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Experimental Research Article

Combined effects of SH-SY5Y–derived exosomes and glutamic acid on neuroinflammation and functional recovery after MCAO in rats

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ABSTRACT

Introduction: Ischemic stroke and brain trauma are significant causes of death and disability in the West. Inflammasomes induce pro-inflammatory cytokine production in cerebral ischemia, thereby driving inflammation. The current study investigates the effects of glutamic acid, N-methyl-D-aspartate receptor agonist, and SH-SY5Y Neuroblastoma-Derived Exosomes in the rat model of cerebral ischemia.

Methods: Sixty adult male Wistar rats were randomly assigned to five groups. After MCAO, rats were treated with glutamic acid, SH-SY5Y neuroblastoma-derived exosomes, and both. Neurological deficits were assessed using the Garcia scale 7 days after ischemia. Neuronal cell death was evaluated using cresyl violet staining. We examined gene and protein levels of BDNF, NLRP3, and NMDAR in brain tissue using real-time PCR and immunohistochemistry. TTC staining was used to determine cerebral infarct volume in brain tissue, and ELISA tests to measure IL-1β and IL-18 levels.

Results: Cresyl violet staining demonstrated that glutamic acid+exosomes significantly reduced neuronal degeneration. Garcia's test results showed that glutamic acid, independently or in combination with exosomes, significantly improved sensory and motor functions. The results of IHC and Real-Time PCR clearly indicated that the expression of the BDNF gene and protein, as well as the NMDAR gene, increased, whereas the expression of the NLRP3 gene and protein, and the Caspase-1 protein, decreased. ELISA results showed a significant drop in IL-1β and IL-18 levels in MCAO groups treated with glutamic acid, exosomes, or both.

Conclusion: The use of glutamic acid with SH-SY5Y neuroblastoma-derived exosomes exerts neuroprotective benefits by enhancing neurotrophic factors and diminishing apoptosis in the MCAO model.

Keywords:
Brain Ischemia
Glutamic Acid
Exosomes
NLRP3 Inflammasome
Caspase

Introduction

Stroke, or cerebral ischemia, is a leading cause of mortality and long-term disability worldwide (Feigin et al., 2021). An ischemic stroke happens when a blood clot

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blocks a blood vessel in the brain, reducing blood flow to brain tissue and leading to a loss of energy in the brain, neuronal death, and a cascade of pathological events, including excitotoxicity, oxidative stress, and inflammation (Ismael et al., 2018; Kuriakose and Xiao., 2020; Salaudeen et al., 2024). Today, various animal models are used to investigate potential treatments for cerebral ischemia. These models are divided into two types: local and global cerebral ischemia. Local cerebral ischemia can be induced by blocking the middle cerebral artery by opening the skull and injecting an embolus or by using the filament method (Casals et al., 2011). When glutamate accumulates at synaptic and postsynaptic sites, it disrupts ion channel function, causes membrane and DNA damage, induces inflammation, and eventually leads to neuronal death and ischemic brain injury (Belov Kirdajova et al., 2020) ischemic injury is a prominent cause of death and disability worldwide. Brain ischemia stems from cardiac arrest or stroke, both responsible for insufficient blood supply to the brain parenchyma. Glucose and oxygen deficiency disrupts oxidative phosphorylation, which results in energy depletion and ionic imbalance, followed by cell membrane depolarization, calcium (Ca2+).

The most common treatment for acute stroke is intravenous thrombolytic therapy with recombinant tissue plasminogen activator (rt-PA). However, its therapeutic window is limited to 3 to 4.5 hours, and it carries a 6-7% risk of intracerebral bleeding, which confines its use to just 5% of stroke patients (Cheng and Kim 2015). As a result, there is a pressing need to develop safer and more effective treatments.

Over the past several decades, substantial research efforts have enhanced our understanding of stroke pathology (Kuriakose and Xiao 2020). Various therapeutic strategies, including NMDA receptor antagonists, calcium channel blockers, and antioxidants, have been explored (Gupta and Chauhan 2011). NMDA receptors are excitatory glutamate receptors widely distributed throughout the nervous system and play a crucial role in neuronal remodeling, brain growth, learning, and memory formation (Olivares et al., 2012; Rajani et al., 2020; Ramanathan et al., 2016). Excessive NMDA receptor activation during a stroke initiates a cascade leading to neuronal death. Preclinical studies indicate that NMDA receptor antagonists protect neurons from ischemic damage and promote the expression of BDNF, which mediates neuroplasticity, neuroprotection, and neurogenesis (Constantino et al., 2014; Jeanblanc et al., 2014; Kotlęga et al., 2017).

Cell-derived vesicles known as exosomes, which carry proteins and nucleic acids, could be considered a new therapeutic potential (Liu et al., 2021a). Exosomes are nanoscale extracellular vesicles secreted by living cells into the extracellular fluid. Multiple studies demonstrate that exosomes are present in biofluids such as blood, urine, and cerebrospinal fluid (Zhang et al., 2015). These nanocarriers regulate several cellular functions, most notably immunological regulation, and include the primary mediators of intercellular communication, including proteins, lipids, and nucleic acids (Vanni et al., 2017; Zhang et al., 2019). In addition, some studies have shown that plasma exosomes from young individuals have anti-inflammatory effects in ischemia (Lee et al., 2018). Recent investigations have demonstrated that SH-SY5Y neuroblastoma-derived exosomes regulate genes associated with neuronal function (Huo et al., 2021).

The central nervous system has a natural defense mechanism that responds to several pathogens and to signals of cell damage. However, an inflammatory mechanism is needed to eliminate threats and aid in cell repair (Chen et al., 2018). Cells deprived of oxygen in areas of cerebral ischemia elevate DAMP levels, leading to a severe inflammatory response (Franke et al., 2021). However, uncompressed non-compressive neuritis may lead to further nerve damage and dysfunction (Chen et al., 2018). One of the crucial factors in neuroinflammation that researchers have recently focused on is a complex set of intracellular proteins known as the inflammasome complex, which is activated by several substances (Song et al., 2017). DAMPs in ischemic regions trigger severe inflammatory responses, mediated in part by the inflammasome complex (Feng et al., 2020). The NLRP3 inflammasome is a key component in CNS inflammation. Activation of NLRP3 leads to the conversion of procaspase-1 into caspase-1, which activates pro-interleukin-1β and interleukin-18 and triggers inflammation (Shi et al., 2017). This process activates microglia, causing further brain damage and producing harmful substances, ultimately leading to rapid cell death via pyroptosis (Latz and Xiao 2013; Zhu et al., 2022).

The current study aims to investigate the combined effect of exosomes with glutamic acid on neurological

function using the Garcia score, a neurological test, aiming to explore novel strategies for neuroprotection and functional recovery.

