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Original Article

Rhapontici Radix Extract Mitigates Cerebral Ischemia-Reperfusion Injury in Rats via Down-Regulating PERK/ATF-4 and Apaf-1/IRE1α Signaling Pathways

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ABSTRACT

Background: Ischemic stroke is a major cause of long-lasting disability and even mortality globally, driven by the sudden blockage of cerebral blood flow and the subsequent pathophysiological events that ultimately lead to neuronal damage and apoptosis. **Objectives:** The major focus of this research study is to determine the neuroprotective properties of the Rhapontici Radix (RR) against ischemic stroke rat model. **Materials and Methods:** The rats were underwent through the Middle Cerebral Artery Occlusion (MCAO) technique to initiate cerebral ischemia/reperfusion injury. The rats received oral treatment of ethanolic extract of RR (EERR) at 100 and 200 µg/kg dosages following 2 hr post-MCAO procedure. The infarction level, neurological deficit score, and brain water of the experimental rats were evaluated. The inflammatory cytokines and biomarkers, oxidative stress markers, Acetylcholine esterase (AChE), and PERK/ATF-4 and Apaf-1/IRE1α pathway proteins were analyzed with commercial kits. **Results:** The present results evidenced that EERR treatment effectively alleviated neurological impairments, brain water content, and infarction level in the cerebral ischemic-reperfusion injury-induced rats. The EERR treatment decreased the pro-inflammatory cytokine levels and mitigated oxidative stress via increasing antioxidants. In addition, the PERK/ATF-4 and Apaf-1/IRE1α pathways were effectively down-regulated by the EERR treatment in the cerebral ischemia-reperfusion injury-induced rats. **Conclusion:** This work demonstrated the neuroprotective activities of EERR in cerebral ischemia-reperfusion injury-induced rats. These findings may facilitate the advancement of EERR as novel therapeutic agent to avert or mitigate brain injury resulting from ischemic stroke.

Keywords: PERK/ATF-4 Pathway, Ischemic Stroke, Acetylcholine Esterase, RR, Inflammation.

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INTRODUCTION

Stroke, a common neurological condition, remains a significant public health concern worldwide. Ischemic stroke is the major type of stroke, signifying over 70% of all stroke incidences. Ischemic stroke, a severe neurological disorder, transpires when blood supply to a particular region of the brain is obstructed, usually due to the formation of a blood clot or the constriction of blood vessels. This stoppage in blood supply deprives the affected brain of nutrients and oxygen, resulting in the apoptosis of brain cells and potentially permanent neurological deficits.[1] The incidence of stroke has been on the rapid increase globally, with significant implications for both individual and societal well-being. It has been shown that ischemic stroke responsible for the majority of stroke incidences, with an increased global prevalence. The high burden of ischemic stroke is further compounded by its associated long-term disabilities, which can develop vast mental and financial hardships for families and society.[2] The etiology of ischemic stroke is multifaceted, with various risk factors contributing to its development. This encompasses modifiable causes including hypertension, diabetes, obesity, less physical activity, and poor dietary habits, alongside non-modifiable causes like age, gender, and genetic susceptibility. Understanding the underlying causes of ischemic stroke is critical to develop the targeted prevention and management methods.[3]

The underlying mechanisms of ischemic stroke are multifaceted and involve intricate signaling pathways that operate in a spatiotemporal manner. At the core of the ischemic lesion, the rapid depletion of oxygen and glucose triggers a rapid necrotic cell death due to the inability to generate sufficient ATP through oxidative phosphorylation. In the surrounding penumbra, a more delayed form of cell death ensues, driven by a complex interplay of factors like excitotoxicity, oxidative stress, inflammation, and apoptosis.[4] The early disruption of blood flow leads to local

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tissue hypoxia, which impairs ATP production and results in the disruption of ion homeostasis, increased intracellular calcium, and the release of excitatory neurotransmitters. This excitotoxicity further exacerbates calcium influx, triggering the calcium-dependent enzymes and the generation of free radicals, which can show direct cellular injury.[5] Concurrently, the ischemia-induced disturbance of the Blood-Brain Barrier (BBB) allows the invasion of inflammatory cells and mediators, perpetuating the inflammatory cascade and contributing to edema formation and tissue injury. In the reperfusion phase, the restoration of blood flow can paradoxically worsen the injury through the accumulation of ROS, the activation of complement, and the disruption of the microvascular integrity leading to hemorrhagic transformation.[6]

Plant-derived bioactive compounds have appeared as an useful avenue to treat ischemic stroke, offering potential neuroprotective effects and the possibility of addressing the limitations of existing approaches.[7] The pathogenesis of stroke implicates a complex cascade of events, including disrupted microcirculation, impaired energy production, and the accumulation of harmful substances like free radicals and excitatory neurotransmitters. These insults can lead to further neuronal damage and cell death, ultimately contributing to the severe consequences of ischemic stroke.[8] Current therapeutic methods for stroke mainly focus on reestablishing blood flow to the infarct area, either through the use of thrombolytic agents or mechanical thrombectomy. While these interventions can be useful in few incidences, they were often restricted by narrow therapeutic windows, the risk of hemorrhagic complications, and the potential for further injury to the BBB. The exploration of plant-derived bioactive compounds offers a promising alternative approach to the treatment of stroke.[9]

Rhapontici Radix (RR) is the dried root of *Rhaponticum uniflorum*, a plant species of the Asteraceae family. *R. uniflorum* roots are a traditional Chinese medicine employed to alleviate several diseases. RR is predominantly produced in China and Korea. The decoction of R. uniflorum has been utilized in traditional herbal treatment throughout several Asian nations.[10] It has been already reported that RR demonstrated various biological activities, including antitumor,[11] cardioprotective,[12] anti-inflammatory,[13] and anticancer[14] activities. Apart from these biological activities, the neuroprotective effects of RR was not reported yet. Hence, the major focus of this research work is to examine the neuroprotective properties of the RR against ischemic stroke-induced rat model.

MATERIALS AND METHODS

Preparation of Ethanolic Extract of RR (EERR)

RR was acquired commercially as a dried herb from a local herbal market. A 30 g of the dried herb were extracted using 400 mL of 70% ethanol in a shaking incubator at 37°C and 100 rpm for 24 hr. The extract solution was filtered with 185 mm filter paper (Whatman, USA) and concentrated utilizing a rotary vacuum evaporator (Buchi, Japan). Samples were lyophilized and preserved in a desiccator at 4°C prior to use.

