miR-429 and GATA4 may participate in cerebral ischemic stroke by regulating autophagy and apoptosis: the impact of Chlorogenic acid

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Conflict of interests

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Running title: Chlorogenic acid modulates autophagy and apoptosis in stroke

Abstract

Autophagy is a double-edged sword for maintaining neural system homeostasis during development of cerebral ischemia. However, the potential molecular mechanisms behind that remain unclear. The miR-429 and its target GATA4 changes, along with autophagy mediators

and apoptosis in ischemic stroke, were examined in this research. Additionally, the study looked at 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.), IR+CGA (30 mg/kg, ip; 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, we observed increased miR-429 gene expression ($P<0.05$) and c-Caspase-3/p-Caspase-3 ratio ($P<0.01$) as well as decreased GATA4 protein expression ($P<0.001$) in IR group. In addition, the brain of CCAO rats displayed significantly increased autophagy activation as evidenced by an increased ratio of LC3-II/I and Beclin1 protein expression and decreased expression of p62 after 24 h of reperfusion ($P<0.001$). Also, studies using immunohistochemistry revealed 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 as well as apoptosis and reversed aforementioned molecule levels. Taken together, these results suggested that ischemic insult can increase autophagic activities and apoptosis possibly by miR-429 and GATA4 alterations in the brain cortex after cerebral IR insult which can be alleviated by CGA as a potential therapy for someone afflicted with ischemia.

Keywords: ischemia-reperfusion; brain; chlorogenic acid

1. Introduction

Ischemic stroke is a serious cause of morbidity, mortality, and permanent disability in aging populations all over the world. It is addressed through intravenous or intra-arterial thrombolysis as the only clinically helpful therapeutic strategy for ischemic stroke. Nerve cell damage worsens upon restoration of blood flow, a condition known as ischemia/reperfusion (IR) injury (1). Central nervous system (CNS) damages have limited repair capacity (2). Therefore, it is urgent to elaborate delicate mechanisms of stroke injury in order to establish a potential target for effective therapy.

Autophagy is known as an intracellular degradation system that allows 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 an excess of autophagy could result in extensive neuronal death during cerebral ischemia. (3). The development of autophagosomes is a vital stage in the process of autophagy. Beclin-1, LC3, and p62 are important proteins involved in autophagy, with Beclin-1 aiding in the transport of other autophagic proteins, LC3 being converted to LC3-II during autophagosome formation, and p62 regulating the degradation of misfolded proteins. Levels of these proteins can be used as biomarkers to assess autophagic activity (4).

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

MicroRNAs (miRNAs, miRs) are small non-coding RNAs that are single-stranded and can typically influence physiological or pathological functions by targeting the 3′-UTR of specific mRNAs. miRNAs have recently been identified as important regulatory factors that could potentially be used as prognostic biomarkers for cerebral ischemia-reperfusion injury. They achieve this by controlling apoptosis and autophagy (5).

miR-429 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 situations. 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 affects the autophagy process during myocardial ischemia/reperfusion injury and could be a potential target for treating myocardial infarction (8). Interestingly, its involvement in ischemic stroke has been recognized in research. Jie et al stated that decreasing miR-429 significantly lessens neuronal harm caused by cerebral ischemia and reperfusion injury in a laboratory setting by increasing the expression of GATA-binding protein 4 (GATA4) (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 the fetal and adult central nervous system and plays a significant role in both proliferation and apoptosis (9). Kobayashi et al previously suggested that GATA4 was able to protect 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, whether miR-429 and GATA4 have participated in cerebral ischemia and reperfusion damage remains undefined. CGA, also known as chlorogenic acid, is a phenolic compound found extensively in plants, fruits, different kinds of vegetables, as well as in tea and coffee beverages (12, 13). Recently, there has been growing evidence suggesting that CGA is often utilized 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 impact against ischemia-reperfusion injury due to its antioxidative, anti-inflammatory, and anti-apoptotic properties (12). It's not clear whether CGA mitigates cerebral ischemia-reperfusion injury by controlling autophagy and its potential mechanism, which requires clarification. In this research, our aim was to examine the changes in autophagy-related molecules, apoptosis, and miR-429/GATA4 in the brain cortex following ischemia/reperfusion in rats. Additionally, we assessed the impact 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 and 3-4 months of age) were purchased from standard laboratory animal house of Urmia University of Medical Sciences. The rats were maintained under standard laboratory conditions, at room temperature (21±2°C) on a 12-hour/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 (with an Ethical Code: IR.UMSU.REC.1399.298).

