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ORIGINAL RESEARCH

4-methylumbilliferon (4-MU) as a Potential Treatment Against Cerebral ischemia and Reperfusion Injury in Rats; An Experimental Study

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Abstract: **Introduction:** Ischemic stroke (IS) is one of the three main fatal disorders and is a major health challenge. 4-methylumbelliferone (4-MU) is one of the coumarin derivatives (7–hydroxy-4–methylcoumarin) with antioxidant and anti-inflammatory impact. This study was conducted to elucidate the neuroprotective effects and anti-inflammatory impact of 4-MU in a rodent model of IS. **Methods:** The IS model was induced by middle cerebral artery occlusion (MCAO) for 1 hour and reperfusion was established for 24 hours. 44 Male Wistar rats were divided into four groups: 1) Sham, 2) MCAO, 3) MCAO + Vehicle, and 4) MCAO + 4-MU (25 mg/kg). Evaluation of neurological deficit was performed using Garcia's score. 2,3,5-triphenoyl-2H-tetrazolium chloride (TTC) staining was employed to measure infarct size. Nissl staining was applied to determine neuronal loss. Moreover, western blotting was utilized to detect the expression of the proteins relevant to the TLR4/NF-KB/NLRP3 axis (p–NF–KB p65, TLR4, NLRP3, IL-1β, IL-10, IL-18, ASC, and Caspase-1). **Results:** It was observed that MCAO caused neurological deficit (P<0.0001), infarct (P<0.0001), and neuronal loss (P<0.002); up-regulated NLRP3 (P<0.0001), TLR4 (P<0.0001), p–NF–KB p65 (P<0.0005), IL-1β (P<0.0014), IL-18 (P<0.0001), ASC (P<0.0027), and Caspase-1 (P<0.0052); and reduced IL-10 concentrations (P<0.0024). Administration of 4-MU (25 mg/kg) quickly after reperfusion reduced neurological deficit (P<0.0001), infarct size (P<0.0001), neuronal loss (P<0.0058), and down-regulated NLRP3 (P<0.0257), TLR4 (P<0.0001), p–NF–KB p65 (P<0.0075), IL-1β (P<0.0106), IL-18 (P<0.0005), ASC (P<0.0072), and Caspase-1 (P<0.0315), and increased IL-10 concentrations (P<0.0215). **Conclusions:** These results indicate that 4-MU can attenuate injury after MCAO by suppressing the TLR4/NF-KB/NLRP3 axis. Our findings show that 4-MU can be considered a novel therapeutic compound to cure IS.

Keywords: Ischemic stroke; Inflammation; sensation

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1. Introduction

Ischemic stroke (IS) is one of the three main fatal disorders and is a major challenge to health since a growing number of individuals worldwide will be above the age of 65 until 2050, which in turn increases the risk of this disease (1, 2). There are 13.5 million new subjects and 5.5 million mortalities due to this disease annually around the world (3). In addition, IS is one of the major reasons for disability across the globe, and hence, survivors need a long period of care and rehabilitation, which in turn results in creating a major social and economic burden on individuals, families, and societies (4). IS is caused by inadequate blood supply to the brain tissue and is responsible for 87% of all strokes (5).

Although reperfusion or re-establishment of blood oxygen to the ischemic tissue is considered a therapeutic option, it may aggravate injury and promote neuronal death. Mechanical thrombectomy and thrombolysis with recombinant tissue plasminogen activator are considered as other therapeutic options and their use can be limited by severe complications such as enhancing/promoting oxidative stress/inflammatory response and a narrow therapeutic window (6-8). Energy depletion and hypoxia confer neuronal loss after ischemia. Reactive astrocytes are activated by apoptotic or necrotic neurons and thereby, mediate the attraction and migration of microglia to the location of inflammation. It has been documented that microglia-mediated neuroinflammatory responses display powerful roles in enhancing injury following reperfusion (9, 10).

Microglia-secreted galectin-3 has demonstrated the ability to act as a Toll-like receptor 4 (TLR4) ligand and facilitate mi-

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croglial proliferation and activation during brain ischemia (11). Afterward, activation of the NOD-like receptor protein 3 (NLRP3) inflammasome in the activated microglia gives rise to secretion of inflammatory cytokines which in turn result in exacerbation of the damage in the early phase following IS (12). More production of inflammatory mediators and reactive oxygen species (ROS) is mediated by inflammatory cytokines, which confer neurovascular injury and disturbance of blood-brain barrier (BBB) (13). Moreover, NLRP3 inflammasome can be activated by TLR4/nuclear factor kappa B (NF-KB) axis, which mediates converting caspase-1 into cleaved-caspase-1.

