Xenon-helium gas mixture at equimolar concentration of 37.5% protects against oxygen and glucose deprivation-induced injury and inhibits tissue plasminogen activator

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Abstract

Xenon (Xe) is considered to be the golden standard neuroprotective gas. However, Xe has a higher molecular weight and lower thermal conductivity and specific heat than those of nitrogen, the main diluent of oxygen in air. These physical characteristics could impair or at least reduce the intrinsic neuroprotective action of Xe by increasing the patient's respiratory workload and body temperature. In contrast, helium (He) is a cost-efficient gas with a lower molecular weight and higher thermal conductivity and specific heat than those of nitrogen, but is far less potent than Xe. In this study, we hypothesized that mixing Xe and He could allow obtaining a neuroprotective gas mixture with advantageously reduced molecular weight and increased thermal conductivity. We found that Xe and He at the equimolar concentration of 37.5% reduced oxygen-glucose deprivation-induced increase in lactate dehydrogenase in brain slices, an *ex vivo* model of acute ischemic stroke. These results together with the effects of Xe-He on the thrombolytic efficiency of tissue plasminogen activator are discussed.

Key words: xenon; helium; inert gases; gas mixtures; synergistic effects; neuroprotection; tissue plasminogen activator

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Introduction

Previous research has shown that the chemically and metabolically inert gases xenon (Xe) and helium (He) have neuroprotective properties in models of hypoxic-ischemic insults, brain ischemia, and traumatic brain injury.[1-19] In line with the critical role played by the N-methyl-D-aspartate (NMDA) receptor in the mechanisms of neuronal death induced by these types of brain insults,[20-23] Xe that is thought to provide neuroprotection by inhibiting the NMDA receptor[24,25] is considered the golden standard neuroprotective gas on the basis of preclinical studies. However, Xe has a molecular weight of 131 g/mol that is higher than that of nitrogen, the main diluent of oxygen in air which molecular weight is 28 g/mol, and further possesses a thermal conductivity of 5.5 mW/m/K and specific heat of 0.16 kJ/kg•K (at 298°K or 25°C) that are lower than those of nitrogen, which thermal conductivity and specific heat are 25.8 mW/m/K and 1.04 kJ/kg•K, respectively,[26] conditions that could impair or at least reduce the intrinsic neuroprotective properties of Xe by increasing the critical care patient's respiratory workload[27,28] and body temperature (unpulished). In addition, in line with its scarcity, Xe suffers an excessive cost of production that is a major obstacle to its clinical development. In contrast, He has a molecular weight of 4 g/mol, which is

lower than that of nitrogen, and a thermal conductivity of 155.3 mW/m/K and specific heat of 5.19 kJ/kg•K,[26] which are higher than those of nitrogen, but unfortunately it is far less neuroprotective than Xe.

Mixing Xe and He would allow reducing the cost of treatment and obtaining a gas mixture with reduced molecular weight and increased thermal conductivity and specific heat as compared to Xe alone. However, although potentially interesting, such a strategy would require that such a gas mixture contains at least 37.5% of Xe, the minimum concentration of Xe shown to possess neuroprotective properties in relevant models of thromboembolic stroke.[2] To determine whether a gas mixture containing 37.5% Xe in combination with 37.5% He (the highest dose of He that can be added to 37.5% Xe while maintaining oxygen at 25%) could allow providing neuroprotection, we investigated the neuroprotective effects of Xe and He at equimolar concentrations of 37.5% on cell injury induced by oxygen-glucose deprivation (OGD) in acute brain slices. In addition, because Xe and He are known to interact with tissue plasminogen activator (tPA),[1,2] whose recombinant form (rtPA) is the only approved drug therapy of ischemic stroke to date, we further investigated *in vitro* and *ex vivo* the effects of Xe-He on the catalytic activity and thrombolytic efficiency of rtPA. These effects of Xe-He were compared to those of 37.5% He, 37.5% Xe, and 50% Xe, the concentration of Xe shown to provide maximal neuroprotection in various *ex vivo* and *in vivo* mechanical and thromboembolic models of acute brain ischemia.[2,18,19]

Materials and Methods

Animals

All animal-use procedures were performed in accordance with the *Declaration of Helsinki*, the French legislation for the use of animals in biomedical experimentation, and the corresponding European Communities Council Directive issued on 24 November 1986 (86/609/EEC). Adult male Sprague-Dawley rats (Janvier, Le Genest Saint-Isle, France) were used. Before being used, rats were housed at 21 ± 0.5°C in Perspex home cages with free access to food and water and lights on from 8:00 p.m. to 8:00 a.m.