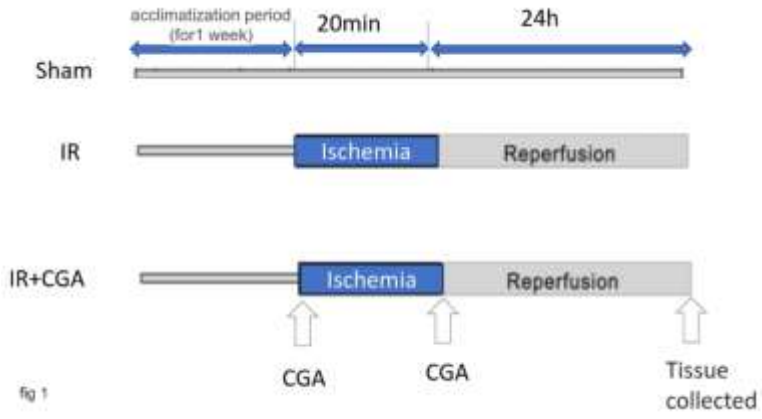
2.2.Animal Groups and treatment

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

1- Sham: Rats were given an equal volume of PBS solution intraperitoneally.

2- IR (ischemia-reperfusion): The cerebral IR was established by bilateral common carotid occlusion for 20 minutes. Also, rats were given an equal volume of PBS solution intraperitoneally.

3- IR±CGA (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). The 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 create the cerebral IR in the same manner as previously explained (17). In summary, all animals were given anesthesia through the use of ketamine (60 mg/kg, intraperitoneal) and xylazine (8 mg/kg, intraperitoneal). Throughout the experiment, their body temperature was upheld at 37◦C using a thermometric blanket. The

surgical area was uncovered, and a vertical incision in the neck, approximately 1.5 cm long, was made to expose both common carotid arteries, freeing them 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 same surgical procedure was carried out in the sham group, but without clamping the carotid arteries.

Tissue preparation

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

2.4.Quantitative real-time PCR

miR-429 expression levels in cortex samples were evaluated using the 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 was calculated using the 2-(ΔΔCt) 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.

Immunohistochemical staining

Following tissue dehydration, the specimens were embedded in paraffin and then 5 μm thick sections were prepared for immunohistochemical evaluation of LC3 expression. In brief, the sections underwent three 5-minute washes with 0.01 mol/L phosphate-buffered saline (pH 7.2-7.4). Subsequently, the sections were treated with 10% normal rabbit serum to block nonspecific binding at 37°C for approximately 1 hour. 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, the sections were subjected to incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution, Beijing Zhongshan Biotechnology Co., Ltd) at 37°C for 1 hour. The sections were washed using 0.1 mol/L Tris-HCl buffer for about 5 minutes. Subsequently, the sections were incubated in DAB (0.05%) and 0.05 mol/L Tris–HCl buffer, as well as 3% hydrogen peroxide with 1 to 2 drops for 5 to 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. For the positive control, rat cerebral cortex was utilized, and a 1:400 dilution of normal rabbit serum was employed for the negative control.

2.5.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 then 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 min. The collected supernatant was used for protein detection. The proteins were separated using 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 provided in table 1.

Table 1

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

2.6.**Statistical analysis**

The data were reported as mean ± SEM, and data analyses were conducted using SPSS version 16.0. We assessed the normality of all parameters using the one-sample Kolmogorov-Smirnov test. To examine statistical differences among the experimental groups, we conducted one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1.miR-429 expression in cerebral cortex

In order to first 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 findings showed that there was a significant ($P<0.05$) increase in the expression of miR-429 (1.95 ± 0.34) in the cerebral cortex of animals subjected to ischemia-reperfusion compared to the sham group. It was further proved to be decreased (0.68 ± 0.29) significantly ($P<0.05$) by the administration of CGA (Figure 2).

