IN VIVO *39*: 742-748 (2025)
doi: 10.21873/invivo.13878

# Differential Expression of NRF2 in the Cortex and Hippocampus Following Bilateral Common Carotid Artery Occlusion

MIN SEON CHOI[1], HYO-IN HWANG[2], YOON YOUNG CHUNG[3], HYE-KYOUNG SHIN[3], DONG-JOON KIM[4] and YONG HYUN JUN[2]

[1]Department of Pediatrics, Chosun University Hospital, Gwang-ju, Republic of Korea;
[2]Institute of Well-Aging Medicare, Chosun University, Gwang-ju, Republic of Korea;
[3]Department of Anatomy, School of Medicine, Chosun University, Gwang-ju, Republic of Korea;
[4]Department of Anesthesiology and Pain Medicine, Chosun University Hospital, Gwang-ju, Republic of Korea

## Abstract

*Background/Aim:* Vascular dementia is the second most common cognitive disorder after Alzheimer's disease. Bilateral common carotid artery occlusion (BCCAO) is a widely used model of vascular dementia associated with chronic cerebral hypoperfusion. Previous studies have reported a beneficial role of nuclear factor erythroid 2-related factor 2 (NRF2) in BCCAO. This study aimed to investigate NRF2 expression in the cortex and hippocampus at 3 and 14 days after BCCAO.

*Materials and Methods:* Unoperated male Sprague–Dawley rats were assigned to the control group, while rats that underwent surgery were assigned to the BCCAO group. The right and left common carotid arteries were exposed beneath the esophagus, separated from the vagus nerve and occluded using 4-0 silk sutures. The cerebral cortex and hippocampus were isolated under anesthesia, 3 and 14 days post-surgery. The expression of NRF2 protein was evaluated using western blot analysis.

*Results:* NRF2 expression in the cerebral cortex increased 3 and 14 days after BCCAO, compared to control group. In the hippocampus, NRF2 expression of BCCAO group mice was increased at 3 days, but no difference was observed at day 14 compared to the control group.

*Conclusion:* Chronic hypoperfusion induced by BCCAO altered the protein expression levels of NRF2 in the cortex and hippocampus, suggesting that NRF2 may have a role in cognitive impairment.

**Keywords:** BCCAO, NRF2, cortex, hippocampus, vascular dementia.

Yong Hyun Jun, Department of Anatomy, School of Medicine, Chosun University, 375 Seosuk-dong, Dong-Gu, Gwangju 501-759, Republic of Korea. Tel: +82 1028862973, Fax: +82 0622341474, e-mail: jyh1483@chosun.ac.kr

Received November 25, 2024 | Revised December 17, 2024 | Accepted January 2, 2025

## Introduction

Vascular dementia (VD) is the second most common cognitive disorder after Alzheimer's disease (1). VD has diverse mechanisms, making it difficult to identify diagnostic criteria for its detection (2). Chronic cerebral hypoperfusion (CCH) closely contributes to the development and progression of vascular cognitive impairment, including VD (3). Increased production of reactive oxygen species (ROS) in CCH is a major contributor to the pathogenesis of vascular cognitive impairment (4). Oxidative stress leads to blood-brain barrier disruption, causing mitochondrial dysfunction, microglial activation, and inflammation (5). In CCH models, endogenous antioxidants decrease, disrupting homeostatic interactions (6).

Nuclear factor erythroid 2-related factor 2 (NRF2) regulates antioxidant enzymes (7) and maintains cellular redox homeostasis (8). Under homeostatic conditions, NRF2 remains within the cytoplasm in an inactive form attached to Kelch-like ECH-associated protein 1 (KEAP1) (9). Under oxidative stress, NRF2 is separated from Keap1 and binds to the antioxidant response element (ARE) (10). Cordaro *et al.* reported that NRF2 activation reduced neuroinflammation *via* NF-κB inactivation after bilateral common carotid artery occlusion (BCCAO) (11). BCCAO is one of the most common models of vascular dementia associated with CCH (12). In the BCCAO model, increased NRF2 expression reduced neuronal cell death *via* autophagy regulation (13). However, the expression levels of NRF2 after BCCAO have not been well investigated.

