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Effects and mechanisms of long-acting glucagon-like peptide-1 receptor agonist semaglutide on microglia phenotypic transformation and neuroinflammation after cerebral ischemia/reperfusion in rats

Rulin Mi[1], Huifeng Cheng[2], Rui Chen[1], Bo Bai[1], An Li[1], Fankai Gao[1], Guofang Xue[1]

Abstract:

BACKGROUND: The optimal method for addressing cerebral ischemic stroke involves promptly restoring blood supply. However, cerebral ischemia-reperfusion injury (CIRI) is an unavoidable consequence of this event. Neuroinflammation is deemed the primary mechanism of CIRI, with various activation phenotypes of microglia playing a pivotal role. Research has demonstrated that long-lasting agonists of the glucagon-like peptide-1 receptor can suppress neuroinflammation and microglial activation.

METHODS: A transient middle cerebral artery occlusion (tMCAO) rat model was established to investigate the effects of semaglutide. Neurological impairments were evaluated utilizing modified neurological severity score on days 1, 3, and 7 postinterventions. Brains were stained with 2,3,5-Triphenyltetrazolium Chloride to determine infarct volume. To assess the expression of various microglia activation phenotypes and neuroinflammatory biomarkers, we utilized immunohistochemistry and immunoblotting.

RESULTS: The study demonstrated that semaglutide in the tMCAO model could decrease neurological deficit scores and reduce the size of cerebral infarcts. In addition, we observed low levels of cluster of differentiation 68 (CD68, an indicator of M1 microglial activation) and tumor necrosis factor alpha (a pro-inflammatory mediator). Moreover, the results indicated a rise in the levels of CD206 (an indicator of M2 activation) and transforming growth factor beta (an anti-inflammatory mediator), while simultaneously reducing P65 levels in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling cascade.

CONCLUSION: In the CIRI model, semaglutide exhibits notable neuroprotective effects on rats, reducing neuroinflammation through the regulation of microglia phenotype transformation and inhibition of NF-κB activation.

Keywords:

Cerebral ischemia-reperfusion, glucagon-like peptide-1, M1/M2 microglia, neuroinflammation, nuclear factor kappa-light-chain-enhancer of activated B-cell signaling pathway

[1]Department of Neurology, Second Hospital, Shanxi Medical University, Taiyuan, Shanxi Province, [2]Department of Geriatric Medicine, Southern University of Science and Technology Hospital, Shenzhen, China

Address for correspondence:
Prof. Guofang Xue, Department of Neurology, Second Hospital, Shanxi Medical University, No. 382, Wuyi Road, Taiyuan 030001, Shanxi Province, China.
E-mail: xueguofangty@163.com

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Introduction

Currently, strokes afflict 80 million individuals globally, placing them second in terms of prevalent fatalities and the primary contributor to disability.[1,2] In China, approximately 2.4 million new stroke cases emerge each year, with the majority of patients experiencing some degree of neurological impairment. This

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imposes a substantial burden on individuals, their families, and society as a whole.[3] There are two primary approaches to treating ischemic stroke (IS): conventional pharmacological management (such as antiplatelet drugs, improving circulation, and stabilizing blood pressure) and revascularization treatment (comprising intravenous clot dissolution and mechanical thrombus removal). While revascularization treatment restores nutrient and oxygen supply and promotes cellular metabolism, it also results in the accumulation of harmful substances such as oxygen-free radicals and excitatory amino acids. This accumulation may lead to apoptosis and neuronal necrosis, a phenomenon known as "ischemia-reperfusion injury (IRI)."[4-7] Therefore, there is a pressing need to investigate therapeutic targets and develop novel neuroprotective therapies for cerebral IRI (CIRI) to enhance prognoses.

Microglia, the immunoreactive cells of the central nervous system (CNS), serve as the primary defense against harmful stimuli.[8,9] The inflammatory response plays a crucial role in the pathology of stroke, and the proliferation and activation of microglia are key aspects of this process. Neuroinflammation is an immune response activated by glial cells in the CNS, which typically arises in response to stimuli such as neural injury, infection, toxin, or autoimmune processes. While a moderate inflammatory response can be protective, excessive inflammation may lead to tissue damage and disease pathology.[10,11] During the pathological progression, microglia rapidly migrate to sites of tissue damage, acting as a "double-edged sword." On the one hand, they clear debris and secrete anti-inflammatory factors that suppress inflammation and promote nerve cell repair. On the other hand, they release inflammatory mediators that can exacerbate tissue damage. The dual role of microglia is largely determined by their activation phenotypes: the pro-inflammatory M1 and the anti-inflammatory M2.[12]

The inflammatory responses of microglia, which include both the pro-inflammatory M1 and anti-inflammatory M2 phenotypes, coexist and interact throughout disease progression. This balance effectively regulates the mechanisms of disease-related injury, thereby influencing the prognosis of IS.[13-15] However, the dominance of the M1 or M2-like phenotypes of microglia varies at different stages of pathology, with some degree of interconversion observed. Research indicates that activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling cascade promotes the polarization of microglia into the M1-like phenotype, leading to the continuous secretion of inflammatory mediators by these M1 microglia. These mediators further activate NF-κB, perpetuating inflammatory cascades and ultimately resulting in cell death.[16-18] Therefore, inducing microglia to shift towards the M2-like phenotype or preventing their conversion to the M1-like phenotype after IS presents a promising new avenue for stroke therapy research.

On ingestion of food, intestinal endocrine cells produce glucagon-like peptide-1 (GLP-1), which interacts with the GLP-1 receptor (GLP-1R) on pancreatic β-cells, stimulating insulin release. However, due to rapid inactivation by dipeptidyl peptidase IV, these molecules have a short duration of activity.[19-21] Currently, long-acting GLP-1R agonists, which are resistant to proteases, are used clinically for the treatment of diabetes and obesity, and they are also recommended for managing cardiovascular disease.[20,22] Incretins from peripheral sources can cross the blood-brain barrier, and their receptors are widely distributed throughout the CNS, including regions such as the cerebral cortex, thalamus, and hypothalamus.[21,23,24] Exendin-4 (Ex-4), a GLP-1R agonist, has been used in the management of type 2 diabetes. Mice pretreated with Ex-4 before middle cerebral artery occlusion (MCAO) showed improved brain function and reduced infarct volume, indicating potential neuroprotective effects.[25] In animal models of various neurological conditions, liraglutide, a long-acting GLP-1 analog, has demonstrated neuroprotective benefits.[24,26-29] Semaglutide, a drug that modifies antiprotease activity, prolongs the survival of the peptide in the bloodstream.[30] Weekly subcutaneous injections of semaglutide have been successful in treating diabetes. Research suggests that semaglutide may induce neuroprotection by inhibiting microglial activation.[19] However, whether semaglutide can modulate microglial polarization and inhibit the neuroinflammatory response in a rat model of CIRI remains unexplored.

