



OPEN Time-dependent expression of neural oxidative and inflammatory biomarkers in ischemic stroke using a transient MCAO rat model

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Ischemic stroke is globally acknowledged as a prominent reason for disability and mortalities. Oxidative stress, neuroinflammation, and autophagy have been implicated in its pathogenesis. A model of middle cerebral artery occlusion (MCAO) in male rats was conducted for this investigation and the brain cortex was sampled taken after at 1, 6, 12, 24, and 48 h of IRI. The results revealed time-dependent decreases in cortical GSH with rises in MDA and NO levels. Additionally, marked upregulations of inflammatory cytokines were observed and increased as time progressed. Besides, significant upregulations in TLR4, GFAP, Iba-1, and BDNF mRNA expressions were noticed along with increases in the P62 and decreases in LC3 immune expression levels. The pathological findings of cerebral sections showed vacuolations in the neuropil, perivascular edema, and shrinkage of neurons which also increased gradually by time. Taken together, these biomarkers could be reliable indicators for determination of time elapsed after IRI.

Keywords Autophagy, Cerebral ischemia, Inflammation, Oxidative stress, Forensics

Ischemic stroke is a destructive cerebrovascular disease caused by occlusion of the cerebral artery that results in inadequate oxygen and nutrient supply to the brain¹. Globally, ischemic stroke accounts for about 10% of the worldwide mortalities and its incidence may increase significantly in the next years because of increased risk factors². Therefore, the early evaluation, diagnosis, and intervention for ischemic brain stroke is crucial³. The ischemic injury is evoked in multiple brain areas and accompanied by diverse morphological and biochemical changes based on its type, duration, and blood flow⁴.

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Most available diagnostic approaches are conducted to rule out neurological or metabolic disorders even with a strong suspicion of ischemic stroke⁵. Additionally, routine histopathological examinations may be insufficient, particularly when death occurs in earlier stages of ischemia^{6,7}. Hence, biochemical markers of tissue injury represent a revolutionary strategy for stroke evaluation⁸. Biomarkers are biological molecules that can be substantially quantified as early sentinels of various biological processes to predict normal or pathological function in the body^{9,10}. These biomarkers allow a temporal classification of ischemic injuries from the forensic point of view to elucidate the duration, time, and pathophysiological mechanisms of ischemic damage as well as the cause of death^{11,12}. Consequently, intensive insight is essential to improve the validity and reliability of cerebral ischemic biomarkers¹³.

Recently, investigating certain biomarkers can predict the outcomes of ischemic stroke in a time-dependent manner based on the pathophysiology of stroke such as biomarkers related to excitotoxicity, oxidative stress, endothelial dysfunction, astrocyte activation, inflammation, fibrinolysis, neuroplasticity, and metabolism^{14,15}. Studies have reported that ischemic injury can cause oxidative stress in the brain that possibly induces neural inflammation¹⁶. Ischemic stroke enhances the activity of glial cells such as microglia and astrocytes which results in the secretion of pro-inflammatory cytokines including TNF- α and IL-1 β , and neuronal damage¹⁷. Following MCAO, the expressions of Iba-1 and GFAP were markedly increased as they are common markers for activated microglia and astrocytes, correspondingly¹⁸. In addition, Xing et al. reported that TLR4 expression is upregulated under the condition of ischemia, which is implicated in the progress of neuroinflammation¹⁹. The expression of TLR4 in human plasma was associated with vigorous inflammatory response and poor outcomes in acute brain ischemia²⁰.

Over the past few years, a notable advancement has been accomplished in clarifying the molecular mechanisms of IRI which mainly involve a series of events including neuronal excitotoxicity, energy failure, oxidative stress, inflammatory reaction, neuronal necrosis, and apoptosis^{21–23}. However, the reports that focused on the time of ischemia are scanty. Therefore, we explored in this study the expression of various neural, oxidative, and inflammatory biomarkers using a MCAO model in rats in relation to the time of stroke occurrence.

Materials and methods

Experimental animals

Male Wistar rats, weighing 200 ± 10 g on average, were received from the animal house, Faculty of Veterinary Medicine, Mansoura University, Egypt. All rats were raised in a controlled environment with free access to food and water, at 23 ± 2 °C and 50–60% humidity, on a 12-h light/dark cycle. The Guide for the Care and Use of Laboratory Animals was followed when handling any of the animals.

After 2 weeks of acclimatization, transient focal cerebral ischemia was induced through middle cerebral artery occlusion (MCAO) as formerly described^{24,25}. In brief, animals were anesthetized by pentobarbital (100 mg/kg i.p.), and the ventral surface of the neck was dissected through a midline incision. The external carotid was tied with a 4-0 nylon suture and the common carotid was occluded using an artery clip. A 3-0 nylon monofilament suture was gently introduced into the lumen of the internal carotid until a slight resistance was felt which indicated intraluminal blockage of the origin of the MCA. After 30 min, reperfusion was established under anesthesia, and the suture was gently withdrawn. The ischemic rats were assigned into five subgroups with different time points of post-ischemic reperfusion (1, 6, 12, 24, and 48 h of reperfusion). At the end of the study, all animals were euthanized humanely by cervical dislocation.

