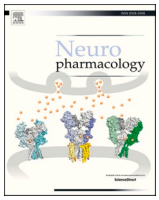
Neuropharmacology 279 (2025) 110602

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm



The mitochondria-targeted hydrogen sulfide donor AP39 reduces cortical stroke volume and improves motor function in a photothrombotic stroke model in mice in a sex-dependent manner

Jakub Jurczyk [a,c], Zuzanna Guzda [a], Alicja Skórkowska [c], Żaneta Broniowska [b], Małgorzata Piechaczek [b], Aleksandra Więcek [d], Emilia Schulze [a], Angelika Ziaja [d], Roberta Torregrossa [e], Matthew Whiteman [e], Michel Soares Mesquita [f], Bartosz Pomierny [c,a], Lucyna Pomierny-Chamioło [b,*]

[a] Department of Toxicology, Jagiellonian University Medical College, Medyczna 9, PL 30-688 Kraków, Poland
[b] Department of Biochemical Toxicology, Jagiellonian University Medical College, Medyczna 9, PL 30-688 Kraków, Poland
[c] BioImaging Laboratory, Center for the Development of Therapies for Civilization and Age-Related Diseases (CDT-CARD), Jagiellonian University Medical College, Medyczna 9, 30-688, Krakow, Poland
[d] Student Scientific Group of Toxicology, Student Scientific Society of Jagiellonian University Medical College, Medyczna 9, PL 30-688, Kraków, Poland
[e] St. Luke's Campus, University of Exeter Medical School, EX1 2LU, Exeter, United Kingdom
[f] L&M Data Science Ltd, 124 City Road, London, EC1V 2NX, United Kingdom

ARTICLE INFO

Keywords:
AP39
Photothrombotic stroke in mice
Skilled reaching task
MRI imaging
Mitophagy

ABSTRACT

Despite many years of research, the treatment for patients affected by ischemic stroke remains very limited and insufficient. Currently, new neuroprotective treatment strategies are being sought to reduce brain tissue damage in the penumbra zone. One of these strategies involves the use of a hydrogen sulfide donor, the compound AP39, which mitochondria-targeted and, in very low concentrations, has shown a favorable action profile in preclinical studies across various disease models.

In this study, we evaluated whether the administration of AP39, given 10 min after photothrombotic focal cerebral stroke, affects motor performance in a skilled reaching task in both female mice and male mice. We also assessed cerebral blood flow using laser speckle contrast analysis, stroke volume via MRI at 24 h, 3 days, and 6 days post-stroke, as well as the expression of mitochondrial proteins TOMM20, COX4, PINK1 and Parkin as markers of mitophagy in cells.

Our results showed significant improvement in motor function, increased blood flow and noticeably lower stroke volume, and TOMM20 and COX4 expression with concomitant upregulation of PINK1 and Parkin expression at day 6 in male mice treated with AP39 after focal cortical stroke. In females, the beneficial effect was limited, with only a slight reduction in stroke volume observed, without any impact on skilled task performance. These results indicate that AP39 has neuroprotective potential, but it is sex dependent.

1. Introduction

Stroke is the second leading cause of death and the third leading cause of disability worldwide. Approximately 87 % of strokes are ischemic, caused by vessel occlusion in the brain (Hatano, 1976; Beal, 2010; World Health Organization (WHO), 2020). Despite decades of research, there is still no effective pharmacotherapy for ischemic stroke. The only available form of pharmacological intervention aimed at restoring circulation is the administration of recombinant tissue plasminogen activator (rt-PA), but it is burdened with many exclusion criteria (Del Zoppo et al., 2009). Immediately after an episode of ischemia, for example, due to occlusion of the middle cerebral artery

* Corresponding author. Department of Toxicology, Jagiellonian University Medical College, Medyczna 9, PL 30-688, Kraków, Poland.
E-mail addresses: jakub.jurczyk@doctoral.uj.edu.pl (J. Jurczyk), zuzanna.guzda@uj.edu.pl (Z. Guzda), alicja.skorkowska@uj.edu.pl (A. Skórkowska), malgorzata.piechaczek@doctoral.uj.edu.pl (M. Piechaczek), aleksandra.wiecek@student.uj.edu.pl (A. Więcek), emilia.schulze@uni-bonn.de (E. Schulze), angelika.ziaja@student.uj.edu.pl (A. Ziaja), r.torregrossa@exeter.ac.uk (R. Torregrossa), M.Whiteman@exeter.ac.uk (M. Whiteman), michel.mesquita@lmdatascience.com (M.S. Mesquita), bartosz.pomierny@uj.edu.pl (B. Pomierny), lucyna.pomierny-chamiolo@uj.edu.pl (L. Pomierny-Chamioło).

https://doi.org/10.1016/j.neuropharm.2025.110602
Received 7 January 2025; Received in revised form 14 July 2025; Accepted 24 July 2025
Available online 26 July 2025

(MCA), neurons cannot maintain their membrane potentials and homeostasis. This event triggers several processes, such as excitotoxicity, oxidative stress, inflammation, apoptosis and necrosis, which ultimately lead to cell death (Baron, 1999; Bandera et al., 2006; Ouyang et al., 2007; Xu et al., 2010). Ischemic stroke can cause varying degrees of severity of symptoms, depending on the degree of restriction of blood flow in different parts of the brain. The area of the brain closest to ischemia (core) is characterized by irreversible neuronal damage due to necrosis. However, in areas farther away from the core, known as the penumbra, more metabolically active neurons are present. Here, cell death processes occur more slowly, allowing these neurons to survive after circulation is restored (Bayir and Kagan, 2008; Niizuma et al., 2010).

The epidemiology of human ischemic stroke exhibits significant sex differences, with incidence rates being generally higher in men compared to women. Women experience a large impact of stroke-related mortality and long-term disability. There are significant differences in the impact and significance of stroke risk factors specific to women. In addition, differences in stroke symptoms, treatment response, and overall outcomes underscore the unique challenges women face in stroke management and recovery (Shajahan et al., 2023). Sex-dependent differences in the pathophysiology and treatment response of ischemic stroke make it essential to seek new treatment strategies for ischemic stroke that take these factors into account (Roy-O'Reilly and McCullough, 2018).

Mitochondria are crucial organelles for the survival of neurons, which have an increased energy demand compared to other cell types. On the other hand, a characteristic feature of mitochondria in ischemic stroke is their involvement in cell death mechanisms (Rodríguez, Ramón Rama Bretón; Julio, 2012). In the ischemic brain, mitochondrial functions are altered due to the excitotoxicity phenomenon mentioned above (Pekkurnaz et al., 2014). Excessive glutamate cell stimulation leads to overload of the endoplasmic reticulum and mitochondrial matrix with calcium. The buffering capacity of mitochondria is altered, the potential difference disappears, causing mitochondrial permeability and activation of lipases and proteases, as well as inhibition of mitochondrial respiratory chain complexes I, III, and IV, together with the release of cytochrome C (Moore et al., 2003). This is followed by massive production of reactive oxygen species (ROS), peroxidation, energy deficit, and DNA damage, resulting in cell death through mechanisms of apoptosis, necrosis, or necroptosis (Borutaite, 2010; Pathak et al., 2010; Schwarz, 2013). Mitochondria are dynamic organelles that require continuous remodeling and transport to maintain their function in the cell. This process is regulated by various molecular mechanisms (Pathak et al., 2010; Schwarz, 2013; Pekkurnaz et al., 2014). In the case of ischemic stroke, the mitochondrial dynamics is disrupted, leading to the accumulation of dysfunctional mitochondria in damaged neurons. Due to the significant role of mitochondria in the pathogenesis of stroke, these organelles appear to be a promising therapeutic target in neurodegenerative diseases.

Mitochondrial quality control is essential for maintaining neuronal integrity, especially under pathological conditions such as ischemic stroke. One of the key mechanisms involved in this process is mitophagy, a selective form of autophagy responsible for the removal of damaged mitochondria. The PINK1 and Parkin (E3 ubiquitin ligase) proteins play a central role in the initiation of mitophagy. PINK1 accumulates on dysfunctional mitochondria at the outer mitochondrial membrane, where it recruits and activates Parkin, leading to the ubiquitination of mitochondrial outer membrane proteins and subsequent elimination of dysfunctional mitochondria via the autophagy - lysosome pathway (Youle and Narendra, 2011; Pickrell and Youle, 2015). This pathway is critical for maintaining mitochondrial fidelity and preventing the accumulation of defective organelles that could contribute to oxidative stress and neuronal death.

Hydrogen sulfide (H_2S), traditionally perceived as a toxic gas, can be classified as a neurotransmitter that acts as an intracellular messenger and neuromodulator (Kamoun, 2004). Research on H_2S has discovered its role in various physiological processes (Goodwin et al., 1989; Warenycia et al., 1989), including its role in the metabolism of brain cells, as well as its neuroprotective effects in ischemic stroke (Goubern et al., 2007; Theissen and Martin, 2008; Marutani et al., 2021). H_2S modulates the activity of the electron transport chain by becoming a source of electrons through irreversible oxidation by sulfide quinone reductase (Goubern et al., 2007; Theissen and Martin, 2008). H_2S is believed to reduce oxidative stress, inflammation, and excitotoxicity, as well as promote mitochondrial function and biogenesis (Goubern et al., 2007; Theissen and Martin, 2008; Yu et al., 2015; Marutani et al., 2021). However, it has been reported that when H_2S concentrations reach more than 50 μM, the electron transport chain can be inhibited, leading to redox imbalance, inhibition of cellular proliferation, and a change in metabolism toward reductive carboxylation (Mancardi et al., 2009). Although, more research is needed to fully understand the mechanisms underlying the beneficial effects of H_2S in ischemic stroke and to develop safe and effective therapies based on H_2S.

AP39 is one of several H_2S donors that have been developed to meet this challenge. AP39 [(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl) phenoxy) decyl) triphenyl phosphonium bromide] is designed to release H_2S in the mitochondrial space of cells, where it can potentially protect these organelles from damage and promote their proper functioning. It consists of a triphenylphosphonium (TPP) lipophilic cation and a dithiolethione group that acts as a carrier of H_2S. The TPP group targets mitochondria due to their negative membrane potential, allowing for selective delivery of the H_2S -releasing group, but the exact mechanism of H_2S release from AP39 is not fully understood (Szczesny et al., 2014). Research on models of myocardial infarction has demonstrated that AP39 reduces the size of the lesion in a manner a NO-independent manner and cytosolic kinases, while concurrently leading to a decrease in the production of mitochondrial reactive oxygen species (ROS) (Ravani et al., 2024), but the exact mechanism of action of AP39 in ischemic brain stroke has not yet been fully elucidated. Our previous studies have shown that a 7-day pretreatment with low doses of AP39 reduced infarct volume and alleviated neurological deficits in rats subjected to the MCAO (middle cerebral artery occlusion) model by decreasing brain neuroinflammation and activating pro-survival neurotrophic factors BDNF-TrkB and NGF-TrkA. Although free H_2S levels remained at baseline, the levels of bound forms of H_2S were found to be increased (Pomierny et al., 2021). Administration of AP39, 10 min after a 90-min occlusion followed by reperfusion of the middle cerebral artery, not only reduced infarct volume and neurological deficits but also lowered glutamate levels within 3 days post-stroke (Skórkowska et al., 2024).

