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*Research Article*

# Cerebral Ischemia-Reperfusion Induced Neuronal Damage, Inflammation, miR-374a-5p, MAPK6, NLRP3, and Smad6 Alterations: Rescue Effect of N-acetylcysteine

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**Abstract**

***Background:*** Ischemic stroke (IS) is still a major cause of neurological disability. This study aimed to ascertain potential markers closely related to IS diagnosis and treatment and then we examined the neuroprotective effect of N-acetylcysteine (NAC) in a transient cerebral ischemia.

***Methods:*** Male Wistar rats were randomly divided into three groups (n=6), including sham, IR (ischemia-reperfusion), IR+NAC (150 mg/kg, ip; intraperitoneally, 1 hour prior to ischemia and 5 min before reperfusion). The infarct volume was evaluated by 2,3,5-triphenyl tetrazolium chloride staining. H&E and Nissle staining were performed to evaluate cerebral ischemia-reperfusion injury. miR-374a-5p gene expression, MAPK6, NLRP3, smad6, TNF-α, and IL-1β protein levels were determined by Real-time PCR, Western blot, and Elisa in the cerebral cortex exposed to IR.

***Results:*** Herein, we found that IR increased infarct volume and pathological damage to the cerebral cortex after global cerebral artery occlusion/reperfusion. In addition, miR-374a-5p gene expression decreased, while MAPK6, NLRP3, and smad6 protein expressions increased in the IR group. TNF-α and IL-1β protein levels increased in the ischemic cortex. Treatment with NAC significantly attenuated infarct size, inflammation and reversed aforementioned molecule levels.

***Conclusion:*** Taken together, these results suggested that ischemic insult can increase infarct size, neuronal damage, and inflammation may in part by modulating miR-374a-5p, MAPK6, NLRP3, and smad6 pathway in the brain cortex after cerebral IR insult and providing new clues to molecular mechanisms and treatment targets in IS. It can be alleviated by NAC as a potential therapy for someone afflicted with ischemia.

## Introduction

Cerebral artery occlusion leads to insufficient oxygen and nutrient supply to brain tissue and subsequent destructive cerebrovascular disease.[1] Embolic arterial occlusion is the major cause of ischemic stroke, but specifically, oxidative stress and inflammation are identified to be the critical pathogenic mechanisms of brain injury.[2] Accumulating evidence suggests that in hibiting the inflammatory cytokines may be protective against ischemic stroke.[3] Nevertheless, the molecular mechanisms of the reperfusion-induced inflammation are not well clarified.

MicroRNAs (miRNAs) are an extensively studied class of non-coding RNAs that can act as a key point in regulating gene expression at the post-transcriptional level. They play desirable diagnostic or prognostic biomarkers in many cellular events including physiological and pathological processes.[4,5] Several miRNAs have been related to neuroinflammation which is a major pathological event during an ischemic stroke.[6-8]

Among them, miR-374a-5p can regulate inflammatory responses, in various disorders including Hypoxic–ischemic encephalopathy[9-11] hypoxia-induced damage of PC12 cells[12,13] and ischemic stroke.[14] miR-374a-5p can regulate the release of inflammatory cytokines by targeting NLR Family Pyrin Domain Containing 3 (NLRP 3) inflammasome as a pro-inflammatory intracellular receptor that contributes to inflammatory response in microglia[15] and Hypoxic–ischemic encephalopathy.[11] These findings suggest that miR-374a-5p may play an important role in the pathophysiology of ischemic stroke. SMAD Family Member 6 (Smad6) as an inhibitory smad family protein is a target gene of miR-374a-5p, which could prevent neuronal damage in Hypoxic–ischemic encephalopathy [11] and also increased after asphyctic preconditioning in fetal brain tissue.[16] Another target gene of miR-374a is Mitogen-activated protein kinase 6 (MAPK6) which can protect

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against ischemia-reperfusion injury in myocardial cells in vivo and in vitro.[17]

Some researchers demonstrated that the MAPK6 pathway was related to neurotoxicity and then, has an important role in the pathogenesis of some neurodegenerative diseases, such as Alzheimer's and Parkinson's.[18,19] However, there is no experimental study to verify miR-374a-5p expression and its target molecules in ischemic stroke to provide an early marker in the early diagnosis and targeted treatment of ischemic stroke. Emerging evidence suggests that a protective strategy against oxidative stress and subsequent neuroinflammation may be useful to mitigate ischemic injury after stroke.

N-acetylcysteine (NAC) as a precursor of glutathione and a powerful antioxidant has been proven to combat oxidative stress and inflammation.[20] Accumulating evidence described the neuroprotective effect of NAC in ischemic brain injury invivo[20-23] and invitro.[24] This protection is mediated by Tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS) reduction,[21] increased interaction of Heat shock protein 90 (Hsp90) with Hypoxia-inducible factor 1-alpha (HIF-1α),[25] MLK3 activation[26] and reduced size of infarct size.[21]

However, it is needed to provide more details for molecular alteration in ischemia-reperfusion injury and NAC treatment for opening a new therapeutic window in ischemic stroke. Considering these points mentioned above, the current study aimed to investigate mir-374a, NLRP3, smad6 and MAPK6 alterations in a rat model of cerebral I/R involved in neuronal damage, and inflammation. This study provides evidence regarding the effect of NAC and whether it can reduce structural changes in the rat cerebral cortex and its corresponding molecular mechanisms caused by common carotid artery occlusion (CCAO).

## Methods

### *Animals, CCAO model, and NAC treatment*

MaleWistar rats were kept in cages with a 12-hour light/12-hour dark cycle at room temperature (21±2°Cand were given unlimited food and water accessibility. The rats were ( 250±20 g in weight and 3-4 months old. All animal procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care Committee, the Urmia University of Medical Sciences (Ethical Code: IR.UMSU.AEC.1400.008).

The rats were divided into three groups (six in each group): sham, IR (ischemia-reperfusion), IR±NAC. According to the protocol of previous studies, the bilateral CCAO approach was used to establish a transient global ischemia model.[27] Ketamine (60 mg/kg) and xylazine (4 mg/kg) were used intraperitoneally to cause anesthesia. The common carotid arteries were exposed by performing a 1.5 cm long vertical midline incision after shaving the neck skin.

After separating the vagal nerves from the arteries, vascular clamps were used to block the carotid arteries. The clamps were withdrawn 20 min after the induction of ischemia to allow for 24 hours period of reperfusion. Rats in the sham group had the same treatmentexcept for having their common carotid arteries tied. During the experiment, a thermometric blanket was used to maintain a constant body temperature of 37 ± 0.2°C .

