BJA

British Journal of Anaesthesia, 126 (1): 256–264 (2021)

doi: 10.1016/j.bja.2020.08.027
Advance Access Publication Date: 22 September 2020
Neuroscience and Neuroanaesthesia

Efficacy of acute administration of inhaled argon on traumatic brain injury in mice

Federico Moro[1], Francesca Fossi[1,2], Aurora Magliocca[3], Rosaria Pascente[1], Eliana Sammali[1], Federico Baldini[1], Daniele Tolomeo[4], Edoardo Micotti[4], Giuseppe Citerio[2], Nino Stocchetti[5,6], Francesca Fumagalli[3], Sandra Magnoni[7], Roberto Latini[3], Giuseppe Ristagno[3,5] and Elisa R. Zanier[1,*]

[1]Laboratory of Acute Brain Injury and Therapeutic Strategies, Department of Neuroscience, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, [2]School of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy, [3]Department of Cardiovascular Medicine, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, [4]Laboratory of Biology of Neurodegenerative Disorders, Department of Neuroscience, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, [5]Department of Pathophysiology and Transplants, University of Milan, Milan, Italy, [6]Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy and [7]Santa Chiara Hospital, Azienda Provinciale per i Servizi Sanitari Della Provincia di Trento-APSS, Trento, Italy

*Corresponding author. E-mail: elisa.zanier@marionegri.it

This article is accompanied by an editorial: Argon: a noble, but not inert, treatment for brain trauma? by Edge & Dickinson, *Br J Anaesth* 2021:126:41–43, doi: 10.1016/j.bja.2020.09.028

Abstract

Background: Whilst there has been progress in supportive treatment for traumatic brain injury (TBI), specific neuroprotective interventions are lacking. Models of ischaemic heart and brain injury show the therapeutic potential of argon gas, but it is still not known whether inhaled argon (iAr) is protective in TBI. We tested the effects of acute administration of iAr on brain oedema, tissue micro-environmental changes, neurological functions, and structural outcome in a mouse model of TBI.

Methods: Anaesthetised adult C57BL/6J mice were subjected to severe TBI by controlled cortical impact. Ten minutes after TBI, the mice were randomised to 24 h treatments with iAr 70%/O_2 30% or air (iCtr). Sensorimotor deficits were evaluated up to 6 weeks post-TBI by three independent tests. Cognitive function was evaluated by Barnes maze test at 4 weeks. MRI was done to examine brain oedema at 3 days and white matter damage at 5 weeks. Microglia/macrophages activation and functional commitment were evaluated at 1 week after TBI by immunohistochemistry.

Results: iAr significantly accelerated sensorimotor recovery and improved cognitive deficits 1 month after TBI, with less white matter damage in the ipsilateral fimbria and body of the corpus callosum. Early changes underpinning protection included a reduction of pericontusional vasogenic oedema and of the inflammatory response. iAr significantly reduced microglial activation with increases in ramified cells and the M2-like marker YM1.

Conclusions: iAr accelerates recovery of sensorimotor function and improves cognitive and structural outcome 1 month after severe TBI in adult mice. Early effects include a reduction of brain oedema and neuroinflammation in the contused tissue.

Keywords: argon; brain protection; neuroinflammation; neuroprotection; traumatic brain injury

Received: 6 January 2020; **Accepted:** 10 August 2020

Editor's key points

- The effects of administration of inhaled argon on brain oedema, tissue inflammation, neurological function, and brain structural outcome were investigated in a mouse model of traumatic brain injury.
- Treatment with argon 70% initiated 10 min after traumatic brain injury accelerated sensorimotor recovery and reduced cognitive deficits 1 month after injury.
- Early administration of inhaled argon was associated with a reduction in pericontusional vasogenic oedema and inflammatory markers, and with less white matter damage.
- The neuroprotective and anti-inflammatory properties of inhaled argon may provide a useful therapy to improve outcome after traumatic brain injury.

Supportive treatment for the management of traumatic brain injury (TBI) has progressed over the past 20 years, but specific neuroprotective strategies are lacking.[1–3] There is an urgent need for new therapeutic interventions.

Noble gases, including argon and xenon, have emerged as promising neuroprotective agents in different models of acute brain injury.[4–7] Advantages of inhaled argon (iAr) over xenon include the possibility of open-flow ventilation, the absence of anaesthetic properties under normobaric conditions, low cost (~3 cents L^{-1}), and absence of cerebral vasodilatory effects,[6] a key limiting factor in TBI patients at risk of intracranial hypertension.[8] Neuroprotective effects of iAr were first observed in an *in vitro* model of hypoxic/ischaemic injury by oxygen–glucose deprivation in neuronal culture[9] and have since been confirmed *in vivo* after brain ischaemia,[10] hypoxia,[11,12] and subarachnoid haemorrhage.[7] However, no data are available on TBI *in vivo* models. Preliminary evidence of protection from biomechanical damage was obtained in an *ex vivo* model of hippocampal slices subjected to stylus drop and treated immediately after injury with argon 50%-enriched culture medium. The reduction in injury varied, ranging from ~30% to 80% at 72 h after trauma in two different studies.[9,13] This suggests that argon 50% is the lower limit for neuroprotection, thus leading to variable results.

iAr exerts neuroprotection after experimental hypoxic/ischaemic insults[7,11,14,15] by reducing inflammatory and pro-apoptotic signalling and attenuating cellular stress.[14,16,17] Oxidative stress, apoptotic processes, and neuroinflammation are key modulators of secondary damage after TBI.[18] Thus, iAr might exert neuroprotection and induce lasting functional and structural benefits after TBI. The aim of the study was to test whether iAr 70% administered acutely for 24 h after severe TBI in mice exerted neuroprotection.

Methods

Study design

Figure 1 shows the timeline of the experimental plan. We first evaluated the effects of iAr administered for 24 h, 10 min after injury, on sensorimotor deficits, brain oedema, and inflammatory changes within a week from TBI in mice (Experiment 1). In a second experiment, we analysed the early effects on sensorimotor deficits, and tested whether there was lasting protection against functional and structural outcomes by analysing sensorimotor and, cognitive functions together with MRI up to 6 weeks after TBI (Experiment 2).