Experimental rats

This study utilized male Sprague-Dawley rats weighing around 200-230 g. The rats were maintained in regulated conditions. The temperature was maintained at a constant 22±2°C, with humidity regulated at 55±5%. The rats were underwent to 12-hr light/dark series. The institutional ethics council for animal research validated and sanctioned all experimental protocols using animals.

Middle Cerebral Artery Occlusion (MCAO)-induced model

The rats were anesthetized using pentobarbital sodium (1%; 0.45 mL/kg). The animals were thereafter kept under anesthesia by administering 1.5% isoflurane and 80% oxygen. A surgical operation was conducted to access the middle cerebral artery and insert an 18-mM filament through a 5-mM incision in the common carotid artery. The filament was subsequently advanced to the internal carotid artery to deliberately occlude the middle cerebral artery. Following 120 min of occlusion, the string was carefully detached, facilitating the reestablishment of blood flow. The identical procedure was conducted on the sham-operated rats (control) without the implantation of the filament. During the MCAO procedure, the rat's body temperature was meticulously maintained at a stable 37±0.5°C utilizing a thermostatic surgical tray.[15] Post-operation, the rats were positioned on the surgical tray until they regained consciousness, after which they were returned to their cages. The experimental rats were assigned into 4 groups, each containing six rats (n=6). Group I comprised sham-operated control rats, group II included rats subjected to MCAO, while groups III and IV consisted of rats that underwent MCAO and received oral treatment with 100 and 200 µg/kg of EERR, respectively, 2 hr post-MCAO procedure.

Analysis of neurological deficit score

After 24 hr of ischemia-reperfusion induction, the neurological score was evaluated. The scoring technique was defined as Grade 0, signifying the lack of neurological damage. Grade 1 denotes the incapacity to fully stretch the left forepaw. Grade 2 indicates recurrent leftward circling. Grade 3 entails a leftward falling. Grade 4 is marked by the lack of spontaneous ambulation and a diminished level of consciousness.

Brain water content

The wet/dry weight technique was utilized to assess the cerebral edema. Subsequent to a neurological deficit evaluation, animals were sacrificed, and their brains were immediately separated and weighed (wet weight). After a 72-hr drying period at 70°C, the

brains were reweighed to evaluate their dry weight. The extent of brain water was evaluated using an equation:

$$\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%$$

Analysis of cerebral infarction

The harvested brains were processed with saline solution. The membranes protecting the brain were subsequently excised and sectioned to a width of 2 mM. The brain sections were immersed in a 0.5% TTC solution for 15 min and thereafter treated with 4% paraformaldehyde for fixation. The brain tissues were analyzed and categorized based on their coloration as follows: The color white was used to represent the infarcted area, whereas red was employed to signify the non-infarcted area. The cerebral infarction area was measured utilizing ImageJ software.

Analysis of oxidative stress and inflammatory biomarkers

The concentrations of oxidative stress markers and inflammatory cytokines was evaluated in the brain tissues of the rats. The brain tissue was mechanically homogenized in a Tris buffer solution comprising 1% Triton X-100, 10% glycerol, 150 mM NaCl, and 50 mM Tris-HCl, along with a protease inhibitor solution for 20 min. The suspension was subsequently centrifuged at 10,000 g for 20 min. The supernatant was employed to measure the Glutathione (GSH), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Malondialdehyde (MDA) and an inflammatory cytokines TNF-α, IL-6, IL-10, and IL-1β concentrations. These marker levels were analyzed with commercially available assay kits. The manufacturer's method (MyBioSource, USA) was adhered to for the estimations conducted in triplicate.

Analysis of biochemical markers

The levels of biochemical parameters were assessed in the brains of the rats. The supernatant of the brain tissue homogenates was employed to examine the concentrations of biochemical parameters, including Acetylcholinesterase (AChE), ATP, NF-κB, PGE-2, and COX-2 levels. The marker levels were assessed with commercially available assay kits. The assessments were performed in triplicate according to the protocol outlined by the manufacturer (Abcam, USA). The concentrations of Hypoxia-Inducible Factor 1-α (HIF-1α), apoptotic protease activating Factor-1 (Apaf-1), Inositol-Requiring Enzyme-1α (IRE1α), Activating Transcription Factor 4 (ATF-4), and protein kinase RNA-like ER kinase (PERK) in the rat brain hippocampus was assessed using the kits. The experiments were done in triplicates using the manufacturer's guidelines (MyBioSource, USA).

Statistical analysis

The results were analyzed utilizing GraphPad software. The data are presented as the mean±SD of three triplicate tests, and results was evaluated using one-way ANOVA and *Duncan's post hoc* test. The significance was fixed with a $p<0.005$.

RESULTS

Effect of EERR on the brain water, infarction level, and neurological deficit scores in experimental rats

Figure 1 illustrates the EERR's effect on the brain water content, infarction level, and neurological deficit scores in the cerebral ischemic-reperfusion-induced rats. The experimental rats with cerebral ischemic-reperfusion injury displayed markedly increased brain content, infarction level, and higher neurological scores than the sham-operated group. Interestingly, the EERR treatment at 100 and 200 μg/kg dosages effectively diminished the brain water, infarction level, and neurological scores in rats with cerebral ischemic-reperfusion injury.

Effect of EERR on oxidative stress biomarkers in experimental rats

Figure 2 presents the concentrations of the oxidative stress biomarkers, namely GSH, CAT, SOD, GPx, and MDA in the experimental rats. The present results indicated that, in comparison to the sham-operated rats, the cerebral ischemic-reperfusion-induced rats exhibited reduced CAT, SOD, GSH, and GPx concentrations and elevated MDA levels in their brain hippocampal tissues. Fascinatingly, the findings of the EERR treatment exhibited the markedly elevated antioxidant levels while reducing MDA concentration in the cerebral ischemic-reperfusion-induced rats.

Effect of EERR on AChE and ATP levels in brain tissues of experimental rats

The concentrations of AChE and ATP in the brain hippocampus of the rats were evaluated, with the results illustrated in Figure 3. The present findings demonstrate a significant increase in AChE activity with decreased ATP concentration in the rats with cerebral ischemic-reperfusion injury. Conversely, the EERR treatment at 100 and 200 μg/kg dosages has successfully decreased the AChE activity while concurrently elevating ATP levels in the hippocampus tissue of cerebral ischemic-reperfusion-induced rats.

