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Evaluation of the effects of transplantation of rat umbilical cord matrix stem cells (RUCMSCs) on ischemic tolerance in MCAO stroke model

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Abstract

Objective: Ischemic stroke is a major cause of mortality and long-term disability worldwide, and effective therapeutic options remain limited. Rat umbilical cord matrix stem cells (RUCMSCs) have demonstrated significant neuroprotective potential by secreting trophic and regenerative factors. This study evaluated the protective effects of RUCMSC transplantation in a rat model of focal cerebral ischemia.

Method: Transient middle cerebral artery occlusion (MCAO) was induced in adult male Wistar rats. Animals were assigned to sham, MCAO, and RUCMSC-treated groups. RUCMSCs were stereotactically transplanted into the right striatum. Neurological deficits, infarct volume, blood–brain barrier (BBB) permeability, cerebral edema, antioxidant enzyme activities (SOD and GSSG), and the expression of GRIN1 and NOS1 genes were assessed 24 h after reperfusion.

Results: RUCMSC transplantation significantly reduced infarct volume, cerebral edema, and BBB disruption compared with untreated MCAO animals. Treatment also enhanced SOD and GSSG activities while significantly downregulating GRIN1 and NOS1 expression in ischemic brain regions. These effects were associated with improved neurological scores in the acute phase after ischemia.

Discussions: RUCMSC transplantation attenuates ischemic brain injury through antioxidant, anti-inflammatory, and neuroprotective mechanisms. The observed reduction in tissue damage and

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preservation of neurological function suggests that RUCMSCs may represent a promising cell-based therapeutic strategy for ischemic stroke.

Keywords*:* Ischemic Stroke; Mesenchymal Stem Cell Therapy; MCAO; Neuroprotection; Oxidative Stress, Cerebral Edema

1. Introduction

Stroke is one of the leading causes of death and long-term disability worldwide, imposing a substantial socioeconomic burden on healthcare systems[1]. Approximately 80–85% of all strokes are ischemic in origin and result from an interruption of cerebral blood flow, leading to neuronal death, blood–brain barrier (BBB) disruption, cerebral edema, oxidative stress, and neuroinflammation[2]. Despite significant advances in understanding stroke pathophysiology, therapeutic options remain limited, and the clinical benefits of thrombolytic therapy are restricted by a narrow treatment window and potential adverse effects[3]. Given the vast body of knowledge in stroke pathophysiology over the last 25 years, the only treatment remains tissue plasminogen activator (tPA). Therefore, only 5-10% of patients with ischemic stroke can benefit from it due to the time limit of using the drug and side effects[4].

One of the recent methods for reducing stroke damage is cell therapy. The aim of this treatment is to preserve cells in the penumbra and reduce the effects of brain tissue injury[5]. Mesenchymal stem cells (MSCs) have demonstrated considerable therapeutic potential owing to their immunomodulatory properties, secretion of trophic factors, stimulation of angiogenesis, and ability to enhance endogenous repair mechanisms. Among the various MSC sources, umbilical cord–derived mesenchymal stem cells are particularly attractive because of their high proliferative capacity, low immunogenicity, ease of isolation, and potent paracrine activity[6]. In 1988, the results of the first studies on stem cell-based stroke treatment were published. These studies and subsequent studies showed that stem cell transplantation will have positive effects on the healing process of neurodegenerative diseases such as Alzheimer's, ALS, Huntington's syndrome, and stroke[7].

Previous studies have shown that umbilical cord-derived MSCs improve neurological outcomes and reduce infarct size in experimental models of ischemic stroke. These beneficial effects have been attributed to the release of neurotrophic and growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and glial cell line-derived neurotrophic factor (GDNF). Furthermore, MSCs may attenuate oxidative stress and inflammatory responses, thereby preserving BBB integrity and limiting secondary brain injury[8, 9].

In addition, oxidative stress and glutamate-mediated excitotoxicity are among the principal mechanisms responsible for neuronal injury following cerebral ischemia and reperfusion. Cerebral ischemia leads to excessive extracellular glutamate accumulation and overactivation of N-methyl-D-aspartate (NMDA) receptors, resulting in calcium overload, mitochondrial dysfunction, and neuronal death. The GRIN1 subunit, an essential component of the NMDA receptor complex, has been implicated in the amplification of excitotoxic signaling during cerebral ischemia[10]. In parallel, ischemic injury enhances neuronal nitric oxide synthase (NOS1)

activity, leading to excessive nitric oxide production and subsequent formation of reactive nitrogen species that contribute to oxidative damage and blood–brain barrier disruption[11]. Moreover, the excessive generation of reactive oxygen species overwhelms endogenous antioxidant defenses, including superoxide dismutase (SOD) and glutathione-dependent systems, thereby exacerbating neuronal injury and infarct progression[12]. Therefore, therapeutic interventions capable of modulating GRIN1- and NOS1-mediated pathways while enhancing antioxidant capacity may provide effective neuroprotection following ischemic stroke.

Although the therapeutic effects of several mesenchymal stem cell populations have been investigated in ischemic stroke, the neuroprotective potential of rat umbilical cord matrix stem cells (RUCMSCs) and their effects on oxidative stress, BBB integrity, cerebral edema, and GRIN1/NOS1 gene expression remain insufficiently characterized.

Therefore, the present study aimed to evaluate the effects of intracerebral transplantation of RUCMSCs in a rat model of middle cerebral artery occlusion (MCAO) and to determine whether these cells could reduce ischemic brain injury through modulation of oxidative stress, inflammatory signaling, blood–brain barrier permeability, and neurological dysfunction. Therefore, the present study aimed to evaluate the effects of intracerebral transplantation of RUCMSCs in a rat model of middle cerebral artery occlusion (MCAO) and to determine whether these cells could reduce ischemic brain injury through modulation of oxidative stress, inflammatory signaling, blood–brain barrier permeability, and neurological dysfunction.

2. Materials and Methods

Animals and maintenance conditions

Adult male Wistar rats weighing between 250 and 350 grams were purchased from the Pasteur research production complex in Iran and kept under standard conditions (22 ± 2°C, 12-hour light/dark cycle starting at 7 am). Animals had free access to water and food throughout the experiment. In this research, an attempt has been made to use at least rats. All the experimental steps were carried out according to the protocol approved by the Ethics Committee of Islamic Azad University. This research was done experimentally. Before starting the experiments, one week time was allocated for the adaptation of the animals to the laboratory environment.