Material and Methods

Grouping

Sixty male Wistar rats (230–300 g) were procured from the Pasteur Institute (Tehran, Iran). Animals were housed in a controlled environment (25 ± 3°C, 35–60% humidity, and a 12-hour light/dark cycle) with unrestricted access to rat chow and water. A power analysis, considering inclusion, exclusion, and mortality rates, determined that 12 rats were needed for each of the five experimental groups. The groups were as follows: (1) ischemia (MCAO) group, (2) vehicle control (vehicle+ MCAO), (3) exosome (E+MCAO), (4) glutamic acid (GLU+MCAO), and (5) combined exosome plus glutamic acid (E+GLU+MCAO) groups. Heart rate and rectal temperature were monitored throughout the MCAO procedure. For groups 3 and 5, 10 µL of vehicle or SH-SY5Y neuroblastoma-derived exosomes were injected into the CA1 region of the hippocampus 24 hours after MCAO. The CA1 region of the hippocampus was selected because it is highly vulnerable to ischemic injury and plays a critical role in synaptic plasticity, learning, and memory. Moreover, CA1 neurons are susceptible to glutamate-mediated excitotoxicity, making this region highly relevant for evaluating neuroprotective and anti-inflammatory interventions following cerebral ischemia (Cavelle and Greenamyre 1995). The stereotaxic coordinates for the CA1 region (AP −1.5 mm, ML +3.2 mm, DV −5.3 mm) were determined using rat brain atlas data. Groups 4 and 5 received an intraperitoneal injection of 10 mg/kg glutamic acid two hours post-MCAO initiation (Cai et al., 2002; Van Hemelrijck et al., 2005).

Development of MCAO Rat Model

The middle cerebral artery occlusion (MCAO) model was established to simulate ischemic stroke. Rats were anesthetized with 2% isoflurane in a 30% oxygen and 68% nitrogen mixture. A midline neck incision was made under a surgical microscope (Olympus SZX12, Germany) to expose the right common carotid artery, external carotid artery, and internal carotid artery. The MCAO procedure was performed according to standardized protocols.

Cell Culture and Flow Cytometry

SH-SY5Y neuroblastoma cell lines procured from Pasteur Cell Bank (Tehran, Iran) were grown in DMEM-F12 at 37°C with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mm l-glutamine. The cell suspension was centrifuged at 250×g for 5 min, washed in 6 ml of DMEM-F12 with 10% FBS, and incubated in a 25-cm^2 plastic flask at 37 °C, 5% CO_2, and 90% humidity. After reaching 80% confluence after the third passage, SH-SY5Y cells were trypsinized, washed, centrifuged, and resuspended in PBS. Flow cytometry (FACSCalibur; BD, Alaska, MN, USA) was used to evaluate SH-SY5Y cells treated with CD9 and CD63 antibodies (Neomarker, CA, USA).

Exosome isolation and verification

Exosomes were extracted from the supernatant using the Exocib kit (Sib Biofan) according to the instructions. The conditioned medium was centrifuged at 3,000 g for 10 minutes to remove cells and debris. The conditioned medium and kit reagents were mixed at a 1:4 ratio. A one-minute vortexed mixture was thereafter incubated at 4°C for 2 hours, followed by centrifugation at 10,000g for 60 minutes to isolate the exosomes. The pellet containing exosomes was subsequently resuspended in 1 mL of PBS and subjected to centrifugation at 3000g for 10 minutes using an Exosome Purification Filter. Preserved the functionality of exosomes; all centrifugation procedures were conducted at 4°C. The exosome weight was quantified using a BCA kit according to the manufacturer's guidelines. exosomes were isolated and stored at -80°C until subsequent analysis (Wei et al., 2019). To investigate the biodistribution of DiI-labeled exosomes in the rat brain, brain sections were stained with DAPI, and pictures were captured using an immunofluorescence microscope.

Garcia test

Neurological deficits were assessed using the Garcia scale seven days after ischemia. The scale, ranging from 3 to 18, evaluates forelimb outstretching, climbing, proprioception, vibrissae touch response, and spontaneous activity. The symmetry of limb movement was also assessed. All tests were conducted in a low-light, quiet environment, and scoring was performed by an observer blinded to group allocation, following the method of

TABLE 1: Real-time PCR primers used in gene expression analysis (F: Forward Primer- R: Reverse Primer)

Gene	Primer
BDNF	*BDNF-F*: GTCCCTTCTACACTTTACCTCT
	BDNF-R: TCTTTCACCCTTTCCACTCC
NMDAR	*NMDAR*-F: GGAGGCAGGTGGAGTTGAG
	NMDAR-R: GGAGTTGAGTATGGACAGGGA
NLRP3	*NLRP3*-F: GGAGTGGATAGGTTTGCTGG
	NLRP3-R: GGTGTAGGGTCTGTTGAGGT
GAPDH	*GAPDH*-F: AGGTCGGTGTGAACGGATTTG
	GAPDH-R: TGTAGACCATGTAGTTGAGGTCA

Garcia et al (Garcia et al., 1995).

Quantification of Infarct Volume

At the end of the experiment, rats were euthanized with deep isoflurane anesthesia, and their brains were rapidly removed. Brains were sectioned into 2-mm coronal slices and stained with 2.0% TTC (Sigma, St. Louis, MO, USA) for 10 minutes at 37°C. Digital images of brain slices were captured with a Canon camera (Japan) and analyzed using ImageJ software (version 6.0). Infarct volume was calculated.

Cresyl violet staining

Paraffin-embedded parietal cortex samples were sectioned into 5-µm coronal slices using a rotary microtome (Licka, USA). Sections were deparaffinized, rehydrated, and stained with 2% cresyl violet. Stained sections were mounted with Entellan adhesive and imaged under a Labomed USA light microscope at 400× magnification. Neuronal cell density, pyknotic nuclei, and Nissl body homogeneity were assessed using ImageJ software.

Gene Expression Analysis

Total RNA was extracted from brain samples using the TriPure isolation reagent (Sigma-Aldrich, Germany). RNA purity and concentration were assessed using a NanoDrop ND-100 spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using the RevertAid cDNA synthesis kit (Fermentas, Germany). Real-time PCR was performed to quantify gene expression levels of BDNF, NLRP3, and NMDAR using SYBR Green chemistry. Primers were designed using GeneRunner software and verified against NCBI BLAST (Table. 1). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Protein Expression Analysis

Immunohistochemistry (IHC) was performed to detect the expression of BDNF, NLRP3, and Caspase-1. Brain sections were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) and blocked with 10% goat serum (Abcam) for 30 minutes. Primary antibodies against BDNF, NLRP3, and Caspase-1 (1:100, Abcam) were incubated overnight at 4°C. Sections were washed and incubated with FITC-conjugated secondary antibodies (1:150, Abcam) at 37°C for 1.5 hours. Nuclei were counterstained with DAPI. Fluorescence images were captured using a Labomed TCS400 microscope.

Pro-inflammatory Cytokines Analysis

Pro-inflammatory cytokines IL-1β and IL-18 were quantified from penumbra tissue using ELISA kits (Shanghai, China). Tissue was homogenized in ice-cold saline (1:10 dilution) and centrifuged at 3000 rpm for 15 minutes at 4°C. Supernatants were analyzed according to the manufacturer's protocol.

Statistical Analysis

Normality was assessed using the Kolmogorov–Smirnov test, while homogeneity of variances was evaluated using Bartlett's test in SPSS version 28.0.0.0. The results confirmed that the data were normally distributed and that the variances were independent. Statistical analysis was conducted using GraphPad Prism (version 8.0). One-way ANOVA followed by Tukey's post hoc test was used for group comparisons. A p-value of less than 0.05 was considered statistically significant.