Effect of EERR on inflammatory cytokines in brain tissues of the experimental rats

The inflammatory cytokines in the brain hippocampus of the rats were illustrated in Figure 4. The results indicate a notable elevation in TNF-α, IL-1β, and IL-6 and a diminution in IL-10 level in the brain tissues of rats subjected to cerebral ischemic-reperfusion injury. Captivatingly, the 100 and 200 μg/kg of EERR treatment

to the cerebral ischemic-reperfusion-induced rats resulted in decreased TNF-α, IL-1β, and IL-6 concentrations, while enhancing IL-10 in their brain tissues.

Effect of EERR on inflammatory biomarkers in brain tissues of experimental rats

The concentrations of COX-2, NF-κB, and PGE-2 in the brain hippocampal tissues were examined in the rats, with the findings presented in Figure 5. The rats subjected to cerebral ischemic-reperfusion injury exhibited elevated COX-2, NF-κB, and PGE-2 concentrations in their brain tissues compared with sham-operated group. Nonetheless, the EERR at varying dosages 100 and 200 μg/kg effectively diminished these inflammatory biomarkers in the brain of cerebral ischemic-reperfusion-induced rats.

Effect of EERR on the HIF-1α, Apaf-1, and IRE1α levels in brain tissues of experimental rats

The influence of EERR on the levels of HIF-1α, Apaf-1, and IRE1α in the brain tissues were assessed, and the findings are presented in Figure 6. The HIF-1α, Apaf-1, and IRE1α concentrations were considerably increased in the brain tissues of the cerebral ischemia-reperfusion-induced rats when compared with sham-operated rats. Nonetheless, the EERR at 100 and 200 μg/kg concentrations effectively decreased the HIF-1α, Apaf-1, and IRE1α concentrations in the brain tissues of the cerebral ischemic-reperfusion-induced rats.

Effect of EERR on ATF-4 and PERK levels in the brain tissues of experimental rats

Figure 7 illustrates the effect of EERR treatment on the levels of both ATF-4 and PERK in the hippocampus of the rats. The concentrations of the ATF-4 and PERK was remarkably elevated in the brain tissues of the cerebral ischemic-reperfusion injury-induced rats. Whereas, the EERR treatment at 100 and 200 μg/kg dosages significantly diminished the ATF-4 and PERK concentrations in the brain tissues of the cerebral ischemic-reperfusion-induced rats.

DISCUSSION

Ischemic stroke occurs when a blood flow to the brain was blocked, often due to a blood clot, leading to a lack of oxygen and nutrients to the affected brain tissue. The burden of stroke is substantial, both in terms of mortality and long-term disability. The affected individuals often face significant physical, cognitive, and emotional challenges, which can have profound impacts on their quality of life and place a substantial burden on their families and healthcare systems.[16] The management of stroke presents unique challenges. The high incidence of severe

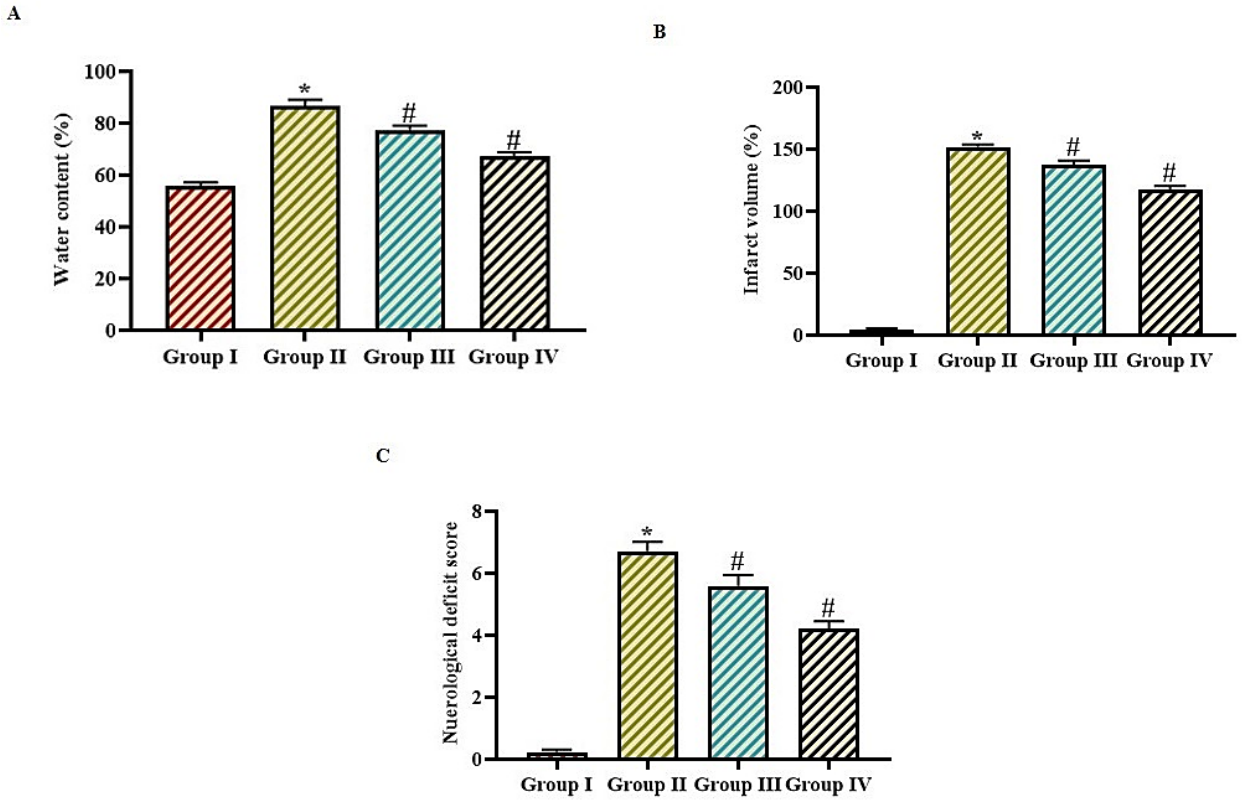


Figure 1: Effect of EERR on the brain water, infarction level, and neurological deficit scores in the experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$. A: Water content; B: Infarct volume; C: Neurological deficit score.