Animals

Male C57BL/6J mice (9 weeks of age) from Envigo (Horst, Netherlands) were used. They were housed in a pathogen-free vivarium with a constant temperature (21 [1]°C) and relative humidity (60 [5]%) with a 12 h light/dark cycle and free access to pellet food and water.

All animal experiments were designed in accordance with Animal Research: Reporting of *In Vivo* Experiments guidelines,[19] with a commitment to refinement, reduction, and replacement; minimising the numbers of mice; and using biostatistics to optimise mouse numbers (as in our previous work with the mouse TBI model[20,21]). For statistical validity, 10 mice were assigned to the iAr or control group using randomisation lists (https://www.random.org/lists/) for Experiments 1 and 2. Treatment, behavioural, imaging, and histological assessments were done by researchers blinded to the experimental groups.

Ethics statement

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the Istituto di Ricerche Farmacologiche Mario Negri (IRFMN), Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy, which adhere to the principles set out in the following laws, regulations, and policies: Italian Governing Law (D.lgs 26/2014; Authorization no. 19/2008-A issued March 6, 2008 by the Ministry of Health), IRFMN Institutional Regulations and Policies providing internal authorisation for persons conducting animal experiments (quality management system certificate: UNI EN ISO 9001:2008, Registration no. 6121), and the EU directives and guidelines (European Economic Community Council Directive 2010/63/UE). They were reviewed and approved by the IRFMN Animal Care and Use Committee, which includes *ad hoc* members for ethical issues, and by the Italian Ministry of Health (Decree nos D/07/2013-B and 301/2017-PR; authorisation no. 801/2018-PR). Animal facilities meet international standards and are regularly checked by a certified veterinarian who is responsible for health monitoring, animal welfare supervision, experimental protocols, and review of procedures.

Traumatic brain injury

The mice were anaesthetised with isoflurane (induction 3 vol %; maintenance 1.5 vol%) in an N_2O/O_2 (70%/30%) mixture and placed in a stereotaxic frame. Rectal temperature was maintained at 37°C. The mice were then subjected to craniectomy, as described.[22,23] Controlled cortical impact brain injury was induced using a 3-mm-diameter rigid impactor driven by an electromagnetic controlled impact device (Impact One™; Leica, Buffalo Grove, IL, USA) rigidly mounted at an angle of 20° to the vertical plane and applied vertically to the exposed dura mater, between bregma and lambda, over the left parieto-temporal cortex (antero-posteriority −2.5 mm; laterality −2.5 mm), at impactor velocity 5 m s^{-1} and deformation depth 2 mm, resulting in severe TBI.[24] The craniotomy was then covered with a cranioplasty and the scalp sutured.

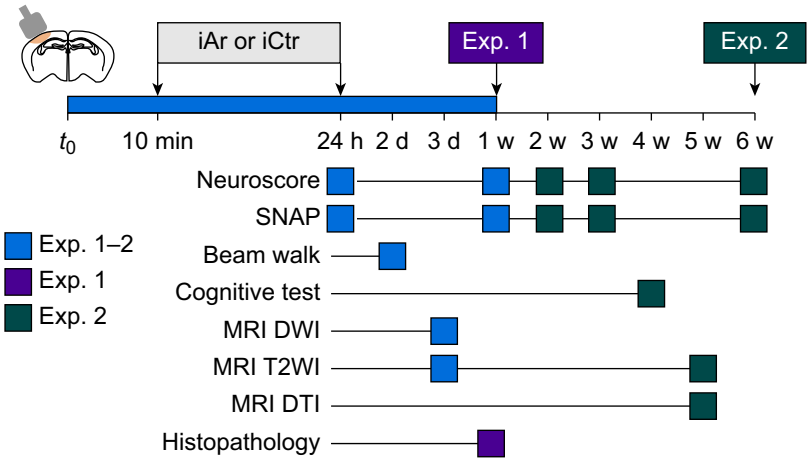

Fig 1. Experimental design. Mice were assigned to two groups (Experiments 1 and 2). Traumatic brain injury (TBI) was induced by controlled cortical impact on the left parieto-temporal cortex in anaesthetised mice. Ten minutes after injury, the animals were treated with argon 70%/O_2 30% (iAr) or room air (iCtr) for 24 h. The violet box indicates measurements in Experiment 1 and the green box in Experiment 2; blue boxes stand for both (Experiments 1–2). d, day; DTI, diffusion tension imaging; DWI, diffusion-weighted imaging; Exp., experiment; SNAP, Simple Neuroassessment of Asymmetric Impairment; T2WI, T2-weighted imaging; w, week.

Treatment

Ten minutes after TBI, an independent operator randomly assigned mice to experimental groups. Argon 70%/O_2 30% (premixed gas from SIAD, Bergamo, Italy), N_2 70%/O_2 30%, or room air was delivered through a 200 L chamber prefilled with the gas (temperature: 21 [1]°C; standard light/dark cycle, in which the mice were kept in their home cage with food/water available) for 24 h (Supplementary Fig. S1a). Total gas flow was measured with a flowmeter and was the same for all treatment groups (saturation: 10 L min^{-1}; maintenance: 1 L min^{-1}). F_{IO_2} was monitored at several times inside the chamber by a portable oxygen analyser (Handi+; Maxtec, Salt Lake City, UT, USA) to confirm that F_{IO_2} concentrations in the chamber were constantly in the range of normoxia (Supplementary Fig. S1b). Soda lime was added as a CO_2 scavenger. Core body temperature measured at the end of the treatment showed that all animals were normothermic (Supplementary Fig. S1c).

In a preliminary experiment, we compared the effects on sensorimotor functions and neuroinflammation for iAr or room air or N_2 70%/O_2 30%-exposed TBI mice. Room air and N_2 70%/O_2 30% TBI mice behaved similarly, and both had significantly worse deficits than iAr mice when tested at 24 h or 7 days (Supplementary Fig. S2a and b), and a comparable increase in ionised calcium-binding adaptor molecule 1 (IBA1)-stained area in the ipsilateral cortex and hippocampus (Supplementary Fig. S2c and d). After confirming the comparable main outcomes of air or F_{IO_2} 30%-exposed TBI mice, we used room air as control for subsequent experiments and referred to it as iCtr.

