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ORIGINAL ARTICLE

Eriocitrin Alleviates the Arterial Occlusion-Mediated Cerebral Ischemic-Reperfusion Injury through the Modulation of Apoptotic Proteins and Immune Markers in Mice

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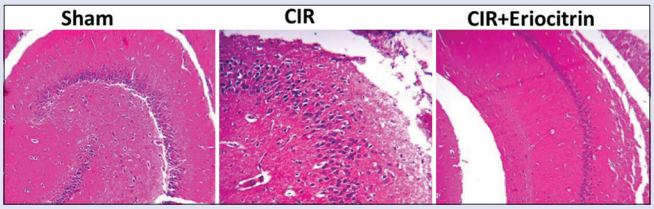
ABSTRACT

Background: Stroke is ranked on top of the persistent ailments associated with the brain. The incidence of ischemic stroke is rapidly rising along with intensified mortality as well as permanent disability conditions. However, tissue plasminogen activator (tissue-type plasminogen activator) is the only FDA-approved treatment for the treatment of stroke with decreased success rate. Eriocitrin, a flavonoid compound of *Citrus limon* with incredible therapeutic values, was active in the current investigation to explore the neuroprotective capability in arterial occlusion-provoked cerebral ischemic reperfusion injury (CIRI) in mouse replica. **Materials and Methods:** The neurological defects were considered through the standard scoring method and the expression status of apoptotic proteins, i.e., nuclear factor kappa B (NF-ƙB), caspase-3, and caspase-9, was examined via western blotting test. The expression of neuronal nitric oxide synthase (nNOS) was scrutinized through real-time polymerase chain reaction and standard ELISA kits were employed to examine the status of the proinflammatory markers. **Results:** The arterial occlusion provoked cerebral ischemic animals displayed the severely elevated status of inflammatory markers namely interleukin-6 (IL-6), IL-1β and tumor necrosis factor α, and the expression statuses of apoptotic proteins, as well as nNOS, were also uplifted while comparing to the sham subjected animals. Amazingly, eriocitrin (100 mg/kg) treatment revealed the declined inflammatory markers level, besides, the expression patterns of apoptotic proteins and nNOS were also reduced noticeably in the cerebral ischemic-reperfusion-operated animals. **Conclusion:** The novel findings of this investigation were demonstrated that the Eriocitrin delivered an astounding neuroprotection in the arterial occlusion-mediated CIRI in mouse replica, which possibly a promising neuroprotectant in cerebral ischemia.

Key words: Arterial occlusion, cerebral ischemia, eriocitrin, inflammatory markers, reperfusion, stroke

SUMMARY

- The cerebral ischemia-reperfusion injury is a harsh ailing condition of the brain which often results in severe neural afflictions and irrevocable neural necrosis
- Eriocitrin treatment ameliorated the too much expressions of apoptotic proteins that is (p) nuclear factor kappa B, caspase-3 and caspase-9 in the cerebral ischemic-reperfusion animals.



Abbreviations Used: tPA: Tissue plasminogen activator; TLR: 4-toll-like receptor; CIRI: Cerebral ischemic reperfusion injury; nNOS: Neuronal nitric oxide synthase.

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INTRODUCTION

An ischemic stroke is a critical illness that results in incurable physiological inabilities as well as fatality; it was caused through the unforeseen blocking of cerebral blood supply along with consequent neural cell necrosis. The occlusion of carotid arteries which carries the blood to the major portions of the brain along with the successive reperfusion is the pivotal feature of the pathological progression of ischemic stroke. Stroke accounted for the second most reason for permanent physical disabilities as well as death worldwide. A recent report from the World Health Organization stated that every year, >15 million individuals are affected from stroke worldwide, among them almost 6 milling stroke victims died and over half of survivors are living with incurable physical disabilities.[1-4] Stroke ranked on top among the recurrent cerebrum associated ailments. Stroke also placed on second between the incurable ailments as well as first on disabling or incapacitating diseases throughout the world. The hypoxic-ischemic stroke accounted for over 70%–85% of the whole incidences of strokes. Ischemic stroke directs to the injury of brain cells as well null function of malfunction as an outcome of unpredicted immediate interference of brain blood vessels.[1,5]