The rats were administered 150 mg/kg of NAC intraperitoneally in heparinized normal saline (pH 7.4) 1 hour prior to cutting off the blood supply and 5 minutes prior to reperfusion in the treatment group. Instead of NAC, the same volume of heparinized normal saline was given to the animals in the ischemia alone and sham groups at the exact period.[20,21]

### *Infarct area assessment*

The rats were sedated (ketamine 60 mg/kg, xylazine 4 mg/kg, ip) and killed by decapitation 24 hours after CCAO, and their brains were promptly removed. Coronal sections were cut into 2mm thick slices and dyed with 1% 2,3,5triphenyltetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA) for 30 minutes at 37 C before being fixed for 10 minutes with 10% paraformaldehyde. ImageJ software was used to determine the size of the infarction. (Version1.54; National Institutes of Health). The following equation was used to calculate the size of infarct regions: Infarct rate (%) = infarct volume/total volume multiplied by 100.

### *Histopathologic analyses*

Hematoxylin-eosin and Nissl staining were applied to paraffin-embedded, formalin-fixed brain tissue sections for a comprehensive examination of general histologic morphology. Consequently, the sections were deparaffinized and hydrated through an alcohol gradient. The portions were then rinsed using distilled water. The nuclei were then stained with hematoxylin for two minutes before being washed with running water for one minute. The slices were stained with eosin for 20 seconds, dehydrated, and then mounted with neutral resins following differentiation with 0.3% acid alcohol. Lastly, the images were captured using a light microscope (Olympus, Japan). For the Nissl Staining, the sections were stained for 10 minutes at room temperature with 1% toluidine blue dye. After washing the pieces for one minute in distilled water, they were dehydrated in gradient alcohol and mounted with neutral resins.

Results were examined under a light microscope (Olympus, Japan). Four randomly selected high-power fields were analyzed, and vacuole formation (HE staining) and Nissl positive cells (Nissl Staining) were counted using OLYSIA Autobioreport Software (Olympus Optical, Co. LTD, Tokyo, Japan) in a blinded manner. Results are reported as the average number of vacuoles and neurons in each staining.

### *Quantitative real-time PCR*

The qRT-PCR method was used to assess the expression levels of miR-374a in homogenate cerebral cortex samples. The miRCURYTMRNA isolation kit (Exiqon, Vedbaek, Denmark) and cDNA synthesis kit were used to extract miRNA and synthesis cDNA from collected materials. Then, using the standard SYBR Green master mix, cDNA was used as a template for the construction of microRNA quantitative real-time PCR (Exiqon, Vedbaek, Denmark). Real-time PCR results were analyzed using the Bio-Rad iQ5 detection System (Bio-Rad, Richmond, CA, USA). For miRNA RT-PCR, U6 was used as an endogenous control, and the relative expression of miRNA was calculated using the precise $2^{-(\Delta\Delta Ct)}$ method.[28] As a result, the fold-change relative to the housekeeping gene was reported. The subsequent primers were used: mir-374a forward, 5-ATATAATACAACCTGCTAAGTG-3; mir-374a reverse, 5-GAACATGTCTGCGTATCTC-3; U6 sense: 5-CTGCTTCGGCAGCACATATACTAA-3; and U6 antisense:3-AGGGGCCATGCTAATCTTCTCT-5. GenBank was used to acquire sequences. Using Gene Runner software, the primers were confirmed (Syngene, Cambridge, UK). The specificity of novel primer sets was evaluated using the software Oligo 7.6.

### *Western blot*

As previously described, the MAPK6, NLRP3, and smad6 protein levels in homogenate cerebral cortex tissue were evaluated by Western immunoblotting.[29] In summary, the obtained tissue was homogenized and then sonicated in a cold lysis buffer comprising 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 50mM Tris hydrochloride (Tris-HCl), pH 7.5, 0.3M sucrose, 5mM Ethylenediaminetetraacetic acid (EDTA), 2mM sodium pyrophosphate, 1mM sodium orthovanadate, and 1mM phenylmethylsulfonyl fluoride, supplemented with a complete protease inhibitor cocktail. To collect the supernatant and detect proteins, homogenized tissues were centrifuged for 15 minutes at 1000 g and 4 °C. Through SDS-PAGE, the proteins were separated and transferred to a PVDF membrane. Upon blocking with skim milk, anti-MAPK6 (catalog number sc-374239, 1:1000 dilution, SANTA CRUZ), anti-NLRP3 (catalog number ab91413, 1:100 dilution, ABCAM), and anti-smad6 (catalog number sc-25321, 1:500 dilution, SANTA CRUZ) antibodies were used to precisely determine the quantity of the proteins.[29] Using Image J software, immunoreaction density was measured. Final results are estimated as a ratio of the target protein to β-actin protein.

### *ELISA*

For determination of the cerebral cortex TNF-α and IL-1β content, the supernatant sample was subjected to a quantitative sandwich enzyme immunoassay using a relevant ELISA kit (China; Cat. NO. CSB-E10526r) and (China; Cat. NO. CSB-EL006328RA) based on the manufacturer's instructions.[30] Therefore, microplates were coated with a specific protein antibody. Then, standards and samples were pipetted into the wells, and an immobilized antibody was used to attach any particular proteins present. After removing all unbound compounds, Horseradish Peroxidase (HRP) coupled with biotin was added to the wells. The wells were then treated with a substrate solution to generate a colour proportional to the amount of protein bound. After terminating the colour development, the color's intensity was evaluated.

### *Determination of CK-BB and LDH activities*

Levels of CK-BB and LDH were measured spectrophotometrically using an autoanalyzer (Roche Cobas-Integra 800) and commercially available test cassettes (Roche). CK-BB levels were also measured spectrophotometrically employing an immunoinhibition technique and an autoanalyzer (CobasIntegra 800).[31]

### *Statistical analysis*

The findings were reported as mean ± SEM, and data analyses were conducted using SPSS 25. All parameters were examined for normality by using the one-sample Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to analyze the data, followed by Tukey's test. The level of significance was denoted by $p < 0.05$.

## Results

### *Cerebral infarction volume*

To assess the effect of NAC on infarct volume, we performed TTC staining. The normal brain tissue appeared red, with infarct area stained white. In sham group, no infarction was observed. CCAO and reperfusion (26.6 ± 2.78) increased the infarct volume ratio in model group, and NAC treatment significantly decreased (10 ± 1.37) the infarct volume ratio in cerebral cortex of rats ($P < 0.001$) (Figure 1).

