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EXPERIMENTAL PAPERS

Vasoactive and Neuroprotective Effects of c-Jun N-Terminal Kinase Inhibitor in Rats with Chronic Cerebral Hypoperfusion

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Abstract—The aim of this study was to evaluate the vasoactive and neuroprotective effects of c-Jun N-terminal kinase inhibitor IQ-1 (11*H*-indeno[1,2-*b*]quinoxalin-11-one oxime) in chronic cerebral hypoperfusion caused by irreversible bilateral common carotid artery ligation [two-vessel occlusion (2VO) model]. Cerebral blood flow was measured quantitatively (hydrogen clearance method) simultaneously in the parietal cortex, hippocampus, substantia nigra, and striatum of the brain of awake rats. It was found that 2VO caused a decrease in blood flow in the brain regions with a more pronounced decrease in the cortex (by 48% of the initial level) and with a minimum drop in the substantia nigra (by 25% of the initial level). The reduced level of blood flow persisted for 14 days of measurements. The responses of the cerebral vessels to hypercapnic probes (5% CO_2) were lost during the 2-week hypoperfusion period, and the neurological status of the animals did not improve. The administration of IQ-1 (50 mg/kg, intraperitoneally, every 48 h for 14 days) was accompanied by an increase in blood flow in all brain regions. A maximum increase in blood flow was observed in the striatum and a minimum in the substantia nigra. After the administration of IQ-1, the sensitivity of the cerebral vessels to the hypercapnic stimulus was restored, and the neurological state of the animals significantly improved by the end of the second week of cerebral hypoperfusion. The results show that the use of the JNK inhibitor can reduce cerebrovascular disorders and related neurological disorders in hypoperfusion brain injury.

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Keywords: c-Jun N-terminal kinase inhibitors, chronic cerebral hypoperfusion, cerebral blood flow, neuroprotection, hypercapnic probe, striatum, substantia nigra

INTRODUCTION

c-Jun N-terminal kinases (JNKs) belong to the mitogen-activated protein kinase (MAPK) family, which are activated in response to a variety of stressors and damaging factors. JNKs include

10 isoforms encoded by three genes: *JNK1* (4 isoforms), *JNK2* (4 isoforms), and *JNK3* (2 isoforms) [1, 2]. JNK1 and JNK2 are present in all cells of the organism, while JNK3 is expressed predominantly in the heart and brain [3]. The transcription factor c-Jun is a JNK substrate and can trigger apoptosis in neuronal cells after phosphorylation [4]. JNKs are involved in the regulation of inflammation, play an important role in the signaling pathways leading to apoptosis and necrosis, regulate some transcriptional as well as non-transcriptional cellular processes, which largely determine the damage to brain neurons and cardiomyocytes during ischemia and reperfusion [5, 6]. JNKs are involved in the pathogenesis of diabetes, atherosclerosis, stroke, Alzheimer's disease, Parkinson's disease [7, 2], tumor growth [3], inflammatory diseases, myocardial infarction, heart failure, and myocardial hypertrophy [5]. JNK inhibitors have attracted widespread attention as potential therapeutic agents for the prevention and treatment of ischemic lesions [8, 9].

We synthesized a new JNK inhibitor, IQ-1 (11*H*-indeno[1,2-*b*] quinoxaline-11-one oxime), which has an increased affinity to the JNK3 isoform [10], as well as a pronounced neuroprotective effect and the ability to penetrate through the blood-brain barrier (BBB) [11]. Using a filamentous middle cerebral artery occlusion (MCAO) model of local ischemia/reperfusion, we demonstrated that the size of the brain necrosis zone in mice was significantly reduced by IQ-1 administration compared to the control values [11]. A pronounced neuroprotective effect of IQ-1 was also revealed in total ischemia/reperfusion of the rat brain [12]. One of the possible mechanisms of the inhibitor's neuroprotective action may lie in an increase in cerebral blood flow due to nitric oxide (NO) release during the biotransformation of the IQ-1 molecule [11]. The goal of the present work was to study the vasoactive and neuroprotective effects of the JNK inhibitor IQ-1 in animals with chronic cerebral hypoperfusion caused by irreversible ligation of two common carotid arteries [two-vessel occlusion (2VO) model [13]]. It is well known from clinical practice that such a chronic limitation of cerebral blood supply can develop due to heart failure, systemic arterial hypotension, cerebral vascular pathology (malformation, atherosclerosis), or the effects of other pathogenetic factors [13, 14]. In addition, chronic cerebral hypoperfusion inevitably entails the loss of cognitive functions in animals [15, 16], which opens the possibility of a quantitative assessment of neurological deficits. A hypoperfusion brain injury is underlain by a slow, progressive death of cerebral nerve cells, leading to such clinical manifestations as memory loss, dementia, motor disorders, Alzheimer's disease, and other neurological pathologies [13].