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The cerebral ischemic brain injury was triggered through the disarrays of blood flow and oxygen delivery to cerebrum, in addition it is a major common cause of brain damage that can escorts to the irreparable or enduring mental disabilities, e.g., malfunction of cognition, cerebral palsy and epilepsy as well as physical difficulties, in some cases it may end with death.[6] If on any occasion, the inclined portions of the brain tissue, like the CA1 segment of the hippocampus were dented that leads to the mutilation of cognition whether in both humans as well as animals.[7] The inflammation at neurons accounted as an essential provider for the pathological process of ischemic brain injury. Extensive research investigations were signified that the momentous actions of the immune cells were endorsed damage of brain tissue as well successive repairing of brain cells and amending in assorted levels of ischemic brain injury.[8,9] A broad research finding highlighted challenges that the tensed neurons have a capable of stimulating the microglial cells along with encouraging the discharge of pro-inflammatory mediators, those can donate to the progressive secondary neuronal as well oligodendroglial damage.[10,11]

Inflammation is a very complicated mediator at the pathological process of cerebrum infarction. Inflammation is regarded as an essential arbitrator that can direct to brain cells injury followed to the ischemic stroke. The stimulation of transcriptional moderators is able to initiate the inflammatory reactions that sequentially directs to the discharging of widespread pro-inflammatory mediators.[12] The nuclear factor kappa B (NF-ƙB) is a major transcriptional modulator of inflammation as well as, the activation of the NF-ƙB transcriptional pathway is regarded as a major factor in the stimulation of inflammation on neurovascular units.[13] The heterodimer complexes of NF-ƙB p50/p65 along with their inhibitory mediator specifically IƙBα beneath the usual state and becomes an inactive state at the cytoplasm. Subsequently, the damage of ischemic cerebral reperfusion, the IκB kinase-β quickly gets phosphorylated and triggered for persuading to IκBα phosphorylation thereby degradation. Accordingly, p50/p65 dimers were discharged as well as carried to the nucleus thereafter it endorsed the expression of pro-inflammatory moderators, for instance, tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β).[4]

The cerebral ischemic stroke can begin toward the deprived oxygen deliver or cerebral hypoxia and later cause the severe injury to the central nervous system (CNS). The reperfusion on the ischemic brain directs toward the surge of pathological progression which results in secondary injuries and accounted to the ischemic reperfusion injury.[14] Progression from ischemic brain injury is mainly associated with the inflammation; free radicals mediated neural damages and excitotoxicity.[15] The preclinical and clinical experimentations were showed that status of oxidative stress markers and inflammatory mediators were surprisingly elevated in the ischemic stroke condition as well. The TNF-α, IL-6, and IL-1 are few of the most distinguished cytokines in the pathogenesis of ischemic stroke.[16,17]

The medication of tissue plasminogen activator (tPA) is the only recognized and approved drug by the USFDA for the efficient therapy to treat the stroke. It also accountable for their some superfluous effects like chances to hemorrhage at intra-cerebrum and restricted thrombolytic time consumption. It has been reported that only 3%–5% of stroke victims were actually benefited by tPA.[18] The NF-ƙB and toll-like receptor-4 was regarded as to assist the inflammation reaction in the ischemic cerebrum and infarction.[19,20] The initiation of expression of pro-inflammatory mediators, for instance, TNF-α, IL-1β, IL-6, and NO was stimulated through these signals, which donates to the ischemic stroke.[19] Thereby, the repression of those inflammatory signs regulated through NF-ƙB can be a auspicious strategy for the healing of ischemic stroke.[21] The preceding literature mentioned the neuroprotective potential of citrus flavonoids.[22] Eriocitrin is a flavonoid compound of *Citrus limon* which influenced the numerous therapeutic values. Eriocitrin possessed anti-inflammatory, antioxidant and anti-hepatoxicity activities.[23-25] Even though there is null evidence for proving the curative powerful of eriocitrin in cerebral ischemic-reperfusion damages. Thereby, this current investigation was envisioned to explore the therapeutic values of eriocitrin on arterial occlusion-mediated cerebral ischemia-reperfusion in mice.

