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Research article

Therapeutic Effects of Intranasal Administration of Resveratrol on the Rat Model of Brain Ischemia

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ABSTRACT

Background: Resveratrol is a natural phenolic compound widely found in plants. Previous studies have suggested its neuroprotective role in cerebral ischemia due to its anti-oxidative, anti-inflammatory, and anti-apoptotic effects. Intranasal administration of resveratrol enhances its capacity to penetrate the blood-brain barrier, increasing therapeutic efficacy and safety.
Objective: We aimed to examine the therapeutic potential of intranasal administration of resveratrol treatment in rats exposed to cerebral ischemia.
Methods: Sixty-four male rats were divided into three groups: the sham group, which was exposed to only surgical stress; the vehicle and resveratrol groups, which received intranasal vehicle or 50 mg/kg resveratrol for 7 days following middle cerebral artery occlusion, respectively. We assessed the modified neurologic severity scores, wire hanging tests, blood-brain barrier disruption, brain water content, and infarct volume. Levels of matrix metalloproteinase-9, nuclear factor-kappa B, B-cell lymphoma protein 2, and B-cell lymphoma protein 2-associated X messenger RNA expression were examined.
Results: At 3- and 7-days post-ischemia, rats receiving intranasal resveratrol had lower modified neurological severity scores and a smaller brain infarct volume than the rats receiving vehicle. Additionally, the intranasal resveratrol-treated rats showed significantly prolonged wire-hanging performance at the 7-day mark post-ischemia compared to the vehicle group. The blood-brain barrier disruption and brain water content were significantly lower in the resveratrol group than in the vehicle group. Furthermore, the resveratrol-treated group displayed lower expression of Matrix Metalloproteinase-9 and Nuclear Factor-Kappa B in contrast to the vehicle group, while the difference in expression levels of B-cell lymphoma protein 2-associated X and B-cell lymphoma protein 2 were not significant.
Conclusion: Intranasal administration of resveratrol showed neuroprotective effects on ischemic stroke by improving neurobehavioral function, reducing blood-brain barrier disruption, cerebral edema, and infarct volume. This treatment also downregulated Matrix Metalloproteinase-9 and Nuclear Factor-Kappa B expression, indicating its potential as a therapeutic option for ischemic stroke.

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1. Introduction

Ischemic stroke (IS) occurs when arterial occlusion restricts cerebral blood flow, leading to depleted Adenosine triphosphate and inadequate delivery of oxygen and glucose to brain tissue. It stands as the primary cause of mortality and disability globally [1]. Recent studies have extensively investigated the diverse pathological mechanisms and potential therapeutic targets of IS. These include excitotoxicity, oxidative and nitrative stress, neuroinflammation, cerebral edema, apoptosis, and the disruption of the blood-brain barrier (BBB) integrity [2]. Currently, thrombolysis and thrombectomy are the main treatment options for IS. The narrow therapeutic window, increased risk of cerebral bleeding, and failure to recover the dying neurons are major limitations of these treatment options [3].

Resveratrol (3,4,5-trihydroxystilbene) is a phenolic compound found in Polygonum cuspidatum, red wine, peanuts, and red grapes. Its anti-apoptotic, anti-inflammatory, anti-cancer, and antioxidant effects have been demonstrated in previous studies [4,5]. Research conducted *in vitro* and *in vivo* has demonstrated that resveratrol is a strong neuroprotective agent and that it can pass the BBB [5–9]. Resveratrol has demonstrated neuroprotective properties and has shown promise in improving pathophysiological and neurobehavioral functions in various central nervous system (CNS) disorders, including spinal cord injury, traumatic brain injury, and stroke [5,8,10]. Several investigations have also shown that resveratrol offers a variety of protective benefits on neurons in rats that have undergone middle cerebral artery occlusion (MCAO) [11–16]. Furthermore, studies have indicated that resveratrol possesses antihypertensive properties that may be advantageous for stroke patients [17].

There are currently several methods of drug delivery for brain disorders. Numerous drugs cannot effectively cross the BBB due to their composition, and invasive injection techniques are not practical. Therefore, improved routes of administration, such as intranasal drug delivery, have been developed. Intranasal drug administration is a novel, non-invasive, direct, and rapid means of treating disorders of the central nervous system. It can cross the BBB through the nasal mucosa while avoiding first-pass metabolism [18]. Even though it has been demonstrated that resveratrol can enter the brain from the bloodstream in animals and humans, its distribution in the CNS appears to be quite poor in comparison to other tissues [19].

Matrix metalloproteinase-9 (MMP-9) disrupts the BBB by breaking down extracellular matrix proteins, collagen, tight junction proteins, and capillary basal lamina proteins [20]. It is widely confirmed that MMP-9 promotes ischemic brain damage, and its