This study aimed to determine the potential beneficial effects of AP39 on motor function, assessed through the skilled reaching task, its neuroprotective effect in limiting stroke volume, and its impact on mitochondrial function following focal cerebral ischemia in both female and male mice. A mouse model of photothrombotic focal ischemic stroke targeting the motor cortex was used. The animals were trained in the skilled reaching task and received AP39 administration 10 min after ischemia induction. Twenty-four hours after surgery, neurological deficits were evaluated, and training continued for the following 6 days. Stroke volume was assessed 6 days post-stroke after the animals were sacrificed. The expression of mitochondrial protein markers, TOMM20 and COX4, and mitophagy related proteins PINK1 and Parkin was examined in brain sections. Additionally, we assessed the effect of AP39 on stroke volume at 24 h, 3 days, and 6 days post-stroke using MRI imaging.

2. Methods

2.1. Animals and experimental design

All experiment were performed on 30 eight-month-old female and 30

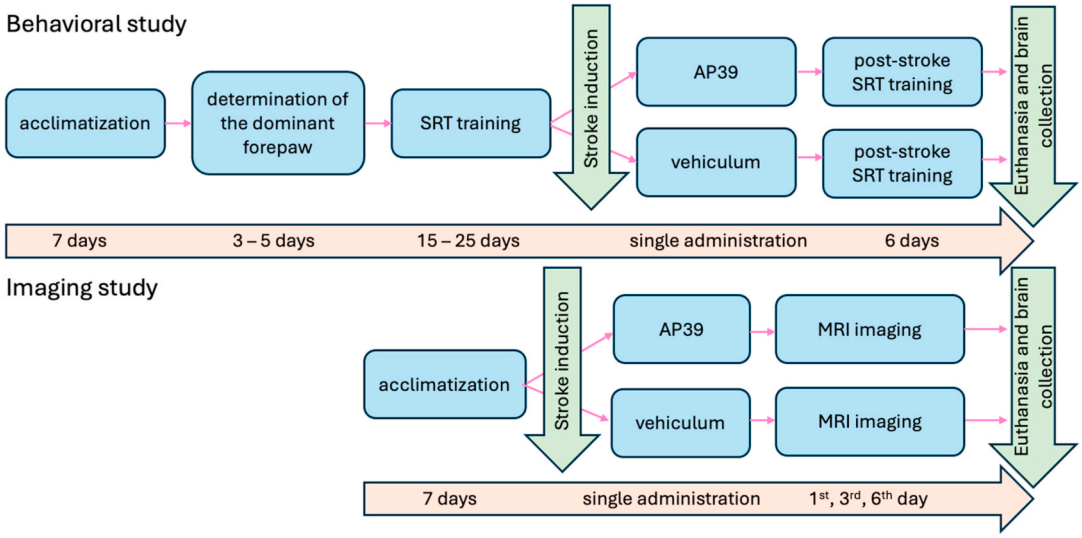


Fig. 1. Experimental design of behavioral and imaging studies. Behavioral study n = 15 females and 15 males/group; MRI imaging study n = 5 females and 5 males/group; AP39 (100 nM/kg, *ip*).

male mice C57BL/6J obtained from an accredited house at the Faculty of Pharmacy, Jagiellonian University Medical College, Krakow, Poland. Animals were kept in groups of 2–3 mice in plastic standard cages (37 cm × 21 cm × 15 cm) in a controlled environment (i.e., constant room temperature (22–24 °C), adequate humidity (55 ± 10 %), and a 12 h light/dark cycle). Access to maintenance feed was unlimited during the acclimatization, handling and gentling period (7 days). To maximize the synchronization of the estrous cycle in females, they were kept in conditions that allowed for olfactory contact, and scent marks from male urine were placed in the cages. At the time of stroke induction, the animals were over 9 months old. Given the age-related irregularities in estrous cycling typically observed at this stage, the phase of the estrous cycle was determined using daily vaginal smears. Stroke induction was consistently performed during the diestrus phase. During the behavioral tasks, all mice were kept on a mild food restriction (90 % of the target body weight according to the table values for the specific developmental period) by providing 3.5–4.5 g of pelleted food per mouse per day. The deficit was compensated by full-value milk pellets given during the behavioral sessions. Since food restriction in mice must be strictly monitored by body weight and animal behavior to avoid compromising their welfare, the amount of food in the cages was adjusted individually within the specified range. Food restriction is a necessary condition for maintaining the reward value of the food pellets (20 mg, BioServ) used in the Skilled Reaching Task (SRT). Pellets, consumed at a rate of about 20 per day, provided an additional source of food for 5 days a week, which compensated for the reduced maintenance feed. On the remaining 2 days, the amount of feed was supplemented to 5 g per mouse. The weight of restricted animals was monitored daily (7 days a week). Animals had free access to water ad libitum at all times, except during training/testing sessions (approximately 30 min daily, 5 days a week). Behavioral procedures were performed between 8 a.m. and 2 p.m. by a trained observer blind to the treatments. Animals were selected randomly for the treatment groups. Each group consisted of 15 mice. In this study, we employed an internal control model, where the baseline performance results obtained from the animal prior to stroke induction serve as a reference for the post-stroke results. Procedures involving animals were conducted according to the current European Community and Polish legislation on animal experimentation with the approvement of 1 Local Ethics Committee in Krakow (number 683/2022). Limiting the number of animals per cage was necessary due to the need to monitor body weight under food restriction. The C57BL/6J strain is characterized by a pronounced social hierarchy, so housing animals collectively under food restriction would likely result in intra-group aggression and difficulty in obtaining food for some individuals. Aggressive individuals were isolated into separate cages while maintaining visual and olfactory contact. The environment of the animals in each cage was enriched with wooden chewing blocks, nesting material, wooden huts, hammocks, paper tunnels, strips of gray paper, wooden ladders, cotton rags, and aspen corners. The enrichment items were cyclically replaced whenever the animals lost interest in them, ensuring that a different type of enrichment material was introduced each time. Nesting material and wooden blocks were present in the cages throughout the duration of the experiment. Experiments on males and females were performed in two separate sets. MRI imaging of stroke volume and brain edema was performed on separate groups of females and males (n = 5). Graphic scheme of the experiment is presented in Fig. 1.

2.2. *Skilled reaching task*

The reaching apparatus was made of clear plexiglass (19.5-cm long, 8-cm wide, and 20-cm high) with a 1-cm wide slit located in the center of the front wall (Farr and Whishaw, 2002). Food items that the mice reached for were pellets (BioServ, 20 mg, 20 pieces/day) placed singly in a divot of a shelf. At this location, it was difficult for the animal to obtain food with their tongue, but it can be obtained with the contralateral forelimb. Food restricted mice, maintained at 90 % body weight, were habituated to the reaching apparatus by placing them in the box for 10-min for seven days. Initially pellets were placed on the box floor, then within tongue distance on the shell and gradually further away in the slit. For 3–5 days, the pellet was placed in the center and observed to see which paw the mouse used to reach for it. This way, the dominant paw was determined. Pellets were then placed in the divot on the side opposite the dominant paw. Animals that used their left and right paws alternately were excluded from the experiment at this stage. Mice received twenty pellets in each training session, and each pellet presentation defined a trial. Alternatively, the session was interrupted after 20 min. SRT training was deemed complete when each mouse's success rate stabilized over three consecutive days of training. The mice's reaching behavior was analyzed using endpoint measures. Four endpoint measures were applied to the task of reaching for a single pellet: Medal (A) referred to the success rate of the first attempt, which was the number of trials in which the mouse successfully grasped the pellet on the first try and brought it to its mouth; Success (B) was defined

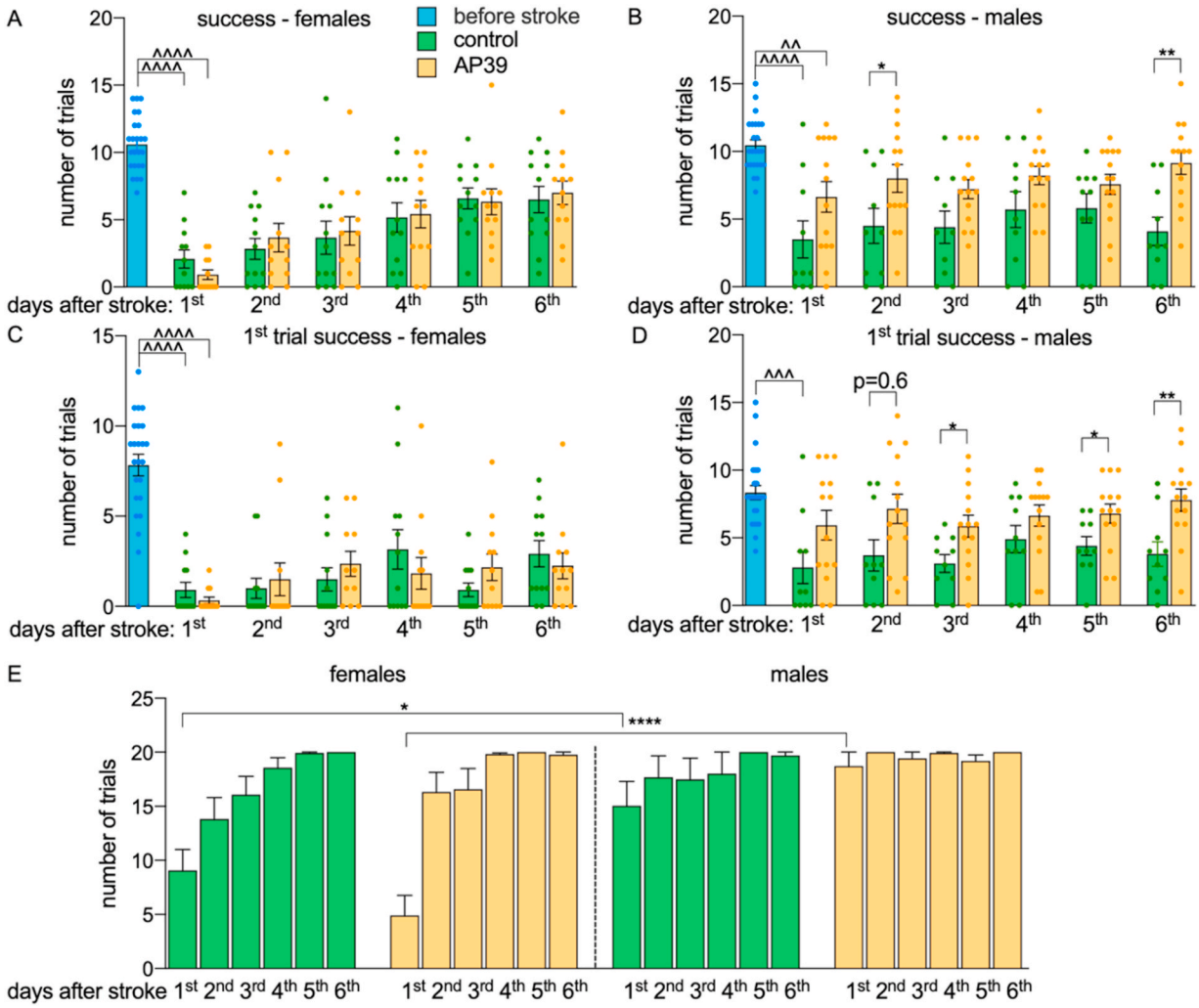


Fig. 2. The effect of AP39 on behavioral task performance in skilled reaching task in females and males before and after ischemic stroke: A - number of successful attempts in females; B - number of successful attempts in males; C - number of successful attempts on the first trial in females; D - number of successful attempts on the first trial in males; E – total number of attempts in the subsequent training days after the stroke. Blue bars – results before stroke induction; green bars – control groups with vehiculum; orange bars – groups treated with AP39. Data are expressed as means (±SEM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

as a reach in which the mouse successfully grasped the food pellet and brought it to its mouth to eat; Drop (C) occurred when the mouse reached for the pellet but it fell before reaching the mouth; and Failure (D) was any reach in which the mouse put its paw through the opening but touched the pellet without grabbing it. After achieving the stability criterion, the animals underwent a procedure to induce localized photothrombotic ischemia in the motor cortex of the dominant forelimb. Twenty-four hours post-procedure, they resumed their daily training, which continued for an additional six days.

