https://doi.org/10.15360/1813-9779-2023-3-46-53

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Experimental Study of Neuroprotective Properties of Inhaled Argon-Oxygen Mixture in a Photoinduced Ischemic Stroke Model

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For citation: *Ekatherine A. Boeva, Denis N. Silachev, Elmira I. Yakupova, Marina A. Milovanova, Lydia A. Varnakova, Sergey N. Kalabushev, Sergey O. Denisov, Victoria V. Antonova, Ivan A. Ryzhkov, Konstantin N. Lapin, Alexandra A. Grebenchikova.* Experimental Study of Neuroprotective Properties of Inhaled Argon-Oxygen Mixture in a Photoinduced Ischemic Stroke Model. *Obshchaya Reanimatologiya = General Reanimatology.* 2023; 19 (3): 46–53. https://doi.org/10.15360/1813-9779-2023-3-46-53 [In Russ. and Engl.]

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Summary

Acute ischemic stroke is a serious problem for healthcare systems worldwide. Searching for the optimal neuroprotector is a contemporary challenge. Various studies have demonstrated neuroprotective properties of argon in ischemic brain damage models. However, the published data are inconsistent.

The aim of the study was to evaluate the effect of 24-hour argon-oxygen mixture (Ar 70%/O_2 30%) inhalation on the severity of neurological deficit and the extent of brain damage in rats after a photoinduced ischemic stroke.

Material and methods. The experiments were carried out on male Wistar rats weighing 430–530 g (*N*=26). Focal ischemic stroke was modeled in the sensorimotor cortex of the rat brain using photochemically induced vascular thrombosis. The animals were randomly divided into 3 groups: sham procedure + N_2 70%/O_2 30% inhalation (SP, *N*=6); stroke + N_2 70%/O_2 30% inhalation (Stroke, *N*=10); Stroke + Ar 70%/O_2 30% inhalation (Stroke+iAr, *N*=10). The limb placement test (LPT) was used for neurological assessment during 14 days. Additionally, on day 14 after the stroke, brain MRI with lesion size morphometry was performed. Summarized for days 3,7 and 14 LPT scores were lower in the Stroke and Stroke + iAr groups as compared to the SP group.

Results. Statistically significant differences in LPT scores between SP, Stroke, and Stroke+iAr groups were revealed on day 3 post-stroke: (scores: 14 (13; 14), 6.5 (4; 8), and 5 (3; 8), respectively, *P*=0.027). However, there was no statistical difference between the Stroke and Stroke+iAr groups.

Conclusion. 24-hour inhalation of argon-oxygen mixture (Ar 70%/O_2 30%) after stroke does not reduce the extent of brain damage or the severity of neurological deficit.

Keywords: argon; neuroprotection; photochemically induced ischemic stroke; organoprotection

Conflict of interest. The authors declare no conflict of interest.

Introduction

Stroke is the second leading cause of morbidity and mortality worldwide. The incidence of stroke is increasing due to the prevalence of diabetes mellitus and obesity [1, 2]. The pathophysiology of ischemic brain injury involves the activation of several signaling cascades. Oxygen deprivation leads to the cessation of energy-dependent ion pumps and channels, resulting in the release of neurotransmitters and subsequent neuronal death. Evidence suggests that post-ischemic inflammation is the major cause of a secondary brain damage, which determines the severity of stroke outcome [3]. Therefore, the search for clinically effective neuroprotective agents is relevant. Many therapeutic agents are currently being evaluated in preclinical studies using ischemic injury models [3, 4].

Research with inert (noble) gases is a promising direction in the search for neuroprotective agents. Xenon has been approved for clinical use as a general anesthetic and its neuroprotective properties have been confirmed in numerous *in vitro* and *in vivo* studies [5–13]. Argon may be another promising neuroprotective agent. Over several decades, data on cardio-, neuro-, and nephroprotective properties of argon in various diseases have been obtained in experimental models *in vivo* and *in vitro* [14–20].

A literature review revealed conflicting data on the neuroprotective properties of argon in different models [21–35].

