
Archives of Razi Institute Journal

Volume 80, NO. 4, 2025

Journal homepage: https://archrazi.areeo.ac.ir

Original Article

Mir-429 and GATA4 May Participate in Cerebral Ischemic Stroke by Regulating Autophagy and Apoptosis: the Impact of Chlorogenic Acid

Rahil Salimi[1], Alireza Shirpoor[2], Roya Naderi[2,3*]

1.Student Research Committee, Urmia University of Medical Sciences, Urmia, Iran.
2.Department of Physiology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran.
3.Neurophysiology Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran.

Article Info:

Received: 27 April 2024
Revised: 22 July 2024
Accepted: 29 July 2024

Keywords:

Ischemia-Reperfusion, Brain, Chlorogenic Acid.

Corresponding Author:
naderi.r@umsu.ac.ir

ABSTRACT

Autophagy is a double-edged sword for maintaining neural system homeostasis during the development of cerebral ischemia. However, the potential molecular mechanisms behind this remain unclear. Changes in miR-429 and its target GATA4, along with autophagy mediators and apoptosis in ischemic stroke, were examined in this research. Additionally, the study investigated these factors in combination with chlorogenic acid (CGA). Male Wistar rats were separated into three categories. (n=8): sham, IR (ischemia-reperfusion, Induction of transient cerebral ischemia via occlusion and reperfusion of the common carotid artery.) and IR+CGA (30 mg/kg, ip; administered intraperitoneally, 10 minutes before the onset of ischemia and 10 minutes prior to reperfusion). Levels of miR-429, GATA4, c-Caspase-3 / p-Caspase-3 ratio, LC3-I, LC3-II, Beclin1 and p62 were assessed using Real- time PCR and Western blot assays. At the end of the experiment, increased miR-429 gene expression ($P<0.05$) and c-Caspase-3/p-Caspase-3 ratio ($P<0.01$), along with decreased GATA4 protein expression ($P<0.001$), were observed in IR group. In addition, the brains of CCAO rats displayed significantly increased autophagy activation, as evidenced by an increased LC3-II/I ratio and Beclin1 protein expression, and decreased p62 expression after 24 h of reperfusion ($P<0.001$). Immunohistochemistry studies has alsorevealed that the ratio of overall LC3 immunoreactivity in the cortex tissue of male rats was significantly increased by cerebral IR ($P<0.001$). Treatment with CGA significantly attenuated autophagic activity and apoptosis, reversing the aforementioned molecule levels. Taken together, these results suggested that ischemic insult can increase autophagic activities and apoptosis, possibly through miR-429 and GATA4 alterations in the brain cortex following cerebral IR insult, which can be alleviated by CGA as a potential therapy for individuals affected by ischemia.

How to cite this article: **Salimi R, Shirpoor A, Naderi R. Mir-429 and GATA4 May Participate in Cerebral Ischemic Stroke by Regulating Autophagy and Apoptosis: the Impact of Chlorogenic Acid. *Archives of Razi Institute*. 2025;80(4):945-955.** DOI: 10.32592/ARI.2025.80.4.945

1. Introduction

Ischemic stroke is a major cause of morbidity, mortality, and permanent disability in aging populations worldwide. It is currently addressed through intravenous or intra-arterial thrombolysis,which remains the only clinically helpful therapeutic strategy for ischemic stroke. However, nerve cell damage often worsens upon restoration of blood flow, a condition known as ischemia/reperfusion (IR) injury (1). The central nervous system (CNS) damages have limited repair capacity (2) making it urgent to elaborate delicate mechanisms of stroke injury to identify a potential target for effective therapy.

Autophagy is an intracellular degradation system that facilitates the phagocytosis of intracellular macromolecules and damaged organelles (3). It has been described that autophagy participates in different pathophysiological processes including inflammation, apoptosis, cancer, and IR injury (4). Autophagy has been shown to be a significant regulatory pathway for IR injury, and excessive autophagy could lead in extensive neuronal death during cerebral ischemia. (3). The development of autophagosomes is a critical stage in the process of autophagy. Beclin-1, LC3, and p62 are important proteins involved in the autophagy: Beclin-1 facilitates the transport of other autophagic proteins, LC3 is converted to LC3-II during autophagosome formation, and p62 regulates the degradation of misfolded proteins. The levels of these proteins serve as biomarkers for assessing autophagic activity (4).

Increased autophagy markers have been observed in rat cortical neurons following ischemic stroke, and suppression of neuronal autophagy induces a neuroprotective effect on rats subjected to IR (4). Therefore, the targeting neuronal autophagy induced by ischemic stroke mediators is assumed as an attractive strategy for ischemic stroke treatment.