2.3. Photothrombotic focal ischemic stroke technique

In this study, the sensorimotor cortex of the hemisphere contralateral to the dominant forepaw was the target of focal photothrombotic ischemia (Winship and Murphy, 2009). To induce photothrombotic ischemia, Bengal Rose, a photosensitizer (Na^+ salt, R3877; Sigma–Aldrich Corp., St. Louis, MO, USA), was first dissolved in saline to a concentration of 20 mg/ml. Anesthetized with 5 % isoflurane for induction and 2–2.5 % for maintenance mice on a stereotaxic platform were then treated with 0.1 ml Bengal Rose solution through the tail vein (Talley Watts et al., 2015). During the entire surgical procedure, the mouse was lying on a temperature-controlled heating pad to maintain the body temperature (37 ± 0.5 °C) and eyes were covered with Vidisic gel to prevent dryness while under anesthesia. After injection of Bengal Rose solution, the skull was exposed through a midline scalp incision under local anesthesia with bupivacaine (2 mg/kg, *s. c.*), and the skin was retracted. The exposure area was limited by a ring with a window of 1 mm diameter and the center of the circular region was AP = 0.5 mm and ML = 2.5 mm from the Bregma. The cold light illuminator was turned on for 7 min, then cerebral blood flow was assessed using laser speckle contrast analysis (LASCA) and the wound was sutured using 6.0 monofilament. AP39 was dissolved in saline/DMSO solution (13:1) and administered once, 10 min after verified stroke induction at the dose of 100 nM/kg, *ip*. Control mice received the same volume of vehicle. Then buprenorphine (0.05 mg/kg/12 h, *s. c.* For 24 h) was administered for pain management. After recovery from anesthesia, the mouse was returned to the recovery cage until it was fully awake. Mice were returned to their home cage once recovered and monitored daily for weight and post-surgical signs for the duration of the chronic experiment.

2.4. Verification of brain blood flow and stroke volume

Ten minutes after inducing ischemic stroke in the motor cortex,

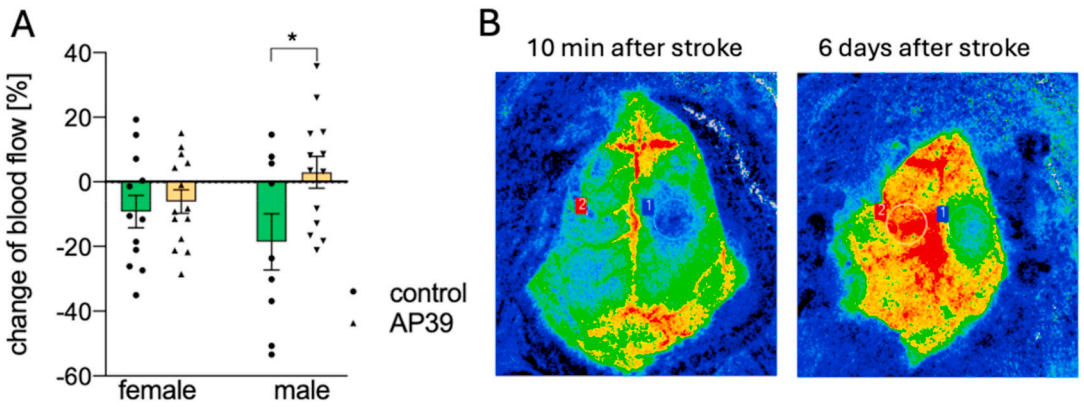


Fig. 3. The effect of AP39 on the blood flow in the ischemic area in females and males presents as a change between flow 10 min after stroke induction and 6 days after ischemic stroke; Data are presented as mean with SEM (A). Example images of cerebral blood flow from laser spot contrast analysis (LASCA) (B).

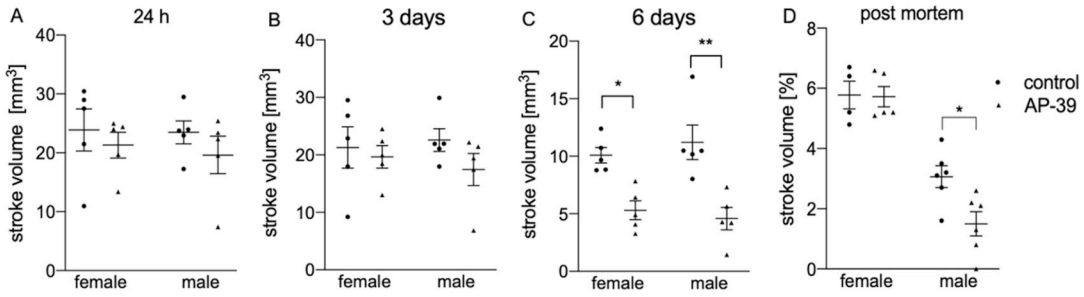


Fig. 4. The effect of AP39 on the stroke volume in T2 sequence of MRI 24h (A), 3 days (B) and 6 days (C) after ischemic stroke; n = 5. Results from TTC staining of post-mortem stroke volume (D). Stroke volume after TTC staining calculated as % of ipsilateral hemisphere; n = 6. Data are presented as mean with SEM.

blood flow at the stroke site was assessed in comparison to the same area in the contralateral hemisphere using a non-contact laser speckle contrast analysis system, which provides real-time, high-resolution blood flow images. A follow-up measurement was conducted six days post-ischemia, followed by animal euthanasia. The change in blood flow was calculated considering the results obtained 10 min after the stroke and after 6 days. Post-mortem analysis of the volume of the necrotic area was performed using the TTC staining method as previously described (Pomierny et al., 2021). The images were analyzed using ImageJ software, and the stroke volume was calculated as a percentage of the ipsilateral hemisphere.

2.5. Immunofluorescence

After transcardial perfusion with 4 °C 0.01M PBS for 5 min and subsequent, 4 °C 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.2, for 15 min, fixed brain tissue was immersed successively in 10 %, 20 % and 30 % sucrose in PBS for dehydration. The brains were then placed in 4 % PFA at 4 °C for 24h and stored at −80 °C. The tissue sections for the detection of Parkin and PINK1 proteins were subjected to antigen retrieval using a citrate buffer prior to incubation with primary antibodies. 30 mm sections on a glass slides were permeabilized with 0.3 % Triton X-100 in PBS (T-PBS) during 10 min and then treated with 10 % goat serum for 1 h and incubated overnight at 4 °C with the primary antibody *anti*-TOMM20 (rabbit, IgG, Affinity Biosciences, Cat. No. AF5208, 1:700), *anti*-COX4 (rabbit, IgG, Affinity Biosciences, Cat. No. AF5468, 1:700), PINK1 (mouse monoclonal, IgG_1, Santa Cruz, Cat. No. #G2322, 1:500) or Parkin (mouse monoclonal, IgG_{2b}, Santa Cruz, Cat. No. #D2222, 1:500) in 2 % goat-serum with antibody anti-MAP-2 (chicken, IgG, Invitrogen, Cat. No. PA1-10005, 1:1000) as a neuronal marker. The sections were washed two times with 0.01M PBS with 0.3 % Triton X-100 and incubated 1 h in the dark box, at room temperature (RT) with the secondary antibody Alexa Fluor - 488 (goat anti-rabbit IgG, Invitrogen, Cat. No. A11034, 1:400), Alexa Fluor – 488 (goat anti-mouse, IgG, Invitrogen, Cat. No. A32723, 1:400) and Alexa Fluor - 594 (goat anti-chicken IgG, Invitrogen, Cat. No. A32759, 1:400) diluted in 2 % goat serum. The sections were washed four times with PBS and cover slipped with mounting medium VectaShield with DAPI (Vector Laboratories). Isotype-matched immunoglobulins were used as a negative control. Images were taken using Leica Stellaris 8 WLL, DLS confocal microscope equipped with a 40×/0.75 and 20×/0.8 Plan Apo lens. ImageJ software (NIH ImageJ, USA) was used for all the quantifications. The imaging parameters for the experimental and control groups were the same. The fluorescence intensity in the ipsilateral areas surrounding the necrotic zone of brain sections was measured by a person unaware of the membership in the experimental groups. The analysis was conducted on 6 animals per each group.

2.6. MRI imaging and infract area quantification

In randomly selected animals not undergoing training, the course of ischemia was monitored by MRI n = 5/group), which was acquired on 7.0 T Bruker BioSpec Maxwell 70/17 MRI/PET with a surface loop coil. Animals were anesthetized with isoflurane (5 % induction, 2 % maintenance) mixed with oxygen. Following scout scans, high-resolution images were acquired with a 2.40 -minute T2-weighted turbo fast spin-echo sequence with fat suppression, oriented coronally. Imaging parameters included TR/TE = 2500 ms/33 ms, 2 averages and the voxel resolution of 0.063 mm × 0.078 mm x 0.7 mm with no interslice gaps. A respiratory sensor and rectal temperature probe were used to monitor physiological parameters (SA Instruments). Body temperature was maintained at 36.6–37.0 °C by means of heated water circuit. Ischemic stroke and its evolution were confirmed at 24h, 3d and 6d after the stroke induction, by comparing, between experimental groups, the area

