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RESEARCH COMMUNICATION

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miR-23b/TAB3/NF-κB/p53 axis is involved in hippocampus injury induced by cerebral ischemia–reperfusion in rats: The protective effect of chlorogenic acid

Shiva Roshan-Milani[1,2] | **Parisa Sattari**[3] | **Firouz Ghaderi-Pakdel**[1,2] | **Roya Naderi**[1,2]

[1]Neurophysiology Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran

[2]Department of Physiology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran

[3]Student Research Committee, Urmia University of Medical Sciences, Urmia, Iran

Correspondence
Roya Naderi, Neurophysiology Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran.
Email: naderi.r@umsu.ac.ir

Abstract

Apoptosis is the main pathological aspect of neuronal injury after cerebral ischemia–reperfusion (I/R) injury. However the detailed molecular mediators are still under debate. The aim of this study is to explore the effect of cerebral I/R on miR-23a/TGF-β-activated kinase 1 binding protein 3 (TAB3)/nuclear factor kappa B (NF-κB)/p53 axis in rat hippocampus alone and in combination with chlorogenic acid (CGA). Common carotid artery occlusion (CCAO) was performed by nylon monofilament for 20 min to establish a model of ischemic brain injury. CGA (30 mg/kg) was administered intraperitoneally (ip), 10 min prior to ischemia and 10 min before reperfusion. Examination of hippocampus neurons by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining showed that the number of apoptotic neurons was elevated at 24 h after reperfusion. At the molecular levels, I/R injury resulted in an increased protein expression of p53 with a concomitant upregulation of cleaved-caspase3/phosphorelated-caspase3 ratio and cytochrome c level. Further miR-23b gene expression was significantly downregulated after 24 h of reperfusion. Also, we observed increased TAB3 and NF-κB protein expressions after 24 h following CCAO. Treatment with CGA significantly reduced the apoptotic damage and also reversed miR-23b gene expression, TAB3 and NF-κB protein expressions in hippocampus neurons in I/R rats. In conclusion our data suggest that miR-23b/TAB3/NF-κB/p53 axis could play a regulatory role in hippocampus cell death, which provide a new target for novel therapeutic interventions during transit ischemic stroke. It also demonstrated that CGA could reverse these molecular alterations indicating an effective component against hippocampus apoptotic insult following acute I/R injury.

KEYWORDS

apoptosis, chlorogenic acid, hippocampus, ischemia–reperfusion

Abbreviations: Bcl-2, B-cell lymphoma 2; CCAO, common carotid artery occlusion; CGA, chlorogenic acid; c-caspase3/p-caspase3 ratio, cleaved-caspase3/phosphorelated-caspase3; IR, ischemia/reperfusion; ip, intraperitoneally; miR, microRNAs; miR-23b, miRNA-23b; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TAB3, TGF-β-activated kinase 1 binding protein 3; TBI, traumatic brain injury; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

1 | INTRODUCTION

Stroke is one of the main causes of disability, mortality and death all over the world.[1] It can be divided into two subgroups including ischemic or hemorrhagic. Ischemic stroke is the most common type of stroke lead to sever reduction of blood flow to the brain.[2] That is vital to restore brain blood flow to the ischemic area as soon as possible; however reperfusion of blood can cause fatal disturbances called ischemic–reperfusion (IR) injury including inflammation, apoptosis, and oxidative stress burst.[3]

Recently, remarkable studies have been made in exploring the molecular mechanisms of IR injury and neuroprotection against it[1,4,5]; however, effective therapies remain unsatisfactory. Therefore, elucidating the exact molecular mechanism of reperfusion injury and developing new targets to mitigating the damage is immediately recommended.[6]

Apoptosis has been considered as the key event for neuronal damage in cerebral ischemia.[1] The hippocampus neurons are the most vulnerable cells to ischemic hypoxia in the brain, ultimately leading to neuronal death or apoptosis.[1,7] Caspase3, cytochrome c, and p53 have a critical role in the apoptotic pathway in neuronal damage.[1,8,9] Previously, various studies reported that caspase3 and p53 knockouts mitigated ischemic injury and infarct size in hippocampus tissue.[10–12] However, the molecular mediators are not yet fully elucidated.

MicroRNAs (miRNAs) are a natural noncoding RNAs that was known as a regulator of gene expression at the posttranscriptional level. These small molecules play a key role in various biological processes such as cell proliferation and differentiation, apoptosis, and other biological events[13]

Accumulating evidence has displayed the significance of miRNAs as potential therapeutic targets in the regulation of neurological pathway and ischemic injury.[14–17] Among them miR-23b alteration was considered as a better neuronprotective effects in several neuropathic impairments including IR injury by attenuating apoptosis and improving neuronal function.[18–20] Accordingly, the expression levels of miR-23b were also decreased in the plasma, cerebral cortex and hippocampus of rats after neurological impairments.[19,21] Previously it was shown that miR-23b diminished apoptosis process by reducing the expression of TGF-β-activated kinase 1 binding protein 3 (TAB3). TAB3 is highly expressed in central nervous system and is a regulator of nuclear factor kappa B (NF-κB) which has been mainly detected in cancers. NF-κB was expressed highly in the hippocampus tissue following vigorous neuronal damage.[22] It can interact with p53 as a key regulator of cell death to activate neuronal death or apoptosis after cerebral IR model.[23] But whether miR-23b and its target molecules are involved in the pathogenesis of hippocampus injury after cerebral IR remains unclear.

