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

Glossary

CA	Cardiac Arrest
HIE	Hypoxic-ischemic encephalopathy
TBI	Traumatic brain injury
CPR	Cardiopulmonary resuscitation
CT	Computer tomography
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
GABA	γ-Aminobutyric acid
OOHCA	Out-of-hospital cardiac arrest
HO-1	Haem-oxygenase 1
NQO-1	NADPH dehydrogenase (quinone 1)
GST	Gluthatine S-transferases
SOD-1	Superoxide dismutase 1
OGD	Oxygen glucose deprivation
tMCAO	Transient middle cerebral artery occlusion
ARE	Anti-oxidant response element
FIO2	Fraction of inspired oxygen
ROS	Reactive oxygen species
TLR	Toll-like receptor
tMCAO	Temporary middle cerebral artery occlusion
OGD	Oxygen-glucose deprivation
ICU	Intensive care unit
VF	Ventricular fibrillation

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.[1] Most die during their initial hospital admission.[2] Furthermore, two-thirds of patients who survive have moderate to severe cognitive deficits three months after their CA.[3] 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.[4-7] Poor functional outcomes from such injuries are commonplace, meaning around half never return to work.[8] These statistics highlight the importance of developing therapeutic strategies which are aimed at providing 'neuroprotection'.[9]

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.[10] Public education has significantly improved rates of early and appropriate bystander cardiopulmonary resuscitation (CPR).[11 12] 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.[13] 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.[14-17] Furthermore, despite cooling, half of neonates with HIE still die or suffer significant neuro-developmental disability.[6 18 19] 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[20], neuroinflammation within the salvageable penumbra,[21] breakdown of the blood-brain barrier[21], disruption and remodelling of the cerebral microvasculature,[22] excitotoxic injury[23] and oxidative stress owed to excessive free-radical production.[23-26 27-30]

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.[31] 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.[32] Xenon, with the most outer electrons, has the greatest polarising effect. This has been correlated with its anaesthetic ability.[32] 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[33]). Like all gases, it can quickly cross the blood-brain barrier, increasing its clinical potential.[34]

Xenon is thought to function in neuroprotection by antagonism of the NMDA receptor, thereby preventing glutamate mediated excitotoxic stress.[35 36] Most current evidence suggests xenon acts by the competitive inhibition of glycine, a co-agonist of the NMDA receptor;[37] although some propose involvement of the adenosine triphosphate dependent potassium channels.[34] The addition of exogenous glycine tends to abolished neuroprotection by xenon *in vitro*.[36 38] However, at least two mutations in the NMDA receptor eliminate xenon binding without affecting the affinity of glycine.[39] Another study demonstrated inhibition of NMDA currents irrespective of glycine concentration.[40] 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.[33 41] Laitio *et al.* demonstrated encouraging radiological evidence of benefit with xenon in humans after out-of-hospital cardiac arrest (OOHCA),

combined with therapeutic hypothermia.[42] 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.[43-47] 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.[48-52] There is significant regional variation in the price of xenon from $60/L in US to $30/L in France,[48] far higher than the often miss-quoted $10/L, which is now historic.[53] The price of xenon is inelastic: increases in consumption beyond its fixed capacity (or supply ceiling) will increase its price.[54] 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.[55 56]

	Cost and utilisation To maintain a 50-70% Xe or Ar concentration	**Approximate cost pre-hospital**	**Approximate cost in hospital**
Argon 2.71¢/L	**Open flow rate:** Steady state use: 10L/min or 6,000L/hr[a] = 27.1¢/min (at 2.71¢/L) = $16.3/hour **Closed circuit flow rate:** no data	**Open flow rate** for ~2-4 hours[b]: = 24,000L over 4 hours Cost: $65	**Open flow (24h):** 20,880L Cost: $390 **Closed circuit (24h):** ~211L[c] Cost: $5.72
Xenon $30-60/L	**Open flow rate:** not feasible **Closed circuit flow rate[d]:** Steady state use: 5.0-12.5L/h Averaged use of 8.8L/h, at $30/L = $264/h	Use not feasible	**Open flow (24h):** not feasible **Closed circuit (24h):** ~211L[e] Cost: $6,330 (at $30/L)

Table 1 - A cost-comparison of argon and xenon using the current data available

[a]Approximate figure, inferred from historic use oxygen and xenon in the literature as part of a 15L/min non-rebreathe flow rate [b] Approximate time to xenon administration in Phase II trial.[42] [c] 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. [d] 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 - 53] 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[52] and has not been integrated into this graphic. [e] 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.[57] This was in contradistinction to a review in 2014,[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.[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.[55] The cost of argon has been cited as approximately 9¢/L in previous literature (e.g. Loetscher *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[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.[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.[40] More specifically, argon does not appear to mediate neuroprotection through effects at GABA[61]or NMDA[36] receptors, or TREK1[36] and KATP[62] channels.