This study aimed to evaluate the potential neuroprotective effects of semaglutide by examining its capacity to inhibit the expression of M1 phenotypes and/or facilitate the emergence of M2 phenotypes in microglia, with the objective of mitigating neuroinflammatory responses. We assessed neurological deficit scores, cerebral infarct volume, various activation phenotypic markers of microglia, and biomarkers of neuroinflammation (Cluster of Differentiation 68 [CD68], CD206, tumor necrosis factor alpha [TNF-α], transforming growth factor beta [TGF-β]), as well as the NF-κB signaling cascade-related protein p65 in the transient MCAO rat model. Semaglutide, administered at effective doses previously demonstrated to have neuroprotective effects, was tested on rats with permanent cerebral ischemia (pMCAO) and on mice with Parkinson's disease (PD) in our experiments.

Materials and Methods

Animals

Male SD rats, healthy and adult, weighing between 280 and 300 g, were obtained from Beijing Changyang Xishan Farm in Beijing, China. The rats were housed in an environment

with 50%–55% humidity, a temperature maintained at 22°C ± 3°C, and a 12-h light/dark cycle. Blood glucose levels were measured via the tail vein after an 8-h fasting period.

All animal procedures were conducted in accordance with the guidelines set forth by the National Institutes of Health (NIH) (NIH Publication No. 85–23, revised 1985) and were approved by the Ethics Committee of Shanxi Medical University. We made every effort to minimize rat distress and reduce the number of rats used in the experiments.

Peptide

Semaglutide, produced by Chinapeptides Ltd. in Shanghai, China, has a purity of 97% and contains 0.9% NaCl. Its molecular weight is 3765, as determined by mass spectrometry and high-performance liquid chromatography. The peptide sequence of semaglutide is as follows:[19]

H**X**EGTFTSDVSSYLEGQAAKN6-(N-(17-carboxy-1-oxoheptadecyl)-L-gamma-glutamyl-2-(2-(2-aminoethoxy) ethoxy) acetyl-2-(2-(2-aminoethoxy) ethoxy) acetyl) EFI-AWLVRGRG-OH where X represents aminoisobutyric acid.

The cerebral ischemia-reperfusion model

A total of 126 rats were randomly divided into three groups. Group 1 underwent sham surgery, while Groups 2 and 3 underwent MCAO surgery. The treatment protocol for each group was as follows: Group 1 received sham treatment; Group 2 (I/R) was administered saline in the same volume; Group 3 (Sema + I/R) received semaglutide at a dosage of 10 nmol/kg through intraperitoneal injection (IpI) every other day. Additionally, rats in Groups 2 and 3 were further subdivided into three subgroups, each corresponding to different time intervals (1 day, 3 days, and 7 days).

To establish the MCAO model, significant modifications were made to the tether approach. Prior to surgery, the animals were fasted overnight. Anesthesia was induced by IpI of 5% chloral hydrate at a dose of 5 ml/kg. A heat lamp was used to maintain body temperature at 37.0 ± 0.5°C. The animals were positioned in a supine posture, and a central incision was made in the neck to expose the right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA). Each blood vessel was carefully isolated. A rounded-tip monofilament nylon thread was inserted into the ECA and advanced into the ICA until it reached the middle cerebral artery to induce blockage. Rats with successful MCAO models were re-anesthetized 2 h later, and the filament was withdrawn approximately 8–10 mm to establish a reperfusion model. Animals were euthanized at 1-, 3-, and 7-days post-reperfusion. Figure 1 illustrates the experimental procedure.

The postoperative rats were scored for neurological deficits according to the Longa 5-grade 4-point scale. The scoring criteria were as follows: 0 points: normal activity without any motor disorder; 1 point: when lifting the tail, the left forelimb was flexed and could still walk in a straight line; 2 points: without any interference, it turned to the left side and couldn't walk in a straight line; 3 points: it involuntarily tilted to the left side when walking; 4 points: it lay on its left side and had no activity. Those with scores of 1-3 were assessed as successful modeling and included in the study, and were randomly divided into the cerebral ischemia/reperfusion model group and the semaglutide intervention group.

Blood glucose assessment

Blood glucose levels were assessed by extracting blood from the tail veins of the rats. Before euthanasia, blood glucose concentrations were monitored using a Sannuo blood glucose meter, purchased from Sinocare Inc. (China). Blood glucose levels were measured in mmol/L.

Modified neurological severity score study

A Modified neurological severity score (mNSS) was used to evaluate the extent of neurological impairment. Neurological functions, including motor skills, sensory perception, reflexes, and balance, were assessed in rats

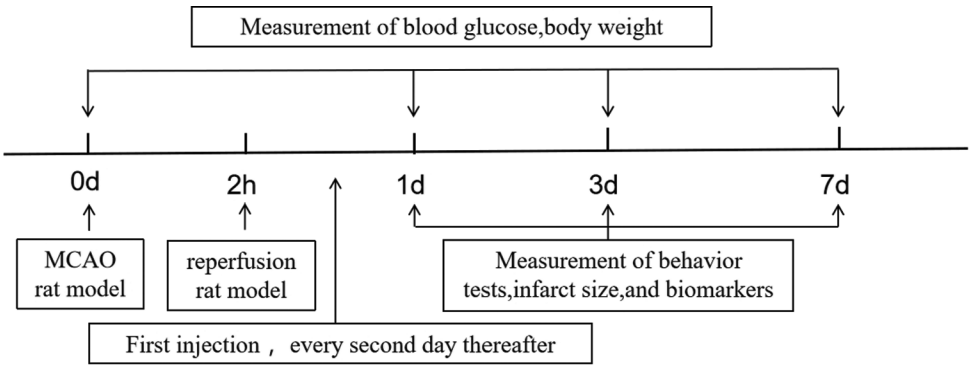

Figure 1: A schematic illustration of the research plan

using the mNSS at 1, 3, and 7 days following successful modeling. The mNSS scale ranged from 0 (normal) to 18 (maximum deficit), with higher scores indicating more severe neurological deficits.