In a previous study, we reported that the number of mature neurons in the hippocampus after 14 days after BCCAO was lower than control group (14). In this study, we sought to investigate the effect of BCCAO on NRF2 expression in the cortex and hippocampus after BCCAO.

## Materials and Methods

*Experimental design.* Six- to seven- week-old male Sprague–Dawley (SD) rats were obtained from Damul Laboratory Animals (Daejeon, Republic of Korea). The rats were housed in a controlled environment at a constant temperature of 25˚C and were fed ad libitum. All animal procedures were approved by the Chosun University Institutional Animal Care and Use Committee.

*Animal surgery.* Bilateral common carotid artery occlusion was performed as described in previous studies (15). Rats were anesthetized using inhalation sevoflurane (1.0-2.0%, end-tidal concentration). After shaving the surgical area, the neck region was exposed. The right and left common carotid arteries were located beneath the esophagus. Both arteries were separated from the vagus nerve and occluded using 4-0 silk sutures. After surgery, the rats were housed under aseptic conditions. The surgery was performed on 46 rats (BCCAO group) and 57 unoperated rats were assigned to the control group.

*Western blot analysis.* The cerebral cortex and hippocampus of rats were isolated under anesthesia, at days 3 and 14 post-surgery. The cortex and hippocampal tissues were homogenized with RIPA buffer (50 mM Tris pH8.0, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS) containing 1 tablet of protease inhibitor cocktail (1183617001, Roche, Basel, Switzerland) in 10 ml of lysis buffer. Total protein was measured by Bradford method (#5000006, Bio-Rad, Hercules, CA, USA), and 30 μg of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). Following incubation with the primary antibodies anti- NRF2 (1:2,000; NBP1-32822, Novus, Centennial, CO, USA) and anti-actin-beta (1:2,000, 66009-1-Ig, Proteintech, Rosemont, IL, USA) in TBS containing 0.05% Tween-20 and 5% skim-milk, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5,000, 111-035-003, Jackson ImmunoResearch, Pennsylvania, PA, USA) and HRP-conjugated goat anti-mouse secondary antibody (1:5,000, 115-035-003, Jackson Immuno Research).

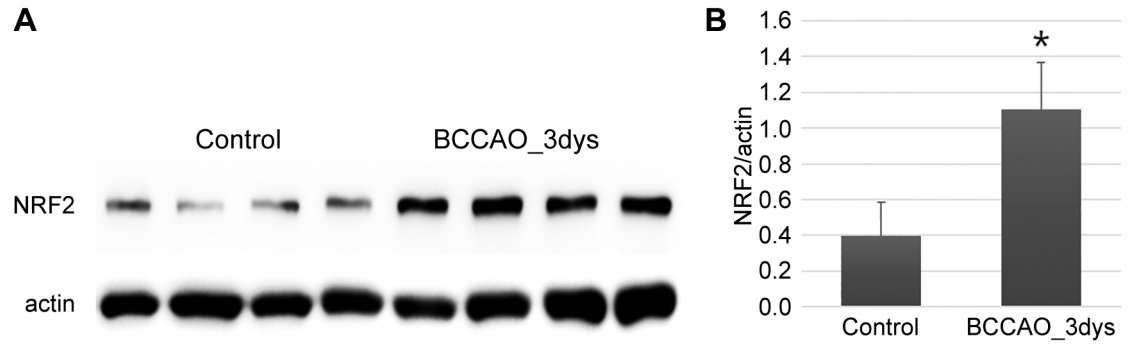


Figure 1. *Nrf2 expression in the parietal cortex 3 days after surgery. The expression of Nrf2 protein in the parietal cortex was measured by western blot 3 days post-surgery (A). The results were quantified and presented normalized to actin-beta expression (B). BCCAO: Bilateral common carotid artery occlusion. *p<0.05.*