Results

Characterization of Exosomes

Fig. 1A illustrates that SH-SY5Y neuroblastoma cells

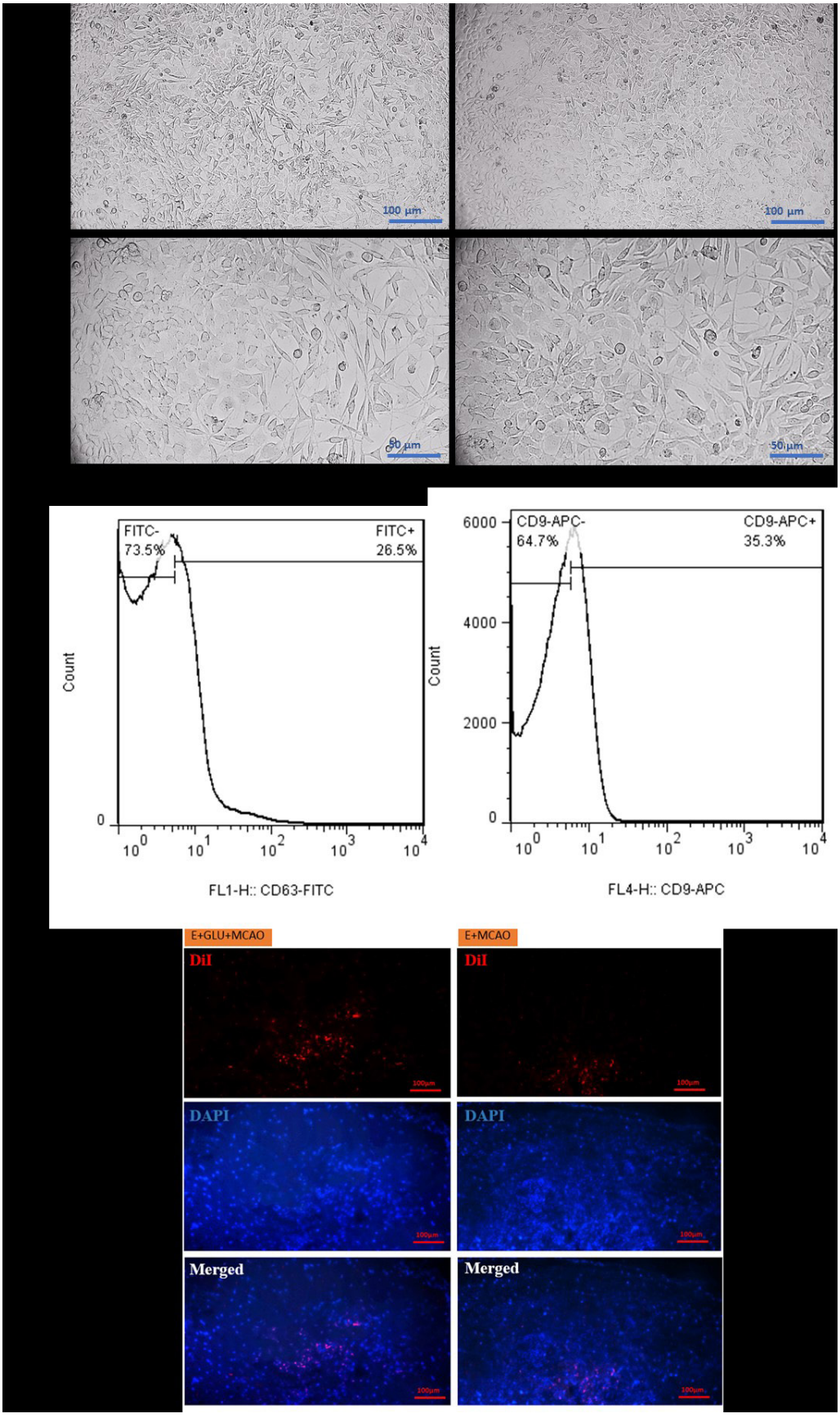

FIGURE 1. Characterization of exosomes derived from SH-SY5Y neuroblastoma cells. (A) An inverted fluo-rescence microscope observed cellular morphology (P1, P3) of SH-SY5Y. (B) Expression level of the surface antigens (CD9 and CD63) in SH-SY5Y cells. (C) The injected exosomes were de-tected in both E+GLU+MCAO group and E+MCAO group.

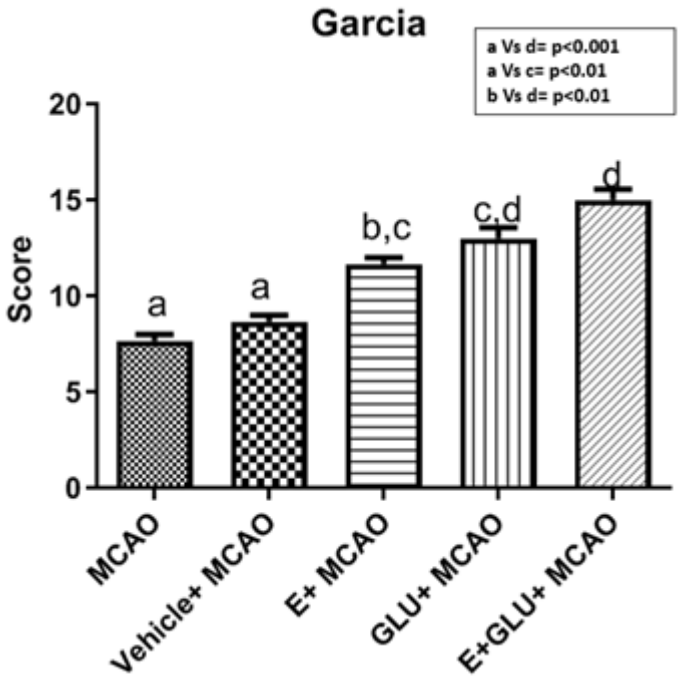

FIGURE 2. Neurological deficits measured 7 days after MCAO induction. Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

at first passage (P1) exhibited spindle-shaped, fusiform, and polygonal forms. However, at the third passage (P3), the cells displayed a consistent spindle-shaped morphology. Based on their spindle-shaped form and plastic adhesion properties, the cells were identified as SH-SY5Y neuroblastoma cells. Flow cytometry analysis revealed that SH-SY5Y cells tested positive for the exosomal markers CD9 and CD63 (Fig. 1B), indicating the successful extraction of exosomes derived from SH-SY5Y neuroblastoma cells.

Behavioral Tests (Garcia Test)

Compared with the MCAO group, treatment with exosomes and glutamic acid (GLU) significantly increased the Garcia score ($p < 0.05$) (Fig. 2).

Cerebral Infarct Volume

Fig. 3 shows the semi-quantitative TTC staining results for total cerebral infarction volume. The E+MCAO, GLU+MCAO, and E+GLU+MCAO treatment groups exhibited a significant reduction in infarct volume compared to the MCAO group, with the E+GLU+MCAO group showing the most pronounced reduction ($p<0.05$). In contrast, no significant differences in brain edema were observed between any of the groups. (Fig. 3C).

Cresyl Violet Staining

Cresyl violet staining revealed that treatment with exosomes, glutamic acid, and a combination of both (E+GLU+MCAO) significantly reduced neuronal cell death compared to the MCAO group ($p<0.05$) (Fig. 4). The E+GLU+MCAO group showed the most pronounced reduction.