Evaluation of the size of cerebral infarction

Following the neurological function assessments, the rats were euthanized, and their brains were extracted for infarction measurement. The brains were quickly removed and cooled in saline at −20°C for 15 min. Subsequently, the cerebellum, lower brainstem, and olfactory bulbs were removed. The brain was then sectioned into 2-mm-thick coronal slices using a brain matrix. The slices were stained for 30 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, USA), with rotation every 8 min. After staining, the tissue slices were immersed in 4% paraformaldehyde (PFA) for 3–5 h to enhance chromogenic development. Infarcted tissue appeared white, while healthy tissue appeared red. The infarct area and total size of the brain slices were measured using ImageJ software, with images captured by a digital camera. The percentage of infarcted area was then calculated.

Biomarker study using Western blot

Rats were anesthetized with IpI of 5% chloral hydrate (5 ml/kg). Following anesthesia, their blood was flushed out via cardiac perfusion with 0.9% cold NaCl. Samples from the ischemic penumbra of the cerebral cortex were then homogenized using RIPA lysis buffer from China Beyotime. The buffer contained 150 mM NaCl, 50 mM Tris (pH 7.4), 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 2 mM sodium pyrophosphate, 0.5 μg/ml leupeptin, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS. After homogenization, samples were centrifuged at 4°C for 10 minutes at 12,000 rpm (11,200× *g*) using an Eppendorf 5810R centrifuge (Germany). The supernatant was collected and stored at −80°C for further analysis. Protein concentration in the supernatant was determined using the BCA Protein Detection Kit from Boster (China). Protein samples were mixed with an equal volume of 5× loading buffer and denatured in hot water for 10 min. Twenty microgram of total protein was loaded into each lane and separated using SDS-PAGE (Boster) for 2 h at 80 V. After separation, proteins were transferred to a PVDF membrane and incubated at 200 mA for 1 h. To block nonspecific binding, the membrane was incubated with 5% skim milk for 1 h at 37°C. Membranes were then incubated overnight at 4°C with the following primary antibodies: rabbit anti-rat P65 (1:1000), β-Actin (1:5000), TGF-β (1:1000), CD68 (1:1000), CD206 (1:1000), and TNF-α (1:1000), all purchased from Bioworld Technology (China). Following three washes of 10 min each, the membrane was incubated with goat anti-rabbit IgG peroxidase (1:3000, Boster) for two hours at room temperature. Protein bands were detected using an enhanced chemiluminescence substrate from Boster, and further analysis was conducted with Image Lab 3.0.

Immunohistochemistry

For anesthesia, rats received an IpI of 5% chloral hydrate at a dose of 5 ml/kg. Following anesthesia, blood was flushed out by cardiac perfusion with 0.9% cold NaCl and 4% PFA. The brain was then isolated and fixed in 4% PFA at 4°C for 12 h. After fixation, the tissue was embedded in paraffin using an automated tissue processor (Lycra, Germany) following dehydration in a gradient alcohol bath. Coronal sections, each 4 μm thick, were cut continuously using a Leica microtome (Germany). The sections were incubated with primary antibodies: TNF-α (1:200, Abcam, UK), CD68 (1:800, Abcam), CD206 (1:1000, Abcam), and P65 (1:100, Bioworld Technology) for 2 h at 37°C. This was followed by incubation with secondary antibodies: goat anti-rabbit and goat anti-mouse IgG antibodies conjugated with horseradish peroxidase (Boster), for 30 min at 37°C. Staining was developed using a DAB kit from Boster, and sections were counterstained with hematoxylin. Images of the ischemic penumbra in the cerebral cortex were captured using a digital camera and a Leica DM1000 microscope (Germany). Three slices from each brain were analyzed according to unbiased stereology guidelines. Six randomly chosen, non-overlapping sections from each slice were analyzed using Image-Pro Plus 6.0 (American Media Cybernetics) for automatic cell counting. The average value from the images of each rat was used for statistical analysis. All analyses were performed by researchers blinded to the treatment conditions.

Statistical analysis

Quantitative data, expressed as mean ± standard deviation (SD), were analyzed using GraphPad Prism 9. To ensure normal distribution, data underwent logarithmic transformation. Groups were analyzed by one-way analysis of variance (ANOVA), followed by the Fisher's Least Significant Difference (LSD) *post hoc* test.

Statistical significance was determined with a threshold of $P < 0.05$, with $\alpha = 0.05$ serving as the cutoff for significance.

Results

Level of fasting tail vein blood glucose

No statistically significant differences in fasting tail vein blood glucose levels were observed among the sham, I/R, and Sema + I/R groups at any of the measured time points ($P > 0.05$) [Figure 2].

Modified neurological severity score study

Under semaglutide treatment, rats exhibited a statistically

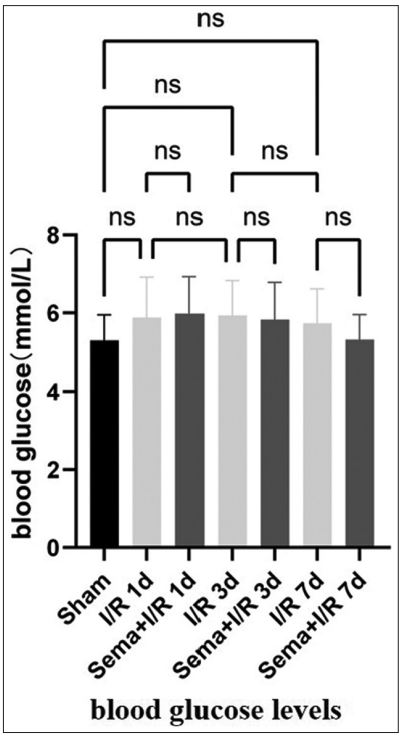

Figure 2: Blood glucose levels were monitored throughout the trial. A one-way analysis of variance indicated no significant variation among the groups, $n = 6$