OGD studies in acute brain slices

Preparation of brain slices

Rats weighing 250–280 g were decapitated under halothane anesthesia. The brains were removed and placed in ice-cold freshly prepared artificial cerebrospinal fluid (aCSF). Coronal brain slices (400 µm thickness; anteriority from bregma: +1.2 to +2 mm) were cut using a tissue chopper (Mickie Lab. Engineering Co., Gomshall, Surrey, UK).

Measurement of cell injury with lactate dehydrogenase activity assay

The effects of gas mixtures containing Xe and/or He on acute brain slices subjected to OGD, an *ex vivo* model of brain ischemia,[29,30] were assessed by measuring the release of lactate dehydrogenase (LDH), a marker of cell injury,[31] as detailed previously[19]: Brain slices were transferred into individual vials with 1.3 mL of freshly prepared oxygenated aCSF containing 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM D-glucose and 30 mM HEPES, and allowed to recover at room temperature for 45 minutes. Then, brain slices were placed at 36 ± 0.5°C into individual vials containing 1.3 mL of freshly prepared aCSF continuously bubbled with 100% oxygen (25 mL/min per vial). After a 30-minute period, aCSF solution was renewed with oxygenated aCSF maintained at 36°C, and the slices were then incubated for 1 hour to allow recording of LDH basal levels. Whereas sham slices were incubated for an additional 20-minute period in the same conditions, OGD slices were incubated in a glucose-free solution continuously bubbled with 100% nitrogen. After that, to mimic reperfusion and treatment, the medium was replaced with freshly prepared aCSF, saturated and continuously bubbled with medical air (control slices) or gas mixtures containing Xe and/or He (n = 28–29 per group).

In vitro tPA catalytic activity assay

The effects of Xe and/or He gas mixtures on the catalytic activity of rtPA were assessed as detailed previously.[2] rtPA (Actilyse®; Boehringer Ingelheim, Ingelheim am Rhein, Germany) and its specific chromogenic substrate methylsulfonyl-D-phenyl-glycil-arginine-7-amino-4-methylcoumarin acetate (Spectrozyme® XF, product 444; American Diagnostica, Stamford, CT, USA) were diluted separately in 1 mL distilled water in 1.5-mL sterile tubes. Each tube containing 0.4 µM rtPA or 10 µM rtPA substrate was saturated for 20 minutes with air (controls) or gas mixtures containing Xe and/or He (n = 12 per group). The catalytic efficiency of rtPA was assessed by the initial rate method by incubating 50 µL rtPA with 50 µL substrate in a spectrofluorometer microplate reader set at 37°C.

Ex vivo thrombolysis experiments

The effects of gas mixtures containing Xe and/or He on the thrombolytic efficiency of rtPA were assessed as detailed previously.[2] Male Sprague-Dawley mature rats weighing 600–650 g (n = 6) were used. Whole blood samples of 500 mL volume were transferred in preweighed sterile tubes of 1.5 mL, and incubated at 37°C for 3 hours. Saline solution (45 mL) was prepared in a laboratory flask of 50 mL volume whose cap was drilled with two holes of 2 mm in diameter, and saturated for 30 minutes with medical air or

Xe and/or He (with the remainder being oxygen at 25% and nitrogen as needed; see below Gas Pharmacology section) at a flow rate of 80 mL/min through microtubing (2 mm in diameter) and a cylinder bubble stone that was introduced down to the bottom of the container through one of the two holes previously drilled. After clot formation and total serum removal, each tube was weighed to determine the clot weight. To reduce variability, we selected blood clots in the same weight range (0.268 ± 0.023 g). Then, each tube was fully filled (including the cap) with saline solution containing 1 mg/mL of rtPA in the form of Actilyse previously saturated with Xe and/or He or medical air (n = 10–14 per group), quickly closed to avoid Xe, He, or Xe-He desaturation, and incubated at 37°C for an additional 90 minutes period. Then, the fluid was removed, and the tubes were weighed again to assess the percentage of clot lysis induced by rtPA in the presence of medical air, or Xe and/or He. Particular attention was paid to avoid gas desaturation by maintaining Xe and/or He at bubbling in saline while filling the tubes containing the blood clots with saline saturated with Xe and/or He.