MicroRNAs (miRNAs, miRs) are small, single-stranded non-coding RNAs that influence physiological or pathological functions by targeting the 3′-UTR of specific mRNAs. miRNAs have recently been identified as important regulatory factors and potential prognostic biomarkers for cerebral ischemia-reperfusion injury, primarily through their roles in controlling apoptosis and autophagy (5). miR-429 has been recognized as a novel miRNA that contributed to neuronal damage induced by oxygen–glucose deprivation and reoxygenation (6).

MiR-429 belongs to the miR-200 family, and its activation can be induced by hypoxia-inducible factor 1α, which is associated with hypoxic conditions. Xiao et al. have indicated that miR-429 plays a significant role in reducing neuronal damage following the oxygen-glucose deprivation/ reoxygenation process (7).

The latest information suggests that miR-429 modulates the autophagy process during myocardial ischemia/reperfusion injury and could be a potential therapeutic target for treating myocardial infarction (8). Interestingly, its involvement in ischemic stroke has been recognized. Jie et al. stated that decreasing miR-429 significantly reduces neuronal damage caused by cerebral ischemia and reperfusion injury by increasing the expression of GATA-binding protein 4 (GATA4) in a laboratory setting (7). According to Xia et al., bioinformatic analysis revealed that miR-429 regulates GATA4 gene expression by targeting the 3'-untranslated region of GATA4. When overexpressed, miR-429 alleviates neuronal injury induced by oxygen-glucose deprivation and reoxygenation (7).

GATA4 was known as an anti‑autophagic factor and a target gene of miR-429. Zinc finger transcription factor GATA4 (7) has been observed in both fetal and adult central nervous system and plays a significant role in both proliferation and apoptosis (9). Kobayashi et al. previously suggested that GATA4 protected cardiomyocytes from hypoxia-induced ischemia/reperfusion damage both in vitro and in vivo. (10). In addition, it was identified that GATA4 overexpression prevented autophagy following anti-tumor drug in cultured neonatal rat cardiomyocytes (11). However, the roles of miR-429 and GATA4 in cerebral ischemia- reperfusion damage remains undefined. Chlorogenic acid (CGA) as, is a phenolic compound widely found in plants, fruits, different kinds of vegetables, and beverages such as tea and coffee (12, 13).

Recently, there has been growing evidence suggesting that CGA is often used in the treatment of various central nervous system (CNS) conditions, such as depression (14) and neurodegenerative insults (15). In prior research, scientists have concentrated on CGA's protective impacts against ischemia-reperfusion injury due to its antioxidative, anti-inflammatory, and anti-apoptotic properties (12).

However, whether CGA mitigates cerebral ischemia-reperfusion injury by controlling autophagy and its

potential mechanism remains unclearand requires clarification and investigation.

In this research, we aimed to examine changes in autophagy-related molecules, apoptosis, and miR-429/GATA4 in the brain cortex following ischemia/reperfusion in rats. Additionally, we assessed the impacts of CGA on these factors in a rat model of cerebral ischemia/reperfusion.

2. Materials and Methods

2.1. Animals

Twenty-four male Wistar rats (250±20 gr body weight aged 3-4 months) were supplied from the standard laboratory animal house of Urmia University of Medical Sciences. The rats were housed under standard laboratory conditions at room temperature (21±2°C) on a 12-hour light/dark cycle, with free access to rat chow and tap water. The procedures involving animals were conducted in accordance with the guidelines of the Ethics Committee of Urmia University of Medical Sciences (Ethical Code: IR.UMSU.REC.1399.298).

2.2. Animal Groups and Treatment

The animals were equally and randomly divided into 3 groups (n = 8 per group) (Figure 1):

1. Sham group: Rats were given an equal volume of PBS solution intraperitoneally.
2. IR group (ischemia-reperfusion): Cerebral IR was established by bilateral common carotid occlusion for 20 minutes. Rats also were given an equal volume of PBS solution intraperitoneally.
3. IR±CGA group (ischemia-reperfusion+Chlorogenic acid). The ip injection of CGA (30mg/kg) occurred 10 minutes before the onset of local ischemia and 10 minutes prior to reperfusion (16). CGA was dissolved in a solution containing phosphate-buffered saline (PBS) (1 mL of PBS was used to dissolve every 3 mg of CGA powder).

2.3. Induction of local Cerebral I/R of Rats

The bilateral common carotid occlusion (CCAO) was used to induce the cerebral IR, following previously described methods (17). In summary, all animals were anesthetized using ketamine (60 mg/kg, intraperitoneal) and xylazine (8 mg/kg, intraperitoneal). Throughout the procedure, their body temperature was maintained at 37◦C using a thermometric blanket.