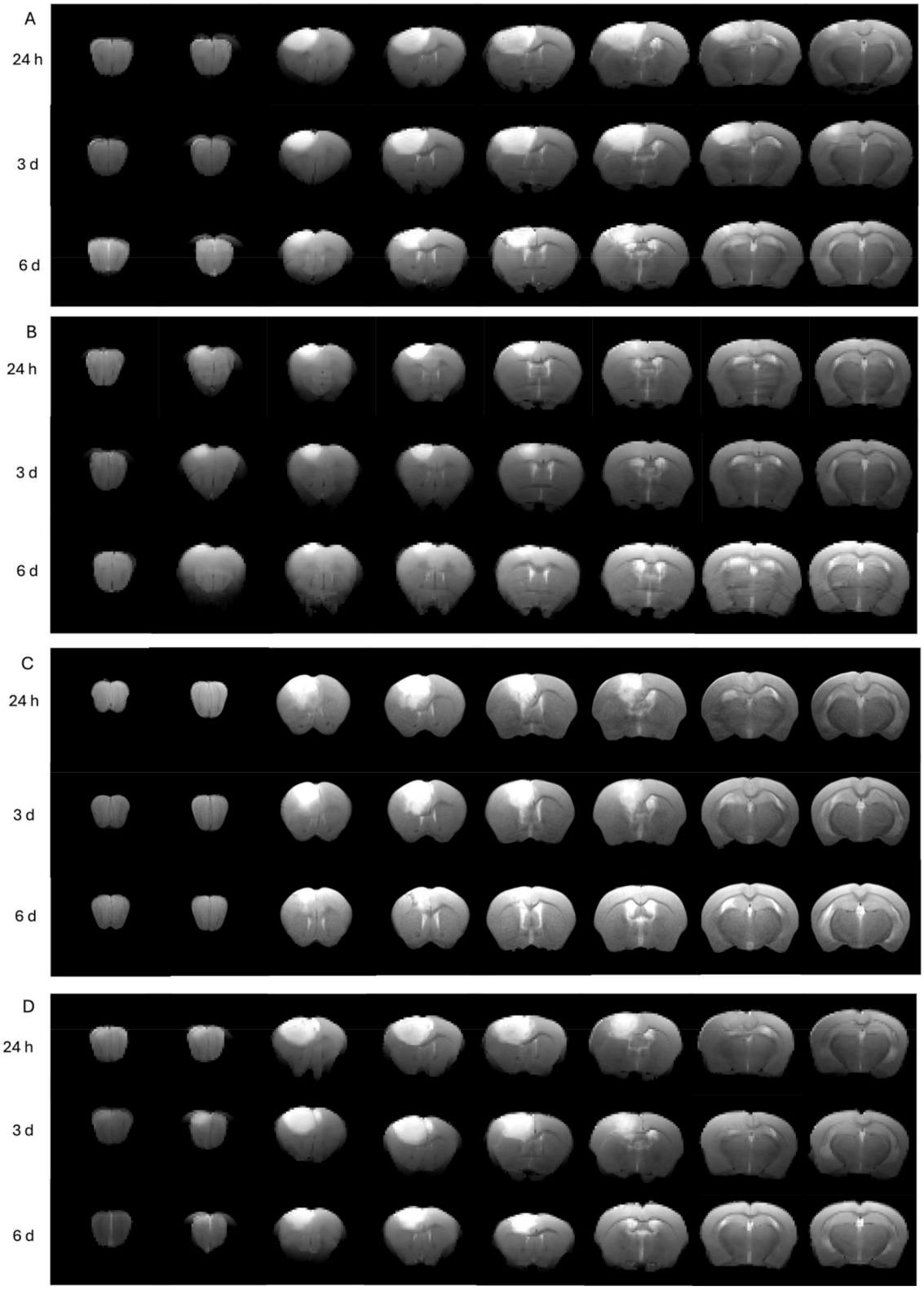


Fig. 5. Representative T2-weighted MRI images 24h, 3 and 6 days after ischemic stroke in males mice A-control group; B-AP39 treated group; and females C-control group; D -AP39 treated group. n = 5/group.

of the hyperintense voxels indicating the infarction in T2W images. For each T2W image, the brain was masked using Jim9 (JIM V.9_21, Xinapse systems, Aldwincle, UK, http://www.xinapse.com) and the mask was then used for skull stripping executed with FSL Maths (the FMRIB Software Library). Next, ANTs (Advanced Normalization Tools) was used for a rigid body registration between the masked brain and the template brain (NITRC). The infarct volume was quantified by the person blinded to the experimental groups using ITK-SNAP 4.2 by outlining the zone with hyperintense regions in each brain slice, and the total infarct area was obtained by summation of the infarcted areas multiplied

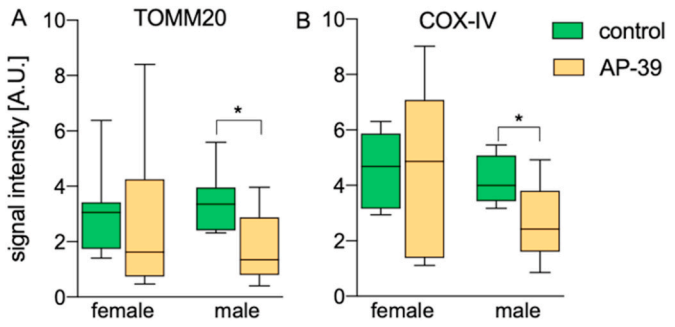


Fig. 6. Results of the study on the effect of AP39 on the expression of mitochondrial markers TOMM20 (A) and COX4 (B) in female and male mice in a photothrombotic cortical ischemic stroke model; Plot: min to max; n = 6.

by the slice thickness and presented in mm^3.

3. Statistical analyses

Statistical analysis was performed using GraphPad Prism 8 software (San Diego, CA, USA). To evaluate differences between pre- and post-stroke performance in the pellet reaching task a One-Way ANOVA was used, followed by Dunnett test post hoc analysis. The normality of the distribution in the studied groups was confirmed using the Shapiro-Wilk test. Differences between results from group AP39 and control were assessed by Wilcoxon matched-pairs signed rank test. Due to the lack of normal distribution, the differences in the total number of attempts made by males and females were assessed using the non-parametric Mann-Whitney *U* test. A 2-way ANOVA with Sidak's multiple comparisons test was used to assess the impact of AP39 on the difference in vascular flow in the ischemic area and stroke volume between females and males. The analysis of immunohistochemical results was performed using a *t*-test, following the verification of normality distribution. The level of significance was set at $p < 0.05$.

4. Results

4.1. Skilled reaching task

The average number of training sessions required to achieve 3 days of stable results was 13 days for females (min. 7; max. 23) and 20 days for males (min. 14; max. 25). Out of the 30 males designated for the experiment, the dominant forelimb could not be determined for one and two did not cooperate during the skilled reaching task training. Similarly, in two out of 30 females, the dominant forepaw could not be determined, and one was not interested in the training.

One -way ANOVA revealed that in ischemic female and male animals, the total number of success and 1st trial success significantly changed during the training at 24h after stroke (female success - $F_{(2,45)} = 129.3$; $p < 0.0001$ (Fig. 2A); female 1st trial success - $F_{(2,45)} = 61.49$; $p < 0.0001$ (Fig. 2C); male success - $F_{(2,45)} = 16.92$; $p < 0.0001$ (Fig. 2B); male 1st trial success - $F_{(2,45)} = 10.04$; $p = 0.0002$ (Fig. 2D)), while *post hoc* analysis showed significant reduction in number of total success trials and 1st trial success in both group of females ($p < 0.001$). Total number of success trials also importantly decreased ($p = 0.0027$ for AP39 group and $p < 0.0001$ for control group), whereas number of 1st trial success was significantly lower only in control group ($p = 0.0001$) Fig. 2.

Males in the group that received AP39 after stroke induction had a statistically significant higher total number of successful attempts, compared to the group that received the vehicle. This difference was observed on the 2nd ($U = 35.5$; $p = 0.0418$) and 6th ($U = 17.5$; $p = 0.0012$) day of post-stroke training. The number of successful first attempts was also higher on day 5 (2nd ($U = 38,5$; $p = 0.0654$), 3rd ($U = 31$; $p = 0.0203$), 5 th ($U = 32.5$; $p = 0.0254$) and 6th ($U = 25$; $p = 0.0065$)). It should also be noted that 2 males in the control group died within 24 h after ischemia induction. These results indicate a beneficial effect of a single administration of AP39 after ischemic cortical stroke on fine motor skills in males. The final number of males in poststroke training was 14 in the AP39 group and 10 in the control group.

It was surprising that no statistically significant differences were observed in females between the group administered AP39 and the control group during six subsequent days of skilled reaching task training, both in the number of successful attempts (Fig. 2 A.) and in 1st trial success (Fig. 2. C).

Sex-dependent differences were also observed in the number of attempts made during the first training session after the stroke, with females in both the experimental ($U = 13.50$; $p < 0.0001$) and control groups ($U = 30.50$; $p = 0.0457$) showing a significantly lower number of attempts compared to males (Fig. 2E.), while the difference between AP39 female and male groups was more statistically significant. This highlights the importance of sex in the response to the administration of the investigated compound. Three females in the control group died within 24 h after ischemia induction. The final number of females in poststroke training was 12 in both groups.

4.2. Blood flow imaging

Statistical analysis of blood flow results demonstrated a significant effect of AP39 treatment on the change in vascular blood flow evaluated 6 days after the induction of ischemic stroke ($F_{(2,45)} = 5.2$; $p = 0.0273$). However, *post-hoc* analysis revealed a significantly increased flow in males ($p = 0.0198$), while the change in females was not observed (Fig. 3A and B).

4.3. Stroke volume MRI imaging and TTC staining

No significant effect of AP39 treatment on stroke volume measured by MRI was observed 24 h or significant reduction 3 days after stroke induction. However, a significant effect was noted after 6 days ($F_{(1,16)} = 31.66$; $p < 0.0001$), with the changes being more pronounced in males ($p = 0.0003$) than in females ($p = 0.0153$) (Fig. 4A and B). Representative T2-weighted MRI images are shown in Fig. 5. The results of necrosis volume measurements in post-mortem analysis are comparable to the MRI results in males ($p = 0.0152$), while no significant reduction was observed in females (Fig. 4C.).

4.4. COX IV, TOMM20, PINK1 and parkin expression

Imaging of mitochondrial protein expression, TOMM20 (Fig. 6A) and COX4 (Fig. 6B), using confocal microscopy revealed a slight but significant decrease in their expression in the group of males treated with AP39 (TOMM20: $t = 2.459$, $df = 10$, $p = 0.0301$; COX4: $t = 2.422$, $df = 10$, $p = 0.0308$). In females, no significant differences were observed between the experimental and control groups; however, the experimental group showed a notable variability in the expression levels of both markers. Representative images are shown in Figs. 7 and 8.

Immunofluorescent analysis of mitochondrial dynamic proteins PINK1 and Parkin (Fig. 9A and B), revealed a significant increase in Parkin expression in the group of males treated with AP39 compared to the vehicle-treated group ($t = 5.429$, $df = 10$, $p = 0.0004$) with a less significant but also increased expression of the Pink1 protein ($t = 2.492$, $df = 10$, $p = 0.0343$). In contrast, Parkin and PINK1 expression levels in females did not differ significantly between the two groups. However, both proteins expression in females exhibited considerable variability, suggesting potential sex-dependent effects. Representative images are shown in Figs. 9 and 10.