Behavioural tests

Sensorimotor deficits were rated with the Neuroscore,[23] Simple Neuroassessment of Asymmetric Impairment (SNAP),[25] and beam walk tests,[23] as described, at the times indicated in Figure 1. Cognitive deficits, including learning and memory, were evaluated in the Barnes maze.[26] Methodological details related to behavioural assessments can be found in Supplementary methods.

MRI acquisition and analysis

Anaesthetised mice (isoflurane 1.5–2 vol% in an N_2O/O_2 (70%/30%) were positioned in the magnet at the times indicated in Figure 1. Respiratory frequency was monitored throughout the experiment and body temperature was maintained at 37°C with a heating pad. Brain imaging was done on a 7T small-bore animal scanner (BioSpec®; Bruker, Ettlingen, Germany) running ParaVision 6.01 and equipped with a quadrature 1H CryoProbe™ (Bruker, Ettlingen, Germany) surface coil as transmitter and receiver. Diffusion-weighted imaging (DWI), diffusion tension imaging (DTI), and T2-weighted imaging (T2WI) were obtained to quantify oedema, white matter (WM) damage, and contusional volume,[27] respectively, as detailed, in Supplementary methods.

Tissue processing for histopathological analysis

Mice were euthanised by deep anaesthesia (ketamine 150 mg kg^{-1} and medetomidine 2 mg kg^{-1}, i.p.) and perfused transcardially with 30 ml of phosphate-buffered saline (PBS) 0.1 mol L^{-1}, pH 7.4, followed by 60 ml of chilled paraformaldehyde 4% in PBS. The brains were removed from the skull and post-fixed overnight at 4°C, then dehydrated with sucrose 30% in PBS 0.1 mol L^{-1} for 24 h at 4°C, then frozen in *n*-pentane for 3 min at −45°C, and stored at −80°C until use. Serial coronal brain sections (20 μm thick) were cut on a cryostat (+1 to −4 mm from bregma) at 200 μm intervals for histological analysis. The ipsilateral cortex was analysed over an area 200 μm deep from the edge of the contusion containing the sensorimotor cortex. Ipsilateral hippocampus (il-Hipp), body of the corpus callosum (il-CCB), and fimbria (il-Fi) were selected manually (as detailed in Supplementary Fig. S3a) and quantified on a single slice (−1.6 mm from bregma). Images were analysed using Fiji software

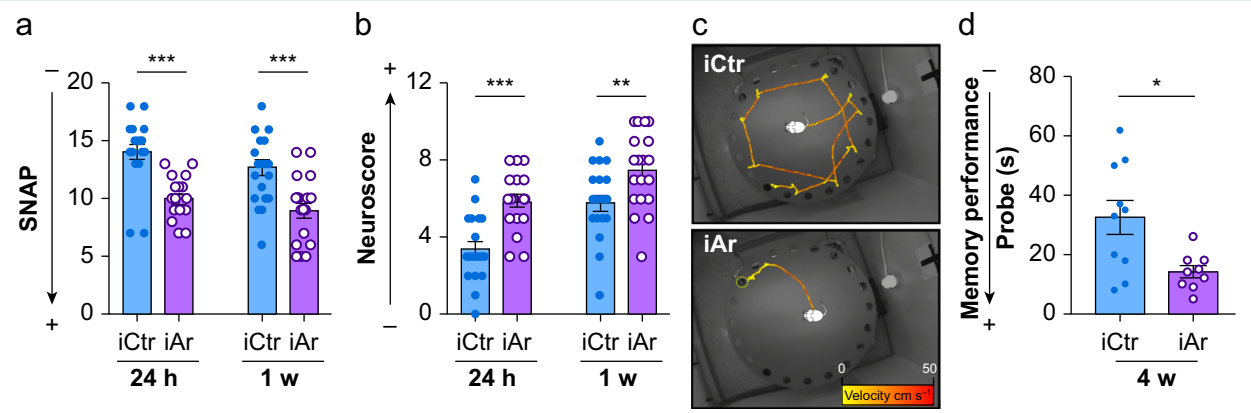

Fig 2. Behavioural deficits. Sensorimotor deficits were rated with the Simple Neuroassessment of Asymmetric Impairment (SNAP) and NeuroScore 1 and 7 days after traumatic brain injury (TBI) in Experiments 1 and 2. Inhaled argon (iAr)-treated mice had a significantly better (a) SNAP score and (b) Neuroscore on both days. Spatial learning and memory were assessed in the Barnes maze 4 weeks after TBI. Barnes maze tracings show the memory performance of a representative animal in the (c) iCtr and iAr groups. (d) Memory performance in the probe trial was better in iAr than iCtr TBI mice. Data are presented as mean (standard error of the mean); n=20 (pooled data from Experiments 1 and 2). SNAP and Neuroscore were analysed by two-way analysis of variance for repeated measurements followed by Tukey's *post hoc* test; probe trial in the Barnes maze by unpaired t-test. *P<0.05; **P<0.01; ***P<0.001. w, week.

(https://fiji.sc/). Neuronal cell loss was evaluated on Nissl-stained sections, whilst WM damage on luxol fast blue (LFB)-stained sections using standard histological protocols.[21,23]

For immunohistochemistry, we used anti-IBA1 (1:200; Wako, Neuss, Germany) and anti-YM1 (1:400; STEMCELL Technologies, Vancouver, BC, Canada) to detect microglia/macrophage activation and polarisation. Immunoreactivity was tested using 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA).[22] Histopathological analysis was done on three 20-μm-thick brain coronal sections per mouse, −0.4, −1.6, and −2.8 mm from bregma. The brain sections were acquired at 20× with an Olympus BX-61-VS microscope (Olympus, Tokyo, Japan). YM1-positive cells were expressed as total number of cells within the defined boundaries over the three sections considered.

Microglia/macrophage morphology was analysed, as described.[28] A size threshold of >25 μm^2 was used to select cells to be analysed for area and aspect ratio. Mean single-cell values for each parameter were used for statistics.