MATERIALS AND METHODS

Drugs

All chemicals including eriocitrin, analytical reagents, ELISA kits, and other diagnostic kits were commercially bought from the Sigma-Aldrich, St. Louis (USA) and used for this study.

Experimental Animals

The 7-week-old male C57BL/6J mice (weighing from 22 to 25 g) were bought from Institutional Animal House and sustained in the polypropylene confines beneath the good lab conditions (26°C ± 1°C, 60%–70% air wetness, and 12 h light and dark sequence). Animals were fed with a regular pellet diet with *ad libitum*. All animals were adapted to laboratory situations for up to 7 days before experimentation. The study was carried out according to the procedures of the Institutional animal ethics committee (2019-071).

Surgical method and experimental setup

The cerebral ischemic-reperfusion was proficient by adopting the procedure given by Ni *et al.*[26] Initially, the animals were anesthetized through injecting the 60 mg/kg of ketamine and 90 mg/kg of xylazine via intra-peritoneal route. Afterward, the central region of the neck was notched and then the bilateral carotid arteries were identified and separated. Cerebral ischemic condition was initiated through the occlusion of carotid arteries along with surgical loops for 1 h. Followed by 25 min, the cerebral ischemia-reperfusion was stimulated by taking away the loops from arteries. Body temperature of the investigational animals were kept constant at 37°C by using thermostatically controlled heating blanket till the mice improved from the surgery. The sham mice were subjected to identical surgery without the occlusion of arteries. Surgical actions were done through sterile principles.

The mice were randomly alienated into 3 groups along with 12 mice in each. Group-I supplied as sham-subjected with saline treatment. Group-2 was cerebral ischemic-reperfusion stimulated group through occlusion of carotid arteries followed by reperfusion for 24 h. Group-III was cerebral ischemic-reperfusion stimulated and treated with 100 mg/kg[24] of eriocitrin through oral gavage route on 30 min later of initiation of reperfusion and after 12 h the second dose of Eriocitrin (100 mg/kg) was supplemented through the same route. After 24 h reperfusion injury, the anesthetized animals were killed through cervical displacement and the brain portions were separated from animals and processed quickly with saline buffer and stored at 4°C for additional biochemical and histopathological examinations.

Determination of neurological defects

The neurological defects of animals were measured after 24 h of cerebral ischemic-reperfusion stimulation and it was according to score ranging from five points (0–5) scale, where, 0 means null defects, 1 means defect in extension of forelimb, 2 means cannot extend the forelimb, 3 means slight circling, 4 means harsh circling and 5 means lessening towards bilateral side.[27] The higher score representing the harshness of neural defects, all experimental mice were assessed through and the scores were noted.

Determination of inflammatory cytokine level

The status of inflammatory mediators, namely TNF-α, IL-1β, and IL-6 in the brain tissues were measured through ELISA kits (BioCompare, USA) according to the manufacturer's guidelines.

Quantitative real-time polymerase chain reaction study

The whole RNA was extracted from the hippocampus tissues of the brain later than 24 h of reperfusion with the aid of commercial RNA extracting kit (ZymoResearch, Irvine, CA) based on the manufacturer's protocol. The purified RNA samples were employed for the manufacturing of the cDNA with the aid of the cDNA synthesis kit (Takara, Japan). Afterward, real-time polymerase chain reaction (RT-PCR) was performed through a commercial PCR kit (Invitrogen Life Technologies) according to the manufacturer's protocol. The utilized primers for the detection of neuronal nitric oxide synthase (nNOS) is 5'GTGGCCATCGTGTCCTACCATAC3' (forward) and 5'GTTTCGAGGCAGGTGGAAGCTA3' (reverse), for detection of β-actin is 5'CCGTTTCTCCTGGCTCAGTTTA3' (forward) and 5'CCCCAATACCACATCATCCAT3' (reverse). The endogenous β-actin gene was utilized as a reference and for the negative control, water excepting DNA was used. All reactions were done in triplicate.

