Timely and Appropriate Administration of Inhaled Argon Provides Better Outcomes for tMCAO Mice: A Controlled, Randomized, and Double-Blind Animal Study

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Abstract

Background: Inhaled argon (iAr) has shown promising therapeutic efficacy for acute ischemic stroke and has exhibited impressive advantages over other inert gases as a neuroprotective agent. However, the optimal dose, duration, and time point of iAr for acute ischemic stroke are unknown. Here, we explored variable iAr schedules and evaluated the neuroprotective effects of acute iAr administration on lesion volume, brain edema, and neurological function in a mouse model of cerebral ischemic/reperfusion injury.

Methods: Adult ICR (Institute of Cancer Research) mice were randomly subjected to sham, moderate (1.5 h), or severe (3 h) transient middle cerebral artery occlusion (tMCAO). One hour after tMCAO, the mice were randomized to variable iAr protocols or air. General and focal deficit scores were assessed during double-blind treatment. Infarct volume, overall recovery, and brain edema were analyzed 24 h after cerebral ischemic/reperfusion injury.

Results: Compared with those in the tMCAO-only group, lesion volume (p < 0.0001) and neurologic outcome (general, p < 0.0001; focal, p < 0.0001) were significantly improved in the group administered iAr 1 h after stroke onset (during ischemia). Short-term argon treatment (1 or 3 h) significantly improved the infarct volume (1 vs. 24 h, p < 0.0001; 3 vs. 24 h, p < 0.0001) compared with argon inhalation for 24 h. The concentration of iAr was confirmed to be a key factor in improving focal neurological outcomes relative to that in the tMCAO group, with higher concentrations of iAr showing better effects. Additionally, even though ischemia research has shown an increase in cerebral damage proportional to the ischemia time, argon administration showed significant neuroprotective effects on infarct volume (p < 0.0001), neurological deficits (general, p < 0.0001; focal, p < 0.0001), weight recovery (p < 0.0001), and edema (p < 0.0001) in general, particularly in moderate stroke.

Conclusions: Timely iAr administration during ischemia showed optimal neurological outcomes and minimal infarct volumes. Moreover, an appropriate duration of argon administration was important for better neuroprotective...
Introduction

Acute ischemic stroke (AIS) is a major global disease characterized by a high incidence, disability rate, and mortality rate and is one of the main reasons for admission to the neurological intensive care unit [1–3]. In recent decades, neuroprotective agents have shown efficacy and safety in experimental animal models of AIS. However, most candidate neuroprotective drugs have failed in preclinical and clinical trials [4, 5]. In recent years, medical gases, such as xenon, helium, argon, volatile anesthetics (sevoflurane, isoflurane), H₂S, H₂, etc., which are used as neuroprotective agents and are alternative or adjuvant methods for thrombolysis and endovascular therapy during multiple phases of AIS, have gained considerable attention [6–10]. Unlike other therapeutics, medical gases can easily diffuse into target tissue, cross the blood–brain barrier, and reach all parts of the brain, with the advantages of rapid onset and offset and titratability. Recently, inhaled argon (iAr) has become a promising choice because of encouraging neuroprotective effects demonstrated in multiple species and a range of experimental models of stroke, hypoxic-ischemic encephalopathy, cardiac arrest, and trauma [11–25], with the unique advantages of being readily available and easy to administer and transport, lacking anesthetic properties under normal pressure, and not inhibiting the thrombolytic efficacy of tissue plasminogen activators at high concentrations (75%) [26–31].

