Moving to human trials for argon neuroprotection in neurological injury:
A narrative review

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Short running title: Argon neuroprotection in neurological injury
Summary

Despite the global burden of brain injury, neuroprotective agents remain elusive. There are no clinically effective therapies which reduce mortality or improve long-term cognitive outcome. Ventilation could be an easily modifiable variable in resuscitation; gases are relatively simple to administer. Xenon is the prototypic agent of a new generation of experimental treatments which show promise. However, use is hindered by its prohibitive cost and anaesthetic properties. Argon is an attractive option, being cheaper, easy to transport, non-sedating and mechanistically distinct from xenon. *In vitro and in vivo* models provide evidence of argon reducing brain injury, with improvements in neurocognitive, histological and biomarker metrics, as well as improved survival. Current data suggests the effect of argon is mediated via the toll-like receptors 2 & 4, the ERK 1/2 and PI-3K-AKT pathways. Ventilation with argon appears to be safe in pigs and preliminary human trials. Given recent evidence that arterial hyperoxia may be harmful, the supplementation of high-concentration argon may not necessitate changes to clinical practice. Given the logistic benefits, and the evidence for argon neuroprotection summarised in this manuscript, we believe that the time has come to consider developing Phase II clinical trials to assess its benefit in acute neurological injury.
Keywords:
Acute brain injuries
Argon
Neuroprotection
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CA</td>
<td>Cardiac Arrest</td>
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<td>HIE</td>
<td>Hypoxic-ischemic encephalopathy</td>
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<td>TBI</td>
<td>Traumatic brain injury</td>
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<td>CPR</td>
<td>Cardiopulmonary resuscitation</td>
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<td>CT</td>
<td>Computer tomography</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<tr>
<td>OOHCA</td>
<td>Out-of-hospital cardiac arrest</td>
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<td>HO-1</td>
<td>Haem-oxygenase 1</td>
</tr>
<tr>
<td>NQO-1</td>
<td>NADPH dehydrogenase (quinone 1)</td>
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<td>GST</td>
<td>Glutathione S-transferases</td>
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<td>SOD-1</td>
<td>Superoxide dismutase 1</td>
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<td>OGD</td>
<td>Oxygen glucose deprivation</td>
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<tr>
<td>tMCAO</td>
<td>Transient middle cerebral artery occlusion</td>
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<td>ARE</td>
<td>Anti-oxidant response element</td>
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<tr>
<td>FIO2</td>
<td>Fraction of inspired oxygen</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>tMCAO</td>
<td>Temporary middle cerebral artery occlusion</td>
</tr>
<tr>
<td>OGD</td>
<td>Oxygen-glucose deprivation</td>
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<td>ICU</td>
<td>Intensive care unit</td>
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<td>VF</td>
<td>Ventricular fibrillation</td>
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Introduction: Neuroprotection with conventional pharmacological agents has failed.

Neuroprotection for brain injury, though conceptually attractive and experimentally effective, has been a clinical failure. Neurological injury is the mode of death after two-thirds of out-of-hospital cardiac arrests (CA) and one-third in hospital. Most die during their initial hospital admission. Furthermore, two-thirds of patients who survive have moderate to severe cognitive deficits three months after their CA. Neurological injuries such as hypoxic brain injury following CA, stroke, ischaemic stroke, neonatal hypoxic-ischemic encephalopathy (HIE) and traumatic brain injury (TBI) are among the leading causes of global mortality and morbidity. Poor functional outcomes from such injuries are commonplace, meaning around half never return to work. These statistics highlight the importance of developing therapeutic strategies which are aimed at providing ‘neuroprotection’.

In any acute neurological insult, we can attempt to reverse the primary process which leads to brain injury (e.g. thrombolysis for ischaemic stroke, or surgical or medical therapy of intracranial hypertension). Early thrombolysis in stroke can greatly improve functional outcomes, despite significant contra-indications and drawbacks including haemorrhagic transformation. Public education has significantly improved rates of early and appropriate bystander cardiopulmonary resuscitation (CPR). However, there are virtually no therapies which protect injured brain tissue (described generically as ‘neurons’) that have been starved of oxygen (hypoxia), and consequently reduce mortality or improve long-term cognitive outcome. While established as an effective treatment in neonatal hypoxic-ischemic encephalopathy (HIE), the benefits of therapeutic hypothermia in adults is still uncertain, particularly regarding precise temperature thresholds for benefit. Furthermore, despite cooling, half of neonates with HIE still die or suffer significant neuro-developmental disability. New strategies are needed. Altering how patients are ventilated could be an easily modifiable variable in resuscitation. More accurately targeting oxygen administration (preventing hyperoxia), along with additive gaseous agents, have been suggested.

Pathophysiological mechanisms of secondary neurological injury

The causes of brain injury are heterogeneous. Broadly, they include primary brain injury either caused by a lack of intra-cerebral vascular supply (i.e. stroke), trauma, and hypoxic ischaemic injury following cardiac arrest. While this review will mention specific causes of brain injury, each with varying mechanisms, substantial commonality exists in the pathophysiology that
drives secondary neural injury following each of these insults, suggesting a basis for common therapies aimed at these.

Secondary neuronal injury drives ongoing damage occurring after the initial insult has occurred. Proposed mechanisms include: reperfusion injury following a period of ischaemia, neuroinflammation within the salvageable penumbra, breakdown of the blood-brain barrier, disruption and remodelling of the cerebral microvasculature, excitotoxic injury and oxidative stress owed to excessive free-radical production.

The noble gases represent plausible neuroprotective candidates; xenon is the prototypic agent

The noble gases, including argon and xenon, have acquired significant interest as potential neuroprotective agents with promising in vitro and in vivo data. While typically thought of as ‘inert’ because of their filled valence shell, noble gases can create physiological effects by Van der Waals forces, providing enough energy to form a bond in a given binding site. Xenon, with the most outer electrons, has the greatest polarising effect. This has been correlated with its anaesthetic ability. Atomically heavier than argon, xenon has been used as an anaesthetic, as a contrast agent in both computer tomography (CT) and magnetic resonance imaging (MRI), as well as a candidate neuroprotectant (especially, in neonates). Like all gases, it can quickly cross the blood-brain barrier, increasing its clinical potential.

Xenon is thought to function in neuroprotection by antagonism of the NMDA receptor, thereby preventing glutamate mediated excitotoxic stress. Most current evidence suggests xenon acts by the competitive inhibition of glycine, a co-agonist of the NMDA receptor; although some propose involvement of the adenosine triphosphate dependent potassium channels. The addition of exogenous glycine tends to abolished neuroprotection by xenon in vitro. However, at least two mutations in the NMDA receptor eliminate xenon binding without affecting the affinity of glycine. Another study demonstrated inhibition of NMDA currents irrespective of glycine concentration. These data suggest a complex and yet incompletely elucidated mechanism of xenon.

