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Effects of *Vaccinium arctostaphylos* L. Seeds Oil on the Cerebral Stroke in Rat: A Biochemical, Immunohistochemically and Molecular Approach

Efectos del Aceite de Semillas de *Vaccinium arctostaphylos* L. en el Ataque Cerebrovascular en Ratas: Un Enfoque Bioquímico, Inmunohistoquímico y Molecular

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SUMMARY: Cerebral ischemic stroke -reperfusion (CIS/R) injury poses a significant threat to cognitive function. In this study, we explored the effects of *Vaccinium arctostaphylos* L. seeds oil (VASO) on a CIS/R model in Wistar rats and investigated its underlying mechanisms. Fifty Wistar rats were randomly divided into five groups: a normal group, a VASO-supplemented group (receiving 400 mg/kg VASO), an ischemic stroke-reperfusion (IS/R) group, and two IS/R groups treated with 200 mg/kg and 400 mg/kg VASO, each comprising 10 rats. Cognitive performance was assessed using the Y-electric maze test. Additionally, we employed enzyme-linked immunosorbent assay (ELISA) and real-time PCR to examine the expression of proteins and genes associated with neurotrophic growth, inflammation, and apoptosis in the brain. Compared to the normal group, rats subjected to IS/R exhibited a significant delay in meeting the maze criteria. The IS/R group also demonstrated markedly elevated levels of pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), alongside reduced levels of anti-inflammatory cytokines (IL-4 and IL-10) and neurotrophic growth factors [brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF)]. The group treated with 400 mg/kg VASO after IS/R displayed improvements, with increased levels of neurotrophic growth factors, and a favorable shift in the protein and gene expression profiles of inflammatory and apoptotic markers (including glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF-6), Bcl-2, Bax, and p53) compared to the IS/R group. These results suggest that VASO extract may mitigate cognitive impairments following IS/R in rats, potentially through its modulation of microglia-secreted inflammatory factors.

KEY WORDS: ***Vaccinium arctostaphylos* L. seeds oil; Inflammation; Apoptosis; Cerebral ischemic stroke -reperfusion.**

INTRODUCTION

Cerebral stroke (CS) is a leading global cause of death and disability, triggered by interrupted brain blood flow, leading to rapid cell death. Ischemic strokes, which account for 85 % of cases, result from blood clots, while hemorrhagic strokes stem from ruptured vessels. Risk factors include age, hypertension, and diabetes. Ischemic strokes are particularly challenging, often causing early neurological decline and long-term disability (Das & Rajanikant, 2018). They induce excessive reactive oxygen species (ROS) production, leading to oxidative stress, apoptosis, and cell death in cerebral ischemia-reperfusion injury (CIS/R). Key damage mechanisms include excitotoxicity and inflammatory responses driven by cytokines like IL-1β and TNF-α. Current treatments are limited to aspirin and thrombolytic therapy for select patients. Despite advances in understanding CS and CIS/R, effective therapies remain elusive due to severe side effects, narrow treatment windows, and additional damage from reperfusion. However, animal studies show promise in targeting these pathways to mitigate brain injury (Sarkar *et al*., 2019).

CS and CIS/R involve complex mechanisms, including apoptosis, neurotrophic factors, and stress responses. Key apoptotic pathways include Bax, Bcl-2, p53, and caspase-3, which regulate neuronal death. Studies in rodent models show that manipulating these pathways, such as increasing Bcl-2 or inhibiting p53, reduces brain damage (Yan *et al*., 2021). Neurotrophic factors like BDNF and

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GDNF play crucial roles in neuronal survival and recovery post-stroke, with therapies targeting their upregulation showing promise (Mokhtari *et al*., 2017). GRP78 is a chaperone protein that helps manage ER stress by assisting in protein folding and preventing apoptosis. In rodent models of ischemic stroke, GRP78 is upregulated as a protective response. Enhancing GRP78 expression has been shown to reduce neuronal apoptosis and infarct size, suggesting its potential as a therapeutic target (Louessard *et al*., 2017). ATF-6 is a transcription factor activated during ER stress, leading to the expression of protective genes like GRP78. ATF-6 activation has been observed in animal models following CIS/R, with studies indicating that modulation of ATF-6 can influence the extent of brain injury. Targeting the ATF-6 pathway might offer a new approach to mitigating ER stress-related damage in stroke (Celik *et al*., 2020). Animal models, particularly rodents, have been instrumental in studying these pathways, providing insights into potential therapeutic strategies, such as antioxidants, neurotrophic factor therapy, and ER stress modulation, to mitigate CIS/R-induced brain injury (Louessard *et al*., 2017; Celik *et al*., 2020).

Interest in traditional herbal medicines has surged, driven by their potential in drug development due to their multi-target capabilities, synergistic effects, and holistic approach, offering advantages over the conventional single-target, single-compound methods (Duan *et al*., 2022). *Vaccinium arctostaphylos* L. seed oil (VASO), derived from a plant in the Ericaceae family, is native to Eastern Europe, Asia, and Northern Africa, thriving in humid to semi-humid regions with blooming pinkish-white flowers from July to September. VASO is rich in proteins, vitamins, carbohydrates, and minerals, making it valuable in food products, antimicrobial films, and quality oil production. LC-ESI-MS/MS analysis reveals that VASO contains bioactive compounds like isoflavonoids (daidzein, luteolin), flavonoids (apigenin, quercetin), saponins, carotenoids, essential fatty acids, anthocyanins, and amygdalin derivatives (Shamilov *et al*., 2022). Predominantly used in Asia, particularly China, VASO is effective in treating ischemic heart diseases, providing anti-anginal effects, and enhancing antioxidant and anti-inflammatory responses, as well as coronary flow velocity (Akhone *et al*., 2022). Studies indicate that VASO can prevent acute myocardial infarction in rats by reducing infarct size and suppressing myocardial cell apoptosis (Jaafar, 2021). VASO's anti-inflammatory and anti-apoptotic effects are mediated by inhibiting interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), leading to downregulation of nuclear factor kappa B (NF-κB) and modulation of apoptotic pathways involving mitogen-activated protein kinases (MAPKs) and the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) signaling pathways (Zhang *et al*., 2017). Flavonoids in VASO, such as apigenin, quercetin, and daidzein, primarily contribute to reducing inflammation and pain related to central nervous system diseases by inhibiting cyclooxygenase-2 and lipoxygenase, key enzymes in the inflammatory cascade (Charalabopoulos *et al*., 2019). Pharmacokinetic studies have confirmed that seven compounds from VASO are absorbed into the bloodstream following oral administration in rats.

This study aims to further explore which bioactive compounds reach injured brain tissue and contribute to neuroprotection after VASO extract administration. The neuroprotective effects of VASO pretreatment, particularly its anti-apoptotic and anti-inflammatory properties, were examined using the Morris water maze to assess its impact on rat brain function.

MATERIAL AND METHOD

Preparation of VASO. Fresh *Vaccinium arctostaphylos* L. seeds (8000 g) were first dried at 32 °C in a dark setting after being authenticated by a botanist. The seeds were then ground into a fine powder using a soil grinder. This powder was mixed with a 50:50 ethanol/acetone solution (v/v) and incubated at 38 °C for 48 h. The mixture was then filtered using a paper filter and concentrated through a rotary evaporator. The final extract (430 grams) was stored at 4 °C (Bazm *et al*., 2018).