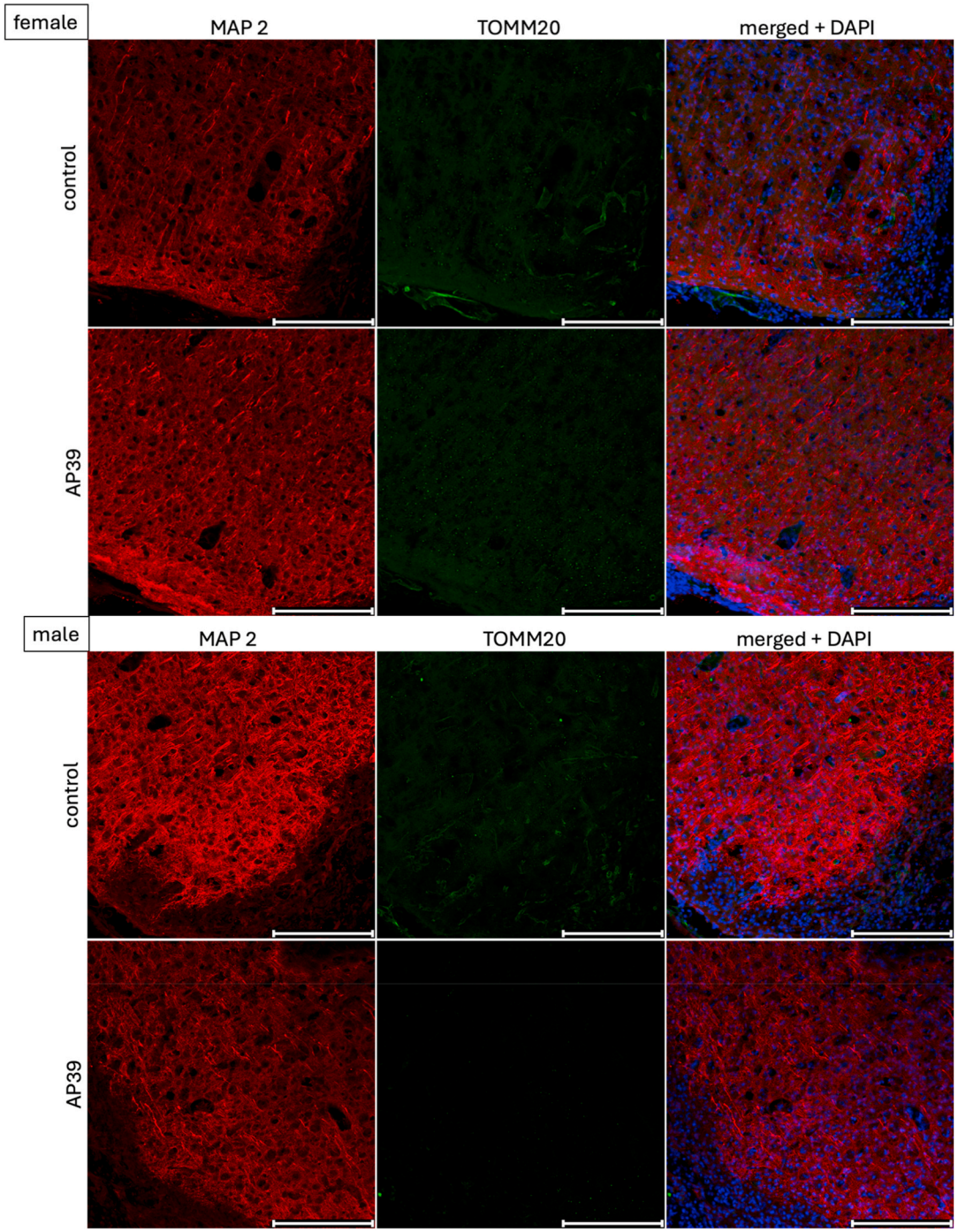


Fig. 7. Representative images of the analysis of TOMM20 mitochondrial protein expression in females and males from the control and experimental groups. Magnification ×20, scale bar: 200 μM.

5. Discussion

The skilled reaching task is a well-known and valued behavioral test for assessing motor deficits not only in neurodegenerative diseases but also after stroke (see Fig. 11). The reaching ability in rodents is homologous to reaching ability in humans, which makes it easy to translate the results of such a test to human conditions (Klein et al., 2012). In this experiment, we found that the hydrogen sulfide donor AP39, administered 10 min after photothrombotic-induced cortical stroke, improved motor performance in a skilled reaching task from the first days post-stroke and reduced infarct volume in males, as measured on day 6 post-ischemia, compared to the control group. Additionally, in the AP39 treated group of males, the number of attempts made on the first day post-stroke was higher, though not significantly so. This difference was particularly noticeable when analyzing the number of successful attempts during the first try at reaching for the pellet. A successful repeated attempt was often the result of compensatory paw movements, which enabled the animal to grasp the food. However, the ability to perform such movements shortly after the stroke may also indicate better condition in the animals (Estrada-Bonilla et al., 2020). In the MRI study, the neuroprotective effect in the form of reduced stroke volume was not observed during the first 3 days, while on day 6, stroke volume was significantly smaller in males who received AP39. The TTC study proved to be less precise compared to MRI. In males, the significance of

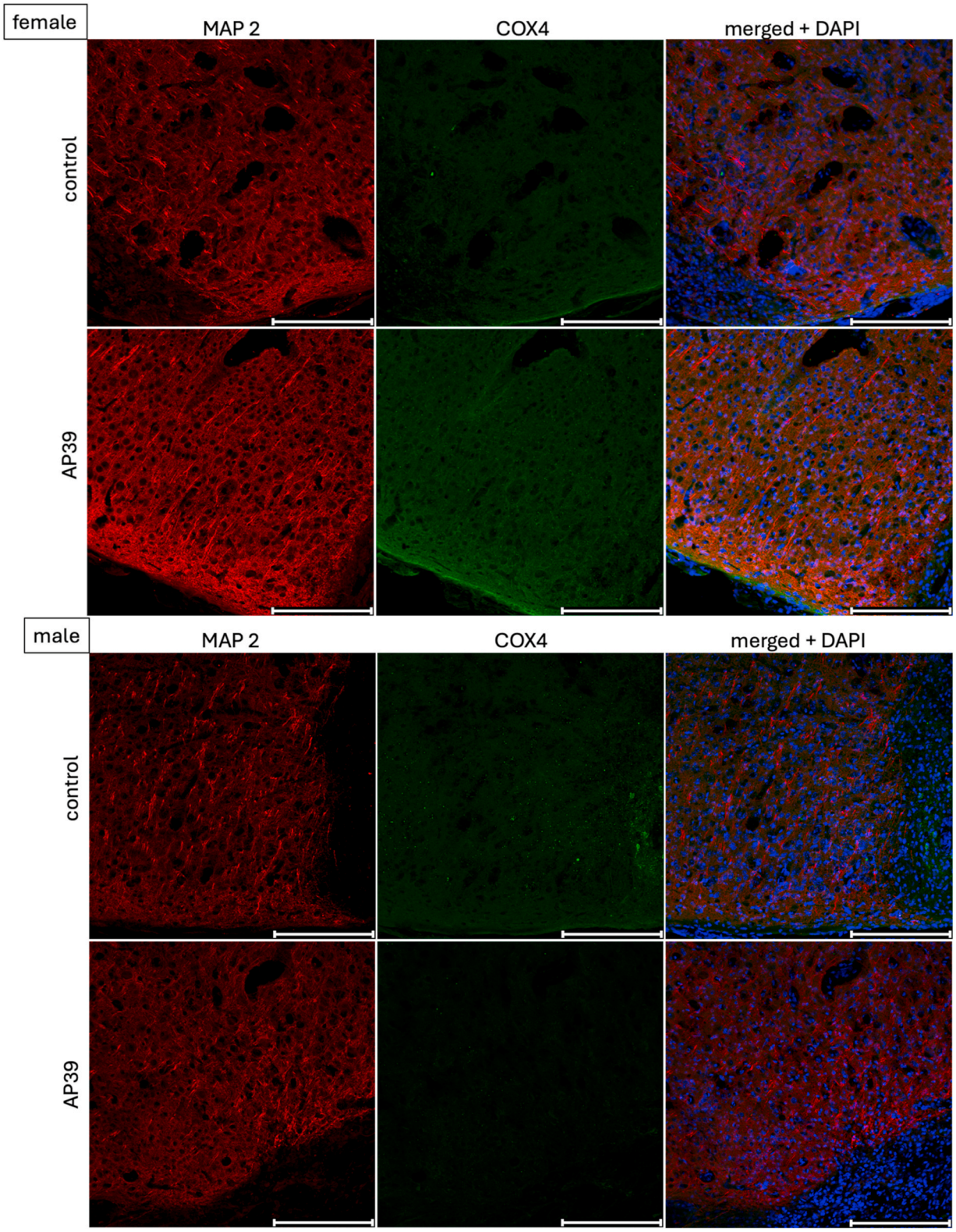


Fig. 8. Representative images of the analysis of COX4 mitochondrial protein expression in females and males from the control and experimental groups. Magnification ×20, scale bar: 200 μM.

the stroke volume difference was lower, while in females, the difference was not statistically significant. This may be due to the thickness of the analyzed sections. In TTC imaging, the slice thickness was 2 mm, while in MRI it ranged from 0.35 to 0.7 mm, depending on the sequence and projection.

The results obtained from the study conducted on males are very promising; however, such an improvement was not observed in females. Our previous studies focused on investigating the effect of brain conditioning on ischemia and the protective action of AP39 administered 10 min after reperfusion in the MCAO model. Both studies showed a

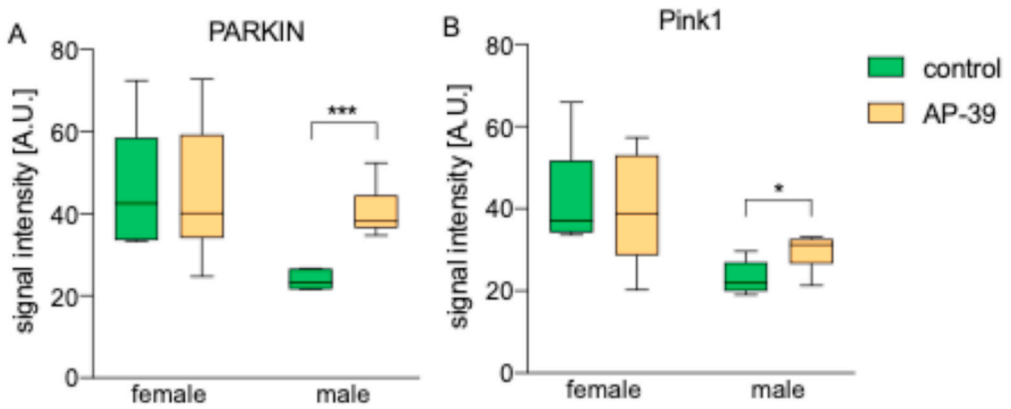


Fig. 9. Results of the study on the effect of AP39 on the expression of mitochondrial dynamic proteins Parkin (A) and PINK1 (B) in female and male mice in a photothrombotic cortical ischemic stroke model; Plot: min to max; n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