### *Histopathologic findings*

HE staining was employed to evaluate brain histological alterations. As indicated in Figure 2, histopathology was normal and no neuronal damage was found in the sham surgery group. In the CCAO model group, however, most cells were haphazardly ordered and morphologic abnormalities were identified, including cell swelling and extensive vacuole formation. The treatment with NAC, however, ameliorated these pathological anomalies. The number of viable cortical neurons was then determined using Nissle staining. Histological examination revealed that cerebral ischemia injury resulted in fewer Nissle-positive neurons than sham-operated rats, but NAC therapy preserved the neurons ($P<0.05$).

### *mir-374a expression in the cerebral cortex*

Real-time PCR was applied to determine the effects of cerebral IR injury and NAC therapy on the expression of mir-374a in the cerebral cortex. In the present

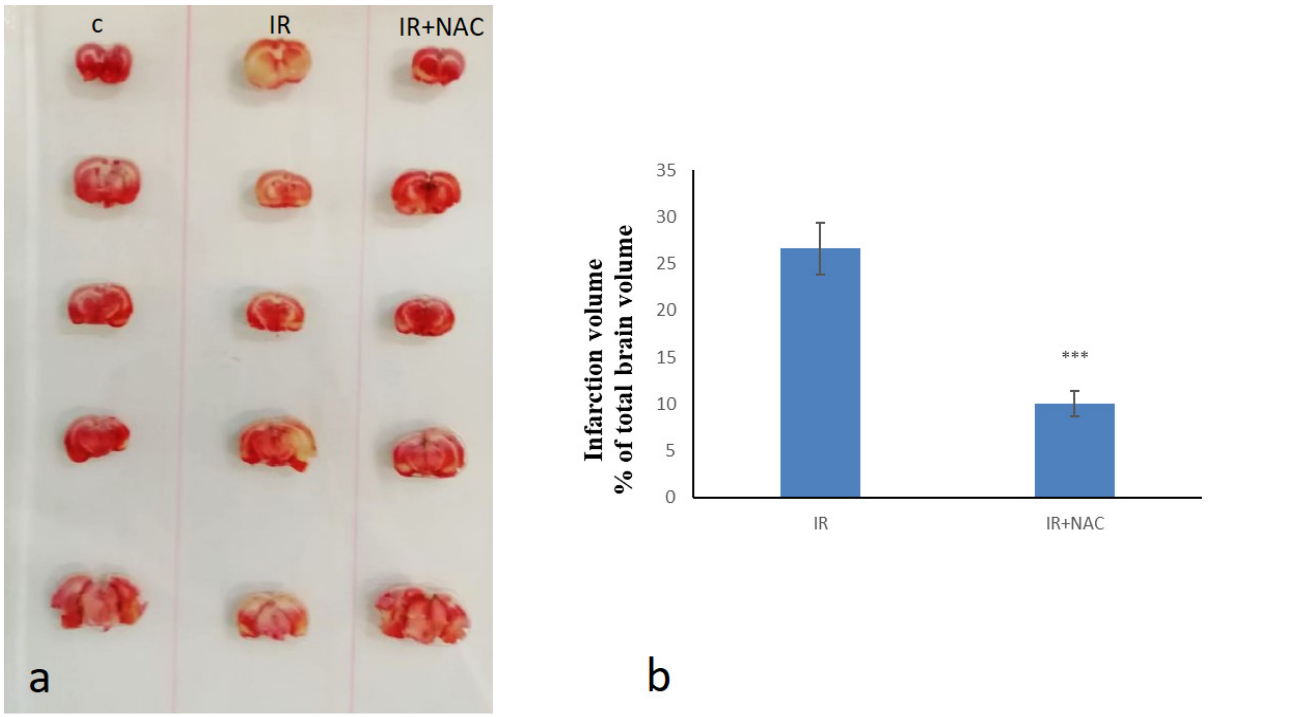


**Figure 1.** NAC treatment reduces the brain infarct volume upon CCAO. (A) Representative micrographs of TTC-stained brain sections (B) Infarct volume quantitation. *** P<0.001, vs IR group (One-way ANOVA followed by Tukey's test). All data are expressed as the means ± SEM (n=6). Sham, IR: ischemia reperfusion, IR+NAC: ischemia-reperfusion+ N-acetylcysteine.