For immunofluorescence, we used anti-CD11b (1:200, Bio-Rad, Hercules, CA, USA) and anti-YM1 (1:400; STEMCELL Technologies). The fluoro-conjugated secondary antibodies were Alexa 488 anti-rat and Alexa 594 anti-rabbit (all 1:500; Invitrogen, Carlsbad, CA, USA), as described.[29] Brain sections were acquired at 40× by a Nikon A1 confocal scan unit managed by NIS-Elements software (Nikon, Tokyo, Japan) with 10% overlapping for stitching, z-axis 10 μm.

Statistical analysis

Data are presented as mean (standard error of the mean [SEM]). Differences between groups over time (group-by-time interaction) for continuous variables were tested using two-way analysis of variance for repeated measurements (time points) followed by Tukey's *post hoc* test. For two groups, unpaired t-test was used; P-values <0.05 were considered significant. Assumptions of normality were checked using the Kolmogorov–Smirnov test. A standard software package was used (version 6.00; GraphPad Prism, La Jolla, CA, USA).

Results

There was no mortality associated with the model or iAr treatment. iAr gave measurable neuroprotective effects both in neurobehavioural and histological outcomes.

iAr accelerated recovery of sensorimotor functions

Three independent tests showed significant improvement of sensorimotor deficits within the first week after injury (Fig 2a and b; Experiments 1 and 2; SNAP: P<0.001 and NeuroScore: P<0.01; beam walk: P<0.05; Supplementary Fig. S4). When long-term sensorimotor deficits were evaluated (Experiment 2), SNAP showed lower deficits in iAr-treated mice up to 2 weeks (P<0.05), but no difference at later times (Supplementary Fig. S5a and b).

iAr improved memory performance after TBI

Four weeks after TBI, we performed the Barnes maze test to evaluate spatial learning and memory function. All animals were able to learn, as reflected by decreasing latencies to find the escape box over the 3day test period (Supplementary Fig. S5). On the probe trial, iAr TBI mice performed significantly better than iCtr (mean latency: 14 [2] and 32 [6] s, respectively; P<0.05), indicating improvement in spatial memory (Fig 2c and d).

iAr reduced acute brain oedema and attenuated delayed WM damage

Three days after TBI, apparent diffusion coefficient (ADC) maps were obtained by DWI MRI (Fig 3a and b). The maps were significantly lower in iAr than iCtr mice both in terms of total

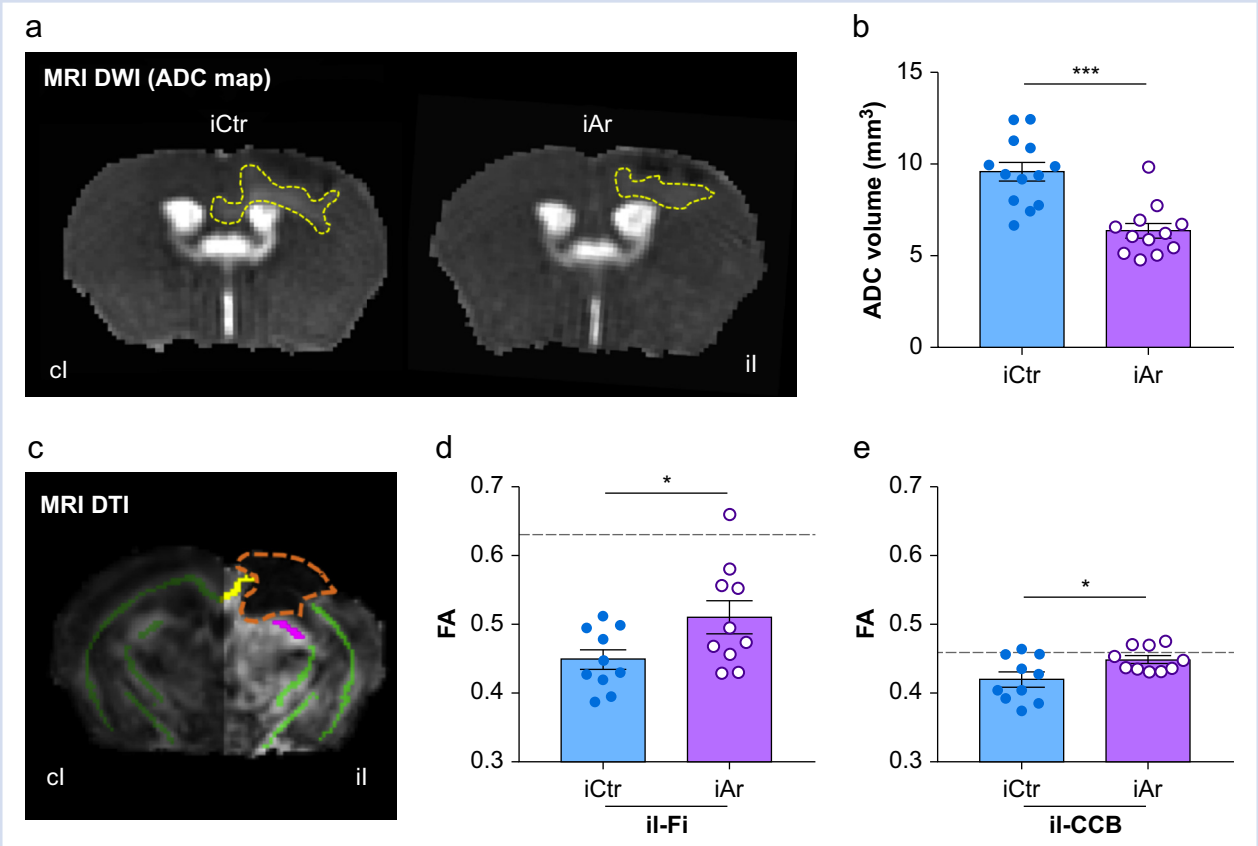

Fig 3. MRI analysis. Diffusion-weighted imaging (DWI) MRI was done 3 days after traumatic brain injury (TBI). Representative apparent diffusion coefficient (ADC) maps show a smaller hyper-intense region reflecting unrestricted water diffusion (vasogenic oedema) around the contusion edge of (a, right) inhaled argon (iAr) compared with (a, left) iCtr TBI mice. (b) iAr reduced vasogenic oedema. Diffusion tension imaging (DTI) MRI was done 5 weeks after TBI. Representative (c) brain coronal section shows ipsilateral (il) white matter tracts (highlighted in pink, yellow, and green), analysed by DTI. DTI metrics at 5 weeks after TBI show a reduction of white matter damage in the ipsilateral fimbria (il-Fi; pink) and in the body of the corpus callosum (il-CCB; yellow), but not in more distal white mater tracks (in green; data not shown). The dottedline indicates the level of the corresponding contralateral white matter region. Data are presented as mean (standard error of the mean); ADC volume: n=13 (pooled data from Experiments 1 and 2); fractional anisotropy (FA): n=10 (Experiment 2). Unpaired t-test. *P<0.05; ***P<0.001.

