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Original Research Article

Matairesinol Shows Neuroprotective Activity Against Cerebral Ischemia/Reperfusion Injury via Downregulating TLR4-NLRP3 Inflammasome Pathway

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Abstract

Background: Stroke, a formidable global health issue, is characterized by high disability and mortality rates, thus necessitating advances in acute treatment strategies and preventative medical regimens. Ischemic stroke (IS), the most prevalent type, emerges due to the interruption of blood flow to the brain, resulting in a series of events that may induce irreparable cerebral damage.

Objectives: The present work was conducted to study the neuroprotective properties of matairesinol in *in vivo* and *in vitro* models.

Materials and Methods: The experimental rats were subjected to middle cerebral artery occlusion (MCAO) to induce ischemia/reperfusion (I/R) injury. Rats were pretreated orally with 25 and 50 mg/kg of matairesinol for 7 days earlier to the MCAO. The brain edema, neurological score, and infarction level of the rats were assessed. The concentrations of oxidative stress markers, inflammatory cytokines, and acetylcholinesterase (AChE) concentrations were assessed using kits. The levels of toll-like receptor 4 (TLR4) and NOD-like receptor protein 3 (NLRP3) were also studied using test kits. The *in vitro* studies were evaluated using oxygen-glucose deprivation/reoxygenation (OGD/R)-stimulated human neuroblastoma cell line (SH-SY5Y) cells. The effect of matairesinol on the viability and inflammatory markers was assessed in OGD/R-stimulated SH-SY5Y cells.

Results: The oral pretreatment of matairesinol significantly diminished the brain edema, neurological scores, and infarction level in the rats subjected to MCAO. The matairesinol treatment considerably diminished pro-inflammatory cytokine levels, enhanced antioxidant concentrations, and diminished the AChE in the brain tissues of MCAO-induced rats. Matairesinol treatment also effectively reduced the TLR4 and NLRP3 levels in the brains of MCAO-induced rats. The findings of *in vitro* studies evidenced that the matairesinol increased viability and reduced inflammatory markers in OGD/R-stimulated SH-SY5Y cells.

Conclusion: The research highlighted the neuroprotective property of matairesinol in rats with MCAO-induced cerebral I/R injury. These results may facilitate the advancement of matairesinol as a novel therapeutic agent to treat IS.

Keywords

TLR4/NLRP3 pathway, ischemic brain injury, matairesinol, SH-SY5Y cells, acetylcholinesterase

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Introduction

Cerebral stroke, a type of cerebrovascular disease affecting the central nervous system (CNS), is a predominant global health issue due to its high morbidity and mortality rates. Stroke ranks as the second most common cause of mortality and a primary source of disability globally, imposing a significant burden on affected individuals, their families, and society (Saini et al., 2021). Ischemic stroke (IS), the more prevalent subtype, emerges from a blockage of blood supply to the brain, resulting in a lack of nutrients and oxygen,

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which subsequently causes tissue damage and neurological deficits. The impact of stroke extends beyond mortality, as a significant proportion of survivors experience long-term disabilities, which can significantly decrease their quality of life (Endres et al., 2022). IS is a critical neurovascular condition defined by brain ischemia resulting from the occlusion of a cerebral blood vessel. It is responsible for nearly 87% of all stroke incidences and shows a significant medical and social burden. The causes of IS are diverse, but they primarily involve conditions that result in the obstruction or diminution of blood flow to the brain (Goodman et al., 2023).

The pathogenesis of IS is multifaceted, encompassing a series of molecular and cellular events that eventually result in neuronal injury and necrosis. It begins with the disruption of cerebral blood flow, leading to a lack of glucose and oxygen, leading to energy failure within brain cells. This energy crisis triggers a series of events, comprising the release of excitatory neurotransmitters such as glutamate, which overstimulate neurons, leading to excitotoxicity and further cellular damage (Majumder, 2024). The ensuing ionic imbalances, particularly the influx of calcium ions, disrupt cellular homeostasis and activate intracellular signaling pathways that promote apoptosis and necrosis. The initial ischemic core, characterized by severe and irreversible tissue damage, is surrounded by a region known as the penumbra, where the blood flow is reduced but the tissue is still potentially salvageable. Inflammatory responses also play a crucial role in the onset of IS, with the stimulation of resident immune cells and the immune cell influx contributing to the expansion of tissue damage (Kuriakose & Xiao, 2020). Microglia are rapidly activated after ischemia, and their activation reflects the severity of the ischemic injury. The breakdown of the blood–brain barrier further exacerbates the inflammatory response, permitting the influx of immune cells into the brain parenchyma. These intricate molecular and cellular mechanisms involved in IS pathophysiology present potential targets for therapeutic interventions aimed at neuroprotection and recovery (Maida et al., 2024).

Current treatment strategies primarily revolve around pharmacological interventions aimed at restoring blood flow and preventing secondary complications, alongside rehabilitative approaches to facilitate functional recovery. Recombinant tissue plasminogen activator (rt-PA) is the primary IS treatment that converts plasminogen to plasmin, thereby dissolving the thrombus obstructing cerebral blood flow (Patil et al., 2022). The therapeutic efficacy of tPA is critically dependent on timely administration, ideally within a narrow therapeutic window of 4.5 h from symptom onset. Nevertheless, thrombolysis is not without its limitations, as a significant proportion of patients may not be eligible due to contraindications such as recent surgery, bleeding disorders, or uncontrolled hypertension. Furthermore, even in eligible patients, tPA therapy carries a risk of symptomatic hemorrhagic transformation, wherein the ischemic infarct undergoes bleeding, potentially worsening neurological outcomes (Bathla et al., 2023). Furthermore, there is a significant gap in the development of neuroprotective therapies that can effectively mitigate the inflammatory and oxidative stress responses associated with IS. Given the limitations and challenges associated with current treatment options, there is a growing need for alternative therapies that can complement existing approaches and address unmet clinical needs in IS management (Sharma et al., 2024). The search for plant bioactive compounds as potential therapeutic interventions for IS holds significant promise, offering a complementary approach to current treatments with the potential for improved neuroprotection and fewer adverse effects (Gomez-Verjan et al., 2023). Matairesinol is a bioactive dibenzylbutyrolactone lignan compound widely present in linseed, sesame seeds, several grains, soybeans, and berries (Rodríguez-García et al., 2019). It has already been reported that matairesinol demonstrated anti-allergic (Sung et al., 2016), anti-cancer (Mahajan et al., 2021), anti-angiogenesis (Lee et al., 2012), and anti-osteoclastogenic (Choi et al., 2014) activities. Furthermore, previous studies have demonstrated that matairesinol decreases neuroinflammation in BV2 microglia (Xu et al., 2017) and inhibits sepsis-induced brain injury in rats (Wu et al., 2021). While matairesinol has demonstrated anti-inflammatory and antioxidant properties in various studies, its neuroprotective effects against IS have not been explored. Therefore, the present study aims to investigate the neuroprotective properties of matairesinol in *in vivo* and *in vitro* models of IS, addressing the existing research gap in the development of effective neuroprotective therapies for IS.