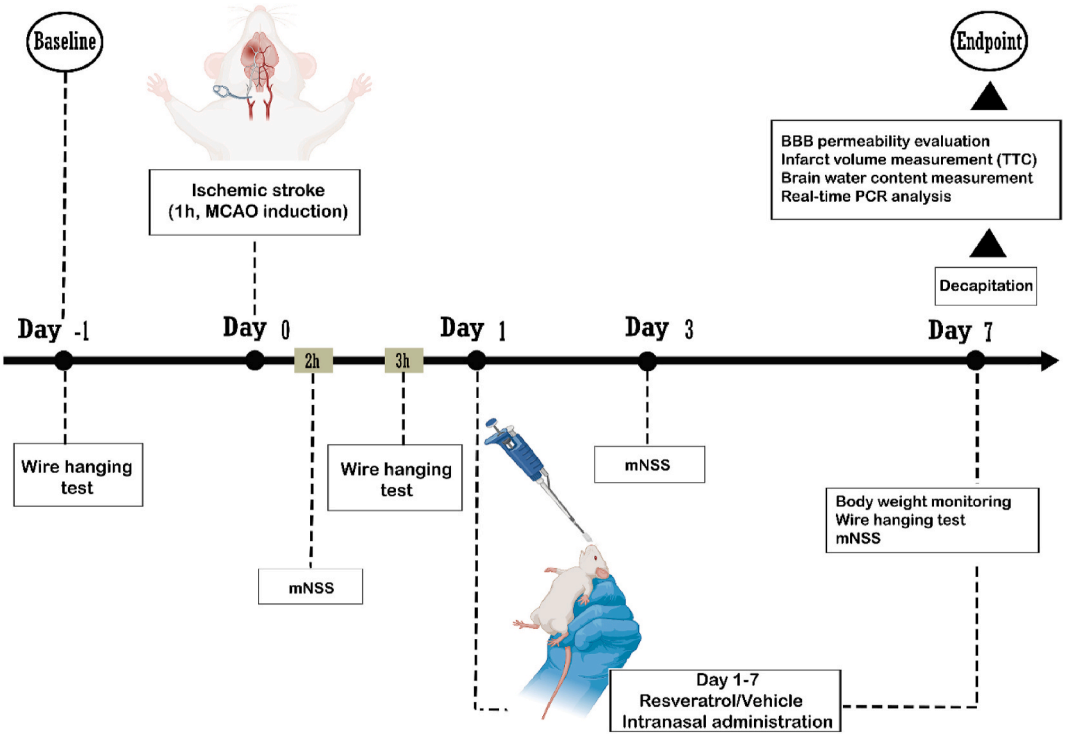

Fig. 1. Schematic diagram of the experimental design to evaluate the therapeutic effects of intranasal administration of resveratrol on the rat model of brain ischemia. Rats were subjected to 1 h of ischemia followed by 7 days of reperfusion with neurological evaluations including the wire hanging test (1 day prior to MCAO induction, 3 h after MCAO induction, and day 7) and mNSS (2 h, 72 h, and 7 days after MCAO induction). Intranasal administration of resveratrol or vehicle was applied from day 1 to day 7. On day 7, after body weight monitoring and behavioral tests, all rats were euthanized for BBB permeability, infarct volume, brain water content evaluations, and real-time PCR analysis (MMP-9, NF-KB, Bax, and Bcl2 mRNA expression levels)

MCAO, middle cerebral artery occlusion; mNSS, modified neurologic severity score; BBB, blood-brain barrier; MMP-9, Matrix metalloproteinase-9; NF-KB, nuclear factor-kappa B; Bcl2, B-cell lymphoma protein 2; Bax, B-cell lymphoma protein 2-associated X.

inhibition appears to be a potential therapeutic target for IS by inhibiting BBB breakdown and cerebral edema [21]. Nuclear Factor-Kappa B (NF-κB) modulates the expression and activation of several pathological, inflammatory, and apoptotic genes [22]. NF-κB is upregulated in the ischemic brain regions in both human and animal models of stroke [23]. Inhibition of NF-κB is a potential therapeutic agent because it inhibits inflammation and apoptosis [22,24]. The B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax) protein initiates a chain of events that ultimately leads to cell death by releasing cytochrome *c* from the mitochondria during the apoptotic process. Bcl-2 prevents Bax from releasing cytochrome *c*, which in turn inhibits the activation of the downstream apoptotic machinery [5]. Several studies have reported a substantial increase in the Bax/Bcl-2 ratio following cerebral ischemia [25]. Elevated Bcl-2 levels and reduced Bax levels present promising therapeutic strategies for mitigating ischemic neuronal damage.

In the current investigation, we examined the possible therapeutic benefits of intranasal administration of resveratrol on neurobehavioral performance, brain edema, BBB permeability, and infarct volume in rats undergoing brain ischemia. The therapeutic effects of resveratrol may suppress the MMP-9-dependent BBB disruption, cerebral edema, and NF-κB-dependent inflammation; hence, their expression levels have been evaluated. We also assessed levels of Bax and Bcl-2 gene expressions which are associated with apoptosis.

2. Materials and methods

2.1. Experimental animals

The Comparative and Experimental Medical Center at Shiraz University of Medical Science provided adult male Sprague-Dawley rats weighing 250–300 g. The animals were kept in cages in a 21–23 °C environment with a 12-h light–12-h dark cycle (lights on at 8:00) and ad libitum access to food and water. All the experimental protocols were approved by the local Ethics Committees of the Behbahan Faculty of Medical Sciences, Behbahan, Iran (Ref. No. IR.BHN.REC.1401.061) and were in accordance with the guidelines set by "Principles of Laboratory Animal Care". Following the adaptation phase, 64 rats were randomly separated into three groups: sham (n = 18), vehicle-treated MCAO or control (n = 23), and resveratrol-treated MCAO groups (n = 23). The sham group underwent surgical stress without MCAO or administration of any medication. After MCAO, in the vehicle control and resveratrol groups, vehicle (0.05 percent dimethyl sulfoxide (DMSO) in phosphate-buffered saline, Sigma-Aldrich, St. Louis, MO, USA) or 100 μl of a 135 mg/ml (13.5 mg/rat) resveratrol solution (~50 mg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) [26] in 0.05 percent DMSO were inoculated through drip with a micropipette into each nostril of the rat for intranasal administration once a day for 7 consecutive days, while the rat was in the head-back supine position with a 70°–90° tilt to enhance drug absorption from the nasal cavity and facilitate uptake into the brain, while minimizing drainage into the esophagus and trachea (Fig. 1) [27]. We evaluated neurobehavioral functioning 2, 72 h, and 7 days after inducing cerebral ischemia. The animals were euthanized in deep anesthesia at 7-day intervals after cerebral ischemia, and their brains were removed to assess brain water content (BWC) (each group; n = 5), BBB integrity (each group; n = 5), 2,3,5-triphenyl tetrazolium chloride (TTC) staining evaluation (n = 2 for the sham group, n = 7 for vehicle- and resveratrol-treated MCAO groups), and gene expression quantification (each group; n = 6). The experimental design is shown as a schematic diagram in Fig. 1.

2.2. Middle cerebral artery occlusion induction

We utilized the MCAO method to induce transient focal cerebral ischemia, as described previously by Koizumi [28]. To induce profound anesthesia, rats were administered an intraperitoneal dose of ketamine and xylazine (80 and 12 mg/kg body weight, respectively) [29]. An incision in the neck was made to reveal the right common carotid artery, as well as the external carotid artery and internal carotid arteries. A 3-0 monofilament nylon suture that was heat-rounded and silicone-coated was inserted into the internal carotid arteries through the right common carotid artery (~ 20 mm) until it obstructed the blood flow to the right middle cerebral artery. Reperfusion was established by withdrawing the suture an hour after the MCAO. After suturing, an oxytetracycline spray was used to make the neck incision sterile. With the use of an electric blanket, the rats were kept at 37.8 °C throughout and after surgery. Throughout this process, we kept track of the rats' physiological variables, including body temperature, heart rate, and respiration rate.

2.3. Body weight monitoring

Before surgery (day 0) and on the seventh postoperative day, a digital scale (Acculab ALC210.4, USA) was used to monitor the body weight. The body weights of the animals (n = 15 rats per group) were determined.

2.4. Neurological scores examination

A combination of sensory (visual, tactile, and proprioceptive), motor, balance, and reflex are all included in the modified neurologic severity scores (mNSS), with a scale of 0 (normal) to 18 (maximal neurologic deficit) [30]. One score point was given for not being able to do the test or not having a tested reflex. Therefore, a higher score indicates a more serious injury. mNSS was performed blindly in each group for neurological deficit assessment at three time points: 2, 72 h, and 7 days following MCAO induction (n = 15 rats per group). If an animal's score dropped below 3 or if it passed away from cerebral ischemia, it was removed from the trial, and a new animal was used to maintain the integrity of the study.

