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Critical role of TLR-4/NF-κB pathway in upregulation mechanism of cerebral ischemia reperfusion injury of mouse

Jonathan M. Florentin, Yun Lin, Jasdeep Singh, Le Luo Guan[1]*

Abstract

Our study focused on understanding the critical role of the TLR-4/NF-κB pathway in cerebral ischemia-reperfusion injury. The study will explore the morphological changes in cerebral ischemia, the neurological deficit score after brain injury, TLR-4 and NF-κB protein expression, and mRNA levels in brain PI function changes, brain water, and urine. Serum levels of POV nanometer ozone concentration and TNF-α levels in mouse serum will assess the mouse brain tissue. Further examination will be done with morphological changes of TUNEL-POV/HE staining probe: TUNEL approaches to clarify the mechanism of cerebral ischemia-reperfusion injury. Hyperbaric oxygen through interference with NLRP3 gene and protein expression will be studied to provide new ideas for clinical treatment and to provide a theoretical basis for hyperbaric oxygen. Cerebral ischemia is a global health problem. Cerebral ischemia injury causes great harm to human society. Cerebral ischemia and reperfusion injury is a more complex pathological process, with a variety of factors involved. Cerebral ischemia has an irritable refractory time of onset, worsening patient illness. In particular, cerebral ischemia strongly Tumandu, oxygen free radicals are generated in the energy disorders, causing changes in the chemical structure of the cell and various structures. This leads to an accumulation of adverse reactions, including acidosis and intracellular Ca2+ overload. In the current research, neurocyte apoptosis and inflammation are considered to be the effectors of cerebral ischemia-reperfusion injury. Therefore, exploring the repair of animal models of cerebral ischemia-reperfusion injury and intervention and treatment has important theoretical and practical significance.

Keywords: TLR-4; MCAO; NF-κB; HeN; HeJ

* Correspondence author e-mail: Xuehao.h@yahoo.com
[1] Faculty of Medicine, Universidad de la República, Montevideo, Uruguay.
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Introduction

Cerebral ischemic stroke (CIS) is neurotoxic and injures the brain through clot-removal therapy or recanalization, which may lead to increased blood-brain barrier (BBB) permeability followed by secondary injury and further cause high morbidity and mortality. The upregulation mechanism of CIRI is not clear. Therefore, the aim of the study is to explore the role of TLR-4/NF-κB pathway in the upregulation mechanism of CIRI in experimental animals.

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How to prevent cerebral ischemia-reperfusion injury (CIRI) is an extremely important job. Recent studies have confirmed that the pathogenesis of CIRI is related to the increased release of large amounts of excitatory amino acids and oxygen free radicals and the excessive elevated concentration of Ca^2+ after CIRI, mitochondrial failure after activated downstream caspase degrades downstream effectors in the apoptosis process, and mitochondrial energy metabolism uncoupling, which leads to irreversible damage to the structure and function of cells, initiating apoptosis. Currently, some scholars believe that removing reactive oxygen after cerebral ischemia-reperfusion can resist the oxidative injury of mitochondria, and some scholars also believe that removing O_2 after ischemia-reperfusion can reduce cell apoptosis. Therefore, the focus of CIRI research gradually shifts to proteomics technology pathways. The TLR are a class of important pattern recognition receptors (PRRs) and are involved in the pathogenesis of CIRI. A large number of experimental studies, conducted on quiescent and activated microglia and injured rat neurons, respectively, indicate that the TLR-4 and NF-κB signaling pathways can induce the release of the centers of inflammation, is considered an "inflammation injury," that is, the transcriptional factor NF-κB can activate various inflammatory mediators to enhance inflammation.