Instead, argon appears to prevent cell death via upregulation of both the ERK 1/2 and PI-3K-AKT pathways (Figure 1). [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 *in vivo*.[64-68] Cytotoxic damage itself also

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stimulates the ERK 1/2 pathway, in the context of *in vivo* models of hypoxic brain injury.[65] Small molecule inhibition of the pathways increased the quantity of cell death and the expression of pro-apoptotic mediators.[65 69] The PI-3k-AKT pathway induces a cellular survival signal in opposition to apoptosis.[70 71] 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.[6 72]

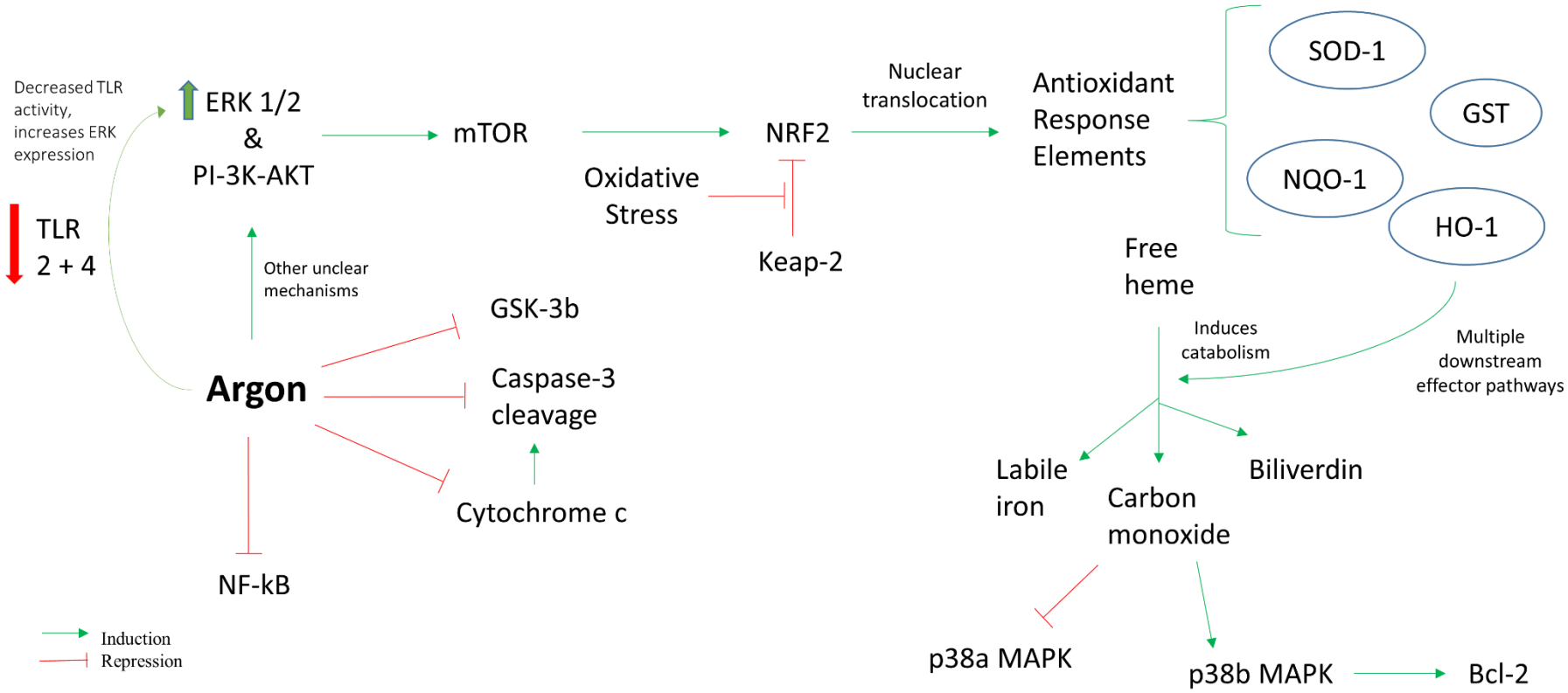

Figure 1 - A cartoon to illustrate the hypothesised molecular mechanisms of argon, synthesising the current available literature.[6, 59, 60-76]

