression level of protein. (B) Protein expression bands. *P<0.05 vs. sham-operation group, #P<0.05 vs. model group.

cal cause of neurological deficit and memory impairment (cognitive impairment) in rats with brain injury.

It has been confirmed that penehyclidine hydrochloride, a novel selective anti-cholinergic drug, has favorable effects on microcirculation improvement, capillary dilatation and brain tissue protection. Studies have uncovered that penehyclidine hydrochloride is capable of alleviating and preventing brain injury to some extent, as well as ameliorating cognitive impairment following brain injury [18,19]. The results of this research also verified that penehyclidine hydrochloride could improve neurological deficit and memory impairment (cognitive impairment) in rats with brain injury. Besides, it facilitated the phosphorylation of CREB in the cAMP/CREB signaling pathway and increased the expressions of BDNF and NGF that have neuroprotective effects.

5. Conclusions

It can be seen that penehyclidine hydrochloride stimulates the expressions of BDNF and NGF by up-regulating the cAMP/CREB signaling pathway, thus exerting neuroprotective effects and improving the cognitive function of rats with brain injury.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Fudan University Animal Center.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Yuting Wang, Wen Zhao: Conceptualization, methodology, writing original draft preparation. Shuhui Zhu: Investigation, software, statistical analysis. Bin Du: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

Funding

None.

References

1. Rohlwink UK, Figaji AA (2014) Biomarkers of brain injury in cerebral infections. Clin Chem 60:823-834. doi: 10.1373/clinchem.2013.212472
2. Ma MW, Wang J, Zhang Q, Wang R, Dhandapani KM, Vadlamudi RK et al (2017) NADPH oxidase in brain injury and neurodegenerative disorders. Mol Neurodegener 12:7. doi: 10.1186/s13024-017-0150-7
3. Jin WN, Shi SX, Li Z, Li M, Wood K, Gonzales RJ et al (2017) Depletion of microglia exacerbates postischemic inflammation and brain injury. J Cerebr Blood F Met 37:2224-2236. doi: 10.1177/0271678X17694185
4. Wang JW, Qiu YR, Fu Y, Liu J, He ZJ, Huang ZT (2017) Transplantation with hypoxia-preconditioned mesenchymal stem cells suppresses brain injury caused by cardiac arrest-induced global cerebral ischemia in rats. J Neurosci Res 95:2059-2070. doi: 10.1002/jnr.24025
5. Mallard C, Tremblay ME, Vexler ZS (2019) Microglia and Neonatal Brain Injury. Neuroscience 405:68-76. doi: 10.1016/j.neuroscience.2018.01.023
6. Wang YP, Li G, Ma LL, Zheng Y, Zhang SD, Zhang HX et al (2014) Penehyclidine hydrochloride ameliorates renal ischemia-reperfusion injury in rats. J Surg Res 186:390-397. doi: 10.1016/j.jss.2013.07.041
7. Cao HJ, Sun YJ, Zhang TZ, Zhou J, Diao YG (2013) Penehyclidine hydrochloride attenuates the cerebral injury in a rat model of cardiopulmonary bypass. Can J Physiol Pharm 91:521-527. doi: 10.1139/cjpp-2012-0329
8. Nakhjavan-Shahraki B, Yousefifard M, Oraii A, Sarveazad A, Hosseini M (2017) Meta-analysis of neuron specific enolase in predicting pediatric brain injury outcomes. Excli J 16:995-1008. doi: 10.17179/excli2017-405
9. Lee ZI, Yu KJ, Lee DH, Hong SK, Woo SB, Kim JM et al (2017) The Effect of Nebulized Glycopyrrolate on Posterior Drooling in Patients with Brain Injury: Two Cases of Different Brain Lesions. Am J Phys Med Rehab 96:e155-e158. doi: 10.1097/PHM.0000000000000669
10. Huang L, Ma Q, Li Y, Li B, Zhang L (2018) Inhibition of microRNA-210 suppresses pro-inflammatory response and reduces acute brain injury of ischemic stroke in mice. Exp Neurol 300:41-50. doi: 10.1016/j.expneurol.2017.10.024
11. Kong X, Gong S, Su L, Li C, Kong Y (2017) Neuroprotective effects of allicin on ischemia-reperfusion brain injury. Oncotarget 8:104492-104507. doi: 10.18632/oncotarget.22355
12. Herz J, Koster C, Reinboth BS, Dzietko M, Hansen W, Sabir H et al (2018) Interaction between hypothermia and delayed mesenchymal stem cell therapy in neonatal hypoxic-ischemic brain injury. Brain Behav Immun 70:118-130. doi: 10.1016/j.bbi.2018.02.006
13. Wang YJ, Chen KY, Kuo LN, Wang WC, Hsu YW, Wong HS et al (2018) The association between BDNF Val66Met polymorphism and emotional symptoms after mild traumatic brain injury. Bmc Med Genet 19:13. doi: 10.1186/s12881-017-0518-0
14. Kheirouri S, Naghizadeh S, Alizadeh M (2019) Zinc supplementation does not influence serum levels of VEGF, BDNF, and NGF in diabetic retinopathy patients: a randomized controlled clinical trial. Nutr Neurosci 22:718-724. doi: 10.1080/1028415X.2018.1436236
15. Ko YH, Kwon SH, Hwang JY, Kim KI, Seo JY, Nguyen TL et al (2018) The Memory-Enhancing Effects of Liquiritigenin by Activation of NMDA Receptors and the CREB Signaling Pathway in Mice. Biomol Ther 26:109-114. doi: 10.4062/biomolther.2016.284
16. Jiang T, Wang XQ, Ding C, Du XL (2017) Genistein attenuates isoflurane-induced neurotoxicity and improves impaired spatial learning and memory by regulating cAMP/CREB and BDNF-TrkB-PI3K/Akt signaling. Korean J Physiol Pha 21:579-589. doi: 10.4196/kjpp.2017.21.6.579
17. Alexaki VI, Fodelianaki G, Neuwirth A, Mund C, Kourgiantaki A, Ieronimaki E et al (2018) DHEA inhibits acute microglia-mediated inflammation through activation of the TrkA-Akt1/2-CREB-Jmjd3 pathway. Mol Psychiatr 23:1410-1420. doi: 10.1038/mp.2017.167
18. Zhao W, Huang X, Zhang L, Yang X, Wang L, Chen Y et al (2016) Penehyclidine Hydrochloride Pretreatment Ameliorates Rhabdo-

myolysis-Induced AKI by Activating the Nrf2/HO-1 Pathway and Alleviating [corrected] Endoplasmic Reticulum Stress in Rats. The. Plos One 11:e0151158. doi: 10.1371/journal.pone.0151158

19. Feng M, Wang L, Chang S, Yuan P (2018) Penehyclidine hydrochloride regulates mitochondrial dynamics and apoptosis through p38MAPK and JNK signal pathways and provides cardioprotection in rats with myocardial ischemia-reperfusion injury. Eur J Pharm Sci 121:243-250. doi: 10.1016/j.ejps.2018.05.023