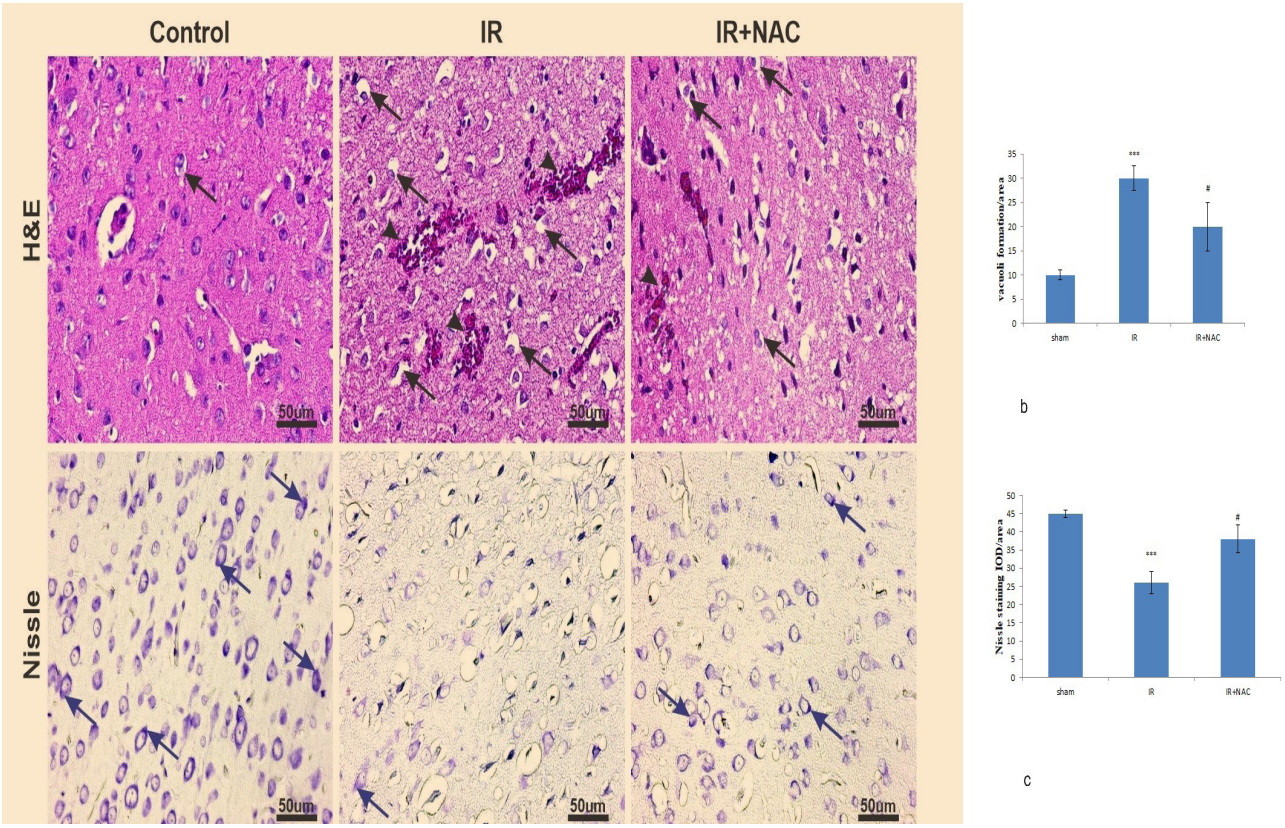


**Figure 2.** NAC treatment rescues neuronal damage upon CCAO. (A) Representative micrographs of H&E staining and Nissle staining (scale bar=50 µm). NAC treatment alleviated morphological changes and increased the number of viable neurons after CCAO. (B) Quantitation of vacuole formation (C) Quantitation of Nissle-stained neurons. *** P<0.001, vs sham group; # P<0.05, vs IR group. All data are expressed as the means ± SEM (n=6) (One-way ANOVA followed by Tukey's test) Sham, IR: ischemia reperfusion, IR+NAC: ischemia-reperfusion+ N-acetylcysteine.

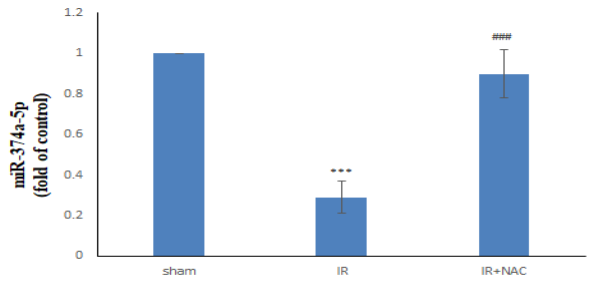


**Figure 3.** The effect CCAO with or without NAC treatment on the mir-374a gene expression in the cerebral cortex in different groups. *** P<0.001 vs sham group, ## P<0.001, vs IR group (One-way ANOVA followed by Tukey's test). All data are expressed as the means ± SEM (n=6). Sham, IR: ischemia-reperfusion, IR+NAC: ischemia reperfusion+ N-acetylcysteine.

investigation, Figure 3 depicts the expression of mir-374a in the rat cortex in experimental groups. Cerebral IR injury led to a significant decrease ($p < 0.001$) in mir-374a expression (0.29±0.08) compared with the sham (1±0.0) group. However, treatment with NAC (0.9±0.12) reversed this effect ($p< 0.001$).

### *MAPK6, NLRP3, smad6 protein levels in the cerebral cortex*

To determine the effect of IR injury and NAC intervention on MAPK6, NLRP3, and smad6 protein levels, Western blot analysis was performed on cerebral cortex tissue taken from separate research groups 24 h after CCAO. In the cerebral cortex, IR damage significantly enhanced the expression of MAPK6 (1.77±0.09), NLRP3 (2.35±0.07) (P<0.001), and smad6 (1.28±0.05) (P<0.01) proteins expression in the cerebral cortex compared to the sham (1±0.0) group (Figure 4). NAC therapy decreased MAPK6 (1.08 ±0.05) (P<0.001), NLRP3 (2.16±0.04) (P<0.05) , and smad6 (1.1±0.05) (P<0.05) protein expressions in the cerebral cortex exposed IR injury.

### *TNF-α and IL-1β level in the cerebral cortex*

Inflammatory cytokines (TNF-α and IL-1β) were highly expressed at 24 h after CCAO occlusion (88.07 ± 5.78, 149 ± 8.2) in IR group compared to sham animals (13.49± 1.39, 31.88± 6.15) as shown by Elisa assay (P<0.001). Treatment with NAC significantly decreased ischemia-induced expression of TNF-α (32.5±3.98) and IL-1β (74.53±7.44) levels at 24 h after reperfusion (P<0.001) (Table 1).

### *Serum level of LDH and CKBB*

Serum LDH and CKBB levels were investigated as indications of brain tissue injury. Since the highest plasma LDH and CK-BB activity was observed at 24 h of reperfusion, we measured these enzymes during this period.[32] Higher levels of LDH (18.65±2.53) and CKBB (286±10.3) were detected in the I/R group compared to the sham group (5.59± 0.9) (121± 16) (P<0.001), and these elevations were considerably mitigated by NAC therapy (12.8±0.93, P<0.05; 164±12.3, P<0.001 respectively) (Figure 5).

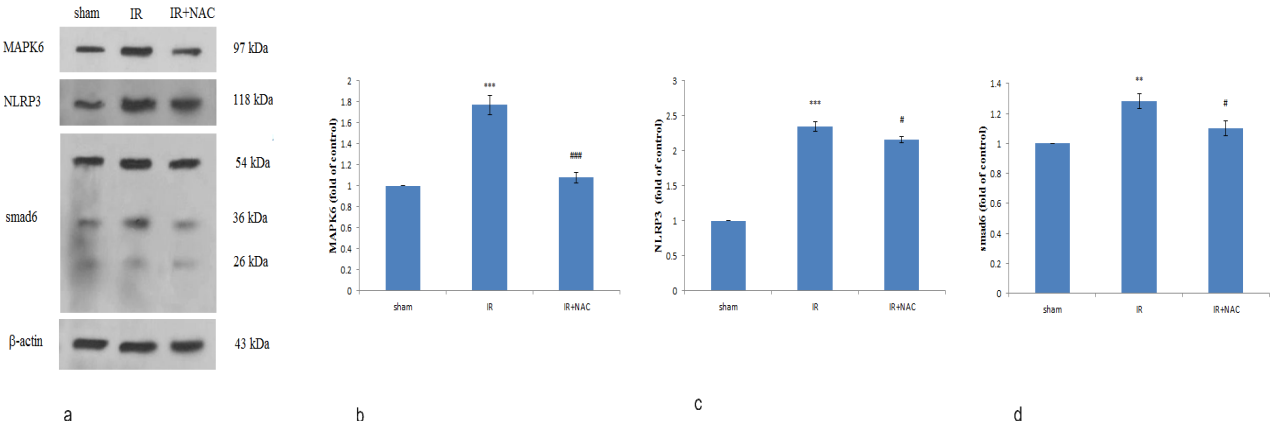


**Figure 4.** The effect of CCAO with or without NAC treatment on the MAPK6, NLRP3 and smad6 protein expressions in the cerebral cortex of all experimental groups. The blotting images of MAPK6, NLRP3 and smad6 (a). The bar charts represent the quantitative analysis of the protein levels of MAPK6, NLRP3 and smad6 (b, c, d) normalized against β-actin. **p < 0.01, ***p < 0.001 vs sham group; # P<0.05, ### p < 0.001 vs IR group (One-way ANOVA followed by Tukey's test). All data are expressed as the means ± SEM (n=6). Sham, IR: ischemia-reperfusion, IR+NAC: ischemia-reperfusion+ N-acetylcysteine.