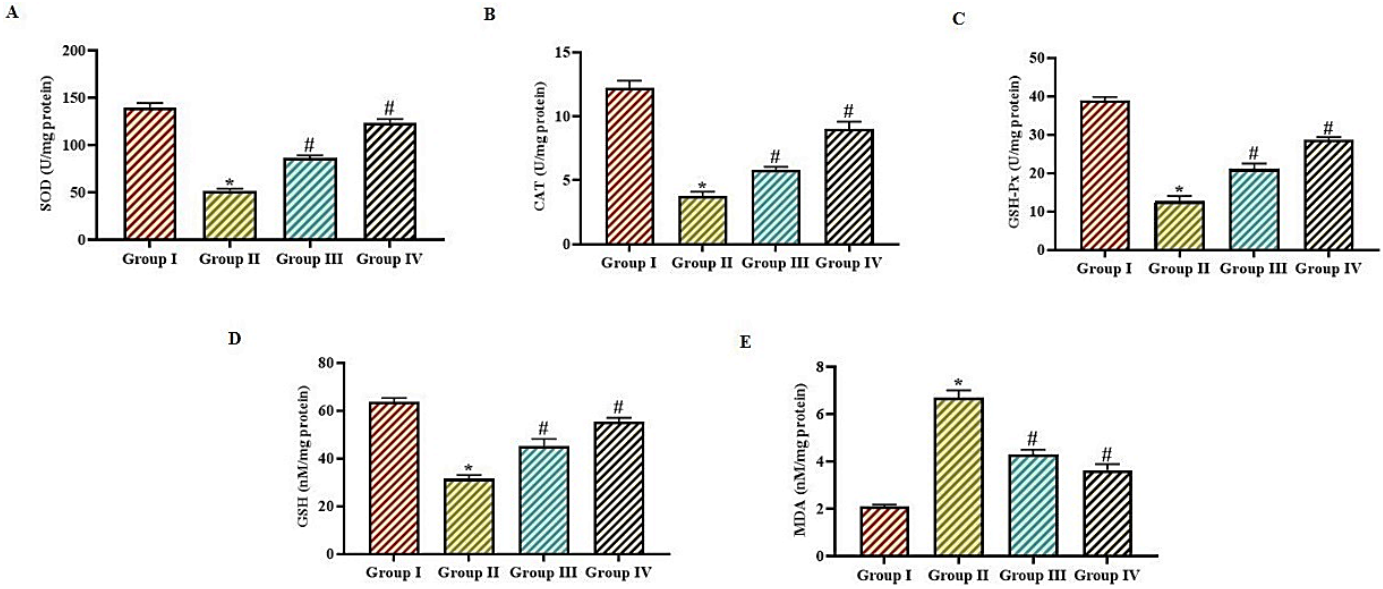


Figure 2: Effect of EERR on the oxidative stress biomarkers in the experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$. A: SOD; B: CAT; C: GSH-Px; D: GSH; E: MDA.

stroke and its early mortality rate in such settings demonstrate the urgent need for improved stroke management strategies. One useful avenue to address this challenge is the exploration of plant-based alternative therapies for stroke. As the global burden of stroke continues to rise, the exploration of plant-based alternative therapies offers a promising avenue for addressing this public health challenge.[17] The complex and multifaceted nature of ischemic stroke has presented considerable challenges in the translation of plant-based interventions from bench to bedside. A comprehensive knowledge of the fundamental molecular causes and the development of more focused and effective therapeutics continues to be a paramount priority in ischemic stroke research.[18]

It has been already highlighted that oxidative stress plays an essential role in the pathogenesis of ischemic stroke, contributing to the several mechanisms that eventually results in the neuronal damage and impaired brain function. Oxidative stress denotes the disparity between the accumulation of ROS and the body's ability to neutralize these detrimental entities or rectify the resulting oxidative injury. In the context of stroke, oxidative stress is initiated by the abrupt cessation of blood flow, leading to the production of free radicals and other reactive species that can harm cellular constituents, including lipids, proteins, and DNA. The brain is especially susceptible to oxidative stress because of its elevated metabolic rate, substantial lipid composition, and insufficient antioxidant defenses.[19] It has been already highlighted the prognostic value of measuring specific oxidative stress biomarkers in stroke patients. These markers, including MDA and antioxidants can offer useful insights into the extent of oxidative damage and the body's response to it. MDA is a widely used biomarker of lipid peroxidation, a process in which free radicals attack and damage cell membranes. Higher levels of MDA have been connected with poorer clinical results in the stroke patients.[20] CAT and SOD are important antioxidant enzymes that help neutralize ROS. Reduced activity of these enzymes has been observed in ischemic stroke patients, indicating an impaired antioxidant defense system. GSH and GPx play crucial roles in the body's protection against oxidative stress. Depletion of GSH and decreased GPx activity have been linked to increased oxidative damage and worse prognosis in ischemic stroke patients.[21] The evaluation of these oxidative stress biomarkers can provide valuable information about the extent of cerebral injury and the potential for neurological recovery. Understanding the role of oxidative stress in the onset of stroke and the prognostic significance of these biomarkers can help develop more targeted interventions to alleviate the devastating effects of this condition.[22] The present findings indicated the elevated MDA level and reduced CAT, GSH, GPx, and SOD concentrations in the brain tissues of the cerebral ischemic-reperfusion injury-induced rats. Captivatingly, the EERR treatment successfully increased the antioxidant concentrations while reducing MDA level, which suggests the antioxidant properties of the EERR.

The pathophysiology of ischemic stroke is complicated, comprising intricate sequences of cellular and molecular processes that result in neuronal injury and cell death. Two key factors that have gained much interest in the context of ischemic stroke are AChE activity and ATP levels. AChE is an enzyme that degrades the neurotransmitter ACh, which plays an essential role in several physiological mechanisms, including neuronal activity and regulation of cerebrovascular tone. Disruption of the delicate balance between ACh synthesis and breakdown by AChE was associated with the onset of ischemic stroke. During the ischemic stroke, the decreased oxygen delivery and blood flow