2.5. Assessment for motor function using wire-hanging test

The animals' gripping abilities and durability were evaluated using the wire-hanging test (n = 7 rats per group) [31]. The rats were allowed to grasp with their forelimbs the middle of a steel wire (2 mm in diameter and 60 cm in length) between two columns at a 50 cm height above a foam pad. The latency to fall, i.e., the time (in seconds) in which the rats remained suspended (for a maximum of 5 min), was measured at three time points: 24 h before the induction of ischemia (baseline), 3 h (day 0), and seven days after MCAO. This test was recorded from three trials with a 5-min recovery period between trials, and the average outcome for each animal was calculated.

2.6. Blood-brain barrier permeability evaluation

Evans blue (EB) permeability in brain tissue (n = 5 rats per group) was measured to determine BBB disruption [32]. Seven days after reperfusion, the right femoral vein was cannulated. EB 2 % was diluted in saline and administered at a rate of 4 ml/kg; i.v. EB circulation was allowed for 2 h. Then, the chest wall was opened under deep anesthesia, and the animals were decapitated. To remove EB from the brain's blood flow, 250 ml of 0.9 % saline was injected transcardially for 20 min. The cerebral hemispheres were separated and weighed after the brains were removed. To precipitate the protein, 2.5 ml of 60 percent trichloroacetic acid (Merck, Darmstadt, Germany) was added to the hemispheres before they were homogenized. The hemispheres were then centrifuged at 3500 rpm for 30 min to separate the supernatant. An Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) was used to measure the extracted EB absorbance at a wavelength of 610 nm using the supernatant (0.2 ml). The content of EB dye in the tissue was calculated using a standard linear curve derived from known amounts of the dye. It was expressed in micrograms per gram (μg/g) of brain tissue depending on wet weight and quantified in accordance with a standard curve using external standards in the same solvent.

2.7. Brain water content measurement

Based on prior studies, we measured BWC to have a better understanding of the amount of cerebral edema (n = 5 rats per group) [33]. Following 7 days of ischemia/reperfusion, animals were sacrificed by decapitation, and the brains were immediately removed and dissected into right (ischemic) and left (nonischemic) hemispheres. The tissue samples were weighed separately and dried for 24 h at 105 °C in an oven. Each sample's BWC was determined by calculating [(wet weight – dry weight)/(wet weight)] × 100 [33].

2.8. Infarct volume measurement

We euthanized the animals with an overdose of ketamine/xylazine seven days following reperfusion, and the brains were then removed. We prepared 2 mm-thick coronal sections of the brain and stained them with TTC (Sigma, St. Louis, MO, USA). The slices were placed in a 2 % TTC Petri dish at 37 °C for 30 min, shaken frequently to ensure that the slices were not on the bottom, and then immersed in 10 % formalin overnight. Using the image analysis system Image J software (NIH, Bethesda, MD, USA), the infarct area of each TTC-stained section in the vehicle (n = 7), resveratrol (n = 7), and sham (n = 2) groups was calculated. Infarct volume calculation was performed by multiplying the sum of the infarct regions across all brain slices by their respective thicknesses (2 mm). The equation volume correction = (infarct volume contralateral volume)/ipsilateral volume was used to calculate the edema correction of the infarct volume. Both hemisphere volumes were determined, and the edema volume was then determined by subtracting the contralateral volume from the ipsilateral volume [34].

2.9. RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction

Total RNA of the ipsilateral ischemic cerebral cortex was extracted with the QIAShredder and RNeasy Mini kits (Qiagen, Iran), according to the manufacturer's protocol instructions, and further quantified using NanoDrop (Thermo Fisher Scientific, USA). Total RNA was reverse transcribed into cDNA with the SuPrime Script RT Premix (2X) cDNA Synthesis Kit (GeNet BIO Inc.; Daejeon, South Korea). Quantitative real-time polymerase chain reaction (qRT-PCR) with the use of a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and the Power SYBR Green PCR Master Mix were performed. β-actin was employed as an internal loading control. The expression of target genes was normalized to the β-actin gene and reported as $2^{-\Delta\Delta Ct}$ [35]. The primer sequences were designed via primer BLAST and described in Table 1.

Table 1
Primers for real-time PCR used for gene amplification in the rat model.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
MMP-9	AAA TGT GGG TGT ACA CAG GC	TTC ACC CGG TTG TGG AAA CT
C	GCGCATCCAGACCAACAATAAC	GCCGAAGCTGCATGGACACT
Bcl-2	AAGCTGTCACAGAGGGGCTA	CTCACTTGTGGCCCAGGTAT
Bax	CTGCAGAGGATGATTGCTGA	GATCAGCTCGGGCACTTTAG
Actb	TGGAATCCTGTGGCATCCATGAAC	TAAAACGCAGCTCAGTAACAGTCCG

2.10. Statistical analysis

Statistical analyses were conducted using SPSS 18.0 software. All data were analyzed and presented as mean ± standard error of mean (SEM). The comparison of three groups was carried out using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. All tests were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of resveratrol on body weight

There were no statistically significant differences in pre-MCAO body weight between the groups ($P > 0.05$, Fig. 2A). Rats in the MCAO vehicle group lost significantly more body weight than the sham group seven days after cerebral ischemia ($P < 0.01$). Additionally, the body weight of the MCAO rats receiving intranasal resveratrol was notably lower than that of the sham group ($P < 0.05$). In contrast, there was no discernible difference in body weight change between the MCAO rats receiving vehicle and the control group ($P > 0.05$, Fig. 2A).

3.2. Effect of resveratrol on neurobehavioral function

The sham group showed no neurobehavioral deficits and was not included in the mNSS assessment. All MCAO rats had a mean mNSS score of 13.53 on day 0 (2 h after MCAO), indicating that the MCAO procedure successfully induced cerebral ischemia. As shown in Fig. 2B, the difference in mNSS between the two groups at day 0 was not statistically significant ($P > 0.05$). Moreover, the improvement in neurobehavioral function (reduction in mNSS) was observed in both resveratrol-treated and vehicle-treated MCAO groups during the treatment period. It is worth noting that MCAO rats receiving intranasal resveratrol showed a significant decrease in mNSS at 3 and 7 days after ischemia-reperfusion injury compared with the MCAO vehicle group ($P < 0.05$ and $P < 0.001$, respectively, Fig. 2B).

3.3. Effect of resveratrol on wire hanging tests

At baseline (before surgery), mean latencies to performance did not differ significantly between the groups (n = 7 rats/group). At two time points of 3 h (day 0) and 7 days after MCAO, the latency to fall off the wire hanging in the MCAO vehicle and resveratrol-treated groups was significantly lower than in the sham group ($P < 0.001$). Interestingly, the effects of intranasal resveratrol therapy at the 7-day time point exhibited a significantly prolonged period of wire-hanging performance compared with the MCAO vehicle group ($P < 0.05$, Fig. 2C).