Nrf2, a basic leucine zipper transcription factor, appears to mediate this pathway.[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.[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).[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^{2+}), carbon monoxide and biliverdin.[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).[75] Despite evidence for an important role of HO-1 in argon-mediate neuroprotection,[6] others have shown an initial reduction in HO-1 protein concentration in retinal ganglion cells after argon ventilation.[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.[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 *in vitro* to mediate apoptosis; inhibition of it appears to protect against apoptosis.[78 79] Similarly, Ulbrich *et al.* used rotenone to induce oxidative damage, which induced upregulation of the TLR 2 and 4 receptors. *In vitro* retinal ganglion culture, and *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.[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.[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.[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 neurons[82] 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.

First author	Disease Model	Animal	Treatment protocol	Treatment group gas mix	Control group gas mix	Cell survival data	Histopathological data	Biomarker analysis
Zhao 2016	OGD	Cortical neuronal cultures (gestational day 16 rat foeti)	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.	75% Ar, 20% O_2, 5% CO_2	75% N_2, 20% O_2, 5% CO_2	Increased percentage of live cells by around 50%	Improved cellular and dendritic cell morphology. Reduced caspase 3 positive areas.	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.
Zhao 2016	OGD	Cortical neuronal cell culture (gestational day 18 rat foeti)	OGD for 90 min with treatment gas bubbled through media, then incubated with treatment for a further 24h	70% Ar, 25% O_2, 5% CO_2	70% N_2, 25% O_2, 5% CO_2		Preserved neuronal morphology and cell viability using Ar, assessed via MTT assay.	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).
Ulbrich 2015	Oxidative stress	Human neuroblastoma cell line (SH-SY5Y)	Rotenone treatment for 4h, then exposure to Ar for 2 or 4h	75%, 50% or 25% Ar, with 21% O_2, made up with N_2	Room air	Maximal benefit observed using 75% Ar, reducing apoptosis to baseline. Benefit of Ar time and dose dependent.	Significantly reduction in TLR-2 & TLR-4 receptor density and protein expression with Ar treatment.	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.

David 2012	OGD	*Ex vivo* rat striatal brain slices	Glucose free solution saturated with 100% nitrogen for 20 min; 3h treatment.	25-75% Ar, 25% O_2, remaining percent N_2	Medical air (75% N_2, 25% O_2)			Significant reduction in LDH release, as a marker of cell injury, using 37.5-75% Ar (maximal at 50%, after 3h).
Fahlenkamp 2012	N/A	Mouse primary cortical neurons, microglia and astrocytes	Exposure to humified gas mixture for 15, 30, 60 or 120 min.	50% Ar, 24% N_2, 21% O_2, 5% CO_2	Humified room air			Significant induction of ERK 1/2 in microglia, maximal at 30 min. A similar induction in neurons and astrocytes did not reach statistical significance.
Jawad 2009	OGD	Mouse cerebral cortices, dissociated neurons	OGD for 90 min with nitrogen (95%, with 5% CO_2) or noble gas, then treatment for further 24h.	75% noble gas (e.g. Ar), 20% O_2, 5% CO_2	75% N_2, 20% O_2, 5% CO_2			Cell viability (judged by reducing ability via MTT assay) preserved after OGD using Ar & Xe, but not neon, krypton or helium. In the absence of OGD, Ar even improved cell viability.
Loetscher 2009	OGD and TBI	*Ex vivo* mouse hippocampal tissue	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.	Media had 25%, 50% and 74% Ar, with 21% O_2, 5% CO_2 and N_2 to top-up	Humified room air	Ar decreased tissue injury significantly after OGD and TBI; maximum benefit observed using 74% & 50%, respectively. Reduced benefit after a delay in treatment, although still significantly reduced injury.		
Yarin 2005	Hypoxia	Cultured organ of Corti cells from rats	Nitrogen or Ar hypoxia; Toxic drug exposure with cisplatin or gentamycin	Hypoxia: 95% Ar, 5% CO_2, Drug exposure: 74% Ar, 21% O_2, 5% CO_2	Hypoxia: 95% N_2, 5% CO_2, Drug exposure: 74% N_2, 21% O_2, 5% CO_2	Approximate 20-30% reduction in cell death with Ar treatment during hypoxia, cisplatin and gentamycin exposure.		