Irrespective of this, good neuroprotective data have also been demonstrated with Xenon, to the point of phase II human clinical trials. Laitio et al. demonstrated encouraging radiological evidence of benefit with xenon in humans after out-of-hospital cardiac arrest (OOHCA),
combined with therapeutic hypothermia. In this study, the severity of white matter injury on diffusion tensor MRI was significantly reduced at 16 hours after rewarming. Global fractional anisotropy (a marker of white matter integrity), the primary outcome measure, was 3.8% higher in the xenon group compared to controls. Anisotropy has previously been shown to be one of the best independent predictors of mortality after CA. While radiological evidence alone is not sufficient to draw significant conclusions about human efficacy, Phase II studies are being planned, and these data provide conceptual evidence for neuroprotection from noble gases.

However, clinical use of xenon is limited by its expense and that it is an anaesthetic at sea level. There have been several human studies into surgical xenon use, including cost-analyses. There is significant regional variation in the price of xenon from $60/L in US to $30/L in France, far higher than the often miss-quoted $10/L, which is now historic. The price of xenon is inelastic: increases in consumption beyond its fixed capacity (or supply ceiling) will increase its price. Further increases in production will be expensive. Closed-circuit recirculation and recycling ventilation systems are therefore required for cost efficiency (Table 1). Consequently, xenon is unlikely to be feasible in emergency care settings where ease of use is a priority. This is especially true in a pre-hospital setting where limited equipment is available. Irrespective of the context, a xenon based ventilator system is currently not commercially available. Additionally, the sedative effects of xenon could confound assessment of conscious level and neurological state.
<table>
<thead>
<tr>
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<th>Cost and utilisation</th>
<th>Approximate cost pre-hospital</th>
<th>Approximate cost in hospital</th>
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<tbody>
<tr>
<td><strong>Argon</strong></td>
<td><strong>Open flow rate:</strong> Steady state use: 10L/min or 6,000L/hr⁶ = 27.1¢/min (at 2.71¢/L)</td>
<td><strong>Open flow rate</strong> for ~2-4 hours ⁷: 24,000L over 4 hours ³ Cost: $65</td>
<td><strong>Open flow (24h):</strong> 20,880L Cost: $390</td>
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<tr>
<td></td>
<td><strong>Closed circuit flow rate:</strong> no data</td>
<td></td>
<td><strong>Closed circuit (24h): ~211L³</strong> Cost: $5.72</td>
</tr>
<tr>
<td><strong>Xenon</strong></td>
<td><strong>Open flow rate:</strong> not feasible</td>
<td>Use not feasible</td>
<td><strong>Open flow (24h):</strong> not feasible</td>
</tr>
<tr>
<td></td>
<td><strong>Closed circuit flow rate</strong> ⁴: Steady state use: 5.0-12.5L/h</td>
<td></td>
<td><strong>Closed circuit (24h): ~211L⁵</strong> Cost: $6,330 (at $30/L)</td>
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<td>Averaged use of 8.8L/h, at $30/L = $264/h</td>
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**Table 1** - A cost-comparison of argon and xenon using the current data available ⁴² ⁴³ ⁴⁸ ⁵⁰ ⁵¹ ⁵²

⁴² Roughly equivalent to xenon: Broad et al. (2016) describe use of 4–5L/minute to ventilate piglets; adult humans would require more. Alderliesten et al. (2014) report a similar rate in personal communication. ⁴³ Ventilation flow rate inconsistently reported (variables include the ventilation equipment and its scavenging efficiency, the gas-mix required, and the use of other anaesthetic agents); data primarily from elective adult surgery using Xenon as an anaesthetic agent. ⁴⁸ ⁵⁰ One additional publication reports flow rates of as low as 0.52 L/h using a custom-made ventilation device, but this was in the context of neonates with neonatal encephalopathy and has not been integrated into this graphic. ⁵¹ Based 24 hours of steady state flow at 8.8L/h, not accounting for higher rates possibly required in the induction phase.
Argon is an attractive alternative and is likely to be mechanistically distinct from xenon

Argon is a potential alternative to xenon. A comparison of the neuroprotective properties of the two agents in past publication suggested the superiority of xenon, but only included a limited number of argon studies.\textsuperscript{57} This was in contradistinction to a review in 2014,\textsuperscript{58} which more fully summarised the data on argon neuroprotection available up to 2013, and suggested that argon showed promise as a clinical neuroprotectant. We build on these two reviews by providing a more up to date survey of the literature (our search strategy is included as Supplementary data) and suggest the next steps for translational neuroprotection studies in this manuscript.

Nearly two decades have passed since Soldatov first demonstrated the protective effects of argon in hypoxia.\textsuperscript{59} Since then, growing evidence has suggested argon could have a role in human neuroprotection. Argon fulfils the criteria of being easy to administer and transport, cheap (Table 1) and with few proven side-effects.\textsuperscript{55} The cost of argon has been cited as approximately 9¢/L in previous literature (e.g. Loetscher \textit{et al.} 2009). However, as of 3.1.17 The BOC Group plc (U.K.) cost for medical grade argon (suitable for patient consumption; 99.99% purity\textsuperscript{1}) was £233.25 for a 50L cylinder containing 10,570L gas. This equates to 2.21p/L (GBP). Equivalent, on the same date, to 2.71¢/L (USD). Argon is far more abundant, a fact that explains low costs and, consequently, negates the requirement for expensive and cumbersome rebreathing circuits. In addition, it has no hypnotic effects at sea-level is also only an anaesthetic at hyperbaric pressures.\textsuperscript{56,60}

Argon appears to have a spectrum of anti-apoptotic qualities, which are distinct from the modulation of neurotransmitters (NMDA) and prevention of excitotoxicity attributed to xenon.\textsuperscript{40} More specifically, argon does not appear to mediate neuroprotection through effects at GABA\textsuperscript{61} or NMDA\textsuperscript{36} receptors, or TREK1\textsuperscript{36} and KATP\textsuperscript{62} channels.