Gas pharmacology

Gases of medical grade were purchased from Air Liquide Santé (Paris, France). Medical air composed of 75% nitrogen and 25% oxygen, gas mixtures containing He at 37.5% (He-37.5), Xe at 37.5% (Xe-37.5), Xe at 50% (Xe-50), and Xe-He at equimolar concentration of 37.5% (Xe-He-37.5), with the remainder being 25% oxygen and nitrogen as needed, were obtained using computer-driven gas mass flowmeters (Aalborg) and an oxygen analyzer for double checking.

Statistical analysis

Data are given as the mean ± the standard error to the mean. The effects of Xe-He were analyzed using Statview software (SAS Institute, Cary, NC, USA) and compared to those of control experiments, Xe and He alone using non-parametric Mann-Whitney *U*-test.

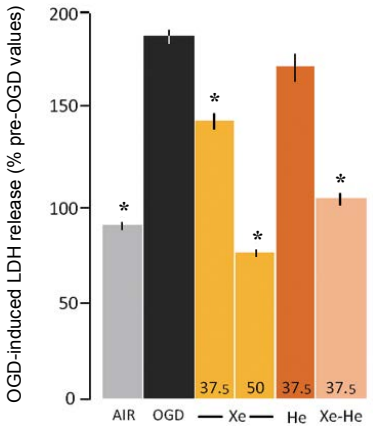

Figure 1: Effects of xenon (Xe) and helium (He) alone or in combination on the increase in lactate dehydrogenase (LDH) release induced by oxygen-glucose deprivation (OGD).

Note: Xe-He-37.5 approximately reduced OGD-induced LDH release to a similar extent than Xe-50. Part of the data with xenon was obtained from a previous study.[19] Data are expressed as the mean ± the standard error to the mean, and analysed by non-parametric Mann-Whitney *U*-test. *P < 0.0001, *vs.* OGD slices.

Results

Control slices exposed to OGD and air exhibited an increase in LDH release (P < 0.0001) compared to sham slices exposed to oxygen (instead of OGD) and air (see above Materials and Methods section). As illustrated in **Figure 1**, Xe-37.5 and Xe-50, but not He-37.5, did provide neuroprotection, leading to a significant difference between Xe-treated slices and air-treated control slices (P < 0.0001). Combining Xe and He at 37.5%, allows reducing OGD-induced LDH release to a similar extent than Xe-50 (P < 0.0001).

Alternatively, because Xe and He have been shown to interact with rtPA, the only approved drug therapy of ischemic stroke to date, we investigated the effects of Xe and

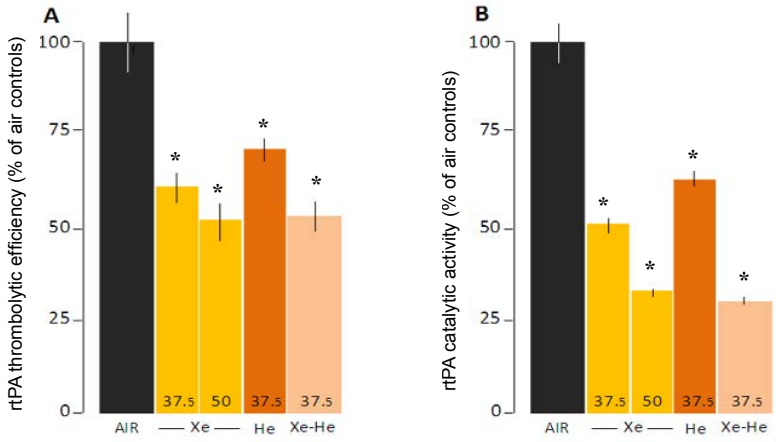

Figure 2: Effects of xenon and helium alone or in combination on the catalytic activity (A) and the thrombolytic efficiency (B) of tissue plasminogen activator (rtPA).