3.4. Effect of resveratrol on BBB permeability

Extravasation of EB dye in the ipsilateral ischemic (right) hemisphere and contralateral (left or control) hemispheres was measured

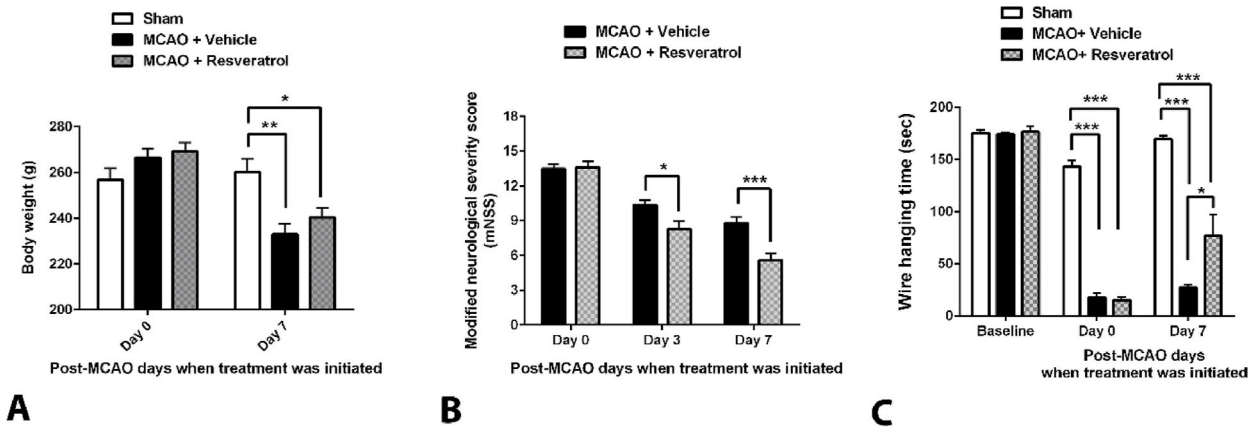

Fig. 2. - A, Body weight in intranasal resveratrol-treated and vehicle-treated MCAO rats, as well as the sham group, on days 0 (before MCAO) and 7 post-ischemia-reperfusion injuries. Data is expressed as mean ± SE.M (n = 15 rats per group); B, significant neurobehavioral improvement in mNSS in the MCAO intranasal resveratrol treatment group compared to the MCAO vehicle group on days 3 and 7 post-ischemia–reperfusion injury. Data is expressed as mean ± S.E.M (n = 15 rats per group); C, fall latency from wire hanging tests of the sham-operated, intranasal resveratrol, and vehicle-treated MCAO rats at baseline (before surgery), 3 h (day 0), and 7 days after MCAO. Data is expressed as mean ± S.E.M (n = 7 rats per group); Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. *$P<0.05$, **$P< 0.01$, ***$P<0.001$
MCAO, middle cerebral artery occlusion; mNSS, modified neurologic severity score.

2 h after EB injection to assess BBB dysfunction (Fig. 3A and B). EB dye extravasation in the MCAO groups for the ipsilateral ischemic hemisphere was significantly higher compared with the contralateral hemisphere to cerebral ischemia 7 days after MCAO (data not shown). EB content in the ipsilateral hemisphere for cerebral ischemia was statistically increased in the vehicle-treated MCAO group in contrast to the sham group ($P < 0.001$). In addition, EB content was significantly reduced in rats with MCAO treated intranasally with resveratrol compared with the vehicle-treated MCAO group ($P < 0.05$, Fig. 3C). This result also shows that this medicine was unable to prevent BBB disruption at the level of the sham group ($P < 0.05$, Fig. 3C).

3.5. Effect of resveratrol on brain water content

Cerebral ischemia leads to a significant increase in BWC% in comparison with the sham group ($P < 0.001$), while intranasal resveratrol treatment attenuates the edema formation ($P < 0.05$, Fig. 4). This drug was unable to be equal to the sham group level ($P < 0.05$).

3.6. Effect of resveratrol on infarct volume

TTC staining causes infarcts to turn white, whereas viable tissues stay deep red, caused by intact mitochondrial activity (Fig. 5A). Rats in the sham group did not show any obvious lesions (Fig. 5A and B). As shown in Fig. 5B, the volume of cerebral infarct was 41.63 ± 2.802 % in the MCAO vehicle-treated group and 27.21 ± 2.725 % in the MCAO resveratrol-treated group. Intranasal treatment with resveratrol can significantly decrease cerebral infarct volume following MCAO ($P < 0.01$).

3.7. Resveratrol effect on MMP-9 and NF-κB expression

MCAO significantly increase MMP-9 expression levels in the ipsilateral ischemic cortex ($P<0.0001$). However, intranasal resveratrol therapy for cerebral ischemia for 7 days significantly decreased MMP-9 levels as compared to the MCAO vehicle-treated group ($P < 0.01$, Fig. 6A).

We also demonstrated that the NF-κB expression level was significantly upregulated in the MCAO vehicle group compared to the sham group ($P < 0.0001$). Moreover, intranasal administration of resveratrol for 7 days following MCAO significantly attenuated the expression of this gene compared to the vehicle-treated MCAO rats ($P < 0.05$, Fig. 6B).