Middle cerebral artery occlusion (MCAO). The rats were weighed and then anesthetized intraperitoneally with chloral hydrate (400 mg/kg) from Merck, Germany. The MCAO procedure was performed following the method described by Wang *et al*. (2017). During the procedure, a 3–0 silicone-coated nylon suture was microscopically inserted through the stump of the external carotid artery. The suture was advanced into the internal carotid artery approximately 20–22 mm past the carotid bifurcation until slight resistance was felt, indicating the tip had reached the anterior cerebral artery, thereby obstructing blood flow to the middle cerebral artery. After 60 minutes of ischemia, reperfusion was initiated by carefully retracting the suture. Throughout the surgery, rectal temperature was monitored using a Citizen-513w thermometer and maintained at 37.0 °C with surface heating and cooling (Wang *et al*., 2017).

Experimental design. Fifty male Wistar rats, each weighing 170±25 g, were randomly divided into five groups (n=10 per group). Before the study began, the rats underwent a 24-h acclimation period to adjust to the study environment, including temperature, food, and water conditions. They were housed in propylene cages maintained at 23±4 °C with a relative humidity of 30±5 %, under a 12-h light/dark cycle. The rats had free access to standard pellets and tap water.

All procedures for their care and euthanasia were conducted with the approval and oversight of the Dalian Medical University ethics committee, in strict compliance with established protocols for laboratory animal care. The normal group and the ischemic stroke-reperfusion (IS/R) group received intraperitoneal injections of 0.5 ml PBS. The co-treatment groups (IS/R +200 and IS/R +400 VASO) received oral doses of 200 mg/kg and 400 mg/kg of VASO, respectively. Additionally, the normal+400 VASO group received 400 mg/kg of VASO orally. The LD50 method, alongside preliminary studies and existing research on VASO, was used to determine the non-toxic effective dose. VASO was administered daily at a consistent time (9 am) for 50 days (Akbari Bazm *et al*., 2020; Khordad *et al*., 2024).

LD_{50} for VASO. The LD_{50} of VASO was assessed using Lork's two-step procedure. Initially, nine rats were divided into three groups and given VASO at doses of 30, 300, and 3000 mg/kg. These animals were monitored for signs of mortality or toxicity over a 24-h period. Following this, an additional set of three rats per group received VASO at doses of 10, 100, and 1000 mg/kg, with similar monitoring for toxicity. The LD_{50} was determined using Lork's formula, which calculates the median lethal dose based on the lowest dose causing mortality (D toxic) and the highest dose without causing observed mortality (D safe). The formula used was:

$$LD_{50} = (D\ safe \times D\ toxic)^{1/2}$$ (Choudhary & Jain, 2021).

Morris water maze test. The Morris water maze, a well-established method for evaluating spatial learning and memory (Morris *et al*., 1982), was utilized in this study from day 20 to day 40 post- IS/R. The maze consisted of a circular tank (120 cm in diameter and 50 cm deep) located in a dark room, quiet room with distinct visual markers. The water temperature was kept at 23 ± 4 °C. A black, round platform (10 cm in diameter) was submerged 2 cm below the water's surface in a fixed position at the center of one quadrant throughout the training period. Rats' swimming paths were recorded using a video camera connected to a computer, with data analyzed by image software. Each rat underwent training twice daily for five consecutive days. In each trial, the rat was placed into the pool facing the wall from one of four randomly assigned starting points and allowed to swim. The time taken to locate the hidden platform (escape latency) was recorded, with a maximum duration of 90 s. If a rat did not find the platform within this time, it was guided to it and allowed to remain there for 15 s, with the escape latency noted as 90 s. One day after the final training session, the platform was removed, and a 90-s probe test was conducted to assess memory retention. The frequency with which the rats crossed the area where the platform had been positioned was also recorded during this probe trial (Othman *et al*., 2022).

Glutathione peroxidase (GPx), Catalase (CAT), and Superoxide dismutase (SOD) serum activity. We employed a sandwich ELISA kit tailored for rodents, provided by Cusabio from China, to measure the serum levels of SOD (Catalog No.: CSB-EL022397RA), CAT (Catalog No.: CSB-E13439r), and GPx (Catalog No.: CSB-E12146r) according to the manufacturer's instructions (Famurewa *et al*., 2023).

Serum levels of nitric oxide (NO). To assess serum nitric oxide (NO) levels, which are key indicators of lipid peroxidation and oxidative stress, we employed the Griess colorimetric method. Specifically, 500 ml of serum samples were combined with 6 mg of zinc oxide, mixed thoroughly, and then centrifuged at 10,000 *g* for 15 min. The supernatant was subsequently mixed with 500 ml of Griess reagent. After incubating the mixture for 60 min at 37 °C, the absorbance was measured using a Stat Fax ELISA reader (303 microwell reader, Awareness Technology, USA) at wavelengths of 540 nm and 630 nm.

Liver tissue thiol, lipid peroxidation levels [thiobarbituric acid reactive substances (TBARS)], and total antioxidant capacity (FRAP levels) levels. To evaluate the total antioxidant capacity, we employed the FRAP assay. For this, 100 mg of brain tissue was homogenized at 4 °C and mixed with 200 µl of cold PBS. A 100 µl aliquot of this mixture was then combined with 10 µl of FRAP reagent. After incubating for 15 min at 25 °C and centrifuging at 12,000 *g* for 10 min, the absorbance of the supernatant was measured at 593 nm using a Stat Fax ELISA reader (303 microwell readers, Awareness Technology, USA) (Badawi, 2022).

Lipid peroxidation levels were assessed by measuring TBARS in brain tissue. Here, 100 µl of homogenized brain tissue was mixed with 100 µl of TBARS solution, incubated at 37 °C for 30 min, and then centrifuged at 12,000 *g* for 5 min. The absorbance of the supernatant was read at 593 nm with an ELISA reader (Badawi, 2022).

For determining thiol levels, a key antioxidant marker, 100 µl of homogenized brain tissue was combined with 20 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The mixture was incubated at 37 °C for 15 min and centrifuged at 12,000 *g* for 5 min. Absorbance of the supernatant was measured at 412 nm using an ELISA reader (Badawi, 2022).

Serum concentrations of GDNF, BDNF, TNF-α, IL-4, IL-1β, IL-10, and IL-6. The serum was obtained by centrifuging the blood at 10,000 *g* for 20 min. Levels of GDNF (Cat. No.: ab244211) and BDNF (Cat. No.: ab108319) in the serum were measured using ELISA kits from Abcam (Abcam, USA). The assays were conducted according to the manufacturer's instructions and protocols.

To evaluate the anti-inflammatory effects of VASO, we measured the levels of various cytokines using rodent-specific sandwich ELISA kits from Novus Biologicals (USA). Specifically, we quantified anti-inflammatory cytokine IL-10 (Cat. No.: R1000) and anti-inflammatory cytokine IL-4 (Cat. No.: NBP1-91171) along with pro-inflammatory cytokines IL-1β (Cat. No.: RLB00), TNF-α (Cat. No.: NBP2-DY410), IL-6 (Cat. No.: M6000B), and, as well as the, following the provided protocol (Lucini *et al*., 2018).

Glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF-6), Bcl-2, Bax, and p53. Total RNA was isolated using Trizol according to the manufacturer's protocol (Ambion, China). Five micrograms of this RNA were converted to cDNA with random primers. Quantitative real-time PCR was performed with SYBR Green (Vazyme, China) on an ABI7900 fluorescence PCR system (CA, USA). The PCR conditions were set as follows: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 30 s at 95 °C and 30 s at 60 °C. Gene expression levels were normalized to GAPDH. The primer sequences used were:

GAPDH: Forward: TGAAGGTCGGAGTCAACGG; Reverse: AGAGTTAAAAGCAGCCCTGGTG
GRP78: Forward: ATGAGTCCACACCCAGAA; Reverse: TCACTGCGGATAGCAGAG
Bcl-2: Forward: AGGAGGAGTGTGAGGAGGAG; Reverse: TGGAGGAGGAGGAGGAGAG
Bax: Forward: GCCGAGGATGATTGCTGAC; Reverse: TCTCCAGCCATGATGGGTT
P53: Forward: GGAAGACAGGCCCAGACTAT; Reverse: GCTCGACGCTAGGATCTGAC
ATF-6: Forward: ATGAGCGGATCCGCGAGAC; Reverse: TCAGGAGCAGCTGTTGTCCT

Relative gene expression levels were calculated using the threshold cycle (Ct) method, along with ΔΔCt and fold change formulas.

The fold formula change = $2^{-\Delta\Delta CT}$; ΔΔCt = [(Ct sample - Ct GAPDH gene) - (Ct sample – Ct control)] (Yardim *et al*., 2020).

Expression ATF-6, GRP78, and p53 proteins in brain with western blotting. The samples were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors for 30 min. Protein concentration was measured using a BCA protein assay kit (Beyotime, China). Following this, 40 μg of protein from each sample was separated by electrophoresis on a 10 % SDS–polyacrylamide gel and subsequently transferred to a PVDF membrane. The membranes were blocked for 1 hour with a solution containing 5 % (w/v) skim milk powder, and then incubated overnight at 4 °C with the following antibodies from Abcam, UK: anti-ATF-6 (Cat. No. ab37149; 1:200), anti-GRP78 (Cat. No. ab76-E6; 1:200), and anti-p53 (Cat. No. ab154036; 1:200). After three washes with PBST, the membranes were probed with an HRP-conjugated secondary antibody (1:5000) for 1 h. Protein bands were detected using ECL (Amersham Pharmacia Biotech, Piscataway, NJ), and their intensities were quantified with ImageJ gel analysis software. Each experiment was performed in triplicate (Yardim *et al*., 2020).

Brain histopathology. In this study, methylene blue staining was used to examine brain tissue, highlighting neuronal structures and cellular details. Brain tissues were first fixed in 10 % paraformaldehyde for 24-48 h, followed by washing with phosphate-buffered saline (PBS). They were then dehydrated through a graded series of ethanol solutions and cleared with xylene or an alternative clearing agent. The samples were embedded in paraffin, sectioned into 5-10 μm slices using a microtome (LEICA SM2010RV1.2, Germany), and rehydrated. Sections were stained with a 1 % methylene blue solution for 10-20 min, rinsed in PBS, re-dehydrated, cleared, and mounted with a mounting medium and coverslip. Histological analysis was conducted using a light microscope at ×100 and ×400 magnifications, with images captured by a BX61TRF calibrated light microscopy system (Olympus, Japan) and processed using ImageJ software (Lin *et al*., 2017).

Statistical analysis. Statistical analysis was performed using graphpad prism 7.0 (san diego, ca, usa). The data are presented as mean ± standard deviation (sd) and were assessed using one -way analysis of variance (anova). Statistical significance was considered at a p value of less than 0.05.

RESULTS

LD_{50} of VASO. After a 24-hour observation period for the groups treated with VASO, it was determined that a safe dose (D safe) was 1000 mg/kg, while the toxic dose (D toxic) was 3000 mg/kg. Using Lork's formula, the LD_{50} for VASO was calculated to be 1732 mg/kg. This suggests that doses below this LD_{50} value are appropriate for use in animal studies.

VASO improved IS/R -induced spatial cognitive deficits. To evaluate the impact of IS/R and VASO treatment on cognitive function, we subjected the animals to the Morris water maze test after 50 days of treatment. During daily training, all rats showed a progressively decreased escape latency, with notable differences between the groups. One-way ANOVA revealed that starting from day 4, the normal group had a significantly shorter latency in locating the platform compared to the IS/R group ($p < 0.05$), with similar results observed on day 5 ($p < 0.05$). These findings indicate memory impairment due to IS/R. On days 4 and 5, the escape

latency was significantly prolonged in the IS/R +400 VASO group ($p < 0.05$), although IS/R +200 VASO treatment did not show a statistically significant difference compared to the IS/R group ($p > 0.05$). This suggests that IS/R +400 VASO treatment effectively improved spatial learning across the 5-day training period. Additionally, in the probe test, where the platform was removed, rats in the IS/R group crossed the previous platform area fewer times compared to the normal group ($p < 0.05$). Rats treated with IS/R +400 VASO crossed the platform location more frequently than those in the IS/R group ($p < 0.05$), indicating improved spatial memory (Fig. 1).

Effects of IS/R and VASO on serum concentrations of GDNF and BDNF. IS/R inhibited the secretion of neurotrophic factors in the brain by triggering apoptotic pathways in glial cells, leading to a significant reduction ($p < 0.05$) in serum concentrations of GDNF and BDNF compared to the normal group. VASO treatment, however, elevated serum levels of GDNF and BDNF in a dose-dependent manner, highlighting VASO's potent ability to stimulate and protect glial cells. Notably, at a 400 mg/kg dose, VASO significantly ($p < 0.05$) increased GDNF and BDNF levels in the IS/R +400 VASO group compared to the IS/R group (Fig. 2a).

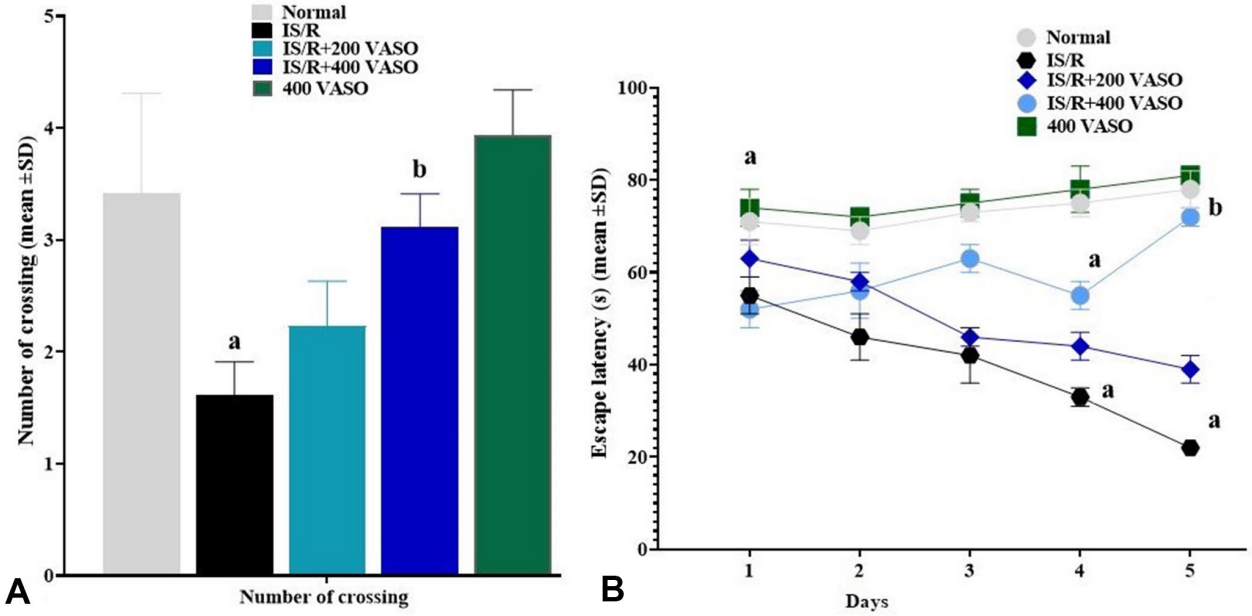

Fig. 1. (a) The number of crossing and (b) Escape latency (s) (means ± SD; n=10/group) in experimental groups. a ($p<0.05$) IS/R *vs*. normal groups; b ($p<0.05$) IS/R +200 and 400 VASO treated *vs*. IS/R groups.