ARRIVE guidelines statement

This study aligns with the ARRIVE guidelines to ensure rigorous, transparent, and detailed reporting of experimental design, methodologies, and results in animal research.

Dissection, homogenization, and sample preparation

The brains were dissected out and washed with ice-cold phosphate buffer saline (PBS). The brain tissue of the ischemic hemisphere was homogenized in 0.1 M of ice-cold PBS (pH 7.4). Then centrifuged $10,000 \times g$ for 10 min at 4 °C and the supernatant was collected and stored at -80 °C for further biochemical analysis.

Estimation of oxidative stress biomarkers

Measurement of malondialdehyde (MDA)

MDA was performed using thiobarbituric acid reactive substances (TBARS) assay²⁶. Briefly, the brain tissue supernatant (0.25 mL) was mixed with 0.5 mL of 5% chilled TCA and 0.5 mL of 0.67% thiobarbituric acid then the mixture was centrifuged at $4000 \times g$ for 10 min. Afterward, the supernatant was collected and placed for 10 min in a boiling water bath then cooled and absorbance was recorded at 535 nm using a spectrophotometer (Shimadzu-1601; Kyoto, Japan). The MDA level was estimated and presented as nmol/g tissue.

Estimation of reduced glutathione (GSH) levels

GSH was measured according to the Ellman method with slight modifications²⁷. A mixture of supernatant (0.1 mL), 1.7 mL PBS (0.1 M, pH 7.4), and 0.2 mL 5'5' dithiobisnitro benzoic acid (DTNB) was vortexed, and the absorbance of the yellow-colored reagent was recorded against blank sample at 412 nm. The GSH content was calculated and presented as nmol of GSH/mg protein.

Estimation of nitric oxide (NO) levels

NO was estimated from nitrite generation as assayed previously²⁸ using Griess reagent (sulfanilic acid and with *N*-(1-naphthyl)-ethylenediamine). The absorbance of the produced azo dye was recorded spectrophotometrically at 540 nm. NO level was expressed as $\mu\text{mol/g}$ tissue.

Estimation of proinflammatory cytokines

The supernatants of the brain tissue homogenate were collected to detect proinflammatory cytokines levels using ELISA kits for TNF- α (E-EL-R2856), IL-6 (E-EL-R0015), and IL-1 β (E-EL-R0012), (Elabscience, Texas, USA). All ELISA analyses were carried out in triplicate using a Bio-Rad microplate reader (Bio-Rad, CA, USA) at 450 nm in compliance with the guidelines provided by the manufacturer. The concentrations of TNF- α , IL-6, and IL1 β were calculated through a standard curve and expressed as pg/ml.

RNA extraction, cDNA transversion, and qRT-PCR assay

Trizol reagent (iNtRON Biotechnology, Inc., South Korea) has been used to separate total RNA from brain tissues in accordance with the manufacturer's guidelines. Then, the concentration of RNA was determined by Nanodrop (Uv-Vis spectrophotometer Q5000/Quawell, Quawell Technology, Inc., San Jose, CA, USA). Next, the cDNA was synthesized using the Fast Hisenscript™ RH (–) RT PreMix cDNA synthesis kit (iNtRON Biotechnology, Inc., South Korea) following the manufacturer's instructions.

In the current experiment, mRNA primers for glial fibrillary acidic protein (GFAP), Ionized calcium-binding adapter molecule 1 (Iba1), brain-derived neurotrophic factor (BDNF), and toll like receptor 4 (TLR4) were used (Table 1). YBR Green PCR Master Mix (SensiFast™ SYBR Lo-Rox kit, Bioline) was employed to evaluate the expression of the targeted genes using a RT-PCR. The thermocycling conditions were 95 °C for 10 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 1 min, and finally 72 °C for 20 s. The mRNA expression folds of each target gene were standardized and normalized against the β -actin using the $2^{-\Delta\Delta Ct}$ method²⁹.

Histopathological and immunohistochemical examination

Hematoxylin and eosin staining (H&E) was used to do a histopathological evaluation of the brain tissue³⁰. Briefly, brain tissues were fixed in formalin (10% v/v) overnight. Afterward, brain tissues were processed, and embedded in paraffin wax, and 5 μ m thick slices were prepared through a microtome (Radical Scientific, India), followed by staining with H&E. Sections were observed under a light microscope equipped with a digital camera (Nikon Corporation, Japan).

For immunohistochemical evaluation, brain Sects. (5 μ m thickness) were deparaffinized and rehydrated. Next, they were boiled with 0.01 M of citrate buffer at 105 °C for 20 min for antigen retrieval then treated with H₂O₂ (3%) for endogenous peroxidases deactivation. Next, brain sections were incubated with a primary specific polyclonal antibody for LC3 (ab192890) and P62 (ab155686), (Abcam, UK) at 4 °C overnight then washed with PBS thoroughly and incubated with biotinylated secondary antibody labeled streptavidin peroxidase complex then visualized with 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin based on manufacturer instructions. Brain sections were captured using an optical microscope equipped with a digital camera (Nikon Corporation, Japan).

Statistical analysis

The data are presented as mean \pm SE. One-way ANOVA was used for statistical analysis, along with Tukey's post hoc test, and statistical significance was set at $P < 0.05$. Statistical analysis was handled by the SPSS 13.0 software (SPSS Inc.; Chicago, USA); where, all graphs were generated by GraphPad Prism software (version 8.4.3., GraphPad Software Inc., MA, USA).