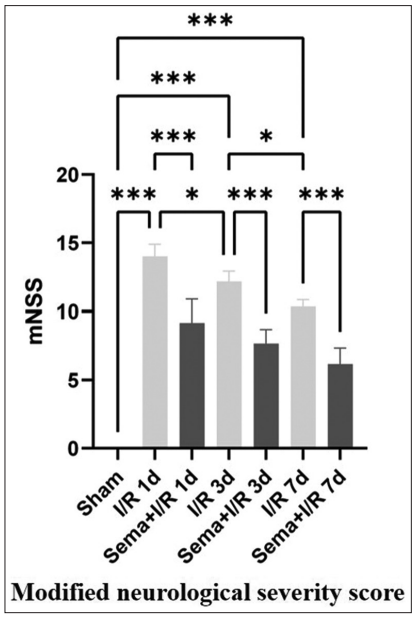

Figure 3: The modified neurological severity score (mNSS) were assessed for each group. A one-way analysis of variance revealed significant differences among all groups. *Post hoc* analysis using the least significant difference test identified variations between groups. The mNSS scores in the Sema + I/R group at the same time point is lower than that in the I/R group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

significant improvement in the degree of neurological deficits at the same time point. A notable difference in mNSS scores was observed between the Sema + I/R and I/R groups (P below than 0.001). Both the Sema + I/R and I/R groups showed a marked reduction in neurological deficits over time [Figure 3].

Infarct volume measured as a percentage

A notable reduction in the infarcted area was observed in the Sema + I/R group compared to the corresponding time-point I/R group ($P < 0.05$). In addition, both the I/R and Sema + I/R groups showed a significant decline in infarct area percentage over time [Figure 4].

Protein expression levels linked with microglial activation

M1-like phenotypic microglial activation marker CD68 expression levels

Immunohistochemistry (IHC) results revealed that the I/R group exhibited a significantly higher number of CD68-positive cells in the cerebral ischemic semi-dark band compared to the Sham group. This increase was most pronounced seven days after I/R (3 days vs. 1 day, $P > 0.05$; 7 days vs. 3 days, $P < 0.001$) and began one day after I/R ($P < 0.001$). The ischemic semi-dark band cortex in the I/R group contained significantly more CD68-positive cells than the Sema + I/R group at 1 day, 3 days, and 7 days ($P < 0.05$) [Figure 5].

Western blot (WB) analysis also showed that CD68 expression in the I/R group was significantly higher than in the Sham group (*P* below than 0.001), with expression levels increasing from the initial day postsurgery and peaking after 7 days (3 days vs. 1 day, $P > 0.05$; 7 days vs. 3 days, $P < 0.001$). This difference was statistically significant. Additionally, CD68 expression in the cerebral ischemic semi-dark band of the Sema + I/R group remained significantly lower than in the I/R group at 1 day, 3 days, and 7 days postsurgery ($P < 0.001$) [Figure 6].

M2-like phenotypic microglial activation marker CD206 expression levels

IHC revealed an increase in the number of CD206-expressing cells within the cerebral ischemic semi-dark band of rats in the I/R cohort one day post-surgery ($P < 0.001$). This count peaked at 3 days and then showed a declining trend by day 7 (3 days vs. 1 day, $P < 0.001$; 3 days vs. 7 days, $P < 0.001$). Notably, on the initial day post-I/R, the Sema + I/R cohort exhibited a higher count of CD206-positive cells compared to the I/R group ($P < 0.001$). However, differences in CD206-positive cell counts between the Sema + I/R and I/R groups at 3 days and 7 days post-I/R were not statistically significant ($P > 0.05$) [Figure 7].

WB analysis showed that CD206 expression in the I/R cohort remained significantly higher compared to the Sham cohort (*P* below than 0.001). Similarly, one day after treatment, CD206 expression in the Sema + I/R

group was higher than in the I/R cohort ($P < 0.05$); however, this difference was not statistically significant at 3 days and 7 days postsurgery ($P > 0.05$). A significant increase in CD206 expression was observed starting from 1 day postintervention ($P < 0.05$), peaking at 3 days, and declining by 7 days (3 days vs. 1 day, $P < 0.05$; 3 days vs. 7 days, $P < 0.05$) [Figure 6].

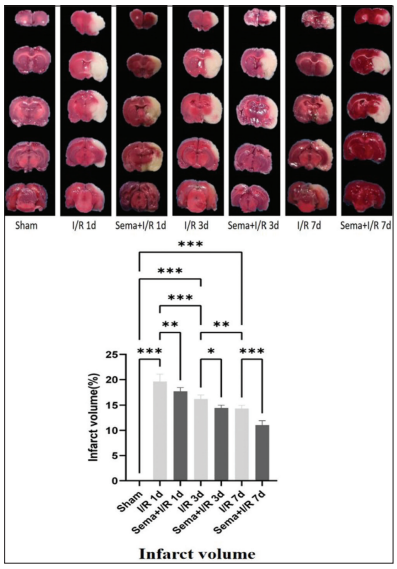

Figure 4: 2,3,5-triphenyltetrazolium chloride staining revealed no infarct area in the sham group, while the I/R and Sema + I/R groups displayed noticeable infarct sizes. Over time, infarct regions in both groups decreased, with the Sema + I/R group showing smaller infarct areas compared to the I/R group at the same time point. Differences among all groups were identified by one-way analysis of variance, and *post-hoc* group distinctions were detected using the least significant difference test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

The levels of tumor necrosis factor alpha

IHC results indicated a significant increase in TNF-α-positive cells within the cerebral ischemic semi-dark band of rats in the I/R cohort compared to the Sham cohort ($P < 0.001$). The number of TNF-α-positive cells peaked at 3 days postsurgery and declined by day 7 (3 days vs. 1 day, $P < 0.001$; 3 days vs. 7 days, P below than 0.001). At 1 day, 3 days, and 7 days postsurgery, the Sema + I/R cohort exhibited significantly fewer TNF-α-positive cells within the cerebral ischemic semi-dark band compared to the I/R cohort ($P < 0.001$) [Figure 8].