**Table 1.** The effect of CCAO with or without NAC treatment on TNF-α and IL-1β protein levels in the cerebral cortex in different groups.

| | Sham | IR | IR+NAC |
|---|---|---|---|
| TNF-α (pg/mg protein) | 13.49±1.39 | 88.07±5.78*** | 32.5±3.98### |
| IL-1β (pg/mg protein) | 31.88±6.15 | 149±8.2*** | 74.53±7.44### |

***p < 0.001 vs sham group, ### p < 0.001 vs IR group (One-way ANOVA followed by Tukey's test). All data are expressed as the means ± SEM (n=6). Sham, IR: ischemia reperfusion, IR+NAC: ischemia reperfusion+ N-acetylcysteine

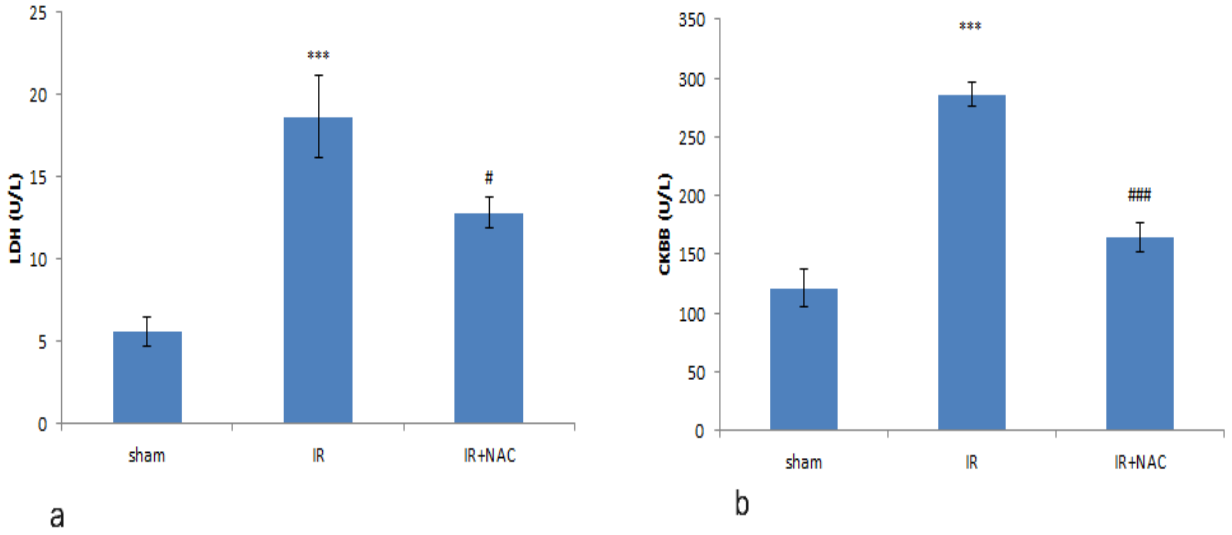


**Figure 5.** The effect of CCAO with or without NAC treatment on LDH and CKBB levels in the serum of different groups. ***p < 0.001 vs sham group, # p < 0.05, ### p < 0.001 vs IR group (One-way ANOVA followed by Tukey's test). All data are expressed as the means ± SEM (n=6). Sham, IR: ischemia-reperfusion, IR+NAC: ischemia reperfusion+ N-acetylcysteine.

## Discussion

In the present study, we demonstrated for the first time that ischemic insult increased infarct size, neuronal damage, and inflammation may in part be associated with regulating miR-374a-5p, MAPK6, NLRP3, and smad6 signaling pathways in the brain cortex. Interestingly, remarkable amelioration of the molecular alterations in the cerebral cortex and, subsequently, the reduction of neuronal damage to those observed in the ischemic animals appeared in the NAC-treated rats.

Based on our analysis, morphologic change of neurons including widespread vacuole formation and reduced staining intensity of Nissl substances in the cerebral cortex as well as increased infarct area upon ischemic insult was found in the present study which appeared after 24 hours of ischemia. Other studies are in line with our findings suggesting that ischemia reperfusion induced histopathological changes in the cerebral cortex which could be recovered by NAC treatment.[21,23,33]

Despite many advances in this area, the mechanisms underlying IR injury remain largely unclear, hence no effective therapy has been described yet.[1,34] The major pathway leading to neuronal damage through activating neurotoxic cascades after initial stroke is related to elevation of glutamate and calcium, resulting in a generation of free radicals and inflammation reaction.[35] Inflammation has been identified as an important leading cause of brain injury and subsequent neurological dysfunction in all stages of the ischemic cascade including ischemia and reperfusion.[36,37] Inflammatory cytokines especially TNF-α and IL-1β upregulated in stroke resulting in neuronal cell death through induction of free radicals in glial cells.[20] In this study, we found high expression of these cytokines after 24 hours of reperfusion which was in agreement with earlier studies.[20,38-40] Here, we address the molecular mechanisms of ischemic stroke which may trigger inflammatory cascade to be a promising candidate for effective therapy in acute stroke.

Numerous miRNAs are found in the brain tissue and may be considered as diagnostic or prognostic biomarkers in the management of ischemic stroke insult.[41,42] Previous reports indicated that miR-374a-5 was decreased in the umbilical cord blood of infants with hypoxic- ischemic encephalopathy,[9] and overexpression of miR-374a-5p attenuated apoptosis of PC12 cells treated by oxygen/glucose deprivation in vitro.[12] Interestingly, in this study, miR-374a-5p decreased in the cerebral cortex 24 hours after IR injury. Thus, this study for the first time notified down-regulation of miR-374a-5p levels of cerebral cortex tissue following IR injury in rats and further provided new insights as the potential target for the treatment of ischemic insult. Other studies are in line with our results. Chen *et al.*,[11] reported that miR-374a-5p was reduced in the brain tissue of hypoxic-ischemic encephalopathy (HIE) rats. Thus, overexpression of miR-374a-5p significantly mitigated pathological damage to the neonatal brain, and inhibited the release of pro-inflammatory factors (IL1β, TNF-α and IL-6) by regulating NLRP3 inflammasome in hypoxic-ischemic encephalopathy rat model in vivo. NLRP3 inflammasome as a pro-inflammatory intracellular recognition receptor is known as a central component in the inflammatory cascade, and triggers an inflammatory response in microglia.[43-45] Microglia and astrocytes are the main factors of neuroinflammation in ischemia which can produce some cytotoxic factors including TNF-α, IL-1β, IL-6, and NO leading to neuronal dysfunction.[46] NLRP3 protein expression was elevated in our study after IR insult in male rats which is in consistent with above mentioned researches.