4-methylumbilliferon (4-MU) is a coumarin derivative (7–hydroxy-4–methylcoumarin) and an inhibitor of hyaluronan (HA) production. 4-MU is capable of inhibiting HA synthesis via elimination of cellular uridine diphosphate (UDP)-glucuronic acid, a building ingredient of HA (14). Some prior investigations have indicated that 4-MU can exert antitumor actions and anti-inflammatory properties by activation or inhibition of various cellular pathways (15-18). A prior report displayed that 4-MU is capable of suppressing inflammation by targeting TLR4 in rat primary astrocytes treated with lipopolysaccharide (LPS), reducing the levels of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-), interleukin 6 (IL-6), and interleukin-1 beta (IL-1β), and elevating levels of anti-inflammatory cytokine of IL-10 (19). Here, in vivo experiments using a rodent model of IS were used to examine whether 4-MU has a neuroprotective impact against IS by targeting inflammation. In addition, we decided to identify the possible mechanisms underlying the neuroprotection of 4-MU in the middle cerebral artery occlusion (MCAO) rats.

2. Methods

2.1. Study design and setting

Healthy male Wistar rats, weighing 260–290 g, were provided by the Iran University of Medical Sciences (IUMS), Tehran, Iran. The rats were maintained on controlled humidity (50 ± 10 %), temperature (22 ± 2 °C), and a light/dark schedule (12 hours/12 hours). They had free access to standard food and water ad libitum throughout the study. Random housing for animals was performed.

The experimental approaches were according to the Research Ethics Committee of IUMS and conformed to the Guide for the Care and Use of Laboratory Animals, based on the National Institutes of Health in the USA (No.80–23, revised in 1996). The rats were randomly divided into four groups: 1) sham-operated group: Healthy animals were exposed to surgical procedure except for induction of MCAO (Sham). 2) MCAO group: The rats were exposed to the occlusion of the middle cerebral artery (MCA) for 60 minutes and re-circulation was created for 24 hours.

3) MCAO + Vehicle group: the animals were exposed to the ligation of the MCA for 60 minutes and received 0.9% dimethyl sulfoxide (DMSO) immediately at the beginning of reperfusion. 54) MCAO + 4-MU group: the rats were exposed to the occlusion of the MCA for 60 minutes and received 4-MU (25 mg/kg) immediately at the beginning of reperfusion. 4-MU was purchased from Sigma-Aldrich Chemical Co., USA. Figure 1 shows the experimental design of this study.

2.2. Induction of MCAO/reperfusion (R) model

The MCAO/R model of male Wistar rats was created as described previously (20). In brief, the rats were subjected to a combined solution of ketamine (60mg/kg) and xylazine (5mg/kg); placed in supine position; and cut via the neck skin to observe the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). First, the proximal end of the CCA was occluded and then ECA was tied and cut at the distal end. Afterward, a silicone-tipped monofilament was placed into the ICA via the right CCA to ligate the MCA for 60 minutes. Insertion of the monofilament was ceased when resistance was felt. After 1hour, the monofilament was eliminated and the bloodstream was re-established for 24hours. To keep normal body temperature at almost 37°C during the procedure, a heating lamp was used. The rats in the sham group were exposed to a similar procedure, except for the insertion of monofilament. The mortality rate was 3 animals in the MCAO group, one animal in the vehicle group, and one in the MCAO + 4-MU group. We performed surgery again to have equal samples in each group.

2.3. Neurological deficit scores (NDS)

At 24 hours following re-circulation, NDS was detected based on the Garcia score (21). In this test, the severity of NDS in rats was examined from six aspects: moving autonomously, climbing movement, forelimb extension function, bilateral body touch, posture symmetry, and bilateral beard touch reaction. The score ranges from 3 to 18 and a greater score indicates lower neurological injury. NDS were assessed by an examiner, blinded to the experimental groups.

2.4 2,3,5-triphenoyl-2H-tetrazolium chloride (TTC) staining

To evaluate infarct size, anesthesia was induced and the animals were decapitated. The brains were immediately separated and then, they were sliced into 2 mm thick coronal slices, and placed in 2% TTC (Sigma Aldrich, St. Louis, MO, USA) at 37 C for 20 minutes in the dark.

Afterward, slices were fixed in 4% paraformaldehyde for 24 hours and then a digital camera was utilized to obtain photos. Image J (National Institutes of Health) software was used to quantify the infarct rate as explained below:

Corrected infarct volume (%) = Left hemisphere volume - (Right hemisphere volume – infracted volume) × 100.

2.4. Histological assessment

Brain tissues in all four groups (n=4) were quickly separated from the skull bone at 24hours after reperfusion. After fixing

in 10% formalin; embedding in paraffin and cutting into 5m thick sections, the samples were stained with the Nissl staining method, using cresyl violet to determine neuronal cell density in the prefrontal cortex region. Five random fields for each sample were selected and counting the number of intact neurons was carried out by an investigator blinded to experimental groups.