positive effect; however, these projects were conducted solely on young males (2–3-month-old) and observations were short-term. In this study, we decided to evaluate the therapeutic effects on motor function in 8-month-old males and females. In the case of females, this is the age at which a decline in fertility may be observed, which is reflected in the extension of the diestrus phase and undoubtedly can increase the translational relevance of the obtained results. Studies on females are rarely conducted due to methodological challenges in monitoring the estrous cycle. However, they are increasingly recommended because of sex-based differences in pathophysiology (Roy-O'Reilly and McCullough, 2018) and treatment response in ischemic stroke (Li and McCullough, 2009; Herson et al., 2013). There are no comparative studies in the literature regarding the activity of AP39 in females and males. So far, the anti-inflammatory activity has been investigated in females using a mouse model of respiratory tract inflammation, showing positive effects (Karaman et al., 2023). In this study AP39 prevented bronchial hyperreactivity and reduced the levels of TNF-alpha and IL-6 in bronchoalveolar lavage fluid. Whereas in animal models mimicking premature ovarian failure in women, multiple (14–44 days) intraperitoneal administrations of AP39 did not show a therapeutic effect on the normalization of reproductive parameters related to ovarian function (Karaman et al., 2023). In a model of ischemic stroke in females, one might expect an even greater therapeutic effect due to the protective nature of estrogens, which makes our result all the more surprising. This suggests that the neuroprotective mechanism of AP39 is at least partially related to its influence on sex-dependent cell death pathways. The poly (ADP-ribose) polymerase 1 (PARP-1) is one of the identified sex-dependent molecules (along with inducible and neuronal nitric oxide synthase and others (Herson et al., 2013)) involved in cell death signaling pathways. The neuroprotective effect of hydrogen sulfide donor administration via the PARP-1 pathway in ischemic brain injury was described by (Yu et al., 2015) in both in vitro and in vivo models, utilizing the inorganic molecule NaHS. Our recent studies on the neuroprotective mechanism of AP39 in a rat MCAO model with reperfusion also have shown that it leads to a significant inhibition PARP1 activation (Bartosz, 2023). PARP-1, a nuclear enzyme activated by DNA damage, is thought to aid in DNA repair and reduce chromatid exchange. In ischemic stroke, there is a dramatic increase in the activity of this enzyme (Andrabi et al., 2006). However, excessive activation of PARP-1 can triggers cell death, and PARP-1 has been identified as a key factor in neuronal death following excitotoxic events, ischemia-reperfusion, oxidative stress, and similar damaging conditions (Alano et al., 2010). In the study of the neuroprotective effects of minocycline, a PARP-1 activity inhibitor, in the MCAO model, it was shown that this effect was beneficial only in males, while no improvement was observed in females (Li and McCullough, 2009) or led to an unexpected exacerbation in ischemic injury in female mice, and this effect was independent of estrogen (McCullough et al., 2005). In our study, we did not observe a significant deterioration in the health of females following AP39 administration; however, the course of the disease over the 6-day observation period post-stroke was significantly worse in females in both the control and experimental groups compared to males. This aligns with available statistical data, which clearly indicate that women experience a more complicated course of ischemic stroke, higher levels of disability, and greater mortality (Shajahan et al., 2023). A slight beneficial effect of AP39 in females was observed in MRI images, where the stroke volume six days post-stroke was smaller than in the control group. The difference was not as pronounced as in males but was statistically significant. The basis for such changes could be the effect of AP39 on caspase-3 levels, which decrease in response to this molecule (Pomierny et al., 2021). Despite the lack of a therapeutic effect from inhibiting the PARP-1 pathway in female stroke models, the inhibition of caspase activity had a protective effect in females. Furthermore, this effect was also observed in females after ovariectomy (Liu et al., 2011; Roy-O'Reilly and McCullough, 2018).

In our study, we also found sex differences in response to AP39 at the molecular level by examining the expression of mitochondrial and mitophagy-related proteins TOMM20 and COX4. TOMM20 is part of the mitochondrial outer membrane translocase complex and plays a role in transporting proteins targeted to the mitochondria into the mitochondrial matrix. It has been widely used as a marker of mitochondrial mass and metabolic activity (Fu et al., 2020; Wen et al., 2021). COX4, a component of the cytochrome *c* oxidase complex, also reflects mitochondrial respiratory function. Mitochondrial autophagy, or mitophagy, is a selective degradation process of damaged mitochondria, generally regarded as an adaptive metabolic response that prevents excessive ROS production and cell death (Shao et al., 2020). Although a comprehensive theory explaining the role of mitophagy in cerebral ischemia is still lacking, accumulating evidence suggests its involvement in post-ischemic pathology. Some studies have shown that enhancing mitophagy can reduce ischemia-reperfusion damage (Li et al., 2014; Di et al., 2015; Shen et al., 2017; He et al., 2019; Wu et al., 2021) while others report benefits of mitophagy inhibition (Feng et al., 2018; Lan et al., 2018; Su et al., 2018) (for review see: (Tang et al., 2016; Shao et al., 2020). We found that AP39 reduced the expression of TOMM20 and COX4 in the peri-infarct region six days after stroke, but this effect was observed only in males. In females, the expression levels of these mitochondrial markers remained comparable to those in the control group. The improvement in motor performance and the reduction in infarct volume seen in our study suggest that lowering mitochondrial marker expression may reflect a beneficial modulation of mitochondrial turnover and quality control mechanisms in this model. The observed

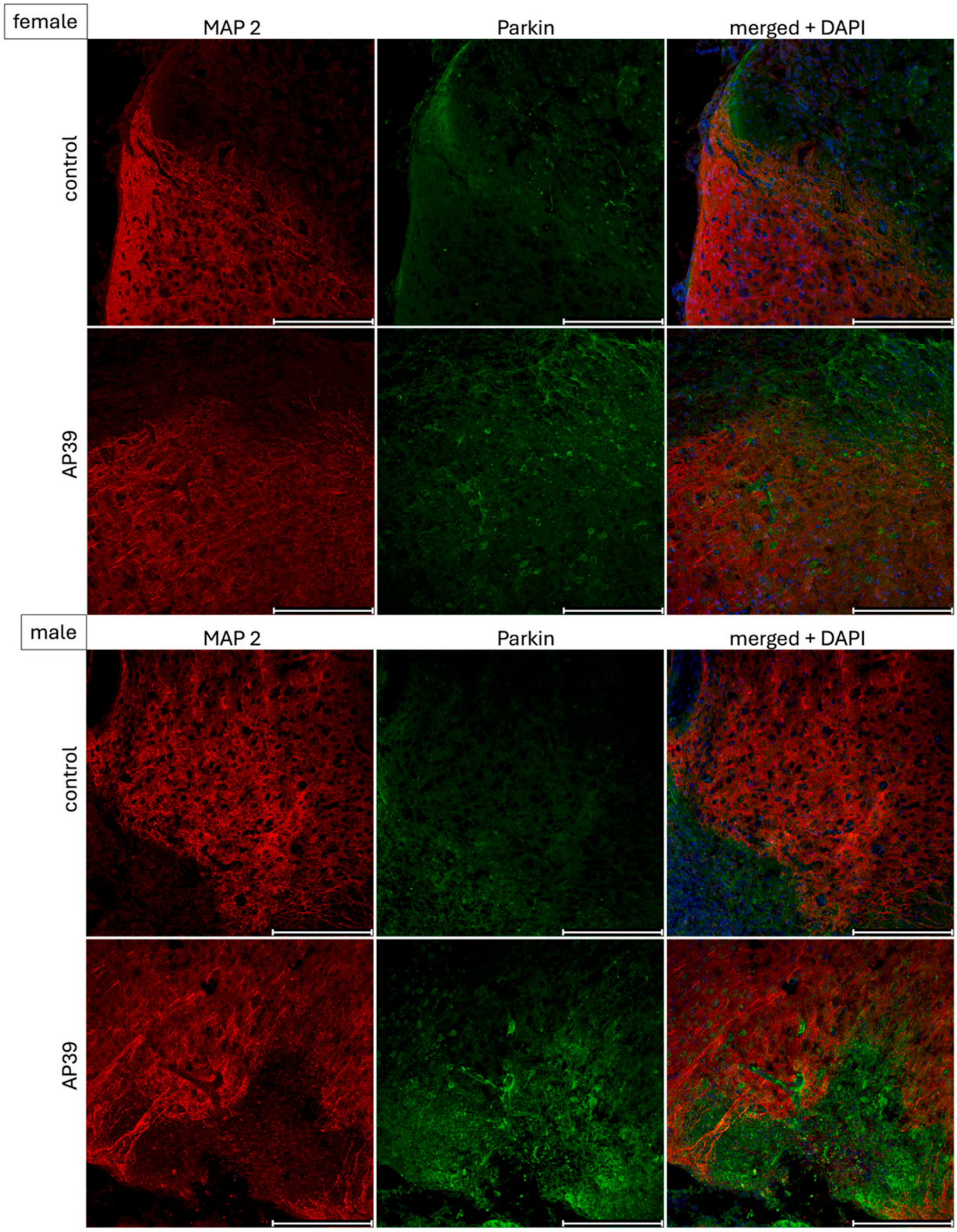


Fig. 10. Representative images of the analysis of Parkin protein expression in females and males from the control and experimental groups. Magnification ×20, scale bar: 200 μM.

sex-specific effects may be related to inherent differences in mitochondrial dynamics and lysosomal function (Demarest et al., 2016). Importantly, the expression of PINK1 and, most prominently, Parkin was upregulated in the peri-infarct area at the same time point, indicating persistent activation of mitophagy. These proteins are central regulators of the mitochondrial quality control pathway: PINK1 accumulates on the outer membrane of depolarized mitochondria and recruits Parkin, an E3 ubiquitin ligase, which tags damaged mitochondria for autophagic degradation (Ashrafi and Schwarz, 2013). The decrease in TOMM20 and COX4 may thus result from Parkin-mediated degradation of

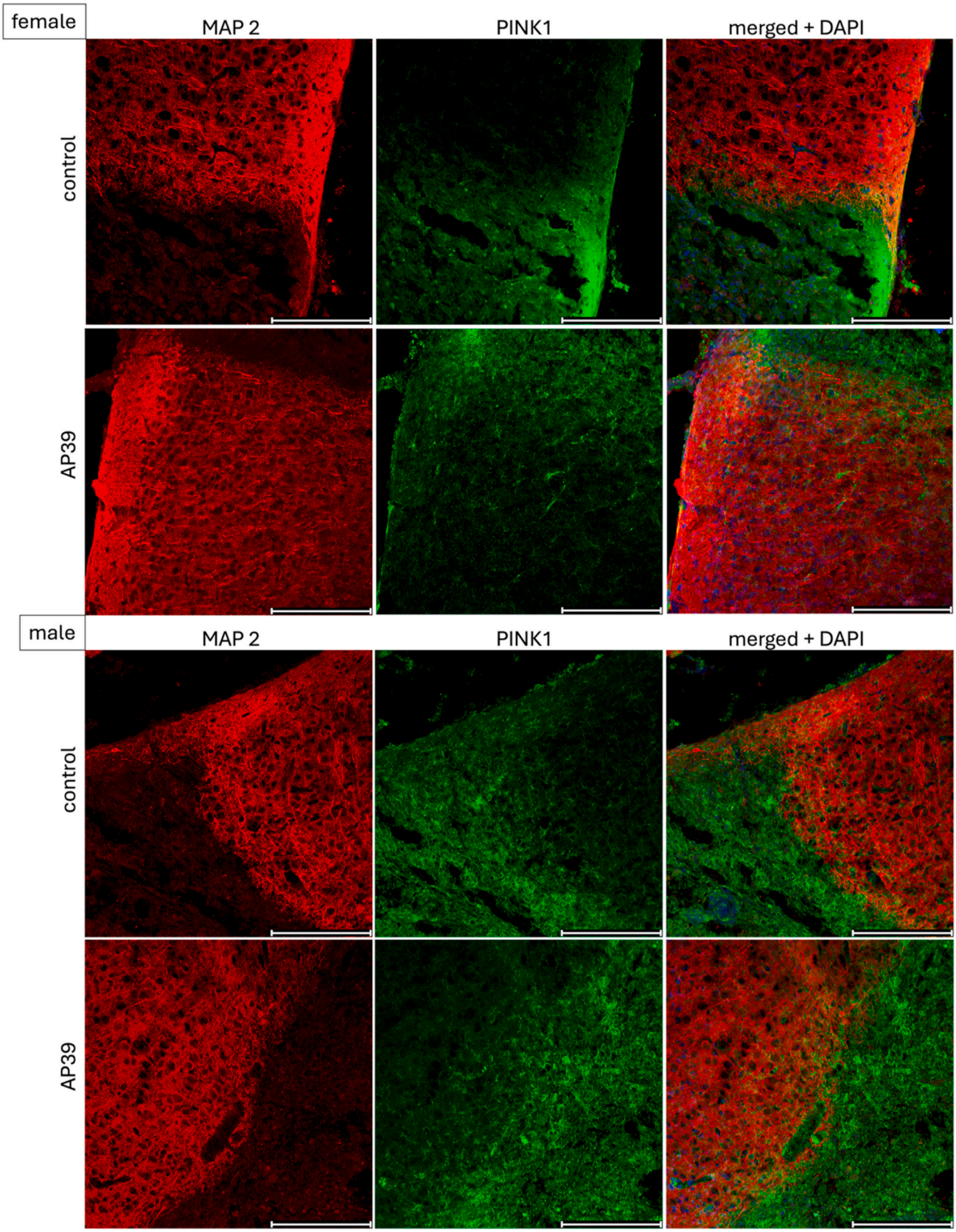


Fig. 11. Representative images of the analysis of PINK1 protein expression in females and males from the control and experimental groups. Magnification ×20, scale bar: 200 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dysfunctional mitochondria. The spatial context of our measurements, performed in the peri-infarct zone rather than the necrotic core, is critical, as this region contains metabolically compromised but potentially salvageable tissue (Dirnagl et al., 1999; Moskowitz et al., 2010). This suggests that the observed changes reflect an active mitochondrial remodeling process rather than irreversible damage.