Table 2 - A comprehensive summary of the literature with the results of *in vitro* data pertaining to the use for argon ventilation for neuroprotection

TBI: traumatic brain injury, OGD: oxygen-glucose deprivation, Ar: Argon, TLR: Toll-like receptor

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,[84] 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).[84] These data, and a dose dependency of argon neuroprotection in this model, were replicated in a larger sample size by the same group.[62] 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.[85] 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.[86] 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[56 86] or cerebral oxygen saturation and brain electrical activity.[56]

A more recent study from Zuercher et al (2016) failed to replicate the neuroprotective benefits of argon.[87] Compared to Brucken[85] and Ristagno[86] *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.[87] 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.[65] 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.[63 88] 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%.[89] However, these results are confounded using 100% O_2 as an anaesthetic carrier, which may have had independent deleterious consequences, given the association with hyperoxia worsening reperfusion injury.[90 91]

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.[60] 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.[6] 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.[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.[6] Argon also reduced weight loss and cerebral reactive gliosis.[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.[6 66] Argon's apparent benefit in reducing histopathological damage and inducing 'anti-apoptotic' expression were blocked by *in vivo* inhibitors of either PI-3K-AKT (Wortmannin) or ERK 1/2 (U0126),[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).[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.[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 *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.

First author	Disease Model	Animal	Treatment protocol	Treatment group gas mix	Control group gas mix	Mortality data	Neuro-cognitive assessment	Histopathological data	Biomarker analysis
Ischaemic Stroke									
Broad 2016	Stroke	Piglet	Bilateral carotid artery occlusion for 12.5 minutes. Initiation of ventilation from 2h till 26h. Simultaneously cooled to 33.5°C	50% Ar, 29% N_2, 21% O_2 (with variable oxygenation to maintain SaO_2 8-13kpa.)	Not specified			Reduced TUNEL+ staining. However, only noted in certain brain areas, e.g. putamen not periventricular white matter.	White matter lactate significantly lower in hypothermia and Ar group. Increased whole brain ATP and phosphocreatine at 48 hours. Improved mean hourly EEG recordings.
Ulbrich 2015	Stroke (retinal ischaemia)	Rat	1h ischemia caused by increased ocular pressure. Then 1h of treatment, immediately or with a 1.5 or 3h delay.	75% Ar, with 21% O_2, 4% N_2	Room air			Inhibition of ERK 1/2 attenuates Ar-mediated protection	Ar ventilation significantly increased ERK 1/2 expression and HSP 70. In contrast, decreased protein levels of HO-1 and HSP-90
Ulbrich 2014	Stroke	Rat	1h ischemia followed by 1h of treatment, immediately or without a 1.5 or 3-h delay.	75%, 50% or 25% Ar, with 21% O_2, made up with N_2	Room air			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.	Ar inhibited NF-kB, Bax and Bcl-2 expression significantly Ischemic induced leucocytosis reduced significantly
Fahlenkamp 2014	Stroke	Rat	tMCAO for two hours, ventilation started 1h later for 1h	50% Ar 50% O_2	50% N_2 50% O_2	No survival benefit noted	Improved composite behaviour outcomes	Reduced infarct volumes	Mixed cytokine production on a spectrum of neuroprotection (TGF- β, NGF) and pro-inflammation (IL-1β, IL-6)
David 2012	Stroke	Rat	Stereotaxic striatal injection of NDMA Or 1h tMCAO, followed by 3h of treatment	15-75% Ar, 25% O_2, remaining percent N_2	Medical air (75% N_2, 25% O_2)		Minimal difference in behavioural outcomes between groups.	Reduced neuronal damage with all concentrations of Ar, maximally at 50% (NMDA condition). Ar reduced cortical brain damage (35%) but increased subcortical	