Recent researches recommended that some active components in natural medicine can attain powerful effect on treating cerebral IR injury, thus can be taken for new drug development as pharmaceutical medications.[24]

Chlorogenic acid (CGA; 5-*O*-caffeoylquinic acid) as one of the most abundant polyphenols of *Coffea canephora*, *Coffea arabica* L., and Mate (*Ilex paraguariensis* A. StHil.), performs vital roles in many physiological processes as a powerful antioxidant and anti-inflammatory agent.[25] Evidence suggests that CGA has a significant neuroprotective effect against IR injury.[26] Previously, it was documented that CGA attenuated apoptosis in cerebral cortex cells.[26,27] In addition, it can attenuate the neuronal apoptosis in hippocampus after alcohol exposure in neonatal rat.[28] However, there have no data to report the protective effect of CGA against hippocampus apoptosis after cerebral IR injury in our best knowledge. At first, this research is intended to illustrate, the effect of cerebral IR on neuronal apoptosis in hippocampus and involvement of miR-23a/TAB3/NF-κB/p53 pathway in a rat model of cerebral IR. The second aim of this study was to find out whether CGA treatment has an impact on the above mentioned parameters in the hippocampus tissue after common carotid artery occlusion (CCAO).

2 | MATERIALS AND METHODS

2.1 | Animals, CCAO model, and CGA treatment

Twenty-four male Wistar rats (250 ± 20 g weight, 3–4 months old) were housed in cages with a 12-h light/12-h dark cycle at room temperature (21 ± 2°C) and performed ad libitum access to food and water. All experimental protocols were approved by the Ethics Committee of Urmia University of Medical Sciences (Ethical Code: IR.UMSU.REC.1399.073). The rats were assigned to three groups ($n = 8$): sham, IR, IR ± CGA.

Transient global ischemia model was established by using the bilateral CCAO method according to the protocol of previous studies.[29]

The rats were anesthetized using intraperitoneal injection of ketamine (60 mg/kg, ip) and xylazine (4 mg/kg, ip). After shaving the neck skin, a vertical midline-incision (∼1.5 cm length) was performed to expose both common carotid arteries. The vagal nerves were isolated from the arteries then the carotid arteries were blocked by vascular clamps. 20 min after the

induction of ischemia, the clamps removed to allow for 24 h of reperfusion. The same treatment was performed on rats in sham group except the ligation of the common carotid arteries. The body temperature (37 ± 0.2°C) was controlled by using a thermometric blanket during the experiment.

Rats in the IR ± CGA group were administered twice at doses of 30 mg/kg of CGA (ip)[30,31] at 10 min before ischemia and 10 min before the beginning of reperfusion. The dose and the injection time of CGA were applied based on the previous studies.[30–32] Accordingly, Lee et al. reported that administration of CGA at 3, 10, and 30 mg/kg ip could reduce brain infarct volume in a dose dependently manner, while only CGA at 30 mg/kg had significant neuroprotective effect.[30] In addition, another study reported that CGA at 30 mg/kg can consider as a potent neuroprotective treatment by modulating the apoptotic-related proteins.[31] The CGA was dissolved in phosphate-buffered saline (PBS) solution. Also, the sham and IR groups received PBS solution.

2.2 | Tissue preparation

Twenty-four hours after CCAO, the rats were anesthetized (ketamine 60 mg/kg in combination with xylazine 4 mg/kg, ip) and sacrificed by decapitation. The brains of rats were removed, and the two cerebral hemispheres were detached from each other. Right hippocampi from the right hemisphere were carefully isolated on ice cold plates for measurement of hippocampal gene and proteins. The left hemisphere was immersed in 10% formaldehyde for 24 h. On the next day, the left hippocampus was separated from the hemisphere and soaked in 70% ethanol solution before being dehydrated in ascending concentrations of ethanol, cleared with toluene, infiltrated, and ultimately embedded in a paraffin block.

2.3 | Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

Apoptosis of the hippocampus tissue was evaluated by the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) method.[1] The sections (5-μm thick) of formalin-fixed paraffin-embedded testis tissue were applied for TUNEL staining with In Situ Cell Death Detection Kit (Cat. Number 11684817910; Roche Molecular Chemical). The tissue sections were deparaffinized, rehydrated, and covered with 20 mg/ml proteinase K (Roche) for 15 min. Endogenous peroxidase activity was inhibited via 3% hydrogen peroxide. Samples were then mixed with 50 μl of TUNEL solution (In Situ Cell Death Detection Kit; Roche) for 1 h at 37°C. Staining was discovered using 3,3diaminobenzidine (Sigma) chromogen. The slides were then rinsed in water and mounted. Finally, the sections were assessed by a light microscope.

