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Neuroprotection (and lack of neuroprotection) afforded by a series of noble gases in an *in vitro* model of neuronal injury

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ABSTRACT

Xenon-induced neuroprotection has been well studied both *in vivo* and *in vitro*. In this study, the neuroprotective properties of the other noble gases, namely, krypton, argon, neon and helium, were explored in an *in vitro* model of neuronal injury. Pure neuronal cultures, derived from foetal BALB/c mice cortices, were provoked into injury by oxygen and glucose deprivation (OGD). Cultures were exposed to either nitrogen hypoxia or noble gas hypoxia in balanced salt solution devoid of glucose for 90 min. The cultures were allowed to recover in normal culture medium for a further 24 h in nitrogen or noble gas. The effect of noble gases on cell reducing ability in the absence of OGD was also investigated. Cell reducing ability was quantified via an MTT assay and expressed as a ratio of the control. The OGD caused a reduction in cell reducing ability to 0.56 ± 0.04 of the control in the absence of noble gas ($p < 0.001$). Like xenon (0.92 ± 0.10; $p < 0.001$), neuroprotection was afforded by argon (0.71 ± 0.05; $p < 0.01$). Neon and krypton did not have a protective effect under our experimental conditions. Helium had a detrimental effect on the cells. In the absence of OGD, krypton reduced the reducing ability of uninjured cells to 0.84 ± 0.09 ($p < 0.01$), but argon showed an improvement in reducing ability to 1.15 ± 0.11 ($p < 0.05$). Our data suggest that the cheap and widely available noble gas argon may have potential as a neuroprotectant for the future.

The term 'noble gas' is a literal translation from the German 'edelgas', meaning 'exclusive gas'. This exclusivity has come about due to their constantly full outer electron shell, making them monatomic gases. A summary of the main properties of the noble gases is shown in Table 1 [16,27]. Noble gases are known as 'inert' gases, but this is far from the common belief that they are unreactive. The term inert refers to their inability to form covalent bonds with other molecules; however, they are capable of interacting with amino acids in the active sites of enzymes or receptors, stabilizing them in an active or inactive form to produce a biological effect [24]. The most well-known effects are the change of voice pitch by inhaling helium, and the general anaesthetic effect of xenon.

Xenon provides fast, smooth induction and rapid emergence, and is therefore an ideal agent to be used in theatre although its relative rarity has limited its routine use in hospitals [12,13].

In 1998 the noble gas xenon was found to antagonise the NMDA receptor [11]. Xenon was much safer than other pharmacological neuroprotectants, with very few side-effects. A number of experiments have been conducted both *in vitro* and *in vivo* to look at neuroprotection by xenon in difference settings. In perinatal hypoxia-ischaemia, *in vitro* experiments have found that exposure of neurons and glia to xenon either before or during oxygen and glucose deprivation dose-dependently reduced the damage caused to the cells [2,17,19,28], and *in vivo* studies have also shown that xenon provides short-term neuroprotection in neonatal rats [10]. Xenon has also been shown to be neuroprotective following cardiac bypass-induced neurocognitive dysfunction [20], and in acute brain injury [1,8,15]. Other pharmacological avenues for neuroprotection have included the use of erythropoietin. Spandou et al. [26] found it to be organoprotective, and xenon has already been found to upregulate erythropoietin expression as well as hypoxia-inducible factor-1α, a gene that promotes cell survival [18].

The aim of the current study was to investigate our hypothesis that like xenon, other noble gases krypton, argon, neon and helium also attenuate neuronal injury provoked by oxygen and glucose deprivation *in vitro*.

This study was approved by the Home Office (UK) and conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986. All efforts were made to minimise animal suffering and the number of animals used.

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Table 1
Summary of the key physical properties of the noble gases under investigation.

	Helium	Neon	Argon	Krypton	Xenon
Electron configuration	$1s^2$	$[He]2s^22p^6$	$[Ne]3s^23p^6$	$[Ar]3d^{10}4s^24p^6$	$[Kr]4d^{10}5s^25p^6$
Melting point (°C)	−272.0	−248.5	−189.6	−157.0	−111.5
Boiling point (°C)	−268.8	−245.9	−185.8	−151.7	−106.6
Abundance (ppm)	5.24	18.18	9340	1.14	0.09
Atomic radius (nm)	0.05	0.07	0.09	0.11	0.13
MAC in rats	–	–	27	7.3	1.6
Enthalpy of binding (kcal/mol)	3.83	3.84	2.87	2.10	1.01

MAC = minimum alveolar concentration of anaesthetic agent at one atmosphere pressure producing immobility in 50% of subjects exposed to a standard painful stimulus.

Obtaining pure neuronal cultures was based on previously described methods [3,4] with some minor modifications. Cerebral cortices (devoid of basal ganglia or hippocampi) were removed from foetal BALB/c mice at 18 days gestation. After trypsination and resuspension, the cells were plated at a density of 2.5×10^5 cells/cm^2 on 24-multiwell plates coated with poly-D-lysine (Becton Dickinson Labware, MA) and cultured in Neurobasal medium (Gibco, Paisley, UK) supplemented with glutamine, B27 (Gibco, Paisley, UK) and antibiotic antimycotic solution (final concentrations when added to the cultures: penicillin 100 U/ml; streptomycin sulphate 100 μg/ml; Amphotericin B 0.25 μg/ml). The media was refreshed every 24–48 h. The cells reached confluence after 7 days. Forty-eight hours before *in vitro* experimentation, cytosine arabinoside hydrochloride (Sigma, UK) was added to the cells to halt glial proliferation and produce a pure neuronal cell culture. Only neuronal cultures at day 7 were used for experimentation.