Isolation and culture of rat umbilical cord mesenchymal stem cells RUCMSCs

Immediately after delivery, rat umbilical cords were transferred to the cell culture laboratory under sterile conditions. The amniotic membrane was carefully removed, and the Wharton’s jelly tissue was dissected into small fragments under a laminar flow hood. After washing with antibiotic-containing culture medium, the tissue samples were cultured using the explant method. Briefly, the tissue fragments were placed in cell culture flasks and allowed to adhere to the flask surface before the addition of DMEM/F12 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

The cultures were maintained under standard incubation conditions (37°C in a humidified atmosphere containing 5% CO_2). After approximately two weeks, the tissue explants were removed, and the adherent cells were further cultured in complete medium. Upon reaching an appropriate level of confluence, the cells were detached using trypsin and subcultured into new flasks for expansion.

Because additional phenotypic characterization assays were not performed in the present study, the identification of RUCMSCs was based primarily on their adherence to plastic surfaces and their characteristic spindle-shaped morphology. The absence of immunophenotypic characterization represents a limitation of the current study.

Prepare cells for injection

To inject cells, RUCMSCs were incubated with Hoechst solution for 5 minutes after reaching the appropriate density, then trypsinised, centrifuged, and the supernatant removed. The cells were counted with trypan bluedye, and half a million cells were prepared for injection.

Stereotaxic surgery

In this research, we were required to perform stereotaxic surgery to inject cells into the right striatum. After anaesthetizing, the animal's head was placed in a stereotaxic machine (Stoelting, USA), and its hair was shaved, and its head was fixed by a stereotaxic machine so that surgery could be performed. Then the head area was disinfected, and a longitudinal incision was made on the scalp with a scalpel blade. After that, the subcutaneous tissues are removed to expose the skull bone. Then, by using the coordinates of the right striatum of the brain obtained from the Paxinus atlas, the desired location was marked (Bregma=0.96, Lateral=3mm, DV=5mm)[12]

The cells were injected into the right striatum at the coordinates described above. After that, the injection site was sutured, and the animals were transferred to the animal room after recovery.

Creating MCAO stroke model

At first, the animal was anesthetized with 400 mg/kg chloral hydrate intraperitoneally [13]. A 5-cm-long 0-3 nylon suture, whose tip was pre-rounded with a flame and marked with dye at the 2-cm mark, was passed through the incision site on the common carotid, then with the help of pliers, the thread was moved forward until there was resistance to the passing of the thread, which is actually the place where the branch of the middle cerebral artery (MCA) separates from the circle of Willis. The thread was left in place for 60 minutes, then slowly pulled back and removed. The thread entry site, where a thread had already been placed in the upper part of the nylon thread entry into the vessel, was blocked. After removing the thread, blood flow to the right hemisphere was restored through the loop of Willis[14]

It should be noted that the 24-hour time point was selected to evaluate the acute

neuroprotective effects of RUCMSC transplantation on oxidative stress, blood–brain barrier disruption, and early ischemic injury.

Neurological Deficit Survey (NDS)

Scoring of neurological deficits 24 hours after MCAO was performed by professional observers who were blinded to the details of animal grouping (blinded procedure), scoring neurological deficits by referring to the grading scales reported by Chen et al[15]. (0: normal; 1-6: slight damage; 7-12: moderate damage; 13-18: severe damage; 18: complete loss of nerve function).

Assessment of stroke volume (IV)

24 hours after reperfusion, the rats were killed with a high dose of anesthetic and their brains were removed and kept in a refrigerator for 10 minutes in cold physiological serum at 4°C. The brain was then removed from the refrigerator and placed in the brain matrix. A slice of the brain was made in the coronal section, from the frontal to the temporal side, with a thickness of two millimetres. The slices were placed in the Petri dish from the frontal to the temporal side. A 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) was poured onto the slices, which were incubated for 15 minutes in a Bain-Marie at 37°C for staining (21). After 15 minutes, the white color indicates the ischemic areas and the red or pink color indicates the healthy areas. Then photography was done with a Nikon digital camera. Finally, the ischemic area on each slice was measured using the ImageJ program (version 1.46r). In this way, the total volume of brain damage was calculated. Damage to the cortical areas, the piriform cortex-amygdala (Pir-Amy), and the striatum was also measured separately and calculated using the following formula. Finally, the stroke volume of each part was analyzed separately.Corrected volume of the affected area = volume of the left hemisphere – (volume of the right hemisphere – volume of the affected area). Identification of the brain areas of the cortex, parietal cortex-amygdala and striatum was done by Paxinus atlas and with the help of the brain matrix[16].

Cerebral water content measurement method

One of the common methods to evaluate the amount of brain edema is measuring the wet and dry weight of brain samples. After killing the animal and removing the brain, the olfactory bulb, cerebellum and medulla were separated from the brain. Then the right and left hemispheres were separated from the parietal groove by a razor. The brain areas of the cortex, parietal cortex-amygdala and striatum of the right hemisphere were separated. They were weighed by a digital scale and the weight of each brain (WW) was recorded. Then the tissues were placed inside the incubator at 120°C for 24 hours to dry the brain. After 24 hours, the different areas were weighed again and the dry weight (DW) was recorded. Finally, brain water content was calculated based on the formula ([(WW-DW)/WW] ×100)[17]

Measuring the permeability of the blood-brain barrier (BBB)

The strength of the blood-brain barrier is evaluated by measuring the amount of Evans Blue (EB) release. In order to measure the permeability of the blood-brain barrier half an hour after the induction of ischemia, Evans Blue 2% aqueous solution was injected at the rate of 0.4 ml per 100 grams of body weight through the tail vein. 24 hours after reperfusion, the anesthetized animal's chest was opened. Transcardial procedure was performed. Then the brain was removed. The brain hemispheres were separated, and the cortical areas, parietal cortex-amygdala, and striatum of the right hemisphere were dissected. Each one was weighed separately and transferred into a tube. First, the weight of each area is multiplied by 4, and the result is cc of phosphate buffer solution (PBS), with pH = 7.4, added to each tube and stirred for 10 minutes using a stirrer; until the texture is uniform. In the next step, the result of multiplying the weight of the brain regions by 4, in the form of 60% trichloroacetic acid, was added to each microtube to precipitate the proteins, and the mixture was stirred for 10 minutes with a stirrer. The microtubes were vortexed for 2 minutes and then refrigerated at 4°C for 30 minutes. In the next step, the samples were centrifuged at 1000 rpm for 30 minutes. Then, 0.50 cc from each tube containing the brain area was transferred into the blot. The blot is placed inside the ELISA processor. The optical absorbance of the brain solution was then measured at 610 nm. It was compared with the standard concentration curve and the concentration of Evans Blue was calculated per gram of tissue.