Apoptotic index = (number of labeled cells/total number of cells counted) × 100.

2.4 | Quantitative real-time polymerase chain reaction

Expression levels of miR-23b in hippocampus samples were examined through quantitative real-time polymerase chain reaction (qRT-PCR) method. Extraction of miRNA and synthesis of cDNA in collected samples were performed by using miRCURYTMRNA isolation kit (Exiqon) and cDNA synthesis kit. Then, cDNA was provided as a template for development of miR qRT-PCR by using the standard SYBR Green master mix (Exiqon).[33] Bio-Rad iQ5 detection System (Bio-Rad) was used for analysis of real-time PCR reactions. For miRNA RT-PCR, U6 was applied as endogenous control and the relative expression of miRNA was estimated by accurate $2^{-\Delta\Delta Ct}$ method.[34] The result was reported as the fold-change to the housekeeping gene. The following primers were used: miR-23b forward, 5′-GTCTCGAGTCGTATCCAGT-3′; miR-23b reverse, 5′-CCAGTGCAGGGTCCGAGGTA-3′; U6 sense: 5′-GGCAGCACATATACTAAAATTGG-3′; and U6 antisense: 3′-AAAATATGGAACGCTTCACGA-5′. Sequences were obtained from GenBank. The primers were verified using Gene Runner software (Syngene). The specificity of the novel primer sets tested by Oligo 7 software.

2.5 | Western blotting assay

TAB3, NF-κB, p53, cleaved-caspase3 (c-caspase3) and pro-caspase3 (p-caspase3), cytochrome c protein levels in the hippocampus tissue were determined by Western immunoblotting assay as described previously.[1] In brief, the collected tissue was homogenized and then sonicated in cold lysis buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM Tris hydrochloride, pH 7.5, 0.3 M sucrose, 5 mM ethylenediaminetetraacetic acid, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride, supplemented with a complete protease inhibitor cocktail. The homogenized tissues were centrifuged (15 min at 1000 × *g* at 4°C) to obtain the supernatant and detect proteins. The proteins were discreted through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane.

After blocking with skim milk, anti-TAB3, anti-NF-κB, anti-p53, anticaspase3, anticytochrome c antibodies were use and the concentration of the proteins was exactly measured,[1] and immunoreaction density was assessed using ImageJ software. The list of antibodies expressed in Table 1.

2.6 | Statistical analysis

The results were presented as mean ± *SEM*, and SPSS 16.0 were performed for data analyses. All parameters were tested for normality by one-sample Kolmogorov–Smirnov test. Data were statistically examined using one-way analysis of variance followed by Tukey's test. The significant level was indicated at $p < 0.05$.

3 | RESULTS

3.1 | Apoptotic cells in the hippocampus tissue

The apoptosis of hippocampus tissue was evaluated by a TUNEL assay. We found that TUNEL positive cells in IR group (2.8 ± 0.35) were significantly ($p < 0.001$) increased compared with sham (1 ± 0.0) group. Notably, when compared with the IR group, CGA treatment (1.83 ± 0.19) significantly ($p < 0.05$) reduced the number of apoptotic neurons after CCAO and reperfusion (Figure 1A,B).

3.2 | miR-23b expression in the hippocampus tissue

Real-time PCR was performed to elucidate the effects of cerebral IR injury and CGA treatment on hippocampus expression of miR-23b. In the current study, expression of miR-23b in the rat hippocampus in experimental groups is depicted in Figure 2. Cerebral IR injury led to significant decrease ($p < 0.01$) in miR-23b expression (0.8 ± 0.04) compared with the sham (1 ± 0.0) group. However, treatment with CGA (0.93 ± 0.05) has been shown to reverse this effect ($p < 0.05$).

3.3 | TAB3, NF-κB, p53, caspase3, cytochrome c protein levels in the hippocampus tissue

To estimate the effect of cerebral IR injury and CGA treatment on TAB3, NF-κB, p53, cytochrome c, and caspase3 activation, western blot analysis was carried out in hippocampus tissue obtained from different groups of study after 24 h of CCAO. Based on the Western blot results, cerebral IR injury significantly increased TAB3 ($p < .001$) (3.87 ± 0.24) and NF-κB (2.45 ± 0.33) ($p < 0.01$) protein expression in the hippocampus compared to the sham (1 ± 0.0), (1.08 ± 0.08) group (Figure 3A–C).