WB data showed that TNF-α expression in the cerebral ischemic semi-dark band was markedly higher in the I/R cohort compared to the Sham cohort ($P < 0.01$). This elevation peaked at 3 days post-surgery and declined by 7 days (3 days vs. 1 day, $P < 0.05$; 3 days vs. 7 days, $P < 0.05$). The statistical significance of this difference was notable. In addition, TNF-α levels in the Sema + I/R cohort were significantly lower than in the I/R cohort at 1 day, 3 days, and 7 days postsurgery ($P < 0.05$) [Figure 6].

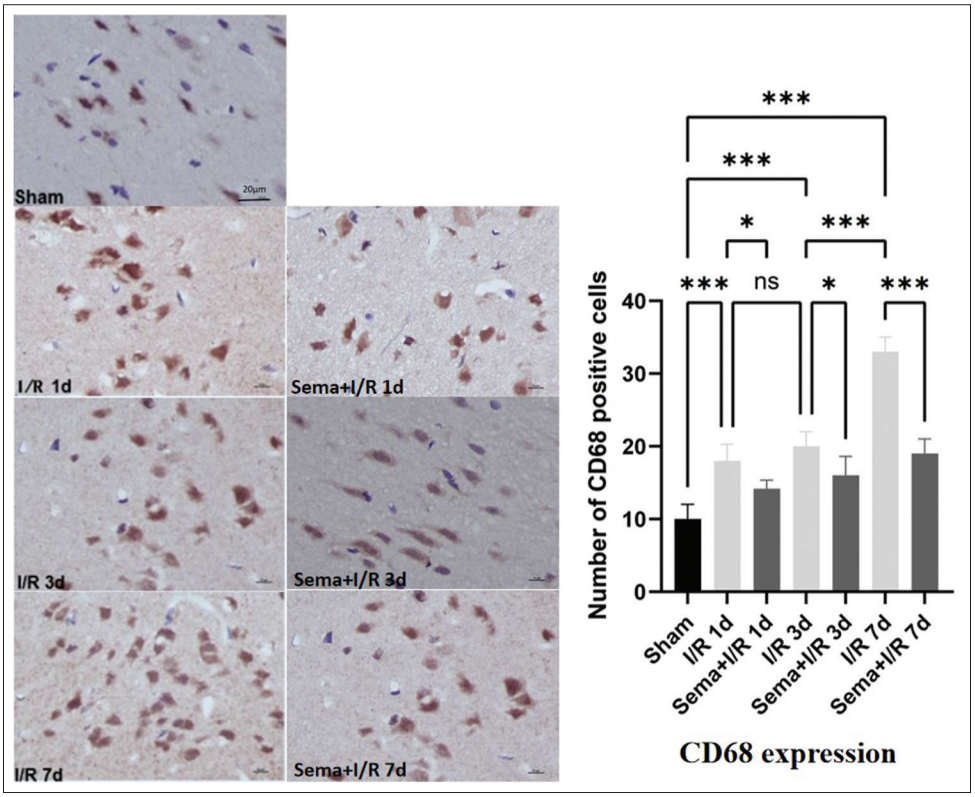

Figure 5: Semaglutide treatment significantly decreased cluster of differentiation 68 levels. For further results, please refer to the results section. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The scale bar represents 20 μm

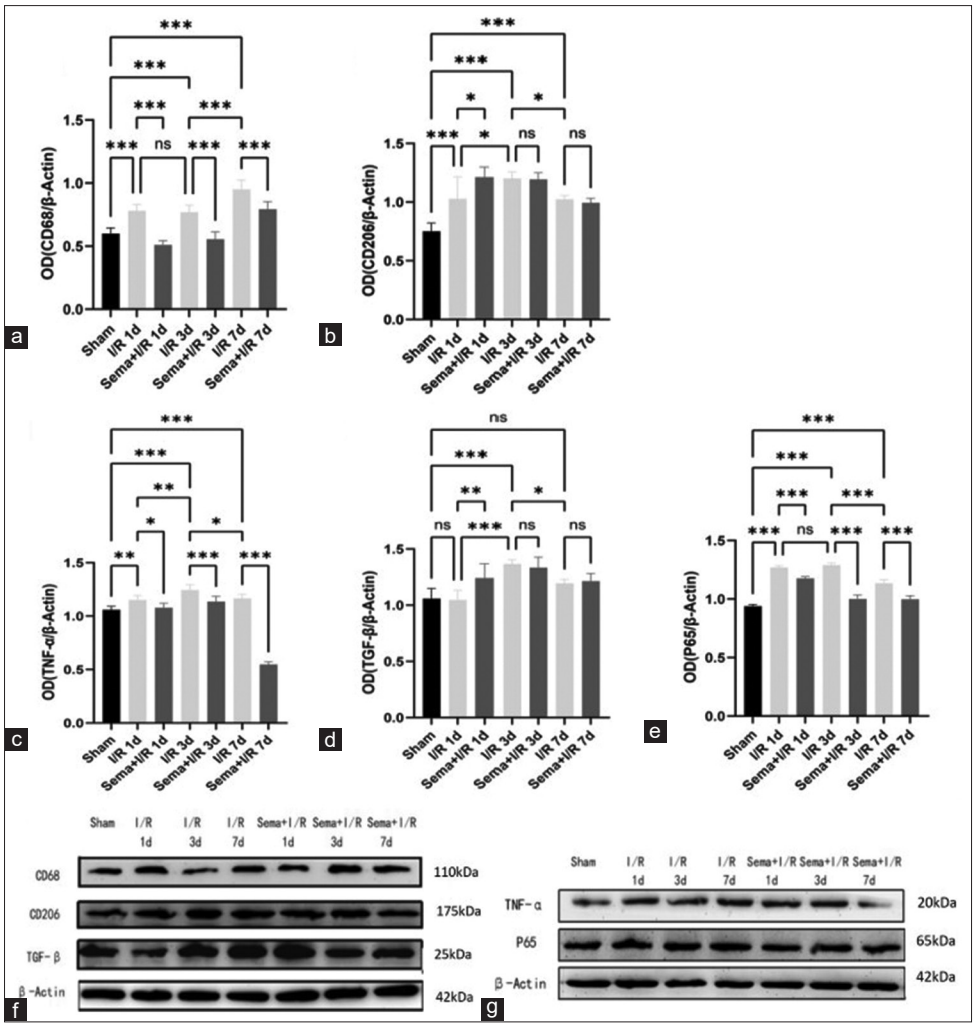

Figure 6: Western blot (WB) analysis was used to assess cluster of differentiation (CD) 68, CD206, transforming growth factor beta, tumor necrosis factor alpha, and P65. (a-e) The relative optical density values of the ischemic penumbra in each group. (f and g) The schematic diagram of the WB analysis. For further results, please refer to the results section. *P < 0.05; **P < 0.01; ***P < 0.001