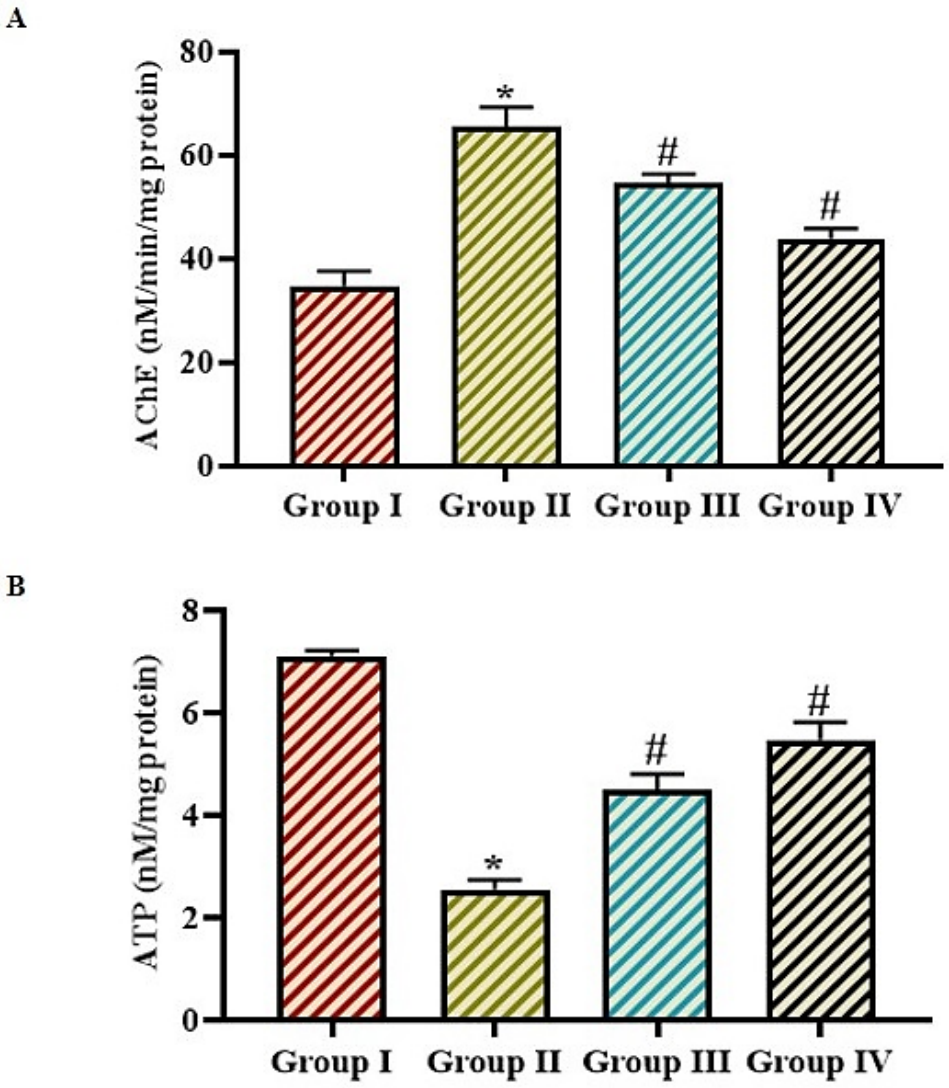


Figure 3: Effect of EERR on the AChE and ATP levels in the brain tissues of experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$. A: AChE activity; B: ATP level.

result in the cascade of events, including the activation of histone deacetylases, which in turn can modulate the activity of AChE. This dysregulation of AChE may contribute to the disruption of cholinergic signaling, impaired cerebrovascular regulation, and ultimately, neuronal injury.[23] In addition to the role of AChE, the depletion of cellular energy stores, as reflected by decreased ATP levels, is a hallmark of stroke. The abrupt disruption of blood delivery to the brain during a stroke event leads to a rapid decline in ATP production, triggering several events that culminate in neuronal dysfunction and death. The disrupted microcirculation and impaired ATP production subsequently lead to a series of insults, including increased intracellular calcium, excitotoxicity, oxidative stress, and inflammation that participates in the onset of stroke.[24] The roles of AChE and ATP levels in the pathogenesis of stroke are crucial and interrelated. The dysregulation of AChE and the depletion of ATP contribute to the complex mechanisms that result in neuronal injury and apoptosis.[25] The present findings evidenced the increased AChE activity and reduced ATP levels in the cerebral ischemic-reperfusion injury-induced rats. Fascinatingly, the EERR treatment considerably reduced the AChE activity while elevating the ATP levels in the hippocampus tissue of cerebral ischemic-reperfusion injury-induced rats.

One of the hallmarks of stroke is the inflammation that is triggered in the affected brain tissue, which can significantly contribute to the overall severity of the injury and influence clinical outcomes. The inflammation in stroke is mediated by a variety of cytokines, including IL-1β, IL-6, TNF-α, and IL-10. These inflammatory cytokines play crucial roles in the pathophysiology of ischemic stroke, initiating and perpetuating the inflammatory cascade that can result in further tissue damage

and neurological impairment.[26] IL-1β is a well-known cytokine that is rapidly upregulated following ischemic brain injury. It facilitates the recruitment of immune cells, including microglia and astrocytes, which may intensify the inflammatory response and induce neuronal cell death. Similarly, TNF-α and IL-6 is a pro-inflammatory cytokines that is also upregulated in the setting of stroke.[27] In contrast, IL-10 is an anti-inflammatory cytokine that has been shown to have a neuroprotective property in the setting of stroke. It can block the generation of pro-inflammatory cytokines and promote the resolution of inflammation, potentially mitigating the extent of brain damage and improving clinical outcomes.[28] The prognostic value of these inflammatory cytokines in ischemic stroke has been extensively studied. Increased IL-1β, IL-6, and TNF-α concentrations have been connected with a poor prognosis and elevated risk of adverse outcomes, like neurological deterioration. Conversely, higher levels of IL-10 have been linked to a more favorable prognosis and improved functional recovery.[29] The current results clearly indicates the elevated TNF-α, IL-1β, and IL-6 concentrations and decreased IL-10 level in the hippocampus of cerebral ischemic-reperfusion injury-induced rats. Captivatingly, the EERR treatment to the cerebral ischemic-reperfusion-induced rats resulted in reduced TNF-α, IL-1β, and IL-6 concentrations, while enhancing IL-10, which evidences the anti-inflammatory activity of EERR.