Examining the activity of GSSG and SOD enzymes

The brain hemispheres were separated and the brain areas of the cortex, parietal cortex-amygdala and striatum of the right hemisphere were separated. Each one was weighed separately and transferred into a tube. GSSG and SOD activity were determined using GSSG (Cat.no. NS-15033) and SOD (Cat.no. NS-15032) detection kits (Nund Salamat, Urmia, Iran), according to the manufacturer's advices[18].

Examining the expression of inflammatory genes

The brain hemispheres were separated and the brain areas of the cortex, parietal cortex-amygdala and striatum of the right hemisphere were separated on ice. Total RNA was extracted from brain tissues according to the manufacturer's instructions (Pars Tous, Tehran, Iran, Cat. A101231) and then converted to cDNA with a cDNA synthesis kit (Pars Tous, cDNA EasyTM, Cat. no. A101161). The PCR reaction was performed using the (2X) SYBR® Green Master Mix kit (Pars Tous, Tehran, Iran, Cat. A101022) in duplicate through the Step One plus device, Applied Biosystems). The Primer Quest™ tool was used for primer design and the primers are (Table #1). The ΔΔCT method was used to calculate the outcomes, and the β-actin gene was used as a housekeeping control to normalize the data.

Table 1. *sequence of primers*

Genes	Sequences		(bp)	Melting (°C)
GRIN1	Forward	5′-CCAG ATGTC CACC AGAC TAAA G-3′	137	62
	Reverse	5′-CCGTA CAGA TCACC TTCTT CAC-3′		
NOS1	Forward	5′-CAAC AGCG TCTCC TCCTA TT-3′	212	
	Reverse	5′-CCCTC ATCTT CAGA ATCCT CTC-3′		
β-Actin	Forward	5′-CAACT GGGA CGAT ATGG AGAA G-3′	206	
	Reverse	5′-CAGA GGCA TACA GGGA CAAC-3′		

Statistical analysis

Data analysis was performed using GraphPad Prism (version 8). Data obtained from NDS were analyzed by Kruskal-Wallis parametric test and Dunn's post hoc test. Infarct volume, BBB permeability, cerebral edema, antioxidant enzyme activity, and gene expression data were analyzed using one-way ANOVA followed by Tukey's multiple-comparison test. The outcomes were reported as Mean ± SEM. The significance criterion for each group was P< 0.05.

3. Results

The effect of RUCMSCs cell transplantation on the neurological deficits of NDS

Neurological examination was performed 24 hours after reperfusion. The scores obtained from all behavioral tests of the sham group compared to the MCAO surgery group also had a significant decrease, which was a completely expected result (P<0.0001). Because in the sham group, no ischemic injury occurred, as a result, behaviours that indicate neurological defects will not appear. The RUCMSCs rat umbilical cord stem cell transplant recipient group showed reduced neurological deficits compared to the MCAO ischemia group, but the difference was not significant (P=0.05). This result is the result of the analysis of the total scores of the behavioral tests in the experimental groups. Also, the scores of the tests were analyzed separately in the experimental groups and are given in Figure 1.

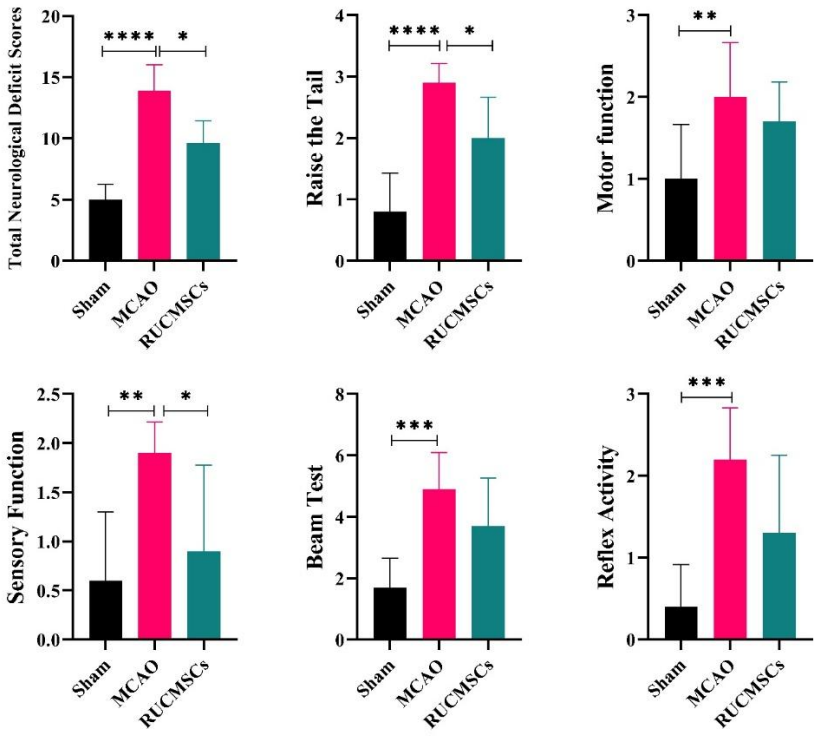

Figure 1. *The effect of RUCMSCs cell transplantation on NDS in the sham, MCAO and RUCMSCs groups 24 hours after MCAO surgery. Each column represents Mean ± SEM. (n=10). (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001) (Kruskal-Wallis nonparametric test).*

The effect of RUCMSCs cell transplantation on stroke volume IV

The effect of RUCMSCs cell transplantation on tissue damage was measured twenty-four hours after reperfusion. The red areas of the brain were positive for the healthy area and the white areas were positive for the damaged area and infarction (Figure 2).