However, CGA treatment alleviated TAB3 (2.64 ± 0.21) ($p < 0.01$) and NF-κB (1.71 ± 0.14) ($p < 0.05$) protein expressions in the hippocampus exposed IR injury. Then, we further investigated p53 protein expression which has the ability to activate intrinsic pathway of apoptosis. As shown in Figure 4A,B, exposure to cerebral IR increased p53 protein expression (3.7 ± 0.23) in hippocampus tissue compared to sham (1.03 ± 0.03) group ($p < 0.001$), which were reversed by CGA administration (2.89 ± 0.18) ($p < 0.05$). This result indicates that p53 protein is involved in CGA-induced antiapoptosis in CCAO. Cytochrome c is a proapoptotic molecule released from mitochondria led to activation of caspase protein cascade. There was a significant increase ($p < 0.001$) of cytochrome c (3.07 ± 0.3) level in hippocampus tissue of global cerebral ischemia reperfusion rat model, whereas, CGA treatment (1.6 ± 0.21) abrogated the effects conferred by CCAO ($p < 0.001$) (Figure 4A,C). As an outstanding mediator of apoptosis, in the present study, we explored the enzymatic activity of the c-caspase3 with respect to p-caspase3 conducting a Western blot analysis.

TABLE 1 The antibodies used in Western blotting assays

Primary antibody	Company	Dilution	Catalog number
TAB3	SANTA CRUZ	1:500	sc-166538
NF-κB	SANTA CRUZ	1:500	sc-8008
p53	SANTA CRUZ	1:500	sc-126
Caspase-3	SANTA CRUZ	1:500	sc-7272
Cytochrome c	SANTA CRUZ	1:500	sc-13156
β-Actin	SANTA CRUZ	1:300	sc-130657

Abbreviations: NF-κB, nuclear factor kappa B; TAB3, TGF-β-activated kinase 1 binding protein 3.

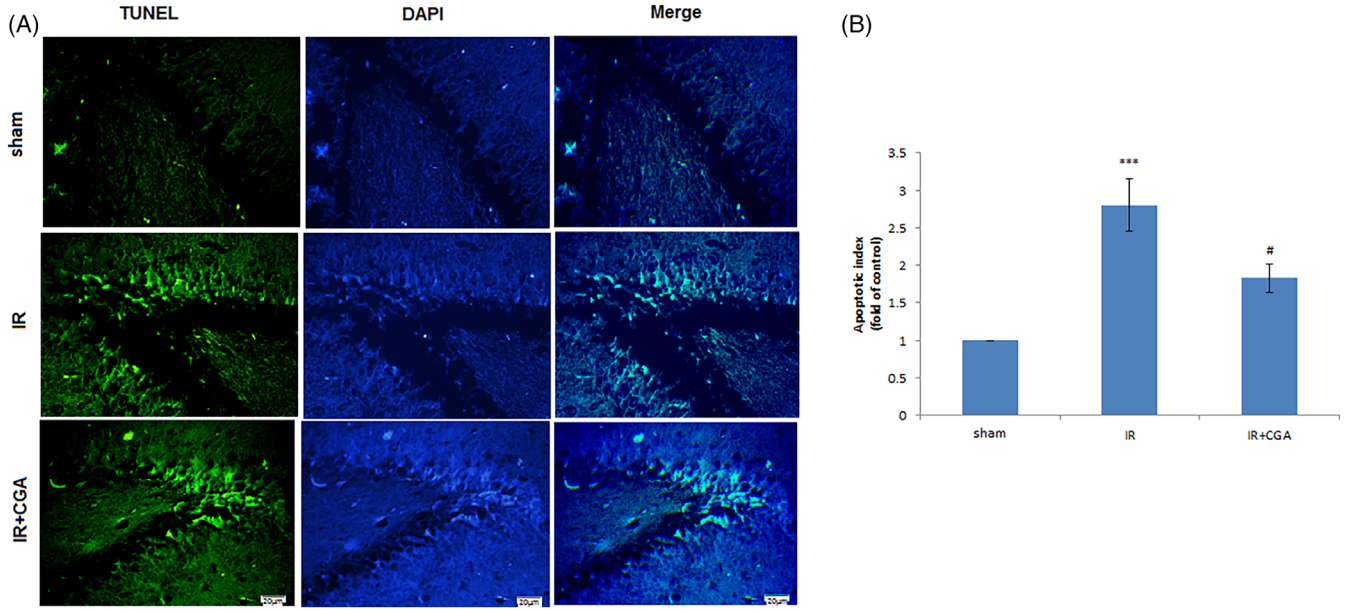
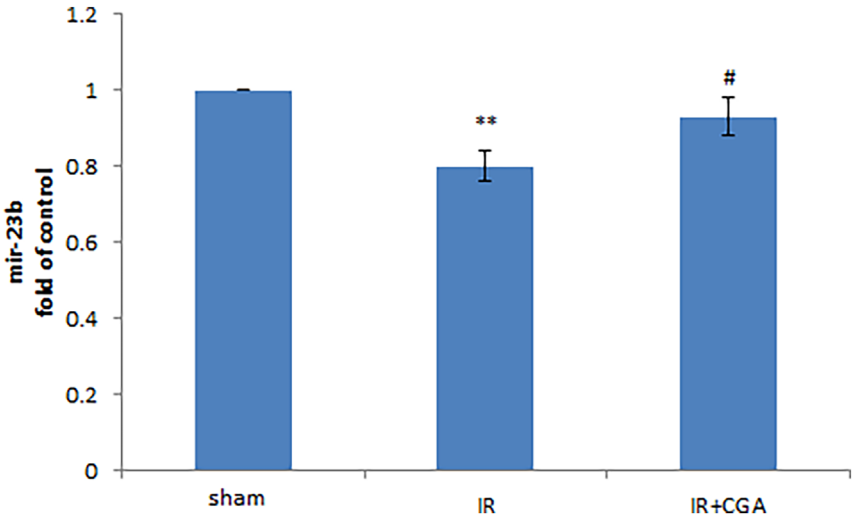