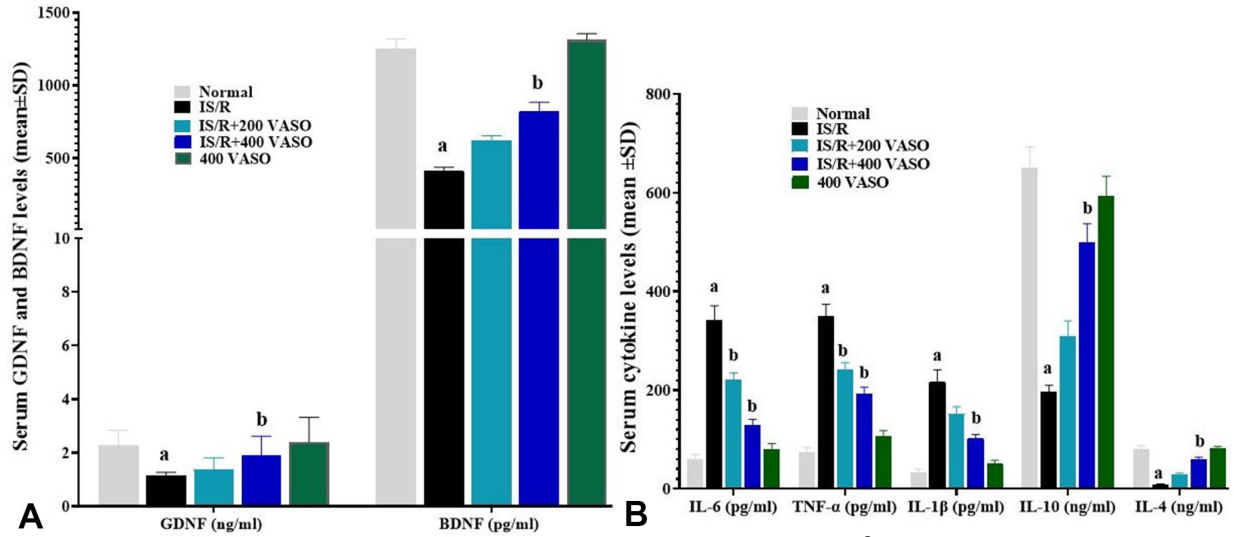

Fig. 2. (a) Serum levels of GDNF (ng/ml) and BDNF (pg/ml) and (b) Serum levels of IL-1β, TNF-α, IL-6 (pg/ml), IL-10, and IL-4 (ng/ml) (means ± SD; n=10/group) in experimental groups. a ($p<0.05$) IS/R *vs*. normal groups; b ($p<0.05$) IS/R +200 and 400 VASO treated *vs*. IS/R groups.

Effects of IS/R and VASO on serum concentrations of TNF-α, IL-6, IL-10, and IL-1β. IS/R triggered inflammatory responses, leading to increased levels of pro-inflammatory cytokines while suppressing systemic anti-inflammatory cytokine activity. This resulted in a significant rise ($p < 0.05$) in the serum concentrations of TNF-α, IL-1β, and IL-6 compared to the normal group, alongside a marked reduction ($p < 0.05$) in IL-10 and IL-4 levels. Treatment with 200 mg/kg of VASO significantly lowered ($p < 0.05$) serum levels of TNF-α and IL-6 compared to the IS/R group, demonstrating VASO's strong anti-inflammatory properties. Specifically, VASO treatment progressively increased IL-10 and IL-4 levels and decreased pro-inflammatory cytokines (IL-1β, TNF-a, and IL-6), with these effects being significant ($p < 0.05$) at the 400 mg/kg dose within the IS/R +400 VASO group compared to the IS/R group (Fig. 2b).

Effects of IS/R and VASO on serum GPx, CAT, and SOD activity alongside serum NO levels. IS/R induced the production of free radicals, resulting in a significant increase in serum NO levels compared to the healthy group. Conversely, VASO treatment led to a dose-dependent decrease in NO concentrations relative to the IS/R group. Administration of VASO at both 200 and 400 mg/kg significantly reduced ($p < 0.05$) serum NO levels, underscoring its potent antioxidant properties. Additionally, IS/R significantly reduced the serum activity of all three antioxidant enzymes compared to normal rats. However, VASO treatment dose-dependently enhanced the serum concentrations of these enzymes, with a significant increase ($p < 0.05$) observed at the 400 mg/kg dose in the IS/R +400 VASO group compared to the IS/R group (Fig. 3a).

Effects of IS/R and VASO on brain thiol, FRAP, and TBARS levels. Thiol, FRAP, and TBARS levels were measured as key indicators of overall antioxidant capacity and lipid peroxidation (LPO). The results revealed that IS/R significantly ($p < 0.05$) reduced the levels of these markers in tissues compared to the normal group. However, due to its potent antioxidant properties, VASO treatment led to a dose-dependent increase in these markers compared to the IS/R group. This increase was significant ($p < 0.05$) at both the 200 and 400 mg/kg doses in the IS/R +200 and 400 VASO groups (Fig. 3b).