Instead, argon appears to prevent cell death via upregulation of both the ERK 1/2 and PI-3K-AKT pathways (Figure 1).\textsuperscript{6,63-66} From these two pathways, a common series of mediators stimulate a set of downstream effectors including anti-oxidant response elements (AREs). Inhibition at the point of ERK 1/2, PI-3K-AKT, the mediator molecules mTOR and nuclear factor (erythroid-derived 2) factor 2 (Nrf-2), or its AREs, using small molecules or SiRNA, all appear to block the neuroprotective effects of argon \textit{in vivo}.\textsuperscript{64-68} Cytotoxic damage itself also

\textsuperscript{1} BOC Product code: 290469-L-PC.
stimulates the ERK 1/2 pathway, in the context of *in vivo* models of hypoxic brain injury. Small molecule inhibition of the pathways increased the quantity of cell death and the expression of pro-apoptotic mediators. The PI-3k-AKT pathway induces a cellular survival signal in opposition to apoptosis. Therefore, it seems likely that argon is stimulating endogenous cellular protection pathways with the result of preventing cellular stress and eventual apoptosis.

Argon has been shown to work synergistically with hypothermia through common pathways, including PI-3K-AKT, in models of neonatal hypoxic brain injury. In contrast, pro-inflammatory pro-apoptotic signalling molecules including GSK3b, cytochrome c and caspase 3 are all inhibited by argon and hypothermia.
Figure 1 - A cartoon to illustrate the hypothesised molecular mechanisms of argon, synthesising the current available literature.5, 59, 60-76
Nrf2, a basic leucine zipper transcription factor, appears to mediate this pathway.\textsuperscript{73,74} Normally Nrf2 is bound to Keap-1 (Kelch-like ECH-associated protein 1), and this leads to Nrf2 ubiquitination and proteasome degradation. However, oxidative stress inhibits Keap-1 repression of Nrf2, and so leaves it free to translocate to the nucleus where it can protect against oxidative stress by upregulating the expression of ARE’s.\textsuperscript{67}

Downstream effector molecules of this common anti-apoptotic pathway include NADPH dehydrogenase (quinone 1), glutathione S-transferases, superoxide dismutase 1 and Haem-oxygenase 1 (HO-1).\textsuperscript{6,73} The last of these, Haem-oxygenase 1, is usually induced by oxidative stress, and catabolises free heme (an oxidant) into labile iron (Fe\textsuperscript{2+}), carbon monoxide and biliverdin.\textsuperscript{6,75,76} Each of these breakdown products elicits individual anti-apoptotic effects. For example, carbon monoxide interacts with the p38 MAPK pathway, leading to the degradation of the pro-apoptotic molecule, p38a; and production of the protective molecule, p38b, which induces Bcl-2 (an anti-apoptotic molecule).\textsuperscript{75} Despite evidence for an important role of HO-1 in argon-mediate neuroprotection,\textsuperscript{6} others have shown an initial reduction in HO-1 protein concentration in retinal ganglion cells after argon ventilation.\textsuperscript{65} Significant work remains to elucidate the downstream effectors of argon. Irrespective, the upregulation of a set of superoxide scavenger molecules explains argons association with reduced signs of oxidative stress.

Other studies show involvement of toll-like receptors (TLR) 2 and 4 upstream of the ERK 1/2 signalling pathway.\textsuperscript{77} Typically characterised as signalling receptors participating in the innate immune system, TLRs are thought to have an important, but unclear, role in neurons. TLR 4 has previously been demonstrated \textit{in vitro} to mediate apoptosis; inhibition of it appears to protect against apoptosis.\textsuperscript{78,79} Similarly, Ulbrich \textit{et al.} used rotenone to induce oxidative damage, which induced upregulation of the TLR 2 and 4 receptors. \textit{In vitro} retinal ganglion culture, and \textit{in vivo} after retinal ischemic reperfusion injury, argon ventilation helped avert oxidative damage. At 75\% argon concentration, there was a marked upregulation of the ERK 1/2 pathway and a decrease in NK-kB expression.\textsuperscript{65,77,80} Blocking of the TLR 2 and 4 signalling pathway by the small molecule OxPAPC prevented argon-mediated cytoprotection and ERK 1/2 expression.\textsuperscript{77,80} It is not clear if argon is acting on the TLRs, potentially preventing their role in apoptosis, or affecting a parallel pathway. Certainly, argon has been shown to downregulate a variety of pro-apoptotic proteins, such as caspase 3.\textsuperscript{80,81}
Efficacy of argon in *in vitro* models of neurological injury

Substantial *in vitro* data support the neuroprotective effectiveness of argon (Table 2), primarily using the oxygen and glucose deprivation (OGD) model of hypoxic-ischemic injury in neuronal culture.55

After OGD, a high-concentration argon-containing media confers a survival benefit to both dissociated cultures of murine cortical neurons82 and *ex vivo* organotypic hippocampal slice preparations,55 with better preservation of cellular and dendritic morphology.6 A dose-dependent improvement in survival has also been demonstrated.55 77 Even during unstressed conditions, cultured neurons survive better in an argon atmosphere compared to nitrogen.82 Intriguingly, neon, krypton and helium either had no benefit or potentiated injury, in model systems.82

Argon shows neuroprotection in related settings. It reduced damage by up to 25% in murine cochlear hair cells subjected to hypoxia, cisplatin or gentamycin toxicity,83 and reduced injury by up to 80% in a stylus drop model of TBI in organotypic hippocampal slices, with protection maintained following a two-hour delay to administration, and some residual protection following a three hour delay.55

Molecular analysis in these *in vitro* models show that argon modulates proposed target pathways, with broad upregulation of anti-apoptotic mediators during and after OGD. This includes the upstream pathways PI-3K-AKT and ERK 1/2, mediators such as mTOR and Nrf2, and downstream effectors like HO-1.6 64-66 77 It appears that inhibition at any stage along this cascade reverses the benefits that argon appears to provide.6 66 Conversely, argon downregulates pro-apoptotic and pro-inflammatory molecules, including cytochrome c, caspase 3, NFkB and GSK3b. Argon treatment appears to reduce the concentration of reactive oxygen species (ROS).66