FIGURE 1 Assessment of apoptosis in different groups. (A) Images of apoptotic cells in the hippocampus tissue of rats after cerebral IR insult and CGA treatment with TUNEL staining. Magnification ×400. (B) Quantitative analysis of the apoptotic index (percentage of TUNEL-positive nuclei, %). All data are represented as the mean ± *SEM* ($n = 8$). ***$p < 0.001$ compared with sham group. $^{\#}p < 0.05$ compared with IR group. Sham; ischemia–reperfusion (IR); and ischemia–reperfusion + chlorogenic acid (IR + CGA). Scale bars are as indicated. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

FIGURE 2 miR-23b gene expression of hippocampal tissues in each group. All data are expressed as the mean ± *SEM* ($n = 8$). **$p < 0.01$ compared with sham group. $^{\#}p < 0.05$ compared with IR group. Sham; ischemia–reperfusion (IR); and ischemia–reperfusion + chlorogenic acid (IR + CGA)



As it was shown in Figure 4A,D, c-caspase3/p-caspase3 protein level markedly enhanced in the IR group (16.79 ± 2.47) in comparison with sham (1 ± 0.0) ($p < 0.001$). Nonetheless, CGA administration could alleviate this ratio (4.57 ± 0.37) in the hippocampus of rats after IR injury ($p < 0.001$).

4 | DISCUSSION

In the current study we delineate that cerebral IR injury was associated with mitochondrial apoptotic pathway, as evidenced by increased protein expression of p53, c-caspase3/p-caspase3 ratio, and cytochrome c level in hippocampus tissue. Further molecular analysis showed that cerebral IR injury elevated miR-23b, TAB3, and NF-κB protein expression in the hippocampus of male rats. Interestingly, treatment with CGA reversed the apoptotic effects of cerebral IR injury as confirmed by TUNEL staining probably mediated through miR-23b/TAB3/NF-κB axis.

Increasing researches have been made to develop a novel therapeutic potential, to reduce brain IR injury, however, there is no effective strategy to attenuating