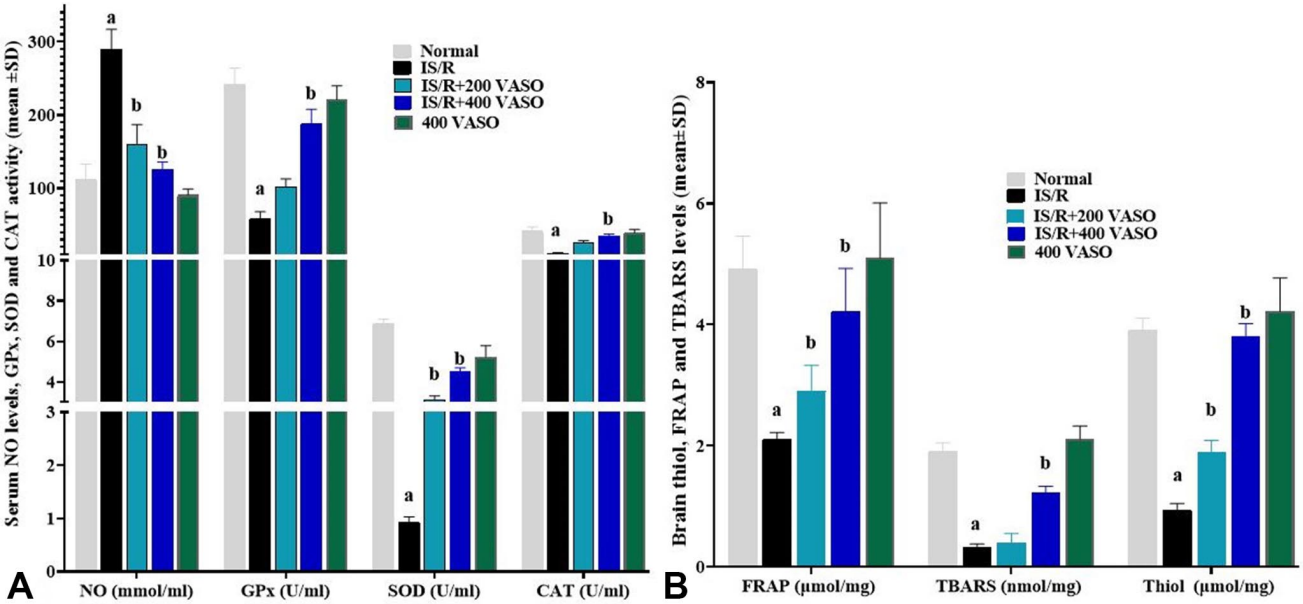

Fig. 3. (a) Serum levels of NO (mmol/ml), alongside the mean serum activity of SOD, CAT, and GPx (U/ml) and (b) Brain tissue levels of TBARS (nmol/mg), thiol (µmol/mg) and FRAP (µmol/mg) (means ± SD; n=10/group) in experimental groups. a ($p<0.05$) IS/R *vs.* normal groups; b ($p<0.05$) IS/R +200 and 400 VASO treated *vs.* IS/R groups.

Effects of IS/R and VASO on expression of brain Bcl-2, Bax, IL-6, GRP78, ATF-6, and p53 genes. Gene expression analysis focusing on apoptosis, oxidative stress, and the metabolism pathways of glial and neural cells revealed that IS/R treatment led to a significant upregulation of Bax, ATF-6, GRP78, and p53, along with a significant downregulation of Bcl-2 in the brain compared to the normal group ($p < 0.05$). In the IS/R +200 VASO group, Bax expression was significantly reduced ($p < 0.05$) compared to the IS/R group. The most substantial changes were observed in the IS/R +400 VASO group, where Bax, ATF-6, GRP78, and p53 expression were significantly ($p < 0.05$) elevated, while Bcl-2 expression was significantly ($p < 0.05$) decreased in comparison to the IS/R group (Fig. 4a).

Effects of IS/R and VASO on expression of brain GRP78, ATF-6, and p53 proteins. To evaluate the impact of IS/R on brain pathways associated with apoptosis, oxidative stress, and metabolism, we examined the protein expression levels of GRP78, ATF-6, and p53. The analysis revealed a significant increase ($p < 0.05$) in the expression of these proteins compared to the normal group. However, in the IS/R +200 VASO group, there was a decrease in GRP78, ATF-6, and p53 protein expression relative to the IS/R group, although these changes were not statistically significant ($p > 0.05$). The most pronounced alterations were observed in the IS/R +400 VASO group, where a significant reduction ($p < 0.05$) in GRP78, ATF-6, and p53 protein expression was noted compared to the IS/R group (Fig. 4b and Fig. 4c).

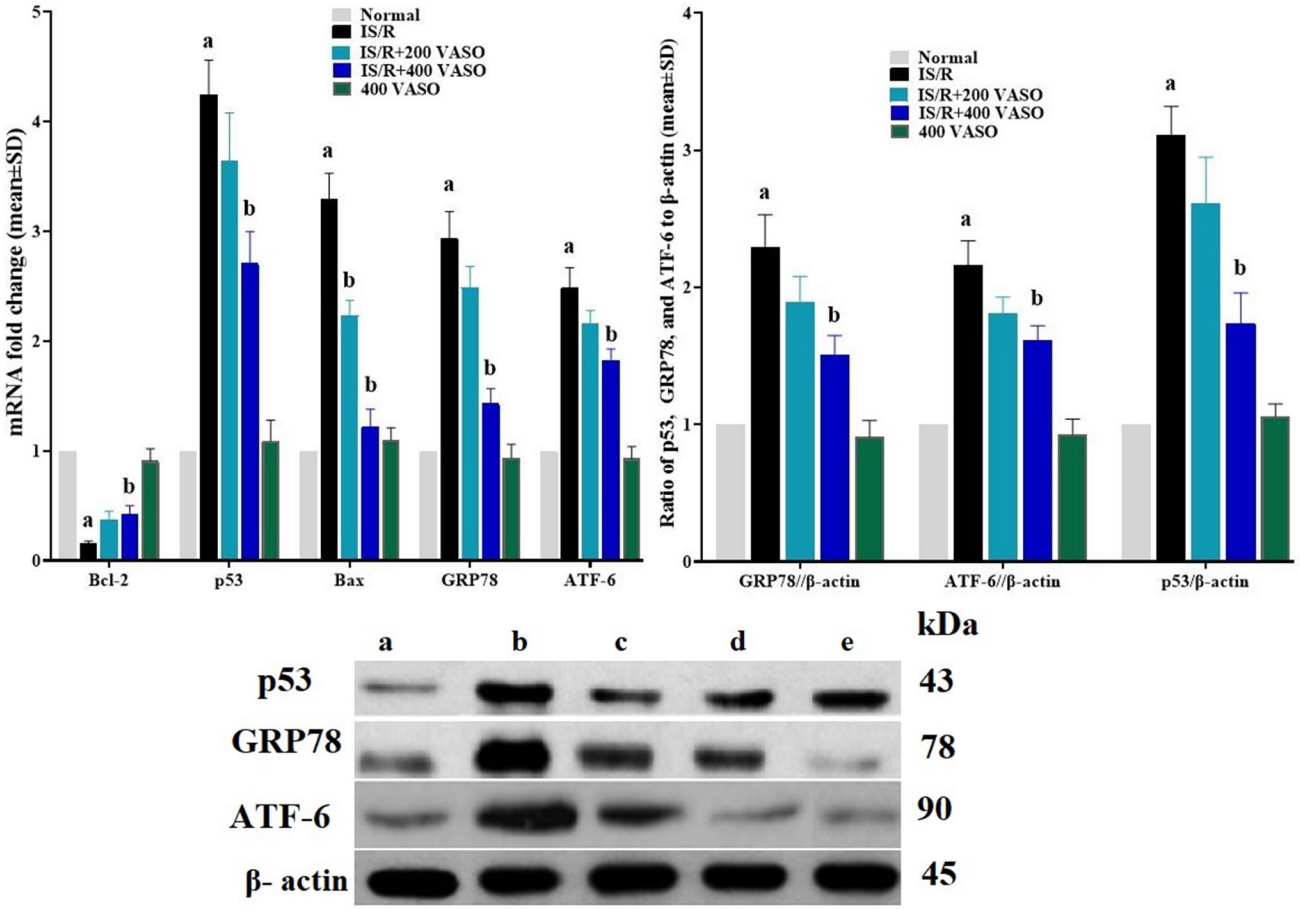

Fig. 4. (a) Bax, Bcl-2, ATF-6, GRP78, and p53 genes expression and (b) and (c) ATF-6, GRP78, and p53 proteins expression in brain (means ± SD; n=10/group) in experimental groups. Normal (a), IS/R (b), IS/R +200 VASO (c), IS/R + 400 VASO (d), and 400 VASO (e) groups. a ($p<0.05$) IS/R *vs.* normal groups; b ($p<0.05$) IS/R +200 and 400 VASO treated *vs.* IS/R groups.