These *in vitro* data support a role for argon neuroprotection, which is dose related and persists in large part despite delays to intervention, and provide a useful basis for translating argon neuroprotection to *in vivo* models.
<table>
<thead>
<tr>
<th>First author</th>
<th>Disease Model</th>
<th>Animal</th>
<th>Treatment protocol</th>
<th>Treatment group gas mix</th>
<th>Control group gas mix</th>
<th>Cell survival data</th>
<th>Histopathological data</th>
<th>Biomarker analysis</th>
</tr>
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<tbody>
<tr>
<td>Zhao 2016</td>
<td>OGD</td>
<td>Cortical neuronal cultures (gestational day 16 rat foeti)</td>
<td>OGD for 90 min with treatment gas bubbled through media, then incubated with treatment for 2h; with (33°C) or without (37°C) hypothermia; incubated in normal media for further 24h.</td>
<td>75% Ar, 20% O₂, 5% CO₂</td>
<td>75% N₂, 20% O₂, 5% CO₂</td>
<td>Increased percentage of live cells by around 50%</td>
<td>Improved cellular and dendritic cell morphology. Reduced caspase 3 positive areas.</td>
<td>Significant upregulation of HO-1 and p-AKT by Ar, maximally at 4h but significantly sustained for 24h. Hypothermia also upregulated HO-1 and p-AKT, which was further enhanced by Ar. Suppression of p-GASK-3β and mitochondrial cytochrome c release was also significantly increased. Inhibition of either HO-1 or p-AKT prevented argon mediated protection.</td>
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<tr>
<td>Zhao 2016</td>
<td>OGD</td>
<td>Cortical neuronal cell culture (gestational day 18 rat foeti)</td>
<td>OGD for 90 min with treatment gas bubbled through media, then incubated with treatment for a further 24h</td>
<td>70% Ar, 25% O₂, 5% CO₂</td>
<td>70% N₂, 25% O₂, 5% CO₂</td>
<td></td>
<td>Preserved neuronal morphology and cell viability using Ar, assessed via MTT assay.</td>
<td>Ar induced upregulation of Erk 1/2, PI-3K and p-mTOR after OGD challenge. Increased expression of Nrf2 and its translocation to nuclei; along with downstream antioxidant response elements (e.g. SOD-1, NQO1). Experimental inhibition of m-TOR and Nrf2 abolished the effect of Ar. Reduced caspase-3 cleavage after OGD with Ar (suggesting increased cell-survival).</td>
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<tr>
<td>Ulbrich 2015</td>
<td>Oxidative stress</td>
<td>Human neuroblastoma cell line (SH-SY5Y)</td>
<td>Rotenone treatment for 4h, then exposure to Ar for 2 or 4h</td>
<td>75%, 50% or 25% Ar, with 21% O₂, made up with N₂</td>
<td>Room air</td>
<td>Maximal benefit observed using 75% Ar, reducing apoptosis to baseline. Benefit of Ar time and dose dependent.</td>
<td>Significant reduction in TLR-2 &amp; TLR-4 receptor density and protein expression with Ar treatment.</td>
<td>Induction of ERK 1/2 pathway by Ar, and caspase-3 activity decreased. Inhibition of TLR signalling prevented this. Small molecule inhibition of ERK 1/2 and TLR receptor signalling prevented the benefits of Ar treatment.</td>
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<tr>
<td>Author</td>
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<td>Condition</td>
<td>Treatment Description</td>
<td>Control Conditions</td>
<td>Results</td>
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<td>David</td>
<td>2012</td>
<td>OGD Ex vivo</td>
<td>Rat striatal brain slices after glucose free solution saturated with 100% nitrogen</td>
<td>Medical air (75% N&lt;sub&gt;2&lt;/sub&gt;, 25% O&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>Significant reduction in LDH release as a marker of cell injury, using 37.5-75% Ar (maximal at 50%, after 3h).</td>
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<tr>
<td>Fahlenkamp</td>
<td>2012</td>
<td>N/A Mouse</td>
<td>Primary cortical neurons, microglia and astrocytes exposed to humified gas mixture</td>
<td>Humified room air</td>
<td>Significant induction of ERK 1/2 in microglia, maximal at 30 min. A similar induction in neurons and astrocytes did not reach statistical significance.</td>
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<tr>
<td>Jawad</td>
<td>2009</td>
<td>OGD Mouse</td>
<td>Cerebral cortices, dissociated neurons exposed to OGD for 90 min with nitrogen (95%, with 5% CO&lt;sub&gt;2&lt;/sub&gt;) or noble gas, then treatment for further 24h.</td>
<td>75% noble gas (e.g. Ar), 20% O&lt;sub&gt;2&lt;/sub&gt;, 5% CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Cell viability (judged by reducing ability via MTT assay) preserved after OGD using Ar &amp; Xe, but not neon, krypton or helium. In the absence of OGD, Ar even improved cell viability.</td>
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<tr>
<td>Loetscher</td>
<td>2009</td>
<td>OGD and TBI Ex vivo mouse hippocampal tissue</td>
<td>OGD for 30 min or subjected to stylus dropped with identical force, then experimental media for 72h, added either immediately or with 2h or 3h delay.</td>
<td>Humified room air</td>
<td>Ar decreased tissue injury significantly after OGD and TBI; maximum benefit observed using 74% &amp; 50%, respectively. Reduced benefit after a delay in treatment, although still significantly reduced injury.</td>
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<td>Yarin</td>
<td>2005</td>
<td>Hypoxia</td>
<td>Organ of Corti cells from rats exposed to Nitrogen or Ar hypoxia; Toxic drug exposure</td>
<td>Hypoxia: 95% Ar, 5% CO&lt;sub&gt;2&lt;/sub&gt;, Drug exposure: 74% Ar, 21% O&lt;sub&gt;2&lt;/sub&gt;, 5% CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Approximate 20-30% reduction in cell death with Ar treatment during hypoxia, cisplatin and gentamycin exposure.</td>
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Table 2 - A comprehensive summary of the literature with the results of in vitro data pertaining to the use for argon ventilation for neuroprotection

Efficacy of argon in whole animal models of HIE, stroke and CA

Several studies, in multiple species and a range of experimental models, provide evidence of argon neuroprotection, using varying concentrations and duration of argon exposure and delays to initiation of treatment (Table 3).

Cardiac arrest models *in vivo*

Post-resuscitation ventilation for 1 hour with a 70% argon + 30% oxygen mixture significantly improved functional and histological outcomes in a ventricular fibrillation model of cardiac arrest in rats, with improved cognitive function during seven days of follow up and less neuronal damage in the neocortex and hippocampus. This benefit was additive to that derived from post-arrest ventilation using controlled oxygen levels (using a 70% nitrogen & 30% oxygen mixture in the comparator group). These data, and a dose dependency of argon neuroprotection in this model, were replicated in a larger sample size by the same group. Furthermore, they have demonstrated that despite a delay in treatment by 3 hours, argon still confers significant neuroprotection, in terms of reduced histopathological damage and improved functional recovery. If observed in humans, this benefit will have significant clinical advantages.

The efficacy of argon has also been replicated pigs using balloon occlusion of the left anterior descending coronary artery to induce ventricular fibrillation. Following an eight-minute period of circulatory standstill, the animals were defibrillated and mechanically ventilated for 4 hours, with either argon/oxygen or argon/nitrogen mixtures. The argon group had significantly better cognitive scores, displaying little cognitive impairment at 24 hours after arrest. After 72 hours, pigs treated with argon had returned to pre-test cognitive levels, whereas control pigs had only recovered 75% of baseline function. Elevation in neuron specific enolase (a neuronal injury marker) and neuropathological evidence of neuronal injury were both attenuated in the argon group. Argon does not appear to have significant independent effects on systemic haemodynamics and ventilation or cerebral oxygen saturation and brain electrical activity.