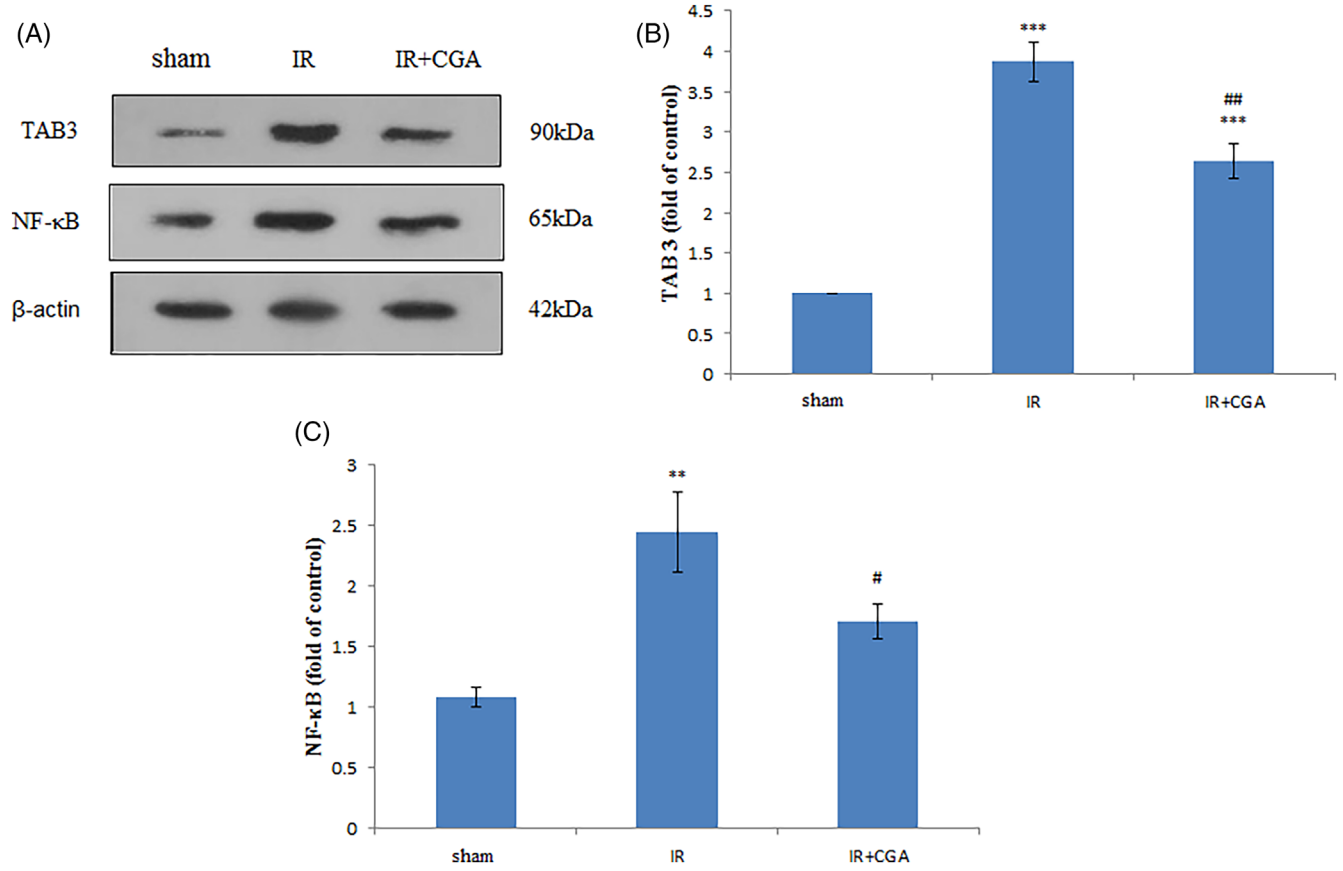


FIGURE 3 TAB3 and NF-κB protein expressions of hippocampal tissues in each group. (A) The blotting images of TAB3 and NF-κB. (B and C) the bar charts represent the quantitative analysis of TAB3 and NF-κB normalized against β-actin (β-actin band in A obtained from the same membrane). All data are expressed as the mean ± *SEM* ($n = 8$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with IR group. Sham; ischemia–reperfusion (IR); and ischemia–reperfusion + chlorogenic acid (IR + CGA). NF-κB, nuclear factor kappa B; TAB3, TGF-β-activated kinase 1 binding protein 3

neuronal damage as yet.[35] Thereupon, for developing a protective agent against ischemic insults, it is urgently needed to elucidate the mechanistic mediators thus, identification new targets and effective drugs to increase the opportunity for ischemic cerebral disease therapy. The main type of neuronal death after ischemic stroke is programmed cell death or apoptosis.[1] It was well-known that the apoptotic cells were detected in the hippocampus tissue following ischemic insult.[36] Numerous studies found that cerebral IR evoked considerable increase in apoptotic cells in the hippocampus tissue.[37–39] In agreement with these observations, in our study an increased number of apoptotic cells by TUNEL staining were detected in hippocampus tissue after cerebral IR injury.

To further investigate the mechanism of apoptotic damage caused by IR injury, we measured apoptosis-related proteins (increased p53, c-caspase3/p-caspase3 ratio, and cytochrome c level) in rat hippocampus. It was illustrating that cerebral IR-induced apoptosis in rat hippocampus via mitochondrial apoptotic pathway. p53 has been distinguished as a principle regulator of cell death by activating mitochondrial apoptotic pathway after neuronal ischemia.[22] The increase of p53 immunoreactivity was reported in hippocampal CA 1 region at an early stage following transient ischemia.[22] Mitochondrial pathway mainly triggers by release of cytochrome c from the mitochondria, activates different caspases as downstream signals.[22,40] Among them, caspase3 is the final step for apoptosis, which may induce nucleus fragmentation with the breaking of nuclear membrane in relation with overproduction of reactive oxygen species, membrane depolarization and increased concentrations of intracellular calcium.[22]

Other studies supported our results. Gao et al. reported that the protein expression of cytochrome c, p53, and caspase3 in hippocampal neuron were significantly increased in hypoxia/reoxygenation animals.[9] Moreover, other studies indicated increased gene expression of caspase3, cytochrome c, and p53 in hippocampus after transient cerebral ischemia.[22,35,41] However, there is no more information about their molecular mediators involved in apoptotic pathway.