volume (6.3 [0.4] and 9.6 [0.5] mm^3; P<0.001; Fig 3b) and mean ADC (0.68 [0.04] and 0.76 [0.01] mm^2 ms^{-1}; P<0.05; Supplementary Fig. S6a), reflecting a reduction of vasogenic oedema 3 days post-TBI. Quantification of T2WI showed that iAr and iCtr TBI mice had comparable contusion volumes both at 3 days (14.7 [1.3] and 17.3 [1.2] mm^3) and 6 weeks (16.8 [1.0] and 18.1 [1.1] mm^3) after TBI (Supplementary Fig. S6d).

Five weeks after TBI, DTI analysis showed a reduction of WM damage in the il-Fi and il-CCB (Fig. 3c–e). Compared with iCtr TBI mice, iAr-treated mice had increased in fractional anisotropy (FA) in the il-Fi and il-CCB (P<0.05; Fig 3d and e) with a concomitant reduction of mean diffusivity (MD) in the same WM tracts (P<0.05; Supplementary Fig. S6b and c). In regions more distal to the biomechanical impact (i.e. splenu and genu of corpus callosum, external and internal capsule, optic tract, and anterior commissure), no differences were found (data not shown).

iAr modulated microglial/macrophage activation

One week post-TBI, we found a marked reduction of the IBA1-stained area at the contusional edge, corresponding to the somatosensory cortex (8.1 [0.5] and 10.4 [0.7]% stained area; P<0.05), il-Hipp (9.9 [0.80] and 7.2 [0.91]% stained area; P<0.05), and il-CCB (17.1 [1.39] and 13.2 [1.26]% stained area; P<0.05) in iAr compared with iCtr TBI mice with a tendency towards a reduction in the il-Fi (13.5 [1.9] and 9.6 [0.77]% stained area; P=0.08) (Fig. 4a–e). Quantification of shape parameters for IBA1-positive cells in the somatosensory cortex indicated a reduction in the area (P<0.01) and an increase in the aspect ratio in iAr *vs* iCtr TBI mice (P<0.05; Fig 4f and g), suggesting a less toxic phenotype of myeloid cells.[28] Analysis of the M2-like marker YM1 in the contusional cortex showed significantly more positive cells in iAr mice (P<0.001; Fig. 5a–c).

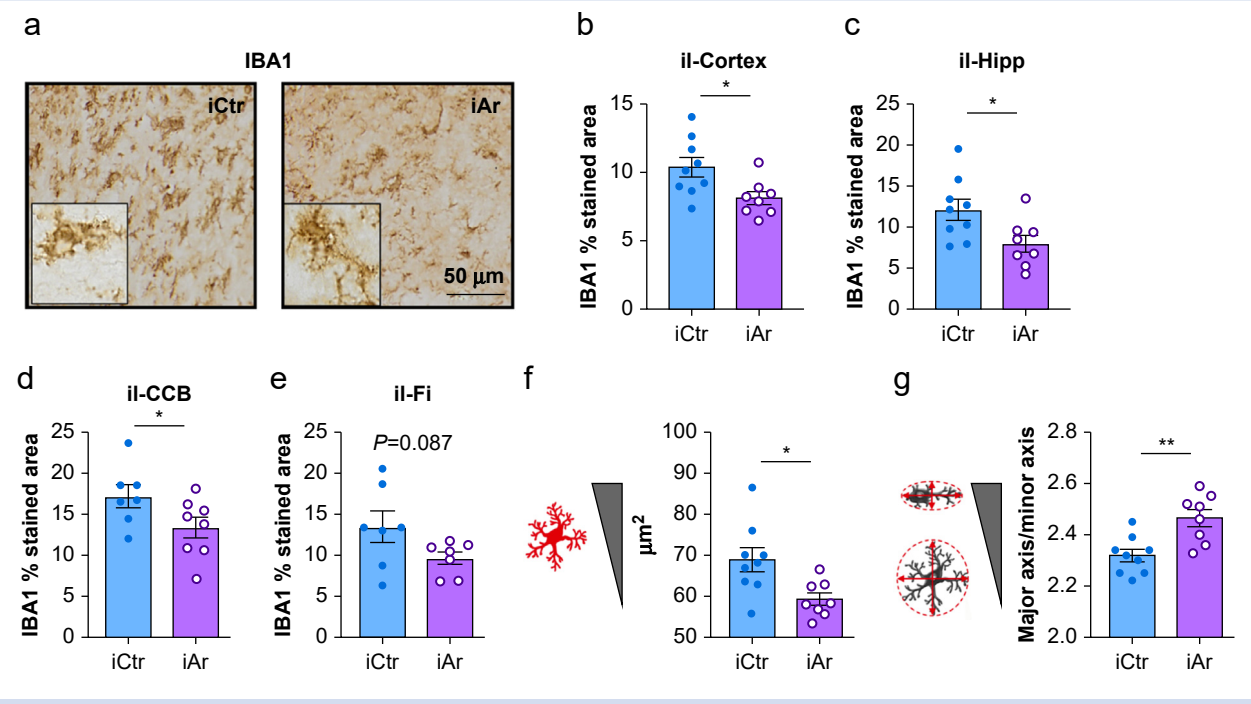

Fig 4. (a) Representative micrographs of IBA1 immunostaining and quantification in the (b) contusional cortex, (c) ipsilateral hippocampus (il-Hipp), (d) body of the corpus callosum (il-CCB), and (e) fimbria (il-Fi) 7 days after traumatic brain injury (TBI). (b–d) The IBA1-stained area was significantly smaller in inhaled argon (iAr) than iCtr TBI mice (e) with a trend towards a reduction in the il-Fi. Shape descriptor parameters, including (f) area and (g) aspect ratio, were quantified in the il-Cortex on IBA1-positive cells. These cells had (f) smaller area with a concomitant increase in the (g) aspect ratio in iAr-treated mice. Data are presented as mean (standard error of the mean). Unpaired *t*-test. *P<0.05; **P<0.01. Bar: 50 µm.