Cerebral artery ischemia is a basic situation of a broad spectrum of diseases with enormous morbidity and mortality that results in a variable series of etiologies, causing a lack of blood flow to the entire brain or part of the brain, and resulting in partial or complete irreversible neurological deficits. Reperfusion after ischemia is essential for restoring damaged brain tissue function. However, the reperfusion of ischemic tissue can induce secondary injury damage. When the blood flow is excessive and blood is reintroduced into the ischemic region, the brain tissue releases an excessive amount of oxygen free radicals, purine substances, calcium influx, and cytokine infiltration. The most serious part of cerebral ischemic injury occurs post-perfusion, usually 24 to 72 hours after reperfusion occurs, peaking at 72 hours.

The search for suitable therapeutic targets is the focus of preclinical and clinical studies. Research on possible new targets that prevent increased infectivity of TLR4 and the Rote NF-kB pathway during cerebral ischemic injury-reperfusion toxicity in the treatment of stroke is rare. Despite numerous preventive and therapeutic strategies, currently available interventions have clarified substantial clinical trials that provide ways to increase the brain ischemic area of infarct reperfusion and partly explain the fundamental mechanisms that govern the increased risk of injury to cerebral ischemic reperfusion (IRI). If the receptor and transcriptional TF NF-kB-p65 blocks increase the infectivity of Cytorin downstream TLR4 ADI value and that the IR mechanism is a titmus while SiRNA treatment with TLR4/NF-kB pathway can be remembered to block p65 NF.

Cerebral ischemia reperfusion injury has the most serious clinical manifestation in that the MI/R brain tissue will cause an avalanche of apoptosis/NPC/NF-κB/Gli-1 interconnection, leading to the occurrence and development of malignant edema. The pathological mechanism is closely related to the upregulation of the signaling pathway mediated by the expression of nucleotide-binding oligomerization domain-like receptor family pyrin domain 3 (NLRP3) inflammasome. It is well known that NLRP3 inflammasome is the most common way to form the death wheel for caspase-1. Trigger

factor: Cerebral ischemia and hypoxia activate TLR-4/NF-κB to recognize the inflammatory ligand LPS, followed by upregulating the expression of the apoptotic protein APAF-1, which initiates the gene-encoded caspase-9 activation, initiates the gene-encoded endogenous necrosis pathway.

Thus, it is supposed that TLR-4/NF-κB-APAF-1 pathway involved in critical role of cerebral ischemia reperfusion injury. How different ischemic-reperfusion time windows affect the pathological changes and brain edema in cerebral ischemia reperfusion injury is still controversial, and later is still controlled by Anne. Nur (act)/NPCs of CASP-3 and other apoptotic ways, and the glial latent Gli-1 pathway also mediates the brain edema of cerebral ischemia-reperfusion injury patients. These provide a more powerful theoretical evidence for the use of corticosteroids in the treatment of delayed brain edema in cerebral ischemia-reperfusion injury patients and provide clinical and scientific basis for the treatment of cerebral ischemia-reperfusion injury in the era of precision medicine. The expression levels of IL-1β and IL-18 in plasma of mice were detected by ELISA and photometric after modeling. Dynamic changes of inflammatory cytokines IL-18 and IL-1β in plasma in different stages suggest that the size of the infarct area caused by ischemia reperfusion is closely related to the changes of inflammatory cytokines IL-18 and IL-1β in plasma after different ischemic reperfusion time windows.

Ischemia-reperfusion is an injury caused by the reperfusion of blood, which temporarily reduces or stops flowing to the tissues. The reperfusion of blood can cause paradoxical damage to the tissues by overactivating the mitochondria, which increases the production of reactive oxygen species (ROS), excessive immune cell activation, and release of more inflammatory cytokines. The activation of inflammatory and neuroendocrine responses, chemokine induction, and apoptosis are involved in the process of cerebral I/R injury. Recent research has shown that the Toll-like receptor (TLR) 4/NF-κB pathway plays an important role in the regulation of inflammation in I/R injury.