The objectives of this work were the following: (a) to study the effect of IQ-1 on cerebral blood flow and cerebral vascular reactivity in animals against the background of chronic cerebral hypoperfusion caused by 2VO, (b) to assess the effect of IQ-1 on the neurological status of rats with chronic cerebral hypoperfusion, (c) to compare a vasomotor action of IQ-1 with the effect of the NO donor S-nitroso-N-acetylpenicillamine (SNAP).

MATERIALS AND METHODS

The following chemicals were used in this study: compound IQ-1 (11*H*-indeno[1,2-*b*]quinoxaline-11-one oxime, synthesized at the N.M. Kizhner Tomsk Polytechnic University, Russia), zoletil (Zoletil Virbac, France), xylanite (Nita-Pharm, Russia), atropine sulfate (Dalkhimpharm, Russia), isoflurane (Laboratorios Inibsa, Barcelona, Spain), SNAP (Sigma-Aldrich, USA), Tween-80 (Sigma-Aldrich, USA).

The experiments were carried out on male Wistar rats weighing 287 ± 19 g, purchased from Rappolovo animal nursery (Leningrad Region, Russia). The experimental protocol was approved by the Bioethics Committee of the IEPhB RAS in accordance with the international recommendations (Code of Ethics) for conducting medical and biological research on animals (CIOMS, Geneva, 1985). When preparing the animals for the experiments, two surgical procedures were performed. In the first stage, platinum electrodes were implanted in the brain to measure cerebral blood flow. At the second stage, two common carotid arteries in animals with implanted electrodes were ligated to create a model of chronic cerebral

hypoperfusion. A week before the experiment, 0.15-mm platinum needle electrodes were inserted under aseptic conditions into the brain regions of an anesthetized rat (parietal cortex, hippocampus, substantia nigra, striatum) according to the atlas of stereotactic coordinates [17]. Electrode implantation was performed under zoletil-xylazine anesthesia (zoletil, 0.3 mg/100 g of body weight i.m.; xylanit, 0.8 mg/100 g of body weight i.m.; atropine sulfate 0.1% solution, 0.01 mL s.c.). The surgical stage of anesthesia was verified by the disappearance of responses to painful needle-prick paw stimulation and corneal reflex suppression.

Each platinum electrode was glass-insulated except for the tip 1.5 mm long, which was coated 3 times with nafion solution (Nafion, Sigma-Aldrich) and dried afterward. With such a size of the active electrode surface, the volume of the brain region in which blood flow was measured averaged 8 mm^3. To secure the electrodes, two stainless steel screws were then screwed bilaterally into the temporal bone of the skull. The electrodes and screws were additionally fixed to the bone with dental cement. The animals were returned to their home cages and provided with appropriate postoperative care.

After 5–7 days, two common carotid arteries were ligated under anesthesia (2VO model [18]). To do this, the rats were placed into an induction chamber connected to a Dvapo isoflurane vaporizer (China). For induction, 4 vol% isoflurane in a fresh oxygen flow of 2 L/min was used while breathing independently. After a loss of motor activity, the rat was transferred onto a heated surgery table, and isoflurane anesthesia continued (1.5 vol.% in a fresh oxygen flow of 1 L/min) through a muzzle mask. After left and right common carotid artery isolation, both vessels were irreversibly ligated with a nylon suture (Ethilon 2/0, Ethicon, USA). The wound was sutured, and the surgical field was treated with an antiseptic (5% iodine solution). Finally, the animals were placed in a box under a heat lamp for 5–7 h.