Smad6 is an oncogenic gene that can participate in promoting hepatocellular carcinoma metastasis.[47] Cox-Limpens *et al.*[16] identified that up-regulation of smad6

could indicate a preconditioning-activated neuroprotective mechanism 96 hour after fetal asphyctic preconditioning. However, Chen *et al.*[11] reported that the expression of Smad6 in Neonatal HIE was up-regulated within 24 h of hypoxia. Taken together, we measured smad6 at 24 h after IR which is in agreement wuth that study. It is considered that this controversy is possibly dependent to the timing of the ischemic cascade in part due to endeavor for recovery from the injury.[48]

Smad6 is a target gene of miR-374a-5p, which was involved in neuroprotection in hypoxic conditions. Chen *et al.*[11] reported that miR-374a-5p significantly inhibited pro-inflammatory cytokines in neonatal rat hypoxic-ischemic encephalopathy model in vivo and also inhibited the activation of NLRP3 inflammatory signals in microglia and the subsequent release of pro-inflammatory factors through Smad6 in vitro. In this study, we found that Smad6 protein level increased in CCAO, suggesting that Smad6 may be considered the direct target gene of miR-374a-5p to regulate the inflammatory cytokines in ischemic stroke.

MAPK6 is a member of Ser/Thr protein kinase family and is mostly expressed in the brain tissue in response to stress.[18] MAPK6 has been involved in the pathogenesis of neurodegenerative diseases and plays an important role in neurotoxicity.[19]

It has recently been identified as a target of miR-374a-5p and has been identified as a critical protein in ischemia/reperfusion in hepatic and cardiac cell injury.[17,49] In this study, we found MAPK6 overexpression in CCAO indicating a new light in the pathogenesis of IR injury in the cortex tissue of ischemic rats.

Based on our results, molecular analysis showed that down-regulation of miR-374a-5p gene expression and up-regulation of NLRP3, Smad6, and MAPK6 levels were observed after 24 h following CCAO in cortex tissue. Our findings support that miR-374a-5p can modulate brain cortex tissue injury through Smad6 and MAPK6 axis, which provide a promising target for diagnostic and therapeutic strategies in cerebral I/R injury.

NAC is a membrane-permeable cysteine precursor that delivers cysteine to the cell. After NAC enters a cell, it undergoes a rapid process of hydrolysis, leading to the release of cysteine. This cysteine then acts as a precursor for the synthesis of glutathione (GSH), a vital antioxidant in the body that plays a crucial role in protecting cells from oxidative damage and maintaining cellular health and function. In light of these observations, NAC has been identified as a potent antioxidant agent that possesses the ability to prevent ischemic injury in several tissue such as cerebral cortex.[20,21,26,35,50,51] It can cause a remarkable increase in glutathione content, elevated hypoxia-inducible factor-1 reduced apoptotic index, and decreased expression of iNOS, IL1β, TNF-α.[20,21,33] Here, in the present study, our results indicated that treatment with NAC could mitigate histopathological damages and inflammation possibly by modulating proinflammatory cytokines (TNF-α, IL-1β), NLRP3 inflammasome, miR-374a-5p, and its targets including MAPK6 and smad6. This finding is in agreement with the study of Khan *et al.*,[20] in which administration of NAC improved neurological score and decreased cerebral infarction through its anti-inflammatory effect by regulating iNOS, IL1β, TNF-α expression. In addition, Keshk *et al.*,[52] reported that NAC could prevent neurodegeneration via improving oxidative stress and neuroinflammation by suppressing NLRP3 in cerebral cortex. Our data indicate for the first time that miR-374a-5p, and its targets including MAPK6, smad6 and NLRP3 may play a crucial role in the inflammatory cascade during I/R. NAC administration as an antioxidant could reverse these aforementioned molecules which can be considered as an effective therapy in cerebral ischemic insult. Future studies are needed to focus on applying molecule inhibitors to confirm the suggested pathway in cerebral IR.

## Conclusion

In conclusion, our research reveals, the underlying mechanism of cerebral IR injury which at least was attributed to miR-374a-5p, MAPK6, smad6, and NLRP3 alterations. This study provides insight into the molecular mediators involved in cerebral IR injury indicating a potential novel therapeutic target for treating cerebral IR. Administration of NAC can protect cerebral cortex injury against IR probably by modulating miR-374a-5p, MAPK6, smad6, and NLRP3. Further clinical or preclinical studies would be required.

## Ethical Issues

All animal procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care Committee, the Urmia University of Medical Sciences (Ethical Code: IR.UMSU.AEC.1400.008).

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## Author Contributions

Hamed Saniei: Data Curation, Formal Analysis. Alireza Shirpoor: Methodology, Writing – Review & Editing. Roya Naderi: Conceptualization, Data Curation, Formal Analysis, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft, Writing - Review & Editing.

## Conflict of Interest

All authors declare no competing interests.

## References

1. Zhou F, Wang YK, Zhang CG, Wu BY. Mir-19a/b-3p promotes inflammation during cerebral ischemia/reperfusion injury via sirt1/foxo3/sphk1 pathway. J