In a study by Grüßer L. (2017), the cytoprotective effect was obtained after argon inhalation for 2 h in a model of traumatic brain injury [36]. In 2021, 2 papers were published evaluating the neuropro-

tective properties of argon in a closed TBI model. In this study, argon inhalation was administered for 24 hours [8, 37]. However, another study [8] showed a significant improvement in neurological status, whereas the study by Creed J. (2021) showed no positive effects [37]. Despite the neuroprotective effect in predominantly ischemic injury, argon did not provide protection after TBI, emphasizing the importance of careful selection of the study model and the time of argon exposure. Studies using models of ischemic injury based on oxygen-glucose deprivation have shown positive results after argon inhalation with different exposure times. Recovery of neurological status and a decrease in the extent of brain injury were observed on histological examination [17–25, 27–50]. Notably, the majority of studies were conducted *in vitro*. Ma S. et al (2019) first performed an *in vivo* study in a model of ischemic injury by middle cerebral artery occlusion with/without reperfusion [48]. The study confirmed the neuroprotective properties of argon, but revealed a discrepancy between the improved neurological outcome and the total area of injury [48]. Given the equivocal results of studies in various models of ischemic injury, photochemically induced thrombosis appears to be one of the most promising experimental models of stroke. Unlike other methods of thrombosis induction, photochemically induced thrombosis can be used in small animals, as this model is characterized by persistent sensorimotor deficits and low postoperative mortality [42].

Thus, based on the literature data, argon may be a promising tool for brain protection against ischemia. However, the lack of consistent results indicates the need for a comprehensive study of this gas as a neuroprotective agent.

The aim of our study was to evaluate the effect of 24 h inhalation of argon-oxygen mixture after photoinduced ischemic stroke on the severity of neurological deficit and the degree of brain injury in rats.

Materials and Methods

Experimental animals. Experiments were performed on male Wistar rats weighing 430–530 g (*N*=26). The animals were deprived of food for 8 h before the experiment, but had free access to water. The study protocol was approved by the Local Ethical Committee of the Federal Research and Clinical Center of Intensive Care Medicine and Rehabilitology, No. 3/22/3 of December 14, 2022. The experiments were performed in accordance with the requirements of Directive 2010/63/EU of the European Parliament and Council of the European Union on the protection of animals used for scientific purposes.

Animals were randomly divided into 3 groups according to the interventions performed:

— sham-operated animals under anesthesia and preparation without stroke + N_2 70%/O_2 30% inhalation (SO group), *N*=6;

— control group with stroke + N_2 70%/O_2 30% inhalation (stroke group), *N*=10;

— experimental group with stroke + Ar 70%/O_2 30% inhalation (stroke+iAr group), *N*=10.

Photoinduced ischemic stroke simulation. Under general anesthesia with sevoflurane 7.0–8.0 ml (2–4 vol%) using the SomnoSuite (Kent Scientific Corporation, USA) low-flow anesthesia system for small laboratory animals with oxygen flow of 1 L/min, ischemic stroke with photochemically induced cortical vascular thrombosis was simulated according to [45]. Photosensitive rose Bengal dye (3%, 40 mg/kg intravenously; Sigma-Aldrich, St. Louis, Missouri, USA) was injected into the jugular vein. The rat head was then fixed in a stereotactic frame (Bregma stereotactic coordinates: 0.5 mm distal and 2.5 mm lateral), and the skull was exposed through a midline incision free of periosteum. The cerebral hemisphere in the area of the sensorimotor cortex was then irradiated with green light at λ=550 nm for 15 min. After skin suture, the rats were placed in a cage under an infrared heating lamp until they recovered from anesthesia. Body temperature was maintained at 37±0.5°C throughout the experiment. The temperature was measured by installing a rectal body temperature sensor, and thermoregulation was maintained in automatic mode by connecting a heating module to a thermoregulator and setting limit values. The sham operation included a paratracheal incision with isolation of the internal jugular vein and exposure of the skull through a midline incision [45].