Absolute values of local cerebral blood flow were measured using a hydrogen clearance method [19]. The method is based on determining the time course of hydrogen clearance in brain tissue pre-saturated with this gas by breathing a hydrogen-containing mixture (2.5% H_2 in air) for 10–15 s. Under continuous measurement of hydrogen tension in cerebral tissue, its washout rate (clearance) directly depends on blood flow intensity, which was laid as a basis for calculating absolute values of cerebral hemodynamics. Hydrogen tension in the brain was measured polarographically via the pre-implanted intracerebral platinum electrodes and a chlorosilver reference electrode as a clip attached to the tail base. Hydrogen clearance curves were recorded by Physioblock-Pulse instruments (St. Petersburg) and used to calculate absolute values of cerebral blood flow (mL/min/100 g of brain tissue) using the WinDaq data acquisition hardware/software system (DC-200, DATA, OH, USA). The hydrogen clearance method allows sequential measurement of cerebral blood flow at intervals of at least 5 min. Neurological deficit resulting from cerebral hypoperfusion was assessed using a McGraw scale modified by I.V. Gannushkina [20, 21], which scores the degree of neurological disorders as mild (1–3 points), moderate (3–6 points), and severe (6–10 points).

A total of 47 rats with implanted platinum electrodes, divided into 3 groups, were used in the experiments. In group 1 (n = 12), striatal blood flow responses were evaluated in rats with intact carotid arteries upon a single intraperitoneal (i.p.) administration of IQ-1 at doses of 25, 50 and 100 mg/kg. The objective of these experiments was to determine a vasoactive dose of IQ-1 for its use in further experiments. In part of group 1 rats, striatal blood flow was measured upon an i.p. administration of the NO donor SNAP at a dose of 2 μg/kg. The remaining 35 animals with implanted electrodes were exposed to irreversible 2VO. Seven rats did not survive the surgery. In the survivors (group 2, n = 14), blood flow measurements were performed simultaneously in the parietal cortex, hippocampus, striatum, and substantia nigra. Blood flow was measured for 2 weeks every 2 days. In part of group 2 animals (n = 7), cerebrovascular reactivity was tested by assessing the changes in local cerebral blood flow in response to a 30-s inhalation of 5% CO_2 in air (hypercapnic test). In all group 2 animals, neurological status was assessed against the background of progressive cerebral hypoperfusion.

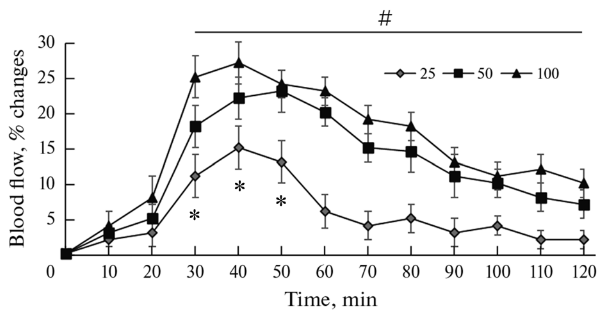

Fig. 1. Dose-dependent changes in striatal blood flow of rats with intact carotid arteries after intraperitoneal administration of IQ-1 at doses of 25, 50 and 100 mg/kg. * $p < 0.05$ vs. basal value for 25 mg/kg, # $p < 0.05$ vs. basal values for 25 and 100 mg/kg. The drug was administered at zero time point.

In group 3 animals (n = 14), blood flow in the four brain regions was measured after i.p. administration of IQ-1 at a dose of 50 mg/kg. This dose was chosen based on the results of our tentative experiments on group 1 animals. The drug (powder) was thoroughly stirred in a water/Tween-80 solution, after which a preset drug amount in 2 mL of the solution was administered i.p. after 2VO. In each experiment, blood flow was measured before and after drug administration for 2 h. Cerebral blood flow measurements during a course IQ-1 administration, i.e. drug infusion every 48 h for 14 days, were carried out on each animal. In part of group 3 rats (n = 6), cerebral blood flow was measured after i.p. administration of the NO donor SNAP (2 μg/kg). Neurological status was assessed in all animals during a 2-week cerebral hypoperfusion against the background of IQ-1 administration. After completion of the experiments, the animals were euthanized by injecting zoletil (3 mg) into the tail vein.

Absolute values of local cerebral blood flow were used for statistical analysis. The data were analyzed using the SigmaPlot 13.0 software package (Systat Software, San Jose, CA). The analysis of variance (ANOVA) was used to compare blood flow values measured within different time intervals of cerebral hypoperfusion in control and IQ-1-treated rats. A paired Student's t-test with Fisher's exact test was used to reveal a statistical significance ($p < 0.05$). All data were presented as $M \pm SEM$.