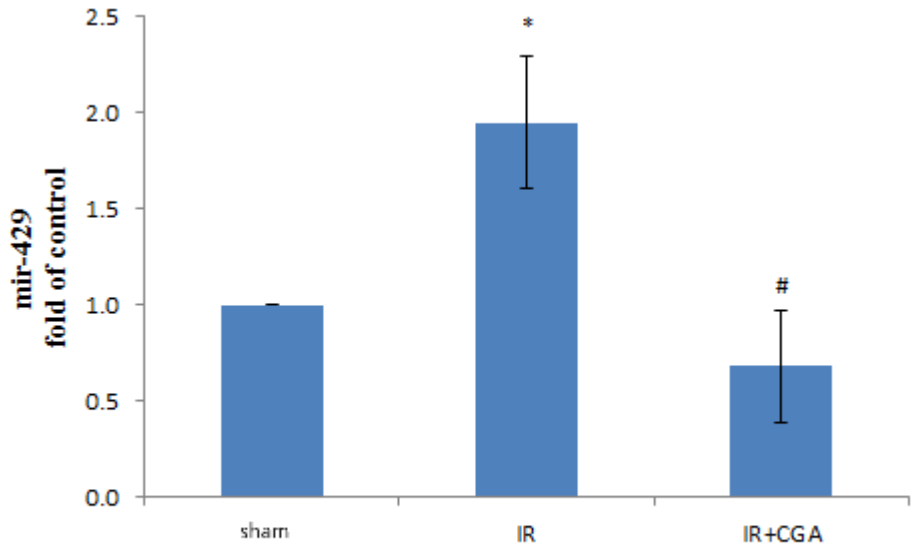


fig 2

3.2.Proteins related to autophagy in the cerebral cortex

To evaluate the impact of CGA in the regulation of autophagy in cerebral I/R injury, visibly the expression of LC3, a usual autophagy marker, was discovered by immunohistochemical staining. In Figure 3, it was evident that 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) by 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 reversed the decrease in p62 level significantly (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.

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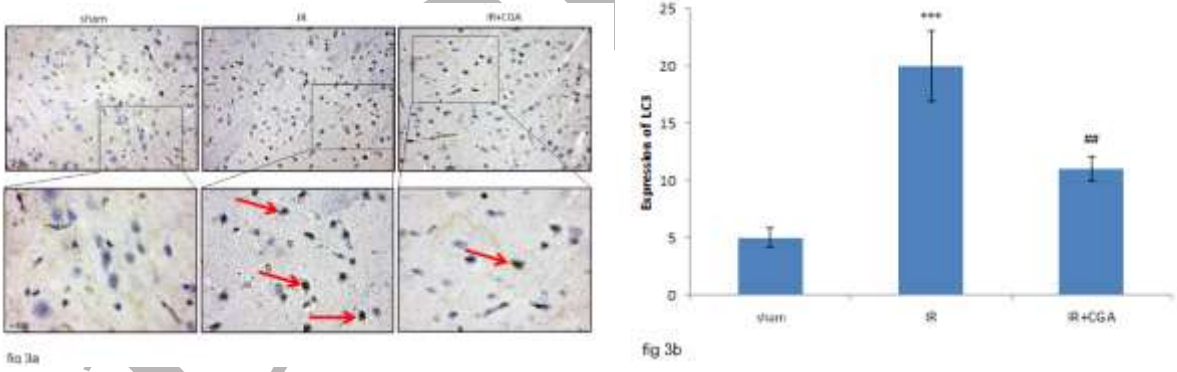