Western blot study

The brain hippocampus tissues were homogenized with saline and spun at 9000 rpm for 6 min to remove the debris, and the resulting supernatant was subjected to whole protein extraction from with the aid of commercial protein extracting kit (Merck Millipore, USA) based on manufacturer protocol. Afterward, protein was purified through SDS-PAGE technique. The separated protein was transferred to nitrocellulose membrane then blocked along with the 5% dry fat-free milk for 3 h, subsequently the membrane was incubated for up to overnight in 4°C along with relevant primary antibodies for caspase-3, caspase-9, NFκB and pNFκB (Boster Bio, CA). In the end, developed bands after addition to the chemiluminescence agent were imagined and documented with the aid of automated software (Biorad Lab, CA, US).

Statistical analysis

All the data were considered statistically through SPSS software (version 19), SPSS Inc., Chicago, IL. The results were analyzed through one-way ANOVA after Dunnett's *post hoc* test and they were depicted as mean ± SD. *P* value ($P < 0.05$) was regarded as statistically significant.

RESULTS

Eriocitrin ameliorates the cerebral ischemic reperfusion injury mediated neurological deficits in mice

The mice subjected to Cerebral Ischemic Reperfusion Injury (CIRI) have showed the distinctive neural defects, as well the forelimb of CIRI animals was completely paralyzed while comparing to the sham-subjected group. Figure 1 proving that eriocitrin substantially protected the animals from CIRI mediated neurological defects. They tested mice supplemented with 100 mg/kg of Eriocitrin were showed a marked amelioration ($P < 0.05$) in the recovering of neural deficits induced by CIRI. The CIRI subjected mice confirmed distinct signs of neural dysfunction, while the sham group displayed null neurological complications [Figure 1]. As a result, it is clear that eriocitrin markedly exhibited the marked neuroprotective potential in CIRI subjected animals.

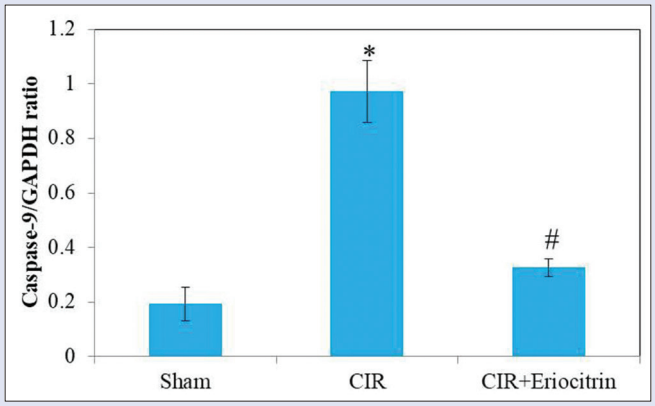

Figure 1: Protective effect of Eriocitrinon Cerebral Ischemic Reperfusion Injury based on neurological deficit score in experimental mice. Data were presented as mean ± standard devitation. A $P \leq 0.05$ was considered statistically significant. Comparisons: *with Sham; # with Cerebral Ischemic Reperfusion

Eriocitrin attenuates the elevated expression of neuronal nitric oxide synthase in the cerebral ischemic reperfusion injury mice model

The status of nNOS expression was studied through the RT PCR. The sham group presented a normal expression level of mRNA of nNOS, conversely, the CIRI subjected group disclosed the drastic elevation in the expression level of nNOS. Figure 2 evidencing that eriocitrin ameliorated the CIRI regulated nNOS expression level while comparing it to the CIRI subjected group. The supplementation of 100 mg/kg of Eriocitrin revealed the astoundingly attenuates the uplifted nNOS expression status, which was mediated by the cerebral ischemic condition. It proved that the Eriocitrin has the therapeutic potential towards cerebral ischemia.