Despite these promising data for argon neuroprotection in various animal models of diseases, several studies have suggested that iAr results in insignificant improvement or even detrimental outcomes under certain prognostic indices or circumstances [14, 18, 19, 32], such as the timing, duration, and optimal dose of applications. In the transient middle cerebral artery occlusion (tMCAO) model, for example, David and colleagues [14] showed that argon at 50 vol% administered for 1 h during reperfusion resulted in an increase in subcortical brain damage but improved infarct volume, in contrast to argon at 50 vol% for 3 h during ischemia, as found by Ryang and colleagues [13]. Another article by Fahlenkamp et al. [33] showed that 50% argon/30% oxygen inhaled for 3 h during ischemia resulted in the elevated expression of not only several neuroprotective growth factors (transforming growth factor β, nerve growth factor, and vascular endothelial growth factor) but also several inflammatory cytokines (interleukin 1β, interleukin 6, and inducible nitric oxide synthase). Similarly, Ma and colleagues [19] found that argon failed to improve infarct size in a recent study in which rats were exposed to 24 h of inhalation of 70% argon/30% oxygen during reperfusion. Thus, the systematic scheme of argon treatment is a key factor required for its therapeutic efficacy. However, few studies have been performed on systematic experimental regimens of argon administration for neuroprotection, especially in tMCAO models.

To explore the optimal treatment regimen for argon inhalation, we investigated the therapeutic efficacy of argon administration in tMCAO-induced severe ischemic stroke at different initiation points, durations, and concentrations of argon by analyzing infarct volume, deficit scores, and overall recovery. We also used brain edema to assess whether argon influences AIS complications in two different stroke models induced by different ischemic durations, mimicking moderate or severe AIS.

Methods

Animals

All applicable institutional and/or national guidelines for the care and use of animals were followed. This study was approved by the Institutional Animal Care and Use Committee of Nantong University (protocol number: S20190920-303). Male ICR (Institute of Cancer Research) mice weighing 25–30 g were obtained from the Experimental Animal Center of Nantong University and housed in a vivarium with a 12-h light/dark cycle and free access to food and water. The body temperature of each animal was maintained at 37 ± 0.5 °C throughout the procedure using a heat pad.

Mouse Model of tMCAO

The mice were anesthetized with isoflurane, and tMCAO was performed as described previously [34]. A midline ventral cervical skin incision was made to expose the right common carotid artery, the external carotid artery, and the internal carotid artery. After ligation at the proximal end of the common carotid artery, a 6–0 monofilament with a silicon-coated tip (6023PK5Re, Doccol Corporation) was inserted and positioned at the origin of the middle cerebral artery to occlude it. The same surgical procedure was performed in sham-operated animals, except for artery occlusion. The signs
of a successful surgery included the following: flexion or reduced grasping ability of the left foreleg and spontaneous circling or toppling to the left. Cerebral blood flow was monitored throughout the surgery using laser Doppler flowmetry. At the time of reperfusion, which varied depending on the experiment, the mice were anesthetized, the neck wound was reopened, and the suture was removed. Body temperature was maintained between 36.5 and 37.5 °C during the procedure. After 24 h of post-tMCAO reperfusion, the mice were killed for the following experiments.

**Gas Exposure System and Experimental Protocols**

The mice were randomly assigned to undergo tMCAO or sham surgery for 1.5 or 3 h using the endoluminal thread model. One or three hours after tMCAO induction, the mice were randomly exposed to 79% iAr (79% argon in 21% oxygen) or 39% iAr (39% argon/40% nitrogen/21% oxygen) for the specified time in a custom-built gas delivery system consisting of a gas intervention box with a total volume of approximately 3.5 L connected to a mixed gas bottle that can display the flow rate. The system was flushed with more than four times the system volume of the mixed gases before the gas transmission circulation system was established. Further protocol details are provided in Fig. 1.

**Protocol 1**

The mice were randomly allocated to the following four groups: sham, tMCAO, iAr treatment 1 h after stroke onset, and iAr treatment 3 h after stroke onset. All mice in each treatment group were administered 79% argon in 21% oxygen ventilation of iAr for 3 h. Two different neurological deficit scores were obtained after tMCAO with reperfusion, after the 3-h iAr application, and at 1 day of recovery. The mice were killed at 24 h after reperfusion, and coronal brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and saved for pathological evaluation.