A more recent study from Zuercher et al (2016) failed to replicate the neuroprotective benefits of argon. Compared to Brucken and Ristagno et al., however, ROSC was achieved in the Zuercher study 20% and 35% quicker, respectively, which was correlated with less severe
impairment in neuropsychological testing. In addition, both Brucken and Ristagno use 70% argon and 30% oxygen and a different method of inducing cardiac arrest (induction of VF by electrical stimulation or balloon occlusion of the left anterior descending coronary artery vs. potassium and esmolol) and ROSC (defibrillation vs. manual CPR). Many of these differences were recognised by Zuercher et al. We speculate that the differences between studies in this context relates to a reduced benefit of argon (and perhaps any neuroprotectant) when the index insult results in relatively mild neuropsychological deficits and/or histological injury.

**Stroke models in vivo**

An ischaemia reperfusion model of retinal artery occlusion showed that argon significantly reduced the damage of retinal ganglion cells. Two studies using the transient middle cerebral artery occlusion (tMCAO) model of stroke in rats demonstrated smaller overall infarct volumes and better composite behaviour outcomes after 50% argon treatment initiated an hour after occlusion (to mimic a clinical timeline). However, there was no survival benefit seen, and cytokine analysis was not conclusive. The effects of argon in this setting seem complex. A further tMCAO study found that 50% argon reduced cortical infarct volume by 35%, but increased subcortical damage by 35%. However, these results are confounded using 100% O₂ as an anaesthetic carrier, which may have had independent deleterious consequences, given the association with hyperoxia worsening reperfusion injury.

Another study modelled subarachnoid haemorrhage using perforation of a cerebral artery, leading to a sharp rise in intracranial pressure and a drop in regional cerebral blood flow. Administration of 50% argon for an hour reduced the risk of premature death by 20% after 3 days. While neurocognitive assessments did not differ, rats in the treatment group appeared to have better general health and less neuronal damage (judged by mean body weight and NeuN positive nuclei, respectively).

**Neonatal HIE models in vivo**

Hypothermia has been successfully used to treat HIE but has limitations. Recent in vivo evidence suggests that argon may not only have a role in neuroprotection after hypoxic brain injury in neonatal mammals, but also enhances the benefit of hypothermia. Carotid artery ligation has been the method most commonly used to model this.
In two studies of neonatal HIE using carotid artery occlusion with hypoxia, Zhao et al. showed that neonatal rats treated with 2 hours of 70% argon had significantly reduced infarction volumes.\textsuperscript{6,66} When combined with hypothermia, the addition of argon therapy reduced the average infarction volume in a temperature dependent manner (by 48% at 37°C and 65% at 33°C), compared to nitrogen at normothermia.\textsuperscript{6} Argon also reduced weight loss and cerebral reactive gliosis.\textsuperscript{6,66} At a molecular level, argon neuroprotection was associated with upregulation of Nrf2, ARE downstream effector molecules (e.g. HO-1) and reduced evidence of oxidant injury (e.g. malondialdelyde) in penumbral tissue.\textsuperscript{6,66} Argon’s apparent benefit in reducing histopathological damage and inducing ‘anti-apoptotic’ expression were blocked by \textit{in vivo} inhibitors of either PI-3K-AKT (Wortmannin) or ERK 1/2 (U0126),\textsuperscript{66} suggesting a requirement for both pathways.

Argon neuroprotection in HIE was replicated by Broad et al. in a neonatal piglet model of HIE, where the addition of 45-50% argon, initiated 2 hours post-insult and administered for 24 hours, resulted in benefit that was additive to induced hypothermia (33.5°C).\textsuperscript{18} Cerebral biomarkers were suggestive of reduced brain injury, including reduced white matter lactate and increased whole brain adenosine triphosphate and phosphocreatine. The argon group also showed improved mean hourly electroencephalogram recordings, suggesting faster recovery of brain activity. Both findings have been associated with better neurodevelopmental outcomes.\textsuperscript{92-94} A survival benefit was not assessed, as piglets were euthanised at 48 hours.