The surgical site was uncovered, and a vertical incision(approximately 1.5 cm long) was made in the neckto reveal both common carotid arteries, which were carefully separated from the surrounding tissues and the vagus nerve. Global ischemia was induced by tying off both carotid arteries. After 20 minutes of occlusion, blood flow was restored through both carotid arteries. The sham group underwent the same surgical procedure without clamping the carotid arteries.

2.4. Tissue Preparation

At 24 hours after reperfusion, animals were anesthetized with a combination of ketamine (60 mg/kg, ip) and xylazine (8 mg/kg, ip). Then brain was gently removed from the skull and washed with a cold saline solution (0.9%). The right hemisphere was placed on ice - cold plates, immediately frozen in liquid nitrogen, and stored at deep freeze (-80°C) for measurement of genes and proteins. The 10% formaldehyde was used to immerse the left hemisphere for immunohistochemical analyses.

2.5. Quantitative Real-Time PCR

miR-429 expression levels in cortex samples were evaluated using qRT-PCR method. The miRCURYTMRNA isolation kit (Exiqon, Vedbaek, Denmark) and a cDNA synthesis kit were utilized for miRNA extraction and cDNA synthesis in the cortex tissues. The microRNA quantitative real-time PCR was carried out using the synthesized cDNA as a template and the standard SYBR Green master mix (Exiqon, Vedbaek, Denmark). Real-time PCR reactions were analyzed using the Bio-Rad iQ5 PCR Detection System (Bio-Rad, Richmond, CA, USA). U6 was used as the internal control for miRNA RT-PCR, and the data were calculated using the $2^{-(\Delta\Delta Ct)}$ method. The primer sequences are as follows:

miR-429 forward, 5-CCAGTGCAGGGTCCGAGGTA -3;

miR-429 reverse, 5-GTCTCGAGGTAATACTGTCTG-3;

U6 sense: 5-GGCAGCACATATACTAAAATTGG; and

U6 antisense: 3-AAAATATGGAACGCTTCACGA -5.

Sequences were acquired from Gen Bank (http://blast.ncbi.nlm.gov/Blast.cgi). The primers were verified by using Gene Runner software (Syngene, Cambridge, UK). I verified the specificity of the new primer sets by utilizing Oligo 7 software.

2.6. Immunohistochemical Staining

Following tissue dehydration, the specimens were embedded in paraffin and sectioned at 5 µm thickness for immunohistochemical evaluation of LC3 expression.

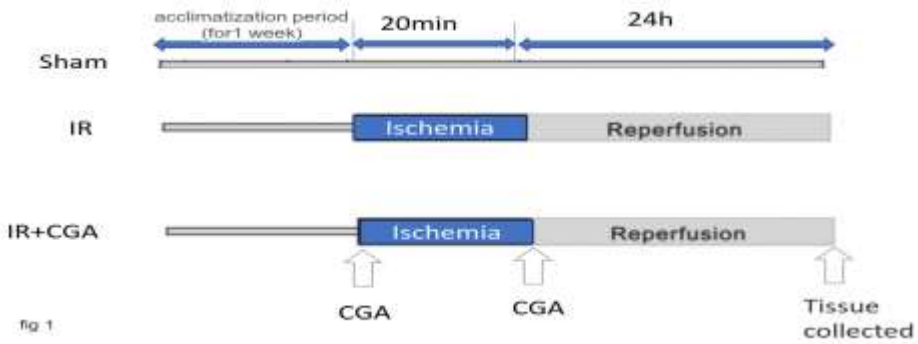

Figure1. Experimental design showing time schedule of CGA administration, I/R, and sampling.

In brief, the sections were washed three times (5 minutes each) with 0.01 mol/L phosphate-buffered saline (pH 7.2-7.4), then treated with 10% normal rabbit serum at 37°C for approximately 1 hour to block nonspecific binding. The sections were then exposed to rabbit anti-rat LC3 primary antibody (1:400 dilution, Sigma) at 37°C for about 4 hours, followed by incubation at 4°C for 48 hours. After rinsing with PBS, sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution, Beijing Zhongshan Biotechnology Co., Ltd) at 37°C for 1 hour.

Sections were washed with 0.1 mol/L Tris-HCl buffer for about 5 minutes, then incubated in DAB (0.05%) and 0.05 mol/L Tris–HCl buffer and with 1 to 2 drops of 3% hydrogen peroxide for 5 - 15 minutes until color change occurred. Following this, the slides were dipped into 0.05 mol/L Tris–HCl buffer to halt the reaction. The slides were allowed to dry and then mounted with a cover slip. Rat cerebral cortex was were used as a positive control, and 1:400 diluted normal rabbit serum served as the negative control.