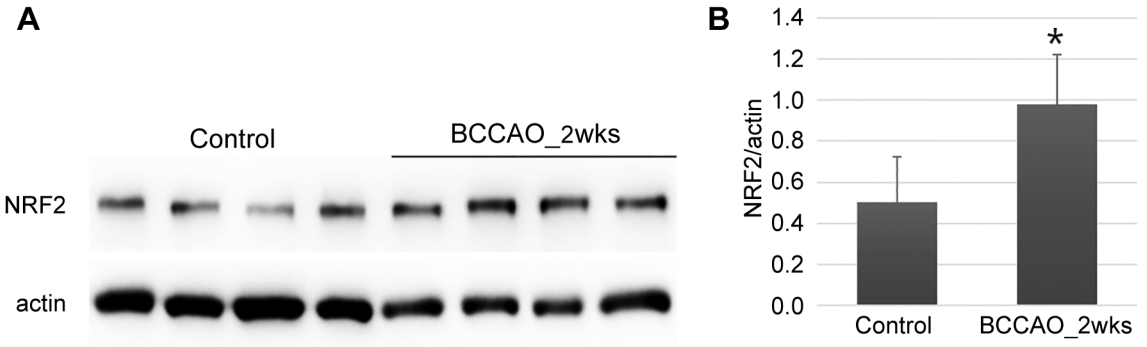


Figure 2. *Nrf2 expression in the parietal cortex 14 days after surgery. The expression of Nrf2 protein in the parietal cortex was measured by western blot 14 days post-surgery (A). The results were quantified and presented normalized to actin-beta expression (B). BCCAO: Bilateral common carotid artery occlusion. *p<0.05.*

Protein signals were visualized using the Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100, Millipore, Burlington, MA, USA). Image J software was used to quantify the signal, actin-beta was used as internal control and the NRF2 protein levels were normalized to actin-beta expression.

*Statistical analysis.* All data are reported as the mean±standard error of the mean (SEM). Two-group comparisons were analyzed using the Student's *t*-test. All analyses were carried out with the Statistical Package for Social Sciences (Information Analysis Systems, SPSS, IBM, Armonk, NY, USA). A *p*-value of <0.05 was considered statistically significant.

## Results

*Cortex findings.* The expression of NRF2 protein was examined in the parietal cortex of rats. NRF2 protein levels were significantly higher in the BCCAO group than in the control group 3 days post-surgery (Figure 1). Two weeks post-surgery, the expression of NRF2 was still increased compared to the control group, similar to the pattern observed at 3 days post-surgery (Figure 2).

*Hippocampus findings.* Three days post-surgery, the expression of NRF2 protein in the BCCAO group was increased, compared to the control group, similar to the cortex findings (Figure 3). However, 2 weeks post-surgery,

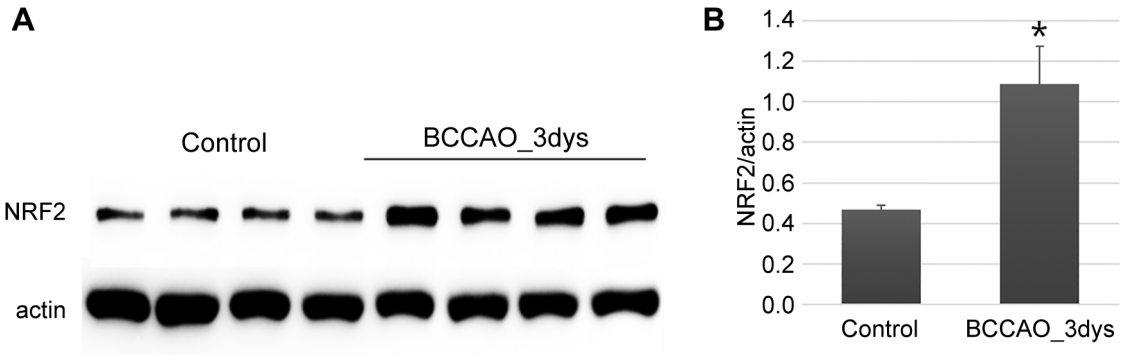


Figure 3. *Nrf2 expression in hippocampus 3 days after surgery. Representative western blot images (A) and quantification of Nrf2 expression (B) in hippocampus 3 days post-surgery. The results were normalized to actin-beta expression. BCCAO: Bilateral common carotid artery occlusion. *p<0.05.*