Put together, these results show a translation of the positive benefits shown \textit{in vitro} to animal CA and stroke models. Arguably most importantly, cognitive testing showed clear beneficial differences in argon treatment groups. This is corroborated by histological and biomarker data. Limitations in the literature, such as small sample sizes and inconsistent experimental conditions, prevent more specific conclusions, for example, about the optimal argon-oxygen mix. Considering its potential, the safety of argon needs to be assessed before human trials can be commenced, and such evidence is now beginning to accumulate.
<table>
<thead>
<tr>
<th>First author</th>
<th>Disease Model</th>
<th>Animal</th>
<th>Treatment protocol</th>
<th>Treatment group gas mix</th>
<th>Control group gas mix</th>
<th>Mortality data</th>
<th>Neuro-cognitive assessment</th>
<th>Histopathological data</th>
<th>Biomarker analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad 2016</td>
<td>Stroke</td>
<td>Piglet</td>
<td>Bilateral carotid artery occlusion for 12.5 minutes. Initiation of ventilation from 2h till 26h. Simultaneously cooled to 33.5°C</td>
<td>50% Ar, 29% N₂, 21% O₂ (with variable oxygenation to maintain SaO₂ 8-13kpa.)</td>
<td>Not specified</td>
<td></td>
<td></td>
<td>Reduced TUNEL+ staining. However, only noted in certain brain areas, e.g. putamen not periventricular white matter.</td>
<td>White matter lactate significantly lower in hypothermia and Ar group. Increased whole brain ATP and phosphocreatine at 48 hours. Improved mean hourly EEG recordings.</td>
</tr>
<tr>
<td>Ulbrich 2015</td>
<td>Stroke (retinal ischaemia)</td>
<td>Rat</td>
<td>1h ischemia caused by increased ocular pressure. Then 1h of treatment, immediately or with a 1.5 or 3h delay.</td>
<td>75% Ar, with 21% O₂, 4% N₂</td>
<td>Room air</td>
<td></td>
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<td>Inhibition of ERK 1/2 attenuates Ar-mediated protection</td>
<td>Ar ventilation significantly increased ERK 1/2 expression and HSP 70. In contrast, decreased protein levels of HO-1 and HSP-90</td>
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<tr>
<td>Ulbrich 2014</td>
<td>Stroke</td>
<td>Rat</td>
<td>1h ischemia followed by 1h of treatment, immediately or without a 1.5 or 3-h delay.</td>
<td>75%, 50% or 25% Ar, with 21% O₂, made up with N₂</td>
<td>Room air</td>
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<td>Retinal ganglion cell loss was reduced by Ar in a dose and time dependent manner. Optimal at 75%, the benefits at this level were largely maintained with a 1.5h delay.</td>
<td>Ar inhibited NF-κB, Bax and Bcl-2 expression significantly Ischemic induced leucocytosis reduced significantly</td>
</tr>
<tr>
<td>Fahlenkamp 2014</td>
<td>Stroke</td>
<td>Rat</td>
<td>tMCAO for two hours, ventilation started 1h later for 1h</td>
<td>50% Ar 50% O₂</td>
<td>No survival benefit noted</td>
<td>Improved composite behaviour outcomes</td>
<td>Reduced infarct volumes</td>
<td>Mixed cytokine production on a spectrum of neuroprotection (TGF-β, NGF) and pro-inflammation (IL-1β, IL-6)</td>
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<tr>
<td>David 2012</td>
<td>Stroke</td>
<td>Rat</td>
<td>Stereotaxic striatal injection of NDMA Or 1h tMCAO, followed by 3h of treatment</td>
<td>15-75% Ar, 25% O₂, remaining percent N₂</td>
<td>Medical air (75% N₂, 25% O₂)</td>
<td>Minimal difference in behavioural outcomes between groups.</td>
<td>Reduced neuronal damage with all concentrations of Ar, maximally at 50% (NMDA condition). Ar reduced cortical brain damage (35%) but increased subcortical damage</td>
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<tr>
<td>Name</td>
<td>Type</td>
<td>Species</td>
<td>Procedure and Treatment Parameters</td>
<td>Outcome</td>
<td>Notes</td>
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<tr>
<td>Ryang 2011</td>
<td>Stroke</td>
<td>Rat</td>
<td>tMCAO for 2h, ventilation started 1h later for 1h</td>
<td>50% Ar 50% N₂ 50% O₂</td>
<td>No survival benefit within 24h</td>
<td>Improved composite behaviour outcomes</td>
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<td>Reduced infarct volume</td>
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<td>Zuercher 2016</td>
<td>Cardiac arrest</td>
<td>Rat</td>
<td>Cardiac arrest induced by potassium and esmolol. 8 minutes arrest, followed by manual chest compressions with intravenous adrenaline. Ventilated for 24h after ROSC</td>
<td>50% Ar, 50% O₂, or 50% He, 50% O₂</td>
<td>50% N₂, 30% O₂</td>
<td>No difference in NDS over 5 days</td>
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<td>Trend (non-significant) to reduced hippocampal neuronal damage in the noble gas treated groups.</td>
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<tr>
<td>Brucken 2015</td>
<td>Cardiac arrest</td>
<td>Rat</td>
<td>VF induced by transoesophageal electrical stimulation. Defibrillation after 7 minutes, ventilated for 1h either 1h or 3h after ROSC</td>
<td>70% Ar, 30% O₂</td>
<td>70% N₂, 30% O₂</td>
<td>Improved NDS at 7 days, in Ar treated group in both the 1 hour and 3-hour delay groups. Significantly more mobile episodes only in the delayed group.</td>
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<td>Significant reductions in neuronal damage index in hippocampal and neocortical samples. Rats in the 3h delay group also showed significant reductions in basal ganglia damage, compared to controls.</td>
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<tr>
<td>Ristagno 2014</td>
<td>Cardiac arrest</td>
<td>Pigs</td>
<td>Balloon Occlusion of LAD to induce VF. Defibrillation after 8 minutes of VF, ventilated for 4h</td>
<td>70% Ar, 30% O₂</td>
<td>70% N₂, 30% O₂</td>
<td>After 72h, pigs treated with Ar had returned to pre-test cognitive levels, whereas control pigs had only recovered 75% of baseline function</td>
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<td>Significantly reduced neuronal damage</td>
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<tr>
<td>Brucken 2014</td>
<td>Cardiac arrest</td>
<td>Rat</td>
<td>VF induced by transoesophageal electrical stimulation. Defibrillation after 7 minutes, ventilated for 1h after 1h</td>
<td>70% Ar with 30% O₂, or 40% Ar with 30% O₂</td>
<td>70% N₂, 30% O₂</td>
<td>Improved NDS at 7 days, with a dose dependent improvement (70% &gt; 40%). Significantly more</td>
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<td></td>
<td>Neocortex and hippocampal slices showed significantly less neuronal damage, irrespective Ar concentration</td>
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<tr>
<td>Brucken 2013</td>
<td>Cardiac arrest</td>
<td>Rats</td>
<td>VF induced by transoesophageal electrical stimulation. Defibrillation after 7 minutes, ventilated for 1h after 1h</td>
<td>70% Ar, 30% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>70% N&lt;sub&gt;2&lt;/sub&gt;, 30% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Improved NDS at 7 days. More mobile episodes in open field test, inconclusive improvement in water maze.</td>
<td>Neocortex and hippocampal slices showed significantly less neuronal damage.</td>
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<tr>
<td>Miscellaneous animal models</td>
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<td>Hollig 2016</td>
<td>Subarachnoid haemorrhage</td>
<td>Rat</td>
<td>Cerebral artery perforation. Treatment started 1h after induction, for 1h</td>
<td>50% Ar, 50% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>50% N&lt;sub&gt;2&lt;/sub&gt;, 50% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>20.6% reduction in ‘premature death’ at 72h</td>
<td>No difference</td>
<td>Mean body weight greater in Ar group. Significantly greater NeuN positive nuclei in some Ar brain slices (dendate gryus, specifically). Cerebral odema equivocal between groups.</td>
<td>HIFα1 increased at 24h ours with Ar, compared to controls (but not 6h or 72h)</td>
</tr>
<tr>
<td>Zhao 2016</td>
<td>Neonatal HIE</td>
<td>Rat (neonatal)</td>
<td>Carotid artery ligation. 1h after induction, 2h treatment, with (33°C or 35°C) or without (37°C) hypothermia</td>
<td>70% Ar, 30% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>70% N&lt;sub&gt;2&lt;/sub&gt;, 30% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>Reduced infarction volume by up to 65% when used synergistically with hypothermia (vs N&lt;sub&gt;2&lt;/sub&gt; and 37°C). Reduced glial activation (GFAP expression)</td>
<td>Ar significantly reduced caspase 3 expression beyond hypothermia alone. Ar induces p-AKT and HO-1 and inhibits NF-κB expression. Inhibition of HO-1 and p-AKT abolishes the benefit of Ar</td>
<td></td>
</tr>
<tr>
<td>Alderliesten 2014</td>
<td>Safety evaluation; hypoxic ventilation mixture administered</td>
<td>Pig</td>
<td>#1 Ventilation with increasing concentrations of Ar, 1h each (30%, 50%, 80%) #2 1h of hypoxia (FiO&lt;sub&gt;2&lt;/sub&gt; 0.08) followed by 3h of 50% Ar #3 As above, with hypothermia (34-35°C)</td>
<td>Immediate euthanasia for histology</td>
<td></td>
<td>No demonstrable increase neuronal injury in normoxia due to Ar</td>
<td>No significant change in heart rate, mean arterial pressure, regional cerebral saturation of oxygen or electroencephalography due to Ar ventilation</td>
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</tr>
<tr>
<td>Zhuang 2012</td>
<td>Neonatal HIE</td>
<td>Rat (neonatal)</td>
<td>Common carotid artery ligation followed by hypoxic</td>
<td>70% Ar, He or Xe, with 30% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>70% N&lt;sub&gt;2&lt;/sub&gt;, with 30% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>No significant difference in</td>
<td>Ar treatment restored the cell number in hippocampi to that of naïve rats; Xe Ar and Xe treated rats sustained their body weight despite carotid ligation.</td>
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</table>
atmosphere (8% O₂) for 90 or 120 minutes. Treatment initiated 2h after, for 1.5h.