2.5. Western blotting assay

The specimens were crushed and homogenized to extract proteins from ischemic brain tissue using 1mL of the RIPA lysis buffer. After centrifugation at 13,000rpm for 15min and gathering the supernatant, protein concentrations were measured using bicinchoninic acid assay (BCA) method. After electrophoresing the 50g of total proteins using 4% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel at a constant voltage (80V for 30min and then 120V for 40min), they were transferred to polyvinylidene fluoride (PVDF) membrane.

Then, non-specific sites in PVDF membranes were blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 (pH 7.6) (TBST). After incubation with primary antibodies overnight, PVDF membranes were incubated with a secondary horseradish peroxidase (HRP)-labelled antibody for 2hours. The color development of the PVDF membranes was performed using the chemiluminescent HRP Substrate (Millipore). The quantification of bands was carried out using Alpha Ease ® FC Imaging System according to optical density.

2.6. Statistical analysis

Analysis of results was conducted using GraphPad Prism version 8.0 according to one-way analysis of variance followed by post hoc Tukey test for comparing multiple groups. Data are presented as mean ±standard deviation and $p < 0.05$ is an indicator of statistically significant difference.

3. Results

3.1. Neurological deficit scores

NDS were markedly reduced in the MCAO group compared to the sham group (P<0.001). The results demonstrated that treatment with 25 mg/kg 4-MU significantly enhanced neurological function in MCAO animals (P<0.001; F: 139.3; degrees of freedom numerator (DFn): 3; degrees of freedom denominator (DFd): 28) (Figure 2).

3.2. Infarct volume

To measure the infarct volume 24 hours after establishing MCAO, we used TTC staining, in which red regions are not at risk and show intact tissue, while white color demonstrates damaged tissue. Compared to the sham group, there was a significantly larger infarct size in the MCAO group (P<0.001). This study indicated that, in contrast to the MCAO group, the infarct size was markedly attenuated in the 25 mg/kg 4-MU-treated group (P<0.001; F: 172.6; DFn: 3; DFd: 12) (Figure 3).

3.3. Neuronal density

In the sham group, pre-frontal cortex neurons were intact, dense, and close with regular arrangement and normal shapes. Reduced number of neurons with widening and swelling of intercellular space and unclear cell contour was seen in the MCAO group compared with sham (P<0.001).

However, an increased number of neurons and reduction in abnormal morphologies were observed following 4-MU (25 mg/kg) administration compared to the MCAO group (P<0.01; F: 30.13; DFn: 3; DFd: 8) (Figure 4).

3.4. Inflammatory cytokines

As seen in Figure 5A-C, the protein amounts of IL-1β, and IL-18 were markedly elevated, whereas the protein levels of IL-10 had significantly reduced in the MCAO group compared to sham. Noteworthily, the protein levels of IL-1β (F: 13.13; DFn: 3; DFd: 12), and IL-18 (F: 36.56; DFn: 3; DFd: 12) were significantly attenuated, and IL-10 (F: 12.67; DFn: 3; DFd: 12) levels were markedly elevated in the MCAO rats treated with 4-MU (25 mg/kg).

3.5. TLR4/NF-KB/NLRP3 axis

To evaluate whether the neuroprotective impact of 4-MU was correlated with the TLR4/NF-KB/NLRP3 axis, we analyzed the levels of these proteins (Figure 6 A-C). Our data indicated that induction of MCAO elevated the protein expression of TLR4/NF-KB/NLRP3 compared to sham.

Moreover, treatment with 25 mg/kg 4-MU down-regulated these proteins (F: 51.29; DFn: 3; DFd: 12, F: 14.37; DFn: 3; DFd: 12, and F: 17.12; DFn: 3; DFd: 12, respectively).

3.6. ASC and caspase1

Relative protein amounts of ASC and caspase1 in brain tissue of rats in the MCAO group increased compared with those in the sham (P<0.01).

Compared with the MCAO group, significantly reduced levels of both ASC (P<0.01; F: 13.43; DFn: 3; DFd: 12) and caspase1 (P<0.05; F: 9.439; DFn: 3; DFd: 12) were seen in the MCAO rats treated with 4-MU (25 mg/kg) (Figure 7 A- B).

4. Discussion

One of the major reasons for mortality and disability worldwide is IS, caused by a blocked cerebral blood vessel, which in turn results in massive social and economic burden on societies (22). Owing to the sophisticated structure of the central nervous system (CNS), many therapeutic agents and approaches for the cure of this disease have failed in clinical applications (23). In addition, improving neurological deficits and brain function after stroke needs a long time and hence exploring and developing safe and effective agents and therapeutic strategies are essential.

4-MU is a key player in suppressing HA production and its anti-inflammatory impact is associated with inhibiting secretory processes that stimulate neutrophil recruitment and

initiation of inflammation (24). The results of tissue staining and assessment of neural function in this work showed that 4-MU (25 mg/kg) is capable of improving neurological function and reducing infarct volume by targeting TLR4/NF-KB/NLRP3 axis.