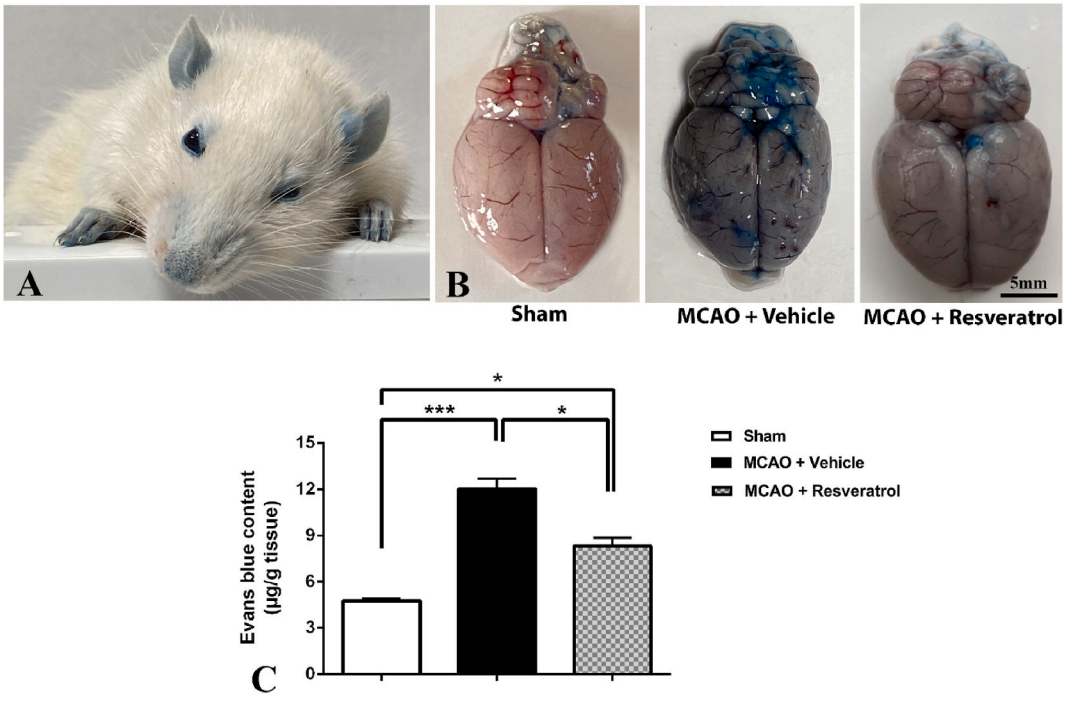

Fig. 3. A, Representative images of rats after administration of EB 2 % at doses of 4 ml/kg by I.V. bolus into the femoral vein. B, Photographs of EB-stained brains from the rats sacrificed 7 days after sham surgery or MCAO treated intranasally with vehicle and resveratrol. Bar = 5 mm. C, Quantitative analysis of the permeability of the blood-brain barrier or EB content (µg/g) from rat brain extracts from the sham-operated, vehicle, and resveratrol-treated MCAO groups 7 days after MCAO (n = 5 rats/group). Data is expressed as mean ± S.E.M (n = 5 rats per group); Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. *$P<0.05$, ***$P<0.001$.
EB, Evans blue; I.V, intravenous; MCAO, middle cerebral artery occlusion.

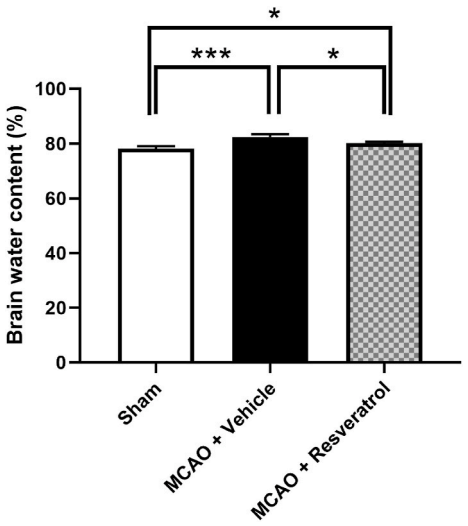

Fig. 4. Quantification of edema or BWC% in the sham-operated, vehicle, and resveratrol-treated MCAO groups 7 days after MCAO using the wet/dry weight technique. Valueswere shown as the mean ± SEM (n = 5 rats/group). Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. *$P < 0.05$, ***$P < 0.001$.
BWC, brain water content; MCAO, middle cerebral artery occlusion.

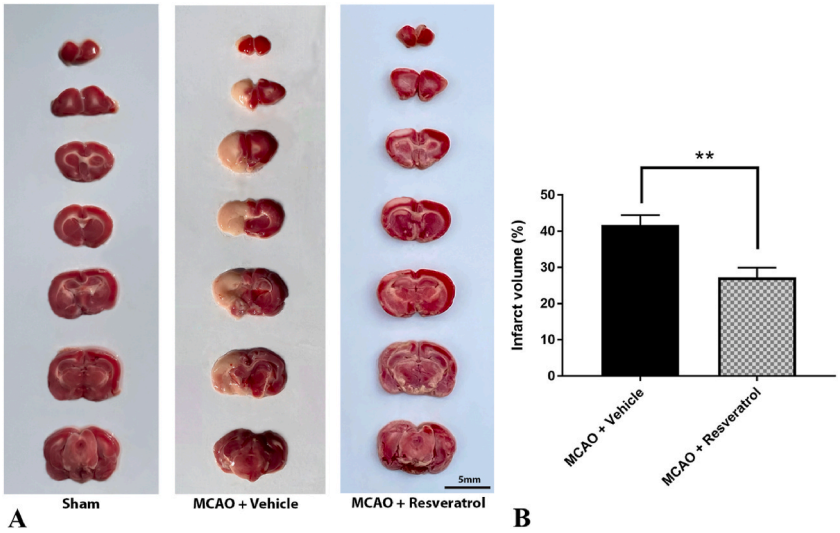

Fig. 5. - A, Representative photographs of TTC-stained brain sections of sham, vehicle, and resveratrol-treated MCAO rats. The non-ischemia regions were stained red, while the ischemic tissue area remained white. Bar = 5 mm
B, Percentage of cerebral infarct volume in the MCAO vehicle-treated, and MCAO resveratrol-treated rats 7 days after MCAO. Valuesare expressed in percentage as mean ± SEM (resveratrol (n = 7), and vehicle MCAO groups (n = 7). Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. **$P < 0.01$.
TTC, 2,3,5-triphenyltetrazolium chloride; MCAO, middle cerebral artery occlusion.

*3.8. Effect of resveratrol on the expression levels of apoptosis-related genes Bax and **Bcl-2***

Our findings indicated a notable decrease in Bcl-2 gene expression ($P < 0.05$, Fig. 7A) and a significant increase in the Bax gene in the MCAO vehicle group ($P < 0.05$, Fig. 7B). However, administration via nasal route with resveratrol resulted in an elevation of Bcl-2 genetic expression and reduction of Bax gene expression when compared with the vehicle-treated MCAO rats, yet these alterations did not show statistical significance. ($P > 0.05$).

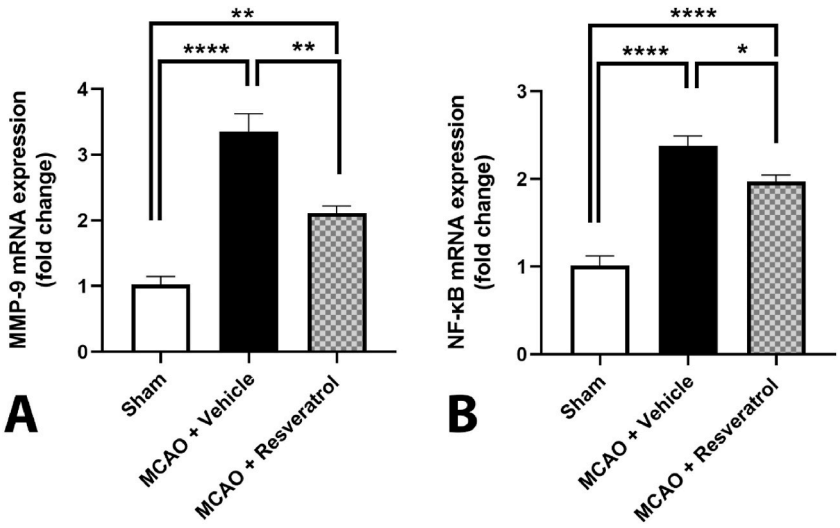

Fig. 6. Quantitative real-time PCR for the mRNA expression of (A) MMP-9 and (B) NF-κB in the ipsilateral ischemic cortex 7 days after cerebral ischemia in sham, vehicle, and resveratrol-treated rats, the results were expressed as fold change over GAPDH. Data are presented as mean ± SEM (n = 6 rats/group). Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. $^{*}P<0.05$, $^{**}P < 0.01$, $^{****}P<0.0001$. MMP-9, Matrix metalloproteinase-9; NF-KB, nuclear factor-kappa B; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