neurological function testing.

and helium to a lesser benefit. Approximate 40% decrease in infarction size by Ar and Xe (no protection by helium).

However, helium treatment further reduced body weight. Bcl-2 expression significantly increased with Ar, and to a lesser degree helium and Xe.

<table>
<thead>
<tr>
<th>Table 3 - A comprehensive summary of the literature with the results of <em>in vivo</em> data pertaining to the use for argon ventilation for neuroprotection</th>
</tr>
</thead>
</table>
The safety of argon in larger animal models and humans

Xenon has been used safely in human anaesthesia,\textsuperscript{49, 51} radiology,\textsuperscript{95} neonatology (up-to 50% xenon concentration)\textsuperscript{52} and now in a phase II clinical trial.\textsuperscript{42} The human use of argon has been far less studied. In a major step forward, Alderliesten and colleagues demonstrated the haemodynamic safety of argon in a porcine model.\textsuperscript{56} Anaesthesia was achieved with a standard human protocol, with argon up to 80% concentration. Oxygen was used to maintain normoxia on arterial blood gas. This was largely kept at 21% O\textsubscript{2}, with occasional temporary increases required during mucus plugging, for example (Groenendaal F., Personal Communication, 2014). The rest of the gas mixture contained nitrogen. Argon did not cause significant changes in heart rate, mean arterial blood pressure, regional cerebral saturation of oxygen or electroencephalography.

Giving pause for caution, two historic studies using argon to achieve pneumoperitoneum in adult pigs demonstrated an increase in systemic vascular resistance.\textsuperscript{96, 97} A negative effect on cardiac output was demonstrated in one,\textsuperscript{96} but then not replicated.\textsuperscript{97} Neither showed any effect from argon on respiratory function, mean arterial pressure, nor acid base-balance. Given these date, further studies into the use of argon for ventilation should carefully monitor for signs of cardiovascular compromise.

Argon has been used before in humans, in the context of developing safe breathing mixtures. Concentrations of 79% were historically (1970’s) used to measure coronary blood flow and is a recognised diving mixture.\textsuperscript{56} In a more recent study, argon was tested to see if it altered the rate of oxygen consumption during physical exercise in a hypoxic environment (15% O\textsubscript{2}).\textsuperscript{98} Seven healthy males cycled whilst breathing mixtures containing either 0%, 30% or 85% argon. Investigators found an increased oxygen consumption with greater argon concentrations, compared to nitrogen-dominated mixtures. However, both at rest and during exercise, there were no differences in the peripheral capillary saturation of blood haemoglobin measured using pulse oximetry. Therefore, it was concluded that differences in pulmonary gas exchange and tissue oxygenation were not the cause of increased oxygen consumption. The authors suggested that this may represent an enhanced ability for humans to tolerate hypoxia while breathing argon-containing mixtures.

Another study evaluated the effect of argon on cognitive performance.\textsuperscript{99} Over six days, four volunteers first breathed ‘normoxic’ mixtures of oxygen, nitrogen, argon and carbon dioxide (14%, 33%, 54% and 0.24%, respectively,). After this, they spent three days in ‘hypoxic’
conditions (10% O₂). Unsurprisingly, breathing ‘normoxic’ gas improved cognitive task performance. Subjectively, study participants reported increased vigour and decreased fatigability in ‘normoxia’. In addition, ‘normoxia’ did not decrease the long-term retention of learnt skills.

These trials provide a basis for further confirmation of the safety of argon in humans. Argon will need to be supplemented only when appropriate oxygen requirements allow it to be used at concentrations that deliver neuroprotection.

Some caveats and confounds for human translation: the impact of hyperoxia

Many trials of argon have used concentrations more than 50%. In the past, this would have required a change in clinical practice as it was common to use high inspired oxygen fractions (FIO₂) as a part of resuscitation, often reaching 100% oxygen. However, more recent concerns regarding hyperoxic injury have led to reduced use of FIO₂ higher than that needed to deliver normal oxygenation.³⁰ ¹⁰⁰-¹⁰⁴ The routine supplementation of oxygen is no longer advised by national guidelines after acute stroke,¹⁰⁵ ¹⁰⁶ though larger trials are ongoing.¹⁰⁷ In myocardial infarction, the recent AVOID trail showed that unnecessary supplementary oxygen exacerbated myocardial injury.¹⁰⁸ ¹⁰⁹

In vivo data show hyperoxia after CA increased ROS production²⁵ and lipid oxidation,¹¹⁰ and exacerbation of neuronal death.²⁴ ¹¹¹ When appropriate, limiting FIO₂ to 50% after CA can reduce oxidative stress, and has shown improvements in myocardial function and survival.²⁹ A canine CA model showed significantly better neurological outcome and neuronal histology having targeted physiologically normal oxygen saturations.⁹⁰ ⁹¹ Consistent experimental evidence,¹¹⁰ ¹¹² ¹¹³ including a meta-analysis published in 2012,¹⁰³ conclude animal evidence clearly points to worse neurological outcomes after hyperoxia.