Results

Changes in oxidative stress biomarkers

As demonstrated in Fig. 1, after incision, there was a marked decrease ($P < 0.05$) in the levels of GSH in brain tissue homogenates in rats after 6 h from IRI in comparison with those sampled after an hour. Additionally, a time-dependent decrease ($P < 0.05$) in GSH levels in ischemic rats after 12, 24, and 48 h related to IRI/6. However, no significant changes were noticed among the groups of IRI after 12, 24, and 48 h.

On the other hand, remarkable time-dependent increases ($P < 0.05$) in the NO levels starting from the time of 1 h and the highest levels after IRI was reached at the time of 48 h. Levels of MDA were the highest ($P < 0.05$) in groups of 48 and 24 h from IRI without any significance in between them. In addition, significant increases ($P < 0.05$) in MDA of the IRI group after 24 h related to the other groups after 1 and 6 h. The IRI groups after 12 and 24 h were not different in MDA levels. Similar results were observed upon comparing the IRI groups sampled after 1 and 6 h from IRI.

Gene	Primers	PCR product	Gene Bank accession number
GFAP	F:5'-GCGAAGAAAACCGCATCACC-3' R:5'-TCTGGTGAGCCTGTATTGGGA-3'	150 bp	XM_039085178.1
Iba1(Aifl)	F:5'-AGCAAGGATTTGCAGGGAGG-3' R:5'-TTGAAGGCCTCCAGTTGGAC-3'	143 bp	NM_017196.3
BDNF	F:5'-GGCTCTCATACCCACTAAGATACATC-3' R:5'-CGGAAACAGAACGACAGAAACAG-3'	107 bp	XM_006234684.4
TLR4	F:5'-GATTGCTCAGACATGGCAGT-3' R:5'-CCCACTCGAGGTAGGTGTTT-3'	137 bp	NM_019178.2
β -actin	F:5'-TCCTCCTGAGCGCAAGTACTCT-3' R:5'-GCTCAGTAACAGTCCGCCTAGAA-3'	153 bp	XM_039089807.1

Table 1. Primer sequences.

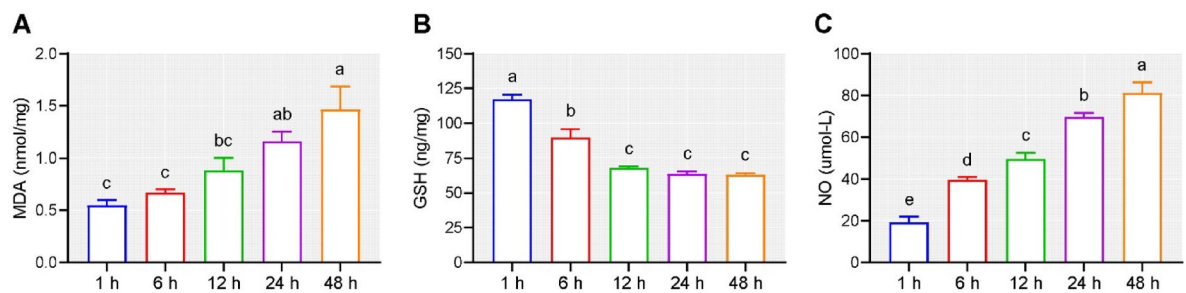


Fig. 1. Changes in the oxidative stress biomarkers (MDA, GSH, and NO) in the brain tissue at different time points in MCAO-induced brain ischemic injury. All values are expressed as the means \pm SE. The different letters represent the statistical significance between different time points ($P < 0.05$).

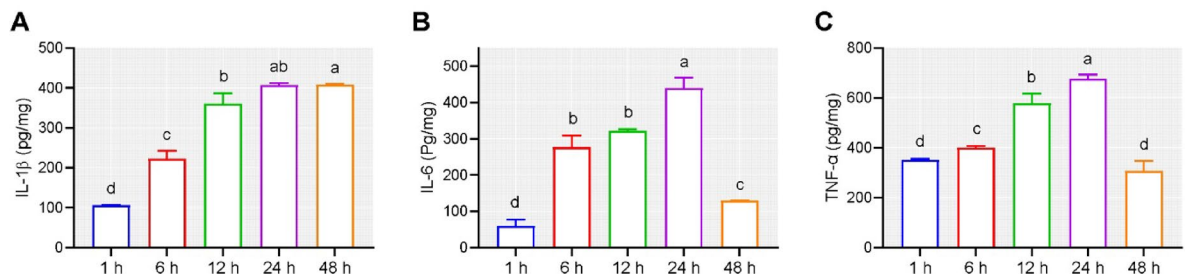


Fig. 2. Changes in the inflammatory biomarkers (IL-1 β , IL-6, and TNF- α) in the brain tissue at different time points in MCAO-induced brain ischemic injury. All values are expressed as the means \pm SE. The different letters represent the statistical significance between different time points ($P < 0.05$).