NLRP3, NMDAR, and BDNF Gene Expression

MCAO induction in rats resulted in reduced expression of NMDAR and BDNF genes while increasing NLRP3 expression (Fig. 5). The E+GLU and GLU+MCAO groups exhibited significantly lower NLRP3 expression compared to the MCAO group ($p<0.05$). The E+GLU+MCAO group showed the largest reduction in NLRP3 gene expression. Conversely, NMDAR and BDNF expression were significantly upregulated in the E+MCAO and GLU+MCAO groups compared to the MCAO group ($p<0.05$).

NLRP3, BDNF and Caspase-1 Protein Expression

The expression levels of NLRP3 and Caspase-1 pro-

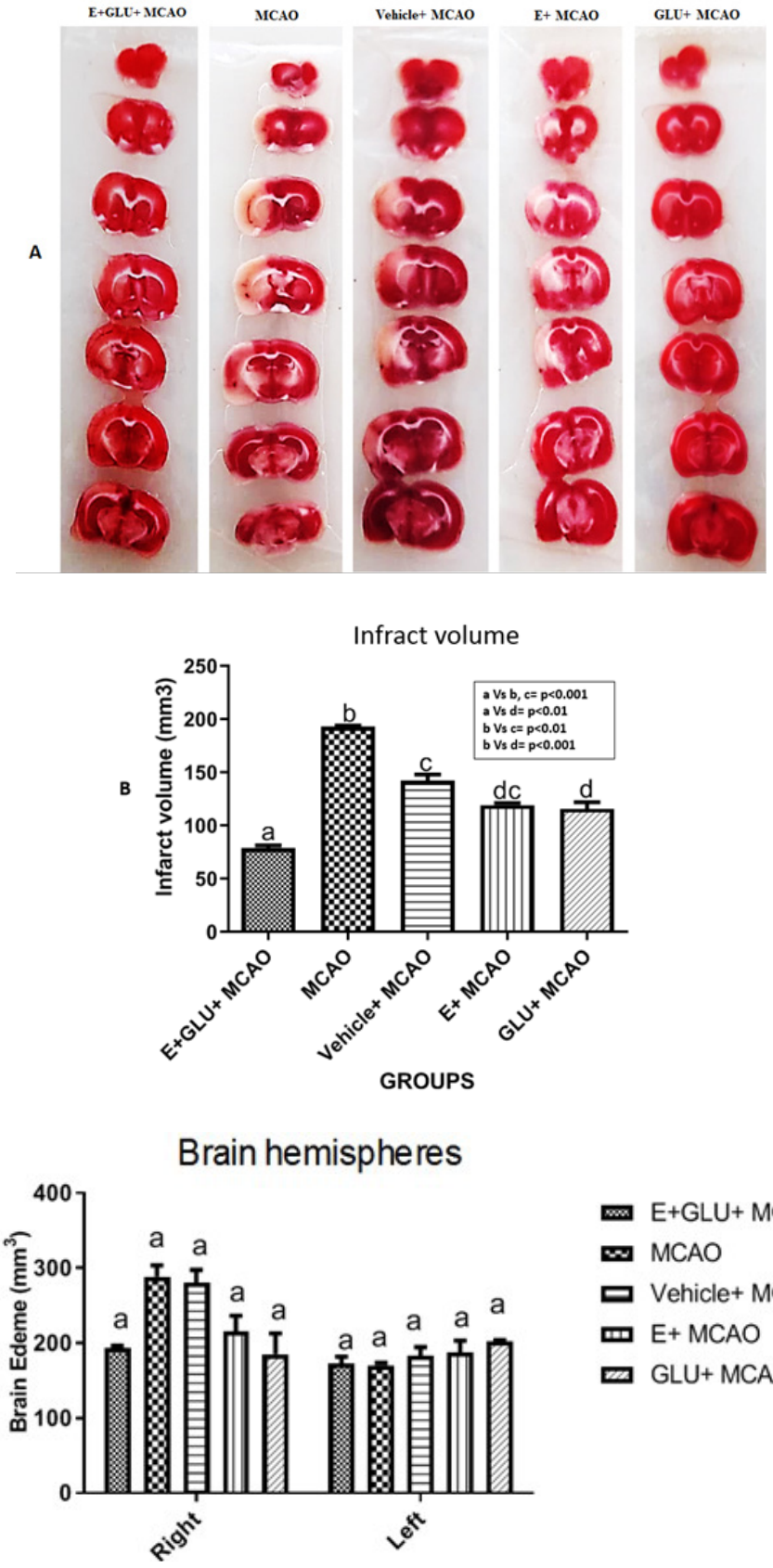

FIGURE 3. The semi-quantitative TTC staining of total cerebral infarction volume. (**A**) Representative pho-tographs illustrating infarct volumes in the different groups following treatments. (**B**) Infarct vol-ume in rats in various groups. (**C**) Brain edema size in the right and left sides of rats' brains. Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