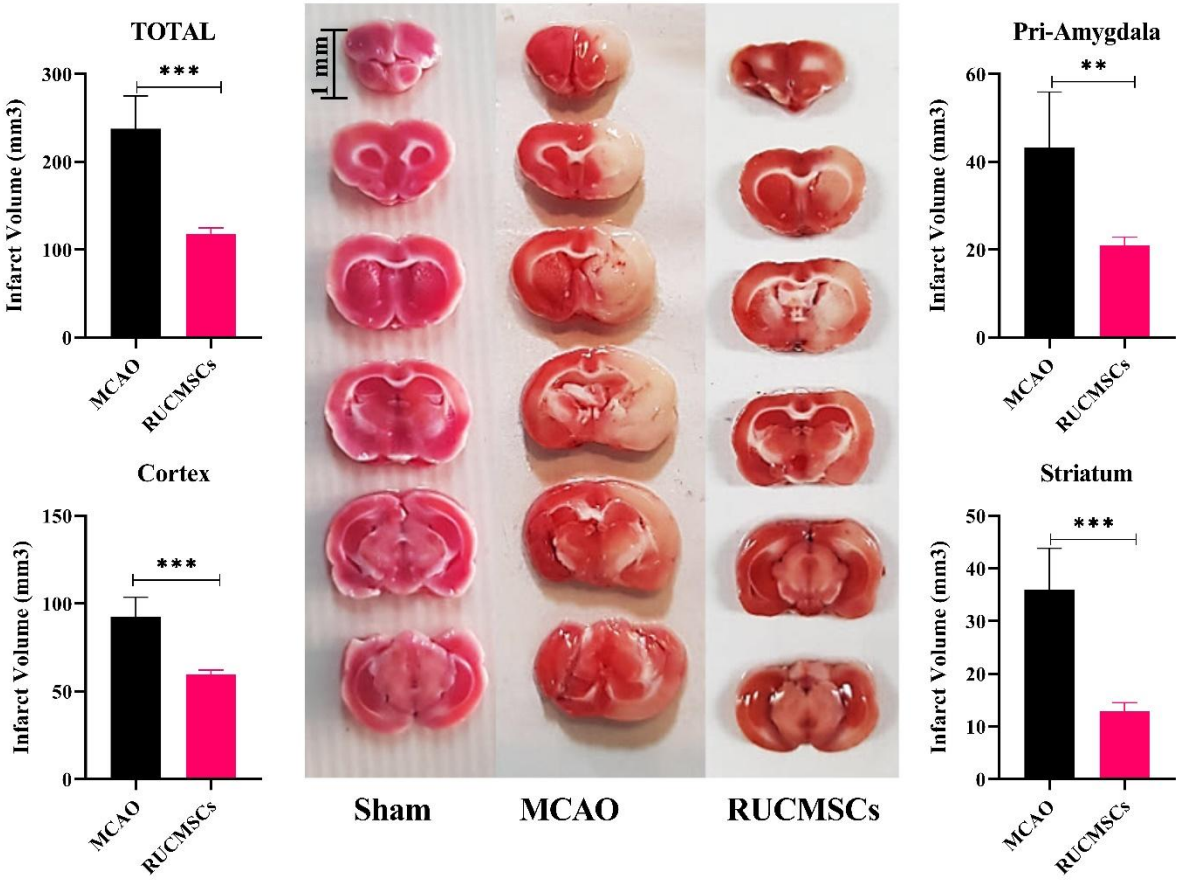

Figure 2. *Effect of RUCMSCs cell transplantation on IV 24 hours after MCAO surgery. Each column represents Mean ± SEM. Staining of brain tissue slides in sham, MCAO, and RUCMSCs groups, characterized by redness in healthy areas and whiteness in infarcted areas. (n=6). (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001) (t test).*

Stroke volume was measured in three parts: total volume, cortex, parietal cortex-amygdala and striatum. In the MCAO group, the total volume of tissue damage was 237.72±12.325 mm. The results show that the use of RUCMSCs, rat umbilical cord mesenchymal stem cells, caused a significant reduction in the total volume of brain damage (118.21±2.372 mm) (P<0.0006). In addition, the recipient group of rat umbilical cord mesenchymal cells, RUCMSCs (P = 0.0006, 31.63±16.31 mm3), significantly increased the infarct volume in the cerebral cortex compared to the cerebral cortex of the MCAO group (48±4.351 mm). /92) decreased. A significant decrease was also seen in the stroke volume by mesenchymal cells of the umbilical cord RUCMSCs in the striatum (P=0.008, 20.96±0.377 mm) compared to the striatum area of the MCAO group

(43.29±5.312 mm). In the preform-amygdala cortex region, the group receiving umbilical cord mesenchymal cells RUCMSCs reduced stroke volume (P=0.0004, 12.84±0.368 mm) compared to the preform-amygdala cortex region of the MCAO group (3.319 mm ±35.98).

The effect of RUCMSCs cell transplantation on brain edema

In the study of brain water content 24 hours after induction of cerebral ischemia, the weights of the cortex, preform-amygdala cortex, and right striatum were measured separately using the formula described in the materials and methods chapter. The results of the comparison between the cortex area of the experimental groups showed that the rat umbilical cord mesenchymal cell transplant recipient group RUCMSCs (P = 0.0003, 79.01 ± 0.83%) caused a significant decrease in cerebral edema compared to the MCAO group (0.42%). ± 83.62). In the statistical comparison between the parietal cortex-amygdala region of the experimental groups, the rat umbilical cord mesenchymal cell transplant recipient group RUCMSCs (P = 0.004, 80.52 ± 0.65%) significantly reduced cerebral edema compared to the MCAO group (18 83.41 ± 0.0%). Also, the group of mesenchymal cells of rat umbilical cord RUCMSCs (78.65 ± 0.84%) significantly reduced the cerebrospinal fluid content in the striatum compared to the striatum of the MCAO group (83.29 ± 0.46%) (03.03). 0=P) (Figure 3).