Brain histopathological evaluations. Histopathological examination of brain tissue showed that IS/R led to lymphocytic infiltration (LI), vascular lesions, neurons with pyknotic nuclei and vacuolated cytoplasm, apoptotic bodies (AP), and degenerated neurons adjacent to necrotic areas (N). The IS/R group exhibited notable neuronal atrophy and a substantial decrease in normal parenchyma density compared to the healthy group. However, VASO treatment improved neuronal parenchyma density and decreased the incidence of apoptotic bodies, neuronal degeneration, and LI in a dose-dependent manner compared to the IS/R group (Fig. 5).

DISCUSSION

Our findings underscore the efficacy of VASO in maintaining normal brain neuron function by promoting antioxidant, anti-inflammatory, and anti-apoptotic mechanisms. This suggests a robust neuroprotective effect of VASO against IS/R-induced damage in brain physiology and structure.

In this study, post-treatment with 200 and 400 mg/kg of VASO led to a significant enhancement in behavioral performance, particularly at 20- and 40-days following

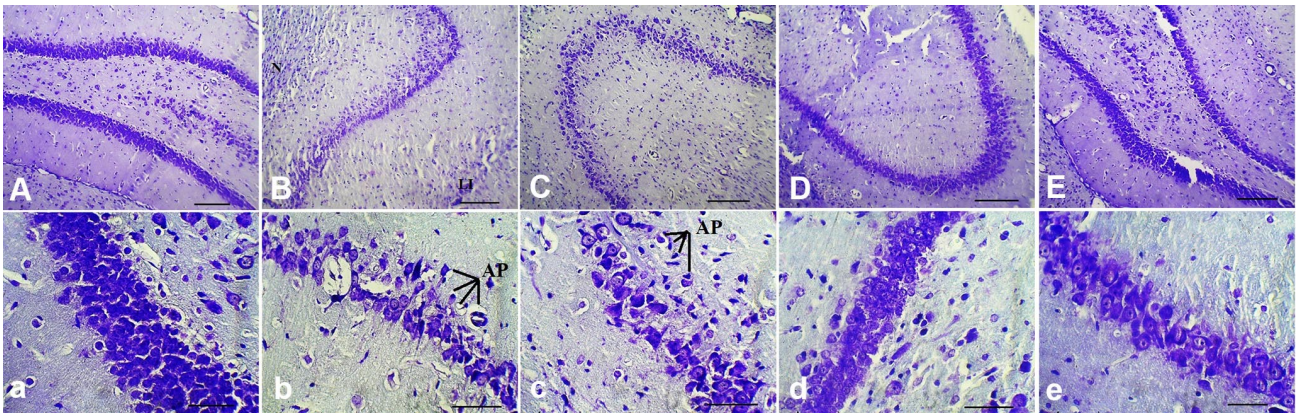

Fig. 5. Histopathological changes in brain tissue in Normal (A, a), IS/R (B, b), IS/R +200 VASO (C, c), IS/R + 400 VASO (D, d), and 400 VASO (E, e) groups [methylene blue staining, Upper raw × 100 (with Scale bar = 200 μm) and lower raw × 400 (with Scale bar = 50 μm)]. Apoptotic bodies (AP), lymphatic infiltration (LI), and degenerated neurons adjacent to necrotic areas (N).

administration. Additionally, there were marked improvements in micro-anatomy at the 400 mg/kg dose. This is the first report, to our knowledge, demonstrating VASO's ability to preserve neuron density after IS/R, especially at the 400 mg/kg dose. IS/R, often resulting from strokes, disrupts nerve impulse transmission in brain neurons by blocking blood flow to specific regions, causing nerve cell degeneration in the affected area. Research has shown that inflammation and oxidative stress can impair neuronal plasticity in the hippocampus, leading to infarct expansion (Choi *et al*., 2024). VASO is recognized for its anti-inflammatory, anti-apoptotic, and antioxidant properties, as supported by various studies Additionally, VASO has been shown to inhibit gliosis and prevent neuronal nucleus destruction in models of arsenic neurotoxicity (Shamilov *et al*., 2022). VASO's anti-inflammatory effects are largely attributed to flavonoids, while its antioxidant benefits are linked to flavonoid and phenolic compounds. Oancea & Calin (2018) demonstrated in an *in vitro* study that VASO, as an anti-cholinesterase and anti-apoptotic prodrug, exhibited neuroprotective effects and increased the survival of PC12 neurons. Shamilov *et al*. (2022), found that VASO enhances the antioxidant capacity of glial cells and brain neurons by increasing the activity of cytochrome C oxidase, superoxide dismutase (SOD), and succinate dehydrogenase. This boost in antioxidant activity protects these cells from oxidative damage and free radical injury associated with cerebral artery permanent occlusion (Shamilov *et al*., 2022). In the study by Ozdemir *et al*. (2023), using the MCAO model, the extract of *Vaccinium myrtillus*—a species within the Vaccinium genus—was shown to increase brain tissue malondialdehyde levels and Na+-K+-ATPase activity by enhancing antioxidant capacity and exhibiting an anti-apoptotic effect, as well as reducing cleaved caspase-3 gene expression (Ozdemir *et al*., 2023). Our findings suggest that administering VASO before an IS/R event can reduce apoptosis and improve performance in behavioral tests. Genistein, a key flavonoid in VASO, has been shown to increase GPx levels and decrease lipid peroxidation by inhibiting the NF-kB pathway in rat brains (Li *et al*., 2022). Moreover, biochanin A, quercetin, and apigenin have been found to elevate norepinephrine and serotonin levels by modulating oxidative stress in an Alzheimer's model. In our study, we induced IS/R and assessed the subsequent neurobehavioral changes in rats using various behavioral parameters.

The IS/R procedure significantly impaired animal behavior, as evidenced by deficits in functional status, neurological impairments, locomotor performance, and exploratory behavior assessed through the Morris water maze test. However, pretreatment with VASO at doses of 200 and 400 mg/kg nearly restored these experimental parameters to normal levels. Animal studies demonstrated that VASO suppresses caspase-9 and cytochrome c, thereby reducing mitochondrial apoptosis and preventing neurodegeneration of hippocampal neurons (Ozdemir *et al*., 2023). The same study also highlighted VASO's role in enhancing cognitive function related to the striatum and hippocampus due to its neuroprotective effects (Chellammal *et al*., 2021). In another study, Khordad *et al*. (2024), explored the protective effects of VASO against oxymetholone-induced liver and kidney injury in BALB/c mice. They found that doses of 200 and 400 mg of VASO significantly increased total antioxidant capacity, enhanced the activity of endogenous antioxidant enzymes, and reduced apoptosis, as indicated by decreased p53 expression in renal tubular cells and liver hepatocytes (Khordad *et al*., 2024). Similarly, Akbari Bazm *et al*. (2020), reported that VASO at 200 and 400 mg/kg effectively reduced lipid peroxidation in seminiferous tubules caused by oxymetholone, thereby suppressing mitochondrial apoptosis through inhibition of the Bex-Bcl-2/p53/caspase-3 pathway in the spermatogenic lineage (Akbari Bazm *et al*., 2020). In