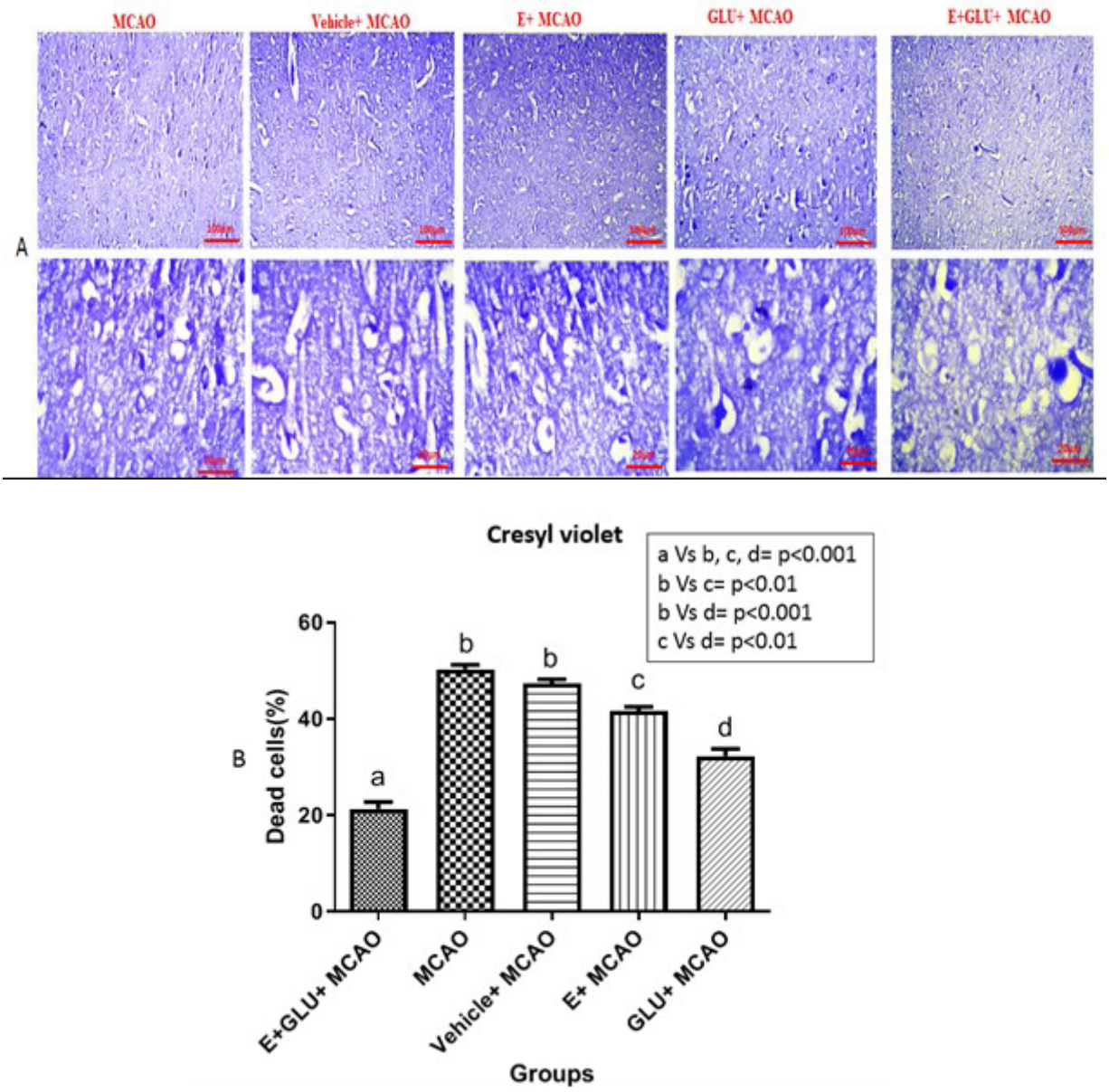

FIGURE 4. (**A**) The cresyl violet staining indicated brain neurodegeneration on MCAO group. (**B**) Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

teins were significantly higher in the MCAO group compared to other groups ($p<0.05$) (Fig. 6A & B). Treatment with exosomes and glutamic acid alone and a combination of both exosomes and glutamic acid and significantly reduced the expression of NLRP3 and Caspase-1 proteins in MCAO rats ($p<0.05$) relative to untreated MCAO rats (Fig. 7A & B). Additionally, BDNF protein expression was significantly lower in the MCAO group than in the other groups ($p < 0.05$). Treatment with exosomes and combined exosome + GLU (E+GLU) significantly increased BDNF protein expression in MCAO rats ($p<0.05$) (Fig. 8A & B).

Expression of Pro-Inflammatory Cytokines (IL-1β and IL-18)

IL-1β and IL-18 levels were significantly higher in the MCAO and MCAO + Vehicle groups compared to treatment groups ($p<0.05$) (Fig. 9). Treatment with exosomes, glutamic acid, and the combination of both significantly reduced the expression of IL-1β and IL-18 in MCAO rats ($p<0.05$).

Discussion

Despite the introduction of several medicines aimed at protecting and regenerating neural tissue post-stroke,

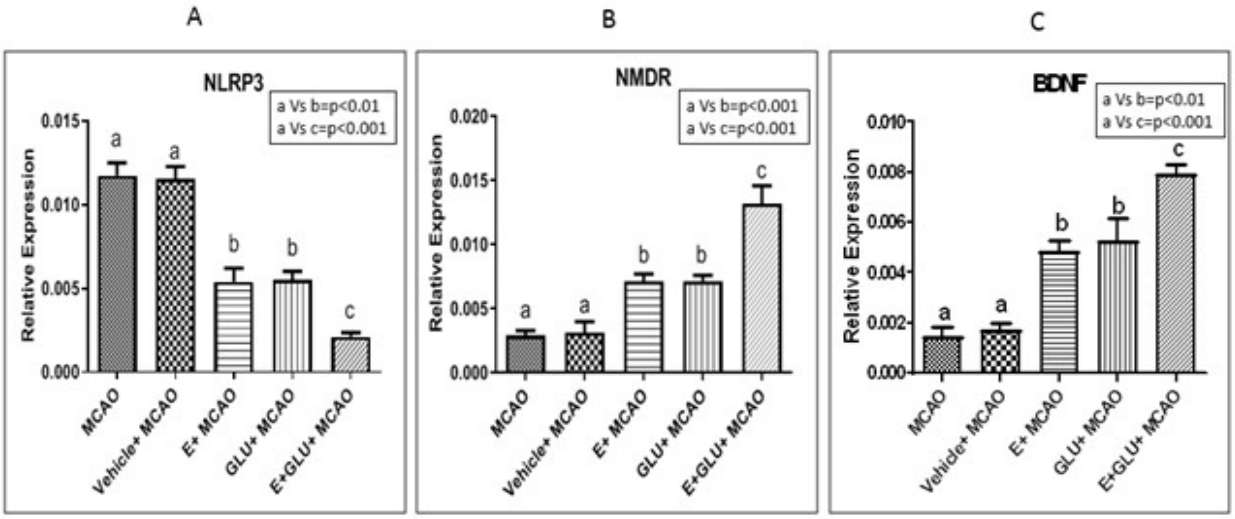

FIGURE 5. Expression levels of the NLRP3, NMDAR and BDNF genes in different groups of study (**A-C**). Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

the enduring long-term impairments resulting from severe side effects and a limited therapeutic window remain an important challenge (Safakheil and Safakheil 2020). This study evaluated the neuroprotective efficacy of a novel combination therapy involving exosome treatment and glutamic acid (E+GLU) in a Middle Cerebral Artery Occlusion (MCAO) model. Our findings demonstrated that the combined therapy elicited greater functional recovery and reduced neuronal damage compared to exosomes alone.

Neuroinflammation is a key contributor to the pathogenesis of cerebral ischemia, leading to progressive neuronal death. While previous studies have examined exosome-based therapies and glutamatergic/NMDA receptor modulation separately, investigations of their combined application, with a focus on inflammasome-related pathways, remain limited. One plausible mechanism for this synergy is glutamic acid's role in enhancing exosome uptake via NMDA receptor (NMDAR)-mediated calcium signaling pathways (Lau and Zukin 2007). Also, glutamate has been suggested to activate pathways that share similarities with exosome uptake (Pocock and Kettenmann 2007). On the other hand, exosomes derived from neural cells contain NMDAR subunits, and their function might increase in glutamate-rich environments (Fauré et al., 2006).

Our results revealed that treatment with exosomes and glutamic acid could enhance the NMDAR in MCAO rats. NMDAR is essential for plasticity and memory formation, indicating that NMDAR stimulation, rather than inhibition, may be helpful in the subacute period following a stroke (Dhawan et al., 2011). Zhen et al. investigated the neuroprotective effects of GLYX-13, a glycine-site partial agonist of NMDAR, using in vitro OGD-induced models and in vivo MCAO models. Their research indicated that different modulations of NMDAR subunit components during the post-ischemia phases resulted in GLYX-13 reducing long-term ischemic potentiation. Mitigating NR2B-containing NMDAR-mediated excitotoxicity may be a potential approach to prevent this phenomenon (Zheng et al., 2017). Many therapeutic attempts using NMDAR antagonists have failed in recent years and, in some cases, have worsened stroke outcomesand sometimes have exacerbated stroke. Recent findings indicate that the continuous decrease in NMDAR concentration after stroke, along with its pivotal role in flexibility and memory structure, suggests that NMDAR stimulation is beneficial. In contrast, inhibition is beneficial in the period after acute stroke (Wu and Tymianski 2018).