2.7. Western Blotting Assay

The expression of LC3-I,II, Beclin1, p62, and GATA4 proteins in the cortex tissue was analyzed using Western immunoblotting, based on previous research. To summarize, the brain tissues were homogenized and sonicated in cold lysis buffer containing: (1% Triton X-100, 0.1% SDS, 50mM Tris-HCl, pH 7.5, 0.3M sucrose, 5mM EDTA, 2mM sodium pyrophosphate, 1mM sodium orthovanadate, and 1mM phenylmethylsulfonyl fluoride, supplemented with a complete protease inhibitor cocktail). Each homogenate was centrifuged at 1000 × g at 4 °C for 15 minutes.

The collected supernatant was used for protein detection. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Following treatment with skim milk, we utilized anti-LC3-I,II, anti-Beclin1, and anti-p62 antibodies to accurately measure the protein concentration. The density of the immunoreactive bands was assessed using Image J software. The details of the antibodies used are listed in table 1.

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2.10. Quantitative Real-Time PCR

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Table1. The antibodies used in Western blotting assays.

Primary antibody	Company	Dilution	Catalog number
LC3B	abcam	1:3000	ab51520
P62	SANTA CRUZ	1:500	sc-10117
Beclin1	SANTA CRUZ	1:500	sc-48341
Caspase-3	SANTA CRUZ	1:500	sc-7272
GATA4	SANTA CRUZ	1:500	sc-25310
β-Actin	SANTA CRUZ	1:300	sc-130657

The miRCURYTMRNA isolation kit (Exiqon, Vedbaek, Denmark) and a cDNA synthesis kit were utilized for miRNA extraction and cDNA synthesis in the cortex tissues. The microRNA quantitative real-time PCR was carried out using the synthesized cDNA as a template and the standard SYBR Green master mix (Exiqon, Vedbaek, Denmark). Real-time PCR reactions were analyzed using the Bio-Rad iQ5 PCR Detection System (Bio-Rad, Richmond, CA, USA). U6 was used as the internal control for miRNA RT-PCR, and the data were calculated using the $2^{-(\Delta\Delta Ct)}$ method. The primer sequences are as follows:

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2.11. Immunohistochemical Staining

Following tissue dehydration, the specimens were embedded in paraffin and sectioned at 5 μm thickness for immunohistochemical evaluation of LC3 expression. In brief, the sections were washed three times (5 minutes each) with 0.01 mol/L phosphate-buffered saline (pH 7.2-7.4), then treated with 10% normal rabbit serum at 37°C for approximately 1 hour to block nonspecific binding.

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Each homogenate was centrifuged at 1000 × g at 4 °C for 15 minutes. The collected supernatant was used for protein detection. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Following treatment with skim milk, we utilized anti-LC3-I,II, anti-Beclin1, and anti-p62 antibodies to accurately measure the protein concentration. The density of the immunoreactive bands was assessed using Image J software. The details of the antibodies used are listed in table 1.

3. Results

3.1. Mir-429 Expression in Cerebral Cortex

To examine the levels of miR-429 in various categories, we analyzed the presence of miR-429 in the brain cortex using Real-time PCR. The results revealed a significan increase t ($P<0.05$) in the expression of miR-429 (1.95 ± 0.34) in the ischemia-reperfusion group compared to the sham group. Notably, administration of CGA significantly reduced miR-429 expression (0.68 ± 0.29, $P<0.05$) (Figure 2).

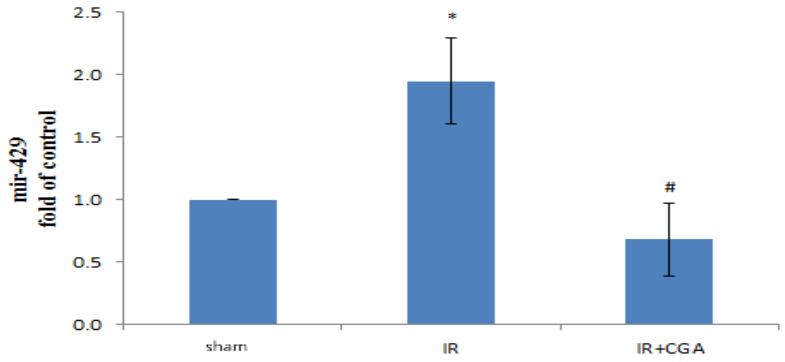

Figure 2. miR-429 gene expression of cerebral cortex tissues in each group. All data are expressed as the means ± SEM (n = 8). *P<0.01 compared with sham group. #P<0.05 compared with IR group. Sham); ischemia reperfusion (IR); and ischemia reperfusion + Chlorogenic acid (IR+CGA).