Argon exposure. Fifteen minutes after the stroke simulation, the animal was placed in a 15 L transparent plastic chamber continuously supplied with a fresh gas mixture (N_2 70%/O_2 30% for SO and stroke groups; Ar 70%/O_2 30% for the stroke+iAr group) at a flow rate of 0.5 L/min per animal. No more than 5 animals of the same group were in the chamber at the same time to avoid hypoxia and hypercapnia.

The exposure time in the chamber was 24 hours. Throughout the experiment, the O_2 and CO_2 levels in the animal chamber were continuously monitored using a closed atmosphere control device (INSOVT, St. Petersburg, Russia). At the end of the exposure period, the general condition of the animal (level of alertness, mobility) was assessed and anesthesia with paracetamol at a dose of 50 mg/kg, subcutaneously, was administered. The animal was then placed in its cage with free access to water and food.

Assessment of neurological status. The neurological status of the animals was assessed one day before the experiment (D0), on day 3 (D3), day 7 (D7), and day 14 (D14) after stroke.

We used a protocol based on the method described by De Rieck et al. (1989) [43] and modified by Yolkkonen J. et al. (2000) [44]. Rats were hand-trained for one week prior to testing. The test consisted of seven tests assessing sensorimotor integration of the forelimbs and hindlimbs in response to tactile, proprioceptive and visual stimulation. Each test was scored as follows: normal performance, 2 points; delayed (>2 s) and/or incomplete performance, 1 point; no performance, 0 points. The scores were summed, and the results were presented as the sum of the test scores.

On day 14 after stroke, animals underwent MRI examination on a 7 Tesla magnetic field induction tomograph with a gradient system of 105 mTl/m (BioSpec 70/30, Bruker, Germany). Anesthesia was performed with isoflurane (1.5–2%), after which the rat was placed in a positioning device with stereotaxis and thermoregulation system as described previously [45].

A standard protocol for rat brain examination was used, including the acquisition of T2-weighted images. A linear transmitter with an internal diameter of 72 mm was used for radiofrequency (RF) signal transmission, and a receiving coil on the rat brain surface was used for RF signal detection. The following pulse sequences (PS) were used: RARE, a spin echo-based PS with the following parameters: TR = 6000 ms, TE = 63.9 ms, 0.8 mm slice thickness in 0.8 mm increments, 256×384 matrix size, 0.164×0.164 mm/pixel resolution. Total scanning time per animal was approximately 25 minutes. The extent of brain injury was assessed by graphical analysis of MRI images with calculation of brain lesion volume. For this purpose, one slide with the largest brain lesion area in a series of MR images was selected. The lesion area in mm^2 was calculated using ImageJ software (National Institutes of Health image software, Bethesda, MD, USA). The brain lesion area was then similarly calculated on four additional slides (two cranial and two caudal). The volume of brain lesions was calculated using the formula: $V = \sum Sn \times d$, where d is the thickness of one section (0.8 mm), $\sum Sn$ is the sum of the lesion areas on five slides (mm^2) [45]. Mortality in the groups of animals was assessed at 24 h, 7 and 14 days after stroke.

Statistical analysis of the data was performed using STATISTICA 7.0 (StatSoft. Inc., USA) and GraphPad Prizm. The distribution of variables was assessed using the Shapiro–Wilk criterion. All data were presented as median and interquartile range. Statistical differences between groups in data with at least one non-normal distribution were analyzed using the Mann–Whitney *U* test with Bonferroni correction for comparison of three or more groups, and the Kruskal–Wallis or Mann–Whitney *U* test for analysis of no more than two groups. The significance level was set at $P<0.05$.