The paradox of why an NMDA receptor agonist, such as glutamate, is used instead of an antagonist lies in the precise timing of administration (after stroke or MCAO modeling) and its very low dose. It is thought that early, dose-controlled administration of glutamate may counteract the neurotoxic effects of excitotoxicity by activat-

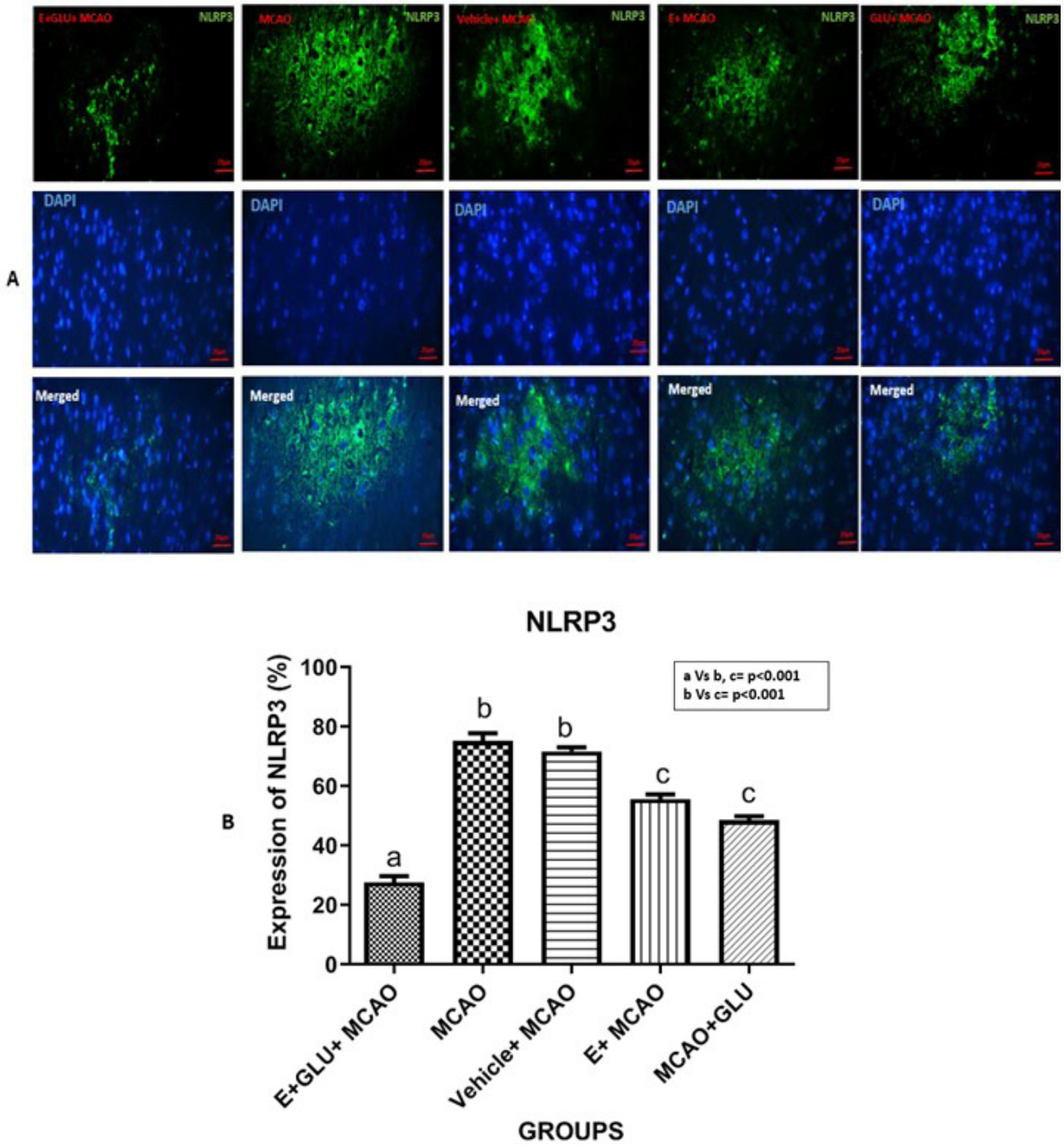

FIGURE 6. Expression levels of NLRP3 protein in different groups of study. (**A**) Fluorescent microscopic picture of NLRP3 protein expression. (**B**) Quantification presentation of NLRP3 protein. Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

ing NMDA-dependent protective signaling pathways (e.g., via specific receptor subunits such as GluN2A) and instead enhance mechanisms of synaptic repair and cell survival. The hypothesis that "the dose makes the poison" is prominent here.

Bordi et al. in the MCAO model of focal ischemia showed the efficacy of the glycine antagonist GV150526 as a neuroprotective. For the first time, this functional and anatomical study showed that GV150526, even when administered six hours after MCAO, has protective effects in focal ischemia. The maintenance of functional responses, as determined by electrophysiological mea-