Materials and Methods

Chemicals

Matairesinol (CAS #: 580-72-3) and other chemicals and reagents utilized in this study were procured commercially from Sigma–Aldrich, USA. For the estimation of biochemical parameters, the assay kits were purchased from MyBioSource, Abcam, and Elabscience, USA, respectively.

Experimental Rats

The current study was performed on 7–8-week-old healthy male Sprague-Dawley rats. The rats were sustained in a regulated laboratory setting with meticulous consideration for their welfare. The conditions were sustained at a temperature of 22°C–26°C, 40%–70% humidity, and a 12-h light–dark series. Throughout the study period, the rats were given purified water and standard rat feed. Before initiating the study, a 1-week acclimatization period was allotted for the rats to adjust to the laboratory setting. The protocols for the animal experimentation were verified and approved by the Institutional Animal Ethics Committee.

Middle Cerebral Artery Occlusion (MCAO)-induced Cerebral Ischemia/Reperfusion (I/R) Injury Model

The rats were anesthetized using intraperitoneal administration of chloral hydrate (10%) and thereafter secured in supine posture. After shaving and sanitizing the neck, a midline cervical incision was performed to expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA), subsequently ligating the ECA and its distal segment. The proximal segments of the ICA and CCA were provisionally occluded. A little incision was performed on the ECA near the common carotid bifurcation, and a silicone-coated suture was introduced. The clip on the ICA was detached, and a suture was carefully introduced into the ICA through the ECA until the development of MCAO. The suture measured 18–20 mm in length. The suture was secured, and the clip on the CCA was withdrawn. Subsequently, the cervical skin was sutured; 2 h post-ischemia, the sutures were excised, facilitating the restoration of blood flow. In the sham (control) group, just the ICA was subjected to dissection, with no other treatments performed.

Experimental Groups

The rats were separated into four experimental groups, each including six rats. Group I contained the sham control rats. Group II contained rats subjected to MCAO procedure, and groups III and IV comprised rats that underwent MCAO operation and were administered oral pretreatment of 25 and 50 mg/kg of matairesinol, respectively, for 7 days.

Analysis of Brain Water Content, Neurological Deficit Score, and Infarction Level

After 24 h of cerebral I/R initiation, the neurological scores were examined. The scaling method for neurological scores was followed as mentioned earlier (Long et al., 1989). The wet/dry weight method was utilized to evaluate cerebral edema. After neurological score examination, the animals were euthanized, and their brains were quickly excised and weighed to get wet weight. Following a 72-h drying at 70°C, the brain was reweighed to assess the dry weight. The volume of brain water was assessed using the equation: (wet weight – dry weight)/wet weight × 100%. The level (%) of cerebral infarction was assessed using the 2,3,5-triphenyltetrazolium chloride (TTC) staining as specified previously (Ridenour et al., 1991).

Analysis of Oxidative Stress Markers and Acetylcholinesterase (AChE) Levels

The oxidative stress markers were assessed in the brains of the rats. The brains were homogenized with Tris buffer along with protease inhibitor solution for 15 min. Then centrifuged at 15,000 rpm for 20 min. The resulting supernatant was employed to measure the malondialdehyde (MDA; #MBS738685), catalase (CAT; #MBS2600683), glutathione (GSH; #MBS265966), superoxide dismutase (SOD; # MBS036924), glutathione peroxidase (GPx; #MBS3807927), and AChE (#MBS725468). The biomarker concentrations were examined using kits. The tests were performed in triplicate using the manufacturer's specified protocol (MyBioSource, USA).

Analysis of Pro-inflammatory Cytokines

The levels of interleukin (IL)-1β (#ab100767), IL-4 (#ab100770), IL-6 (#ab100772), IL-10 (#ab100765), and tumor necrosis factor-alpha (TNF-α) (#ab100784) in the brain tissues of the rats were quantified with commercial test kits from the manufacturer (Abcam, USA), adhering to the prescribed protocols. Each experiment was conducted in triplicate, and the findings are presented in pg/mg protein.

Analysis of Toll-like Receptor 4 (TLR4) and NOD-Like Receptor Protein 3 (NLRP3) Levels

The levels of TLR4 (#MBS2024497) and NLRP3 (#MBS7612469) in the brains of the rats were evaluated using commercial diagnostic kits. The tests were conducted in compliance with the manufacturer's instructions (MyBioSource, USA).

In Vitro Studies

Experimental Cells

The neuroblastoma human neuroblastoma cell line (SH-SY5Y) cells were purchased from ATCC, USA. They were cultured in accordance with ATCC-specified methods. Cells were grown on Dulbecco's Modified Eagle Medium (DMEM) enriched with fetal bovine serum (FBS) (10%) in a 5% CO_2 incubator at 37°C.

Oxygen-Glucose Deprivation/Reoxygenation (OGD/R) Model

An *in vitro* I/R injury model was induced using an OGD/R method as per the previously established procedure (Zhao et al., 2013). SH-SY5Y cells were sustained in hypoxic circumstances with O_2 (1%), CO_2 (5%), and N_2 (94%) for a duration of 4 h. Cells were then relocated to DMEM and maintained under usual conditions for 24 h. SH-SY5Y cells, continuously grown in DMEM and maintained under conventional incubation conditions, served as the negative control.

MTT Assay

The influence of matairesinol on SH-SY5Y cell viability was studied using the MTT assay. The OGD/R subjected cells were treated with 5, 10, and 15 μM/mL of matairesinol for

24 h. Cells without the OGD/R procedure served as a control. Consequently, 20 µL MTT reagent was combined with 100 µL DMEM and incubated for 4 h. After liquefying the developed formazan deposits in DMSO, the absorbance was taken at 570 nm.