Previous investigations have indicated that inflammation plays an important role in the pathogenesis of IS. Pro-inflammatory cytokines such as IL-1β not only display a crucial role in suppressing the process of neurogenesis, but also contribute to the secretion of some endogenous ligands that act through TLRs and cause neurological deficits (25, 26). Inducing secondary brain damage after cerebral ischemia-reperfusion (I/R) is mediated via TLR4-mediated neuroinflammation (27). There are two stages in which NLRP3 is activated during IS. First, it is activated by the TLR4/NF-KB axis to enhance the transcription of NLRP3 components (28). In the next step, the NLRP3 protein creates a complex with ASC and then forms the inflammasome by binding to the cysteine protease caspase-1. Formation of the inflammasome gives rise to activation of caspase-1, which in turn contributes to converting pro-IL-1β and pro-IL-18 to their mature forms (IL-1β and IL-18) and subsequently triggering inflammatory responses and neuronal loss (29, 30). Activation of neuronal NLRP3 inflammasome by TLR4/NF-KB axis after cerebral I/R damage confers release of pro-inflammatory cytokines such as IL-18, IL-1β, which in turn leads to stimulating the activation and M1 polarization of microglia/macrophages and initiation of neuronal pyroptosis (31). In keeping with these reports, we also confirmed that MCAO resulted in activation of the TLR4/NF-KB/NLRP3 axis and secretion of pro-inflammatory cytokines (IL-1β and IL-18), which was reversed by 4-MU (25 mg/kg). It has been reported that a degree of neurological deficit in the early stroke period can be linked to increased amounts of pro-inflammatory cytokines and reduced concentrations of anti-inflammatory cytokines like IL-10 (32). Moreover, another study showed that the NLRP3 inflammasome-induced microglial pyroptosis under ischemic circumstance was responsible for neurological deficits and its inhibition by therapeutic agents could reduce neurological deficits and infarct volume (33). In agreement with these reports, our findings demonstrated that induction of MCAO led to neurological deficits and cerebral ischemic infarct, possibly through the NLRP3 inflammasome-induced microglial pyroptosis, which was reversed by 4-MU (25 mg/kg). TLR4 is expressed on the surface of microglia and plays a pivotal role in activating NF-KB. Activated NF-KB p65 and p50 are capable of the formation of a heterodimer to trigger the inflammation process and thereby, stimulate M1 activation and hamper the M2 phenotype of microglia, which in turn confers neural loss, astrocyte apoptosis, and blood-brain barrier (BBB) disturbance (34). Likewise, these investigations are in consonance with our finding that indicated induction of cerebral I/R injury resulted in neural loss, possibly via activation of TLR4/ NF-KB axis, which was reversed by 4-MU (25 mg/kg). In agreement with our findings, some previous studies have indicated that 4-MU can exert neuroprotective and anti-inflammatory impact (35). For example, 4-MU could promote neuroplasticity in the CNS via endogenous modulation of extracellular matrix (ECM) molecules (36). In addition, it has been found that 4-MU has beneficial impacts on regulating inflammatory astrocyte responses via targeting the NF-kB pathway (19). In another study, it was shown that 4-MU is capable of reducing perineuronal nets and enhancing memory in rodents (37).

4.1. Limitations

The present study has several limitations. A major limitation was that there was no possibility of monitoring the blood flow rate during ischemia and reperfusion due to the lack of a Doppler laser in our laboratory. Another limitation of this study is that the outcome assessments were not performed randomly.

5. Conclusion

With anti-inflammatory properties, 4-MU might pose a promising therapeutic agent for IS. In addition, our results indicate that 4-MU was capable of suppressing TLR4/NF-KB/NLRP3 axis to exert its neuroprotective function. Our finding confirms that 4-MU can be considered as a good candidate for the cure of IS.

6. Declarations

6.1. Acknowledgments

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6.2. Ethics approval and consent to participate

All protocols were approved by Ethical Committee of Iran University of Medical Sciences, Tehran, Iran (Ethics code: IR.IUMS.FMD.REC.1401.014).

6.3. Consent for publication

All authors agree to publish the article in the present form.

6.4. Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

6.5. Competing interests

The authors have no relevant financial or nonfinancial interests to disclose.

6.6. Funding

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6.7. Authors' contributions

N.A participated in the design and interpretation of the study, data analysis, and the review of the manuscript. H.M, A.M and D.N conducted the experiment and collected the tissue and were responsible for the data analysis. H.M performed histopathological analyses. H.M wrote the manuscript, and all authors reviewed, read, and approved the article.

6.8. Using Artificial Intelligence Chatbots

No AI chatbots were used for any part of this study.