Changes in neural inflammatory biomarkers

We aimed to investigate whether the time relapse from induction of IRI has an effect on the levels of inflammatory biomarkers in this model of IRI (Fig. 2). Marked increases ($P < 0.05$) in the levels of neural TNF- α and in a time-dependent manner in IRI groups until 24 h followed by decreases in the 48-h group. A similar trend ($P < 0.05$) was observed in the levels of IL-6 in the brain tissue without significant changes between 6 and 12 h after induction of IRI. On the contrary, IL-1 β levels, throughout the 48 h, displayed marked increases ($P < 0.05$) with prolonged the time to 48 h with no observed differences between IRI groups after 24 and 48 h.

GFAP, Iba-1, BDNF, and TLR4 mRNA relative expression in brain tissues

The relative mRNA expressions of GFAP, Iba-1, BDNF, and TLR4 genes in brain tissues of experimental rats following different periods of brain IRI are shown in Fig. 3. It was speculated that the expressions of these genes were altered as reflected by a significant upregulation ($P < 0.05$) of brain-related genes (GFAP, Iba-1, and BDNF). The upregulation of these genes was concurrent with the increase of sampling time as 48 h of brain IRI revealed the most significant upregulations than other time points (24, 12, 6, and 1 h, respectively). Meanwhile, the expression of neural TLR4 gene showed a different pattern as its expression showed the most significant upsurge ($P < 0.05$) after 24 h of brain IRI followed by its expressions after 12 h, 6 h, and 48 h then 1 h, respectively.

Histopathological findings

Microscopic pictures of HE-stained cerebral cortex sections from IRI groups after 1, 6, 12, 24, and 48 h from reperfusion. Marked vacuolations in the neuropil, perivascular edema, and shrinkage of neurons were observed in stained sections. Remarkably, these pathological findings were increased gradually by increasing the time from infliction of IRI (Fig. 4).

Changes in autophagy-related proteins

Microscopic pictures of immunostained cerebral sections against LC3 from IRI groups after 1, 6, 12, 24, and 48 h showed decreased numbers of positively stained neurons gradually over time (Fig. 5). However, the immunostained cerebral sections against P62 from IRI groups displayed increased numbers of positively stained neurons gradually over time (Fig. 6).

Discussion

Stroke is a severe neurodegenerative disease that is associated with neuronal loss, oxidative stress, mitochondrial dysfunction and neuroinflammation. In our study, the IRI model was performed using adult male albino rats. It was previously documented that the number, shape, and inflammatory responses of microglia are