Lactate Dehydrogenase (LDH) Assay

The LDH release from the SH-SY5Y cells was assessed utilizing the LDH Cytotoxicity Assay kit (#ab102526, Abcam, USA) as per the manufacturer's specifications. The absorbance at 490 nm was quantified using a microplate reader.

Inflammatory Cytokine Analysis

The untreated and treated cells were obtained, lysed, and centrifuged at 6,000 rpm for 15 min, and the resulting supernatant was employed for the estimation of inflammatory markers. The TNF-α (#E-EL-H2306), IL-1β (#E-EL-H0149), and IL-6 (#E-EL-H6156) concentrations were evaluated utilizing assay kits in accordance with the manufacturer's specifications (Elabscience, USA).

Statistical Analysis

The values are analyzed utilizing GraphPad Prism software. The data are given as a mean ± standard deviation (SD) of triplicates and evaluated using one-way analysis of variance (ANOVA) and Dunnett's *post hoc* assay. The significance was fixed with a p value < .05.

Results

Effect of Matairesinol on Brain Water Level, Neurological Score, and Infarction Level in Experimental Rats

Figure 1 depicts the impact of matairesinol on the brain edema, neurological score, and infarction levels in the MCAO-induced rats. The neurological score, brain water level, and infarction level were observed to be elevated in the MCAO-induced rats when compared with sham controls. Fascinatingly, the oral pretreatment with 25 and 50 mg/kg of matairesinol markedly diminished the brain edema, neurological score, and infarction levels in the MCAO-induced rats.

Effect of Matairesinol on Oxidative Stress Markers and AChE Concentrations in Experimental Rats

Figure 2 presents the impact of matairesinol on the AChE and oxidative stress markers in the MCAO-induced rats. In

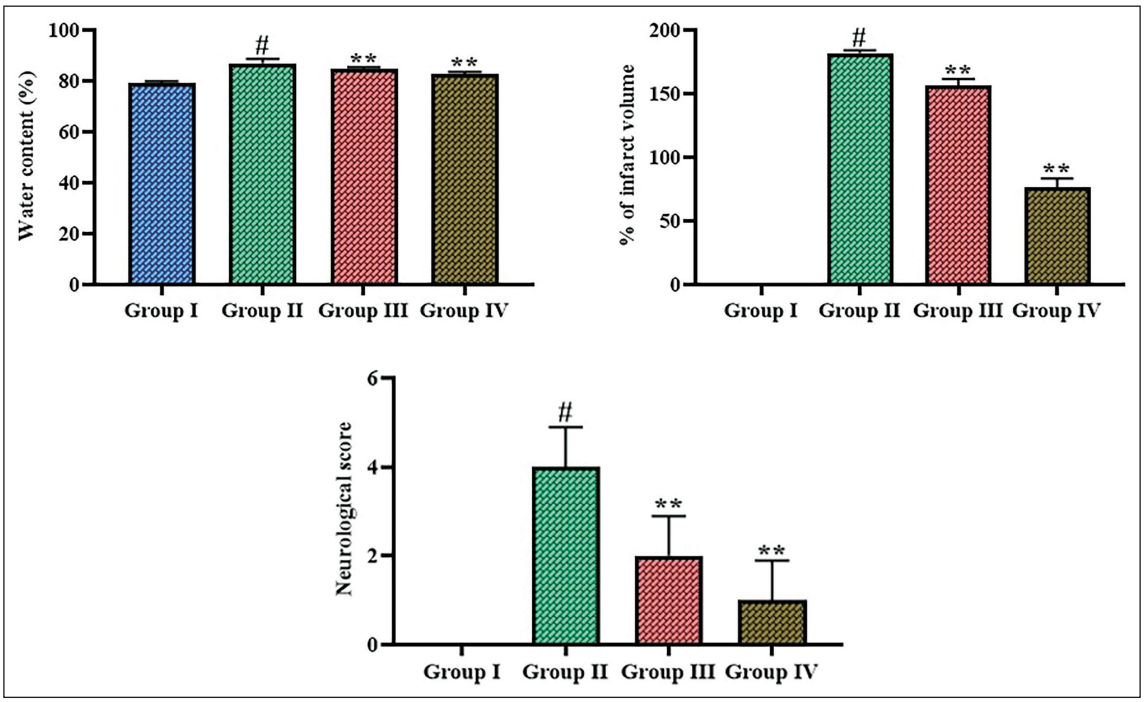


Figure 1. Effect of Matairesinol on the Neurological Score, Brain Water Content, and Infarction Level in the Experimental Rats. The Mean ± Standard Deviation (SD) Values of Triplicates are Given in Each Bar, and Statistical Analysis is Conducted Using GraphPad Prism Software. Values are Assessed Utilizing One-way Analysis of Variance (ANOVA) and Dunnett's *Post Hoc* Analysis. The Symbol "#" Denotes a Statistically Significant Difference (p < .01) Relative to the Control Group. The Symbol "**" Denotes a Statistically Significant Difference (p < .05) Relative to the Middle Cerebral Artery Occlusion (MCAO)-induced Group.