Discussion

We found that iAr 70% administered 10 min after TBI for 24 h (i) accelerated the recovery of sensorimotor function and induced memory improvements up to 4 weeks after TBI; (ii) reduced brain oedema (3 days after TBI) and reduced WM damage (5 weeks after TBI), as shown by MRI; and (iii) ameliorated the neuroinflammatory micro-environment in the contusional cortex, il-Hipp, and il-CCB by reducing microglia/macrophage activation, with pericontusional inflammatory cells displaying markers and morphological parameters of M2-like activated microglia/macrophages.

We characterised the sensorimotor functional impairment up to 6 weeks after TBI. iAr clearly accelerated the recovery of sensorimotor deficits with improvements in the SNAP and Neuroscore already immediately after treatment and lasting up to 2 weeks. Thereafter, the difference between the two groups was no longer clear, although there was a tendency to better performance in iAr-treated mice only in the SNAP test.

As previously reported, mice showed spontaneous partial recovery of sensorimotor functions between 1 and 6 weeks after TBI,[21,30] so it is difficult to detect a treatment effect after the acute phase. However, the data suggest that the effect on sensorimotor functions may be potentiated, so follow-on studies may now be useful to test multiple or longer exposure to iAr. Memory performance at 1 month post-TBI was improved in iAr-treated mice. The ability of iAr to improve cognition after brain ischaemia was shown in pigs 3 days after resuscitation from cardiac arrest.[11] Our results now show that iAr also improves cognition in TBI mice up to 1 month after injury.

Brain oedema is a hallmark of TBI, and it usually aggravates secondary injuries caused by tissue compression around the contusion. While clinically oedema volumes are not routinely utilised, in animal models the extent of oedema is useful to monitor the progression of an intervention.[31,32] Here, we used DWI to reconstruct ADC maps and quantify brain oedema. Hyper-intense areas in ADC maps are associated with increased movement of water molecules outside the cells, reflecting vasogenic oedema.[33] We found a clear reduction of vasogenic oedema around the contusional area in iAr compared with iCtr TBI mice, with no overt effect on contusion volume, indicating that despite a similar degree of tissue loss induced by the biomechanical impact, the blood–brain barrier integrity was preserved better in the surrounding parenchyma in iAr-treated mice.

The effect of iAr on brain water content as an indicator of oedema was previously measured up to 3 days post-injury in subarachnoid haemorrhage rats.[7] The authors found no effect of iAr (50% for 1 h). Whilst pathogenic differences related to the primary injury might explain these contrasting results, it can also be hypothesised that prolonged iAr exposure or a higher iAr concentration may be needed to induce greater neuroprotection after acute brain injury.

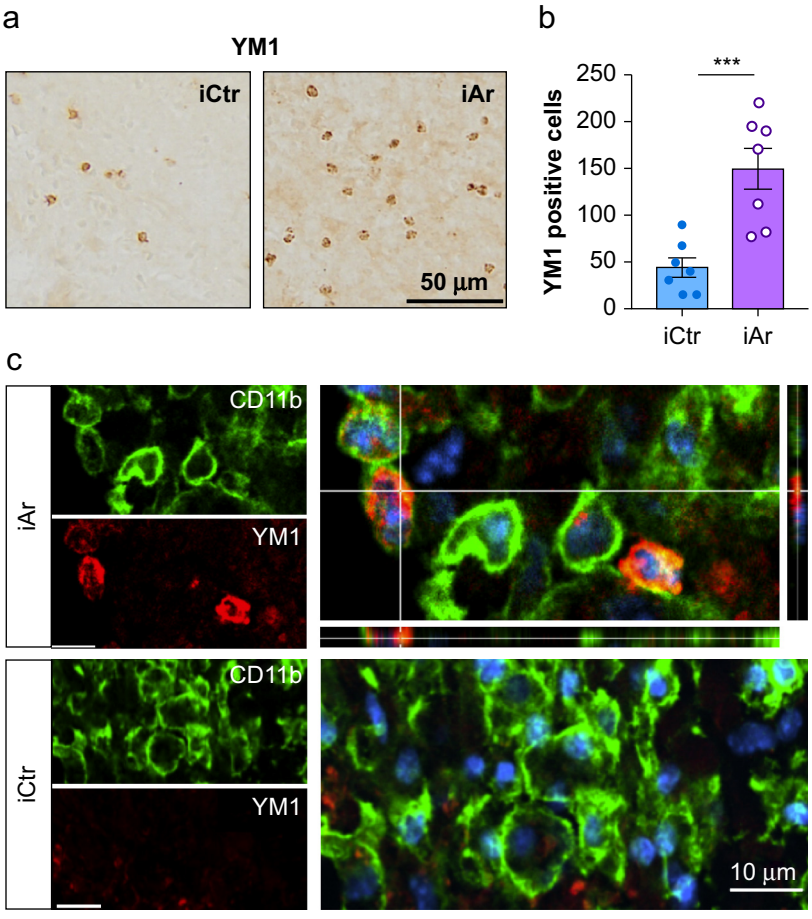

Fig 5. (a) Representative YM1 immunostaining and (b) quantification in the contusional cortex 7 days after traumatic brain injury (TBI). (b) YM1-positive cells increased in inhaled argon (iAr)-treated mice. (c) Microphotographs show co-expression of CD11b (green) and YM1 (red) markers in the contusional cortex of iAr TBI mice 7 days after TBI. Data are presented as mean (standard error of the mean). Unpaired *t*-test. **P*<0.05; ***P*<0.01; ****P*<0.001. Bars: (a) 50 µm and (c) 10 µm.

iAr may mitigate neuroinflammation after brain ischaemia.[10,16] Argon exposure of cortical neuronal cultures subjected to oxygen–glucose deprivation reduced expression of tumour necrosis factor alpha and interleukin 6.[14] More recently, Liu and colleagues[34] showed that short treatment with iAr (50%) had no real effect on microglia/macrophage overall activation, but significantly increased the M2-like marker arginase 1 at the inner boundary of the infarction 7 days after reperfusion in an *in vivo* model of ischaemia in rats. Our data indicate that in TBI, prolonged exposure to iAr leads to a more robust effect on microglia/macrophages, with an overall reduction of IBA1-positive cells in the contusional cortex, il-Hipp, and corpus callosum. At this stage, after TBI myeloid cells display an amoeboid phenotype with enlarged somata and retracted processes.[28] iAr induced morphological changes in IBA1-positive cells in the injured somatosensory cortex towards a more ramified phenotype. Importantly, this morphological switch was associated with a significant increase of the M2-like marker YM1, supporting a role of iAr in mitigating the neuroinflammatory environment, favouring cell polarisation towards a pro-resolving and tissue repair action,[35] and likely contributing to the improvement in sensorimotor function.