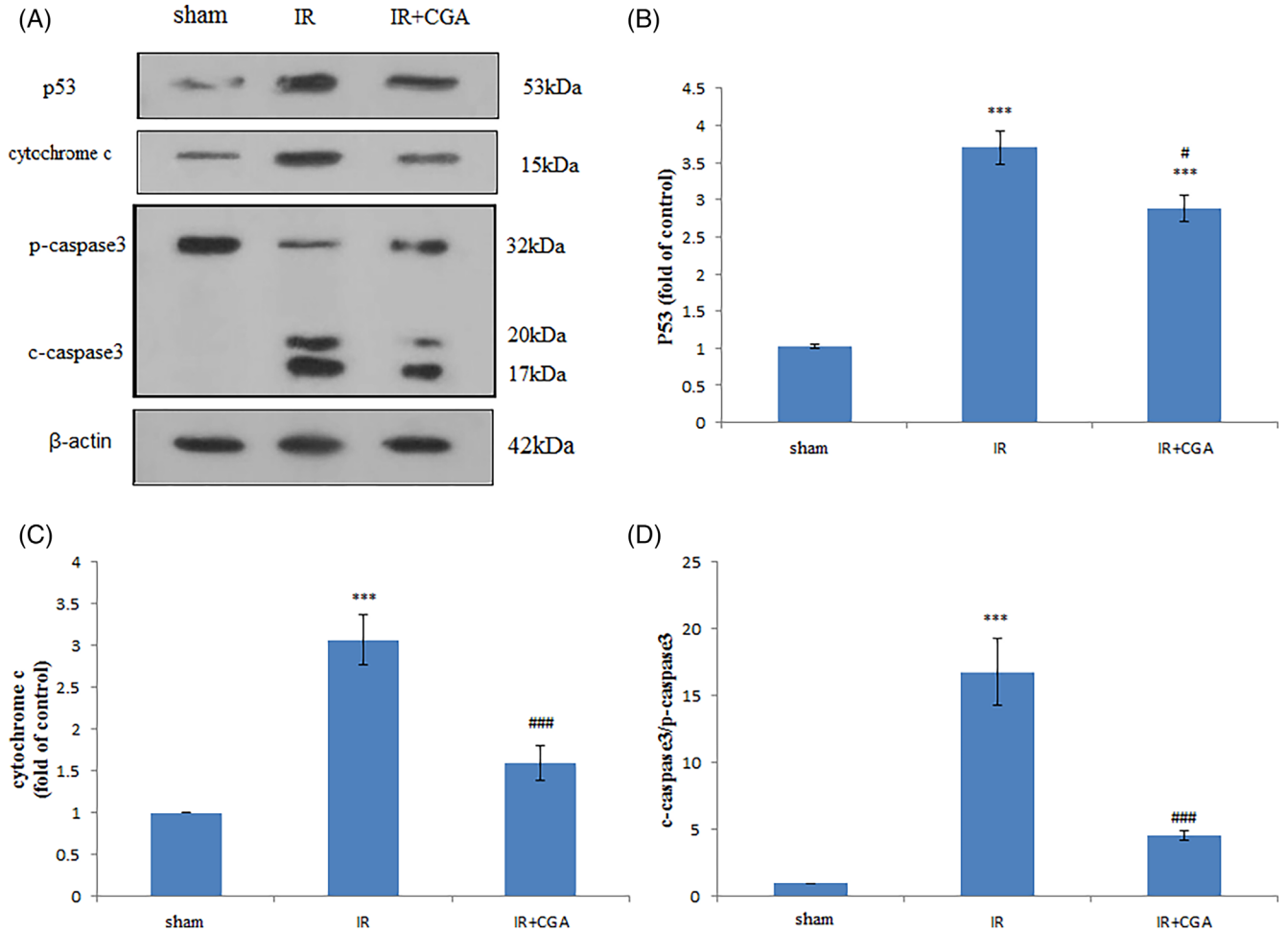


FIGURE 4 p53, Cytochrome c, and c-caspase3, p-caspase3 protein expressions of hippocampal tissues in each group. (A) The blotting images of p53, cytochrome c and also c-caspase3, p-caspase3. (B–D) The bar charts represent the quantitative analysis of p53, cytochrome c and c-caspase3/p-caspase3 protein level normalized against β-actin (β-actin band in A obtained from the same membrane). All data are expressed as the mean ± *SEM* ($n = 8$). ***$p < 0.001$ compared with sham group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with IR group. Sham; ischemia–reperfusion (IR); and ischemia–reperfusion + chlorogenic acid (IR + CGA)

Nowadays, miRs are widely accepted as key regulators of cellular survival, which could also be associated to the pathology of cerebral IR insult.[2] These small noncoding RNA may consider as the novel biomarkers and therapeutic target for ischemic stroke.[21] Previously, it was indicated that reduced expression of miR-23b was attributed to neuronal apoptosis in mouse cerebral cortex exposed to intrauterine hypoxia.[42] Wu et al. asserted that miR-23b-3p expression markedly altered in the serum of ischemic stroke patients probably in response to aggravated neurological deficits.[21] In addition, miR-23b was obviously downregulated in plasma, cerebral cortex and hippocampus of experimental traumatic brain injury (TBI) as well as in plasma of TBI patients[19] and in spinal cord injury model.[20] Zhao et al. suggested that downregulation of the miR-23b inhibited IR injury in arthroplasty and inhibit apoptosis of articular chondrocytes by regulation of the p53 signaling pathway.[43] However, the expression level of miR-23b in hippocampus in cerebral IR still needs rigorous evaluations. It is of interest that in our present study, miR-23b decreased in hippocampus undergoing cerebral IR insult. Thus, this study for the first time clarified downregulation of miR-23b levels of hippocampus tissue in cerebral IR rats and further shed new light on their usage as the potential target for effective therapy of ischemic insult.