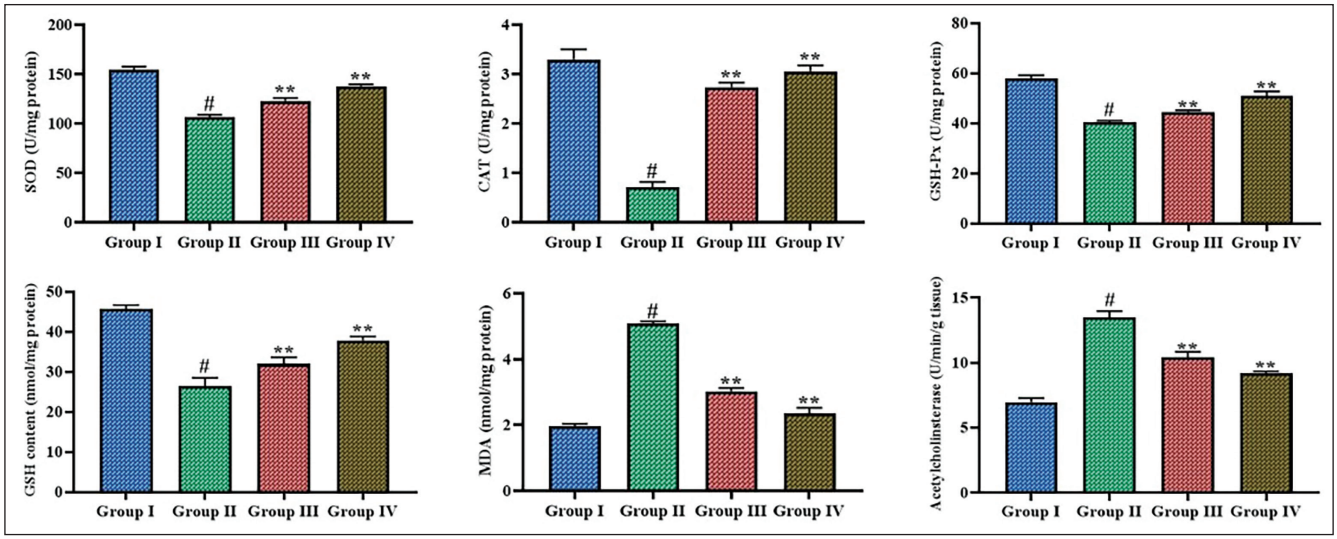


Figure 2. Effect of Matairesinol on the Oxidative Stress Markers and Acetylcholinesterase (AChE) Levels in the Experimental Rats. The Mean ± Standard Deviation (SD) Values of Triplicates are Given in Each Bar, and Statistical Analysis is Conducted Using GraphPad Prism Software. Values are Assessed Utilizing One-way Analysis of Variance (ANOVA) and Dunnett's *Post Hoc* Analysis. The Symbol "#" Denotes a Statistically Significant Difference ($p < .01$) Relative to the Control Group. The Symbol "**" Denotes a Statistically Significant Difference ($p < .05$) Relative to the Middle Cerebral Artery Occlusion (MCAO)-induced Group.

comparison with sham controls, the MCAO-induced rats illustrated increased MDA levels along with diminished antioxidants such as CAT, SOD, GSH, and GPx in their brain tissues. The concentrations of AChE were also found to be elevated in the brains of MCAO-induced rats. Whereas, the oral pretreatment of 25 and 50 mg/kg of matairesinol significantly diminished the MDA levels and subsequently elevated the antioxidant concentrations in the brain tissues of MCAO-induced rats. In addition, the matairesinol also diminished the AChE concentrations in the brains of MCAO-induced rats.

Effect of Matairesinol on Inflammatory Markers of Experimental Rats

Figure 3 depicts the levels of inflammatory biomarkers in control and experimental rats. The brains of MCAO-induced rats demonstrated reduced IL-4 (419.18 pg/mg protein) and IL-10 (159.02 pg/mg protein) concentrations, while exhibiting elevated TNF-α (56.67 pg/mg protein), IL-1β (62.46 pg/mg protein), and IL-6 (137.70 pg/mg protein) concentrations in comparison with sham-operated controls. Nonetheless, the matairesinol pretreatment at 25 and 50 mg/kg concentrations significantly elevated IL-4 (606.11 and 717.32 pg/mg protein, respectively) and IL-10 (254.71 and 282.56 pg/mg protein, respectively) concentrations. Furthermore, the matairesinol pretreatment also diminished the concentrations of TNF-α (40.10 and 35.03 pg/mg protein, respectively), IL-1β (52.83 and 46.53 pg/mg protein, respectively), and IL-6 (111.65 and 87.31 pg/mg protein, respectively) in the brains of MCAO rats.

Effect of Matairesinol on TLR4 and NLRP3 Levels in Experimental Rats

The concentrations of TLR4 and NLRP3 were quantified in the brains of the rats, with the outcomes illustrated in Figure 4. The rats with MCAO-induced cerebral I/R injury displayed a significant upregulation in both TLR4 and NLRP3 concentrations in their brains when compared with sham-operated controls. Nonetheless, the matairesinol pretreatment at dosages of 25 and 50 mg/kg considerably reduced the concentrations of both TLR4 and NLRP3 in the brains of MCAO-induced rats.

Effects of Matairesinol on Growth and LDH Release in the SH-SY5Y Cells

The present results explicitly indicate that the SH-SY5Y cells experienced OGD/R, resulting in a significant decline in their viability when compared with the control. However, the treatment with matairesinol at concentrations of 5, 10, and 15 μM/mL demonstrated a significant enhancement in cell growth of OGD/R-stimulated cells (Figure 5A). In addition, the LDH release was significantly elevated in OGD/R-stimulated cells compared with the control group. Interestingly, LDH release was significantly diminished by the matairesinol treatment in a dose-dependent manner (Figure 5B).

Effects of Matairesinol on Inflammatory Cytokines in the SH-SY5Y Cells

The concentrations of inflammatory cytokines were examined in the SH-SY5Y cells, with the findings given in Figure 6.