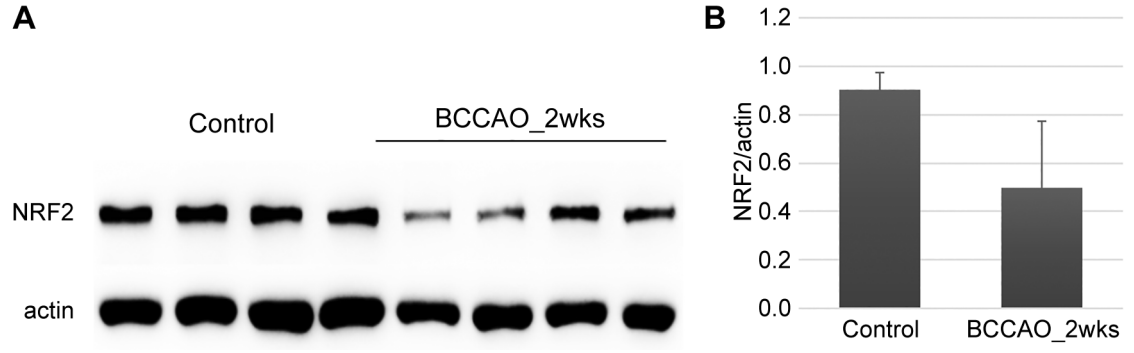


Figure 4. *Nrf2 expression in hippocampus 14 days after surgery. Representative western blot images (A) and quantification of Nrf2 expression (B) in hippocampus 14 days post-surgery. The results were normalized to actin-beta expression. BCCAO: Bilateral common carotid artery occlusion. *p<0.05.*

there was no significant difference between the BCCAO and control groups in hippocampal NRF2 (Figure 4).

## Discussion

Chronic cerebral hypoperfusion leads to neuronal damage and cognitive impairment (16, 17). We measured the protein levels of NRF2 at 3 and 14 days after inducing chronic hypoperfusion. Otori *et al.*, reported that relative cerebral blood flow (rCBF) was significantly reduced to 33–58% in the cortex, 2 days after BCCAO (18). Tae-Kyeong *et al.*, demonstrated that pyramidal cell death occurred 2 days after BCCAO (19). One study reported that cerebral blood flow was reduced at 3 days and this reduction was sustained until 14 days after BCCAO (20). Interestingly, Frantellizzi *et al.* noted that the reduction in blood flow differed between the cortex and hippocampus after BCCAO (21).

NRF2 is known to have an important role in defense against ischemia injury. Li *et al.* suggested that induced NRF2 expression in the ischemic cortex was related to the response to ischemic injury (22). Another study found upregulation of NRF2 at the mRNA level in ischemic cerebral cortex (23), while the NRF2/ARE pathway has been shown to be activated in astrocytes in oxygen-glucose deprivation conditions (24). Zhou *et al.* proposed that silent information regulator 1 (SIRT1) was involved in NRF2 pathway activation following oxidative stress injury (25). Cerebral ischemia induces ROS production, calcium overload, and neuronal inflammation (26, 27). In

the present study, NRF2 expression increased in the cortex at both 3 and 14 days after BCCAO. Wang reported that neuronal damage in cerebral cortex was greatest 3 days after BCCAO, in line with our findings (28). Our results suggest that the increase of NRF2 after BCCAO in the cortex could initiate the endogenous defense mechanism against oxidative stress.

Contrary to the cortex results, in the hippocampus, we found elevated expression of NRF2 at 3 days, but not at 14 days after BCCAO. In a previous study, the survival rate of rats decreased to 50% after 7 days BCCAO (14). Minghua *et al.*, reported that blood flow reduction in the hippocampus persisted up to 21 days after BCCAO (29). Iadecola noted that hippocampus blood flow differs from cortical flow due to the "outside in" pattern of intracerebral circulation (30). Activation of the ERK/NRF2 signaling pathway in the hippocampus is associated with the activation of the endogenous antioxidant defense system (31). Yadav *et al.* suggested that significant reduction in NRF2 expression levels led to the generation of ROS in mitochondria and protein carbonyls (32). Another study showed that the downregulation of NRF2 signaling was associated with dysfunction of antioxidant enzymes (33). Yang *et al.* observed compensatory NRF2 synthesis in the hippocampus of rats (34). However, some studies have shown that reduced NRF2 expression is associated with cognitive dysfunction after 14 days, and that endogenous compensation of NRF2 expression in the hippocampus is insufficient (34, 35).