Imaging studies were also conducted 5 weeks after TBI to evaluate evolution of contusion volumes and WM damage. In line with the acute assessments, iAr had no effect on contusion volume. However, WM tracts in proximity to the contusion edges, including the il-CCB and il-Fi, had higher FA and lower MD values than in iCtr. The fimbria is the main connection to the hippocampal formation and is vital in spatial memory.[36] Its greater integrity in iAr-treated mice may explain their better performance in the Barnes maze test compared with controls.

Fractional anisotropy is a marker of WM damage,[37] and an increase in MD is associated with axonal degeneration or demyelination.[38] The DTI analysis indicated that iAr reduced markers of WM damage close to the contusion. A trend towards greater WM integrity in iAr mice was detectable by LFB in the il-CCB 1 week after injury. All WM regions showed lower microglia activation in iAr-treated mice at this time point. There is evidence from studies in mice[23] and humans[39] that persistent inflammation may underpin subsequent WM

degeneration. Thus, mitigation of WM inflammation early on may explain the reduction of later WM damage and the better functional outcome despite no overt effect on the contusion volume.

Limitations and future perspectives

The control arm was treated with air (F_{IO_2} 21%), whilst the argon group received F_{IO_2} 30%. This potential confound is mitigated by our initial experiment, where sensorimotor deficits and inflammatory changes were similar in animals treated with air and an oxygen–nitrogen mixture at 30% F_{IO_2}. Most importantly, normoxia (F_{IO_2} 21–30%) was maintained for all conditions, and other confounders, such as hypothermia, were excluded. Protective effects of small changes in F_{IO_2} (<10%) in the range of normoxia are not reported in TBI, making it unlikely that the benefit in the treatment group was simply attributable to the F_{IO_2} 30% used in iAr-treated mice.

iAr is easy to administer and transport, so treatment at the scene of the primary injury, en route to emergency care, could occur with no delay to therapy. Our data provide a proof of principle that iAr can be exploited for therapy in TBI. However, we only tested acute administration 10 min after TBI. Further studies are needed to define the therapeutic window and long-term outcome of iAr in TBI. Defining the patient population that may benefit from this proposed treatment is an additional key preclinical step, and the effects of iAr at lower concentrations (40–60%) should be explored, as patients with TBI with concomitant pulmonary injury often require high F_{IO_2}.

Conclusions

Early administration of iAr after experimental TBI for 24 h had anti-oedematous effects, promoted early recovery of sensorimotor function and persistent improvement of memory with WM preservation in a mouse model of TBI. iAr attenuated maladaptive aspects of microglia/macrophage activation in the contused tissue. Thus, the neuroprotective properties of iAr may provide a useful therapy to improve outcome after TBI.

Authors' contributions

Study design/planning: FM, FFo, AM, GR, ERZ
Study conduct: FM, FFo, AM, RP, FB, DT, EM, ES
Data analysis: FM, FFo, RP, ES, FB, ERZ
Data interpretation: FM, FFo, AM, GC, NS, FFu, SM, RL, GR, ERZ
Drafting of paper: FM, FFo, ERZ
All authors contributed to discussion, data analysis, and final drafting of the paper. All authors take responsibility for the contents of the paper.

Acknowledgements

The authors are grateful to E. Sinelli for technical support.

Declarations of interest

The authors declare that they have no conflicts of interest.

Funding

Institutional funding

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2020.08.027.

References

1. Bragge P, Synnot A, Maas AI, et al. A state-of-the-science overview of randomized controlled trials evaluating acute management of moderate-to-severe traumatic brain injury. *J Neurotrauma* 2016; **33**: 1461–78
2. Maas AIR, Menon DK, Adelson PD, et al. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. *Lancet Neurol* 2017; **16**: 987–1048
3. Zoerle T, Carbonara M, Zanier ER, et al. Rethinking neuroprotection in severe traumatic brain injury: toward bedside neuroprotection. *Front Neurol* 2017; **8**: 354
4. Campos-Pires R, Armstrong SP, Sebastiani A, et al. Xenon improves neurologic outcome and reduces secondary injury following trauma in an in vivo model of traumatic brain injury. *Crit Care Med* 2015; **43**: 149–58
5. Campos-Pires R, Hirnet T, Valeo F, et al. Xenon improves long-term cognitive function, reduces neuronal loss and chronic neuroinflammation, and improves survival after traumatic brain injury in mice. *Br J Anaesth* 2019; **123**: 60–73
6. Gardner AJ, Menon DK. Moving to human trials for argon neuroprotection in neurological injury: a narrative review. *Br J Anaesth* 2018; **120**: 453–68
7. Höllig A, Weinandy A, Liu J, Clusmann H, Rossaint R, Coburn M. Beneficial properties of argon after experimental subarachnoid hemorrhage: early treatment reduces mortality and influences hippocampal protein expression. *Crit Care Med* 2016; **44**: e520–9
8. Trudell JR, Koblin DD, Eger EI. A molecular description of how noble gases and nitrogen bind to a model site of anesthetic action. *Anesth Analg* 1998; **87**: 411–8
9. Loetscher PD, Rossaint J, Rossaint R, et al. Argon: neuroprotection in in vitro models of cerebral ischemia and traumatic brain injury. *Crit Care* 2009; **13**: R206
10. Zhuang L, Yang T, Zhao H, et al. The protective profile of argon, helium, and xenon in a model of neonatal asphyxia in rats. *Crit Care Med* 2012; **40**: 1724–30
11. Ristagno G, Fumagalli F, Russo I, et al. Postresuscitation treatment with argon improves early neurological recovery in a porcine model of cardiac arrest. *Shock* 2014; **41**: 72–8
12. Fahlenkamp AV, Rossaint R, Haase H, et al. The noble gas argon modifies extracellular signal-regulated kinase 1/2 signaling in neurons and glial cells. *Eur J Pharmacol* 2012; **674**: 104–11
13. Harris K, Armstrong SP, Campos-Pires R, Kiru L, Franks NP, Dickinson R. Neuroprotection against traumatic brain injury by xenon, but not argon, is mediated by inhibition at the N-methyl-D-aspartate receptor glycine site. *Anesthesiology* 2013; **119**: 1137–48
14. Zhao H, Mitchell S, Ciechanowicz S, et al. Argon protects against hypoxic-ischemic brain injury in neonatal rats through activation of nuclear factor (erythroid-derived 2)-like 2. *Oncotarget* 2016; **7**: 25640–51
15. Brücken A, Kurnaz P, Bleilevens C, et al. Delayed argon administration provides robust protection against cardiac