Neuroinflammation. 2021;18(1):122. doi:10.1186/s12974-021-02172-5
2. Yu J, Wang WN, Matei N, Li X, Pang JW, Mo J, et al. Ezetimibe attenuates oxidative stress and neuroinflammation via the ampk/nrf2/txnip pathway after mcao in rats. Oxid Med Cell Longev. 2020;2020:4717258. doi:10.1155/2020/4717258
3. Anrather J, Iadecola C. Inflammation and stroke: An overview. Neurotherapeutics. 2016;13(4):661-70. doi:10.1007/s13311-016-0483-x
4. Gebert LFR, MacRae IJ. Regulation of microrna function in animals. Nat Rev Mol Cell Biol. 2019;20(1):21-37. doi:10.1038/s41580-018-0045-7
5. Ardekani AM, Naeini MM. The role of micrornas in human diseases. Avicenna J Med Biotechnol. 2010;2(4):161-79.
6. Bansal A, Prathap R, Gupta S, Chaurasia A, Chaudhary P. Role of micrornas in stroke recovery. J Family Med Prim Care. 2019;8(6):1850-4. doi:10.4103/jfmpc.jfmpc_296_19
7. Tahamtan A, Teymoori-Rad M, Nakstad B, Salimi V. Anti-inflammatory micrornas and their potential for inflammatory diseases treatment. Front Immunol. 2018;9:1377. doi:10.3389/fimmu.2018.01377
8. Chen ZJ, Zhao XS, Fan TP, Qi HX, Li D. Glycine improves ischemic stroke through mir-19a-3p/ampk/gsk-3β/ho-1 pathway. Drug Des Devel Ther. 2020;14:2021-31. doi:10.2147/dddt.s248104
9. Looney AM, Walsh BH, Moloney G, Grenham S, Fagan A, O'Keeffe GW, et al. Downregulation of umbilical cord blood levels of mir-374a in neonatal hypoxic ischemic encephalopathy. J Pediatr 2015;167(2):269-73.e2. doi:10.1016/j.jpeds.2015.04.060
10. Looney AM, Ahearne CE, Hallberg B, Boylan GB, Murray DM. Downstream mrna target analysis in neonatal hypoxic-ischaemic encephalopathy identifies novel marker of severe injury: A proof of concept paper. Mol Neurobiol. 2017;54(10):8420-8. doi:10.1007/s12035-016-0330-4
11. Chen Z, Hu Y, Lu R, Ge M, Zhang L. Microrna-374a-5p inhibits neuroinflammation in neonatal hypoxic-ischemic encephalopathy via regulating nlrp3 inflammasome targeted smad6. Life Sci. 2020;252:117664. doi:10.1016/j.lfs.2020.117664
12. Jiang F, Yang M, Wu C, Wang J. Potential roles of mir-374a-5p in mediating neuroprotective effects and related molecular mechanism. J Mol Neurosci. 2019;69(1):123-32. doi:10.1007/s12031-019-01340-w
13. Gong W, Qie S, Huang P, Xi J. Protective effect of mir-374a on chemical hypoxia-induced damage of pc12 cells in vitro via the gadd45α/jnk signaling pathway. Neurochem Res. 2018;43(3):581-90. doi:10.1007/s11064-017-2452-0
14. Feng B, Meng X, Zhou H, Chen L, Zou C, Liang L, et al. Identification of dysregulated mechanisms and potential biomarkers in ischemic stroke onset. Int J Gen Med. 2021;14:4731-44. doi:10.2147/ijgm.s327594
15. Walsh JG, Reinke SN, Mamik MK, McKenzie BA, Maingat F, Branton WG, et al. Rapid inflammasome activation in microglia contributes to brain disease in hiv/aids. Retrovirology. 2014;11:35. doi:10.1186/1742-4690-11-35
16. Cox-Limpens KE, Vles JS, Schlechter J, Zimmermann LJ, Strackx E, Gavilanes AW. Fetal brain genomic reprogramming following asphyctic preconditioning. BMC Neurosci. 2013;14:61. doi:10.1186/1471-2202-14-61
17. Huang ZQ, Xu W, Wu JL, Lu X, Chen XM. Microrna-374a protects against myocardial ischemia-reperfusion injury in mice by targeting the mapk6 pathway. Life Sci. 2019;232:116619. doi:10.1016/j.lfs.2019.116619
18. Zhang Y, Su Z, Liu HL, Li L, Wei M, Ge DJ, et al. Effects of mir-26a-5p on neuropathic pain development by targeting mapk6 in in cci rat models. Biomed Pharmacother. 2018;107:644-9. doi:10.1016/j.biopha.2018.08.005
19. Liu J, Xiang C, Huang W, Mei J, Sun L, Ling Y, et al. Neurotoxicological effects induced by up-regulation of mir-137 following triclosan exposure to zebrafish (danio rerio). Aquat Toxicol. 2019;206:176-85. doi:10.1016/j.aquatox.2018.11.017
20. Khan M, Sekhon B, Jatana M, Giri S, Gilg AG, Sekhon C, et al. Administration of n-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. J Neurosci Res. 2004;76(4):519-27. doi:10.1002/jnr.20087
21. Sekhon B, Sekhon C, Khan M, Patel SJ, Singh I, Singh AK. N-acetyl cysteine protects against injury in a rat model of focal cerebral ischemia. Brain Res. 2003;971(1):1-8. doi:10.1016/s0006-8993(03)02244-3
22. Shen WH, Zhang CY, Zhang GY. Antioxidants attenuate reperfusion injury after global brain ischemia through inhibiting nuclear factor-kappa b activity in rats. Acta Pharmacol Sin. 2003;24(11):1125-30.
23. Liu Y, Liu WC, Sun Y, Shen X, Wang X, Shu H, et al. Normobaric hyperoxia extends neuro- and vaso-protection of n-acetylcysteine in transient focal ischemia. Mol Neurobiol. 2017;54(5):3418-27. doi:10.1007/s12035-016-9932-0
24. Pahan K, Sheikh FG, Namboodiri AM, Singh I. N-acetyl cysteine inhibits induction of no production by endotoxin or cytokine stimulated rat peritoneal macrophages, c6 glial cells and astrocytes. Free Radic Biol Med. 1998;24(1):39-48. doi:10.1016/s0891-5849(97)00137-8
25. Zhang Z, Yan J, Taheri S, Liu KJ, Shi H. Hypoxia-inducible factor 1 contributes to n-acetylcysteine's protection in stroke. Free Radic Biol Med. 2014;68:8-21. doi:10.1016/j.freeradbiomed.2013.11.007
26. Tian H, Zhang Q, Li H, Zhang G. Antioxidant n-acetylcysteine and ampa/ka receptor antagonist dnqx inhibited mixed lineage kinase-3 activation following cerebral ischemia in rat hippocampus.