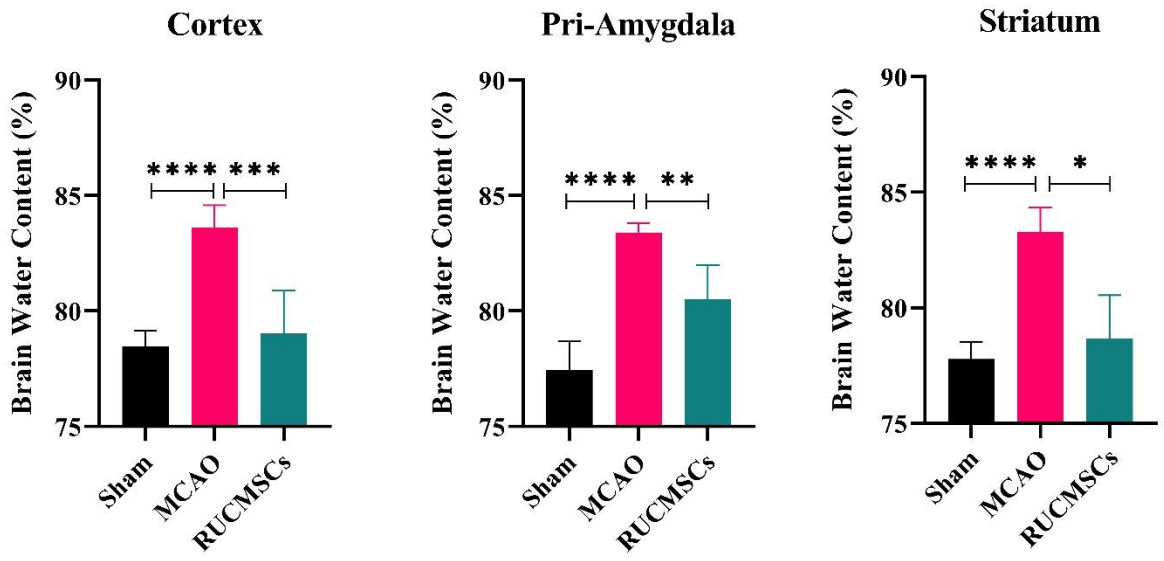

Figure 3. *The effect of RUCMSCs cell transplantation on cerebral edema 24 hours after MCAO surgery. Each column indicates Mean ± SEM. (n=5). (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001) (One-way ANOVA test)*

The effect of RUCMSCs cell transplantation on the strength of the BBB

Observing a high amount of Evans Blue in the brain tissue 24 hours after the induction of cerebral ischemia confirms the breaking of the blood-brain barrier and the release of this colored substance from the cerebral vessels. In the statistical comparison between the parietal cortex-amygdala region of the experimental groups, the rat umbilical cord mesenchymal cell transplant recipient group (RUCMSCs; P = 0.03, 0.23 ± 0.006) showed a significant decrease in Evans Blue concentration compared to the MCAO group (04 was 0.39 ± 0.00). Also, the rat umbilical cord mesenchymal cells group of RUCMSCs (0.27 ± 0.03) significantly reduced the concentration of Evans blue in the striatum area compared to the striatum area of the MCAO group (0.58 ± 0.03) (0.01 P = 0) (Figure 4).

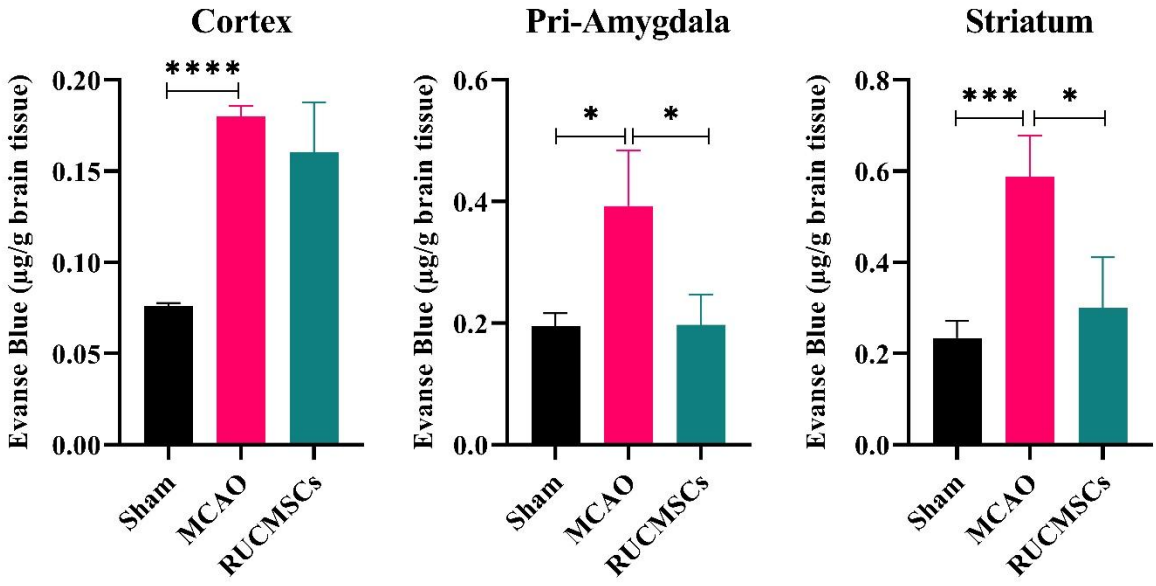

Figure 4. *The effect of RUCMSCs cell transplantation on BBB 24 hours after MCAO surgery. Each column indicates Mean ± SEM. (n=5). (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001) (One-way ANOVA test).*

The effect of transplanting RUCMSCs cells on the activity of GSSG and SOD enzymes

The results of the comparison of the cortical area between the experimental groups showed that the group receiving RUCMSC cells (P=0.0001, 1.9±0.14) showed a significant increase in GSSG enzyme activity compared to the MCAO ischemia group (49±0.09). /0) became in the statistical comparison between the prefrontal cortex-amygdala region of the experimental groups, the group receiving RUCMSCs cells (P=0.0001, 1.54±0.11) caused a significant increase in the enzymatic activity of GSSG enzyme compared to the MCAO ischemia group (0.2 was 0.23 ± 0). Also, the group receiving RUCMSC cells (P=0.0001, 1.01±0.01) showed significantly increased GSSG enzyme activity in the striatum compared to the MCAO ischemia group (0.21±0.03). increased (P=0.01). The results of the comparison between the cortex area of the experimental

groups showed that the RUCMSCs transplant group (P = 0.0001) caused a significant increase in SOD enzyme activity in the RUCMSCs group (P = 0.0001, 54.54 ± 0.3) compared to the MCAO ischemia group (3.5 ± 5.27). 363) became. In the statistical comparison of the prefrontal cortex-amygdala region between the experimental groups, the RUCMSCs cell transplant group (495.99±2.41, P=0.0001) showed a significant increase in SOD enzyme activity compared to the MCAO ischemia group (2.29). ± 328.09). Also, the transplant group of RUCMSCs cells (504.37 ± 0.79) significantly increased the enzyme activity of SOD in the striatum area compared to the striatum area of the MCAO ischemia group (328.53 ± 1.92) (= 0.0001). P) (Figure 5).