**Protocol 2**

On the basis of the result of protocol 1, inhaled 79% argon was used 1 h after stroke onset to provide neuroprotection in this experiment. For this time-dependent study, the treatment group received 79% iAr (79% argon in 21% oxygen) for 1, 3, or 24 h at 1 h after stroke onset. The mice were randomly allocated to the following five groups: sham, tMCAO, 79% iAr for 1 h, 79% iAr for 3 h, and 79% iAr for 24 h. Measurements of the focal deficits to obtain neurological deficit scores were performed 24 h after reperfusion. After evaluation of neurological deficits, the mice were killed for histological assessment performed by staining brain areas.

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Fig. 1 Schematic illustration of the experimental design of inhaled argon iAr. The red box represents the period of ischemia. The blue box represents the period of reperfusion. 39% iAr, inhaled 39% argon–40% nitrogen–21% oxygen; 79% iAr, inhaled 79% argon–21% oxygen; A concentration of 79% iAr was used for all experiments except for the dose–response experiment 3, where the doses are indicated.
Protocol 3
In this dose-dependent study, each treatment group received either 39% or 79% iAr at 1 h after stroke onset. The mice were randomly allocated to the following four groups: sham, tMCAO, 39% iAr for 3 h, and 79% iAr for 3 h. Focal deficits were measured to obtain neurological deficit scores 24 h after reperfusion. The mice were killed at 24 h after reperfusion, and coronal brain slices stained with TTC were saved for pathological evaluation.

Protocol 4
Mice were randomly allocated to sham, tMCAO, and 79% iAr for 3 h in two different ischemia duration models (1.5 h/24 h; 3 h/24 h), inducing moderate and severe ischemic stroke. The mice were subjected to 1.5 h/3 h tMCAO followed by reperfusion, and both groups were exposed to 79% iAr (79% argon/21% oxygen) 1 h after stroke onset for 3 h. After 24 h of reperfusion, two different neurological deficit scores were evaluated, and the mice were then killed for measurement of the infarct volume.

Protocol 5
The argon treatment approach was performed, and neurological deficit scores were evaluated as in protocol 4. The mice were killed at 24 h for determination of brain water content.

Mortality Rates and Evaluation Of Neurological Deficits
Mortality rates were calculated 24 h after tMCAO as a parameter of this study. Mice died of surgery-induced ruptured vessels during the reperfusion stage, which was verified by autopsy, and were excluded from other outcome parameter analyses. The neurologic assessment was conducted by a researcher who was blinded to the experimental groups. The neurological behavior of the mice was scored 24 h after MCAO/Reperfusion according to Clark’s scoring system [35] using a general neurological scale (0–28) and a focal neurological scale (0–28). In the general scoring system, for each of the six general deficits measured, animals received between 0 and 12 points depending on the severity; the scores for the six areas were then summed to generate a total general score ranging from 0 to 28. In the focal scoring system: for each of the seven areas assessed, animals were rated with scores between 0 and 4 depending on the severity; the seven items were then summed to give a total focal score ranging between 0 and 28.

Measurement of Cerebral Infarct Volume
After neurological evaluation, the mice were killed, and the brains were collected for measurement of the infarct volume. The brain was removed rapidly and placed at −20 °C for 20 min. Coronal sections were cut into five 2-mm-thick slices, and the slices were immersed in 1% TTC in phosphate-buffered saline at 37 °C for 20 min followed by overnight immersion in 4% paraformaldehyde. The infarct area of each slice was demarcated and analyzed using ImageJ by an investigator blinded to the experimental groups. Infarct volume was calculated by integration of the infarct areas for all slices from each brain, and each section was measured by a blinded researcher. The infarct area was determined by subtracting the area of noninfarcted tissue in the ipsilateral hemisphere from that of the intact contralateral hemisphere to correct for brain swelling [36].