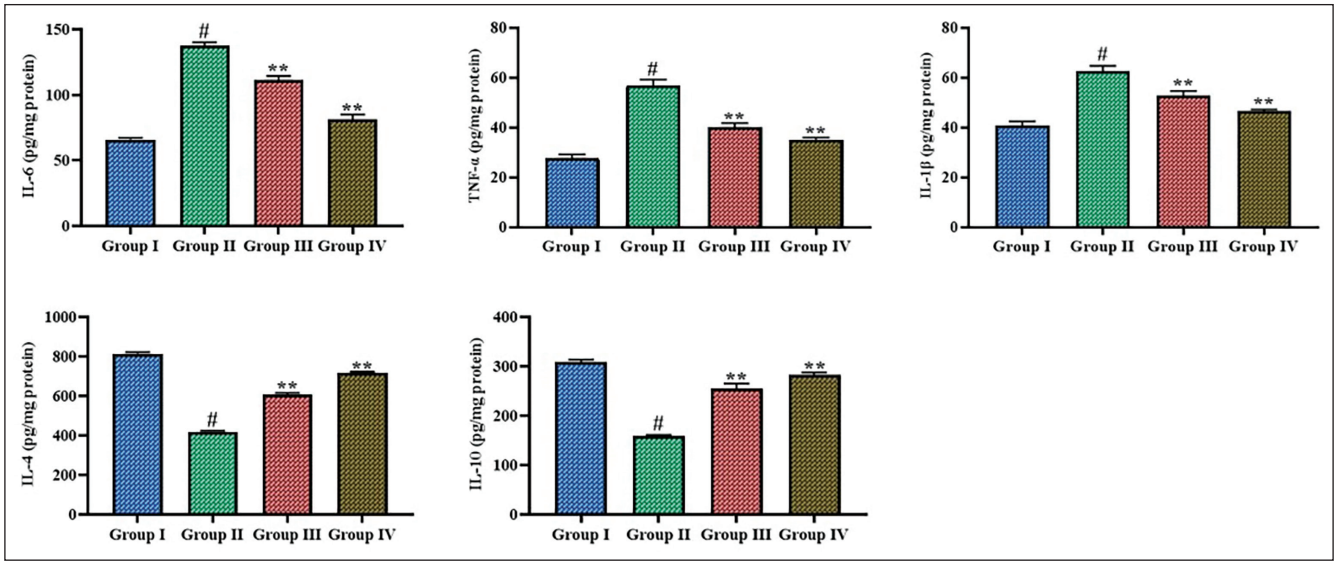


Figure 3. Effect of Matairesinol on the Inflammatory Marker Levels in the Experimental Rats. The Mean ± Standard Deviation (SD) Values of Triplicates are Given in Each Bar, and Statistical Analysis is Conducted Using GraphPad Prism Software. Values are Assessed Utilizing One-way Analysis of Variance (ANOVA) and Dunnett's *Post Hoc* Analysis. The Symbol "#" Denotes a Statistically Significant Difference ($p < .01$) Relative to the Control Group. The Symbol "**" Denotes a Statistically Significant Difference ($p < .05$) Relative to the Middle Cerebral Artery Occlusion (MCAO)-induced Group.

The present findings demonstrate that IL-6 (86.86 pg/mL), IL-1β (75.53 pg/mL), and TNF-α (117.31 pg/mL) concentrations are augmented in the OGD/R-stimulated cells in comparison with the control. Nevertheless, the treatment with matairesinol revealed a drastic reduction in these cytokine levels of OGD/R-induced cells, as depicted in Figure 6.

Discussion

IS, a critical cerebrovascular event, occurs due to an interruption of blood supply to the brain, resulting in glucose and oxygen deficiency, culminating in neuronal damage and subsequent neurological deficits. This complex cascade involves intricate cellular and molecular mechanisms, including excitotoxicity, oxidative stress, inflammation, and apoptosis, which amplify the initial ischemic injury. The current therapies for IS, mainly focused on thrombolysis with tPA and mechanical thrombectomy, focus on reestablishing cerebral blood flow but are often restricted by a narrow therapeutic window and potential hemorrhagic complications (Mosconi & Paciaroni, 2022). The onset of IS encompasses a complex interplay of factors, beginning with reduced cerebral blood flow, which initiates a series of events that cause neuronal injury. This cascade includes the release of excitatory neurotransmitters like glutamate, leading to excitotoxicity, oxidative stress, and inflammation, which ultimately result in cellular damage. The intricate pathogenesis of IS encompasses a complex interplay of molecular and cellular events that contribute to neuronal injury and functional deficits (Qin et al., 2022). Despite advancements in acute stroke management, a significant proportion of patients still experience long-term disability, highlighting the pressing need for novel and effective neuroprotective strategies. Plant-derived bioactive compounds may offer a promising avenue for the development of alternative and complementary therapies for IS. The limited success of conventional treatments and the high incidence of adverse effects have encouraged the exploration of alternative therapies, with plant-derived bioactive compounds gaining considerable attention for their potential neuroprotective properties (Xu et al., 2021). The present results found that the matairesinol pretreatment effectively prevented the I/R-induced neuronal damage in MCAO-operated rats.

Oxidative stress, characterized by the disproportion of ROS generation and the body's antioxidant mechanisms, plays a critical role in the onset of IS, participating significantly to the cascade of events resulting in neuronal injury and functional deficits. The excessive production of reactive oxygen species (ROS), coupled with a compromised antioxidant mechanism, results in oxidative damage to critical biomolecules, including lipids, proteins, and DNA, ultimately exacerbating the ischemic injury. This intricate interplay between oxidative stress and antioxidant defense mechanisms dictates the extent of neuronal damage and influences the overall outcome following IS (Jurcau & Ardelean, 2022). The pathogenesis of IS encompasses a complex interplay of events, where oxidative stress emerges as a central player, initiating and propagating neuronal injury through diverse mechanisms. During ischemia, the disruption of the electron transport chain in mitochondria results in the enhanced

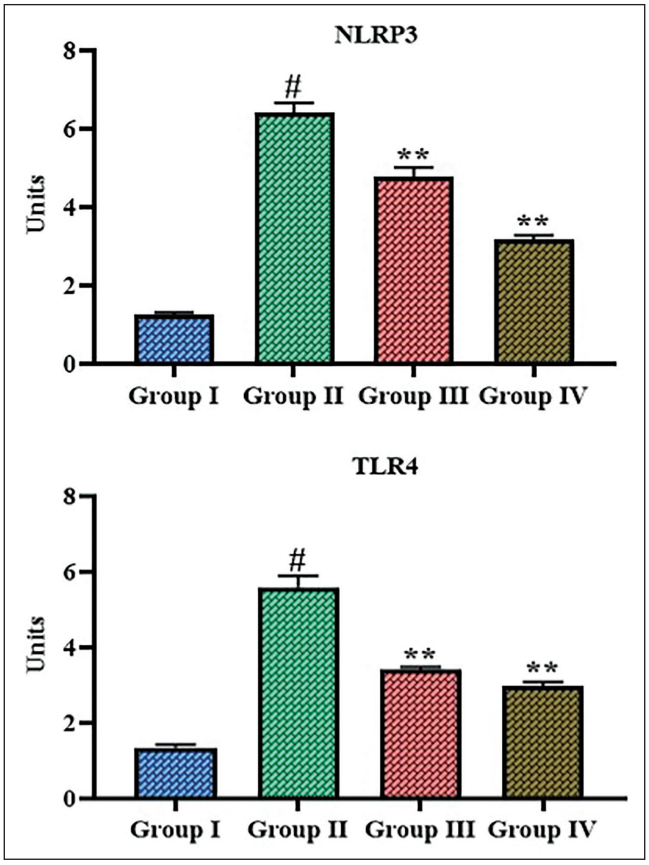


Figure 4. Effect of Matairesinol on the Toll-like Receptor 4 (TLR4) and NOD-like Receptor Protein 3 (NLRP3) Levels in the Experimental Rats. The mean ± Standard Deviation (SD) Values of Triplicates are Given in Each Bar, and Statistical Analysis is Conducted Using GraphPad Prism Software. Values are Assessed Utilizing One-way Analysis of Variance (ANOVA) and Dunnett's *Post Hoc* Analysis. The Symbol "#" Denotes a Statistically Significant Difference ($p < .01$) Relative to the Control Group. The Symbol "**" Denotes a Statistically Significant Difference ($p < .05$) Relative to the Middle Cerebral Artery Occlusion (MCAO)-induced Group.