The complex pathophysiological mechanisms stroke implicates a several molecular mechanisms, including disrupted microcirculation, local tissue hypoxia, increased calcium levels, and excitatory amino acid release. As the primary mediators and modulators of this process, various biochemical and molecular events, including the activation of NF-κB, COX-2, and accumulation of PGE2, play a critical role in the onset of ischemic stroke.[30] NF-κB is a transcription factor that is widely recognized for its involvement in the regulation of inflammation and immune reactions in the ischemic stroke. Upon activation, NF-κB can trigger the numerous pro-inflammatory gene expressions, including COX-2 and other cytokines, resulting in an worsening of the inflammatory cascade.[31] The upregulation of COX-2, an enzyme responsible for the conversion of arachidonic acid to PGE2, is a hallmark of the inflammatory reaction in stroke. PGE2, in turn, can contribute to the disturbance of the BBB, the development of vasogenic edema, and the promotion of neuronal apoptosis.[32] The pathophysiological significance of these mediators extends beyond their immediate involvement in the stroke. The time-dependent propagation of the ischemic penumbra, a region of potentially salvageable tissue, suggests that targeting the biochemical and molecular events, such as the modulation of NF-κB, COX-2, and PGE2, may offer a broader therapeutic window for effective intervention. By elucidating the role of these markers in the pathophysiology of stroke, researchers

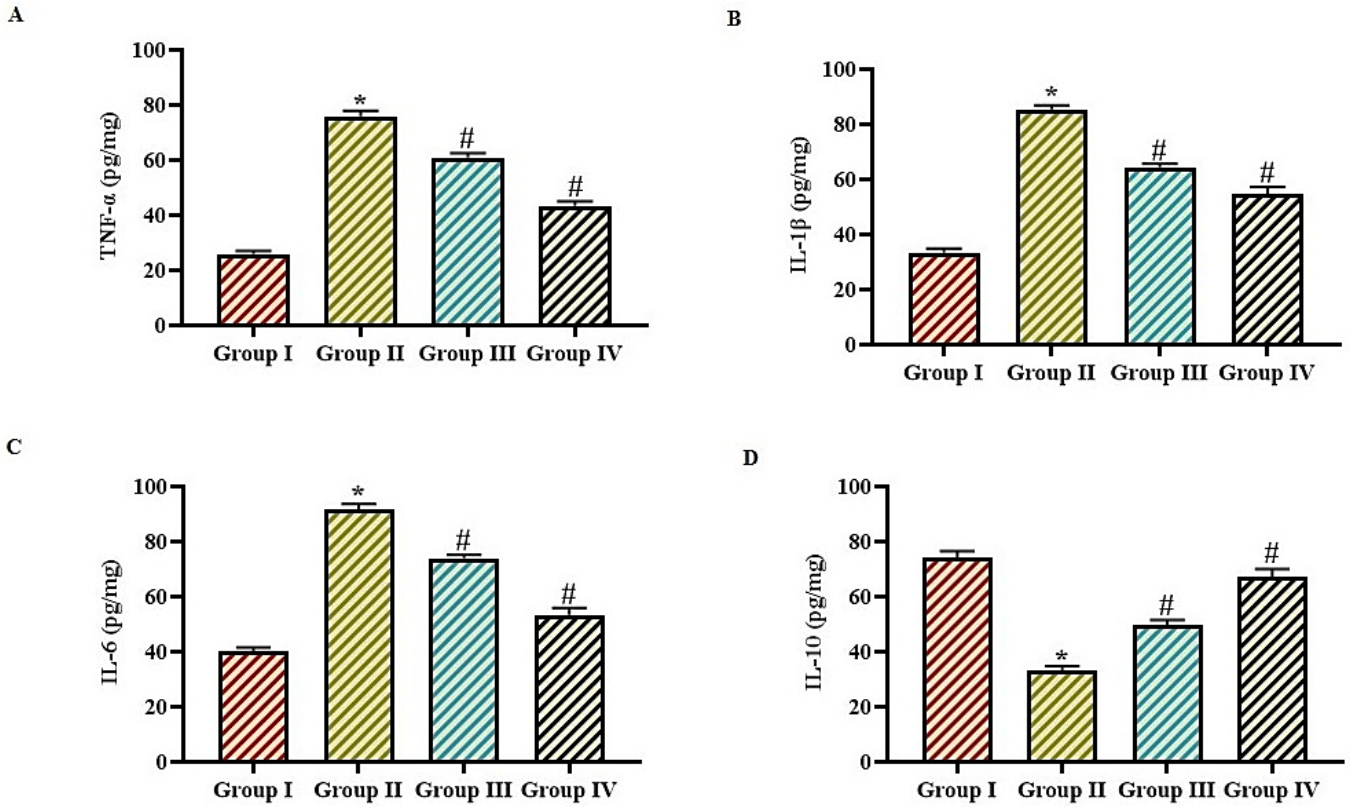


Figure 4: Effect of EERR on the inflammatory cytokines in the brain tissues of the experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$. A: TNF-α level; B: IL-1β level; C: IL-6 level; D: IL-10 level.

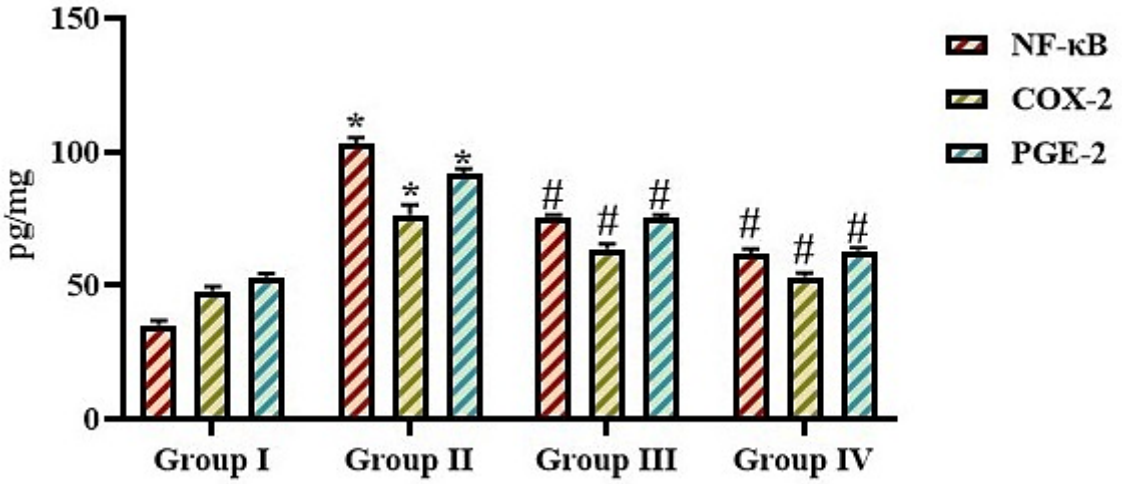


Figure 5: Effect of EERR on the inflammatory biomarker levels in the brain tissues of experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$.