The levels of transforming growth factor beta

WB analysis revealed that TGF-β expression peaked within the cerebral ischemic semi-dark band of the I/R cohort at 3 days postsurgery, followed by a decline at 7 days (3 days vs. 1 day, $P < 0.001$; 3 days vs. 7 days, $P < 0.05$). TGF-β levels in the Sema + I/R cohort were significantly higher than those in the I/R cohort one day postoperatively ($P < 0.01$). However, at 3 days and 7 days post-surgery, the differences in TGF-β levels between the Sema + I/R and I/R cohorts were not statistically significant ($P > 0.05$) [Figure 6].

Levels of the nuclear factor kappa-light-chain-enhancer of activated B cells signaling cascade-related protein P65

IHC results indicated an increase in the number of P65-positive cells within the cerebral ischemic semi-dark band of the I/R cohort compared to the sham-operated cohort on the 1st postoperative day ($P < 0.001$). However, a decreasing trend was observed by day 7 (3 days vs. 1 day, $P > 0.05$; 3 days vs. 7 days, $P < 0.05$). The Sema + I/R cohort showed a reduction in the number of P65-positive cells at 1 day, 3 days, and 7 days post-I/R compared to the I/R cohort ($P < 0.001$) [Figure 9].

WB analysis revealed higher P65 expression in the ischemic semi-dark band at 1d post-surgery in the I/R cohort compared to the sham-operated cohort ($P < 0.001$), with a statistically significant decrease in expression by 7 days (3 days vs. 1 day, $P > 0.05$; 3 days vs. 7 days, $P < 0.001$). P65 levels in the ischemic semi-dark band were significantly lower in the Sema + I/R cohort at all time points compared to the I/R cohort ($P < 0.001$) [Figure 6].

Discussion

Significant neuroprotective benefits of semaglutide were observed in a rat model of brain ischemia-reperfusion.

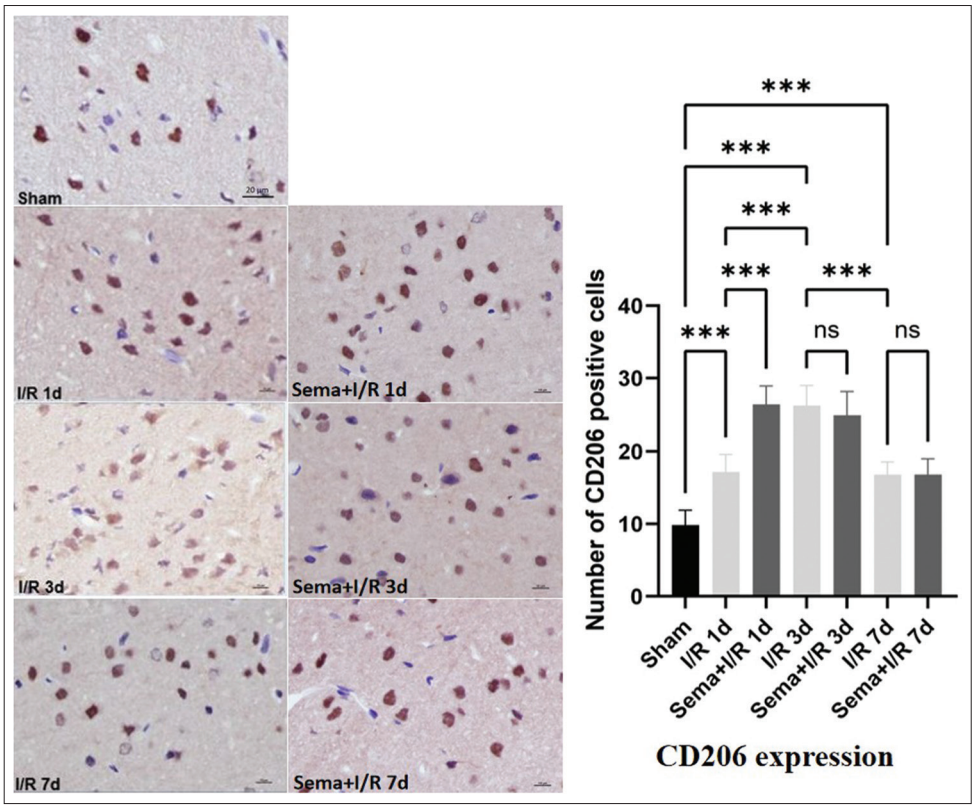

Figure 7: Semaglutide treatment resulted in increased cluster of differentiation 206 levels on the initial day post-I/R. For further results, please refer to the results section. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The scale bar represents 20 μm