the present study, VASO was found to increase endogenous antioxidant enzyme levels by enhancing antioxidant capacity, leading to a decrease in the expression of p53 and Bax genes in hippocampal cells. These protective effects of VASO suggest its potential anti-infarct properties. Previous research has suggested that the observed behavioral recovery and reduction in infarct volume may be linked to VASO's ability to preserve dopaminergic receptors and maintain neurotransmitter levels (Patel *et al*., 2023). However, our study did not examine neurotransmitter levels, and further research is needed to fully understand the mechanism of IS/R in limiting infarct areas. VASO, administered at 200 and 400 mg/kg, also stimulated the secretion of GDNF and BDNF from glial cells. IS/R is known to increase free radical production, leading to oxidative stress and subsequent membrane lipid peroxidation, which compromises membrane integrity and function. Our findings demonstrate that VASO exhibits neuroprotective properties by preserving neuron density in the hippocampus. Shamilov *et al*. (2022), showed that VASO reduces carbonyl content and lipid peroxidation while enhancing antioxidant enzyme levels, such as superoxide dismutase, catalase, and glutathione, which collectively improve the survival of rat brain neurons (Shamilov *et al*., 2022). The increase in hippocampal neuronal density following VASO treatment is likely due to the inactivation of inflammatory mediators by VASO. The interaction between IS/R and hippocampal regions in the injured groups further indicates that VASO enhances hippocampal nerve cell activity by maintaining synaptic plasticity between neurons, aiding in the replacement of damaged areas. In this study, VASO at doses of 200 and 400 mg was also found to strengthen the metabolic/antioxidant pathway dependent on GRP78 and ATF-6, which preserves brain neuron function and structure against ischemic damage caused by IS/R. Consequently, VASO improved animal movement performance and reduced infarct areas by promoting neuronal recovery in the hippocampus. As previously discussed, the active components of VASO, including quercetin, genistein, and apigenin glycosides, are believed to contribute to its neuroprotective effects. These effects correlate with tissue concentrations of FRAP and TBARS levels and serum GPx, CAT, and SOD levels, further supporting the efficacy of VASO treatment.

CONCLUSION

Our study highlights that pre-treatment with VASO can significantly reduce hemorrhage volume and enhance behavioral outcomes in rats subjected to IS/R. The neuroprotective effects observed are likely due to VASO's anti-inflammatory, anti-apoptotic, and antioxidant properties, which are largely attributed to its flavonoid and phenolic content. Notably, a 400 mg/kg dose of VASO was particularly effective in preserving hippocampal neuron density and reducing the extent of IS/R damage. These findings suggest the potential of VASO as a natural therapeutic option for stroke management. Further research is needed to better understand the mechanisms by which VASO limits IS/R-induced damage and apoptosis while promoting neuronal recovery. This study underscores the importance of exploring natural compounds like VASO for developing innovative therapies for IS/R.

Ethical Approval. The experimental protocols of this study were approved by the Dalian Medical University ethics committee.

ZHANG, J. & DONG, B. Efectos del aceite de semillas de *Vaccinium arctostaphylos* L. en el accidente cerebrovascular en ratas: Un enfoque bioquímico, inmunohistoquímico y molecular. *Int. J. Morphol., 42(6)*:1773-1782, 2024.

RESUMEN: La lesión cerebral por ataque cerebrovascular isquémico-reperfusión (CIS/R) plantea una amenaza significativa para la función cognitiva. En este estudio, exploramos los efectos del aceite de semillas de *Vaccinium arctostaphylos* L. (VASO) en un modelo CIS/R en ratas Wistar e investigamos sus mecanismos subyacentes. Cincuenta ratas Wistar se dividieron aleatoriamente en cinco grupos: un grupo normal, un grupo suplementado con VASO (que recibió 400 mg/kg de VASO), un grupo de ataque cerebrovascular isquémico-reperfusión (IS/R) y dos grupos IS/R tratados con 200 mg/kg y 400 mg/kg de VASO, cada uno compuesto por 10 ratas. El rendimiento cognitivo se evaluó mediante la prueba del laberinto eléctrico Y. Además, empleamos un ensayo inmunoabsorbente ligado a enzimas (ELISA) y PCR en tiempo real para examinar la expresión de proteínas y genes asociados con el crecimiento neurotrófico, la inflamación y la apoptosis en el cerebro. En comparación con el grupo normal, las ratas sometidas a IS/R exhibieron un retraso significativo en el cumplimiento de los criterios del laberinto. El grupo IS/R también demostró niveles marcadamente elevados de citocinas proinflamatorias, incluidas la interleucina-1β (IL-1β), IL-6 y el factor de necrosis tumoral-α (TNF-α), junto con niveles reducidos de citocinas antiinflamatorias (IL-4 e IL-10) y factores de crecimiento neurotrófico [factor neurotrófico derivado del cerebro (BDNF) y factor neurotrófico derivado de la línea celular glial (GDNF)]. El grupo tratado con 400 mg/kg de VASO después de la IS/R mostró mejoras, con mayores niveles de factores de crecimiento neurotrófico y un cambio favorable en los perfiles de expresión de proteínas y genes de marcadores inflamatorios y apoptóticos (incluyendo la proteína regulada por glucosa 78 (GRP78), el factor de transcripción activador 6 (ATF-6), Bcl-2, Bax y p53) en comparación con el grupo IS/R. Estos resultados sugieren que el extracto de VASO puede mitigar los deterioros cognitivos posteriores a la IS/R en ratas, potencialmente a través de su modulación de los factores inflamatorios secretados por la microglia.

PALABRAS CLAVE: Aceite de semillas de *Vaccinium arctostaphylos* L.; Inflamación; Apoptosis; Ataque cerebrovascular isquémico cerebral - reperfusión.

REFERENCES

Akbari Bazm, M.; Goodarzi, N.; Shahrokhi, S. R. & Khazaei, M. The effects of hydroalcoholic extract of *Vaccinium arctostaphylos* L. on sperm parameters, oxidative injury and apoptotic changes in oxymetholone-induced testicular toxicity in mouse. *Andrologia, 52(3)*:e13522., 2020.

Akhone, M. A.; Bains, A.; Tosif, M. M.; Chawla, P.; Fogarasi, M. & Fogarasi, S. Apricot kernel: Bioactivity, characterization, applications, and health attributes. *Foods, 11(15)*:2184, 2022.

Badawi, M. S. A study on the antioxidant activity of rosmarinic acid against carbon tetrachloride-induced liver toxicity in adult male albino rats. *Int. J. Morphol., 40(1)*:157-67, 2022.

Bazm, M. A.; Khazaei, M.; Ghanbari, E. & Naseri, L. Protective effect of *Vaccinium arctostaphylos* L. fruit extract on gentamicin-induced nephrotoxicity in rats. *Comp. Clin. Pathol., 27*:1327-1334., 2018.

Celik, H., Kucukler, S., Ozdemir, S., Comakli, S., Gur, C., Kandemir, F. M. & Yardim, A. Lycopene protects against central and peripheral neuropathy by inhibiting oxaliplatin-induced ATF-6 pathway, apoptosis, inflammation and oxidative stress in brains and sciatic tissues of rats. *Neurotoxicology, 80*:29-40, 2020.