3.2. Proteins related to autophagy in the cerebral cortex

To evaluate the impact of CGA on the regulation of autophagy in cerebral I/R injury, visibly the expression of LC3, a usual autophagy marker, was discovered using immunohistochemical staining. As shown in Figure 3, the expression of LC3 protein significantly increased in the cortical region as a reaction to CCAO., which was further decreased by CGA treatment.

In addition, CCAO significantly up-regulated Beclin-1 protein expression (4.24 ± 0.27) and LC3II/LC3I ratio (8.24 ± 1.11), while down-regulated p62 protein expression (0.44 ± 0.02) using western blotting technique (P<0.001). The administration of CGA further reduced the increase in Beclin-1 protein level and LC3II/LC3I ratio, and also significantly reversed the decrease in p62 level (P<0.001) (2.41 ± 0.19) (1.5 ± 0.25) (0.61± 0.06) (Figure 4 a,b,c,d), indicating that CGA alleviated autophagy after cerebral I/R injury in male Wistar rats.

3.3. GATA4, c-Caspase-3 / p-Caspase-3 ratio in the cerebral cortex

To explore the effect of cerebral IR injury and CGA treatment on apoptosis, western blot techniques was performed in the cerebral cortex to reveal Caspase-3 activation. Based on our analysis, cerebral IR injury significantly increased c-Caspase 3 / p-Caspase 3 protein amount (P<0.01) (43.01±11.72) in the IR group in comparison with the sham group (1 ± 0.0). However, CGA treatment could markedly alleviate this ratio (4.82 ±1.05) in the cortical region exposed to IR injury (P<0.01) (Figures 5 a,b).

Then, we further measured GATA4 protein expression ,recognized as an anti-autophagic protein, by western blotting. As indicated in Figures 5 a and 5c, GATA4 protein expression significantly decreased in the cerebral cortex compared to sham group (P<0.001) ,as a result of exposure to cerebral IR (0.56 ±0.06), which were increased after CGA treatment (0.86 ±0.08) (P<0.01).

4. Discussion

The findings of this study demonstrated that cerebral IR at 24 hours activated both autophagy and apoptosis in the cortical region of male Wistar rats subjected to the CCAO model of ischemic stroke. The autophagy and apoptosis induced by cerebral I/R were reduced after the administration of CGA treatment. These results explored a novel mechanism of the neuroprotective function of CGA on IR-induced cerebral damage. To our knowledge, this study is the first to demonstrate that, apart from apoptosis, CGA may significantly reduce IR‐induced autophagy in male Wistar rats.

Cerebral ischemic stroke remains a global health challenge, contributing to high levels of illness, death, and long-term impairment. I/R events inevitably lead to intensive secondary injury in brain cells such as severe inflammation, oxidative stress, autophagy, and apoptosis (13, 18-20). Previous reports have indicated that CGA has been authorized to produce neuroprotective impacts in cerebral ischemia-reperfusion injury by reducing cerebral lesions, BBB disruption, as well as brain edema (12).

Although the precise mechanisms of its protective effects against IR injury remain unclear, previous studies have demonstrated that CGA reduces levels of proinflammatory cytokine, apoptotic markers, calcium, nitrate, and glutamate levels in different regions of the brain (1, 21).

Autophagy is a survival pathway that can promote intracellular protein damages and organelles for maintaining the stability of the intracellular environment.