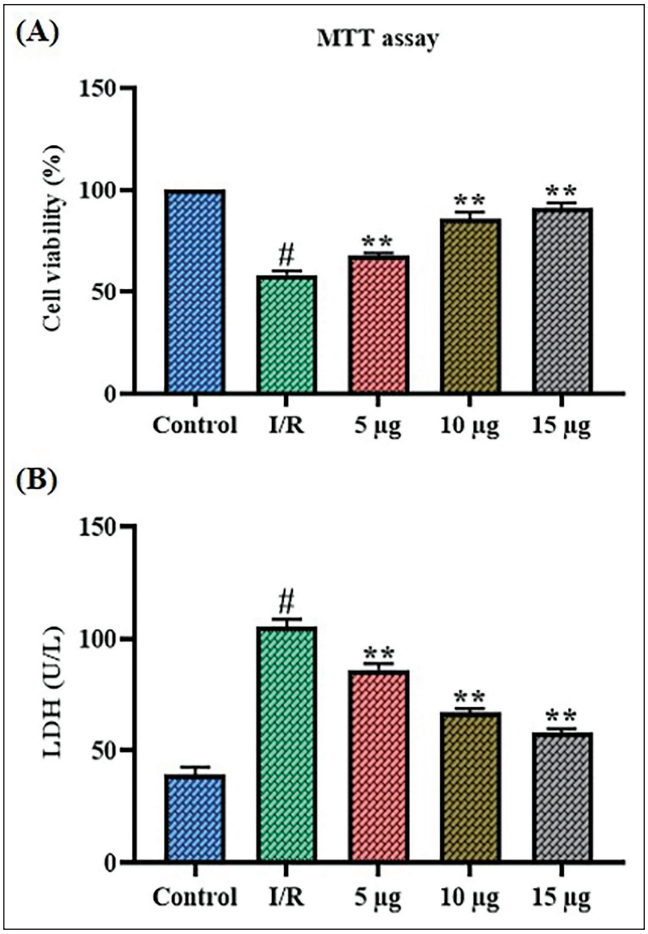


Figure 5. Effects of Matairesinol on the Viability and Lactate Dehydrogenase (LDH) Activity in the Oxygen-Glucose Deprivation/Reoxygenation (OGD/R)-induced Human Neuroblastoma Cell Line (SH-SY5Y) Cells. The Mean ± Standard Deviation (SD) Values of Triplicates are Given in Each Bar, and Statistical Analysis is Conducted Using GraphPad Prism Software. Values are Assessed Utilizing One-way Analysis of Variance (ANOVA) and Dunnett's *Post Hoc* Analysis. The Symbol "#" Denotes a Statistically Significant Difference ($p < .01$) Relative to the Control Group. The Symbol "**" Denotes a Statistically Significant Difference ($p < .05$) Relative to the Middle Cerebral Artery Occlusion (MCAO)-induced Group. (A) Cell Viability; (B) LDH Activity.

secretion of superoxide radicals, which subsequently contribute to the formation of other ROS, like hydrogen peroxide and hydroxyl radicals. The overproduction of these reactive species overwhelms the endogenous antioxidant systems, leading to a state of oxidative stress that promotes lipid peroxidation, protein oxidation, and DNA damage (Jelinek et al., 2021).

The brain's inherent vulnerability to oxidative stress stems from its high metabolic rate, heightened polyunsaturated fatty acid levels, and low concentrations of antioxidants, rendering it particularly susceptible to oxidative damage. The imbalance between prooxidants and the body's scavenging ability causes oxidative stress. GSH, GPx, CAT, and SOD represent crucial constituents of the antioxidant system, functioning synergistically to counteract ROS and lessen oxidative damage. GSH, a tripeptide, acts as a direct scavenger of free radicals and also serves as a substrate for GPx, an enzyme that converts hydrogen peroxide to water (Hou & Brenner, 2024). CAT, another essential antioxidant enzyme, directly converts hydrogen peroxide into water and oxygen, thereby reducing the levels of this reactive species. SOD, on the other hand, catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen, representing the first line of defense against superoxide-mediated oxidative damage. MDA, a lipid peroxidation end product, serves as a widely used biomarker of oxidative stress, reflecting the extent of lipid damage caused by ROS (Wang et al., 2022). The measurement of MDA levels in biological fluids or tissues