Although we examined the expression level of NRF2 protein after BCCAO using a large number of animals, this study did not include any investigation of the mRNA levels of NRF2. More experiments are warranted to show changes in mRNA and protein expression of NRF2.

## Conclusion

NRF2 protein levels were increased in the hippocampus of rats at 3 days following BCCAO; however, this effect did not persist up to 14 days after BCCAO. These findings suggest that chronic hypoperfusion induced by BCCAO affects the expression of NRF2, thus NRF2 may be implicated in cognitive impairment related to CCH.

## Conflicts of Interest

The Authors reported that they have no competing interests.

## Authors' Contributions

YHJ and MSC designed the study. DJK and HKS, YYC participated in surgical procedures. HIH and MSC analyzed the data. MSC performed western analyses. All Authors approved the final article.

## Acknowledgements

This study was supported by research funds from Chosun University (2019).

## References

1 Hobert MA, Hofmann W, Bartsch T, Peters S, Maetzler W: [Diagnosis and treatment of vascular dementia]. Z Gerontol Geriatr 53(7): 687-698, 2020. DOI: 10.1007/s00391-020-01786-3

2 O'Brien JT, Thomas A: Vascular dementia. Lancet 386(10004): 1698-1706, 2015. DOI: 10.1016/s0140-6736(15)00463-8

3 Lin MS, Chiu MJ, Wu YW, Huang CC, Chao CC, Chen YH, Lin HJ, Li HY, Chen YF, Lin LC, Liu YB, Chao CL, Tseng WY, Chen MF, Kao HL: Neurocognitive improvement after carotid artery stenting in patients with chronic internal carotid artery occlusion and cerebral ischemia. Stroke 42(10): 2850-2854, 2011. DOI: 10.1161/strokeaha.111.613133

4 Li N, Chen H, Wang J: DNA damage and repair in the hematopoietic system. Acta Biochim Biophys Sin (Shanghai) 54(6): 847-857, 2022. DOI: 10.3724/abbs.2022053

5 Sandoval KE, Witt KA: Blood-brain barrier tight junction permeability and ischemic stroke. Neurobiol Dis 32(2): 200-219, 2008. DOI: 10.1016/j.nbd.2008.08.005

6 Du SQ, Wang XR, Xiao LY, Tu JF, Zhu W, He T, Liu CZ: Molecular mechanisms of vascular dementia: what can be learned from animal models of chronic cerebral hypoperfusion? Mol Neurobiol 54(5): 3670-3682, 2017. DOI: 10.1007/s12035-016-9915-1

7 Zhang L, Lang F, Feng J, Wang J: Review of the therapeutic potential of Forsythiae Fructus on the central nervous system: Active ingredients and mechanisms of action. J Ethnopharmacol 319(Pt 2): 117275, 2024. DOI: 10.1016/j.jep.2023.117275

8 Ariffianto A, Deng L, Abe T, Matsui C, Ito M, Ryo A, Aly HH, Watashi K, Suzuki T, Mizokami M, Matsuura Y, Shoji I: Oxidative stress sensor Keap1 recognizes HBx protein to activate the Nrf2/ARE signaling pathway, thereby inhibiting hepatitis B virus replication. J Virol 97(10): e0128723, 2023. DOI: 10.1128/jvi.01287-23

9 Farina M, Vieira LE, Buttari B, Profumo E, Saso L: The Nrf2 pathway in ischemic stroke: a review. Molecules 26(16): 5001, 2021. DOI: 10.3390/molecules26165001

10 Yun HR, Jo YH, Kim J, Shin Y, Kim SS, Choi TG: Roles of autophagy in oxidative stress. Int J Mol Sci 21(9): 3289, 2020. DOI: 10.3390/ijms21093289

11 Cordaro M, D'Amico R, Fusco R, Peritore AF, Genovese T, Interdonato L, Franco G, Arangia A, Gugliandolo E, Crupi R, Siracusa R, Di Paola R, Cuzzocrea S, Impellizzeri D: Discovering the effects of fisetin on NF-κB/NLRP-3/NRF-2 molecular pathways in a mouse model of vascular dementia induced by repeated bilateral carotid occlusion. Biomedicines 10(6): 1448, 2022. DOI: 10.3390/biomedicines10061448