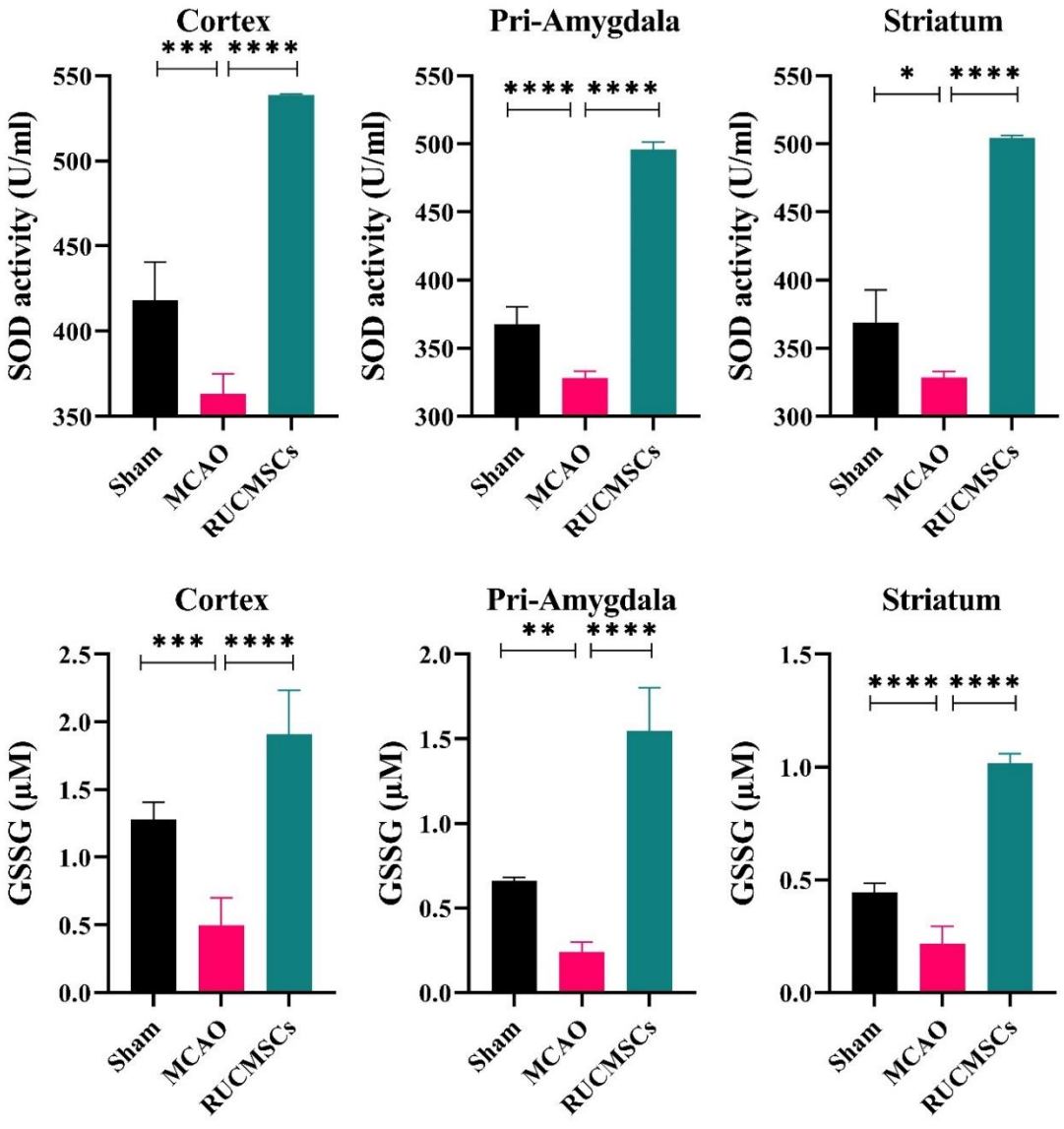

Figure 5. *The effect of RUCMSCs cell transplantation on the activity of GSSG and SOD enzymes 24 hours after MCAO surgery. Each column indicates Mean ± SEM. (n=5). (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001) (One-way ANOVA test).*

The effect of RUCMSCs cell transplantation on the expression of GRIN1 and NOS1

genes In examining the results of the expression level of GRIN1 and NOS1 genes 24 hours after the induction of cerebral ischemia, measuring the expression level of GRIN1 and NOS1 genes in the cortex, preform cortex-amygdala and right striatum areas separately according to the formula mentioned in the materials chapter and The methods were performed and the results showed that cerebral ischemia significantly increased the expression of GRIN1 and NOS1 genes in the ischemic hemisphere, which indicates brain inflammation in the ischemic hemispheres following MCAO surgery. The results of the comparison of the cortical area between the experimental groups showed that the group receiving RUCMSC cells (P = 0.01, 1.01 ± 0.29) showed a significant decrease in GRIN1 gene expression compared to the MCAO ischemia group (08 ± 0.69). /3) became in the statistical comparison between the prefrontal cortex-amygdala region of the experimental groups, the group receiving RUCMSCs cells (0.65±0.05, P=0.02) caused a significant decrease in the level of GRIN1 gene expression compared to the MCAO ischemia group (61. was 2.2 ± 0). Also, the group receiving RUCMSC cells (0.36 ± 0.03, P < 0.002) showed significantly higher GRIN1 gene expression in the striatum than the MCAO ischemia group (1.77 ± 0.18). The comparison of cortical areas between the experimental groups showed that the group receiving RUCMSC cells (0.76 ± 0.01, P = 0.007) had a significant decrease in NOS1 gene expression compared with the MCAO ischemia group (83 ± 0.34). /1) became in the statistical comparison between the preform cortex-amygdala region of the experimental groups, the group receiving RUCMSCs cells (0.93±0.05, P=0.003) caused a significant decrease in the level of NOS1 gene expression compared to the MCAO ischemia group (51. was 2.73 ± 0). Also, the group receiving RUCMSC cells (0.93 ± 0.05, P = 0.008) significantly increased NOS1 gene expression in the striatum compared to the MCAO ischemia group (2.73 ± 0.51). decreased (Figure 6).