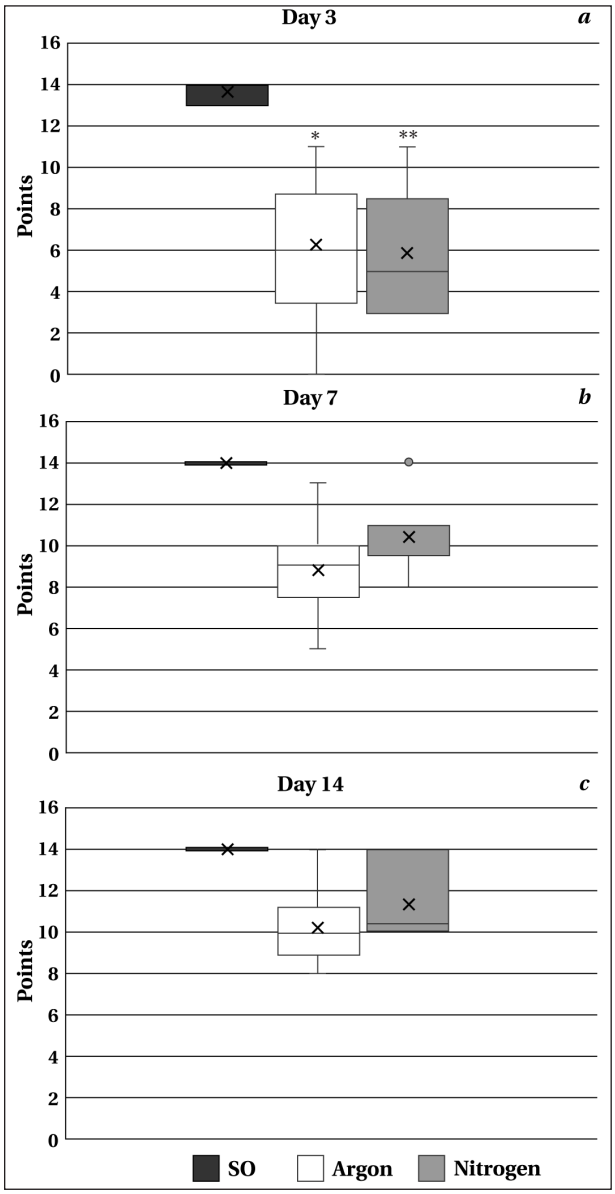

Fig. 1. Results of the limb-placing test (LPT).
Note. *a* — Results on day 3 after simulated stroke; *P*=0.027 between SO and stroke* groups, SO and stroke+iAr** groups. *b* — Results on day 7 after simulated stroke. *c* — Results on day 14 after simulated stroke. Data are presented as median and interquartile range [25%; 75%]. Mann–Whitney *U* test with Bonferroni correction, Kruskel–Wallis test was used to compare three or more groups.

Results

No animals were withdrawn from the study for 14 days and no humane endpoint was reached. There were no lethal outcomes.

Neurological evaluation. The limb-placing test (LPT). At each of the time points (D3, D7, and D14), the sum of LPT scores in animals from both experimental groups was lower than in the SO group. We obtained significant differences between the SO group and the stroke and stroke+iAr groups on day 3 (14 (13; 14), 6.5 (4; 8), 5 (3; 8), respectively, *P*=0.027). The stroke and stroke+iAr groups did

not differ (day 3, *P*=0.57; day 7, *P*=0.70; day 14, *P*=0.71) (Fig. 1).

Over time, the values of this parameter in the SO group of animals did not change from D3 to D14 (Fig. 2, *a*). The changes in LPT scores in the stroke and stroke+iAr groups were almost identical: the lowest values were at time point D1 (5.9 (3; 8) in the stroke group and 6.3 (7; 9.5) in the stroke+iAr group; *P*=0.73). At D7, there was a trend toward increasing the score values in both groups (10.4 (10; 10.8) in the stroke group and 8.8 (8; 10) in the stroke+iAr group, *P*=0.59). At D14, the total LPT score exhibited a trend to be higher in the stroke group (11.4 (10; 14)) (Fig. 2, *b*) and stroke+iAr (10.3 (9; 11)) (Fig. 2, *c*) group compared to both D1 (*P*=0.56 for the stroke group, *P*=0.63 for the stroke+iAr group) and D7 (*P*=0.68 for the stroke group, *P*=0.61 for the stroke+iAr group). However, the differences were not significant.

Brain MRI. The mean lesion volumes in the stroke+iAr group and the stroke group were 9.68 (7.42; 12.2) mm3 and 9.34 (8.74; 12.90) mm^3, respectively. No significant differences were found between the groups (*P*=0.500) (Fig. 3, 4 *a*, *b*, *c*).