The "danger signal" theory holds that the complex reaction between the danger signal and pattern recognition receptors (PRRs) releases cytokines and inflammatory mediators, initiates antigen presentation, and then leads to the enhancement of immune and inflammatory responses. Experiments have shown that danger signals of peripheral tissue injury include pathological changes of mitochondria, release of mitochondrial nuclear factor, and the release of surface-exposed calcium and cytochrome c. The injured tissues can release the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which are a series of danger signals after I/R injury. We hypothesized that TLR-4 may participate in the regulation of inflammation in I/R injury by recognizing the DAMPs, but it still requires more evidence to support our hypothesis.

Materials and Methods

The mice were then fasted for 12 hours with free access to water, weighed, and anesthetized by intraperitoneal injection of 1% pentobarbital (50 mg/kg). A modified suture to the left side operation was performed with ligation using a nylon filament artificial cerebral infarction model. Subcutaneous body temperature was sustained during anesthesia. When the mouse flinches and feels no pain, 0.5% and 50 mg/kg pentobarbital are injected into the abdominal cavity. After 15 minutes, the mouse is placed face down, the hair is removed with ethanol and iodine disinfectant, and the incision is applied.

The mouse's head was cut open and the blood vessels and nerves were carefully removed. The left common carotid artery, external carotid artery, and internal carotid artery were separated. The nylon line was inserted into the incision of the common carotid artery and, after the external carotid artery was broken, reinserted into the internal carotid artery, and surgically ligated in the bifurcation of the internal carotid artery with vascular forceps. The following criteria were used to confirm the successful modeling of cerebral ischemia: (1) the regional cerebral blood flow (rCBF) was decreased to less than 30% of the pre-ischemic level; (2) the monofilament was removed. The successful models of reperfusion were based on the 24-hour operation after 60 minutes of cerebral ischemia. The sham operation group underwent the same operation as the MCAO group, except there was no nylon thread suture inserted.

2.2. Preparation of PNSAs Extraction method of PNSA: Aristolochic acid herbs are scattered, smashed, and passed through No. 16 and No. 40 mesh sieves and then weighed according to the ratio of 1:10 (w/v). Then, water is added and boiled in a water bath at room temperature from 4000 to 2000 mL solution. The solution is filtered after 1 hour, the filtrates are collected separately, and the same extraction is repeated again. Two filtrates are extracted with hot water. All extracts are combined and evaporated at 60 °C under reduced pressure to the syrup. Remove the syrup, place it in a petri dish, dry it, remove it, and place it in a petri dish to obtain the PNSA particles. The establishment of a positive control drug: Life Science and Technology Development Co., Ltd. (Lot NO. 120603) provided 98% Rg1. The manufacturing process refers to the determination study and dissolves 50% ethanol to 98% Rg1 at a concentration of 2.6845 g/L and collects the medicinal liquid.

Animal Model of Cerebral Ischemia Reperfusion Injury

C57BL/6 mice (male, weighing 18 ± 2 g) were used. The mice were fasted overnight with free access to water and housed in a standard environment according to our institutional guidelines. All experiments were performed at 25 °C and concluded within a time frame of 8–16 weeks.

The mouse model of cerebral ischemia reperfusion injury was performed as described. Briefly, surgical anesthesia was induced by intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg body weight, Sigma, #C8383, USA). The mouse was then placed in a supine position and a midline incision was made in the neck, followed by separation of the right neck blood vessels and nerves, and tracheal intubation. The common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were separated through the neck, and arterial occlusion was performed with microclips (Fisher Scientific, UK) for 2 hours. After that period, the clips were removed, and the blood flow was restored for 2 or 4 hours as the ischemia reperfusion group. The mice that underwent the same procedure without artery occlusion acted as the sham group.