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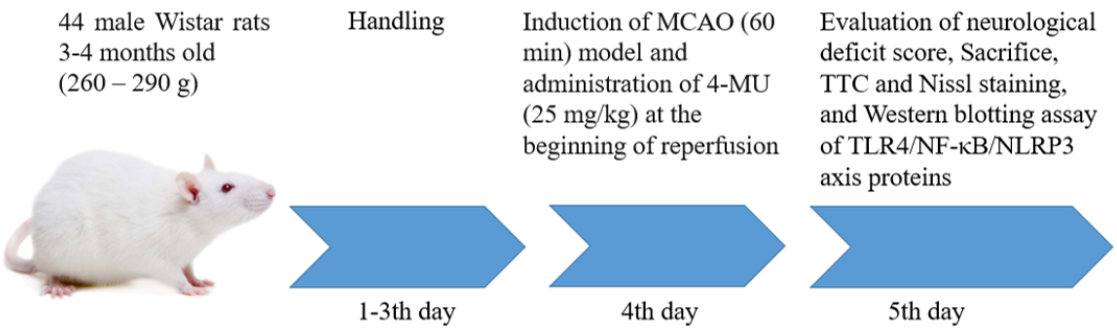

Figure 1: Outline of experimental designs. MCAO: middle cerebral artery occlusion; 4-MU: 4-methylumbelliferone; TTC: 2,3,5-triphenoyl-2H-tetrazolium chloride.

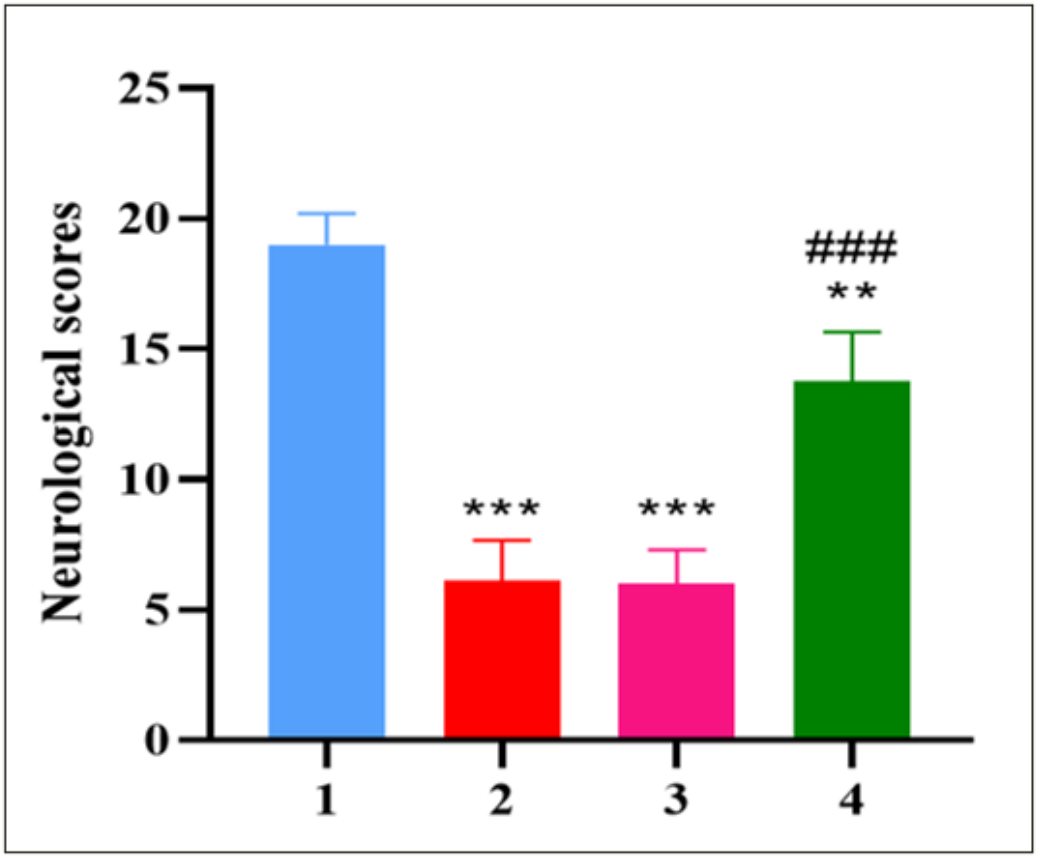

Figure 2: Neurological scores in experimental groups. Data are presented as mean ± standard deviation(SD) (n = 8). 1: Sham group; 2: middle cerebral artery occlusion group (MCAO); 3: middle cerebral artery occlusion + 0.9% dimethyl sulfoxide group (MCAO + Vehicle); 4: middle cerebral artery occlusion + 4-methylumbelliferone (25 mg/kg) group (MCAO + 4-MU). ***p<0.001 and **p<0. 01 vs. 1, ###p<0.001 vs. 2 and 3.