Eriocitrin decreased the cerebral ischemic reperfusion injury induced elevation in inflammatory markers in brain cells of mice

The experimental animals which are subjected to cerebral ischemia were indicated the rapidly elevated status of inflammatory mediators that is TNF-α, IL-6, and IL-1β in the brain tissues of CIRI animals than the sham animals. In this test, it was proven that eriocitrin (100 mg/kg) treatment distressingly decreased the inflammatory mediator's status in the brain tissues, which was elevated by cerebral ischemia [Figures 3-5]. Thus, Eriocitrin can lessen the elevated inflammatory cytokines status in the brain tissues at cerebral ischemic conditions and highlighting the therapeutic value of Eriocitrin against CIRI.

Eriocitrin ameliorates the apoptotic protein expressions in the brain tissues of cerebral ischemic reperfusion injury mice model

The expression status of the apoptotic proteins that are NFκB, caspase-3, and caspase-9 was examined through the western blotting method and the result is depicted in Figure 6. The expression status of NFκB, caspase-3 and caspase-9 proteins, which were related with the apoptotic pathway was severely uplifted during the cerebral ischemia in mice while comparing to the sham subjected group. The Eriocitrin (100 mg/kg) treatment

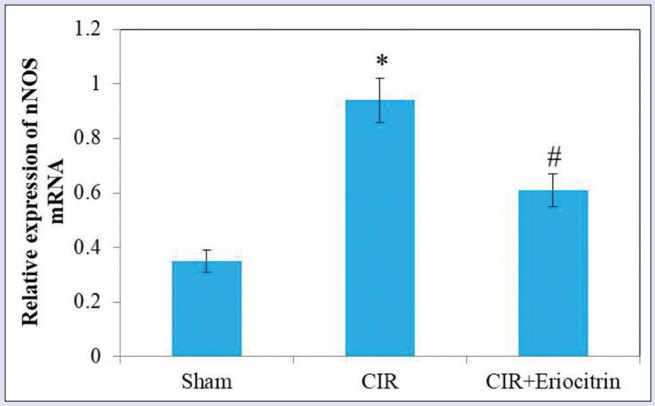

Figure 2: Expression levels of neuronal nitric oxide synthase mRNA in experimental groups of mice. ΔCt = Ct (nNOS) - Ct (β-actin), where Ct = cycle threshold, β-actin is the endogenous reference gene. Relative nNOS mRNA expression: PΔCt = 2ΔCt, representing nNOS mRNA levels. Final data in the graph were magnified to 1000 × 2ΔCt. Data were reported as mean ± standard devitation. A $P \leq 0.05$ (two-tailed Student's *t*-test). Comparisons: *with Sham; #with Cerebral Ischemic Reperfusion

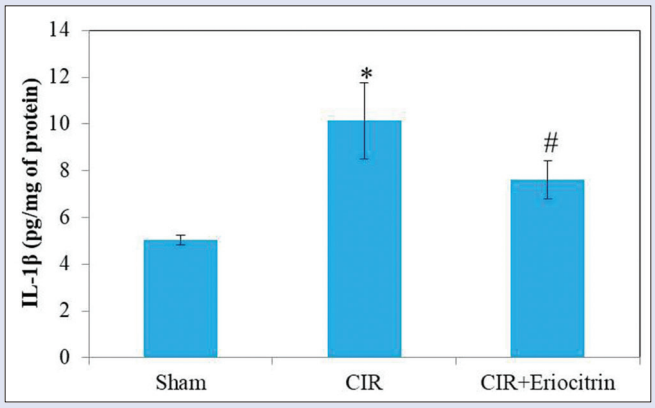

Figure 4: Levels of inflammatory cytokine interleukin-1β on Cerebral Ischemic Reperfusion Injury and treatment by Eriocitrin in experimental mice. Data were presented as mean ± standard deviation. A $P \leq 0.05$ was considered statistically significant. Comparisons: *with Sham; #with Cerebral Ischemic Reperfusion