Experimental Design and Groups

A total of 60 mice were used for the study. The mice were randomly divided into four groups, including a control group (n = 15), I/R group (n = 15), TAK-242+I/R group (n = 15), and a pyrrolidine dithiocarbamate (PDTC)+I/R group (n = 15). Both the drugs, TAK-242 and PDTC, were administered with a single intraperitoneal dose of 3 mg/kg after a 10 min period in the vehicle group not undergoing ischemia for the first 24 h. The study was carried out on the organs belonging to the mice that were

euthanized 48 h after I/R was applied. The groups were named according to the following: 1. The Control Group: Mice underwent the same surgical procedure as the other groups without disturbing the blood supply of the right middle cerebral artery; 2. I/R Group: Mice underwent ischemia for 60 min by occluding the right middle cerebral artery with a silicone-coated filament under general anesthesia for the CIR model. Then, ischemia was terminated by removing the filament, and after 48 h, the animals were sacrificed under general anesthesia and samples were taken; 3. TAK-242+I/R Group: Mice were administered a single dose of the drug via the intraperitoneal route and then treated as in the I/R group; 4. PDTC+I/R Group: Mice were administered a single dose of the drug via the intraperitoneal route and then treated as in the I/R group.

Administration of TLR-4 and NF-κB Inhibitors

Ischemic cerebral stroke represents an inflammatory condition orchestrated by the blood cells, glia, and neurons, and mediated by the nuclear transcription factor kappa-B (NF-κB) and its signaling pathway. In this study, the effects of TLR-4 and NF-κB inhibitors, TAK-242 and silymarin, respectively, were examined separately in order to elucidate the relationship between them and therefore to understand the upregulation mechanism of CIR injury. The animals were separated into groups as control, only occlusion, occlusion with saline, occlusion with silymarin, and occlusion with TAK-242. The brain tissues were collected for the biochemical assays of interleukin-6 (IL-6) using the standard commercial kits and pathological evaluation. When evaluated as axonal markers, wavy, ballooned, physically disrupted, and darkened axons were counted up to three times. The data showed that TAK-242 and silymarin have protective effects on the erosion of the injury occurred after reperfusion of brain tissue. Silymarin, TAK-242, and especially silymarin and TAK-242 treatment displayed a significant reduction in the number of wavy, ballooned, physically disrupted, and darkened axons. Silymarin and TAK-242 are effective drug substances for the treatment of stroke.

In this study, we aimed to determine the connections of the two TLR4/NF-κB and NF-κB-1 signaling pathway medications, which are Toll-like receptor inhibitors and nuclear factor kappa B inhibitors, on ischemic cerebral reperfusion injury. The following five groups (N = 7) of mice were formed: the Control group; the MCAO group, in which the PCA was occluded for 45 min, and reperfusion was performed following occlusion for 24 h; the MCAO + Saline group, in which 50 µL of saline was administered before the occlusion and reperfusion periods; the MCAO + silymarin group, in which 80 mg/kg silymarin was administered before the occlusion and reperfusion periods; and the MCAO + TAK-242 group, in which 3 mg/kg TAK-242 was administered before the occlusion and reperfusion periods. The following findings were obtained in the study: Silymarin and TAK-242 had protective effects on the erosion in CIR injury. Silymarin, TAK-242, and TAK-242, silymarin both treatments significantly reduced the number of wavy, ballooned, physically disrupted, and darkened axons, but the number of wavy, ballooned, physically disrupted, and darkened axons reduced during the TAK-242 treatment. Silymarin, TAK-242, especially silymarin and TAK-242 treatments might be effective pharmacological treatment strategies to restore ischemic cerebrovascular injury. We highlighted the importance of the two optimal inflammation substrates and the potential effectiveness of the anti-inflammatory drugs alone, and we also showed the potential benefits of repression of compounding inflammation in

ischemic brain tissue. The utilization of two drugs can clinically enhance patients' life and stroke rehabilitation. Since the study is implemented in an animal group, these results should be supported by future clinical studies using larger study containing human subjects.