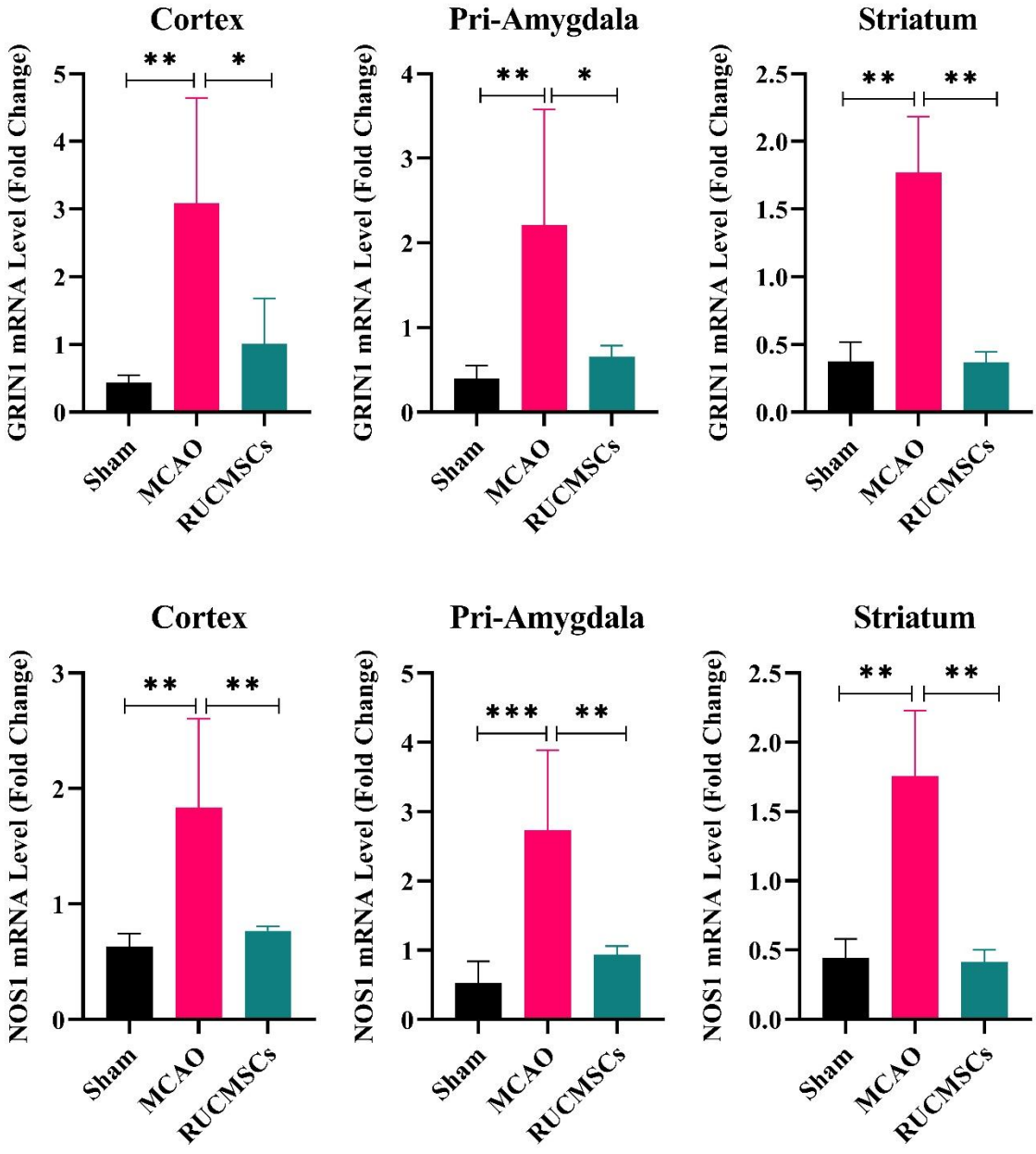

Figure 6. *The effect of RUCMSCs cell transplantation on the expression of GRIN1 and NOS1 genes 24 hours after MCAO surgery. Each column indicates Mean ± SEM. (n=5). (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001) (One-way ANOVA test).*

4. Discussion

The present study demonstrated that RUCMSC transplantation was associated with reduced infarct volume, attenuation of cerebral edema and blood–brain barrier disruption, enhancement of antioxidant defences, and altered expression of GRIN1 and NOS1 in the acute phase following cerebral ischemia. Although these findings support a neuroprotective role for RUCMSCs, the underlying mechanisms remain incompletely understood and require further investigation. Antioxidant enzymes act as the first line of defence against the damage caused by free radicals in the body[19]. SOD enzyme catalyses a series of reactions related to the conversion of superoxide to oxygen and hydrogen peroxide[20]. In the continuation of glutathione peroxidase, Glutathione reductase (GR) catalyses the reduction of GSSG back to reduced glutathione (GSH). GSSG is conserved in most organisms and they all have one gene for this enzyme[21, 22]. The outcomes of the present study show that the transplantation of rat umbilical cord mesenchymal cells RUCMSCs significantly increased the expression of antioxidant-related genes such as GSSG and SOD.

Also, although GRIN1 and NOS1 are generally associated with excitotoxicity and oxidative injury, moderate upregulation may also reflect adaptive neuronal signalling, synaptic plasticity, or tissue remodeling during recovery following ischemic injury[23].. While glutamate binding is necessary for normal physiological functions, including neuronal plasticity, excessive, long-term activity leads to neuronal death. Glutamate binding to Glutamate Ionotropic Receptor NMDA Type Subunit 1 (GRIN1) increases calcium influx into the cell. The high concentration of cellular calcium triggers the activation of phospholipases and proteases, which, in turn, break down the cell membrane and essential proteins that maintain the cell structure[24] . Also glutamate receptors initiate sodium/chlorine flow in the cell, which further leads to cell swelling and cell edema. The role of glutamate metabolic receptors in ischemia-induced cell death has also been proven [25, 26]

During the reperfusion phase, the production of free radicals, such as superoxide and hydroxyl radicals, increases significantly, directly damaging DNA, proteins, lipids, and carbohydrates. Morever, the activation of NOS1 during ischemia increases nitric oxide production. The produced nitric oxide reacts with superoxide to form the powerful oxidant peroxynitrite, which intensifies tissue damage. Free radicals (FR), ROS, and nitrite (RNS) are known reactants in brain damage [27, 28]. Free radicals facilitate the functional destruction of the mitochondrial inner membrane and the oxidation of proteins involved in the electron transport chain, thereby disrupting ATP production[27]. As a result, the permeability of the mitochondrial membrane increases and causes mitochondrial swelling and the production of more free radicals. Due to the abundance of unsaturated fats in the cell membrane, the production of fatty acid peroxidation products increases due to the presence of high amounts of free radicals. These products are effective on the health of the cell membrane, and cell signalling pathways[28] . On the other hand, these cellular radicals play a significant role in initiating cellular inflammatory signals under physiological conditions.