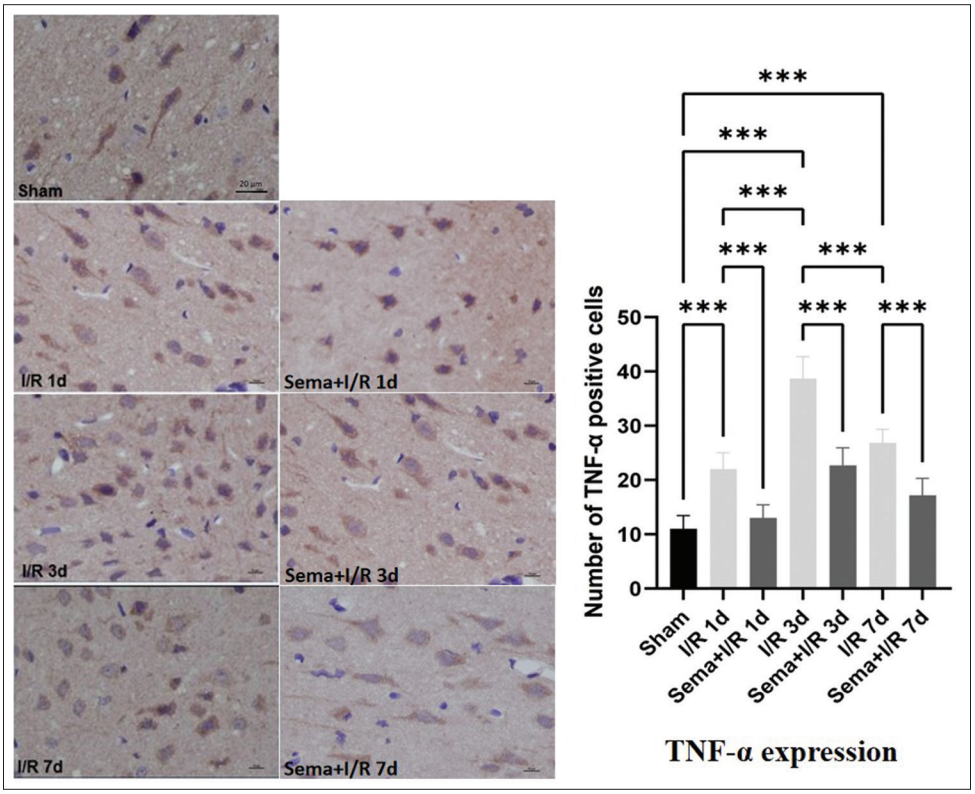

Figure 8: Semaglutide treatment resulted in decreased tumor necrosis factor alpha levels. For further results, please refer to the results section. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The scale bar represents 20 μm

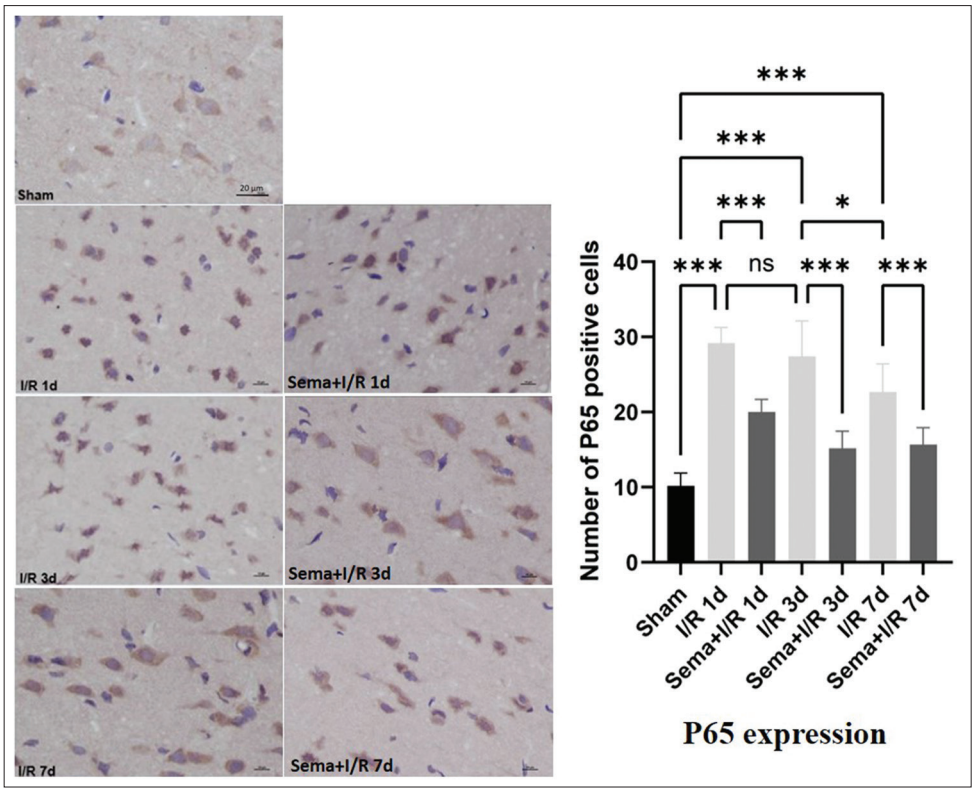

Figure 9: Semaglutide treatment resulted in lower P65 levels. For further results, please refer to the results section. *P < 0.05; **P < 0.01; ***P < 0.001. The scale bar represents 20 μm

Semaglutide modulates the phenotypic transition of microglia by decreasing the levels of the M1 phenotype and increasing the levels of the M2 phenotype. This modulation results in elevated levels of the anti-inflammatory protein TGF-β, reduced amounts of the pro-inflammatory protein TNF-α, and diminished expression of P65, which is associated with the NF-κB signaling cascade. Notably, semaglutide reduces neurological impairment in rats with CIR without affecting blood glucose levels in nondiabetic rats. Microglia exhibiting M1/M2-like phenotypes play crucial roles in the neuroinflammatory response following a stroke.[31,32]

The M1-like phenotype, a form of classical activation, is primarily triggered by interferon-gamma, lipopolysaccharide, and TNF-α. This phenotype releases several inflammatory components, including TNF-α, interleukin-6 (IL-6), IL-1β, and others. In a diseased state, the M1 phenotype induces an inflammatory cascade that exacerbates the inflammatory response and contributes to neuronal death by secreting inflammatory agents and neurotoxic chemicals. Common markers for the M1 phenotype include CD68, CD86, CD16, and CD32. The M2-like phenotype, which can be further categorized into four subtypes – M2a, M2b, M2c, and M2d – represents a form of selective activation. These subtypes are predominantly triggered by factors such as glucocorticoids, TGF-β, and IL-10. The primary function of M2-like microglia is to secrete anti-inflammatory factors such as TGF-β and IL-10 as well as nerve repair factors, thereby exerting anti-inflammatory and pro-repair effects. Surface markers for the M2 phenotype include CD301, CD206, and CD163.[33]