arrest-induced neurological damage. *Neurocrit Care* 2015; **22**: 112–20
16. Zhao H, Mitchell S, Koumpa S, et al. Heme oxygenase-1 mediates Nneuroprotection conferred by argon in combination with hypothermia in neonatal hypoxia-ischemia brain injury. *Anesthesiology* 2016; **125**: 180–92
17. Galluzzi L, Blomgren K, Kroemer G. Mitochondrial membrane permeabilization in neuronal injury. *Nat Rev Neurosci* 2009; **10**: 481–94
18. Loane DJ, Kumar A, Stoica BA, Cabatbat R, Faden AI. Progressive neurodegeneration after experimental brain trauma: association with chronic microglial activation. *J Neuropathol Exp Neurol* 2014; **73**: 14–29
19. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother* 2010; **1**: 94–9
20. Zanier ER, Bertani I, Sammali E, et al. Induction of a transmissible tau pathology by traumatic brain injury. *Brain* 2018; **141**: 2685–99
21. Zanier ER, Marchesi F, Ortolano F, et al. Fractalkine receptor deficiency is associated with early protection but late worsening of outcome following brain trauma in mice. *J Neurotrauma* 2016; **33**: 1060–72
22. Zanier ER, Pischiutta F, Riganti L, et al. Bone marrow mesenchymal stromal cells drive protective M2 microglia polarization after brain trauma. *Neurotherapeutics* 2014; **11**: 679–95
23. Pischiutta F, Micotti E, Hay JR, et al. Single severe traumatic brain injury produces progressive pathology with ongoing contralateral white matter damage one year after injury. *Exp Neurol* 2018; **300**: 167–78
24. Brody DL, Mac Donald C, Kessens CC, et al. Electromagnetic controlled cortical impact device for precise, graded experimental traumatic brain injury. *J Neurotrauma* 2007; **24**: 657–73
25. Shelton SB, Pettigrew DB, Hermann AD, et al. A simple, efficient tool for assessment of mice after unilateral cortex injury. *J Neurosci Methods* 2008; **168**: 431–42
26. Attar A, Liu T, Chan W-TC, et al. A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PLoS One* 2013; **8**, e80355
27. Yushkevich PA, Piven J, Hazlett HC, et al. User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability. *Neuroimage* 2006; **31**: 1116–28
28. Zanier ER, Fumagalli S, Perego C, Pischiutta F, De Simoni M-G. Shape descriptors of the 'never resting' microglia in three different acute brain injury models in mice. *Intensive Care Med Exp* 2015; **3**: 39
29. Pischiutta F, Brunelli L, Romele P, et al. Protection of brain injury by amniotic mesenchymal stromal cell-secreted metabolites. *Crit Care Med* 2016; **44**: e1118–31
30. Fox GB, Faden AI. Traumatic brain injury causes delayed motor and cognitive impairment in a mutant mouse strain known to exhibit delayed Wallerian degeneration. *J Neurosci Res* 1998; **53**: 718–27
31. Badaut J, Adami A, Huang L, Obenaus A. Noninvasive magnetic resonance imaging stratifies injury severity in a rodent model of male juvenile traumatic brain injury. *J Neurosci Res* 2020; **98**: 129–40
32. Rodriguez-Grande B, Obenaus A, Ichkova A, et al. Glio-vascular changes precede white matter damage and long-term disorders in juvenile mild closed head injury. *Glia* 2018; **66**: 1663–77
33. Schaefer PW, Grant PE, Gonzalez RG. Diffusion-weighted MR imaging of the brain. *Radiology* 2000; **217**: 331–45
34. Liu J, Nolte K, Brook G, et al. Post-stroke treatment with argon attenuated brain injury, reduced brain inflammation and enhanced M2 microglia/macrophage polarization: a randomized controlled animal study. *Crit Care* 2019; **23**: 198
35. Perego C, Fumagalli S, Zanier ER, et al. Macrophages are essential for maintaining a M2 protective response early after ischemic brain injury. *Neurobiol Dis* 2016; **96**: 284–9
36. de Bruin JP, Moita MP, de Brabander HM, et al. Place and response learning of rats in a Morris water maze: differential effects of fimbria fornix and medial prefrontal cortex lesions. *Neurobiol Learn Mem* 2001; **75**: 164–78
37. Newcombe VFJ, Williams GB, Nortje J, et al. Analysis of acute traumatic axonal injury using diffusion tensor imaging. *Br J Neurosurg* 2007; **21**: 340–8
38. Shanmuganathan K, Gullapalli RP, Mirvis SE, Roys S, Murthy P. Whole-brain apparent diffusion coefficient in traumatic brain injury: correlation with Glasgow Coma Scale score. *AJNR Am J Neuroradiol* 2004; **25**: 539–44
39. Johnson VE, Stewart JE, Begbie FD, et al. Inflammation and white matter degeneration persist for years after a single traumatic brain injury. *Brain* 2013; **136**: 28–42

Handling editor: Hugh C Hemmings Jr