The process of OGD was based on previously described methods with some slight alterations [28]. The OGD solution is a deoxygenated balanced salt solution devoid of glucose (ionic composition: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, and 26 mM $NaHCO_3$; pH 7.4) which was prepared by bubbling pure gas (N_2 or noble gas) through it for 15 min using sterile Drechsel bottles (Pegasus, Guelph, Canada) to deoxygenate. The culture medium was replaced with the deoxygenated OGD solution and incubated for 90 min in an airtight anoxic chamber, exposed to either 95% N_2 and 5% CO_2 (positive control group) or 75% noble gas, 20% N_2 and 5% CO_2 (noble gas group with OGD). The gas mixture inside the chamber was altered to create an anaerobic environment, and the chamber was placed in an incubator (Air Jacketed, DH auto flow, Automatic CO_2 incubator, NUAIRE) at 37 °C. After 90 min, the media was replaced with supplemented Neurobasal Medium (as described above), and incubated in 75% noble gas or N_2, 20% O_2 and 5% CO_2 for a further 24 h. In the naïve control group, the media was changed to fresh prewarmed supplemented neurobasal media, and left for 24 h in a humidified, 5% CO_2 incubator at 37 °C. In the noble gas group without OGD, the media was replaced with fresh Neurobasal medium and then left for 24 h in 75% noble gas, 20% O_2 and 5% CO_2.

Hank's balanced salt solution, foetal bovine serum, horse serum, murine epidermal growth factor, glutamine, B27, AAS, neurobasal media, and minimum essential media were all purchased from Invitrogen Life Technologies Ltd. (Paisley, UK). DNase and cytosine arabinoside hydrochloride were purchased from Sigma–Aldrich (Poole, UK). Apart from xenon, which was supplied by Air Products Plc (Cheshire, UK), all other gases were obtained from Boc Gases (Surrey, UK).

A purpose built airtight cell culture chamber equipped with inlet and outlet valves and an electric fan was used to produce effective, continuous and homogenous delivery of gases. When connected to the calibrated flow meters for the noble gases, nitrogen, oxygen and carbon dioxide, the desired gas composition was obtained. The chamber was flushed for 75 min before establishing a closed system.

Cell reducing ability was quantified with an MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Merck KGaA, Germany). Cells were incubated with MTT solution for 2 h and then dimethylsulphoxide (DMSO, Fisher Scientific UK Limited, Leicestershire, UK) was added. The resulting purple solution was taken for colorimetric analysis with duplicates. Less injured cells would have a greater ability to reduce the MTT compound from a yellow to a deep purple colour. The deeper colour corresponds to a higher colorimetric value, giving a quantitative value of cell reducing ability.

Data were expressed as a ratio of the naïve control, and analysed by one-way ANOVA followed by Student–Newman–Keuls *post hoc* with Bonferroni correction. Statistical significance was taken to be at $p < 0.05$.

Neuronal cell bodies in cortical cell cultures were readily identifiable using microscope image capture after fixation with paraformaldehyde (2%) in phosphorate buffer (0.1 M) and staining with cresyl violet. The control cultures revealed the presence of a dense neuronal dendrite network and triangular cell bodies (Fig. 1A). After intervention with 90 min of OGD, the neurons lost the dendritic network and the cell bodies condensed (Fig. 1B).

In the naïve control experiment, whereby there was no OGD, the cell reducing ability was high as expected (1.0 ± 0.10). This ability was significantly reduced in the positive control group (nitrogen OGD) to 0.56 ± 0.04 ($p < 0.01$). As has been found in previous work, the damage was recovered when the cells were exposed to xenon 0.92 ± 0.10 ($p < 0.01$). Argon also had a neuroprotective effect on the cells, improving reducing ability to 0.71 ± 0.05 compared to the positive control ($p < 0.01$). Despite this recovery, cell reducing ability was lower than the naïve control ($p < 0.01$) (Fig. 2). The noble gases neon and krypton had no effect on cell reducing ability (0.51 ± 0.05; 0.45 ± 0.15 respectively). Exposing injured cells to helium worsened the injury compared to the positive control (0.42 ± 0.03; $p < 0.05$) (Fig. 2). Overall, neuroprotection was afforded by the noble gas xenon and to a lesser degree argon.

In the absence of OGD, no effect was observed with the noble gases neon and helium (0.98 ± 0.04; 0.91 ± 0.06 respectively). Neurons exposed to krypton showed a lower reducing ability (0.84 ± 0.09; $p < 0.01$) compared to the naïve control. However in the absence of OGD there was an improved cell reducing ability in the argon group (1.15 ± 0.11; $p < 0.05$) compared to the naïve control (Fig. 3).

This work has shown that noble gases are capable of producing a significant biological effect. Both xenon and argon have neuroprotective properties, although this was only partial by argon. Neon and krypton had no effect on neuronal injury in this model. Helium had a detrimental effect on the cells. Krypton is capable of injuring neuronal cells, whereas argon may suppress natural cell deterioration.