Quantification of Brain Water Content
After neurological measurement, we used the wet–dry method to assess the brain water content [37]. The mice were killed under deep anesthesia. Each hemisphere was weighed (wet weight) and left in a desiccating oven at 95 °C overnight, and then the dried hemispheres were weighed again (dry weight). The percentage of brain water content of each part was calculated as [(wet weight − dry weight)/wet weight] × 100%.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism software (GraphPad Prism 8.0.1, GraphPad Software Inc., San Diego, CA). The data are expressed as the mean ± standard error. One-way analysis of variance was used to evaluate the differences among multiple groups if the data exhibited a normal distribution and homogeneity of variance. Two-way analysis of variance was used to measure the effects of two factors simultaneously, with post hoc Bonferroni correction for multivariate analyses. Otherwise, the differences were assessed by a nonparametric test. A p value less than 0.05 was considered indicative of statistical significance.

Results
iAr Administration Initiated During Early Ischemia/Reperfusion Attenuated Brain Injury Better than Administration After Reperfusion
To assess whether argon treatment at different initiation points has neuroprotective effects after severe ischemic stroke induced by delayed reperfusion (ischemic/reperfusion [I/R]) (3 h/24 h) and to explore the best time point for iAr administration, we applied two different treatment strategies that are closer to the actual clinical situation (iAr administered at 1 h after stroke onset; iAr administered at 3 h after stroke onset). We analyzed the brain infarct volume, neurological deficit scores, and weight recovery 24 h after reperfusion to assess the severe ischemic stroke outcome. The mice were...
reperfused 3 h after tMCAO, inducing severe ischemic stroke. Compared with the tMCAO-alone group, the treatment group that inhaled argon 1 h after stroke onset (during ischemia) showed significantly lower volumes ($p<0.0001$) (Fig. 2a, b), improved neurological scores (general, $p<0.0001$; focal, $p<0.0001$) (Fig. 2c, d), and improved weight recovery ($p=0.0029$) (Fig. 2e) at 24 h after reperfusion. The treatment group receiving iAr at 3 h after stroke onset (during reperfusion) exhibited improved focal deficits (neuroscore) compared with the tMCAO-alone group ($p=0.0018$) (Fig. 2d). In this group, however, infarct volume was not reduced, nor was weight recovery improved ($p>0.05$; Fig. 2b, e). iAr administration at the two initial intervention time points showed a trend toward decreased mortality in mice compared with tMCAO alone. However, the trend did not reach significance (Fig. 2f). These results suggested that iAr administration 1 h after stroke onset (during ischemia) improved ischemic brain injury, attenuated the general deficit scores, and improved weight recovery in severe ischemic stroke, but this was not so in animals administered iAr 3 h after stroke onset (during reperfusion).

**iAr Administration Attenuated Brain Injury in a Time-Dependent Manner**

To further evaluate the relationship between the inhalation duration of iAr during ischemia and its neuroprotective effect on tMCAO-induced severe ischemic stroke in mice, we tested the neuroprotective effects of three different durations of iAr (argon application for 1, 3, or 24 h) given 1 h after stroke onset. At 1 day after tMCAO, we sectioned and stained brains with TTC, a substrate for mitochondrial respiration (Fig. 3a). We estimated infarct volumes by assessing the staining of brain areas (Fig. 3a, b). The infarct volume in the brain slices stained with TTC was the lowest in the group receiving 1 h of argon administration, followed by the group receiving 3 h of argon administration, the group receiving 24 h of argon administration, and the control groups, which is indicative of the neuroprotective effect of iAr, with 1–3 h of iAr providing the most substantial effect (Fig. 3b). The
groups administered iAr for both 1 and 3 h showed significantly lower volumes than the group administered iAr for 24 h (1 vs. 24 h, \( p < 0.0001 \); 3 vs. 24 h, \( p < 0.0001 \)) (Fig. 3b), but no significant difference was observed between them (\( p = 0.7346 \)) (Fig. 3b). Treatment with iAr for both 1 and 3 h resulted in improved focal deficits 24 h after reperfusion (1 h vs. control, \( p < 0.0001 \); 3 h vs. control, \( p < 0.0001 \)) (Fig. 3c), but treatment with iAr for 24 h did not (\( p = 0.7109 \)) (Fig. 3c). These results indicated that iAr application during ischemia over a duration range of 1–3 h provided the most efficacious neuroprotection and that prolonged inhalation for 24 h improved infarct volume.