RESULTS

The average striatal blood flow in group 1 rats with intact carotid arteries (n = 12) was 69.0 ± 5.8 mL/min/100 g of tissue. I.p. administration of IQ-1 at doses of 25, 50, and 100 mg/kg caused an increase in striatal blood flow, whose magnitude and duration were dose-dependent (Fig. 1). A significant increase in blood flow was detected 30 min after drug administration and persisted for 2 h of measurements. I.p. SNAP administration at a dose of 2 μg/kg increased striatal blood flow by the same value as with IQ-1 administration at a dose of 50 mg/kg. We chose this IQ-1 dose for use in subsequent studies of the vasoactive and neuroprotective effects of IQ-1 in rats with chronic cerebral hypoperfusion.

In group 2 rats (n = 14), blood flow was measured in four brain regions for 2 weeks after 2VO. The obtained data demonstrate the presence of heterogeneity in cerebral blood supply before 2VO with the highest blood flow levels in the substantia nigra and parietal cortex (Table 1).

Thirty min after 2VO, blood flow significantly decreased in all the studied brain regions and persisted at a significantly low level for 2 weeks, relative to its values before the onset of hypoperfusion. An insignificant increase in blood flow was revealed by the end of the observations, however, the values of these changes were statistically non-significant relative to the cerebral hemodynamics values recorded on the first day of hypoperfusion.

In group 3 rats (n = 14) after 2VO, the hypoperfusion level was assessed by measuring blood flow in four brain regions for 14 days against the background of a course IQ-1 administration (50 mg/kg, i.p.) every 48 h (Table 2).

Table 2 data analysis showed that the blood supply to the four brain regions 30 min after 2VO decreased significantly: maximally in the cortex and minimally in the substantia nigra. IQ-1 administration every 2 days revealed a common trend of gradually increasing cerebral blood flow in all the brain regions studied. However, a statistically significant increase in local cerebral blood supply was detected only 2 weeks after the onset of cerebral hypoperfusion. A comparison of local blood flow values in group 2 and 3 rats after 2-week

Table 1. Blood flow dynamics in four brain regions in rats after bilateral carotid artery ligation (2VO model)

Brain regions	Blood flow (mL/min/100 g tissue)								
	Time of measurement								
	before 2VO	30 min 2VO	2 days	4 days	6 days	8 days	10 days	12 days	14 days
Parietal cortex	82 ± 6	43 ± 4 (–48%)*	42 ± 4*	42 ± 4*	43 ± 5*	45 ± 5*	46 ± 5*	46 ± 5*	47 ± 5*
Hippocampus	59 ± 5	32 ± 4 (–46%)*	32 ± 4*	33 ± 4*	32 ± 3*	36 ± 4*	37 ± 3*	38 ± 4*	39 ± 5*
Substantia nigra	89 ± 8	67 ± 6 (–25%)*	61 ± 7*	65 ± 4*	70 ± 4*	71 ± 4*	72 ± 7*	71 ± 6*	73 ± 7
Striatum	67 ± 6	38 ± 4 (–43%)*	35 ± 3*	31 ± 5*	32 ± 4*	35 ± 4*	40 ± 4*	39 ± 5*	42 ± 5*

* $p < 0.05$ for all blood flow levels vs. the level before 2VO.

Table 2. Blood supply to the brain of rats with a course administration of IQ-1 against the background of cerebral hypoperfusion

Brain regions	Blood flow (mL/min/100 g tissue)								
	Time of measurement								
	before 2VO	30 min 2VO	2 days	4 days	6 days	8 days	10 days	12 days	14 days
Parietal cortex	84 ± 6	46 ± 4 (–45%)*	42 ± 4	45 ± 4	46 ± 5	47 ± 3	50 ± 5	53 ± 4	55 ± 4#
Hippocampus	61 ± 5	35 ± 4 (–43%)*	34 ± 4	36 ± 4	38 ± 3	39 ± 4	40 ± 3	41 ± 5	45 ± 4#
Substantia nigra	90 ± 7	69 ± 5 (–33%)*	70 ± 6	71 ± 4	72 ± 4	75 ± 4	73 ± 5	74 ± 5	82 ± 6#
Striatum	72 ± 6	41 ± 4 (–41%)*	43 ± 4	46 ± 5	45 ± 4	49 ± 4	46 ± 4	51 ± 5#	57 ± 5#

The data represent blood flow values measured every time before IQ-1 administration. * $p < 0.05$ for all blood flow levels vs. the level before 2 VO, # $p < 0.05$ for all blood flow values vs. the level after a 30-min 2VO.

hypoperfusion revealed a significantly higher blood flow level in the animals treated with IQ-1 (Fig. 2).