Charalabopoulos, A.; Davakis, S.; Lambropoulou, M.; Papalois, A.; Simopoulos, C. & Tsaroucha, A. Apigenin exerts anti-inflammatory effects in an experimental model of acute pancreatitis by down-regulating TNF-α. *In vivo, 33(4)*:1133-41, 2019.

Chellammal, H. S. J.; Menon, B. V.; Hasan, M. H.; Azil, A.; Suhaimi, M. T. B.; Chandarasekaran, P. & Murugan, Y. Neuropharmacological studies of ethanolic extract of *Vaccinium corymbosum* on Alzheimer's type dementia and catatonia in Swiss albino mice. *J. Herbmed Pharmacol., 10(2)*:241-8, 2021.

Choi, J. H.; Poli, S.; Chen, M.; Nguyen, T. N.; Saver, J. L.; Matouk, C. & Pile-Spellman, J. Selective brain hypothermia in acute ischemic stroke: reperfusion without reperfusion injury. Front. Neurol., 11:594289, 2020.

Choudhary, G. P. & Jain, A. P. Evaluation of acute, subacute and LD50 values of methanolic extract of *Sphaeranthus indicus* leaves in Albino mice. *Res. J. Pharm. Technol., 14(5)*:2487-92, 2021.

Das, J. & Rajanikant, G. K. Post stroke depression: the sequelae of cerebral stroke. *Neurosci. Biobehav. Rev., 90*:104-14, 2018.

Duan, T.; Li, L.; Yu, Y.; Li, T.; Han, R.; Sun, X.; Cui, Y.; Liu, T.; Wang, X.; Wang, Y.; *et al*. Traditional Chinese medicine use in the pathophysiological processes of intracerebral hemorrhage and comparison with conventional therapy. *Pharmacol. Res., 179*, 106200, 2022.

Famurewa, A. C.; Asogwa, N. T. & Ezea, S. C. Antidiabetic drug sitagliptin blocks cyclophosphamide cerebral neurotoxicity by activating Nrf2 and suppressing redox cycle imbalance, inflammatory iNOS/NO/NF-kB response and caspase-3/Bax activation in rats. *Int. Immunopharmacol., 116*:109816, 2023.

Jaafar, H. J. Effects of apricot and apricot kernels on human health and nutrition: a review of recent human research. *Technium BioChemMed., 2(2)*:139-62, 2021.

Khordad, E.; Akbaribazm, M.; Hosseini, S. M. & Rahmani, Y. Protective Effects of *Vaccinium arctostaphylos* L. against oxymetholone-injured liver and kidney injury in BALB/c mice: an integrated biochemical, stereological, histopathological, and immunohistochemical study. *Pharmacogn. Res., 16(2)*:401-13, 2024.

Li, Y.; Zhang, J. J.; Chen, R. J.; Chen, L.; Chen, S.; Yang, X. F. & Min, J. W. Genistein mitigates oxidative stress and inflammation by regulating Nrf2/HO-1 and NF-κB signaling pathways in hypoxic-ischemic brain damage in neonatal mice. *Ann. Transl. Med., 10(2)*:32, 2022.

Lin, Z. H.; Wang, S. Y.; Chen, L. L.; Zhuang, J. Y.; Ke, Q. F.; Xiao, D. R. & Lin, W. P. Methylene blue mitigates acute neuroinflammation after spinal cord injury through inhibiting NLRP3 inflammasome activation in microglia. *Front. Cell. Neurosci., 11*:391, 2017.

Louessard, M.; Bardou, I.; Lemarchand, E.; Thiebaut, A. M.; Parcq, J.; Leprince, J. & Roussel, B. D. Activation of cell surface GRP78 decreases endoplasmic reticulum stress and neuronal death. *Cell Death Differ., 24(9)*:1518-29, 2017. ?

Lucini, C.; D'Angelo, L.; Cacialli, P.; Palladino, A. & De Girolamo, P. BDNF, brain, and regeneration: insights from zebrafish. *Int. J. Mol. Sci., 19(10)*:3155, 2018.

Mokhtari, T.; Akbari, M.; Malek, F.; Kashani, I. R.; Rastegar, T.; Noorbakhsh, F.; Khansari, M. G.; Attari, F. & Hassanzadeh, G. Improvement of memory and learning by intracerebroventricular microinjection of T3 in rat model of ischemic brain stroke mediated by upregulation of BDNF and GDNF in CA1 hippocampal region. *Daru, 25(1)*:4, 2017.

Oancea, S. & Ca˘lin, F. Phenolics content, *in vitro* antioxidant and anticholinesterase activities of combined berry extracts. *Rom. Biotechnol. Lett., 23(5)*:14025-34, 2018.

Othman, M. Z.; Hassan, Z. & Has, A. T. C. Morris water maze: a versatile and pertinent tool for assessing spatial learning and memory. *Exp. Anim., 71(3)*:264-80, 2022.

Ozdemir, A.; Mercantepe, T.; Erdivanli, B.; Sen, A.; Mercantepe, F.; Tumkaya, L. & Uydu, H. A. Neuroprotective effects of *Vaccinium myrtillus* on damage-related brain injury. *J. Chem. Neuroanat., 127*:102193, 2023.

Patel, M. P.; Vyas, A. S.; Bharadia, P. D.; Patel, J. K. & Patel, D. H. *Urogenital System Disorders and Functional Foods and Nutraceuticals.* In: Mohan, S.; Abdollahi, S. & Pathak, Y. (Eds.). Applications of Functional Foods and Nutraceuticals for Chronic Diseases. Hoboken, CRC Press, 2023. pp 317-40.

Sarkar, S.; Chakraborty, D.; Bhowmik, A. & Ghosh, M. K. Cerebral ischemic stroke: cellular fate and therapeutic opportunities. *Front. Biosci., 24(3)*:415-30, 2019.

Shamilov, A. A.; Olennikov, D. N.; Pozdnyakov, D. I.; Bubenchikova, V. N.; Garsiya, E. R. & Larskii, M. V. Caucasian blueberry: Comparative study of phenolic compounds and neuroprotective and antioxidant potential of *Vaccinium myrtillus* and *Vaccinium arctostaphylos* leaves. *Life, 12(12)*:2079, 2022.

Wang, X., Cheng, J. L., Ran, Y. C., Zhang, Y., Yang, L. & Lin, Y. N. Expression of RGMb in brain tissue of MCAO rats and its relationship with axonal regeneration. *J. Neurol. Sci., 383*:79-86, 2017.

Yan, H.; Huang, W.; Rao, J. & Yuan, J. miR-21 regulates ischemic neuronal injury via the p53/Bcl-2/Bax signaling pathway. *Aging (Albany NY):13(18)*:22242-55, 2021.

Yardim, A.; Kandemir, F. M.; Ozdemir, S.; Kucukler, S.; Comakli, S.; Gur, C. & Celik, H. Quercetin provides protection against the peripheral nerve damage caused by vincristine in rats by suppressing caspase 3, NF-kB, ATF-6 pathways and activating Nrf2, Akt pathways. *Neurotoxicology, 81*:137-46, 2020.

Zhang, A.; Pan, W.; Lv, J. & Wu, H. Protective effect of amygdalin on LPS-induced acute lung injury by inhibiting NF-kB and NLRP3 signaling pathways. *Inflammation, 40(3)*:745-51, 2017.

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