High Concentrations of iAr Alleviated Brain Damage Better After Ischemic Stroke

To further investigate whether the concentration of argon affected the neuroprotective effect of argon during ischemia on tMCAO-induced severe ischemic stroke in mice, we tested the neuroprotective effects of two different concentrations of argon (39% argon/40% nitrogen/21% oxygen vs. 79% argon/21% oxygen) initiated 1 h after stroke onset. The outcomes were brain infarct volume and neurological deficit scores. Compared with the control group, the two treatment groups exhibited reduced volume (\( p < 0.0001 \)) (Fig. 4b) and improved focal neurological deficits (\( p < 0.0001 \)) (Fig. 4c). Notably, significant differences were observed regarding the neuroscore between the two treatment groups (\( p = 0.0274 \)) (Fig. 4c). These results suggested that either 39% or 79% argon improved both infarct volume and the neuroscore 24 h after severe ischemic stroke, but high concentrations of iAr resulted in better neurological outcomes. These results suggested that inhalation of 39% argon and 79% argon during ischemia both showed improved infarct volume and functional outcome 24 h after severe ischemic stroke, but the 79% argon inhalation group exhibited a better neurological outcome assessed by the
general deficits compared with the 39% argon inhalation group.

**iAr Promoted Neurological Recovery in Both Moderate and Severe Stroke Models**

To explore whether iAr during ischemia could improve the outcomes of stroke of varying severity (moderate and severe ischemic stroke) induced by different ischemia durations, especially severe ischemic stroke induced by delayed reperfusion (not previously reported in vivo), and whether there are differences between the two ischemia durations in the argon treatment group, we used two different ischemia duration models (I/R 1.5 h/24 h; I/R 3 h/24 h) at the same treatment time point (1 h after stroke onset) with a 3-h argon therapeutic duration. tMCAO-induced focal cerebral ischemia was performed as a 1.5-h (I/R 1.5 h/24 h) or 3-h (I/R 3 h/24 h) occlusion, followed by 24-h reperfusion (Fig. 5a), and was assessed by the infarct volume, neurobehavioral scores, weight loss, and mortality rate 24 h after reperfusion (Fig. 5b–f). Delayed reperfusion induced a larger infarct volume ($p<0.0001$) (Fig. 5a, b) and more prominent neurological deficits (general, $p<0.0001$; focal, $p<0.0001$) (Fig. 5c, d) than the 1.5-h occlusion and 24-h reperfusion models. Treatment with iAr in both ischemia models resulted in a significant improvement in infarct volume ($p<0.0001$) (Fig. 5b), neurological deficits ($p<0.0001$) (Fig. 5c, d), and weight recovery ($p<0.0001$) (Fig. 5e) compared with the control group. Of note, we found that argon treatment beginning 1.5 h after tMCAO-induced focal cerebral ischemia significantly improved infarct volume ($p<0.0001$) (Fig. 5b) and focal deficits ($p=0.0428$) (Fig. 5d) compared with treatment beginning 3 h after ischemia. No difference in mortality rate was noted between the groups. Together, these results indicated that iAr treatment reduced infarct volume and improved neurological deficits and weight recovery after moderate and severe ischemic stroke induced by different ischemia durations, particularly in moderate stroke.