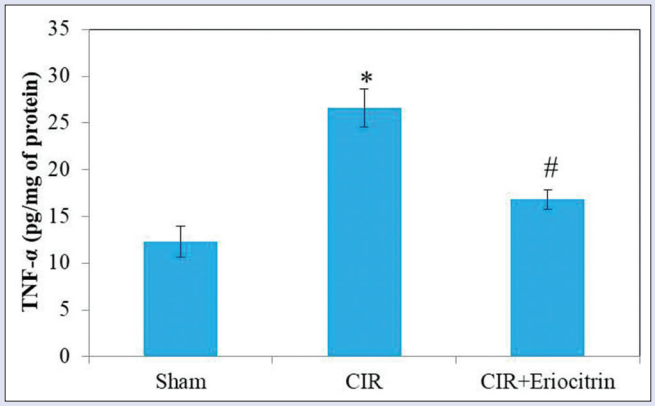

Figure 3: Levels of inflammatory cytokine tumor necrosis factor α on Cerebral Ischemic Reperfusion Injury and treatment by Eriocitrin in experimental mice. Data were presented as mean ± standard devitation. A $P \leq 0.05$ was considered statistically significant. Comparisons: *with Sham; #with Cerebral Ischemic Reperfusion

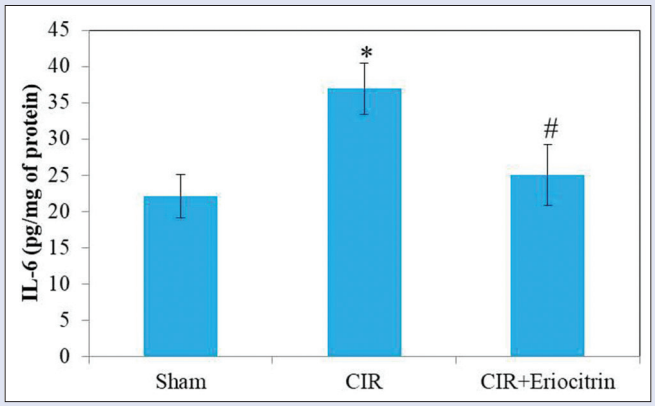

Figure 5: Levels of inflammatory cytokine interleukin-6 on Cerebral Ischemic Reperfusion Injury and treatment by Eriocitrin in experimental mice. Data were presented as mean ± standard decitation. A $P \leq 0.05$ was considered statistically significant. Comparisons: *with Sham; #with Cerebral Ischemic Reperfusion

revealed the striking reduction in the expression apoptotic markers i.e., phosphorylated NFκB as well as caspase-3 and caspase-9. The result of assessment was evidenced that the Eriocitrin exhibited a drastic reduction in the apoptotic marker's expression in the brain tissues of cerebral ischemic mice replica, which representing the therapeutic importance of Eriocitrin.

Eriocitrin prevented the brain tissues in the experimental mice model

Figure 7 exemplifies the cyto-architecture of the brain tissues of experimental animals. The H and E stained brain tissues demonstrated that there were severe changes in the tissue architecture and the loss of neuronal cells in the CIRI operated animals when compared to control. However, the eriocitrin treated animals exhibited almost normal histological structures in the brain tissues. The eriocitrin treatment also improved the viable neuronal cell population in the experimental animals.

DISCUSSION

The CIRI is a harsh ailing condition of the brain, which often results in severe neural afflictions and irretrievable neural necrosis. CIRI occupied in many crucial pathological processes for instance, oxidative stress, excessive intracellular calcium load, inflammatory damages, mitochondrial damage, apoptosis and acidosis, and so it is essential to find an enhanced neuroprotective agent against the CIRI.[28-30] The ischemic stroke takes place at any time if there is a perpetual occlusion in the blood vessels, which delivers blood to the brain tissues that ultimately result in necrosis of neural cells.[1] Though the supplying of blood to whole parts of the body is mandatory, in particular, the brain is thought to more vulnerable to the occlusion of arteries due to the highest metabolic pace and more oxygen demands.[31]