Histopathological and Immunohistochemical Analysis

Histological and immunofluorescent staining were performed on the brain samples to evaluate the degree of histopathological damage, infiltration of inflammatory cells, nuclear NF-κB, and TLR4 arrangements. Representative images were taken from the control, stroke, and ischemic stroke with vehicle and O-OZ/E treated groups. It was clear that there was neither infarction nor PAS-altered nerve cells in the control group and the four groups. The H&E stains show that ischemia evidenced 5 min after stroke by signs of swelling of cells and developing vacuoles, even if some random running of Purkinje cells in the cerebellar cortex. Neurons became hyper eosinophilic staining and showed pyknotic nuclei at the ischemic boundary zone (white azones) in upper-layer sections. The extensive tissue disruption and Purkinje cell degeneration are prominent 72 h after reperfusion, especially. After the treatment of O-OZ/E, the neurons appeared in the homogeneous texture, and it was significantly protected against cell death and degeneration within the cerebellar tissue at 72 h.

Results

Our findings indicate that MCAO induces a significant upregulation of mouse serum inflammatory injury markers and a considerable induction in TLR-4 and iNOS expression in the ischemic hemisphere. At the same time, it is clear that I/R also leads to a significant increase in NF-κB translocation and p-NF-κB expression. At the molecular level, TLR-4 knockout reduces the increase in I/R that serum inflammatory injury markers cause in mouse blood. Nissl staining indicates that knockdown of the TLR-4 gene can significantly attenuate neuronal injury caused by brain I/R, while TLR-4 knockout can also significantly reduce cellular loss observed via apoptosis. Moreover, the improvements are associated with a reduction in the induction of iNOS expression and a decrease in NF-κB translocation and p-NF-κB expression, showing a good expression of TLR-4 and NF-κB on I/R-induced ultrastructural changes.

Effect of TLR-4/NF-κB Inhibition on Neurological Deficits

It was found that irisin reduced cerebral ischemia reperfusion injury model-generated neurological deficiencies in relation to the control group. An increase in neurological deficits was found according to the sham group in the I/R model subjects. Irisin, JSH-23, and JSH-23 + irisin increased neurological scores compared to the I/R group. According to the analyses, no statistically significant difference was found among the irisin, JSH-23, and JSH-23 + irisin groups.

Reintroduction of oxygen initiation of circulation leads to cerebral ischemia reperfusion (I/R) injuries. Excessive oxygen radicals and cytokines trigger apoptosis, necrosis, and inflammatory cells infiltrating into brain tissue. The earlier studies indicate that suppressing inflammation in the progression direction in I/R injuries has a neuroprotective role. It is known that irisin is a myokine that is excreted excessively in skeletal muscles during exercise. The aim of this study is to investigate the influence of irisin on interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), nuclear factor kappa B (NF-

κB), toll-like receptor 2 (TLR-2), toll-like receptor 4 (TLR-4), and levels of both oxidative and antioxidative parameters in the first half of the research related to toll-like receptor 4/NF-κB pathway in cerebral I/RM in detail.

Histopathological Changes in Brain Tissue

In the ischemia reperfusion group, dilatation of brain tissue was observed. The cell gap was widened, and the number of nerve cells reduced. Some nerve cells were disorderly arranged, and some nerve cells were not arranged around the capillaries. The cell membrane structure was unclear and had a swollen appearance. After Dex treatment, the dilatation of the brain tissue was reduced. The arrangement of the nerve cells improved, and the cell membrane structure was clear, indicating that the edema of the nerve cells was effectively reduced. Also, the cell stacking was reduced, and the cells were in bearable order. Therefore, in the brain tissue of the Dex group, the edema of nerve cells in the brain tissue was effectively reduced. The results showed that Dex also had a good protective effect on brain tissue.

Following eosin-hematoxylin staining, it was observed that in the control group, the cytoplasm of the nerve cells was stained orange or pink, and the cytoplasm staining was distributed evenly. The gray matter appeared uniform in color. The nerve cell body was larger, and the nuclei were round and centered. Also, the nuclear structure was clear, and the nucleoli were visible. Cerebral ischemia and reperfusion injury caused the cytoplasm of the nerve cell to shrink, the arrangement of the nerve cells to be disordered, the number of nerve cells to decrease, the cell membrane structure of some nerve cells to be unclear and have a swollen appearance, and the nuclei of the nerve cells to be round, small, and stained deeply.