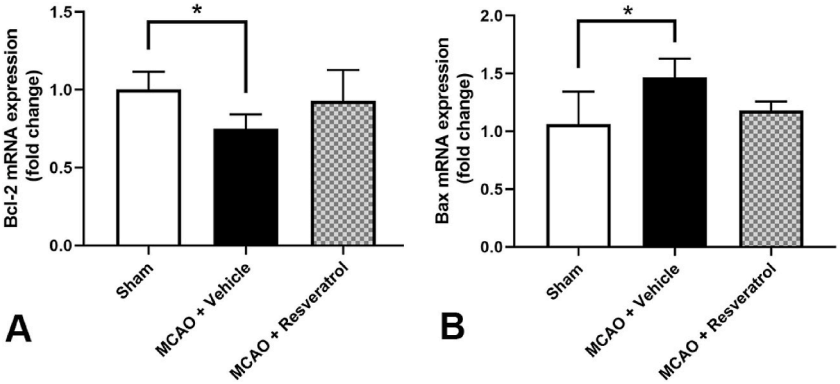

Fig. 7. - Quantitative real-time PCR for mRNA expression of apoptosis-associated genes (A) Bax and (B) Bcl-2 in the ipsilateral ischemic brain cortex 7 days after cerebral ischemia in sham, vehicle-, and resveratrol-treated rats. The results were expressed as fold changes over GAPDH. Data are presented as mean ± SEM (n = 6 rats/group). Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. $^{**}P < 0.001$ Bcl2, B-cell lymphoma protein 2; Bax, B-cell lymphoma protein 2-associated X; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

4. Discussion

Resveratrol has been shown to protect neurons from damage and exert anti-inflammatory and antioxidant effects following cerebral ischemia and reperfusion; however, its exact mechanism of action remains unknown [9]. The therapeutic benefits of resveratrol in IS have been studied after oral, intravenous (IV), and intraperitoneal administration [36]. Furthermore, intranasal administration of resveratrol is expected to increase drug transferability to the brain and improve therapeutic efficacy and safety [37].

This research revealed for the first time, that intranasal administration of resveratrol represents a novel, noninvasive, direct, and rapid treatment approach against brain ischemia in an experimental rat model. The treatment resulted in attenuated neurological deficits, brain edema, BBB disruption, and infarct volume. These effects may be associated with the inhibition of cortical MMP-9 and NF-kB. However, it is noteworthy that intranasal resveratrol did not significantly affect the expression of Bax and Bcl-2.

Intranasal drug delivery is a novel, noninvasive, direct, and rapid method for CNS-targeted medicines to enter the brain parenchyma and cerebrospinal fluid through the trigeminal and olfactory pathways from the nasal cavity while avoiding first-pass metabolism [38,39]. The significant vascularity present in the mucosa, lamina propria, and leaky epithelium provides a significant surface area for drug absorption. Nasal absorption is greatest for the lowest molecular weight and least lipophilic substances, while the mucosa shows low permeability for large polar hydrophilic compounds like peptides and proteins [40]. Resveratrol, a natural polyphenol, faces challenges in being effective when taken orally due to its poor solubility in water, chemical instability, and lipophilicity. This

leads to low levels of the compound in the bloodstream, as it is broken down quickly in the stomach and intestines. Intranasal delivery, however, provides a solution to these issues by passing the digestive system and allowing for direct absorption into the bloodstream, potentially increasing its effectiveness. In our administration method, resveratrol is delivered to the brain through the intranasal route without intestinal absorption and first-pass metabolism by crossing the BBB. According to previously published results, pulmonary administration has been 2.5 times more effective at delivering resveratrol to the bloodstream [41]. Furthermore, it has been demonstrated that resveratrol can cross the BBB and enter cerebral tissue [42]. Neurological deficits carried along by ischemic stroke include sensory and motor impairments. Intranasal treatment of cerebral ischemia with resveratrol significantly reduced neurobehavioral these deficits (Fig. 2B). These results are consistent with a number of previous studies that employed resveratrol to treat brain ischemia. In this regard, resveratrol administration of 10^{-7} g/kg twice intravenously, 15 min before occlusion, and at the time of reperfusion (2 h after occlusion) [9], 30 mg/kg, intraperitoneal injection (i.p.) immediately after MCAO [43], 30 mg/kg i.p. for 4 days after MCAO [16], 30 mg/kg, i.p for 7 days before MCAO [5], 30 mg/kg i.p. for 7 days before MCAO [13], 200 mg/kg, i.p for 7 days before MCAO [44] and 100 mg/kg, but not 10 mg/kg i.p. 2 h after MCAO [45] significantly improved neurological outcomes. The animals' motor functions, neuromuscular strength, and balance were assessed using the hanging wire grip test. Our findings showed that cerebral ischemia significantly reduced the time animals spent hanging from the wire, indicating severe motor deficits following cerebral ischemia (Fig. 2C), which is consistent with a recent study [46]. Moreover, intranasal administration of resveratrol for 7 days following cerebral ischemia significantly enhanced motor function in the wire suspension test compared to the MCAO rats (Fig. 2C). Similar to the current study, Karalis et al. demonstrated that 90 mg/kg resveratrol administered i.p. significantly improved wire hanging ability immediately after cerebral hypoxia-ischemia induction in neonatal rats [47]. Another study also showed that elderly female mice treated with resveratrol via oral gavage for 10 days starting 7 days before MCAO significantly increased wire hanging ability in the wire suspension test [48]. It is worth noting that there are insufficient reports to compare the effects of resveratrol treatment in IS on motor function using a wire-hanging test.

Cerebral ischemia can disrupt the BBB and increase its permeability. When the BBB breaks down, immune cells that have been activated flood the brain, leading to further damage and loss of function. In line with previous research, our findings revealed that MCAO increased the extravasation of EB (Fig. 3C) [33,49,50]. Moreover, intranasal application of resveratrol for 7 days following MCAO significantly reduced EB extravasation in comparison to the vehicle-treated MCAO group (Fig. 3C). Our findings supporting the positive impacts of resveratrol on BBB permeability are consistent with those of Lei et al., who found that EB extravasation was significantly reduced 24 h after cerebral ischemia following treatment with 100 mg/kg resveratrol but not with 10 mg/kg resveratrol [46]. Furthermore, intra-carotid artery administration of resveratrol polymeric nanoparticles showed significant protection against cerebral ischemia, as evidenced by the preservation of the BBB [12]. This is also consistent with a prior study by Dou et al., which showed that 3 days after ischemia, resveratrol treatment (200 mg/kg) significantly reduced EB leakage to the ischemic cortex and striatum. The study also showed that extravasation of EB to the ipsilateral ischemic cortex and striatum was markedly higher in stroke-affected mice than in sham-treated control mice [50]. Furthermore, another study also indicated that the increased EB extravasation caused by ischemia was decreased when 50 mg/kg resveratrol was administered intraperitoneally to rats [49]. Other studies have also described that 200 mg/kg of resveratrol reduced the amount of extravasated EB dye in brain tissue and improved BBB permeability in a rat stroke model [51]. Also in agreement with our findings, the EB extravasation parameter was significantly decreased 24 h after MCAO when resveratrol (1.9 mg/kg; i.v.) was administered at the beginning of reperfusion compared to the control group [33]. Moreover, resveratrol treatment after a single or repeated mild stroke preserves BBB function [14].