**Argon Administration Reduced Edema After Stroke Onset**

Brain edema and neurologic function were measured following argon treatment in moderate and severe AIS. MCAO/Reperfusion mice in the two ischemia models demonstrated significantly higher brain water content and neurologic impairment than those in the sham group ($p<0.0001$) (Fig. 6a–c). Delayed reperfusion induced an increase in both brain water content ($p<0.0001$) (Fig. 6a) and worse neurological deficits ($p<0.0001$) (Fig. 6b, c). The argon-treated group in both models showed significantly less brain edema and

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**Fig. 5** The neuroprotective effect of iAr (79%) in two models of ischemic duration. **a**, Representative images of TTC-stained cerebral coronal sections of mice (scale bar: 10 mm) in the sham, tMCAO, and argon treatment groups in two different time windows between ischemia and reperfusion (I/R 1.5 h/24 h; I/R 3 h/24 h). **b**, Quantification of brain infarct volume ($n=5$ to 23/group), **c, d**, neurobehavioral scores ($n=7$ to 20/group), and weight loss ($n=8$ to 22/group) 24 h after reperfusion in tMCAO- and argon-treated mice. **f**, The mortality rate 24 h after ischemia/reperfusion in two different durations of ischemia. The data are shown as the mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistical comparisons were carried out using two-way ANOVA.
lower neurological deficit scores than the MCAO/R group \((p < 0.0001)\) (Fig. 6a–c). Similarly, treatment with argon beginning 1.5 h after ischemia demonstrated improved brain edema \((p < 0.0001)\) (Fig. 6a) and focal deficits \((p = 0.0414)\) (Fig. 6c) compared with treatment beginning 3 h after ischemia. These results suggested that iAr treatment improved brain edema in moderate and severe ischemic stroke, especially moderate stroke.

**Discussion**

This study is the first to systematically explore the neuroprotective effect of iAr under different therapeutic schedules in tMCAO-induced moderate and severe ischemic stroke models. Our results suggest that compared with the tMCAO group, the groups administered 39% and 79% argon at 1 h after stroke onset for 1 and 3 h showed a significant reduction in infarct volume in the severe cerebral ischemia model, and neurological outcome was obviously improved in a dose-dependent manner with 3 h of treatment. The neuroprotective efficacy of 79% argon administration at 1 h after stroke onset for 3 h was further validated in a moderate stroke model. Moreover, we found that the optimal schedule of argon treatment reduced edema in both models.

**The Neuroprotective Effects of iAr on the Infarct Volume After Ischemic Stroke**

We observed that argon administration improved the infarct volume in severe ischemic stroke at 1 h after stroke onset (during ischemia) but not at 3 h after stroke onset (during reperfusion) in experiment 2. Consistent with our observations during ischemia, Ryang et al. [13] observed improved infarct volume when argon was administered for 1 h during ischemia after tMCAO induction. During reperfusion, David et al. [14] found that argon decreased the subcortical volume but increased the subcortical volumes of damaged brain regions when given 1 h after reperfusion (during reperfusion), which is partly consistent with our results. Liu et al. [18] reported that the infarct volume was not affected by argon treatment with a 3-h delay after stroke onset and 1 h after reperfusion (during reperfusion). Similarly, Ma et al. [19] suggested that the infarct size was not decreased when argon was administered for 24 h during reperfusion. Notably, our results showed that argon administered for 1, 3, or 24 h during ischemia decreased the infarct volume, especially at 1–3 h. We observed that during the trial by Ma et al. [19], the initial application of argon for 24 h occurred during the reperfusion phase, and in our study, it occurred during the ischemic phase in experiment 2. Overall, current evidence seems to show that iAr effectively improves the infarct volume when administered during ischemia, not reperfusion. The reasons for these results are unclear. One possible reason is that application of iAr immediately after reperfusion (3 h after ischemia) may miss the therapeutic window for neuroprotective intervention in the ischemic penumbra. Many animal and human experimental studies suggest that the therapeutic window for neuroprotective intervention in the ischemic penumbra is very brief, often less than 2 or 3 h [38, 39]. Another possible reason is that with the increase in the duration of ischemia, the ischemic core area is further enlarged, and I/R injury may simply counteract or even exceed the protective effect of argon, as first proposed by Ma et al. [19]. Necroptosis and apoptosis are of great importance in cerebral I/R injury, and the activation of necroptosis depends on reperfusion and is activated immediately after ischemic insult on reperfusion [40]. Reperfusion itself followed by tMCAO can lead to neuronal death due to overperfusion and hemorrhagic transformation, which aggravates ischemic brain injury [41, 42]. Zhuang et al. [15] showed the effect of argon on the antiapoptotic signaling pathway in a neonatal rat asphyxia model. Therefore, we speculate...
that the antiapoptotic activity of argon may be offset or even concealed by this reperfusion injury to some extent. Moreover, recent literature [18] has reported that treatment with argon promotes the switch of microglia/macrophage polarization toward the anti-inflammatory M2 phenotype. In addition, cerebral ischemia and ischemia coupled with reperfusion result in differing pathologic mechanisms and microglial morphological responses, and a spatiotemporal relationship exists between microglial morphology and evolving brain injury after ischemic stroke and reperfusion [43]. We speculate that iAr can impact the balance between proinflammatory and anti-inflammatory cytokines toward anti-inflammation after AIS and affect the infarct size under the appropriate combination of treatment timing and duration of treatment (for example, short-term intervention during ischemia). However, this possibility is only a speculation, and additional experiments are needed to validate this hypothesis and further dissect the possible mechanisms involved.

