● PERSPECTIVE

Argon: a novel therapeutic option to treat neuronal ischemia and reperfusion injuries?

Neuronal injury and neuroprotection: Ischemia and reperfusion injuries in neuronal cells such as acute ischemic stroke - represent the third leading cause of death in the world. Current therapeutic concepts mainly aim to re-establish cerebral blood flow within a time window of less than 3 hours with the goal of limiting secondary brain injury. Secondary brain injury of the "penumbra" develops exponentially, causing severe mental and physical disabilities. The resulting morbidity and mortality in patients of all ages leads to an enormous economic burden. Neurons are well-known to possess a limited potential to recover and regenerate their basic function once damaged. Therefore the protection and especially the therapy of neuronal injuries in cells and organ systems has emerged as a major focus of research in recent years. The aim is to maximize neuronal function not only by restoring sufficient blood flow to the areas at risk, but also by protecting neuronal cells with various novel interventions. Many of these interventions, such as intravascular cooling, focus on the reduction of neuronal metabolism in an effort to induce protection.

The term 'neuroprotection' implies a multitude of strategies to reduce the detrimental effects of hazardous stimuli to brain tissue. The additional opportunity to initiate *neurorepair* by endogenous agents opens up the chance to improve neuronal function within the days or weeks following injury by stimulating regeneration. Cellular as well as molecular targets responsible for secondary brain damage are chosen as sites of action, with the objective of promoting neuronal cell survival, stabilizing the penumbra area, reducing cerebral edema, minimizing blood-brain-barrier disruption and antagonizing proteins of the ischemic and inflammatory cascades. Different mechanisms of excitotoxicity have been analyzed regarding possible molecular approaches to achieving neuroprotection (*i.e.*, apoptosis, inflammatory reactions, reactive oxygen species (ROS) scavenging, glutamate release). During the last number of years, the evaluation of many neuroprotective strategies has shown only modest or inconsistent neuronal tissue protection if at all, accentuating the need for further innovative research (O'Collins et al., 2006). All of these neuroprotective strategies have the potential to compliment the already well established thrombolytic therapy.

Experimental agent: The gaseous molecule argon: The characteristics of various "gasotransmitters" including nitric oxide (NO), carbon monoxide (CO), xenon (Xe) and hydrogen sufide (H_2S) have been extensively investigated for decades by scientists, using a huge variety of *in vitro* and *in vivo* models; *i.e.*, transplantation, stroke, myocardial injury, inflammation, cancer, organ failure, *etc.*, (Motterlini and Otterbein, 2010). Different molecular mechanisms of action have been identified and have demonstrated beneficial or even protective (anti-inflammatory and anti-apoptotic) effects in a multitude of models of physiological impairment, when applied as pre- or postconditioning treatment.

In contrast, the noble gas argon just recently emerged into scientific focus. First synthezised using fractioned distillation of liquid air and described by later Nobel Prize winners Lord Rayleigh and Sir William Ramsay in 1895 as a "heavy component of air", the two scientists named the newly discovered moelcule a "lazy" gas (greek: αργόν = argon, meaning lazy) due to its inert nature and chemical inactivity. The latter physico-chemical characteristics are based on the fact, that noble gases present themselves with a full outer shell of electrons, thus preventing formation of covalent protein interactions under physiological conditions. Being uncharged and consequently non-polar, argon may diffuse easily into brain compartments, interacting with amphiphilic protein cavitities.

Up to date no unwanted side effects could be detected. Moreover, there is no data about long term effects after argon administration. The concentration of argon in tissue as well as in peripheral blood has not been measured so far, which makes any direct link between the tissue concentration and resulting effects difficult.

Unlike xenon, argon fortunately does not exert any sedative or even anesthetic properties under normobaric conditions. This, and the fact that argon provides distinct cardiovascular stability (Ryang et al., 2011) makes it tempting to speculate about its application in patients with focal neurologic injury such as stroke. Other major advantages are the low price, the ease of production and the ability to administer the gas without the need for additional equipment.