Consistent with recent studies, we found that MCAO enhanced cerebral BWC or edema in the ipsilateral ischemic hemisphere (Fig. 4) [33]. In the present investigation, intranasal resveratrol delivery significantly decreased BWC compared with the MCAO vehicle-treated group (Fig. 4). This indicates that brain edema was inhibited by this medication. Our result regarding the beneficial effect of resveratrol on BWC is consistent with the results of Alquisiras-Burgos et al. (2013), which showed that the percentage of water content was significantly decreased when resveratrol (1.9 mg/kg; i.v.) was administered at the initiation of reperfusion compared with the vehicle control rats [33]. Resveratrol treatment (25 mg/kg) after a single or repeated mild stroke also decreases BWC and cerebral edema [14]. In addition, Zou et al. previously determined that pretreatment with resveratrol (100 mg/kg; intraperitoneally, 30 min before TBI) significantly reduced the BWC or edema when compared to the sham group [10]. Besides, He et al. reported that administration of resveratrol (100 mg/kg i.p.) at the beginning of reperfusion significantly decreased BWC after 24 h of reperfusion when compared to the MCAO group [52]. In addition, resveratrol administration at doses of 10 mg/kg [53] and 30 mg/kg or 60 mg/kg [54] could reduce BWC in the ipsilateral brain region after intracerebral hemorrhage (ICH), which would decrease ICH-induced cerebral edema. Moreover, resveratrol was given intravenously at doses of 10^{-8}, 10^{-7}, and 10^{-6} g/kg after reperfusion, which significantly decreased the BWC in a dose-dependent manner (10^{-7} and 10^{-6}) [55]. Besides, i.p. injections of 20 mg/kg resveratrol 10 min before the MCAO surgery and 0 and 20 h after reperfusion have also been shown to decrease BWC [56]. A significant decrease in BWC was also observed after i.p. administration of 50 mg/kg resveratrol following MCAO [49]. Besides, resveratrol treatment after MCAO [22,57] or resveratrol pretreatment (20 or 40 mg/kg) [58] significantly reduced BWC and lowered cerebral edema in rats following focal cerebral ischemia. Additionally, another study also illustrated that IV injection of resveratrol twice, 15 min pre-occlusion and at the time of reperfusion (2 h post-occlusion) significantly reduced BWC [9].

In line with other recent studies, we found that intranasal resveratrol administration for 7 days after cerebral ischemia/reperfusion injury significantly decreased infarct size (Fig. 5B). In this regard, resveratrol administration of 125, 250, 500 μg/kg/day, for 10 days, once a day before MCAO, i.p. [59], 30 mg/kg/day for 3 consecutive days before MCAO, oral gavage or i.p. [60], 10^{-7} g/kg, twice: 15 min pre-occlusion and at the time of reperfusion (2 h post-occlusion), i.v. [9], 200 mg/kg, for 7 days before MCAO, i.p. [44], 50 mg/kg resveratrol for 7 days before MCAO, i.p. [61], 20 and 30 mg/kg, once a day for 6 days before MCAO, i.p. (Wan, Zhou et al., 2016), 2×10^{-4}, 2×10^{-3} mg/kg, 30 min prior to MCAO, i.v. [61], 100 mg/kg for 7 consecutive days after MCAO, i.p. [22], 30 mg/kg/day, 5 min

before reperfusion, i.p. (Grewal, Singh et al., 2019), 200 mg/kg for 3 days after MCAO, i.p [62]. significantly reduced infarct volume compared to those treated with vehicle in MCAO/Reperfusion injury *in vivo* and it also ameliorated oxygen/glucose deprivation/reoxygenation (OGD/R)-induced neuronal damage *in vitro* [6,60,62–65].

MMP-9 is an enzyme that is activated during acute IS. It breaks down proteins in the extracellular matrix, tight junctions, and capillary basal lamina, leading to the disruption of the BBB and the development of cerebral edema. This enzyme is considered a potential marker for acute IS [66]. The expression of MMP-9 is notably increased in both animal models and humans experiencing cerebral ischemia. This increase in MMP-9 expression was associated with the breakdown of the BBB, the formation of edema, and the risk of hemorrhagic transformation [20]. Multiple studies have reported that animals lacking MMP-9 or treated with MMP tissue inhibitors exhibit protection of the BBB integrity. Furthermore, these interventions have been shown to decrease infarct volume, apoptosis, neuronal damage, and brain edema following cerebral ischemia [21,67–71]. Our findings align with numerous previous studies indicating that MCAO increases MMP-9 expression (shown in Fig. 6A). This elevated MMP-9 expression has been associated with various adverse outcomes, including an increase in BBB permeability, inflammation, apoptosis, and neuronal damage [49, 71–74]. Our result regarding resveratrol intranasal administration is consistent with previous studies that show resveratrol may decrease MMP-9 expression levels (Fig. 6A) [75]. In alignment with our results, *Gao* et al. reported that pretreatment with resveratrol (50 mg/kg, administered orally for 7 days before surgery) inhibited the increase of MMP-9 in mice 12 or 24 h after the onset of the ischemic insult. This emphasizes resveratrol's potential to modulate MMP-9 expression in ischemic conditions [76]. Additionally, co-administration of resveratrol at a dose of 2.5 mg/kg significantly improves treatment outcomes for patients undergoing delayed recombinant tissue plasminogen activator therapy compared to placebo. This improvement is attributed to resveratrol's significant reduction in plasma levels of MMP-2 and MMP-9 [77]. Furthermore, Cheng et al., demonstrated that resveratrol possesses neuroprotective characteristics through down regulation of MMP-9 in an OGD-exposed neuron model [6]. Our results are consistent with other studies that found treatment with resveratrol (50 mg/kg, i.p.) attenuated BBB dysfunction by reducing MMP-9 expression in treated groups compared to controls [49]. Resveratrol treatment has a therapeutic impact in rats following focal cerebral ischemia by suppressing the upregulation of inflammatory factors such as Toll-Like receptor 4, TNF-α, COX-2, NF-κB p65, MMP-9, and IL-1β that were caused by cerebral ischemia [22]. In addition, resveratrol inhibited the activation of inflammatory mediators and cell apoptosis by downregulating MMP-9 expression in primary cortical neurons [63] and microglia [60] cultures with OGD. Additionally, resveratrol-treated (30 mg/kg, i.p.) rats demonstrated reduced brain damage following subarachnoid hemorrhage due to MMP-9 suppression [24].