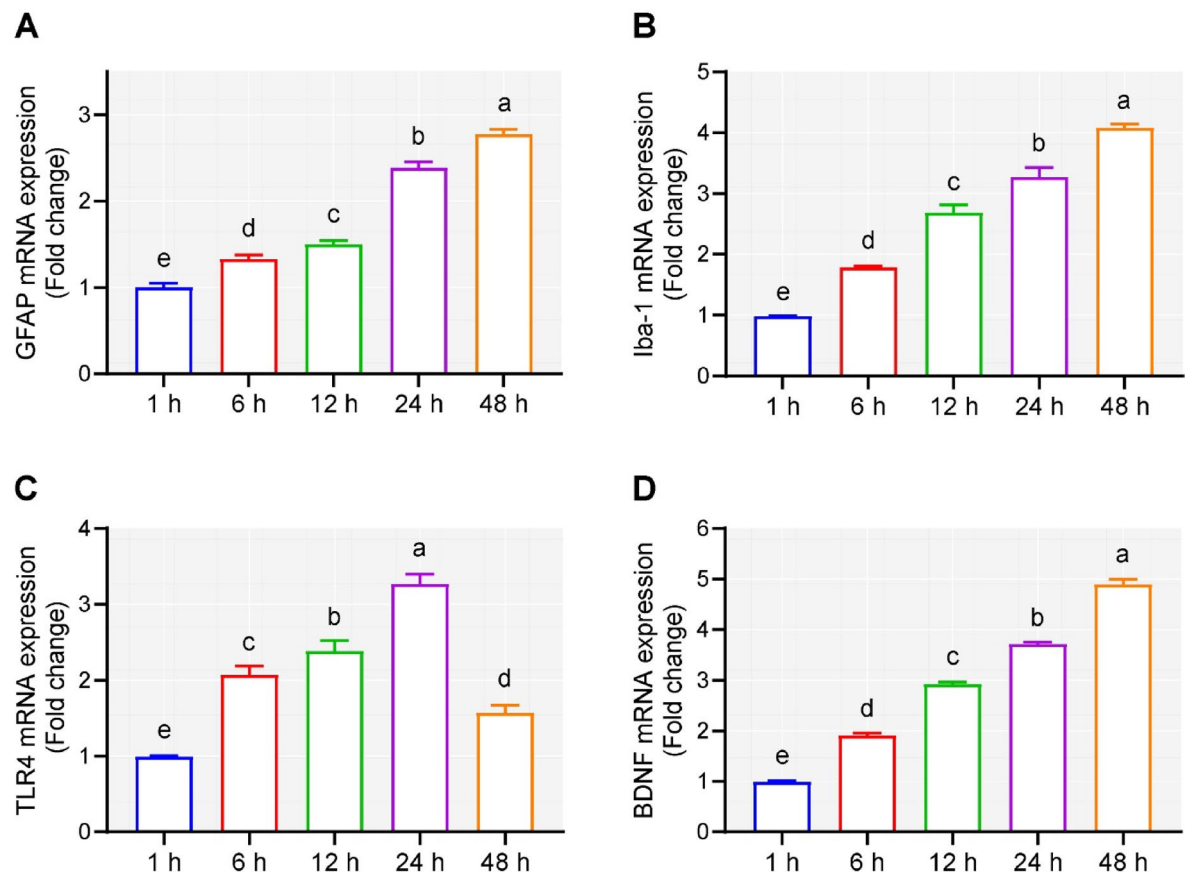


Fig. 3. Changes in the mRNA expression levels of GFAP, Iba-1, TLR4, and BDNF in the brain tissue at different time points in MCAO-induced brain ischemic injury. mRNA expression results were recorded as the means \pm SE in triplicate and β -actin was the housekeeping gene. The different letters represent the statistical significance between different time points ($P < 0.05$).

affected by several factors such as age, brain region, and hormones. Microglia is also responsive to estrogens as it significantly mitigates its immune and inflammatory responses³¹. In former experimental stroke models, female animals displayed less damage from stroke induced damage than males, and this effect can be partially reversed by ovariectomy³². Cell death induced by deprivation of oxygen and/or glucose is less extensive in female astrocytes³³. In addition, male neurons are more vulnerable than female cells to glutamate or peroxynitrite which are used to simulate brain injury³⁴. Given the significant role of microglia in neural damage during ischemic conditions, we examined the effect of IRI on the neural changes in the brain of male rats to exclude the effect of female sexual hormones.

Accumulating evidence has unveiled the crucial role exerted by glial cells in the pathogenesis of cerebral IRI injury³⁵. This injury triggers the activation of microglia and astrocytes that results in increases in blood–brain barrier permeability, strong inflammatory response and further nerve cell damage¹⁷. Iba-1 and GFAP proteins are common biomarkers of activated microglia and astrocytes, respectively. Increased expressions of Iba-1 and GFAP by IRI promotes the proliferation of microglia and astrocytes, stimulates the expression of NF- κ B, and secretion of inflammatory cytokines as TNF- α and IL-1 β ³⁶. GFAP, a type III intermediate filament protein, contributes in the maintenance of the structure, shape and skeletal function as well as mechanical strength of astrocytes. It also plays a vital role in cell communication, astrocyte–neuron interactions, and maintenance of blood–brain barrier functions³⁷. Liedtke et al.³⁸ have reported that GFAP knockout in mice affected negatively on cell structure and resulted in incomplete myelination.

Although the stimulated astrocytes release various neurotrophic chemicals to aid the neurons in surviving, it is believed that severe stimulation generates an inflammatory response that leads to neuronal death and brain injury³⁹. After prolonged stimulation, astrocytes release a range of neurotoxic substances and show increased expression of GFAP⁴⁰. The number and intensity of cells expressing the GFAP gene increase in reactive gliosis. Additionally, GFAP represents a sensitive and early marker for neurotoxic conditions. Mandwei et al.⁴¹ found that rats with spinal cord injury (SCI) displayed marked upregulated gene expression levels of GFAP than that of control group. Following SCI, discrete brain regions may have been exposed differently to increased norepinephrine and glucocorticoids which could explain the variations in GFAP gene expression levels. Thus, GFAP level is an accepted biomarker for assessment of neurological damage in stroke and it can be used as an indicator of reactive astrocytes and neuroplasticity.