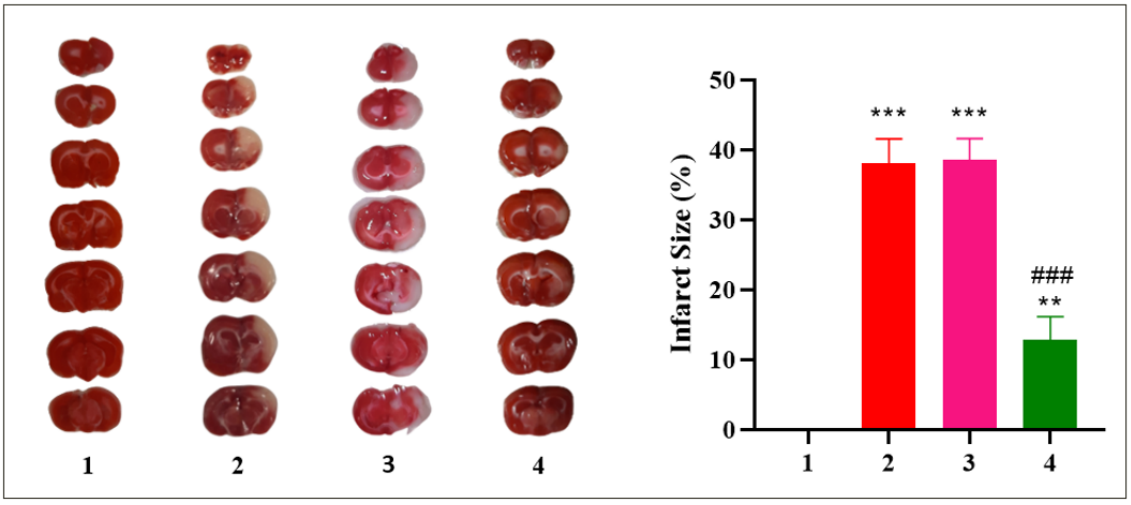

Figure 3: Infarct volume in experimental groups. Data are presented as mean ±standard deviation(SD) (n = 4). 1: Sham group; 2: middle cerebral artery occlusion group (MCAO); 3: middle cerebral artery occlusion + 0.9% dimethyl sulfoxide group (MCAO + Vehicle); 4: middle cerebral artery occlusion + 4-methylumbelliferone (25 mg/kg) group (MCAO + 4-MU). ***p<0.001 and **p<0. 01 vs. 1, ###p<0.001 vs. 2 and 3.