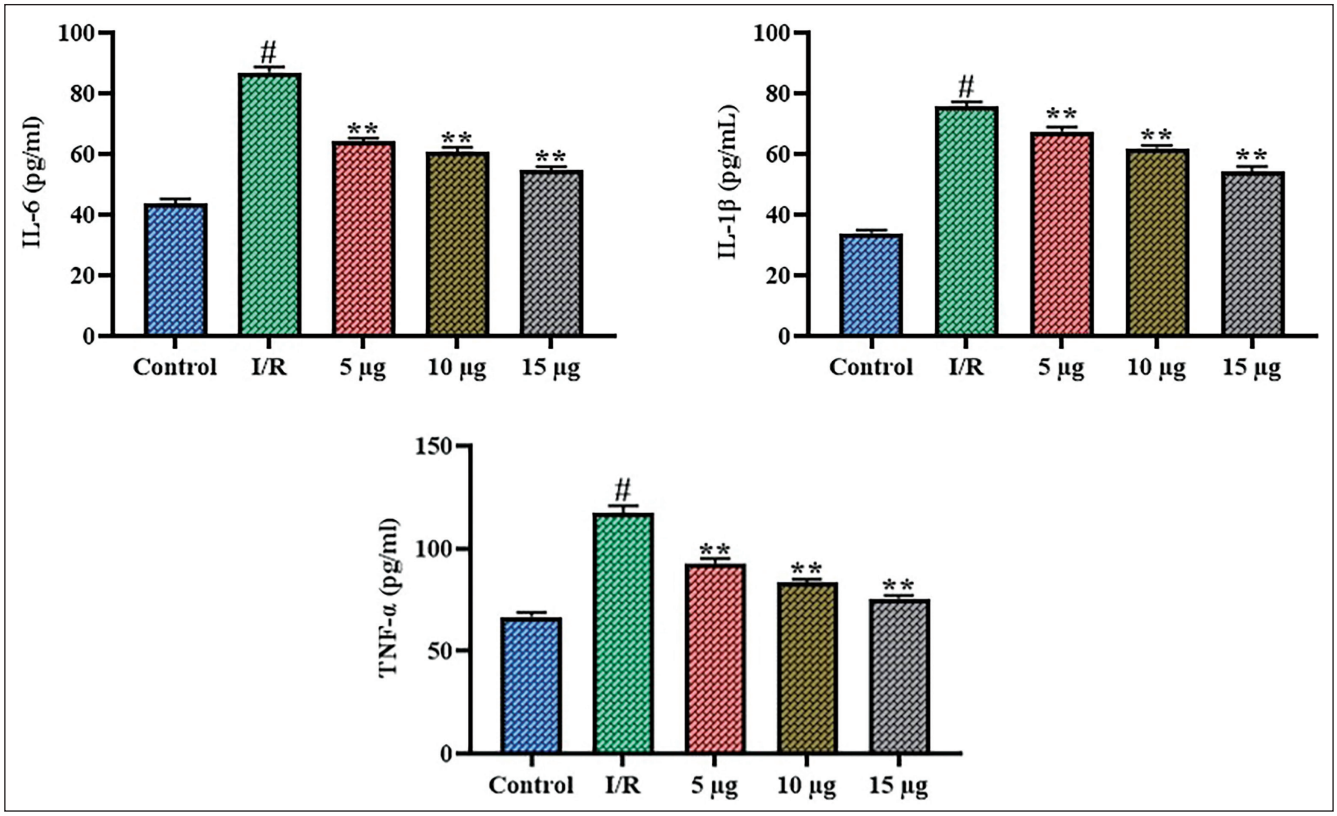


Figure 6. Effects of Matairesinol on the Inflammatory Cytokines in the Oxygen-Glucose Deprivation/Reoxygenation (OGD/R)-induced Human Neuroblastoma Cell Line (SH-SY5Y) Cells. The Mean ± Standard Deviation (SD) Values of Triplicates are Given in Each Bar, and Statistical Analysis is Conducted Using GraphPad Prism Software. Values are Assessed Utilizing One-way Analysis of Variance (ANOVA) and Dunnett's *Post Hoc* Analysis. The Symbol "#" Denotes a Statistically Significant Difference ($p < .01$) Relative to the Control Group. The Symbol "**" Denotes a Statistically Significant Difference ($p < .05$) Relative to the Middle Cerebral Artery Occlusion (MCAO)-induced Group.

indicates the degree of oxidative stress and its impact on lipid-rich structures, such as cell membranes. The concerted action of antioxidants is achieved to ameliorate the harmful effects of oxidative stress. The disruption of redox homeostasis has been implicated in neurodegenerative disorders. The decline in antioxidants may initiate oxidative stress-mediated neuronal loss (Zhang et al., 2017). Here, we have found that the MCAO-induced rats demonstrated elevated MDA levels along with decreased CAT, SOD, GSH, and GPx concentrations in their brains. Remarkably, the pretreatment of matairesinol diminished the MDA and augmented the antioxidant concentrations in the brains of MCAO-induced rats. These results clearly proved the antioxidant effects of matairesinol.

AChE, a crucial enzyme in the nervous system, primarily functions to terminate signal transmission at cholinergic synapses by rapidly hydrolyzing the neurotransmitter acetylcholine (Ach) into choline and acetic acid. This enzymatic action is indispensable for maintaining precise control over cholinergic neurotransmission, preventing overstimulation of postsynaptic receptors, and ensuring the proper functioning of various physiological processes. In the case of IS, several events unfold, resulting in neuronal injury and functional deficits, where the role of AChE becomes multifaceted and intricately linked to the pathophysiology of the condition (Tan et al., 2018). The intricate interplay between the pathological processes and the cholinergic system, particularly AChE activity, has gained attention in recent times, prompting investigations into its efficacy as both a therapeutic target and a diagnostic marker for IS. The contribution of AChE in the onset of IS is complex, with research suggesting both detrimental and potentially protective roles, depending on the specific context and stage of the ischemic cascade (Lin et al., 2016). During the acute stage of IS, excessive release of ACh into the synaptic cleft can occur due to neuronal damage and impaired reuptake mechanisms. This surge in ACh levels can overstimulate cholinergic receptors, contributing to excitotoxicity. The precise mechanisms by which ischemia affects AChE activity are still under investigation. Still, it is hypothesized that oxidative stress, inflammation, and changes in intracellular calcium levels may play a role. Furthermore, studies have explored the potential of AChE inhibitors as

therapeutic agents in IS (Sun & Liu, 2022). Here, the MCAO-induced rats had elevated AChE levels in their brain tissues than sham-operated controls. However, the pretreatment with the matairesinol successfully decreased the AChE concentrations in the brains of MCAO-induced rats.

Inflammation, triggered by less blood flow and the influx of proinflammatory markers from ischemic endothelium and brain parenchyma, might exacerbate tissue damage. Following the onset of cerebral ischemia, elements of the immune system contribute to all phases of the ischemic cascade, ranging from acute intravascular occurrences triggered by the interruption of blood flow to parenchymal processes that cause brain damage and subsequent tissue repair. Understanding the nuances of the inflammation and the specific roles of various inflammatory markers is essential for developing targeted therapies to mitigate ischemic brain injury (Monsour & Borlongan, 2023). Inflammation has emerged as a critical contributor to neuronal damage and functional deficits in IS. The immune system, often perceived as a defender against external threats, plays a multifaceted role in the result of stroke, influencing both the extent of damage and the potential for recovery (Meisinger et al., 2024). The balance between pro-inflammatory and anti-inflammatory signaling pathways dictates the overall outcome of the inflammatory response, tipping the scales toward either neurotoxicity or neuroprotection. The inflammatory process is typically considered a protective mechanism employed by the organism to reinstate homeostasis following cellular damage. However, excessive inflammation can exacerbate tissue injury and hinder the recovery process (Wang et al., 2023).