Also, the brain is a first-ever organ that suffers from the declined blood supply and denied sufficient oxygen level that finally results in neural tissue necrosis. The mechanisms of brain tissues were blocked abruptly after irretrievable injuries at the time of poor oxygen delivery due to the suspended blood circulation rate. Accordingly, the functions of brain tissues depending on effective enough blood deliver to the brain

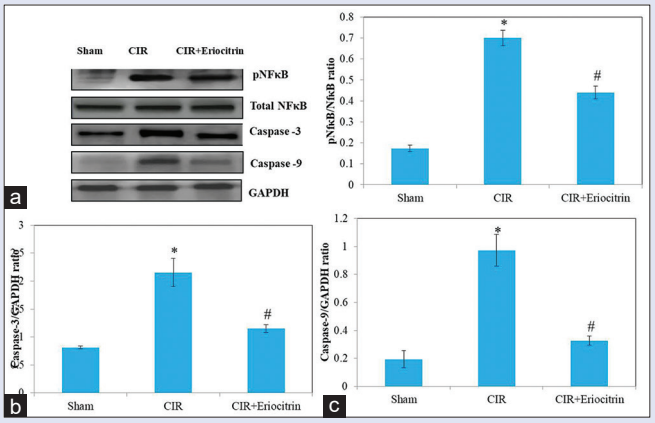

Figure 6: (a-c). Western blot analyses of apoptotic proteins in Cerebral Ischemic Reperfusion injury and Eriocitrintreatment in experimental mice. Data were presented as mean ± standard decitation. A $P \leq 0.05$ was considered statistically significant. Comparisons: *with Sham; #with Cerebral Ischemic Reperfusion

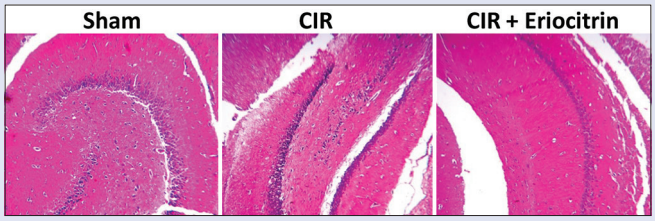

Figure 7: Histopathological analysis of Cerebral Ischemic Reperfusion Injury and Eriocitrin treatment in experimental mice

cells.[31,32] The harsh injuries to the cortex of the cerebrum, cerebellum and the ganglia through ischemic stroke was thought to encourage the prevalent neuronal defects and long-lasting disabilities which frequently illustrious the ailment due to lessen blood deliver to brain.[33,34] The nitric oxide is an imperative one which contributes vital molecules for the controlling of blood supply to cerebrum.[35] In this investigation, the RT-PCR result displayed a severe elevation in the expression of mRNA of nNOS which denotes the cerebral ischemic-reperfusion condition, enchantingly the Eriocitrin treatment declined the unkindly elevated expression status of nNOS in CIRI mice replica [Figure 2].

Though a pathological mechanism of CIRI was regarded as exceeding inflammatory reactions, as well, neural apoptosis, which is mediated through an array of genes was too anticipated to take an essential function on the mutilation of neural mechanisms. Thus, the maintenance of the internal permanence of body organs especially a brain was obligatory.[36] Copious research statements displayed that ischemic-reperfusion damage is a crucial stimulus that can stimulate the expressions of apoptotic genes thereby results in neural apoptosis.[37,38] Caspase-3 is an important protease to breakdown the cytoskeleton and nucleus proteins. It is the most effectual apoptotic regulator in a category of caspase proteins. The expression status of caspase-3 is often marked as a characteristic of apoptosis.[39] In this exploration, the CIRI subjected animals showed the uplifted level of caspase-3 expression, while eriocitrin treatment markedly reduced the expression status of caspase-3 [Figure 6c].