Neurosci Res. 2003;47(1):47-53. doi:10.1016/s0168-0102(03)00186-x
27. Hermawati E, Arfian N, Mustofa M, Partadiredja G. Chlorogenic acid ameliorates memory loss and hippocampal cell death after transient global ischemia. Eur J Neurosci. 2020;51(2):651-69. doi:10.1111/ejn.14556
28. Yousefzadeh N, Jeddi S, Ghiasi R, Alipour MR. Effect of fetal hypothyroidism on myomir network and its target gene expression profiles in heart of offspring rats. Mol Cell Biochem. 2017;436(1-2):179-87. doi:10.1007/s11010-017-3089-7
29. Tian X, An R, Luo Y, Li M, Xu L, Dong Z. Tamibarotene improves hippocampus injury induced by focal cerebral ischemia-reperfusion via modulating pi3k/akt pathway in rats. J Stroke Cerebrovasc Dis. 2019;28(7):1832-40. doi:10.1016/j.jstrokecerebrovasdis.2019.04.017
30. Mohammadi M, Ghaznavi R, Keyhanmanesh R, Sadeghipour HR, Naderi R, Mohammadi H. Voluntary exercise prevents lead-induced elevation of oxidative stress and inflammation markers in male rat blood. ScientificWorldJournal. 2013;2013:320704. doi:10.1155/2013/320704
31. Aras M, Urfalı B, Serarslan Y, Ozgür T, Ulutaş KT, Urfalı S, et al. Protective effects of minocycline against short-term ischemia-reperfusion injury in rat brain. Pediatr Neurosurg. 2013;49(3):172-8. doi:10.1159/000362202
32. Ravindran S, Kurian GA. Eventual analysis of global cerebral ischemia-reperfusion injury in rat brain: A paradigm of a shift in stress and its influence on cognitive functions. Cell Stress Chaperones. 2019;24(3):581-94. doi:10.1007/s12192-019-00990-4
33. Turkmen S, Cekic Gonenc O, Karaca Y, Mentese A, Demir S, Beyhun E, et al. The effect of ethyl pyruvate and n-acetylcysteine on ischemia-reperfusion injury in an experimental model of ischemic stroke. Am J Emerg Med. 2016;34(9):1804-7. doi:10.1016/j.ajem.2016.06.003
34. Goldstein LB. Modern medical management of acute ischemic stroke. Methodist Debakey Cardiovasc J. 2014;10(2):99-104. doi:10.14797/mdcj-10-2-99
35. Cuzzocrea S, Mazzon E, Costantino G, Serraino I, Dugo L, Calabrò G, et al. Beneficial effects of n-acetylcysteine on ischaemic brain injury. Br J Pharmacol. 2000;130(6):1219-26. doi:10.1038/sj.bjp.0703421
36. Ma Y, Wang J, Wang Y, Yang GY. The biphasic function of microglia in ischemic stroke. Prog Neurobiol. 2017;157:247-72. doi:10.1016/j.pneurobio.2016.01.005
37. Pawluk H, Woźniak A, Grześk G, Kołodziejska R, Kozakiewicz M, Kopkowska E, et al. The role of selected pro-inflammatory cytokines in pathogenesis of ischemic stroke. Clin Interv Aging. 2020;15:469-84. doi:10.2147/cia.s233909
38. Iadecola C, Anrather J. The immunology of stroke: From mechanisms to translation. Nat Med. 2011;17(7):796-808. doi:10.1038/nm.2399
39. Amantea D, Micieli G, Tassorelli C, Cuartero MI, Ballesteros I, Certo M, et al. Rational modulation of the innate immune system for neuroprotection in ischemic stroke. Front Neurosci. 2015;9:147. doi:10.3389/fnins.2015.00147
40. Rayasam A, Hsu M, Kijak JA, Kissel L, Hernandez G, Sandor M, et al. Immune responses in stroke: How the immune system contributes to damage and healing after stroke and how this knowledge could be translated to better cures? Immunology. 2018;154(3):363-76. doi:10.1111/imm.12918
41. Mao L, Zuo ML, Wang AP, Tian Y, Dong LC, Li TM, et al. Low expression of mir-532-3p contributes to cerebral ischemia/reperfusion oxidative stress injury by directly targeting nox2. Mol Med Rep. 2020;22(3):2415-23. doi:10.3892/mmr.2020.11325
42. Ma GP, Yang BZ, Zhang YS, Wang B, Wei XH, Zhang RF, et al. Protective effects of mirna-589 on cerebral ischemia-reperfusion injury. J Biol Regul Homeost Agents. 2020;34(4):1269-75. doi:10.23812/20-52-a
43. Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, et al. Nlrp3 is activated in alzheimer's disease and contributes to pathology in app/ps1 mice. Nature. 2013;493(7434):674-8. doi:10.1038/nature11729
44. von Herrmann KM, Salas LA, Martinez EM, Young AL, Howard JM, Feldman MS, et al. Nlrp3 expression in mesencephalic neurons and characterization of a rare nlrp3 polymorphism associated with decreased risk of parkinson's disease. NPJ Parkinsons Dis. 2018;4:24. doi:10.1038/s41531-018-0061-5
45. Chen W, Teng X, Ding H, Xie Z, Cheng P, Liu Z, et al. Nrf2 protects against cerebral ischemia-reperfusion injury by suppressing programmed necrosis and inflammatory signaling pathways. Ann Transl Med. 2022;10(6):285. doi:10.21037/atm-22-604
46. Nayak D, Roth TL, McGavern DB. Microglia development and function. Annu Rev Immunol. 2014;32:367-402. doi:10.1146/annurev-immunol-032713-120240
47. Cai J, Liu Y, Li Q, Wen Z, Li Y, Chen X. Ceramide synthase 3 affects invasion and metastasis of hepatocellular carcinoma via the smad6 gene. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2022;47(5):588-99. doi:10.11817/j.issn.1672-7347.2022.210477
48. Przykaza Ł. Understanding the connection between common stroke comorbidities, their associated inflammation, and the course of the cerebral ischemia/reperfusion cascade. Front Immunol. 2021;12:782569. doi:10.3389/fimmu.2021.782569
49. Hao W, Zhao ZH, Meng QT, Tie ME, Lei SQ, Xia ZY. Propofol protects against hepatic ischemia/reperfusion injury via mir-133a-5p regulating the expression of mapk6. Cell Biol Int 2017;41(5):495-504. doi:10.1002/cbin.10745
50. Martinez PF, Bonomo C, Guizoni DM, Junior

SA, Damatto RL, Cezar MD, et al. Influence of n-acetylcysteine on oxidative stress in slow-twitch soleus muscle of heart failure rats. Cell Physiol Biochem. 2015;35(1):148-59. doi:10.1159/000369683

51. Kang KS, Shin S, Lee SI. N-acetylcysteine modulates cyclophosphamide-induced immunosuppression, liver injury, and oxidative stress in miniature pigs. J Anim Sci Technol. 2020;62(3):348-55. doi:10.5187/jast.2020.62.3.348
52. Keshk WA, Ibrahim MA, Shalaby SM, Zalat ZA, Elseady WS. Redox status, inflammation, necroptosis and inflammasome as indispensable contributors to high fat diet (hfd)-induced neurodegeneration; effect of n-acetylcysteine (nac). Arch Biochem Biophys. 2020;680:108227. doi:10.1016/j.abb.2019.108227