fig 3a

fig 3b

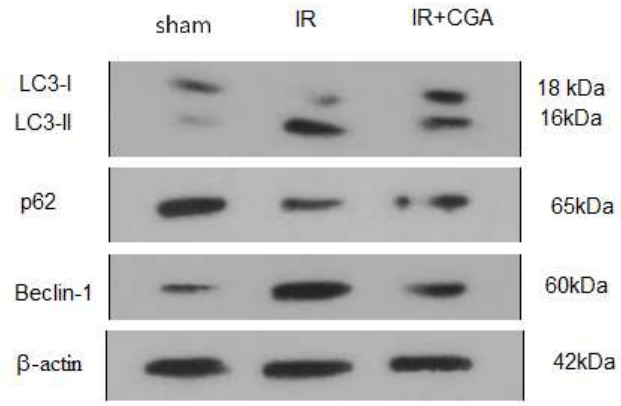

fig 4a

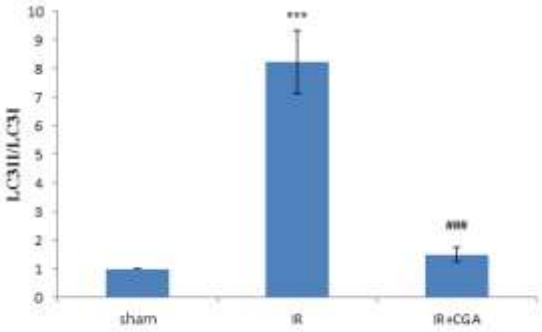

fig 4b

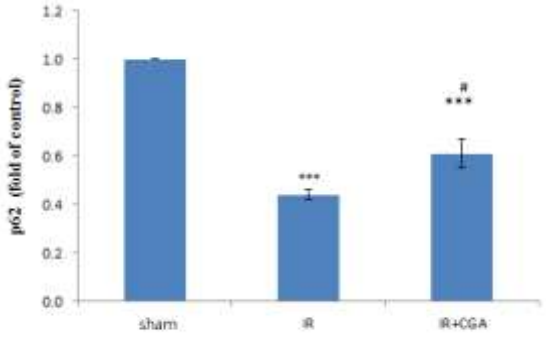

fig 4c

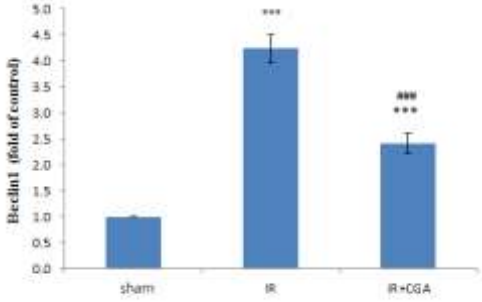

fig 4d

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, the analysis involved western blot techniques 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 in the IR group ($P<0.01$) (43.01±11.72) in comparison with the sham group (1 ± 0.0). However, CGA treatment could alleviate this ratio (4.82 ±1.05) markedly ($P<0.01$) in the cortical region exposed to IR injury (Figure 5 a,b).

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

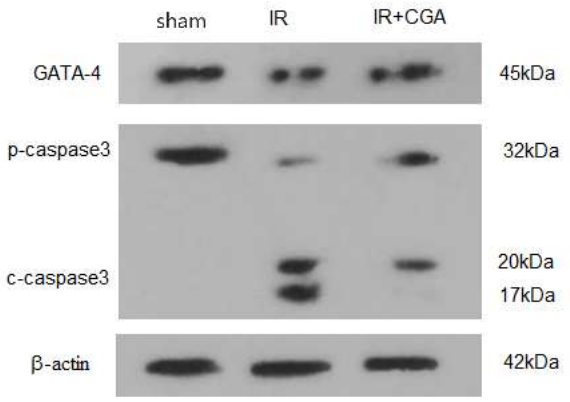

fig 5a

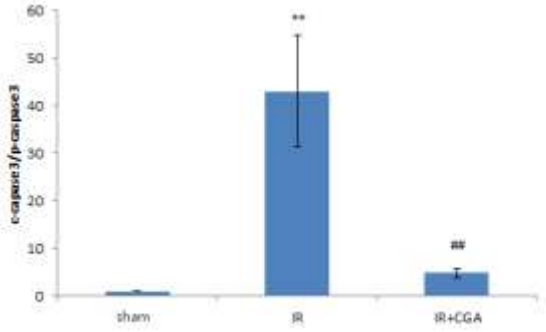

fig 5b

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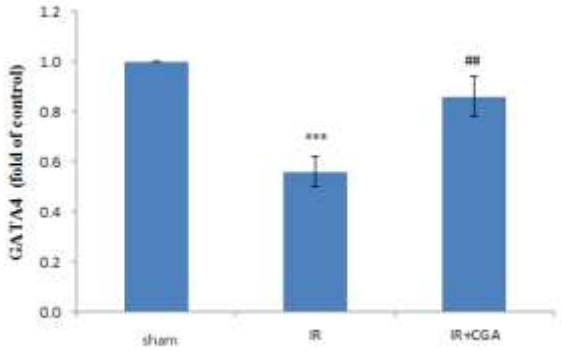