In humans, epidemiological studies amongst intensive care patients have shown arterial hyperoxia is independently associated with an increased in-hospital mortality after stroke,¹¹⁴ TBI,¹¹⁵ CA,¹⁰² and all-comers.¹¹⁶ This data, as well as two recent meta-analyses, provide evidence that arterial hyperoxia is associated with poor functional neurological outcomes, in a dose-dependent manner.¹⁰⁰ ¹⁰¹

Feasibility and safety studies have supported the use of conservative oxygen management in appropriate intensive care patients, associated with shortened lengths of hospital stay.¹¹⁶ ¹¹⁷
This evidence suggests argon can be used safely in high concentrations in acute neurological compromise, with appropriate titration of oxygen in dynamic gas mixtures. Trials into the neuroprotective benefit of argon must include standard-of-practice control groups, ensuring any benefit observed is not simply from a reduced rate of hyperoxia in the argon treated group. Notwithstanding this, a proportion of patients who have compromised gas exchange after neurological injury (e.g. due to pulmonary aspiration or heart failure) may require a high FiO2 to achieve arterial normoxia, and the use of argon for clinical neuroprotection may be inappropriate in these subjects.

**A route to translational neuroprotection with argon**

Both xenon and argon have shown promise as neuroprotective agents, with improvements in histological, biochemical and cognitive outcomes. Xenon has a well-documented track record in this regard, which has culminated in Phase II trials, and a planned Phase III trial. However, xenon is relatively scarce and expensive, which necessitates closed-circuit ventilation systems and limits its use in out-of-hospital settings, thus delaying the onset of neuroprotective benefit. In addition, the anaesthetic effects of xenon provide a benefit in perioperative settings and in contexts where sedation is desirable. However, these properties may hamper clinical neurological assessment. Perhaps more importantly, the fact that xenon may be a cerebral vasodilator limits its use in patients with (or at risk of) intracranial hypertension, thus excluding many patients with acute neurological injury (particularly TBI).

The literature reviewed in this manuscript suggests that argon has significant neuroprotective effects, which may be comparable to xenon. It does not have anaesthetic properties at the concentrations used (and hence, if used in the prehospital setting, would not confound neurological assessment at hospital arrival). Further, although data are limited, it has no known vasodilatory effects on the cerebral circulation (avoiding the risk of exacerbating intracranial hypertension). The key elements of translation are summarised in Figure 2. Perhaps most important of the drivers to attempt to develop argon as a neuroprotective agent is the fact that it is a more logistically tractable agent than xenon. Argon is cheap (3¢/L) and easy to transport, meaning open-flow ventilation would be economically acceptable.
The translational development of argon as a neuroprotective agent could be informed by additional experimental studies that addressed dose–response–relationships, identification of the most effective dose and duration of administration, most effective time-point of administration in relation to the insult. However, this route to clinical neuroprotective translation has not had any signal successes in the past. If argon is safe, an alternative translational philosophy suggests that, given the past failures in neuroprotective translation, there is a case for initiating early clinical studies alongside such preclinical refinement, since the maximum delivered concentration, time point for administration, and duration of administration in clinical studies are constrained by clinical and logistic realities.

A more relevant consideration, however, is to address the practicalities of effectively delivering the intervention. Minimal changes in equipment would be required, although a method of titrating the mixed gases (oxygen and argon) would be needed. In addition, there may be a need to recalibrate ICU ventilators to account for differences between argon and nitrogen, with respect to density, viscosity and thermal conductivity, as has been required with helium.
Robust evidence will be required that titrating oxygen supplementation to peripheral oxygen saturations is safe, as has been demonstrated with xenon.\textsuperscript{42} Direct measurement of argon in gas mixtures is possible,\textsuperscript{124} but not currently clinically routine, and dosing would need to rely on measurement of other gases in inspired gas mixtures. While direct measurement is not essential to proceed with clinical trials, this would be desirable. Safety testing will also need to ensure sufficient ventilation of ambulances (and other closed environments) exist to ensure first-responder paramedics are not at risk of hypoxia. Providing these caveats are addressed, the prehospital use of argon would be plausible.\textsuperscript{86}

Given these logistic benefits, and the evidence for argon neuroprotection summarised in this manuscript, we believe that the time has come to think about Phase II clinical trials to assess its benefit in acute neurological injury. Some initial safety data may be required before such studies can be fully planned and eventually implemented, particularly in terms of longer exposures in larger animals (which would be needed for regulatory approval). Similar human data from healthy volunteers is already available from a small cohort of volunteers (n=4), showing that exposure to 50\% argon is safe for up to six days;\textsuperscript{99} replication and extension of this study would be useful, along with the assessment of effects on cerebral blood flow (using, for example, transcranial Doppler ultrasound, or MRI with arterial spin labelling). Given the potential commercial returns from this initiative, it would not be unreasonable to ask the vendors of noble gases for human use to generate an investigational medicinal product dossier (IMPD) available for argon (which is a prerequisite for entering into clinical trials in humans). While further refinement will clearly be needed, Table 4 provides a starting point for design of Phase II studies in candidate patient groups.
<table>
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<th><strong>Patient group</strong></th>
<th>Primary neuroprotection in patients with cardiac arrest, stroke or traumatic brain injury. Peri-operative neuroprotection in patients undergoing cardiopulmonary bypass or neurosurgical intervention for aneurysmal subarachnoid haemorrhage.</th>
</tr>
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</table>
| **Interventions** | Initial target argon concentration 50% with nitrogen and oxygen  
- Ideally initiated in pre-hospital phase for primary neuroprotective trials, continued for 3-5 days  
- As part of anaesthetic regime for perioperative neuroprotection, and consider ongoing postop use for 3-5 days. |
| **Controls**     | Nitrogen used as a comparator gas. The FiO₂ of the control group needs to be the same as the treatment group. |
| **Outcomes**     | Circulating biomarkers (NSE, S100b; especially for perioperative neuroprotection – may be confounded by injury heterogeneity in primary neuroprotection studies), Neuroimaging (e.g. conventional and diffusion tensor MRI; especially with serial imaging to demonstrate change over period of neuroprotection)  
Clinical outcomes – not likely to be a primary end point in Phase II studies, but will need assessment, using validated instruments tailored to indication (e.g. Glasgow Outcome Score-extended for TBI; modified Rankin scale for stroke) |

**Table 4** - A P.I.C.O. model for a randomised control phase 2 trial into the effectiveness of argon in neuroprotection.

NSE: Neuron-Specific Enolase, MRI: Magnetic resonance imaging, TBI: Traumatic brain injury, FiO₂: Fraction of inspired oxygen
**Author Contributions**

Andrew Gardner (AJG) and David Menon (DKM) were involved in the design of the paper. AJG wrote the initial manuscript and created the figures. This was subsequently edited and revised by AJG and DKM.

**Declaration of Interests**

Neither Dr. Gardner, nor Professor Menon, have competing interests relating to the subject of this review.
Table of references