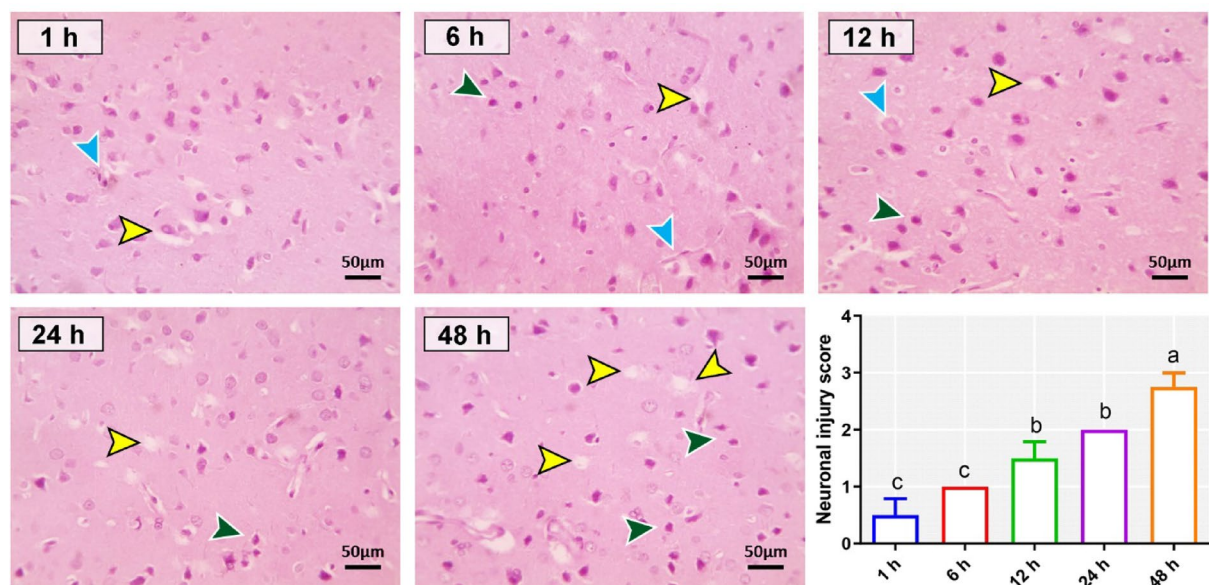


Fig. 4. Representative photomicrographs of HE-stained cerebral sections after 1, 6, 12, 24, and 48 h of induction of cerebral ischemia. Vacuolations in neuropil (yellow arrows), perivascular edema (blue arrows), shrinkage of neurons (dark green arrows) that increased gradually by time are seen. magnification X:400, scale bars = 50 μm. The bar chart shows the semi-quantification of the neuronal injury scores. All data expressed as means ± SE (n = 4). The different letters represent the statistical significance between different time points (P < 0.05).

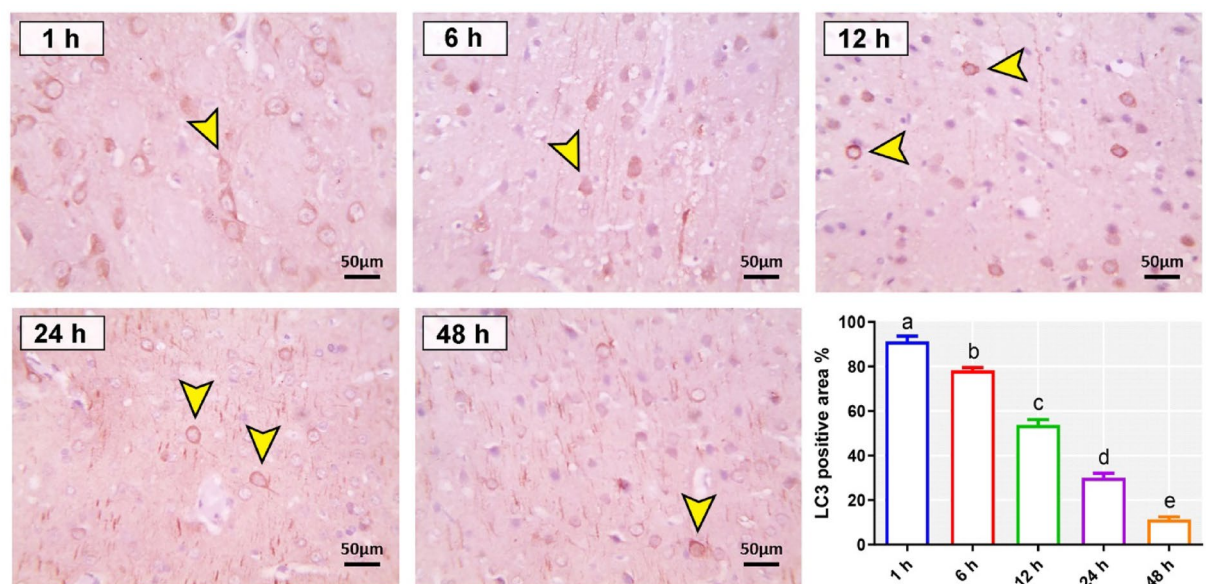


Fig. 5. LC3-immunostained cerebral tissue after 1, 6, 12, 24, and 48 h of induction of cerebral ischemia. Decreased numbers of positively stained neurons (yellow arrows) gradually over time are seen. Sections were counterstained by Mayer's hematoxylin. magnification X:400, scale bars = 50 μm. The bar chart shows the LC3 positive area %. All data expressed as means ± SE (n = 4). The different letters represent the statistical significance between different time points (P < 0.05).

Iba-1 is a specific calcium-binding protein of microglia/macrophages and a marker for activated microglia and is highly expressed in damaged neural tissue³⁷. The increased Iba-1 expression levels indicate the dynamic activation of microglial cells. Marked increases were detected in the protein expression levels of Iba-1 in a rat MCAO model^{17,35,42}. Similar results were also reported by Kim and collaborators⁴³ who found upregulated Iba-1 was reported and correlated with severe neuronal cell death after global cerebral ischemia. Also, similar trend was observed in the cortex samples taken from patients who were dead shortly after aneurysmal subarachnoid