Recovery of Neurological Function with Inhaled Argon After Ischemic Stroke

Functional recovery is one of the main end outcomes of patients with stroke. Clinically, it is customary to divide the neurological impairment of patients with stroke into general functional injury and focal functional injury. The former reflects the patient’s life state and global neurological function, whereas the latter reflects localized functional defects caused by local injury. Therefore, in addition to infarct volume, neurological deficits were assessed in this study according to Clark’s scoring system [35], reflecting the degree of neurological injury after ischemia from the point of view of general functional injury and focal functional injury. Our in vivo results suggested that argon administered at 39 vol% and 79 vol% could improve neurologic outcome in experiment 3 in a dose-dependent manner. In accordance with our results, previous studies suggest that argon exerts neuroprotective effects in a dose-dependent manner in different injury models (hypoxia vs. ischemia), such as models of traumatic brain injury [12], retinal ischemia/reperfusion injury [44], and cardiac arrest [45]. Similarly, Liu et al. [18] demonstrated significantly improved neurological performance using a 6-point neuroscore daily from 24 h to 7 days after reperfusion. In addition, Ma et al. [19] found that neurologic outcome was significantly improved after 24 h of treatment with argon instituted after reperfusion. However, in contrast to its beneficial effect on neurological outcomes, David et al. [14] found that 50 vol% argon inhaled during reperfusion for 3 h failed to improve tMCAO-induced neurologic deficits. The results seem to indicate that even if argon is administered at similar time points (such as after reperfusion), it will lead to significant differences in neurological impairment due to different treatment protocols, such as durations and concentrations. Notably, however, in addition to the initial time point of iAr, the difference in neurologic assessment systems is one factor to be considered.

An interesting aspect of our research is that argon administration for 24 h during ischemia reduced infarct volume, but neurologic outcomes were not improved. Another unanticipated result was that iAr caused dose-dependent improvement of neurological prognosis in our study, which was also inconsistent with observations of infarct volume. These results reflect those of Liu et al. [18], who found that with a 3-h delay after stroke onset and 1 h after reperfusion, argon significantly alleviated neurological deficits during the first week after stroke but failed to reduce the infarct volume. Additionally, these differences corroborate the ideas of Ma et al. [19], who found that neurologic outcome was improved but that infarct size was not reduced when argon administration was delayed 2 h after permanent stroke onset or instituted after reperfusion. One possible explanation of this difference may be that although there is a correlation between the focal functional injury score and cerebral infarction volume of the Clark score, evidence from the recent literature indicates that histologic lesion size is less correlated with improved neurologic outcome, as Ma and colleagues [19] have clearly noted. Additionally, we have to acknowledge that this inconsistency may, in part, be related to several factors, such as differences in the samples and differences in neurological indicators.