When assessing the reactivity of cerebral vessels to a hypercapnic stimulus (inhalation of 5% CO_2), the blood flow in the studied brain regions of rats with intact carotid arteries increased significantly by 19–37% ($p < 0.05$). After 2VO, cerebral blood flow in response to hypercapnic stimulus changed non-significantly. After a single IQ-1 administration against the background of cerebral hypoperfusion, the dilatory responses of cerebral vessels to the inhalation of 5% CO_2 progressively increased and reached statistically significant values of 14–26% ($p < 0.05$) 14 days after 2VO.

To assess the vasoactive potency of IQ-1, the changes in striatal blood flow after IQ-1 administration were compared to cerebrovascular responses to NO donor (SNAP) administration. A comparison revealed unidirectional and equipotent vasomotor effects and SNAP (2 μg/kg) effects at different levels of brain perfusion, although IQ-1 at a dose of 50 mg/kg caused greater cerebrovascular responses under conditions of cerebral hypoperfusion (Fig. 3).

After 2VO, in animals untreated with IQ-1, the degree of neurological deficits on day 8 of hypoperfusion scored 6.93 ± 0.18 points by the McGraw scale, as manifested in torpor, bradykinesia, unilateral and bilateral eyelid ptosis, limb paresis, and hind limb paralysis in 2 of 12 animals. After 14 days of cerebral hypoperfusion, the degree of neurological deficit decreased to 5.73 ± 0.11 points. Against the background of the course IQ-1 administration, neurological deficits after

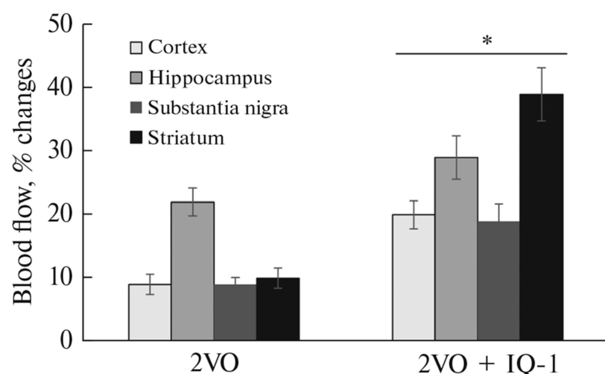

Fig. 2. Changes in cerebral blood flow after 2 weeks of hypoperfusion in rats without pharmacological support (2VO) and with IQ-1 treatment (2VO + IQ-1). *Y-axis*: blood flow changes in % (2VO 14 days/30 min ratio according to Tables 1 and 2). * $p < 0.05$ vs. 2VO.

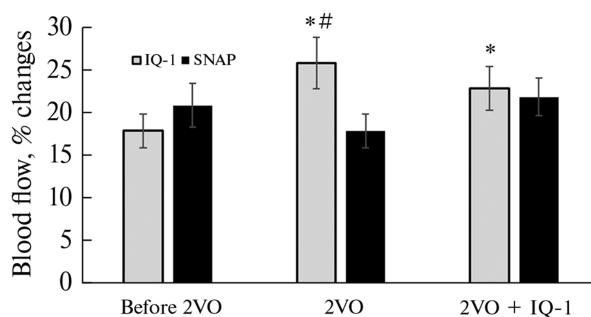

Fig. 3. Changes in rat striatal blood flow in response to the administration of IQ-1 and the NO donor (SNAP). Before 2VO—before bilateral carotid artery ligation; 2VO—after 14 days of cerebral hypoperfusion without pharmacological support (IQ-1 treatment); 2VO + (IQ-1)—after 14 days of cerebral hypoperfusion with pharmacological support (IQ-1 treatment); * $p < 0.05$ vs. "Before 2VO". # $p < 0.05$ IQ vs. SNAP.

8 days of hypoperfusion scored 4.06 ± 0.08 points and 2.13 ± 0.18 points after 14 days, which was significantly different from the same index in group 1 rats that received no pharmacological support ($p < 0.01$).

DISCUSSION

The main findings of the present work are the following: (1) IQ-1, a JNK inhibitor, has vasoactive properties and causes a dose-dependent increase in cerebral blood flow, (2) the vasodilator effect of IQ-1 is enhanced under conditions of chronic cerebral hypoperfusion, (3) course administration of IQ-1 restores cerebrovascular reactivity to hypercapnia in cerebral hypoperfusion, (4) IQ-1 improves neurological status in rats under conditions of chronic cerebral hypoperfusion.