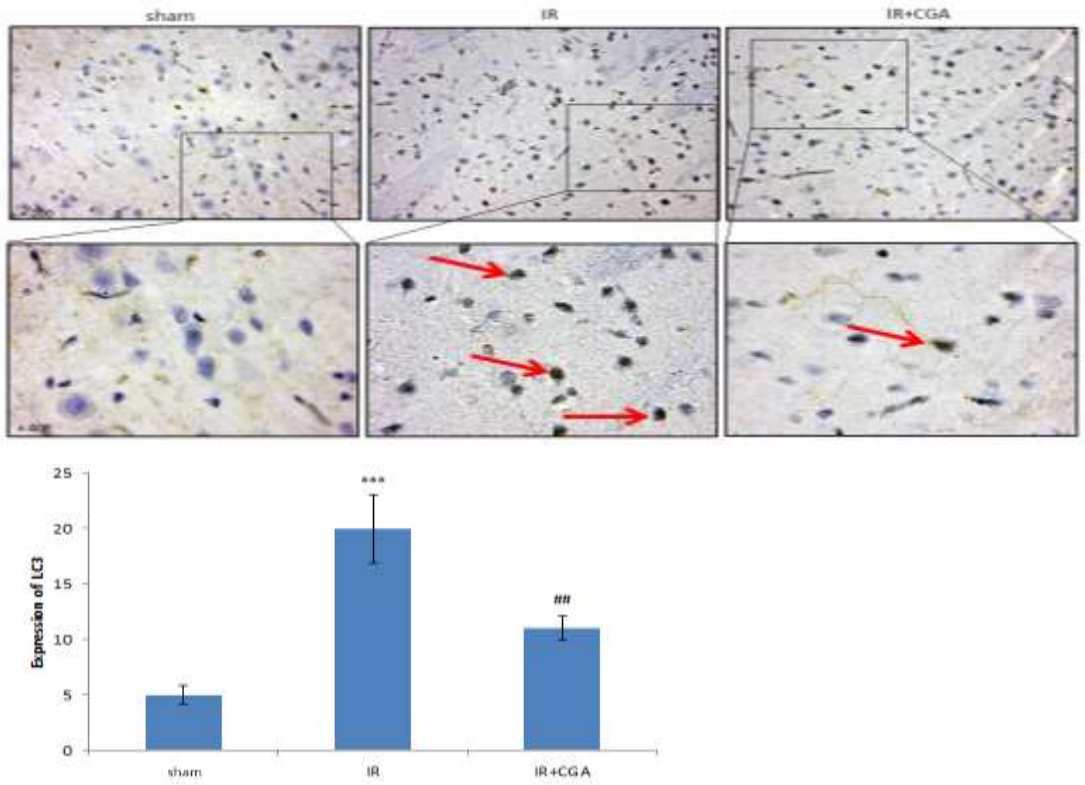

Figure 3. a) Immunohistochemical staining for LC3 in different groups; Sham; ischemia reperfusion (IR); and ischemia reperfusion + Chlorogenic acid (IR+CGA). **b)** Quantitative analysis of LC3-positive stained cells. All data are represented as mean±SEM, * P<0.001 compared with sham group. ## P<0.01 compared with IR group. Scale bars are as indicated. LC3-positive cells (⟶) Magnification = ×400.