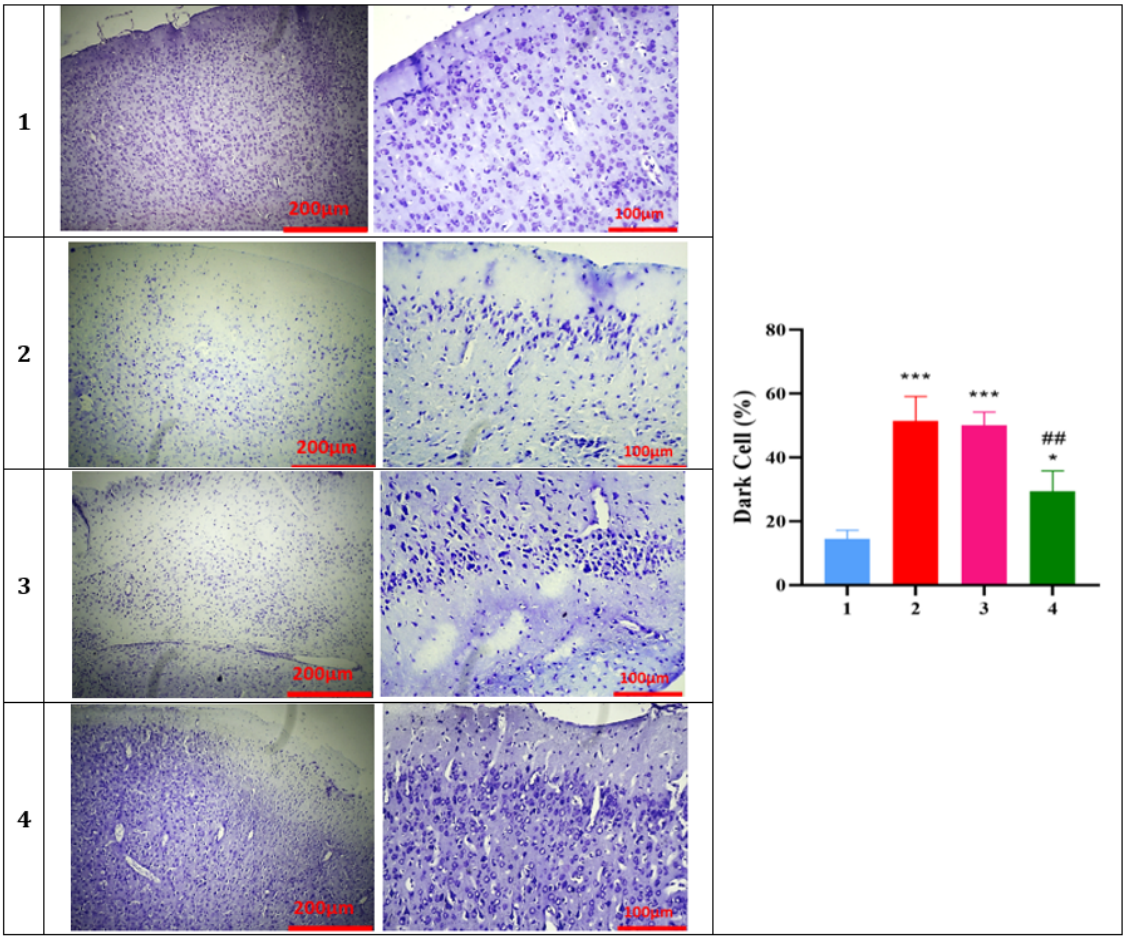

Figure 4: Neuronal density in the frontal cortex region. Data are presented as mean ±standard deviation(SD) (n = 3). 1: Sham group; 2: middle cerebral artery occlusion group (MCAO); 3: middle cerebral artery occlusion + 0.9% dimethyl sulfoxide group (MCAO + Vehicle); 4: middle cerebral artery occlusion + 4-methylumbelliferone (25 mg/kg) group (MCAO + 4-MU). ***p<0.001 and *p<0. 05 vs. 1, ##p<0.01 vs. 2 and 3.

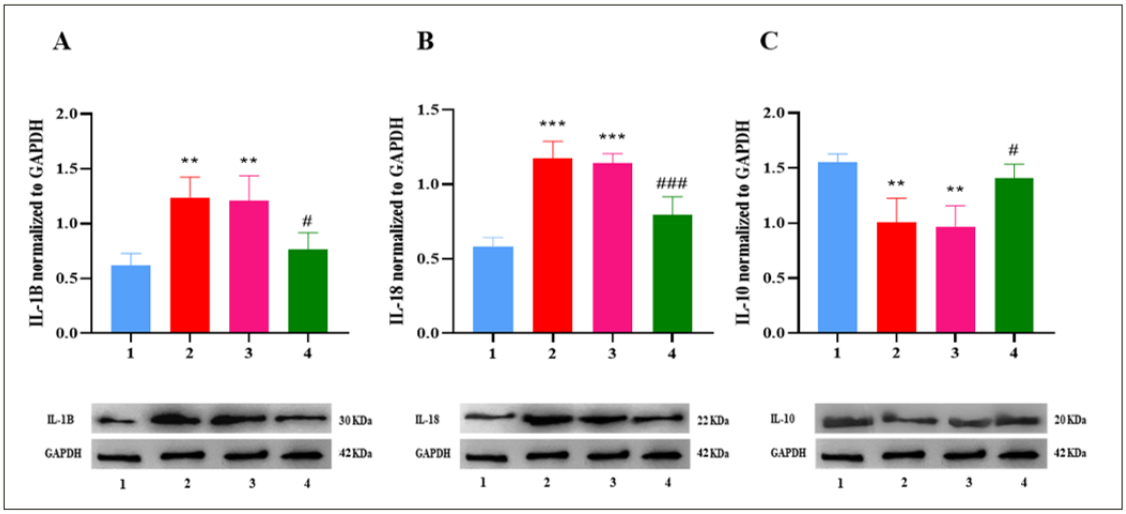

Figure 5: Expression of inflammatory cytokines in experimental groups. A) IL-1β, B) IL-18, and C) IL-10. Data are presented as mean ±standard deviation(SD) (n = 4). 1: Sham group; 2: middle cerebral artery occlusion group (MCAO); 3: middle cerebral artery occlusion + 0.9% dimethyl sulfoxide group (MCAO + Vehicle); 4: middle cerebral artery occlusion + 4-methylumbelliferone (25 mg/kg) group (MCAO + 4-MU). GAPDH expression was measured once for all. ***p<0.001 and **p<0. 01 vs. 1, ###p<0.001 and # p<0. 05 vs. 2 and 3.