								damage (35%) after tMCAO.	
Ryang 2011	Stroke	Rat	tMCAO for 2h; ventilation started 1h later for 1h	50% Ar 50% O_2	50% N_2 50% O_2	No survival benefit within 24h	Improved composite behaviour outcomes	Reduced infarct volume	
Cardiac arrest & global cerebral hypoxia									
Zuercher 2016	Cardiac arrest	Rat	Cardiac arrest induced by potassium and esmolol. 8 minutes arrest, followed by manual chest compressions with intravenous adrenaline. Ventilated for 24h after ROSC	50% Ar, 50% O_2, or 50% He, 50% O_2	50% N_2, 30% O_2		No difference in NDS over 5 days	Trend (non-significant) to reduced hippocampal neuronal damage in the noble gas treated groups.	
Brucken 2015	Cardiac arrest	Rat	VF induced by transoesophageal electrical stimulation. Defibrillation after 7 minutes, ventilated for 1h either 1h or 3h after ROSC	70% Ar, 30% O_2,	70% N_2, 30% O_2		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.	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.	
Ristagno 2014	Cardiac arrest	Pigs	Balloon Occlusion of LAD to induce VF. Defibrillation after 8 minutes of VF, ventilated for 4h	70% Ar, 30% O_2	70% N_2, 30% O_2		After 72h, pigs treated with Ar had returned to pre-test cognitive levels, whereas control pigs had only recovered 75% of baseline function	Significantly reduced neuronal damage	Significantly reduced Neuron-specific enolase
Brucken 2014	Cardiac arrest	Rat	VF induced by transoesophageal electrical stimulation. Defibrillation after 7 minutes, ventilated for 1h after 1h	70% Ar with 30% O_2, or 40% Ar with 30% O_2	70% N_2, 30% O_2		Improved NDS at 7 days, with a dose dependent improvement (70% > 40%). Significantly more	Neocortex and hippocampal slices showed significantly less neuronal damage, irrespective Ar concentration	

							mobile episodes in open field test only using 70% Ar.		
Brucken 2013	Cardiac arrest	Rats	VF induced by transoesophageal electrical stimulation. Defibrillation after 7 minutes, ventilated for 1h after 1h	70% Ar, 30% O_2	70% N_2, 30% O_2		Improved NDS at 7 days. More mobile episodes in open field test, inconclusive improvement in water maze.	Neocortex and hippocampal slices showed significantly less neuronal damage.	
Miscellaneous animal models									
Hollig 2016	Subarachnoid haemorrhage	Rat	Cerebral artery perforation. Treatment started 1h after induction, for 1h	50% Ar, 50% O_2	50% N_2, 50% O_2	20.6% reduction in 'premature death' at 72h	No difference	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.	HIFα1 increased at 24h ours with Ar, compared to controls (but not 6h or 72h)
Zhao 2016	Neonatal HIE	Rat (neonatal)	Carotid artery ligation. 1h after induction, 2h treatment, with (33°C or 35°C) or without (37°C) hypothermia	70% Ar, 30% O_2	70% N_2, 30% O_2			Reduced infarction volume by up to 65% when used synergistically with hypothermia (vs N_2 and 37°C). Reduced glial activation (GFAP expression)	Ar significantly reduced caspase 3 expression beyond hypothermia alone. Ar induces p-AKT and HO-1 and inhibits NF-kβ expression. Inhibition of HO-1 and p-AKT abolishes the benefit of Ar
Alderliesten 2014	Safety evaluation; hypoxic ventilation mixture administered	Pig	#1 Ventilation with increasing concentrations of Ar, 1h each (30%, 50%, 80%) #2 1h of hypoxia (FiO_2 0.08) followed by 3h of 50% Ar #3 As above, with hypothermia (34-35°C)		Immediate euthanasia for histology		No demonstrable increase neuronal injury in normoxia due to Ar	No significant change in heart rate, mean arterial pressure, regional cerebral saturation of oxygen or electroencephalography due to Ar ventilation	
Zhuang 2012	Neonatal HIE	Rat (neonatal)	Common carotid artery ligation followed by hypoxic	70% Ar, He or Xe, with 30% O_2	70% N_2, with 30% O_2		No significant difference in	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.

			atmosphere (8% O_2) 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 3 - A comprehensive summary of the literature with the results of *in vivo* data pertaining to the use for argon ventilation for neuroprotection

NDS: neurological deficit score, ROSC: return of spontaneous circulation, tMCAO: transient middle cerebral artery occlusion, HIE: hypoxic-ischemia encephalopathy, LAD: left anterior descending, VF: ventricular fibrillation, Ar: argon, HSP: Heat-shock protein