Among the key players in the inflammatory cascade are cytokines, a diverse group of signaling molecules that mediate communication between cells and orchestrate the immune response. Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are generated in the ischemic brain and contribute to neuronal damage through various mechanisms. These cytokines amplify the inflammatory response by activating immune cells, increasing the production of ROS, and promoting excitotoxicity, a process in which excessive stimulation of neurons leads to their demise (Li et al., 2022). Whereas, anti-inflammatory cytokines like IL-4 and IL-10 demonstrate protective effects by reducing the secretion of pro-inflammatory markers, promoting tissue repair, and modulating the immune cell activities. While acute neuroinflammation may serve a protective role, chronic neuroinflammation is often detrimental and damaging to nervous tissue. The intricate interplay between pro- and anti-inflammatory cytokines highlights the complexity of inflammation in IS (Xie et al., 2024). The current results evidenced that the brains of MCAO-induced rats illustrated decreased IL-4 and IL-10 concentrations, while demonstrating augmented TNF-α, IL-1β, and IL-6 concentrations. Interestingly, the matairesinol pretreatment successfully increased IL-4 and IL-10 concentrations, and reduced the TNF-α, IL-1β, and IL-6 concentrations in the brains of MCAO rats. The findings of *in vitro* studies also witnessed the reduction of inflammatory cytokines in OGD/R-stimulated SH-SY5Y cells after treatment with matairesinol. These data evidenced the notable anti-inflammatory effects of matairesinol.

TLR4 and NLRP3 are key constituents of innate immunity, playing crucial roles in the inflammatory response following IS. TLR4, a transmembrane receptor, recognizes a wide array of pathogen- and damage-associated molecular patterns, activating pathways that further activate transcription factors like nuclear factor kappa B (NF-κB), thereby inducing the pro-inflammatory cytokines, chemokines, and adhesion molecules (Mao et al., 2023). The inflammatory response, while initially intended to clear debris and promote tissue repair, can become dysregulated, contributing to secondary brain damage. Specifically, the ischemic core, characterized by complete absence of perfusion, suffers irreversible tissue loss within minutes, while the surrounding penumbra experiences limited perfusion and impaired function, with the potential for recovery or progression to infarction. The involvement of TLR4 in post-stroke inflammation is supported by studies demonstrating increased TLR4 expression in the ischemic brain, as well as the protective effects of TLR4 inhibition or knockout in experimental stroke models (Nalamolu et al., 2021).

The NLRP3 inflammasome, a multi-protein complex, works as an essential intracellular sensor of cellular stress and damage, triggering the stimulation of caspase-1, resulting in the amplification of the inflammatory response. Neuroinflammation is a crucial innate immune response inside the CNS, wherein the brain and spinal cord respond to various pathogens and endogenous signals indicative of cellular injury (Puleo et al., 2022). The NLRP3 is activated by various stimuli relevant to IS, including ROS, ionic flux, and mitochondrial dysfunction, which are all hallmarks of the ischemic cascade. The activation of NLRP3 in the context of stroke contributes to neuronal injury and exacerbates brain damage (Jia et al., 2022). Therefore, therapeutic strategies targeting the TLR4/NLRP3 pathway hold promise for mitigating neuroinflammation and improving outcomes after IS. Such therapeutic approaches may involve the use of TLR4 antagonists, NLRP3 inhibitors, or strategies aimed at modulating microglial activation and polarization, which are intended to dampen the detrimental aspects of the inflammatory response while preserving its beneficial functions in tissue repair and remodeling (Zhang et al., 2022). Here, the MCAO-induced rats with cerebral I/R injury demonstrated upregulated TLR4 and NLRP3 concentrations in their brains. However, the matairesinol pretreatment successfully decreased the TLR4 and NLRP3 concentrations in the brains of MCAO-induced rats. Although our study demonstrates the neuroprotective effects of matairesinol in MCAO-induced rats and OGD/R-stimulated SH-SY5Y cells, it has some limitations. The exact molecular mechanisms underlying the neuroprotective effects of matairesinol require further elucidation. Additionally, clinical trials are needed to confirm the

therapeutic efficacy and safety of matairesinol in human stroke patients. Further confirmatory studies are warranted to fully explore the potential of matairesinol as a novel therapeutic agent for IS.

Conclusion

In conclusion, our findings underscore the neuroprotective properties of matairesinol in MCAO-induced rats. The matairesinol treatment successfully reduced the brain edema, neurological score, and cerebral infarction in the MCAO-induced rats. Moreover, matairesinol treatment significantly inhibited oxidative stress and inflammation responses by upregulating the antioxidants in the MCAO-induced rats. The findings of *in vitro* studies also proved that matairesinol effectively increased viability and reduced inflammatory markers in OGD/R-stimulated SH-SY5Y cells. The present data may facilitate the advancement of matairesinol as a novel salutary agent to prevent and treat IS. Moreover, further confirmatory works are still required to understand the therapeutic activity of matairesinol on IS precisely.

Abbreviations

ACh: Acetylcholine; AChE: Acetylcholinesterase; ANOVA: Analysis of variance; CAS: Chemical Abstracts Service; CAT: Catalase; CCA: Common carotid artery; CNS: Central nervous system; ECA: External carotid artery; GPx: Glutathione peroxidase; GSH: Glutathione; ICA: Internal carotid artery; IL-1β: Interleukin-1 beta; IL-4: Interleukin-4; IL-6: Interleukin-6; IL-10: Interleukin-10; I/R: Ischemia/reperfusion; IS: Ischemic stroke; MCAO: Middle cerebral artery occlusion; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa B; NLRP3: NOD-like receptor protein 3; OGD/R: Oxygen-glucose deprivation/reoxygenation; rpm: Revolutions per minute; ROS: Reactive oxygen species; SD: Standard deviation; SH-SY5Y: Human neuroblastoma cell line; SOD: Superoxide dismutase; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor alpha; tPA: Tissue plasminogen activator; TTC: 2,3,5-Triphenyltetrazolium chloride.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

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