Besides, inflammation is an essential route which can direct toward the apoptosis in neural cells. The NFκB is an essential transcriptional factor of inflammation and it can get triggered quickly on the acute cerebral ischemic condition, which highlights the uplifted expression status of NFκB in the cerebral ischemic mice of investigation.[40-42] The NFκB signalling pathway is engaged in acute cerebral ischemic condition since it directs to the accretion of inflammatory regulators including TNF-α, IL-1β, and IL-6 and thereby after further damages. It is highlighted that the despite transient cerebral ischemic replica, the agent which can inoperative the NFκB can markedly reduce the volume of infarction and enhancing the neural defects which highlighting that the aiming the NFκB pathway afterward to cerebral ischemia-reperfusion may be a hopeful healing approach.[43]

The stimulation of the NFκB signalling pathway in neural cells can radically elevate the transcription of inflammatory genes thereby decodes the inflammatory regulators i.e., TNF-α, IL-1 β and IL-6.[44,45] The NFκB is considered the stress-mediated transcriptional modulator that takes a crucial function in the controlling of inflammatory reactions. NFκB signalling cascade is anticipated to take part in essential inflammatory functions in the damages of the CNS, which includes the traumatic brain damage, permanent focal ischemia and ischemic reperfusion.[46] It has already been reported that the NFκB regulated apoptosis and inflammatory signalling pathways were taking a crucial function in the pathological process of CIRI, thereby it activates the microglia after cerebral ischemia and enhancing the post-ischemic inflammatory processes.[47,48]

The TNF-α was illustrious as the highly reactive cytokine and it can be viewed as an imperative proinflammatory regulator along with double functions in the NFκB signalling pathway. In many cases, the IL-1β status was raised drastically after the many inflammatory reactions and contributing to the pathological processes of inflammation.[49] In the ischemic stroke victims, the IL-6 was associated with the deterioration of neurons, increasing infarction levels and deprived long-lasting effects.[50] The highest level of TNF-α in the plasma was as well linked to the volume of infarction and deficits of neurons on the several studied models of ischemic stroke.[41] Besides, at some stages in reperfusion, the elevated level of steroids in serum could worsen the neural damages via perturbing the glucose homeostasis and uplifting the oxidative stress in the brain.[51]

As a result, the amelioration of inflammatory conditions after cerebral ischemic damage will contribute in a defensive function in the treatment of cerebral ischemia. In this investigation, the signalling cascade NFκB was uplifted drastically [Figure 6a and b] and the grades of TNF-α, IL-1β and IL-6 were as well elevated severely [Figures 3-5] in the CIRI subjected animals. Surprisingly, the Eriocitrin treatment ameliorated the too much expressions of apoptotic proteins that is (p) NFκB, caspase-3 and caspase-9 in the cerebral ischemic-reperfusion animals, in that way, the Eriocitrin treatment was as well distressingly attenuated the drastic elevation in inflammatory markers, for instance TNF-α, IL-1β and IL-6 in the CIRI animals, which decoding the therapeutic value of Eriocitrin towards the neuroprotection in CIRI condition through lessening the inflammatory response subsequent to cerebral ischemic condition.

Besides, Wang *et al.*[52] defined that the neuroprotectants was appreciably declined the expression patterns of NF-κB, which signifies the NF-κB was substantially playing an indispensable function in the pathological process of ischemic brain injury in animal test, which highlighting the inflammatory signs regulated through NF-κB was an essential to the ischemic stroke progression.[53] In agreement with this finding, this current investigation was showed that the Eriocitrin was extraordinarily weakened to the cerebral ischemic damage in the mice.

CONCLUSION

The novel findings of this exploration disclosed the astounding neuro-protection value of Eriocitrin in opposition to CIRI, which is provoked through the carotid arterial occlusion in mice replica

via assuaging the elevated expressions of apoptotic proteins and inflammatory regulators. It was concluded that the Eriocitrin, a flavonoid compound of *Citrus limon* can be a promising neuroprotectant for the treatment of cerebral ischemia.

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Conflicts of interest

There are no conflicts of interest.

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