Argon's neuroprotective effects and potential molecular mechanisms: Soldatov et al. (1998) were the first to describe the effects of argon in an animal model. They subjected mice to hypoxia and were able to demonstrate that the inhalation of argon in concentrations ranging from 25 to 77% of volume attenuated brain injury, resulting in an increase of animal survival. In a complex series of *in vitro* experiments, Loetscher et al. (2009) utilized organotypic hippocampal slices of newborn mice pups, subjecting these to oxygen and glucose deprivation (OGD) as a model of ischemia and reperfusion injury. Additionally, traumatic brain injury was produced by driving a stylus 7 mm in the CA1 hippocampal area with a force of 5.26 µJ. After 3 hours, slices of either group were exposed to argon at 25–74% of volume. While comparably high damage was observed after each form of injury, argon treatment showed significant reduction in trauma injury score, revealing a time- and dose-dependency *in vitro* (Loetscher et al., 2009). One of the first investigators to really prove neuroprotection *in vivo* was Ryang et al. (2011), who allocated rats to 2 hours of transient middle cerebral artery occlusion (tMCAO). After 1 hour of cerebral ischemia and 1 hour prior to reperfusion, animals spontaneously breathed either argon + O_2 (50%/50%, v/v) *via* a facemask for 1 hour or N_2 + O_2 (50%/50%, v/v). Cerebral blood flow as well as mean arterial blood pressure, heart rate, temperature and blood gas analysis parameters were comparable in both groups, again demonstrating the distinct cardiovascular stability of argon. Twenty-four hours after thromboembolic middle cerebral artery occlusion (tMCAO), animals were killed. Edema-corrected infarct volume was significantly lower in argon treated animals in the cortex and the basal ganglia but not in the hippocampal area. Overall infarct size was significantly reduced after argon inhalation. Additionally, a neurologic deficit score (NDS) testing was performed 24 hours after reperfusion, applying a 6 point neuroscore. Argon was able to attenuate and shift poor neurological outcome towards a better score (Ryang et al., 2011). Concomitantly, David et al. (2012) were able to confirm Ryang´s results. In a three step experimental setting, at first excitotoxicity was analyzed in coronal brain slices after OGD treatment. Secondly, rats either received an intrastriatal injection of N-methyl-D-aspartate (NMDA) or an tMCAO. For *in vitro* experiments, slices were incubated with argon (25, 37.5, 50 and 75%, v/v) after OGD and lactate dehydrogenase (LDH) release was measured. Interestingly, there was no linear correlation of argon concentration. In contrast, argon inhalation after NMDA treatment resulted in a dose dependent reduction of neuronal cell death. In contrast to the beneficial effects of argon regarding the cortical level, subcortical tissue damage and neurological deficiency scores were increased. Interestingly, this might be due to the fact, that in MCAO, intra-ischemic administration of a possible neuroprotective agent seems superior compared to a post-ischemic treatment (David et al., 2012). In a first approach to investigate the molecular patterns of argon's beneficial effects, Fahlenkamp et al.

(2012) analyzed the mitogen-activated protein kinase ERK1/2 in an *in vitro* model of primary neuronal and astroglial cells while exposed to 50% argon (v/v). They demonstrated, that argon was able to increase ERK1/2 phosphorylation after a very short interference period of approximately 15 minutes. Following lipopolysaccharide (LPS) administration, pro-inflammatory cytokines (tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6) were analyzed in the absence and presence of 50% argon (v/v). Argon significantly reduced IL-1β protein expression, giving a first hint towards anti-inflammatory properties (Fahlenkamp et al., 2012). The neuroprotective effects of argon following cardiac arrest and resuscitation were studied in rats by Bruecken et al (2013). Ventricular fibrillation was induced by esophageal electrical stimulation with subsequent cardiopulmonary resuscitation (CPR). One hour after return of spontaneous circulation, animals were ventilated with 70% argon (v/v). The NDS was calculated every day for 7 days and histopathological analysis was performed on day 7. Although animals in the control group recovered over time, argon treated animals showed a significantly higher NDS from day 1. Histopathological evaluation revealed different neuronal damage indices with benefical effects of argon in the neocortex and the hippocampal area CA3 and CA4 but not in the basal ganglia or stratum granulosum (Brucken et al., 2013). Proceeding with the question of post-resuscitation treatment after cardiac arrest, Ristagno et al. (2014) demonstrated that 70% argon (v/v) lead to a fast and complete neurological recovery 72 hours after CPR in pigs. Moreover, argon inhalation attenuated neuronal loss and degeneration as well as migroglia activation. Neuron-specific enolase and histological brain injury were significantly lower after argon treatment. Additionally, in a large animal model, no adverse effects of argon regarding hemodynamics or respiratory gas exchange were seen. Concerning the mechanism, again Bruecken et al. subjected rats to a K_{ATP}-channel blocker (5-hydroxydecanoate) prior to cardiac arrest and resuscitation. While argon's protective effects were comparable to the results of other researchers, the administration of 5-hydroxydecanoate neither abolished these positive effects on functional recovery nor on histopathological changes and hence does not seem to be part of the mechanisms (Brucken et al., 2014).