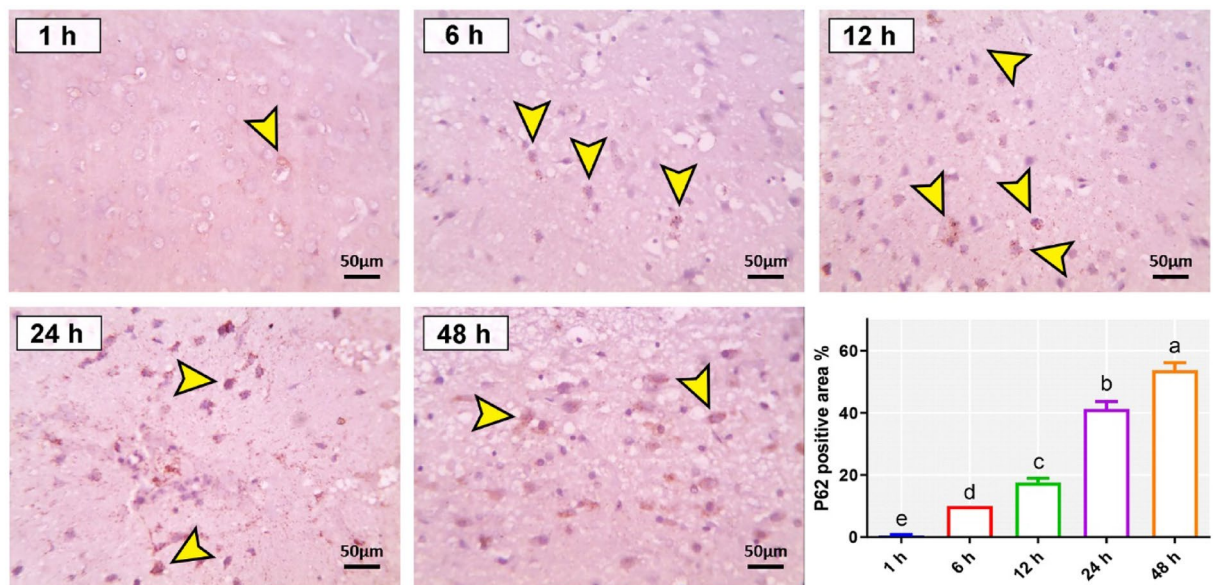


Fig. 6. P62-immunostained cerebral tissue after 1, 6, 12, 24, and 48 h of induction of cerebral ischemia. Decreased numbers of positively stained neurons (yellow arrows) gradually over time are seen. Sections were counterstained by Mayer's hematoxylin, magnification X:400, scale bars = 50 μ m. The bar chart shows the P62 positive area %. All data expressed as means \pm SE (n = 4). The different letters represent the statistical significance between different time points ($P < 0.05$).

hemorrhage⁴⁴. In another study, the spinal cord damage dramatically increased levels of Iba1 gene expression, which is consistent with our results⁴¹.

BDNF is a vital mediator for functional, structural plasticity as well as neuronal development and differentiation in CNS, and it evoked a marked neural protection against various neurological diseases including cerebral ischemia^{45–47}. BDNF is involved in synaptic plasticity in stroke recovery through synaptic reorganization to restore motor and cognitive functions⁴⁷. The BDNF protein and mRNA levels as well as proBDNF are reduced in the post-mortem brain of AD patients⁴⁸. BDNF contributes to the progress of the neural damage during the first stages of a IRI, as it is secreted in large amount to activate the reconstruction of the synapses. This rapid increase of BDNF after cerebral ischemia injury may be related to the initiation of the body's protective mechanism for neuron repair, and regeneration⁴⁹. Moreover, BDNF could be increased with oxidative stress, neuroinflammation, as part of an antioxidant defense supporting neuronal survival and preventing apoptosis⁴⁸.

Excess production of reactive oxygen species (ROS) could trigger a disruption of the antioxidant system, which plays a fundamental role in tissue damage in ischemic stroke^{50,51}. Abundant studies have reported that oxidative stress could provoke lipid peroxidation, DNA damage, neuronal degeneration, and apoptosis in the ischemic cascade^{52–54}. Our current study revealed a notable rise of MDA, a core biomarker of lipid peroxidation, and NO over different time points, which may be attributed to excess oxyradicals generation from infarcted tissue during the acute phase and consistent with the time course of tissue damage^{55,56}. Meanwhile, a temporal decline of GSH, the most important free radical scavenger, has been found to significantly decrease within 24 h of IRI which also correlated with the neurological deficit severity, and infarct size⁵⁷. These findings are in harmony with earlier reports which implied that MDA and NO levels were markedly elevated, alongside a significant reduction of GSH during the acute phase of IRI (24 h)^{58–60}. The time point concentrations of such oxidative biomarkers could explain the pathophysiological mechanism of IRI and the expected outcome since the spontaneous thrombolysis in ischemic stroke through tissue plasminogen activator may be diluted upon exposure to an oxidant environment in the acute phase of IRI^{56,61}.

Neuroinflammation has been reported to play a prime role in cerebral IRI pathogenesis. The recent findings suggest that overexpression of the TLR4 gene was recorded in cerebral ischemic injury and the extent of cerebral ischemia was linked to TLR4 gene expression patterns⁶². TLRs are expressed in the brain's blood vessels by stimuli-responsive endothelium and smooth muscle cells. Inflammation is brought on by ischemia or reperfusion of the brain's tissues. The invasion of microglia and a rise in inflammatory cells, particularly macrophages, result in the release of proinflammatory molecules such as TNF- α and interleukins⁶³. Lamberts et al.⁶⁴ reported that TLR4-induced increases in inflammation led to the upregulation and activation of inflammasomes, which in turn boosted TNF- α and interleukins following cerebral artery occlusion. Likewise, TLR4 gene expression was found to be expressed around three to five days after renal ischemia, as shown by four to five times the increase in TLR4 gene expression⁶⁵. The local release of inflammatory cytokines exacerbates tissue damage and results in the formation of reactive radicals that have been connected to the activation of the TLR4 gene in podocytes. Additionally, this may alter the barrier function of the glomerular capillary wall, increasing albumin permeability and drawing in inflammatory cells. Under ischemic stroke condition, activation of astrocytes/microglia and infiltration of immune cells is prompted by pro-inflammatory cytokines such as TNF α and IL-1 β .