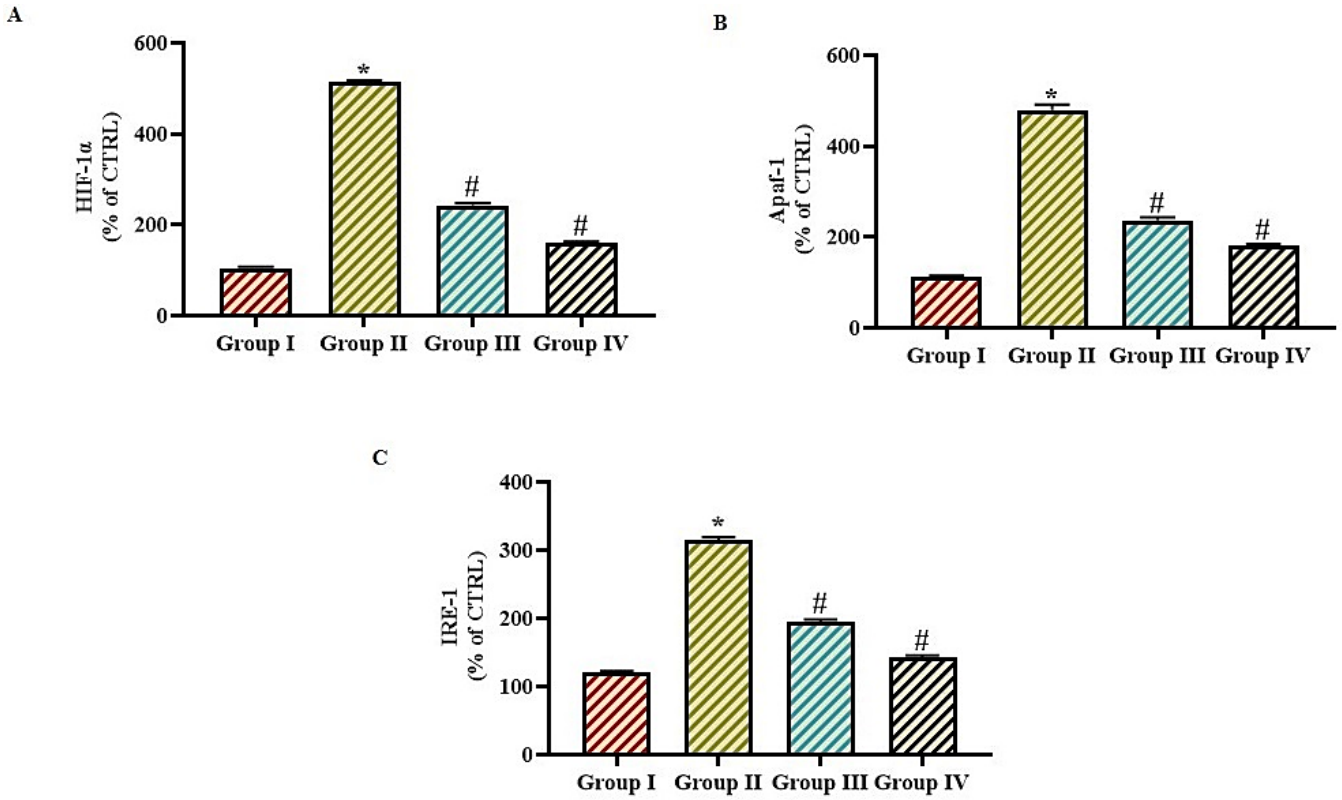


Figure 6: Effect of EERR on the HIF-1α, Apaf-1, and IRE1α levels in the brain tissues of experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$. A: HIF-α level; B: Apaf-1 level; C: IRE-1 level.

can gain useful insights into the prognostic significance of these mediators and explore novel therapeutic approaches that leverage their regulatory functions.[33] In this work, we found that the rats with cerebral ischemic-reperfusion injury displayed augmented COX-2, NF-κB, and PGE-2 concentrations in their brain tissues. Captivatingly, the EERR treatment successfully decreased these inflammatory biomarker levels in the brain tissues of rats with cerebral ischemic-reperfusion injury.

In the complex pathophysiological process of stroke, there are several key molecular players plays a major roles with the significant prognostic implications. HIF-1α is a transcription factor that orchestrates the cellular response to hypoxic

conditions, upregulating the gene expressions implicated in angiogenesis, glucose metabolism, and cell survival. In the context of ischemic stroke, HIF-1α exhibits both neuroprotective and neurotoxic properties. On one hand, it promotes the production of erythropoietin, which activates signaling cascades connected with neuroprotection, while it also induces the VEGF expression, leading to neovascularization and the development of microvascular networks in hypoxic-ischemic brain tissue. The delicate balance between these competing effects of HIF-1α ultimately determines the outcome of ischemic stroke.[34] Apaf-1, a critical component of the intrinsic apoptotic pathway, plays a pivotal role in the pathophysiology of stroke. Upon exposure to ischemic stress, the pro-apoptotic protein Bax accumulates in the mitochondria, leading to the release of cyt-C into the cytosol. Cyt-C then binds to Apaf-1, forming the apoptosome complex, which in turn triggers the caspase cascade, ultimately resulting in programmed cell death. The degree of Apaf-1 activation and the extent of apoptotic signaling have been shown to correlate with the severity of neuronal injury and infarct size.[35] IRE1α, a crucial modulator of the unfolded protein response, significantly contributes to the pathophysiology of stroke. Under conditions of hypoxia and endoplasmic reticulum stress, IRE1α is activated, triggering the gene transcriptions implicated in protein folding, quality control, and eventual cell death. Activation of IRE1α was associated with both neuroprotective and neurodegenerative properties, depending on the duration and severity of the ischemic insult.[36] Elevated levels of HIF-1α, Apaf-1, and IRE1α have all been associated with larger infarct size, poorer functional results, and elevated mortality in ischemic stroke patients. Importantly, the temporal dynamics of their expression and the balance between their pro-survival and pro-apoptotic functions appear to be crucial in determining the ultimate fate of the ischemic brain tissue.[37] The present findings clearly showed that the concentrations of HIF-1α, Apaf-1, and IRE1α were considerably elevated in the brain tissues of the cerebral ischemia-reperfusion-induced rats. Whereas, the treatment with EERR markedly reduced these biomarker concentrations in the brain tissues of the rats with cerebral ischemic-reperfusion injury.