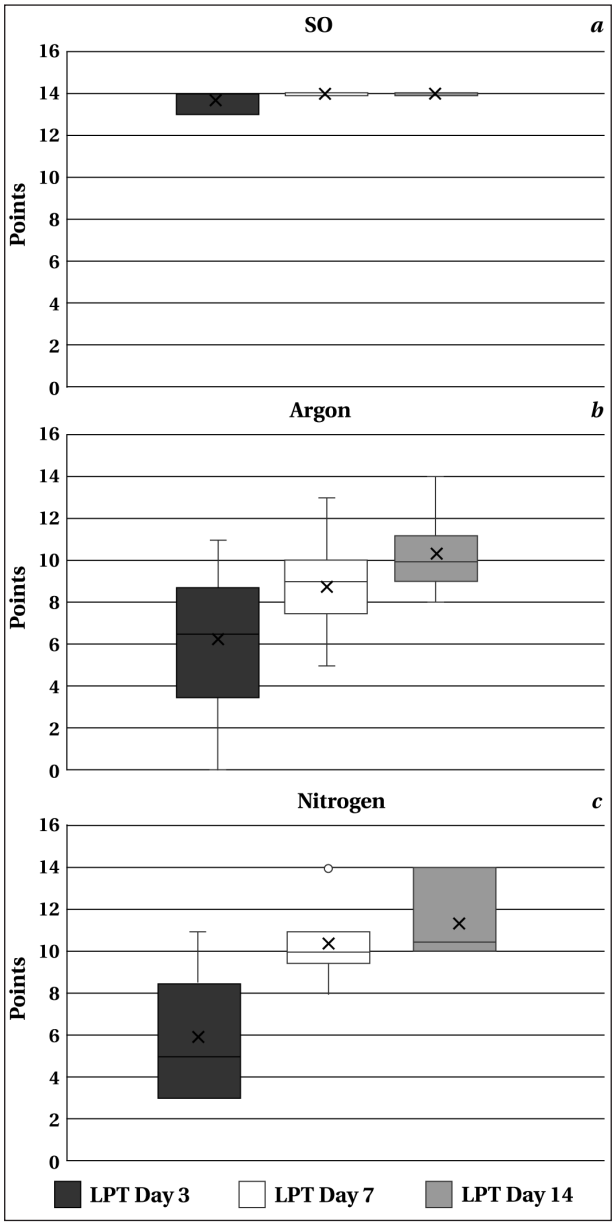

Fig. 2. Limb-placing test.
Note. *a* — changes in LPT results in the SO group; *b* — changes in LPT results in the stroke+iAr group; *c* — changes in LPT results in the stroke group. Data are expressed as median and interquartile range. Mann–Whitney *U* test with Bonferroni correction and Kruskal–Wallis test were used to compare three or more groups.

Discussion

This study was designed to evaluate the neuroprotective effect of argon on important outcome parameters after ischemic stroke. According to the literature, the most pronounced neuroprotective effect of this gas has been demonstrated in models of ischemic neuronal injury *in vitro*. Thus, in an in vitro model of traumatic brain injury [50], 50-percent argon showed a strong neuroprotective profile compared to 6-percent desflurane. Meanwhile, a small number of preclinical studies of the protective effects of argon in vivo have shown conflicting results.

Our study of the neuroprotective effect of 24 h argon inhalation starting from the first hours of photoinduced ischemic stroke in rats showed no significant effect on the severity of neurological deficit during the 2-week postischemic period and on the lesion volume according to MRI data at day 14.

The negative result of the study could be due to several factors.

First, argon, unlike xenon, may not have clinically significant neuroprotective effects in ischemic stroke, which is confirmed by negative results in other in vivo studies [24, 37]. Second, experimental modeling of stroke and other brain injury is almost always performed in anesthetized animals, so it is

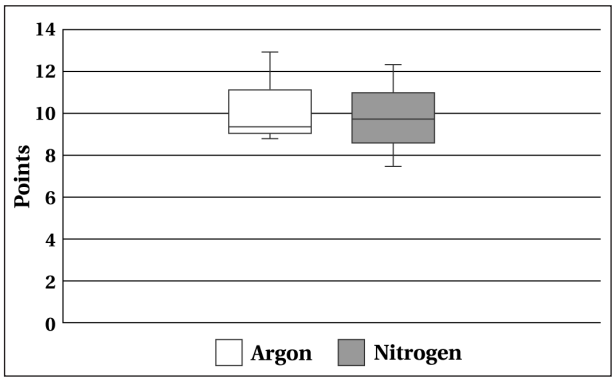

Fig. 3. Extent of brain injury in rats on day 14 of follow-up according to MRI.
Note. The data were presented as medians and quartiles.