Once activated, microglia and macrophages produce more TNF α and IL-1 β ⁶⁶. Their release affects the severity of neurological deficits and neuronal damage via production of reactive radicals¹⁷. TNF α may play a dual role, maintaining an inflammatory environment in neurons while also modulating synaptic strength and excitatory transmission in the cortex⁴⁶. IL-1 β is another pro-inflammatory cytokine that is markedly augmented in cerebral ischemia. It triggers the secretion of inflammatory cytokines and chemokines such as TNF- α , interferon- γ , and IL-6. Furthermore, IL-1 β releases glutamate, which evokes disruption in BBB permeability and neuronal cell death in neurodegenerative diseases³⁷. In our study, inflammatory cytokines show different trends as they elevated significantly until 24 h then decreased at 48 h. This can be explained by the numbers of microglia tended to elevate at 24 h after cerebral ischemia and then declined at 8 h⁶⁷.

Autophagy is a cellular process that identifies and eradicates damaged mitochondria, in order to maintain the stability of this cellular organelle and to get rid of decomposed cellular components⁶⁸. It represents a potent protective mechanism in ischemic conditions as it provides energy by degrading proteins as well as protecting cells by degrading damaged proteins and synthesizing new proteins⁶⁹. Mitophagy may decrease ROS production and promote cell survival under normal and mild stress conditions. However, excessive ROS can over activate the mitophagy (maladaptive mitophagy), which may increase tissue injury and cell death⁷⁰. In the cytoplasm, PINK1 accumulates on the external membrane of damaged mitochondria. Next, P62 binds to LC3 resulting in activation of autophagosomes, which activates the degradation of damaged mitochondria by lysosomes. In our study, we found that P62 increased and LC3 decreased after induction of IRI in a time-dependent manner. Under ischemic conditions, the induction of autophagy provides energy by degrading the damaged proteins in addition to synthesizing new proteins⁷¹. Zeng et al.⁷² reported that significantly activated Drp1 accelerates the formation of P62-containing autophagosomes after cerebral ischemia–reperfusion. Similarly, a marked increase was found in the expression of P62, with a decrease in the expression of LC3II/LC3I in HT22 cells after cerebral I/R injury⁶⁹. In addition, Yu and colleagues⁶⁸ reported that the number of LC3-positive cells decreased, and the number of P62-positive cells increased in a MCAO/R model in rats. In the initial stage, ischemic injury encourages cellular autophagy via increasing the expression of LC-3 II. During the transportation phase, the stimulation of autophagic flux results in the exhaustion of P62. Hence, the increase in P62 level indicates compromised autophagy clearance and transportation. Therefore, p62 and LC3 proteins are helpful indicators to verify autophagy activation⁷³.

The current findings show that the evolution of ischemia injury is mirrored by time-dependent alterations in oxidative stress (MDA, NO), inflammatory cytokines (TNF- α , IL-1 β), and autophagy markers (P62, LC3) during MCAO. In forensic investigations, these biomarkers may act as a molecular clock to determine when a stroke occurred, especially when histology is equivocal (e.g., early-stage ischemia). To help forensic pathologists determine the timing of injury, for example, the increasing accumulation of P62 and depletion of LC3 provide a measurable parameter for the length of ischemia.

Conclusion

From the abovementioned findings, it could be concluded that the levels of oxidative stress biomarkers (GSH, MDA, and NO) displayed significant changes in post-IRI conditions. In addition, TNF- α , IL-1 β , and IL-6 can be considered as age determinant for brain ischemia. The gene expression results revealed high expressions of GFAP, Iba-1, BDNF, and TLR4 genes in the cortical region which correlated by time. Moreover, the IRI injury modulated the autophagy-related biomarkers (LC3 and P62) in brain cortical tissue of rats. Further studies about the expression of these markers in IRI are highly recommended to clarify their useful use in human forensic cases.

The study's limitation and future perspectives

While this study provides detailed insights into the brain pathology induced by MCAO, we acknowledge that assessing neural, oxidative stress, inflammation, and autophagy biomarkers. However, longitudinal imaging of infarct progression would further strengthen the translational relevance of our findings at different time points. Due to the scope and experimental design of this study, we focused primarily on neural, oxidative stress, inflammation, and autophagy markers. Future studies should incorporate multi-time-point imaging of infarcted areas to correlate structural damage with the time progression of brain ischemia.

Data availability

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

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Conceptualization, investigation, and methodology; O.H., A.A., B.M.H., H.M.E., B.O., R.E.A., and M.A. Formal analysis, data curation, validation, and visualization; O.H., A.A., L.M.E., S.F.I., R.O.A., H.A.A., H.F.H., N.A.M., K.S.A., M.A., and H.Z. Writing original draft, review, and editing; all authors. All authors have approved the published version of this manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

All animal treatment and care strictly adhered to the UK Animals Act 1986 (ASPA) and the 3Rs principles (replacement, reduction, refinement). Ethical approval was approved by the Mansoura University Animal Ethical Committee, Egypt (MU-ACC VM.R.23.07.114).

Additional information

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