12 Thangwong P, Jearjaroen P, Tocharus C, Govitrapong P, Tocharus J: Melatonin suppresses inflammation and blood–brain barrier disruption in rats with vascular dementia possibly by activating the SIRT1/PGC-1α/PPARγ signaling pathway. Inflammopharmacology 31(3): 1481-1493, 2023. DOI: 10.1007/s10787-023-01181-5

13 Impellizzeri D, D'Amico R, Fusco R, Genovese T, Peritore AF, Gugliandolo E, Crupi R, Interdonato L, Di Paola D, Di Paola R, Cuzzocrea S, Siracusa R, Cordaro M: Açai berry mitigates vascular dementia-induced neuropathological alterations modulating Nrf-2/Beclin1 pathways. Cells 11(16): 2616, 2022. DOI: 10.3390/cells11162616

14 Kim DJ, Lee MJ, Cho HB, Jo GH, Hwang HI, Shin HK, Chung YY, Jun YH: Differential expression of Pax6 following bilateral common carotid artery occlusion. In Vivo 37(2): 655-660, 2023. DOI: 10.21873/invivo.13125

15 Kim D, Shin Y, Cho S, Kim H, Hwang H, Shin H, Chung Y, Jun YH: Dexmedetomidine stereotaxic injection alleviates neuronal loss following bilateral common carotid artery occlusion *via* up-regulation of BDNF expression. In Vivo 38(1): 184-189, 2024. DOI: 10.21873/invivo.13424

16 Yoshioka M, Takahashi M, Kershaw J, Handa M, Takada A, Takuwa H: Two-photon optogenetics-based assessment of neuronal connectivity in healthy and chronic hypoperfusion mice. Neurophotonics 11(3): 035009, 2024. DOI: 10.1117/1.NPh.11.3.035009

17 Wang D, Zhang X, Huang Z, Li Y, Wang X, Wang J, Zhao Y, Lv Q, Wu M, Zha M, Yuan K, Zhu W, Xu G, Xie Y: Theta-burst transcranial magnetic stimulation attenuates chronic ischemic demyelination and vascular cognitive impairment in mice. Exp Neurol 383: 115022, 2025. DOI: 10.1016/j.expneurol.2024.115022

18 Otori T, Katsumata T, Muramatsu H, Kashiwagi F, Katayama Y, Terashi A: Long-term measurement of cerebral blood flow and metabolism in a rat chronic hypoperfusion model. Clin Exp Pharma Physio 30(4): 266-272, 2003. DOI: 10.1046/j.1440-1681.2003.03825.x

19 Lee TK, Kim H, Song M, Lee JC, Park JH, Ahn JH, Yang GE, Kim H, Ohk TG, Shin MC, Cho JH, Won MH: Time-course pattern of neuronal loss and gliosis in gerbil hippocampi following mild, severe, or lethal transient global cerebral ischemia. Neural Regen Res 14(8): 1394-1403, 2019. DOI: 10.4103/1673-5374.253524

20 Leahy S, Farzad S, Blair NP, Shahidi M: Retinal oxygen delivery, metabolism, and extraction fraction during long-term bilateral common carotid artery occlusion in rats. Sci Rep 10(1): 10371, 2020. DOI: 10.1038/s41598-020-67255-4

21 Frantellizzi V, Pani A, Ricci M, Locuratolo N, Fattapposta F, De Vincentis G: Neuroimaging in vascular cognitive impairment and dementia: a systematic review. J Alzheimers Dis 73(4): 1279-1294, 2020. DOI: 10.3233/jad-191046

22 Li M, Zhang X, Cui L, Yang R, Wang L, Liu L, Du W: The neuroprotection of oxymatrine in cerebral ischemia/reperfusion is related to nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response: role of Nrf2 and hemeoxygenase-1 expression. Biol Pharm Bull 34(5): 595-601, 2011. DOI: 10.1248/bpb.34.595