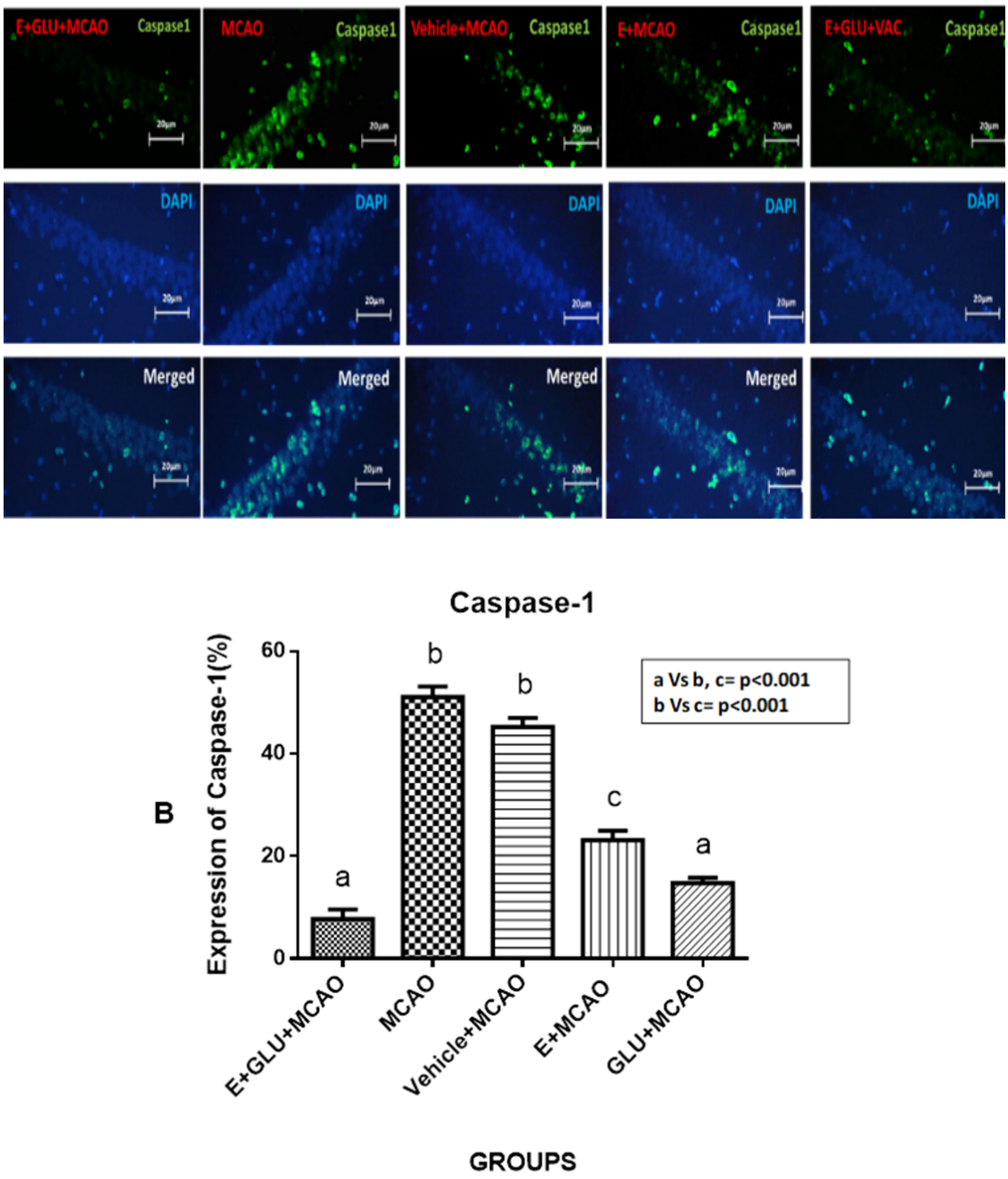

FIGURE 7. Expression levels of Caspase-1 protein in different groups of study. (**A**) Fluorescent microscopic picture of Caspase-1 protein expression. (**B**) Quantification presentation of Caspase-1 protein. Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

surements, is correlated with reduced ischemic damage (Bordi et al., 1997). In focal ischemia models, blocking the NMDA receptor decreases infarct volume, making effective pharmaceutical NMDA receptor blockade the preferred neuroprotective strategy in human stroke studies (Muir and Lees 1995). Noncompetitive open-channel blockers are pharmacologically appealing because they can be focused in ischemic areas where glutamate receptors are most pathologically activated.

According to the Garcia scoring system, GLU, whether administered alone or combined with SH-SY5Y neuroblastoma-derived exosomes, significantly improved sensory and motor functions in rats seven days after MCAO induction. Supporting our findings, Safakheil et al. reported that a combination of exosomes and Rosuvastatin significantly enhanced the Garcia score com-

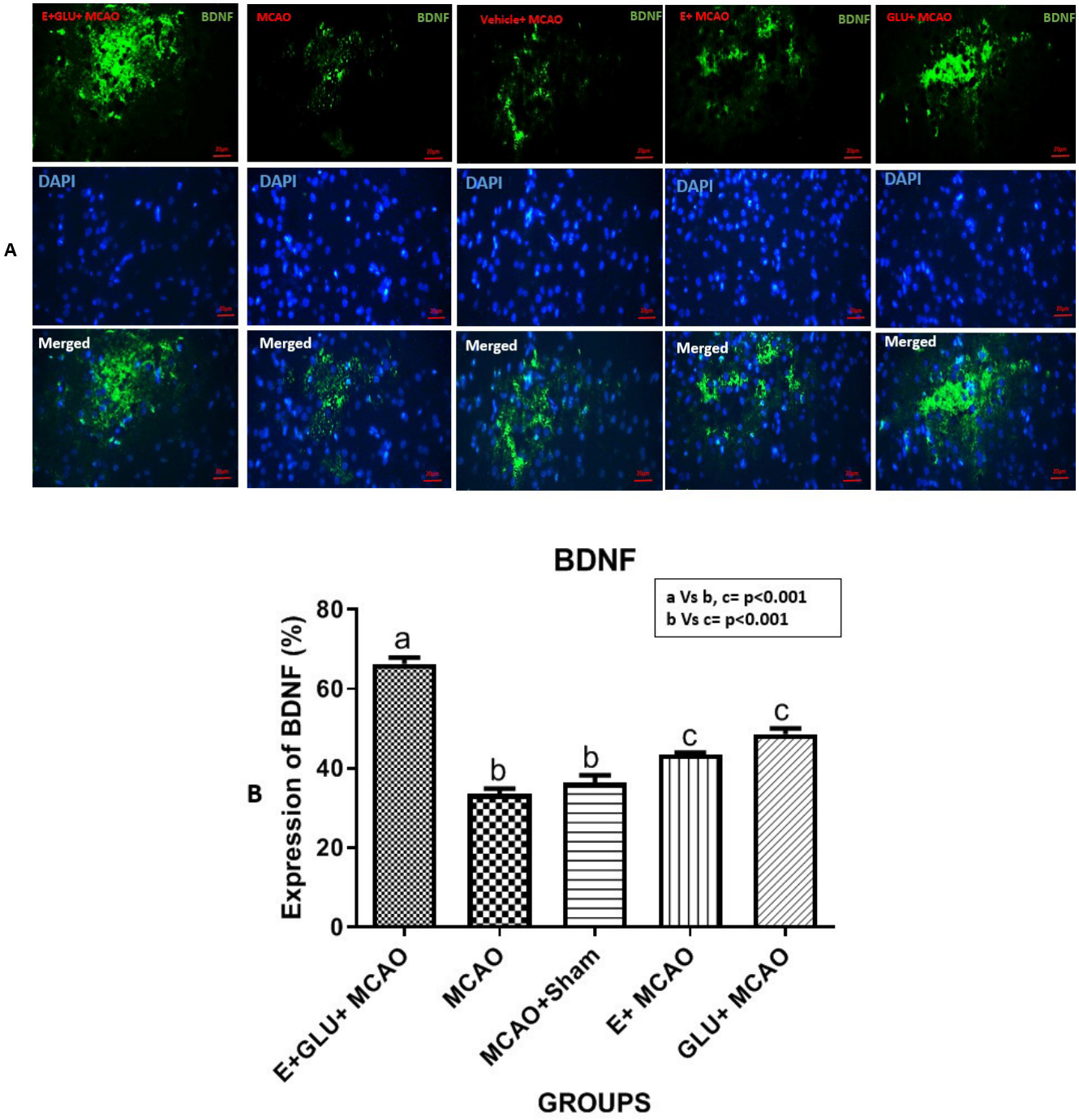

FIGURE 8. Expression levels of BDNF protein in different groups of study. (**A**) Fluorescent microscopic picture of BDNF protein expression. (**B**) Quantification presentation of BDNF protein. Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

pared to the MCAO group (Safakheil and Safakheil 2020).

Cresyl violet staining revealed significantly increased neuronal death in the MCAO group. The number of dead cells decreased substantially following E+ GLU treatment. Therefore, our findings indicate that combining GLU with exosomes derived from SH-SY5Y neuroblastoma cell lines could effectively promote neurogenesis following MCAO in rat brain tissue.