Furthermore, AP39 has been shown to preserve mitochondrial function, reduce oxidative stress, and improve bioenergetics in models of ischemic and oxidative injury (Gerő et al., 2016). In our study, the increased expression of mitophagy-related proteins PINK1 and especially Parkin was observed **only in males** treated with AP39. This suggests that AP39 may promote a compensatory response specifically in male rats, potentially supporting mitochondrial quality control and neuronal survival in the peri-infarct region. In contrast, **females displayed higher basal expression levels of both PINK1 and Parkin**, regardless of treatment, and showed greater interindividual variability, which may have masked any treatment-related effects. These findings indicate that **the molecular response to AP39 is sex-dependent**, with males showing a more pronounced activation of the PINK1-Parkin pathway following treatment. The differential expression patterns observed between sexes may be influenced by hormonal or genetic factors affecting mitochondrial dynamics and should be further explored in future studies.

Despite the promising results demonstrating the neuroprotective potential of the mitochondria-targeted hydrogen sulfide donor AP39 in a rat photothrombotic model of ischemic stroke, our study has several important limitations. Experimental conditions, such as the stroke model, stroke size, and reperfusion, play a significant role. We used a photothrombotic stroke model limited to the motor cortex of the dominant forelimb, without pharmacological or mechanical reperfusion. Enriching the MRI scanning protocol with DWI and PWI sequences was challenging in post-stroke mice without reperfusion due to the prolonged exposure to isoflurane and the resulting hemodynamic instability. However, in future studies using the MCAO model in rats, this approach may provide valuable information on the therapeutic activity of AP39 and cerebral blood flow in post-stroke animals. Moreover, we did not assess mitochondrial ultrastructure, which could provide additional insight into mitochondrial integrity and dynamics in response to treatment. Transmission electron microscopy would allow for the visualization of morphological changes such as swelling, fragmentation, or cristae disruption. It would also be valuable to quantify ATP levels, mitochondrial membrane potential, and brain H_2S dynamics to provide a more comprehensive overview of mitochondrial bioenergetic status following AP39 administration. These functional indicators could also help elucidate the mechanisms behind the observed sex-dependent differences in response to treatment. Moreover, extending the observation period beyond 6 days post-stroke to at least 4 weeks would allow for evaluation of the long-term efficacy of AP39, particularly in terms of recovery and neuroplasticity. However, it is important to note that mice exhibit a relatively rapid and robust recovery following cerebral ischemia, which may limit the ability to detect persistent deficits or delayed therapeutic effects (Schaar et al., 2010; Balkaya et al., 2013). Compared to rats, mice often demonstrate higher spontaneous motor recovery and compensatory behaviors, which complicates the interpretation of long-term functional outcomes (Dirnagl and Endres, 2014). Therefore, future studies aimed at assessing the long-term neuroprotective and pro-regenerative potential of AP39 should consider using rat models of ischemic stroke, which provide greater sensitivity for evaluating chronic motor and cognitive impairments. This approach will help clarify whether the early benefits of AP39 observed in mice are sustained and functionally relevant over extended periods of recovery. Lastly, implementing a repeated dosing regimen, rather than single administration, could offer insights into the sustained or cumulative effects of AP39 in both acute and subacute phases of ischemic injury.

6. Conclusions

In summary, this study demonstrated the beneficial effects of a single dose of the mitochondria-targeted hydrogen sulfide donor, AP39, following photothrombotic ischemic stroke in the cerebral cortex on motor function, cerebral blood flow, and stroke volume in male rats. No improvement in motor performance was observed in females, although stroke volume measured by MRI was slightly lower after AP39 administration compared to the control group. Immunofluorescence imaging of mitochondrial autophagy markers revealed a significant reduction in TOMM20 and COX4 expression in males treated with AP39, suggesting altered mitochondrial turnover or preservation of mitochondrial integrity. In contrast, females showed no such changes, and overall expression levels were more variable. Additionally, expression of key mitophagy regulators PINK1 and, most notably, Parkin was significantly upregulated only in males after AP39 treatment. Females exhibited higher baseline levels of these proteins across both experimental groups, accompanied by high interindividual variability, which may indicate intrinsic sex-related differences in mitochondrial quality control mechanisms. These molecular findings suggest that the neuroprotective effects of AP39 are associated with modulation of mitophagy pathways, particularly via the PINK1-Parkin axis, and are more pronounced in males. The observed sex-specific responses to AP39 emphasize the importance of considering biological sex in both experimental stroke research and the development of targeted neuroprotective therapies.

These results contribute to a better understanding of sex-dependent pathophysiological mechanisms in ischemic stroke and the underlying mitochondrial responses. They support the need for tailored therapeutic approaches that take into account sex differences in mitochondrial regulation and recovery potential following ischemic injury.

CRediT authorship contribution statement

Jakub Jurczyk: Writing – original draft, Methodology, Investigation, Formal analysis. **Zuzanna Guzda:** Methodology, Investigation. **Alicja Skórkowska:** Visualization, Methodology, Investigation. **Żaneta Broniowska:** Investigation. **Małgorzata Piechaczek:** Investigation. **Aleksandra Więcek:** Investigation. **Emilia Schulze:** Investigation. **Angelika Ziaja:** Investigation. **Roberta Torregrossa:** Resources. **Matthew Whiteman:** Resources. **Michel Soares Mesquita:** Visualization, Formal analysis. **Bartosz Pomierny:** Visualization, Methodology, Investigation, Formal analysis. **Lucyna Pomierny-Chamioło:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Conflict of interest

M.W., R.T. and the University of Exeter have intellectual property (patent filings awarded and pending) related to hydrogen sulfide delivery molecules and their therapeutic uses. M.W. is a co-founder and CSO of MitoRX Therapeutics, Oxford. M.S.M is a co-founder and CEO of L&M DataScience Ltd. There are no conflicts of interest to disclose by other authors.

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

During the preparation of this work, the authors used Open Writefull and ChatGpt in order to improve the grammar in our articles. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Founding sources

This work was supported by: the Statutory Funds of the Department

of Toxicology Jagiellonian University Medical College, Poland, number: N42/DBS/000315 and N42/DBS/000430. This research was carried out with the use of research infrastructure co-financed by the Smart Growth Operational Programme POIR 4.2 project no. POIR.04.02.00–00-D023/20. The research for this publication has been supported by a grant number U1C/P04/NO/02.20 from the Priority Research Area qLIFE under the Strategic Programme Excellence Initiative at Jagiellonian University.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matthew Whiteman reports a relationship with MitoRX Therapeutics, Oxford that includes: board membership and consulting or advisory. Michel Soares Mesquita reports a relationship with L&M Data Science Ltd that includes: board membership and consulting or advisory. Roberta Torregrossa has patent issued to Matthew Whiteman. Matthew Whiteman has patent issued to Matthew Whiteman. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Alano, C.C., et al., 2010. NAD+ depletion is necessary and sufficient for poly(ADP-ribose) polymerase-1-mediated neuronal death. J. Neurosci. : the Off. J. Soc. Neurosci. 30 (8), 2967–2978. https://doi.org/10.1523/JNEUROSCI.5552-09.2010.

Andrabi, S.A., et al., 2006. Poly(ADP-ribose) (PAR) polymer is a death signal. Proc. Natl. Acad. Sci. U. S. A 103 (48), 18308–18313. https://doi.org/10.1073/pnas.0606526103.

Ashrafi, G., Schwarz, T.L., 2013. The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death Differ. 20 (1), 31–42. https://doi.org/10.1038/CDD.2012.81.

Bartosz, P., et al., 2023. 'Neuroprotection by Post-Stroke Administration of the Slow-Releasing Hydrogen Sulfide (H2s) Delivery Molecule Ap39: novel Insight into Stroke Therapy'. Neuroscience. https://doi.org/10.21203/rs.3.rs-3100208/v1 under revi (preprint).

Balkaya, M., et al., 2013. Assessing post-stroke behavior in mouse models of focal ischemia. J. Cerebr. Blood Flow Metabol. : Off. J. Int. Soc. Cerebral Blood Flow and Metabolism 33 (3), 330–338. https://doi.org/10.1038/JCBFM.2012.185.

Bandera, E., et al., 2006. Cerebral blood flow threshold of ischemic penumbra and infarct core in acute ischemic stroke: a systematic review. Stroke 37 (5), 1334–1339. https://doi.org/10.1161/01.STR.0000217418.29609.22.

Baron, J.C., 1999. Mapping the ischaemic penumbra with PET: implications for acute stroke treatment. Cerebrovasc. Dis. (Basel, Switzerland) 9 (4), 193–201. https://doi.org/10.1159/000015955.

Bayir, H., Kagan, V.E., 2008. Bench-to-bedside review: mitochondrial injury, oxidative stress and apoptosis–there is nothing more practical than a good theory. Crit. Care 12 (1), 206. https://doi.org/10.1186/cc6779.

Beal, C.C., 2010. Gender and stroke symptoms: a review of the current literature. J. Neurosci. Nurs. : J. Am. Assoc. Neurosci. Nurses 42 (2), 80–87.

Borutaite, V., 2010. Mitochondria as decision-makers in cell death. Environ. Mol. Mutagen. 51 (5), 406–416. https://doi.org/10.1002/em.20564.

Demarest, T.G., et al., 2016. Sex-dependent mitophagy and neuronal death following rat neonatal hypoxia-ischemia. Neuroscience 335, 103–113. https://doi.org/10.1016/j.neuroscience.2016.08.026.

Di, Y., et al., 2015. Methylene blue reduces acute cerebral ischemic injury via the induction of mitophagy. Mol. Med. (Camb.) 21 (1), 420–429. https://doi.org/10.2119/molmed.2015.00038.

Dirnagl, U., Endres, M., 2014. Found in translation: preclinical stroke research predicts human pathophysiology, clinical phenotypes, and therapeutic outcomes. Stroke 45 (5), 1510–1518. https://doi.org/10.1161/STROKEAHA.113.004075.

Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 22 (9), 391–397. https://doi.org/10.1016/S0166-2236(99)01401-0.

Estrada-Bonilla, Y.C., et al., 2020. Compensatory neuromuscular junction adaptations of forelimb muscles in focal cortical ischemia in rats. Brain and Behavior 10 (3), e01472. https://doi.org/10.1002/brb3.1472.

Farr, T.D., Whishaw, I.Q., 2002. Quantitative and qualitative impairments in skilled reaching in the mouse (Mus musculus) after a focal motor cortex stroke. Stroke 33 (7), 1869–1875. https://doi.org/10.1161/01.str.0000020714.48349.4e.