fig 5c

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٢٥٩ **4. Discussion**

٢٦٠ The findings of this study demonstrated that cerebral IR at 24 hours caused activation of
٢٦١ autophagy and also apoptosis in the cortical region in CCAO model of ischemic stroke in male
٢٦٢ rats. 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 is a major problem all over the world experiencing 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, 19, 20). Reports from earlier 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 mechanisms of its protective effects against IR injury remain unclear, previous studies have demonstrated that CGA reduces 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. 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 prevents neuronal injury. (4).

Taking into account these studies and our discoveries, we deduce 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 activation of autophagy and

contributed to apoptosis which is in agreement with other studies (22). But 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 are in line with the other studies to confirm the anti-autophagic activity of CGA in lead-induced developmental neurotoxicity (23), Alzheimer's disease (24), and Parkinson's disease (21). Therefore, it was assumed that over-activated autophagy following cerebral IR could be reversed by CGA treatment and then, alleviated 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 there is growing evidence that miRNAs can impact this process, including 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 have an important role in survival and progression of several diseases (25). For example, overexpression of miR-429 enhanced apoptosis in some pathologies such as, ischemic hippocampal neurons (25). It was reported that down-regulation of miR-429 could improve neurological deficit following traumatic brain injury (5) and also protect brain neurons in hypoxia-ischemia damage in the model of neonatal mice with negatively regulated apoptosis (25). Notably, Zhu et al showed that alleviating anoxia/reoxygenation injury in cardiomyocytes by regulating apoptosis and autophagy is possible through the attenuation of miR-429 antagonism. (8). Consistently, the current study demonstrated that miR-429 remarkably was up-regulated in the brain tissue of CCAO rats, pointing to a pivotal role of miR-429 in the pathology of stroke.

In researches miR-429 was found to target the important gene GATA4. 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 the embryonic and adult CNS (7, 9). Kobayashi 2009 et al, declared that GATA-4 expression was 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 researches, in this study, we developed that miR-429 was up-regulated and GATA4 protein expression was down-regulated significantly in cortical neurons subjected to ischemia, 24 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.

Conclusion

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. CGA can attenuate IR-induced neuronal autophagy and apoptosis. miR-429 and GATA4 might play a role in controlling autophagy during cerebral IR, and this effect could be countered by treatment with CGA. Targeting miR-429 and GATA4 could be an effective treatment approach for ischemic stroke. However, more research is needed to explore the molecular mechanisms using a transgenic animal model.

Ethics Statement

The 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.

Funding

NONE

Author Contributions

Concept and design of the study: Roya Naderi, Alireza Shirpoor

Collection of data: Rahil Salimi

Analysis and interpretation of data: Roya Naderi, Rahil Salimi

Drafting of the manuscript: Roya Naderi

Critically revising the manuscript for significant intellectual content: Roya Naderi

Statistical analysis: Rahil Salimi

Support in the form of administration, technology, and materials: Roya Naderi

Conflicts of Interest

The authors have no competing interests to declare.

Data Availability Statement

The findings of this study are supported by data that can be obtained from the corresponding author upon making a reasonable request.

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Table1. The antibodies used in Western blotting assays

Fig.1. Experimental design showing time schedule of CGA administration, I/R, and sampling.

Fig.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).

Fig 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.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).

Fig.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, ***

٤٤٦ P<0.001 compared with sham group. ##P<0.01 compared with IR group. Sham; ischemia
٤٤٧ reperfusion (IR); and ischemia reperfusion + Chlorogenic acid (IR+CGA).