ATF-4 and its upstream regulator, PERK, are pivotal components in the pathogenesis of stroke. ATF-4 is a transcription factor induced by many cellular stressors, such as hypoxia, nutritional deficiency, and endoplasmic reticulum stress. PERK is a transmembrane protein kinase that is activated by unfolded protein accumulations in the ER, which can occur during ischemic conditions. When activated, PERK phosphorylates the translation initiation factor eIF2α, resulting in the selective translation of ATF-4 and the subsequent activation of its target genes.[38] The role of ATF-4 and PERK in the pathogenesis of stroke has been well reported. During ischemic conditions, the activation of the PERK-ATF-4 pathway can lead to the upregulation of genes involved in the adaptive response to stress, such as those involved in amino acid metabolism, antioxidant defense, and protein folding. However, prolonged or excessive activation of this pathway can also contribute to apoptosis, through the activation of pro-apoptotic genes and the inhibition of protein synthesis.[39] In addition to their role in the acute phase of stroke, ATF-4 and PERK have also been implicated in the long-lasting results of stroke patients. It has been shown that the ATF-4 and PERK expressions in the brain can be used as prognostic markers for predicting the severity of stroke and the likelihood of recovery. Understanding the mechanisms by which these molecules facilitate the onset of ischemic stroke is essential for formulating novel interventions and identifying predictive biomarkers.[40] The present findings evidences the increased concentrations of the ATF-4 and PERK in the brain tissues of the rats with cerebral ischemic-reperfusion injury. Captivatingly, the treatment with EERR successfully reduced the ATF-4 and PERK concentrations in the brain tissues of the cerebral ischemic-reperfusion injury-induced rats.

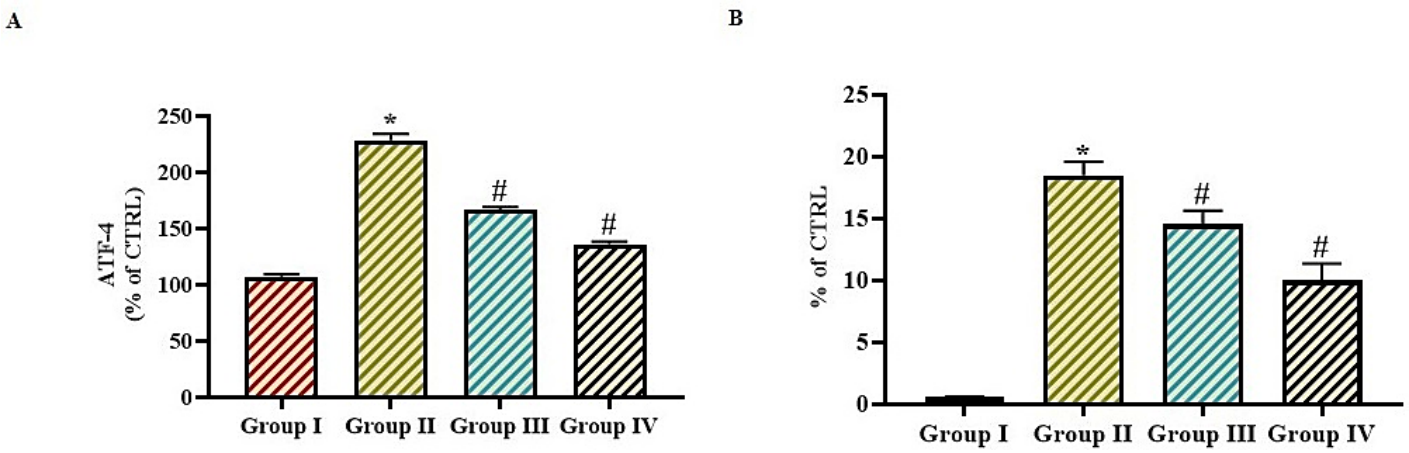


Figure 7: Effect of EERR on the ATF-4 and PERK levels in the brain tissues of experimental rats. Data presented in each bar represent the mean±SD of triplicate assays and were statistically analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Note: '*' reveals that results are significantly differ from the control group at $p<0.001$; '#' reveals that results are significantly differ from the MCAO-induced group at $p<0.005$. A: ATF-4 level; B: PERK level.

CONCLUSION

This study demonstrated the neuroprotective activities of EERR in cerebral ischemia-reperfusion injury-induced rats. The treatment with EERR significantly reduced infarct size, diminished behavioral abnormalities, regulated biochemical markers, and alleviated alterations in the rat's brain. Moreover, EERR treatment considerably diminished inflammatory responses and oxidative stress via upregulating the antioxidants in rats with cerebral ischemic/reperfusion injury. In addition, the PERK/ATF-4 and Apaf-1/IRE1α pathways were effectively down-regulated by the EERR treatment in the cerebral ischemia-reperfusion injury-induced rats. These findings may facilitate the advancement of EERR as a novel therapeutic agent to avert or mitigate brain injury resulting from ischemic stroke. Furthermore, additional works are needed to elucidate the specific therapeutic effects of EERR on the against ischemic stroke.

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ABBREVIATIONS

RR: Rhapontici Radix; **EERR:** Ethanolic Extract of RR; **AChE:** Acetylcholine Esterase; **MCAO:** Middle Cerebral Artery Occlusion; **GSH:** Glutathione; **MDA:** Malondialdehyde; **HIF-1α:** Hypoxia-inducible Factor 1-α; **Apaf-1:** Apoptotic Protease Activating Factor-1; **ATF-4:** Activating Transcription Factor 4.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

This work has approved by the institutional animal ethical committee by Baoji People's Hospital, Baoji City, Shaanxi province, 721000, China.

SUMMARY

Cerebral ischemic stroke occurs when a blood flow to the brain was blocked, often due to a blood clot, leading to a lack of oxygen and nutrients to the affected brain tissue. The burden of stroke is substantial, both in terms of mortality and long-term disability. The present findings demonstrated the neuroprotective activities of EERR in cerebral ischemia-reperfusion injury-induced rats. These findings may facilitate the advancement of EERR as a novel therapeutic agent to avert or mitigate brain injury resulting from ischemic stroke.

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