Note: Xe-He-37.5 reduced the catalytic activity and thrombolytic efficiency of rtPA to a similar extent than Xe-50. Part of the data with xenon or helium alone was obtained from previous studies.[1,2] Data are expressed as the mean ± the standard error to the mean, and analyzed by non-parametric Mann-Whitney *U*-test. *P < 0.0001, *vs.* oxygen-glucose deprivation slices.

He on the catalytic and thrombolytic efficiency of rtPA. As illustrated in **Figure 2**, we found Xe-50 > Xe-37.5 > He-37.5 at reducing the catalytic activity and thrombolytic efficiency of rtPA, leading to significant differences between Xe-37.5, Xe-50, He-37.5 and air controls for both the catalytic activity ($P < 0.0001$) and thrombolytic efficiency ($P < 0.0001$) of rtPA. Interestingly, Xe-He-37.5 reduced the catalytic and thrombolytic activity of rtPA to a similar extent than Xe-50 ($P < 0.0001$).

Discussion

In this study, we confirm and extent previous data by demonstrating that Xe-50, Xe-37.5 and importantly Xe-He-37.5, but not He-37.5, reduce OGD-induced increase in LDH release. As reported previously,[18,19] we found that maximal neuroprotection was provided by Xe-50. As hypothesized, combining Xe-37.5 with He-37.5, the highest concentration of He that can be added to Xe-37.5 while maintaining oxygen at 25%, allows reducing OGD-induced LDH release to a similar extent than Xe-50, thereby demonstrating a synergistic effect between Xe-37.5 and He-37.5 at providing neuroprotection since He-37.5 has no effect alone.

Alternatively, as reported previously,[1,2] we also confirm *in vitro* and *ex vivo* that Xe and He further reduce the catalytic and thrombolytic efficiency of rtPA, the only approved drug therapy of ischemic stroke to date, with Xe-50 > Xe-37.5 > He-37.5. Combining Xe and He at equimolar concentrations of 37.5% did reduce the catalytic and thrombolytic efficiency of rtPA to a similar extent than Xe-50. These results, taken together with the above-mentioned data on neuroprotection, clearly indicate that Xe-He-37.5 can be considered equivalent to Xe-50.

Taken together, from a clinical perspective, the results of the present study suggest: (1) Xe-He-37.5 could be an efficient alternative to Xe-50 with, advantageously, a lower molecular weight and higher thermal conductivity and specific heat than Xe alone; (2) Xe-He-37.5 should not be administered before or together with rtPA therapy due to the risk of inhibiting the beneficial thrombolytic effect of rtPA therapy, as reported previously for Xe and He.[1,2] Whether or not Xe-He-37.5 would inhibit, like Xe-50, the adverse proteolytic effects of rtPA if administered after reperfusion has occurred cannot be concluded from the existing *ex vivo* and *in vitro* studies, and remains to be demonstrated in future *in vivo* studies. However, if one considers, on one hand, the inhibitory action of Xe-He-37.5 on the catalytic efficiency of rtPA shown in the present study and, on the other hand, the previously demonstrated antiproteolytic action of Xe and He when given alone at efficient concentrations,[1,2] it could be hypothesized with reasonable doubt that postischemic Xe-He-37.5 would further exhibit antiproteolytic properties in addition of its neuroprotective action.

If such, it is likely that Xe-He-37.5 could be administered advantageously after rtPA-induced reperfusion has occurred to provide both neuroprotection and reduction of rtPA adverse side effects, mainly brain hemorrhages and disruption of the blood-brain barrier. Therefore, we believed that future studies should investigate the organ protective properties of equimolar concentrations of Xe-He-37.5 (shown to offer similar neuroprotection as Xe-50 with, advantageously, lower molecular weight and higher thermal conductivity and specific heat) in clinically relevant models of thromboembolic stroke, traumatic brain injuries, and renal and cardiac ischemia.

Author contributions

HND and BH performed the experiments, HND and JHA analyzed data, NV, JEB, JJR and JHA wrote the manuscript.

Conflicts of interest

All authors declare no competing interest.

Research ethics

The study protocol was approved by the local ethic committee at Toulon, France.

Data sharing statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check

Checked twice by iThenticate.

Peer review

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