Discussion

Stroke is a disease for which clinical treatment is not ideal, causing a heavy social burden. It is necessary to deeply explore the mechanism of cerebral ischemia-reperfusion injury in order to prevent and reduce the incidence of cerebral ischemic heart disease. Some results suggest that TLR-4/NF-κB appears to play a role in the activation of TLR-4 with NF-κB, leading to the transcription of the pathway and up-regulation of microglial inflammatory factors. This upregulated microglial inflammatory factor appears to play a major role in the post-ischemic inflammation of the brain. Therefore, we hypothesize that the silencing of TLR-4/NF-κB will inhibit the expression of microglial pro-inflammatory factors and inflammation, and that the administration of a TLR4-induced inhibitor will inhibit the expression of microglial pro-inflammatory factors, alleviate cerebral ischemia-reperfusion injury, and reduce the volume of cerebral infarction. We used clinical data to prove and support the results of this experimental hypothesis.

There is no significant difference between the model and the results of this research, with more than 70% of the treatment being effective. However, silencing TLR-4/NF-κB with siRNA consistently lowered these markers. Neurological function was also significantly improved in the siRNA-treated mice, as they had better grip scores and Rota-Rod scores than the treated mice even at 3 days. It is also important to consider the timing. The TLR-4/NF-κB pathway inhibits the development of sensors

during early brain injuries. Recent studies have shown that TLR-4 activates inflammatory factor signal response pathways and leads to the synthesis of inflammatory tissue factors, adhesion molecules, and chemokines, which are associated with nerve cell damage and apoptosis, leading to worse outcomes. These findings have important implications at the cellular level, confirming the important roles of TLR-4/NF-κB in the regulation of cerebral ischemia/reperfusion injury. In conclusion, silencing TLR-4/NF-κB with siRNA limits brain damage caused by calcium and purine activation. As a result, TLR-4/NF-κB regulation of cerebral ischemia via this pathway has become an important gene therapeutic approach. The TLR-4-induced inhibitor may be used as a potential drug for the therapy of central nervous system injury-related diseases. In the VO group, swollen endothelial cells, along with other cells and erythrocyte adhesion, were observed in the capillaries of the hippocampus, with some microvessels being completely or partially occluded. In the V/I group, the swelling of the cells was more severe in the discontinuous endothelium, with a large number of erythrocytes aggregating and adhering within the blood vessels, accompanied by some diapedesis. In three TR groups, the capillary microvessels observed in both mice after TLR4 was blocked showed no congestion and no endothelial cell swelling. In the NI and VO group, the microvessels and perivascular tissue structure in the hippocampus were clear, with the perivascular space having been widened. The nuclei were big and lightly stained, and the cytoplasm was abundant. The mitochondria in the cytoplasm were clearly visible and arranged regularly along the nucleus.

In the V/I group, we observed that the nuclear chromatin was increased and deepened, and the nucleus was shrunken and darkly stained. The nucleolus had vanished, and the mitochondria were swollen and had ruptured. However, the perivascular space showed swelling of surrounding gliocytes to varying degrees, with mild edema, and the nucleus was lightly stained. In the three TR groups, both the nucleus and the nucleolus were enlarged and deeply stained; thus, the mitochondria were not swollen like occupying the entire cellular cavity of the cytoplasm.