23 Liu L, Vollmer MK, Ahmad AS, Fernandez VM, Kim H, Doré S: Pretreatment with Korean red ginseng or dimethyl fumarate attenuates reactive gliosis and confers sustained neuroprotection against cerebral hypoxic-ischemic damage by an Nrf2-dependent mechanism. Free Radic Biol Med 131: 98-114, 2019. DOI: 10.1016/j.freeradbiomed.2018.11.017

24 Zhou Y, Duan S, Zhou Y, Yu S, Wu J, Wu X, Zhao J, Zhao Y: Sulfiredoxin-1 attenuates oxidative stress *via* Nrf2/ARE pathway and 2-Cys Prdxs after oxygen-glucose deprivation in astrocytes. J Mol Neurosci 55(4): 941-950, 2015. DOI: 10.1007/s12031-014-0449-6

25 Zhou Y, Peng L, Li Y, Zhao Y: Silent information regulator 1 ameliorates oxidative stress injury *via* PGC-1α/PPARγ-Nrf2 pathway after ischemic stroke in rat. Brain Res Bull 178: 37-48, 2022. DOI: 10.1016/j.brainresbull.2021.11.001

26 Zhao Y, Zhang J, Zheng Y, Zhang Y, Zhang XJ, Wang H, Du Y, Guan J, Wang X, Fu J: NAD(+) improves cognitive function and reduces neuroinflammation by ameliorating mitochondrial damage and decreasing ROS production in chronic cerebral hypoperfusion models through Sirt1/PGC-1α pathway. J Neuroinflammation 18(1): 207, 2021. DOI: 10.1186/s12974-021-02250-8

27 Thong-Asa W, Bullangpoti V: Neuroprotective effects of tiliacora triandra leaf extract in a mice model of cerebral ischemia reperfusion. Avicenna J Phytomed 10(2): 202-212, 2020.

28 Wang X, Lin F, Gao Y, Lei H: Bilateral common carotid artery occlusion induced brain lesions in rats: A longitudinal diffusion tensor imaging study. Magn Reson Imaging 33(5): 551-558, 2015. DOI: 10.1016/j.mri.2015.02.010
29 Sun M, Wu L, Chen G, Mo X, Shi C: Hemodynamic changes and neuronal damage detected by 9.4 T MRI in rats with chronic cerebral ischemia and cognitive impairment. Brain Behav 12(7): e2642, 2022. DOI: 10.1002/brb3.2642
30 Iadecola C: The pathobiology of vascular dementia. Neuron 80(4): 844-866, 2013. DOI: 10.1016/j.neuron.2013.10.008
31 Zhang D, Xiao Y, Lv P, Teng Z, Dong Y, Qi Q, Liu Z: Edaravone attenuates oxidative stress induced by chronic cerebral hypoperfusion injury: role of ERK/Nrf2/HO-1 signaling pathway. Neurol Res 40(1): 1-10, 2018. DOI: 10.1080/01616412.2017.1376457
32 Yadav A, Sunkaria A, Singhal N, Sandhir R: Resveratrol loaded solid lipid nanoparticles attenuate mitochondrial oxidative stress in vascular dementia by activating Nrf2/HO-1 pathway. Neurochem Int 112: 239-254, 2018. DOI: 10.1016/j.neuint.2017.08.001
33 Duan J, Cui J, Yang Z, Guo C, Cao J, Xi M, Weng Y, Yin Y, Wang Y, Wei G, Qiao B, Wen A: Neuroprotective effect of Apelin 13 on ischemic stroke by activating AMPK/GSK-3β/Nrf2 signaling. J Neuroinflammation 16(1): 24, 2019. DOI: 10.1186/s12974-019-1406-7
34 Yang Y, Zhang J, Liu H, Zhang L: Change of Nrf2 expression in rat hippocampus in a model of chronic cerebral hypoperfusion. Int J Neurosci 124(8): 577-584, 2014. DOI: 10.3109/00207454.2013.863196
35 Lee JM, Lee JH, Song MK, Kim YJ: NXP031 improves cognitive impairment in a chronic cerebral hypoperfusion-induced vascular dementia rat model through Nrf2 signaling. Int J Mol Sci 22(12): 6285, 2021. DOI: 10.3390/ijms22126285