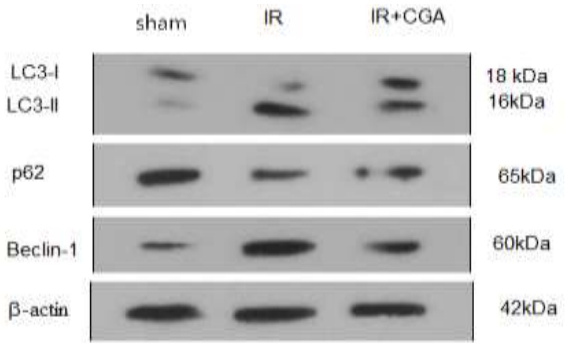

fig 4a

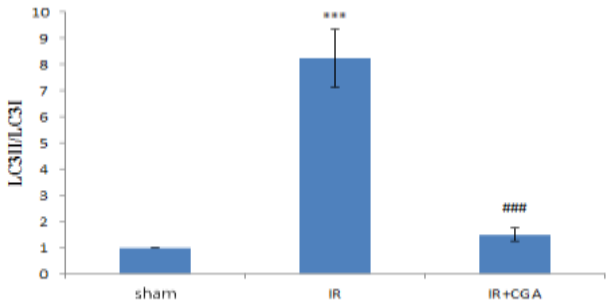

fig 4b

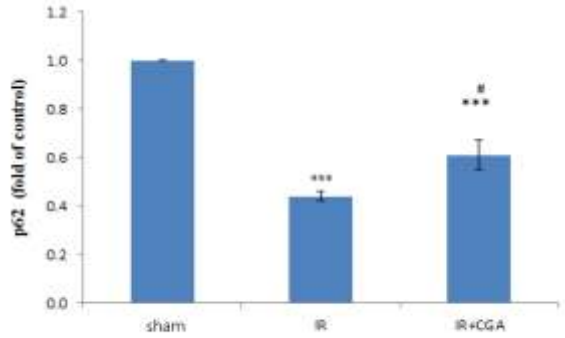

fig 4c

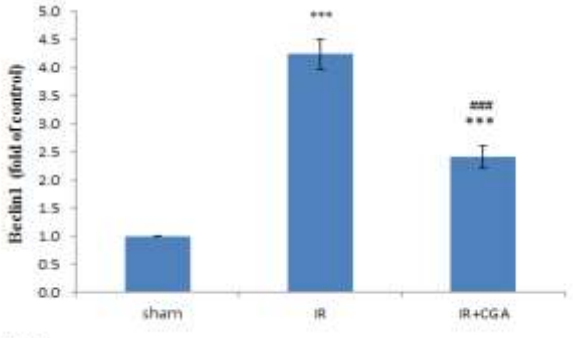

fig 4d

Figure 4. LC3-I/LC3-II ratio. p62 and Beclin1 protein expressions of cerebral cortex tissues in each group a) The blotting images of LC3-I/LC3-II ratio. p62 and Beclin1 b,c,d) The bar charts represent the quantitative analysis of LC3-I/LC3-II ratio. p62 and Beclin1 normalized against β-actin. All data are expressed as the means ± 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).

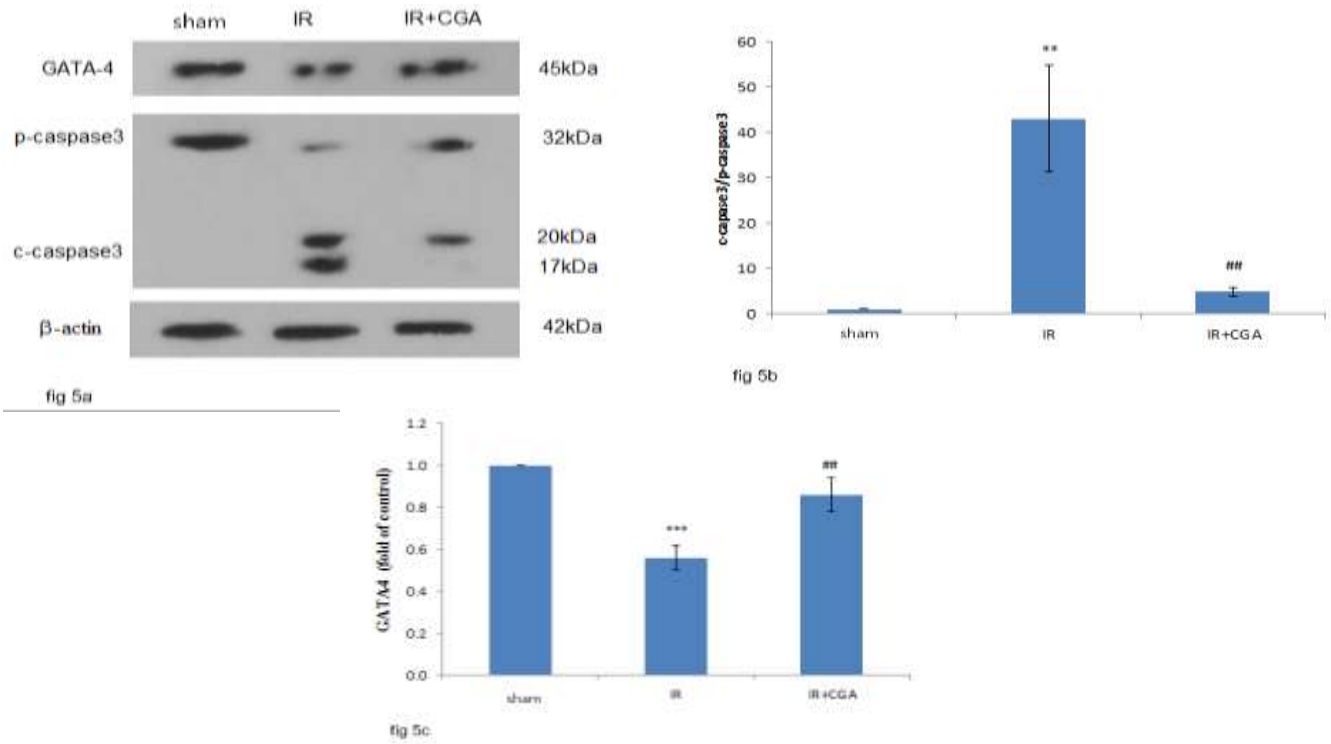

Figure 5. GATA4 and c-Caspase3, p-Caspase3 protein expressions of cerebral cortex tissues in each group a) The blotting images of GATA4 and c-Caspase3, p-Caspase3 b,c) The bar charts represent the quantitative analysis of GATA4 and c-Caspase3, p-Caspase3 protein level normalized against β-actin. All data are expressed as the means ± SEM (n = 8). ** P<0.01, *** P<0.001 compared with sham group. ##P<0.01 compared with IR group. Sham; ischemia reperfusion (IR); and ischemia reperfusion + Chlorogenic acid (IR+CGA).