In our laboratory, we focused on the time- and dose dependency of different concentrations of argon, as well as its molecular mechanism *in vivo* (Ulbrich et al., 2014). Although upstream, the retina is considered part of the central nervous system and rats were subjected to retinal ischemia for 1 hour with consecqutive reperfusion. Spontaneously breathing, argon was inhaled immediately or with a delay of 1.5 and 3 hours after the end of ischemia at concentrations of 25, 50 or 75% (v/v) in combination with 21% O_2 (v/v)and N_2 supplement. Twentyfour-hours after the end of ischemia, argon treated animals demonstrated a significant, time-dependent reduction in caspase-3 mRNA expression and caspase cleavage. In addition, Bcl-2 and Bax mRNA expression were significantly reduced in a time- and dose-dependent manner. Part of a possible mechanism may be the involvement of a central transcription factor, *i.e.*, nuclear factor kappaB (NF-κB); a key regulator in apoptosis and inflammation. Suppression of NF-κB was only seen at 75% argon (v/v) inhalation. Seven days after ischemia and reperfusion, retinae were extracted to analyze the number of vital retinal ganglion cells (RGC). Following ischemia and reperfusion, the number of vital RGCs decreased by around 40%. Immediate inhalation of 75% argon (v/v) completely abolished this effect, protecting RGC from reperfusion injury. Even within a time window of up to 3 hours, argon still protected these neuronal cells significantly, fading with time and reduction in dosage. These effects may be mediated by the Müller cells, a special population of retinal glia cells. Moreover, argon inhalation at high concentrations attenuated ischemia-induced elevation of leukocytes in peripheral blood, which may be an indicator of it's ability to counteract systemic neuroinflammation.

Conclusions: Our increasing comprehension of neurogenesis, neuroplasticity and synaptogenesis in combination with the results of the few studies concerning argon's role in neuroprotection may open up new fields of opportunities. Especially, stroke or neurological post-resuscitation limitations call for alternative options, since current treatments seem insufficient regarding long-term outcome. Yet, the mechanism by which argon exerts its effects remains far from being understood. Further studies regarding the basic sciences are needed for the identification of the exact mechanism. As for now, since no toxic or adverse effects of argon have been reported, clinical safety studies using argon or argon-supplemented inhalation or ventilation in humans seem justified.

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References

Brucken A, Kurnaz P, Bleilevens C, Derwall M, Weis J, Nolte K, Rossaint R, Fries M (2014) Dose dependent neuroprotection of the noble gas aafter cardiac arrest in rats is not mediated by K(ATP)-channel opening. Resuscitation 85:826-832.

Brucken A, Cizen A, Fera C, Meinhardt A, Weis J, Nolte K, Rossaint R, Pufe T, Marx G, Fries M (2013) Argon reduces neurohistopathological damage and preserves functional recovery after cardiac arrest in rats. Br J Anaesth 110 Suppl 1:i106-112.

David HN, Haelewyn B, Degoulet M, Colomb DG, Jr., Risso JJ, Abraini JH (2012) Ex vivo and in vivo neuroprotection induced by argon when given after an excitotoxic or ischemic insult. PLoS One 7:e30934.

Fahlenkamp AV, Rossaint R, Haase H, Al Kassam H, Ryang YM, Beyer C, Coburn M (2012) The noble gas argon modifies extracellular signal-regulated kinase 1/2 signaling in neurons and glial cells. Eur J Pharmacol 674:104-111.

Loetscher PD, Rossaint J, Rossaint R, Weis J, Fries M, Fahlenkamp A, Ryang YM, Grottke O, Coburn M (2009) Argon: neuroprotection in in vitro models of cerebral ischemia and traumatic brain injury. Crit Care 13:R206.

Motterlini R, Otterbein LE (2010) The therapeutic potential of carbon monoxide. Nat Rev Drug Discov 9:728-743.

O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW (2006) 1,026 experimental treatments in acute stroke. Ann Neurol 59:467-477.

Ristagno G, Fumagalli F, Russo I, Tantillo S, Zani DD, Locatelli V, De Maglie M, Novelli D, Staszewsky L, Vago T, Belloli A, Di Giancamillo M, Fries M, Masson S, Scanziani E, Latini R (2014) Postresuscitation treatment with argon improves early neurological recovery in a porcine model of cardiac arrest. Shock 41:72-78.

Ryang YM, Fahlenkamp AV, Rossaint R, Wesp D, Loetscher PD, Beyer C, Coburn M (2011) Neuroprotective effects of argon in an in vivo model of transient middle cerebral artery occlusion in rats. Crit Care Med 39:1448-1453.

Soldatov PE, D'iachenko AI, Pavlov BN, Fedotov AP, Chuguev AP (1998) [Survival of laboratory animals in argon-containing hypoxic gaseous environments]. Aviakosm Ekolog Med 32:33-37.

Ulbrich F, Schallner N, Coburn M, Loop T, Lagreze WA, Biermann J, Goebel U (2014) Argon inhalation attenuates retinal apoptosis after ischemia/reperfusion injury in a time- and dose-dependent manner in rats. PLoS One 9:e115984.