TAB3 is an upstream positive regulator of the NF-κB pathway, which was assumed to be a target of miR-23b.[20] TAB3 can promote the NF-κB activation by binding to polyubiquitin chains.[44] Previously, it has been reported that miR-23b abolished interleukin-17, tumor necrosis factor α (TNF-α), or interleukin-1 beta expression-induced NF-κB activation through TAB3 signaling.[45] NF-κB is associated to inflammatory reaction in the brain after transient cerebral ischemia.[46] Recently, it was demonstrated that activation of NF-κB in glial cells lead to

neuronal cell death of the hippocampus region of the brain tissue in cerebral IR.[22]

Zhao et al. reported that overexpression of miR-23b mitigated the apoptosis by inhibiting TAB3 and NF-κB pathway in microglial cells in SCI model.[20] TAB3 was increased during neuronal apoptosis with an elevated expression of caspase3, caspase8, and decreased expression of B-cell lymphoma 2 (Bcl-2) in the model of intracerebral hemorrhage.[47] More importantly, the expression of apoptotic markers decreased in PC12 cells after knocking down TAB3.[47]

Based on our data, related molecular analysis showed that downregulation of miR-23b gene expression and upregulation of TAB3 and NF-κB levels were exhibited after 24 h following CCAO in hippocampus tissue. Our results support that miR-23b probably is involved in the regulation of apoptotic neuronal cell death via TAB3/NF-κB/p53 axis, which could be a novel therapeutic target for cerebral IR injury.[9]

CGA as a potent neuroprotective agent has discovered that can naturally suppress the lesion of neuronal damage in cerebral IR-induced injury in hippocampus tissue.[25,26,48–50] It reduced hippocampal calcium, nitrate and glutamate as well as TNF-α, nitric oxide synthases and caspase3[25] and also increase the erythropoietin and hypoxia-inducible factor 1-alpha level.[48]

The second point addressed in this study was that the antiapoptotic effect of CGA in hippocampus tissue as evidenced by decreasing c-caspase3/p-caspase3 ratio, cytochrome c, and p53 level. This finding is in agreement with the study of Guo et al. in which administration of CGA exerted an antiapoptotic effect by reduction of caspase3 enzyme activation in alcohol-induced brain damage in neonatal rat.[28] In cerebral IR injury rats, CGA exerts antiapoptotic effect via upregulating caspase3 and caspase9 expressions.[26]

In the models of ischemic stroke, antiapoptosis properties of flavonoid extract including CGA has been widely reported as evidenced by upregulated the expression of antiapoptotic (Bcl-2) and downregulated the apoptotic proteins (p53, Apaf1, Fas, FasL, Bax, Bid, cytochrome c and caspase3, caspase9, and caspase8).[27] On the other hand, Hermawati et al. investigated whether CGA treatment attenuated hippocampal neuronal cell death in rats subjected to transient ischemia induced by CCAO and found no significant differences in caspase3 between the groups.[29] However, we examined not only caspase3 but also cytochrome c and p53 protein level alterations among the groups. These differences could be described by the differences among the models, the time of administration, species, and age of the rats.

At the molecular levels, CGA affected miR-23b, TAB3, and NF-κB proteins, which are possibly, suggest that the protective effects of CGA on hippocampus damage might result, at least in part, from a reduction of apoptotic index by miR-23b/TAB3/NF-κB axis. This is the first study to identify the fact that cerebral IR causes hippocampus apoptosis which is closely related to miR-23b/TAB3/NF-κB pathway. This finding provides a potential and effective strategy to affect miR-23b/TAB3/NF-κB pathway in the setting of cerebral IR injury. However, there are some limitations in our study, that include not applying molecule inhibitors to validate the pathway involved in this hazardous effect of IR injury. Also, the effect of CGA pretreatment was checked only at 24 h after reperfusion and whether this protection continues at later time points remains elusive.

5 | CONCLUSION

Overall, our data describe the pathogenesis of hippocampus injury after cerebral reperfusion is linked to apoptosis partly mediated by miR-23b/TAB3/NF-κB axis. However, CGA treatment could alleviate the hippocampus apoptosis as manifested by caspase3, cytochrome c, and p53 alterations. Of note, CGA can exert this effect probably by modulating miR-23b/TAB3/NF-κB axis and therefore favoring hippocampus neuron survival in the context of cerebral IR injury.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Shiva Roshan-Milani: Conceptualization, methodology, writing – review and editing. **Parisa Sattari:** Data curation. **Firouz Ghaderi-Pakdel:** Review and editing. **Roya Naderi:** Conceptualization, data curation, formal analysis, methodology, project administration, supervision, validation, visualization, writing – original draft, and writing – review and editing.

DATA AVAILABILITY STATEMENT

Data will be available, when it is needed.

ORCID

Roya Naderi https://orcid.org/0000-0001-8529-7306

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