In our experiments, the vasoactive and neuroprotective effects of IQ-1 were studied on the bilateral carotid artery ligation or two-vessel occlusion (2VO) model, which is used to assess the efficacy of pharmacological correction of cerebral hypoperfusion disorders [13]. Irreversible 2VO caused in our experiments an acute blood flow reduction in the parietal cortex, hippocampus, substantia nigra, and striatum, being a maximum in the cortex and a minimum in the substantia nigra. The difference in the degree of blood flow reduction in the above brain regions is due to the fact that in 2VO, blood supply disturbances are more pronounced in the forebrain and less in the medulla oblongata and basal ganglia, where blood circulation is accomplished via two vertebral arteries [22, 23]. The reduced blood flow, loss of cerebrovascular reactivity to the hypercapnic stimulus, and neurological disorders remained stable for 14 days, indicative of a lacking or low efficacy of natural recovery during this period. The course IQ-1 administration during the 2-week hypoperfusion period progressively increased blood flow in all brain regions studied. A maximum increase in blood flow compared to the onset of hypoperfusion was detected in the striatum, and the minimum in the substantia nigra. IQ-1 treatment restored the sensitivity of cerebral vessels to hypercapnia and appreciably improved the neurological status of the animals. These data suggest a pronounced vasomotor effect of IQ-1 under chronic cerebral ischemia, as manifested in a progressive increase in the blood supply to brain structures.

The neuroprotective activity of IQ-1 was confirmed when assessing the neurological status of the animals during the hypoperfusion period. With a course IQ-1 administration, the animals exhibited a less pronounced neurological deficit as soon as 5–7 days of hypoperfusion, and the time of its recovery was noticeably reduced. There was a significant decrease in the mean score of neurological deficits compared to the group of animals untreated with IQ-1. Already within the

first 3–5 postoperative days, there was observed a decrease in the number of animals with severe neurological disorders and, accordingly, an increase in the number of animals with a moderate degree of neurological deficit. Mortality rates were lower in rats treated with IQ-1 since the very first day after 2VO.

The vasoactive properties of IQ-1, revealed in this work, raise the question about the mechanism of its dilatory action toward cerebral vessels. Our previous studies showed that IQ-1 undergoes enzymatic metabolism in the hepatic microsomes with an NO output [11]. These data allow the possibility to consider IQ-1 as a potential NO donor for cerebral vessels. This is indirectly supported by a comparative assessment of the vasoactive IQ-1 and SNAP properties, carried out in this study. The vasodilatory effect of SNAP, based on NO release, is well known with regard to cerebral vessels [24]. In the present study, SNAP administration increased blood flow in different brain regions by 17–27%. IQ-1 evoked an equal vasodilator effect in animals with cerebral hypoperfusion, whereas in intact animals, its vasomotor effect was less pronounced. One of the possible reasons for the different vasomotor effects of IQ-1 may be the state of the BBB in animals with intact vs. ligated carotid arteries. Brain ischemia is known to cause a disruption of the BBB [1], which may increase its permeability for IQ-1 and thus elevate its bioavailability for vasomotor effects. Our recent results suggest that the efficacy of IQ-1 increases against the background of hypothetically elevated JNK activity, which allows pharmacological correction of cerebrovascular and related neurological disorders [12].

In this study, IQ-1 demonstrated pronounced vasoactive and neuroprotective properties in a rat model of chronic cerebral hypoperfusion caused by irreversible carotid artery ligation. We hypothesize that the favorable effects of IQ-1 on the outcome of chronic cerebral hypoperfusion may represent a combined result of both JNK inhibition in brain tissue and NO production that promotes an increase in brain tissue microcirculation, reduction in blood viscosity, and improvement of endothelial function. The obtained results suggest that IQ-1 can be considered a multitarget neuroprotector.

AUTHORS' CONTRIBUTION

Conceptualization and experimental design (S.Yu.Zh., I.T.D., D.N.A.); IQ-1 synthesis (A.I.Kh.); data collection (S.Yu.Zh., I.T.D., T.F.P.); data processing (S.Yu.Zh., I.T.D.); writing and editing the manuscript (S.Yu.Zh., I.T.D., A.I.Kh., I.A.Shc., D.N.A.).

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COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed on animals met the ethical standards approved by the legal acts of the Russian Federation, the principles of the Basel Declaration, and the recommendations of the Bioethics Committee at Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (IEPhB RAS, protocol no. 9/2021 of 23.09.2021).

CONFLICT OF INTEREST

The authors declare that they have neither evident nor potential conflict of interest related to the publication of this article.

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