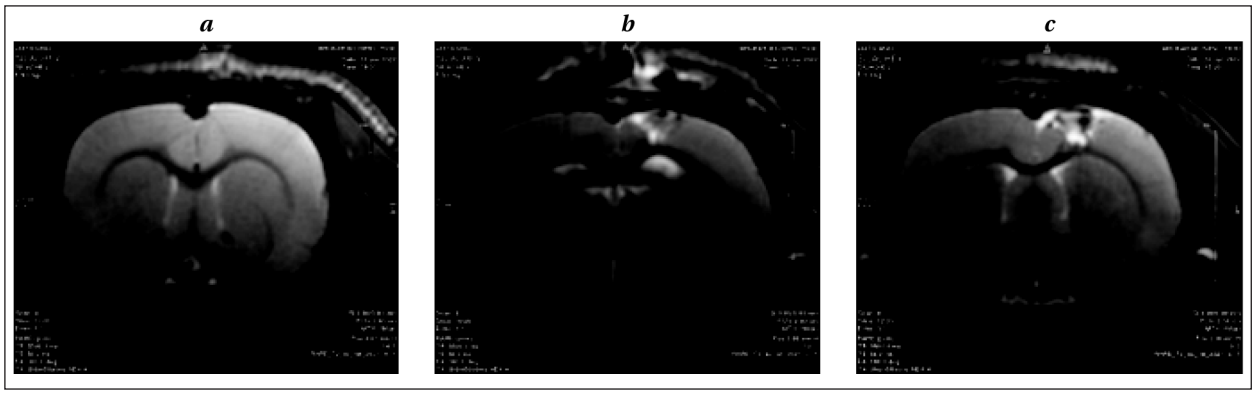

Fig. 4. MRI examination of the rat brain.
Note. *a* — T2-weighted coronal MR image of the animal from the SO group. *b* — T2-weighted coronal MR image of an animal from the stroke group. *c* — T2-weighted coronal MR image of an animal from the stroke+iAr group.

imperative to consider the effects of the anesthetic used. According to the literature, comparative studies of sevoflurane, isoflurane, and argon in the ischemic injury model have not been performed. Both groups of stroke animals had high limb-placing test scores, which may be a manifestation of the neuroprotective effect of sevoflurane. Experimental and clinical studies confirm the strong neuroprotective properties of sevoflurane [51–53]. In this regard, the use of another anesthetic without apparent organoprotective effects (e. g., chloral hydrate [54]) may reveal the neuroprotective effects of argon in a similar experimental model. Another factor that could influence the results of the study is the duration and conditions of exposure. On the one hand, 24 h exposure should have been sufficient to obtain a positive result. However, a number of studies [55, 56] have suggested that argon, due to its specific heat capacity, which is twice lower than that of air, causes moderate hyperthermia, exacerbating ischemic brain injury. In the present study, the temperature of the animals in the postoperative period and the volume of fluid consumed by the animal were not evaluated. In this regard, a long duration of argon inhalation in a closed chamber may have influenced the results obtained. However, the lack of significant differences between the groups suggests that prolonged argon inhalation did not have a deleterious effect.

Considering the data obtained and the review of the literature, we can conclude that further studies with a modified design that takes into account the above-mentioned limitations of this study are needed to evaluate the neuroprotective effect of argon.

Conclusion

Inhalation of an argon-oxygen mixture (Ar 70%/O_2 30%) for 24 hours after photochemically induced stroke does not reduce the extent of brain injury and the severity of neurological deficits.

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Received 25.01.2023
Accepted 01.06.2023