After cerebral ischemia, microglia undergo a dynamic polarization process over time, transitioning primarily from a transient M2-like phenotype to an M1-like phenotype.[34,35] The extent of infarction and the various modeling techniques used can influence the temporal variability in the expression of distinct microglial phenotypes following cerebral ischemia. The M2-like anti-inflammatory phenotype is most prominent from 0.5 to 7 days after brain ischemia, while the M1-like pro-inflammatory phenotype becomes more prominent from 7 to 14 days.[35]

Our findings revealed an increase in CD206 and CD68 levels in the ischemic region starting from day 1 after CIR. CD68 continued to rise through day 7, while CD206 peaked on day 3. The levels of the anti-inflammatory factor TGF-β were closely synchronized with CD206 during this period. Concurrently with the increase in CD68, the level of the pro-inflammatory factor TNF-α peaked 3 days after CIR. These results demonstrated that

CIR activates microglia into both M1-like and M2-like phenotypes. The M2-like phenotype predominated in the early stage, while the M1-like phenotype was more prevalent in the later stage. The M2-like phenotype released anti-inflammatory factors that aided nerve repair, whereas the M1-like phenotype released pro-inflammatory factors, exacerbating nerve damage. Our results are consistent with previous findings. The early peak of TNF-α suggested that it can be released by M1-like microglia as well as through other mechanisms, which warrants further investigation. In summary, IRI and the associated inflammatory response can mutually reinforce each other, creating a vicious cycle that exacerbates nerve injury. Therefore, IRI may involve inhibiting the neuroinflammatory response. This can be achieved by promoting nerve recovery and reducing neuroinflammation through strategies such as transforming microglia into the M2-like phenotype and/or preventing their transition to the M1-like phenotype.

Inflammation and CIR damage are both influenced by NF-κB signaling. The phenotypic changes in M1/M2-like microglia are linked to this pathway.[36,37] NF-κB, a versatile nuclear transcription factor, plays a crucial role in the inflammatory response and is found at the endpoint of several signal transduction pathways.[38] In mammals, the NF-κB/Rel family comprises five members: Rel (cRel), RelB, p50 (NF-κB1), p52 (NF-κB2), and p65 (RelA/NF-κB3). Among these, p65 is closely associated with microglial activation, and its transcriptional activity facilitates the phenotypic transition of microglia.[39] Our findings indicated that in rats subjected to ischemic conditions, p65 expression in the ischemic region began to rise 1 day after CIR and peaked between days 1 and 3 before declining. This peak occurred prior to increases in TNF-α and CD68 levels. This pattern suggested that CIR may enhance the release of TNF-α by promoting p65 expression, which in turn activates microglia into the M1-like phenotype. Concurrently, M1-like microglia further stimulate the levels of TNF-α and p65. These factors are causally related and mutually reinforce the inflammatory response, although the specific mechanisms involved still require further experimental investigation.

GLP-1 has demonstrated neuroprotective effects in clinical trials for Alzheimer's disease and PD.[40,41] Both the US and Europe have authorized semaglutide as a treatment for diabetes, and clinical studies have explored its neuroprotective benefits in PD patients.[41] GLP-1R agonists consistently show protective effects on the nervous system across various animal models of neurological disorders. The mechanism behind this action is actively being investigated, with microglia playing a crucial role. Semaglutide has been shown to reduce inflammatory mediators, prevent cell death, and provide neuroprotection in pMCAO rats by regulating microglial proliferation and the p38 MAPK–MKK–c-Jun–NF-κB p65 pathway.[19] Additional research suggests that exenatide-4 may offer neuroprotective effects by promoting M2 phenotype expression in microglia, which contributes to repair.[42] Our study further demonstrates that following semaglutide intervention, levels of CD206 and TGF-β significantly increased at 1 day, while levels of CD68 and TNF-α markedly decreased at 1 day, 3 days, and 7 days. These findings indicate that semaglutide may inhibit the transition to the M1 phenotype and promote the transition to the M2 phenotype by directing microglial activation. This regulation suppresses the inflammatory cascade, promotes nerve repair, and exerts neuroprotective effects. After semaglutide intervention, p65 expression decreased alongside reductions in CD68 and TNF-α, suggesting that semaglutide may inhibit phenotypic transformation and the release of inflammatory factors in microglia by suppressing p65 expression. This action indirectly promotes the transformation to an M2-like phenotype, mitigates the inflammatory response, enhances nerve repair, and ultimately provides neuroprotection.

Limitations of the study

This study found that Semaglutide exerts neuroprotective effects by regulating microglia phenotypic transformation and NF-κB signaling pathway, which can be further verified by adding corresponding antagonists in the future; in this study, the cerebral ischemia-reperfusion model in rats was used to indirectly assess the success of the model using behavioral science, and it is necessary to observe the cerebral blood flow in rats by animal Doppler blood flow detector.

Conclusion

Our research demonstrates that the long-acting GLP-1 receptor agonist semaglutide, by downregulating p65 expression in the NF-κB cascade, effectively prevents the release of inflammatory components. Semaglutide inhibits the expression of M1 phenotypes in microglia, promotes the expression of M2 phenotypes, and thereby reduces the neuroinflammatory cascade while facilitating nerve repair, ultimately exerting neuroprotective effects. Importantly, semaglutide does not affect blood glucose levels in nondiabetic patients. Consequently, semaglutide presents a potential new therapeutic option for stroke patients and warrants further investigation in clinical trials.

Author contributions

Rulin Mi: Experimental design, Experimental studies, Writing – original draft; Huifeng Cheng: Establishing the cerebral ischemiareperfusion model; Rui Chen: Manuscript preparation; Bo Bai: Statistical analysis; An Li: Immunohistochemistry and western blot analysis;

Fankai Gao: Conceptualization; Guofang Xue: Project administration, Supervision, Writing – review.

Ethical policy and institutional review board statement

This study and included experimental procedures were approved by the Animal Ethics Committee of the Second Hospital of Shanxi Medical University (approval No. DW2024029, dated on March 20th, 2024). All animal housing and experiments were conducted in strict accordance with the institutional guidelines for care and use of laboratory animals.

Data availability statement

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

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Conflicts of interest

There are no conflicts of interest.

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