The influences of Argon on Edema After Ischemic Stroke

Few studies have addressed the effect of iAr on the complications of AIS. However, the complications of AIS have significant effects on the short-term and long-term prognosis of patients, and brain edema is a common complication of AIS and one of the main causes of death. Acute brain injury is linked to cellular edema, characterized by abnormal intracellular accumulation of water in brain cells resulting in cellular swelling [46]. Delayed reperfusion, whether by thrombolysis or endovascular therapy, can result in more severe brain edema and poorer clinical outcomes and can increase the risk of mortality, hemorrhagic transformation, headache, and seizures [47, 48]. Brain edema is also an important prognostic indicator in the assessment of argon efficacy in severe ischemic stroke induced by delayed reperfusion [42]. As previously reported [13, 14, 18, 49, 50], argon has been shown to exert a neuroprotective effect in in vivo tMCAO models induced by ischemia for 1–2 h, consistent with our findings. Our study showed that neuroprotection still existed in severe ischemic stroke induced by an increasing ischemic duration beyond those commonly used at
clinically relevant time windows. The results showed that argon treatment in moderate and severe AIS models led to significantly reduced brain water content, infarct volume, neurologic function, and weight recovery at the same treatment time point, duration, and concentration. Importantly, delayed reperfusion affects the ability of iAr to improve brain edema, infarction volume, and neurologic function following tMCAO. The design of the present study allowed conclusions to be drawn regarding whether a higher concentration of argon inhaled for 3 h during ischemia would improve the complications characterized by brain edema in the tMCAO model. Further studies are needed to clarify the underlying mechanism of this improvement and should pay more attention to argon-induced improvements in other complications of AIS, such as hemorrhagic transformation.

Limitations and Prospective

There are several design limitations in our experiment that deserve special attention. First, an important limitation is the lack of data to measure potential medium- and long-term effects, which are clinically important for the prognosis of patients with AIS. The animals survived for only 24 h after reperfusion, which may contribute to some results that are not wholly consistent with the conclusions of our study at a later sampling time. Longer recovery times should be included in future studies, and functional imaging, such as magnetic resonance imaging [37], can be applied to monitor the evolution of brain injury and treatment. Second, animal models other than rodents should be adopted, including large animal models [51, 52], such as pigs, dogs, and monkeys. Further research should test the same strategies in larger species with basic diseases, such as hypertension or diabetes, and explore the impact of other biological variables, such as sex and age. These factors were not explored in this study because of budgetary limitations. Finally, this study is limited because it considered only the timing, duration, and concentration; however, the specific mechanism involved in this improvement was not investigated, and this is part of our future research contents and direction. Thus, further animal and clinical studies addressing these issues at length are needed. Despite these limitations, this study is the first to demonstrate the effects of argon on cellular edema after tMCAO-induced severe cerebral ischemic stroke in mice. Moreover, our study adopted the synthesis of two kinds of neurobehavioral scores that are suitable for clinical practice, and the behavioral experiments were double-blinded to limit bias.

Conclusions

In this study, it was demonstrated that timely iAr administration during ischemia for appropriate short-term treatment at a higher concentration provides better neuroprotective efficacy. We conceived this study as an instructive guide for the protective effects of argon on AIS and better clinical translation.

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Author Contributions

YZ and XL conceived and designed the study, supervised the work, analyzed and interpreted the data, wrote the manuscript, revised the manuscript, and approved the final manuscript. JH was involved in the experimental conception, the first draft of the manuscript, and analysis and interpretation of the data. KX was involved in data acquisition and analysis and interpretation. JL helped with the tMCAO model and double-blind analysis. JG was involved in data interpretation. BP and LX helped with gas ventilation and animal supply according to the experimental protocol. GW and ZJ helped to design and evaluate the study. All authors read and approved the final manuscript.

Source of support

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Conflict of interest

There are no conflicts of interest.

Ethical Approval/Informed Consent

The research protocol and animal care procedures of this study were inspected and approved by the Laboratory Animal Center and Lab Animal Ethics Committee of Nantong University.

Human and Animal Rights

Animal studies followed and adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines.

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