NF-κB is a transcription factor commonly found in the cytoplasm. Upon activation, it translocates to the nucleus to regulate the expression of genes encoding proteins associated with apoptosis, proliferation, and inflammation. We found that MCAO significantly elevated NF-κB levels (Fig. 6B), which is consistent with other studies that found NF-κB was overexpressed after cerebral ischemia [23]. However, intranasal treatment with resveratrol significantly downregulated the expression level of this gene (Fig. 6B). Our findings are in line with an earlier study that discovered resveratrol exerts neuroprotective effects by inhibiting NF-κB signaling in cortical neuron cultures with OGD as an *in vitro* model of ischemia [23]. In addition, Simão et al. reported that treatment with resveratrol (30 mg/kg, i.p., for 7 days prior to ischemia-reperfusion induction) reduced inflammation via blocking NF-κB activation [78]. Furthermore, resveratrol (10 or 100 mg/kg, i.p.) post-treatment significantly downregulated the expression levels of NF-κB p65, which reduces inflammation-induced ischemic brain damage [22]. Additionally, resveratrol-treated (30 mg/kg, i.p.) rats showed reduced brain damage following subarachnoid hemorrhage by inhibiting the NF-κB activation cascade [24]. Besides, resveratrol reduces brain injury in IS by downregulating inflammatory factors such as NF-κB p65 in rats [22,58]. We also found a significant decrease in anti-apoptotic Bcl-2 expression and an increase in pro-apoptotic Bax expression in the MCAO vehicle group (Fig. 7A and B), which is consistent with other research that found Bcl-2 was downregulated, and Bax was upregulated after cerebral ischemia [5,25, 79,80]. Resveratrol intranasal administration increased the expression of the anti-apoptotic gene Bcl-2 and decreased the expression of the pro-apoptotic gene Bax; however, these changes were not statistically significant (Fig. 7A and B). Accordingly, Kizmazoglu et al., demonstrated that resveratrol (20 and 40 mg/kg, i.p.) significantly enhanced Bcl-2 levels after 30 min of bilateral carotid artery occlusion [80]. In addition, resveratrol treatment (30 mg/kg, i.p.) for 4 days following MCAO [78] and for 7 days before MCAO [5] upregulated the protein Bcl-2 expression and downregulated the protein Bax expression in the cerebral hippocampus and cortex, respectively. Resveratrol injection (5 mg/kg) 3 h after MCAO also increased the anti-apoptotic Bcl-2 gene transcription [80]. Furthermore, hippocampal Bcl-2 in the resveratrol-treated vascular dementia group was significantly increased, while Bax expression was significantly decreased after 4 weeks of resveratrol treatment (20 and 10 ml/kg, i.p.) [25]. In addition, administration of resveratrol showed significant protection against cerebral ischemia/reperfusion injuries, as evidenced by an enhanced Bcl-2/Bax ratio [11,12]. Besides, the effect of resveratrol on the expression of Bax and Bcl2 in PC12 cells receiving oxygen and glucose deprivation (OGD) for 6 h and reoxygenation for 24 h was evaluated, and the results indicated that resveratrol upregulated the expression of Bcl-2 and downregulated the expression of Bax at mRNA and protein levels [81]. Furthermore, western blot analysis conducted 24 h after oxygen–glucose deprivation/reoxygenation (OGD/R) injury showed a significant increase in the anti-apoptotic protein Bcl-2 in both the resveratrol pretreatment and post-treatment groups compared to the sham group [21]. Furthermore, resveratrol administration (50 mg/kg) for 7 days before MCAO upregulated the ratio of Bcl-2/Bax [82]. Interestingly, another study verified resveratrol's anti-apoptotic effects by demonstrating a significant increase in Bcl-2 expression after resveratrol (100 mg/kg, i.p.) administration three times at 0 h, 8 h, and 18 h following hypoxic-ischemic brain damage. However, it significantly inhibited the Bax up-regulation in the rats' cerebral cortex and hippocampus during hypoxia/ischemia [81].

The current study presents several limitations that may potentially influence the comprehensiveness and applicability of the findings. Firstly, the reliance on the minimum necessary sample size of rats could restrict the depth of the analysis. Moreover, the utilization of a single dose of resveratrol may not fully encapsulate its potential therapeutic benefits, necessitating future research to

explore varying dosages and thereby enhance our understanding of its possible advantages. Additionally, the assessment period, confined to specific intervals up to a week post-operation, might curtail the scope of our results. Extending the follow-up duration in subsequent studies could potentially furnish a more nuanced understanding of both the positive and negative implications of the treatments undertaken. Moreover, protein western blot data for MMP9, Bcl2, Bax, and NF-kB vs. cytosolic localization were not feasible, potentially affecting the depth of our conclusions. These data are correlative and cannot demonstrate the cause and effect relations between NF-kB, MMP-9 and reversal of inflammation/BBB breakdown. Future studies should consider incorporating these analyses for a more comprehensive understanding.

5. Conclusion

In conclusion, intranasal administration of resveratrol in an acute brain ischemia rat model resulted in significant improvements in neurobehavioral function, latency to fall off the wire-hanging performance, BBB disruption, cerebral edema, and infarct volume. MMP-9 and NF-kB expression were decreased, while the expression of the pro-apoptotic gene Bax and the anti-apoptotic gene Bcl-2 did not alter substantially in resveratrol-treated MCAO rats. Resveratrol may be a treatment option for IS and further studies are required to confirm its therapeutic potential.

Ethics statement

The study was reviewed and approved by the local Ethics Committees of the Behbahan Faculty of Medical Science, Behbahan, Iran, with the approval number: IR.BHN.REC.1401.061. The experiment was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Every attempt was taken to decrease the amount of animal used and to limit their pain and discomfort.

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Data availability statement

All data used in the generation of the results presented in this manuscript will be made available upon reasonable request from the corresponding author.

CRediT authorship contribution statement

Maryam Owjfard: Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Zahra Rahimian:** Writing – review & editing, Resources, Investigation. **Rezvan Ghaderpanah:** Writing – review & editing, Resources, Investigation. **Elahe Rafiei:** Writing – review & editing, Visualization, Resources, Investigation. **Seyedhassan Sadrian:** Writing – review & editing, Resources, Investigation. **Mohammad Sabaghan:** Writing – review & editing, Software, Formal analysis, Data curation. **Farzaneh Karimi:** Writing – original draft, Validation, Project administration, Methodology, Conceptualization.

Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors did not utilize any AI or AI-assisted technologies for tasks beyond basic grammar and spelling checks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

MCAO middle cerebral artery occlusion
mNSS modified neurologic severity scores

BBB	blood brain-barrier
BWC	brain water content
MMP-9	Matrix metalloproteinase-9
NF-κB	nuclear factor-kappa B
Bcl-2	B-cell lymphoma protein 2
Bax	B-cell lymphoma protein 2-associated X
mRNA	messenger RNA
IS	ischemic stroke
ATP	adenosine triphosphate
DMSO	dimethyl sulfoxide
EB	evans blue
i.p,	Intraperitoneal injection
OGD	Oxygen glucose deprivation

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