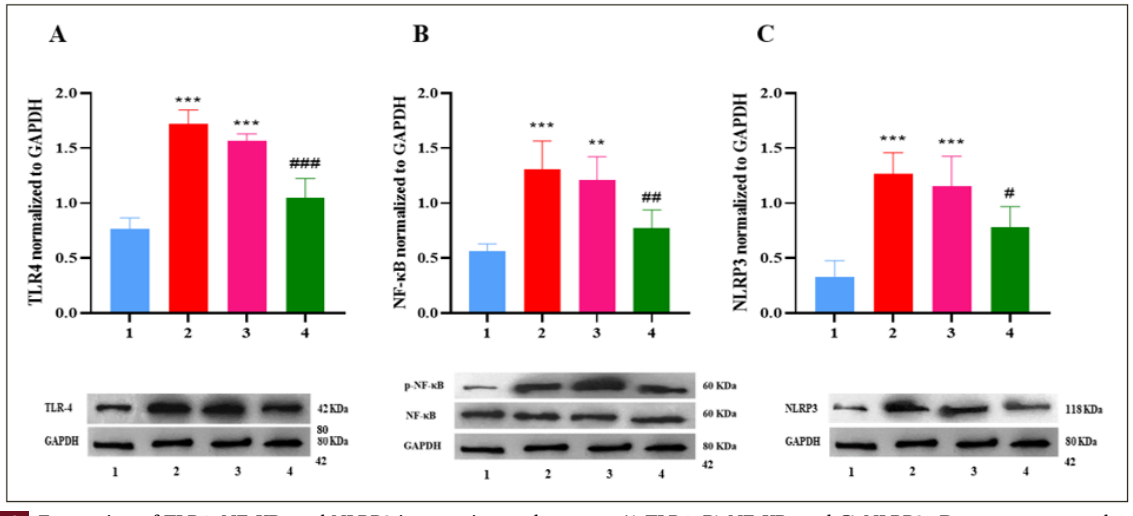

Figure 6: Expression of TLR4, NF-KB, and NLRP3 in experimental groups. A) TLR4, B) NF-KB, and C) NLRP3. Data are presented as mean ±standard deviation(SD) (n =4). 1: Sham group; 2: middle cerebral artery occlusion group (MCAO); 3: middle cerebral artery occlusion + 0.9% dimethyl sulfoxide group (MCAO + Vehicle); 4: middle cerebral artery occlusion + 4-methylumbelliferone (25 mg/kg) group (MCAO + 4-MU). GAPDH expression was measured once for all. ***p<0.001 and **p<0. 01 vs. 1, ###p<0.001, ##p<0.01, and # p<0. 05 vs. 2 and 3.

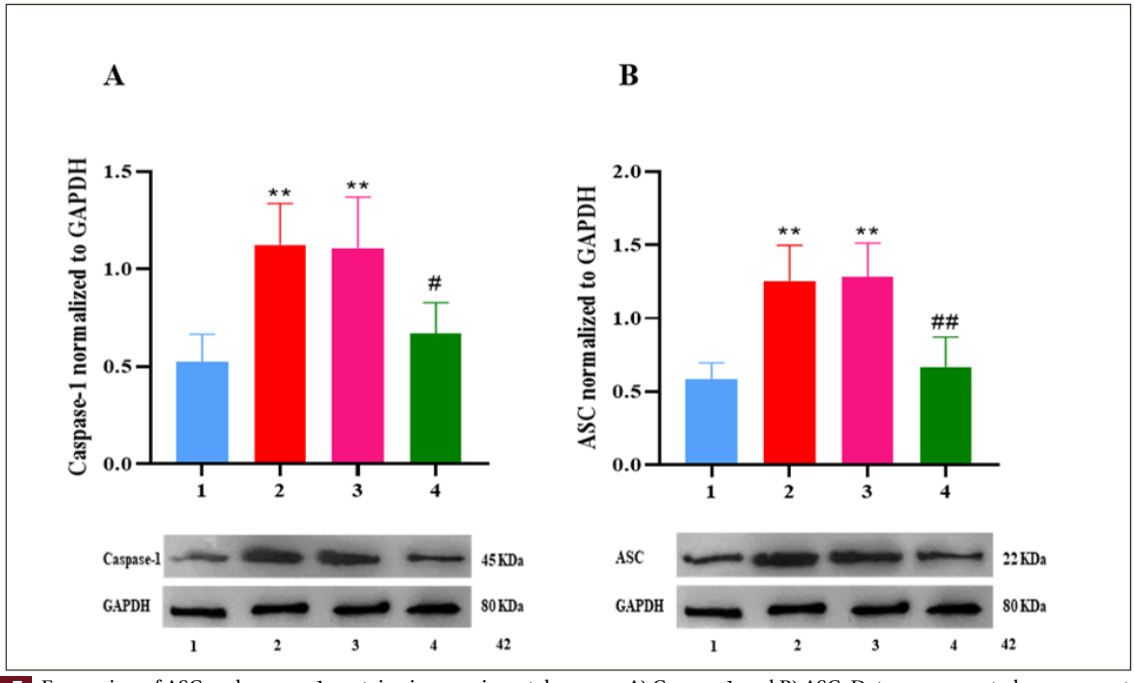

Figure 7: Expression of ASC and caspase1 proteins in experimental groups. A) Caspase1 and B) ASC. Data are presented as mean ±standard deviation(SD) (n = 4). 1: Sham group; 2: middle cerebral artery occlusion group (MCAO); 3: middle cerebral artery occlusion + 0.9% dimethyl sulfoxide group (MCAO + Vehicle); 4: middle cerebral artery occlusion + 4-methylumbelliferone (25 mg/kg) group (MCAO + 4-MU). GAPDH expression was measured once for all. **p<0. 01 vs. 1, ##p<0.01, and # p<0. 05 vs. 2 and 3.