Autophagy is neuroprotective after IR damage by mitochondrial effect, but extreme activation of autophagy may cause cell death and apoptosis mediated by several proteins. several studies have revealed that cerebral IR injury can increase autophagy in neuronal cells, which mediates IR-induced neurotoxicity. Moreover, blocking autophagy with 3-MA has been shown to prevents neuronal injury (4). Considering these studies and our discoveries, we conclude that overactivated autophagy is a pivotal factor for neuronal damage and also apoptosis during cerebral ischemia/reperfusion damage. Therefore, blocking autophagy might be another way to reduce neuronal harm.

In the current study, our results demonstrated that IR triggered the autophagy and apoptosis, which is in agreement with other studies (22). CGA could inhibit cerebral IR-induced autophagy in cortical neurons, hence after CGA treatment, as immunoblotting showed, the ratio of LC3II/LC3I and Beclin-1 reduced while the protein expression of p62 increased in the cortex tissue. Our results align with other studies confirming the anti-autophagic activity of CGA in lead-induced developmental neurotoxicity (23), Alzheimer's disease (24), and Parkinson's disease (21).

Therefore, CGA appears to reverse over-activated autophagy following cerebral IR, thereby alleviatig brain cell death and apoptosis, as evidenced by reducing caspase-3 activation.

The loss of nerve cells is a crucial part of stroke pathophysiology, and growing evidence suggests that miRNAs can play a critical role in regulating apoptosis and autophagy. Small non-coding RNAs can serve as innovative biomarkers for the identification and prediction of ischemic brain damage (7). miR-429 has been noticed to be associated with apoptosis and has an important role in sprogression of several diseases (25). For example, miR-429 overexpression enhanced apoptosis in some pathologies such as, ischemic hippocampal neurons (25) while its downregulation improves neurological deficit following traumatic brain injury (5) and protect brain neurons in hypoxia-ischemia damage in the model of neonatal mice with negatively regulated apoptosis (25).

Notably, Zhu et al. showed that miR-429 antagonism alleviates anoxia/reoxygenation injury in cardiomyocytes by regulating apoptosis and autophagy. (8). Consistently, the current study demonstrated that miR-429 was remarkably up-regulated in the brain tissue of CCAO rats,

pointing to a pivotal role of miR-429 in the stroke pathology.

Previous research has identified GATA4 as a direct target of miR-429. Down-regulation of miR-429 reduces hypoxia- reoxygenation -induced neuronal apoptosis by up-regulating GATA4 expression in-vitro (7). GATA4 was most highly expressed in both embryonic and adult CNS tissues (7, 9). Kobayashi 2009 et al., declared that GATA-4 expression decreased in the cardiomyocytes exposed to Doxorubicin, and preservation of GATA4 mitigates this cardiotoxicity by suppressing autophagy through alteration of the expression of Bcl2 and transcription of autophagy-related genes (11).

In agreement with these studies, in this study, we developed that miR-429 was up-regulated and GATA4 protein expression was significantly down-regulated in cortical neurons subjected to ischemia24 hours after reperfusion, which was reversed by CGA treatment. These results help improve our knowledge of the development of ischemic stroke. So, this is the first study by itself to suggest that miR-429/GATA4 axis is a potential target for therapy in cerebral ischemic insult.

In conclusion, the current research showed that autophagy is induced by cerebral IR in the cortical region of the rat brain, which contributes to IR-induced neuronal death. Treatment with chlorogenic acid (CGA) can attenuate IR-induced neuronal autophagy and apoptosis.

miR-429 and GATA4 might play a role in controlling autophagy during cerebral IR, and that CGA can counteract these effects. Targeting the miR-429 and GATA4 could be an effective treatment approach for ischemic stroke. However, more research using transgenic animal models is needed to explore the molecular mechanisms.

Acknowledgment

This research was supported by the Neurophysiology Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran

Authors' Contribution

Concept and design of the study: R. N, A. S.
Collection of data: R. S.
Analysis and interpretation of data: R. N, R. S.
Drafting of the manuscript: R. N.
Critically revising the manuscript for significant intellectual content: R. N.
Statistical analysis: R. S.
Support in the form of administration, technology, and materials: R. N.

Ethics

All animal procedures were conducted in accordance with the guidelines of the Ethics Committee of Urmia University of Medical Sciences and were approved under Ethical Code: IR.UMSU.REC.1399.298.

Conflict of Interest

The authors declare no competing interests.

Funding

None.

Data Availability

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

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