The safety of argon in larger animal models and humans

Xenon has been used safely in human anaesthesia,[49 51] radiology,[95] neonatology (up-to 50% xenon concentration)[52] and now in a phase II clinical trial.[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.[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_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.[96 97] A negative effect on cardiac output was demonstrated in one,[96] but then not replicated.[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.[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_2).[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.[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% O_2). 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 (FIO_2) as a part of resuscitation, often reaching 100% oxygen. However, more recent concerns regarding hyperoxic injury have led to reduced use of FIO_2 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 production[25] and lipid oxidation,[110] and exacerbation of neuronal death.[24 111] When appropriate, limiting FIO_2 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 FiO_2 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,[42] and a planned Phase III trial.[118] 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.[48 53] 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.[35 51 54] Perhaps more importantly, the fact that xenon may be a cerebral vasodilator[119 120] 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).[32] 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.

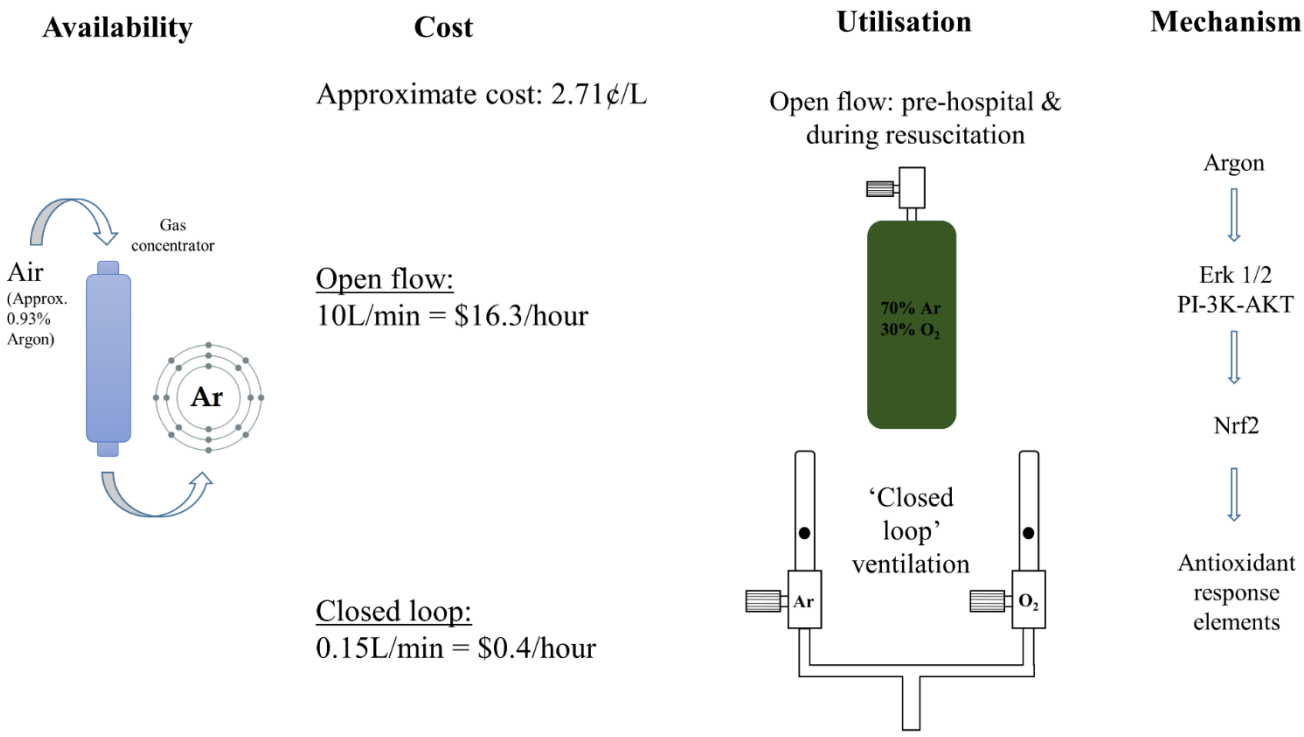

Figure 2 - A graphic demonstrating the important factors in the clinical implementation of argon as a neuroprotective treatment for brain injury

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[121] 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,[122] as has been required with helium.[123]

Robust evidence will be required that titrating oxygen supplementation to peripheral oxygen saturations is safe, as has been demonstrated with xenon.[42] Direct measurement of argon in gas mixtures is possible,[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 pre-hospital use of argon would be plausible.[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;[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.

Patient group	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).
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 FiO_2 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.

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