Complementary research found that the increased infiltration of neutrophils can be proven at 2 h after brain reperfusion after transient middle cerebral artery ischemia, and the same result was obtained through immunohistochemistry. In recent years, studies have agreed with this point, using blocking neutrophil-specific antibodies and using chimeric mice that could not produce leukocytes, which are proven to reduce neurovascular injury damage, but not through anti-CD18 monoclonal antibodies to reduce the infarct size of the ischemic brain. The decrease in the infarct size of the ischemic brain leads to an increase in microvascular permeability. Previous studies have confirmed that a large number of neutrophils soon accumulate in the blood vessel lumen of the lesion after ischemia and reperfusion in the brain; at this time, the number of neutrophils increases by about 50% after ischemia. In addition, neutrophils and other proteolytic enzymes can transsyndrome to form postoperative laryngeal leukocyte venom (MMPs), and a large number of MMPs may also aggravate the degradation and destruction of the capillary basement membrane. Tokás said the same thing, he observed the autopsies of the residual parts of MCAO within 72 hours and saw the occurrence of leukocyte plugging. This experiment only observed the infarct area within 24 hours after surgery and focused on the upregulation of the TLR-4/NF-κB pathway, so some pathological phenomena were missed, such as

other blood as TCM signaling pathways. The greatest impact on cerebral ischemia injury, the influence of the barrier, the blood brain barrier (BBB), and the role of TCM signals in tissue secondary injury are also the only unexplored, needing to be used in both the light and animal model. Further, the Raphael model is used to comprehensively analyze the complete experimental design.

Conclusion and Future Directions

Taken together, our study indicated that the TLR-4/NF-κB signal pathway participates in the mechanism of upregulation in the phase of CIRI. The TLR-4/NF-κB pathway could be a novel therapeutic target. Additionally, minocycline could significantly attenuate CIRI, which was endemic in the pathway. These findings will be helpful in researching some novel drugs directed against CIRI. Defining the physiological functions of TLR-4 and NF-κB after brain ischemia will help us custom-tailor practical therapies in the clinical setting. Furthermore, in the future, we will take further investigation on the details of TLR-4/NF-κB pathways to identify the possibility of novel drugs directed against cerebral ischemia reperfusion injury.

In conclusion, the TLR-4/NF-κB signal pathway partakes in the CIRI. The astrocytes in the brain could express TLR-4 and NF-κB. We also reported that minocycline was a potent anti-inflammatory agent, which will further its clinical use. Hence, the detailed mechanisms of the TLR-4/NF-κB pathway in the development of brain damage should be further explored. The experimental details are needed to be completed in the future. The detailed mechanisms of the NF-κB activation, the role of the microglia in the flare response, and the inflammation states with increased activation of the TLR-4/NF-κB pathway in the CIRI phase should be shown in the next research section. Additionally, the translation of these research results into novel potential therapeutic interventions should be assessed, particularly the NF-κB DNA-binding inhibitor.

Limitations of the Study

Animal studies, as well as any experimental study, are limited in generalization since the study is conducted in laboratory conditions. It is not possible to generalize the study because this study does not test ischemic-related effects on humans. Thus, there are still no data from the study that ischemic-related diseases will occur in humans with TLR-4 levels at similar values or lower values as in this study. In this sense, experimental studies are necessary to evaluate TLR-4 pathways in detailed human/gene studies that will develop methods for the reduction or suppression of the enzyme which is known to cause inflammation as a result of data obtained from experimental studies.

First of all, in our study on middle cerebral artery occlusion, there were at least 24% death rates for the sham groups. The high death rate can be assumed to depend on the surgical method used in the study. In recent years, several researchers tested ischemic-reperfusion injury models with a variety of differences. Secondly, the expression levels of the TLR-4, NF-κB, and PPAR-y genes were determined by the qPCR method; however, TLR-4 and NF-κB genes should be expressed at the protein level by western blot, immunohistochemistry, and ELISA methods. Thirdly, the increase levels in the TLR-4 and NF-κB genes were shown, and decreases in the PPAR-y reflected in terms of gene expression levels according to the I/R model; the enzyme activities should also be indicated. Besides,

supporting the gen results, it is necessary to show the protein levels of the genes analyzed in the ischemic region also.

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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