Feng, J., et al., 2018. Inhibition of peroxynitrite-induced mitophagy activation attenuates cerebral ischemia-reperfusion injury. Mol. Neurobiol. 55 (8), 6369–6386. https://doi.org/10.1007/s12035-017-0859-x.

Fu, Z.-J., et al., 2020. HIF-1α-BNIP3-mediated mitophagy in tubular cells protects against renal ischemia/reperfusion injury. Redox Biol. 36, 101671. https://doi.org/10.1016/j.redox.2020.101671.

Gerő, D., et al., 2016. The novel mitochondria-targeted hydrogen sulfide (H2S) donors AP123 and AP39 protect against hyperglycemic injury in microvascular endothelial cells in vitro. Pharmacol. Res. 113 (Pt A), 186–198. https://doi.org/10.1016/J.PHRS.2016.08.019.

Goodwin, L.R., et al., 1989. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. J. Anal. Toxicol. 13 (2), 105–109. https://doi.org/10.1093/jat/13.2.105.

Goubern, M., et al., 2007. Sulfide, the first inorganic substrate for human cells. FASEB J. : Off. Publ. Federation of Am. Societies for Experimental Biol. 21 (8), 1699–1706. https://doi.org/10.1096/fj.06-7407com.

Hatano, S., 1976. Experience from a multicentre stroke register: a preliminary report. Bull. World Health Organ. 54 (5), 541–553.

He, Q., et al., 2019. Parkin-Dependent mitophagy is required for the inhibition of ATF4 on NLRP3 inflammasome activation in cerebral ischemia-reperfusion injury in rats. Cells 8 (8). https://doi.org/10.3390/cells8080897.

Herson, P.S., Palmateer, J., Hurn, P.D., 2013. Biological sex and mechanisms of ischemic brain injury. Transl. Stroke Res. 4 (4), 413–419. https://doi.org/10.1007/s12975-012-0238-x.

Kamoun, P., 2004. Endogenous production of hydrogen sulfide in mammals. Amino Acids 26 (3), 243–254. https://doi.org/10.1007/s00726-004-0072-x.

Karaman, Y., et al., 2023. The effect of mitochondria-targeted slow hydrogen sulfide releasing donor AP39-treatment on airway inflammation. Eur. J. Pharmacol. 946, 175619. https://doi.org/10.1016/j.ejphar.2023.175619.

Klein, A., et al., 2012. The use of rodent skilled reaching as a translational model for investigating brain damage and disease. Neurosci. Biobehav. Rev. 36 (3), 1030–1042. https://doi.org/10.1016/j.neubiorev.2011.12.010.

Lan, R., et al., 2018. Xiao-Xu-Ming decoction reduced mitophagy activation and improved mitochondrial function in cerebral ischemia and reperfusion injury. Behav. Neurol. 2018, 4147502. https://doi.org/10.1155/2018/4147502.

Li, J., McCullough, L.D., 2009. Sex differences in minocycline-induced neuroprotection after experimental stroke. J. Cerebr. Blood Flow Metabol. : Off. J. Int. Soc. Cerebral Blood Flow and Metabolism 29 (4), 670–674. https://doi.org/10.1038/jcbfm.2009.3.

Li, Q., et al., 2014. Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke. Biochem. Biophys. Res. Commun. 444 (2), 182–188. https://doi.org/10.1016/j.bbrc.2014.01.032.

Liu, F., et al., 2011. Sex differences in the response to poly(ADP-ribose) polymerase-1 deletion and caspase inhibition after stroke. Stroke 42 (4), 1090–1096. https://doi.org/10.1161/STROKEAHA.110.594861.

Mancardi, D., et al., 2009. Physiological and pharmacological features of the novel gasotransmitter: hydrogen sulfide. Biochim. Biophys. Acta 1787 (7), 864–872. https://doi.org/10.1016/j.bbabio.2009.03.005.

Marutani, E., et al., 2021. Sulfide catabolism ameliorates hypoxic brain injury. Nat. Commun. 12 (1), 3108. https://doi.org/10.1038/s41467-021-23363-x.

McCullough, L.D., et al., 2005. Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. J. Cerebr. Blood Flow Metabol. : Off. J. Int. Soc. Cerebral Blood Flow and Metabolism 25 (4), 502–512. https://doi.org/10.1038/sj.jcbfm.9600059.

Moore, P.K., Bhatia, M., Moochhala, S., 2003. Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol. Sci. 24 (12), 609–611. https://doi.org/10.1016/j.tips.2003.10.007.

Moskowitz, M.A., Lo, E.H., Iadecola, C., 2010. The science of stroke: mechanisms in search of treatments. Neuron 67 (2), 181–198. https://doi.org/10.1016/J.NEURON.2010.07.002.

Niizuma, K., et al., 2010. Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. Biochim. Biophys. Acta 1802 (1), 92–99. https://doi.org/10.1016/j.bbadis.2009.09.002.

Ouyang, Y.-B., et al., 2007. Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. J. Neurosci. : the Off. J. Soc. Neurosci. 27 (16), 4253–4260. https://doi.org/10.1523/JNEUROSCI.0211-07.2007.

Pathak, D., Sepp, K.J., Hollenbeck, P.J., 2010. Evidence that myosin activity opposes microtubule-based axonal transport of mitochondria. J. Neurosci. : the Off. J. Soc. Neurosci. 30 (26), 8984–8992. https://doi.org/10.1523/JNEUROSCI.1621-10.2010.

Pekkurnaz, G., et al., 2014. Glucose regulates mitochondrial motility via Milton modification by O-GlcNAc transferase. Cell 158 (1), 54–68. https://doi.org/10.1016/j.cell.2014.06.007.

Pickrell, A.M., Youle, R.J., 2015. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. Neuron 85 (2), 257–273. https://doi.org/10.1016/j.neuron.2014.12.007.

Pomierny, B., et al., 2021. The slow-releasing and Mitochondria-targeted hydrogen sulfide (H(2)S) delivery molecule AP39 induces brain tolerance to ischemia. Int. J. Mol. Sci. 22 (15). https://doi.org/10.3390/ijms22157816.

Ravani, S., Chatzianastasiou, A., Papapetropoulos, A., 2024. Using mechanism-based combinations of H(2)S-donors to maximize the cardioprotective action of H(2)S. N. Schmied. Arch. Pharmacol. 397 (3), 1853–1864. https://doi.org/10.1007/s00210-023-02729-6.

Rodríguez, Ramón Rama Bretón, Julio, C.G., 2012. Excitotoxicity and oxidative stress in acute ischemic stroke. In: Acute Ischemic Stroke. InTech, pp. 29–58. https://doi.org/10.5772/28300.

Roy-O'Reilly, M., McCullough, L.D., 2018. Age and sex are critical factors in ischemic stroke pathology. Endocrinology 159 (8), 3120–3131. https://doi.org/10.1210/en.2018-00465.

Schaar, K.L., Brenneman, M.M., Savitz, S.I., 2010. Functional assessments in the rodent stroke model. Exp. Transl. Stroke Med. 2 (1). https://doi.org/10.1186/2040-7378-2-13.

Schwarz, T.L., 2013. Mitochondrial trafficking in neurons. Cold Spring Harbor Perspect. Biol. 5 (6). https://doi.org/10.1101/cshperspect.a011304.

Shajahan, S., et al., 2023. Sex differences in the symptom presentation of stroke: a systematic review and meta-analysis. Int. J. Stroke : Off. J. Int. Stroke Soc. 18 (2), 144–153. https://doi.org/10.1177/17474930221090133.

Shao, Z., et al., 2020. The role of mitophagy in ischemic stroke. Front. Neurol. 11, 608610. https://doi.org/10.3389/fneur.2020.608610.

Shen, Z., et al., 2017. PARK2-dependent mitophagy induced by acidic postconditioning protects against focal cerebral ischemia and extends the reperfusion window. Autophagy 13 (3), 473–485. https://doi.org/10.1080/15548627.2016.1274596.

Skórkowska, A., et al., 2024. The hydrogen sulfide donor AP39 reduces glutamate-mediated excitotoxicity in a rat model of brain ischemia. Neuroscience 539, 86–102. https://doi.org/10.1016/j.neuroscience.2023.11.008.

Su, S.-H., et al., 2018. Inhibition of excessive autophagy and mitophagy mediates neuroprotective effects of URB597 against chronic cerebral hypoperfusion. Cell Death Dis. 9 (7), 733. https://doi.org/10.1038/s41419-018-0755-y.

Szczesny, B., et al., 2014. AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro. Nitric Oxide : Biol. Chem. 41, 120–130. https://doi.org/10.1016/j.niox.2014.04.008.

Talley Watts, L., et al., 2015. Rose bengal photothrombosis by confocal optical imaging in vivo: a model of single vessel stroke. J. Vis. Exp. 100, e52794. https://doi.org/10.3791/52794.

Tang, Y.-C., et al., 2016. The critical roles of mitophagy in cerebral ischemia. Protein Cell 7 (10), 699–713. https://doi.org/10.1007/s13238-016-0307-0.

Theissen, U., Martin, W., 2008. Sulfide : quinone oxidoreductase (SQR) from the lugworm Arenicola marina shows cyanide- and thioredoxin-dependent activity. FEBS J. 275 (6), 1131–1139. https://doi.org/10.1111/j.1742-4658.2008.06273.x.

Warenycia, M.W., et al., 1989. Acute hydrogen sulfide poisoning. Demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels. Biochem. Pharmacol. 38 (6), 973–981. https://doi.org/10.1016/0006-2952(89)90288-8.

Wen, H., et al., 2021. Hypoxic postconditioning promotes mitophagy against transient global cerebral ischemia via PINK1/Parkin-induced mitochondrial ubiquitination in adult rats. Cell Death Dis. 12 (7), 630. https://doi.org/10.1038/s41419-021-03900-8.

Winship, I.R., Murphy, T.H., 2009. Remapping the somatosensory cortex after stroke: insight from imaging the synapse to network. Neuroscientist : A Review J. Bringing Neurobiol., Neurol. and Psychiatr. 15 (5), 507–524. https://doi.org/10.1177/1073858409333076.

World Health Organization (WHO), 2020. Stroke, Cerebrovascular Accident. WHO.

Wu, M., et al., 2021. Garciesculenxanthone B induces PINK1-Parkin-mediated mitophagy and prevents ischemia-reperfusion brain injury in mice. Acta Pharmacol. Sin. 42 (2), 199–208. https://doi.org/10.1038/s41401-020-0480-9.

Xu, L., et al., 2010. Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia. Glia 58 (9), 1042–1049. https://doi.org/10.1002/glia.20985.

Youle, R.J., Narendra, D.P., 2011. Mechanisms of mitophagy. Nat. Rev. Mol. Cell Biol. 12 (1), 9–14. https://doi.org/10.1038/nrm3028.

Yu, Q., et al., 2015. ROS-dependent neuroprotective effects of NaHS in ischemia brain injury involves the PARP/AIF pathway. Cell. Physiol. Biochem. : Int. J. Experimental Cell. Physiol, Biochem., and Pharmacol. 36 (4), 1539–1551. https://doi.org/10.1159/000430317.

Del Zoppo, G.J., et al., 2009. Expansion of the time window for treatment of acute ischemic stroke with intravenous tissue plasminogen activator: a science advisory from the American heart association/American Stroke Association. Stroke 40 (8), 2945–2948. https://doi.org/10.1161/STROKEAHA.109.192535.