Furthermore, we demonstrated that E+ GLU reduces infarct volume and improves therapeutic outcomes following 7 days of MCAO, but there is no significant

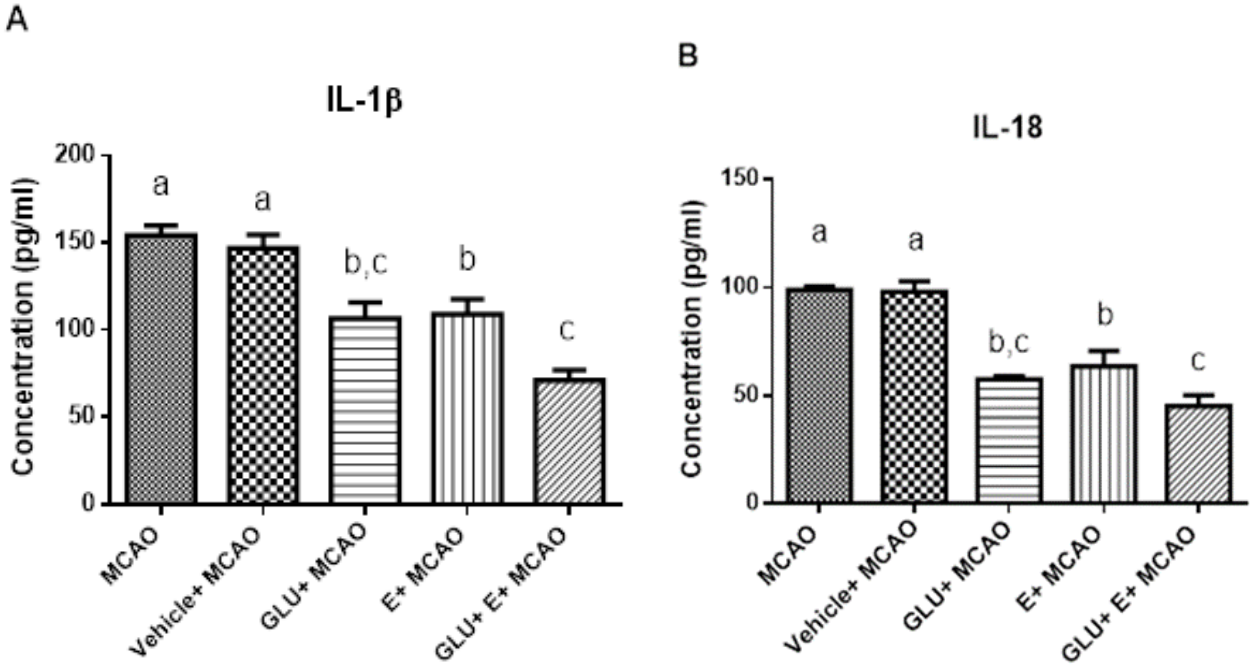

FIGURE 9. The levels of brain IL-1β (**A**) and IL-18 (**B**) in different groups of study. Data were indicated as mean ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$); identical symbols indicate no significant difference.

difference in brain edema. Nazarian et al. show that combining mesenchymal stem cells (MSCs) with modafinil-nanoparticle therapy diminishes cerebral infarct volume and cell death more quickly than either MSCs or modafinil-nanoparticle treatment alone (Nazarian et al., 2020). The NLRP3 inflammasome is significantly regulated by autophagy during ischemic damage, as evidenced by new evidence. Mechanistic studies demonstrated that Resveratrol-loaded nanoparticles reduced the levels of caspase-1, IL-1β, and IL-18 and suppressed the activation of the NLRP3 inflammasome, thereby mitigating cerebral ischemic injury (Liu et al., 2013; Lu et al., 2020). A study on exosomes from bone marrow stem cells combined with Rosuvastatin demonstrated downregulation of inflammasome pathway genes, including NLRP1 and NLRP3, in rats with MCAO, suggesting reduced cell death (Safakheil and Safakheil 2020).

Zhang et al. investigated that stem cell-derived exosomes can significantly ameliorate brain injury caused by cerebral ischemia-reperfusion (Zhang et al., 2021). Another study by Huang et al. indicated that exosomal microRNA derived from mesenchymal stromal cells repaired stroke-induced functional impairments, provided nervous system protection, and promoted MSC differentiation (Huang et al., 2022). Furthermore, a recent study indicated that, after intravenous administration of the generated RGD-EVReN, post-stroke inflammation was suppressed in the ischemic lesion region of the brain (Tian et al., 2021). Our research demonstrated that the expression of NMDAR and BDNF genes decreased significantly after MCAO induction compared with other groups. Recent studies indicated BDNF reduction is associated with delayed neuronal cell death (Salazar-Colocho et al., 2008). Xiaoqiong Liu and colleagues demonstrated that MLNO inhibited AMPK/mTOR in ischemic stroke patients, thereby improving CREB/BDNF-mediated neuroprotection (Liu et al., 2023). On the other hand, NLRP3 gene expression in the MCAO group was significantly increased. However, across treatment groups, a significant decline was observed, with the largest increase in the E+GLU+ MCAO group. In this investigation, we used IHC to examine NLRP3 expression, which was substantially increased in the MCAO group. The E+GLU+MCAO group exhibited the most significant reduction in this protein among the treated groups. Additionally, Liu's research demonstrated that BMSC-Exos reduced brain injury by modulating microglial polarization and inhibiting NLRP3 inflam-

masome-mediated inflammation and pyroptosis (Liu et al., 2021b). Similarly, MCAO groups treated with GLU and E+GLU showed significantly lower Caspase-1 levels than other groups.

One of the main limitations of this study is the use of an animal model (rats), which, although providing valuable information, does not necessarily fully reflect the complex physiological and pathological responses in the human body. Also, the relatively short follow-up period after treatment may not have revealed the long-term effects or possible complications of this combination. In addition, the exact molecular mechanism of interaction between exosomes and glutamic acid, as well as the downstream signaling pathways, remains unclear and warrants further investigation. The lack of investigation into different doses and variable treatment schedules is another limitation that could hinder optimization of the treatment regimen. For future studies, it is suggested that this promising therapeutic combination (SH-SY5Y exosomes with glutamic acid) be investigated in more sophisticated preclinical animal models, such as non-human primates, with long-term follow-up periods to assess its sustained effects and safety. It is also necessary to determine the optimal dose and effective treatment time window and to further investigate the molecular mechanisms using techniques such as proteomics and transcriptomics. Finally, a key step will be to design and conduct phase I clinical studies in patients with ischemic stroke to finally confirm the efficacy and translate this strategy into a practical treatment.

Conclusion

This study suggests that combined treatment with glutamic acid and SH-SY5Y neuroblastoma-derived exosomes may provide greater neuroprotective effects than either treatment alone in a rat model of ischemic stroke. This innovative therapy enhances sensory and motor functions. Moreover, GLU and SH-SY5Y exosomes reduce the extent of brain infarction and the rate of cell death in rats. The medication enhances BDNF gene and protein expression while decreasing NLRP3 gene and protein expression, which may have neuroprotective and immunomodulatory effects, positioning E+-GLU as a novel and effective candidate for post-stroke treatment.

List of Abbreviations

BBB = Blood-Brain Barrier
BDNF = Brain-Derived Neurotrophic Factor
CNS = Central Nervous System
DAMPs = Damage-Associated Molecular Patterns
E = Exosomes (SH-SY5Y Neuroblastoma-derived)
GLU = Glutamic acid
GLYX-13 = NMDAR Glycine Site Partial Agonist
MSCs = Mesenchymal Stem Cells
MCAO = Middle Cerebral Artery Occlusion
NLRP3 = NOD-like receptor protein 3
NMDA = N-Methyl-D-Aspartate
NMDAR = N-Methyl-D-Aspartate Receptor
IHC = Immunohistochemistry
PBS = Phosphate-Buffered Saline
PCR = Polymerase Chain Reaction
rt- PA = Recombinant Tissue Plasminogen Activator
TTC = (2,3,5-Triphenyltetrazolium chloride)

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Human and Animal Rights

No humans were used that are the basis of this study. All the animal experiments were conducted in accordance to the Institutional Animal Care and Use Committee recommendations (Code Number; IR.IAU.K.REC.1400.025).

Authors' Contributions

All authors equally contribute in preparation of this manuscript.

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