The outcomes of the present study show that the transplantation of rat umbilical cord mesenchymal cells RUCMSCs significantly improved neurological defects. The scoring of neurological defects was done based on sensory-motor behavior assessment tests. The outcomes

demonstrate the positive role of umbilical cord mesenchymal cells and their neuroprotective effects on sensory and motor control areas of the brain. In this regard, past studies demonstrate the undeniable effects of umbilical cord mesenchymal cell secretions, including transforming growth factor (TGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and derived neurotrophic factors. of glial cells (GDNF) on reducing the scores of neurological defects[29, 30]. In a study it was shown thatthe injection of IGF at a certain dose improved neurological deficits in an animal model of stroke[29]. Bi-Chen and Olkan et al. , also demonstrated the protective effect of GDNF on motor function deficits in the stroke model[31, 32]. In addition, consistent with previous reports, growth factors such as FGF, VEGF, and TGF-β have been shown to facilitate neuroprotection and functional recovery after ischemic stroke[33-35].

In this study, it was observed that the volume of the stroke in the cortex, prefrontal cortex of the amygdala and striatum, which is actually the transplant site of mesenchymal cells of the rat umbilical cord RUCMSCs, was significantly reduced compared to the MCAO control group. This shows the proper functioning of RUCMSCs cells by creating favorable conditions for the survival of neurons, preventing cell death in the penumbra area and then reducing the infarct volume. The effects observed in the present study can be partially attributed to the factors secreted from stem cells, including growth and neurotrophic factors. Growth factors are especially important in applying the effects of stem cells. Growth factors enable the growth of cells and their survival, and protect the brain from damage resulting from ischemia[36]

The neuroprotective effects observed following RUCMSC transplantation may be partly attributed to the secretion of trophic and angiogenic factors. Previous studies have demonstrated that growth factors such as TGF-β, GDNF, FGF, and VEGF promote neuronal survival, enhance angiogenesis, improve cerebral perfusion, and attenuate ischemic brain injury. Through these paracrine mechanisms, mesenchymal stem cells can support tissue repair and functional recovery, which may, at least in part, explain the reduction in infarct volume observed in the present study [6, 37, 38].Mesenchymal cells of the umbilical cord can also play a role in maintaining BBB integrity by releasing growth and neurotrophic factors, stimulating antioxidant enzymes and promoting angiogenesis. Of course, it is worth mentioning that on the other hand, the growth factors secreted from mesenchymal cells of the umbilical cord, such as IGF, TGF, FGF and VEGF, which are the cause of the relative breakdown of the blood-brain barrier, can also to some extent They prevent the useful function of antioxidants in reducing damage and destruction of the blood-brain barrier in the acute stage of ischemia[39, 40]. Of course, it is important to note that the receptors for some of these cytokine factors, such as FGF, IGF and VEGF, are dose-dependent, and they exhibit different responses based on the amount of these substances in the environment and the timing of stimulation. A high dose of VEGF in the acute stage of ischemia causes damage to the blood-brain barrier, and in low doses it causes optimal angiogenesis[40, 41]. However, the interaction of positive and negative factors that ultimately strengthen the blood-brain barrier can be investigated as suggestions for future studies.

The present study demonstrated a significant reduction in brain water content in the cortex, striatum, and piriform cortex–amygdala regions of RUCMSC-treated animals compared with the MCAO group. This finding is consistent with the observed preservation of blood–brain barrier

integrity, as BBB disruption is a major contributor to edema formation following cerebral ischemia. Reduced BBB permeability likely limited fluid extravasation into the brain parenchyma, thereby attenuating vasogenic edema and secondary tissue injury. Consequently, the reduction in cerebral edema may represent an additional mechanism through which RUCMSCs exert their neuroprotective effects after ischemic stroke[42-44]. (53,54,55). Since in this study, this pathophysiological outcome was measured 24 hours after ischemia, it is possible that the observed edema is of acytotoxic type. Anyway, with the transplantation of RUCMSCs cells, we saw the reduction of edema in the recipient groups. Edema is a dangerous clinical complication as a result of stroke[45]. In fact, following the breakdown of the blood-brain barrier during ischemia, plasma proteins enter the brain tissue, increasing its concentration and drawing water into the tissue by osmosis. The pressure caused by swelling can cause serious damage to the brain[46, 47].In the meantime, substances such as the growth factors defined in this research, which are secreted by the mesenchymal cells of the umbilical cord, GDNF factor is one of the factors known to inhibit brain edema. This factor indirectly reduces the destruction of the blood-brain barrier and thus edema by inhibiting the caspase activation pathway and inflammatory factors[48, 49].

Several limitations of the present study should be considered when interpreting the findings. First, all analyses were performed 24 h after reperfusion; therefore, the observed effects primarily reflect the acute neuroprotective responses of RUCMSC transplantation rather than long-term tissue repair. Consequently, the effects of RUCMSCs on neurogenesis, angiogenesis, tissue remodelling, and sustained functional recovery remain to be elucidated. In addition, the present study evaluated GRIN1 and NOS1 only at the transcriptional level, and further protein-based and mechanistic studies are required to confirm the involvement of these signaling pathways in the protective effects of RUCMSCs.

5. Conclusion

RUCMSC transplantation significantly attenuated ischemic brain injury in a rat model of focal cerebral ischemia. Treatment reduced infarct volume, blood–brain barrier permeability, and cerebral edema, while enhancing antioxidant defenses and improving neurological outcomes. These effects were accompanied by downregulation of GRIN1 and NOS1 expression, suggesting modulation of excitotoxic and nitrosative stress pathways. Collectively, the findings indicate that RUCMSCs exert multifaceted neuroprotective effects and these findings provide preliminary evidence supporting the therapeutic potential of RUCMSCs in experimental ischemic stroke. Nevertheless, further mechanistic studies and long-term preclinical investigations are required to confirm their efficacy and translational potential.

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