Neuroprotective Effects Of The Inert Gas Argon Onexperimental Traumatic Brain Injury *In Vivo* With The Controlled Cortical Impact Model In Mice

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Abstract

BACKGROUND: Argon has shown neuroprotective effects after traumatic brain injury (TBI) and cerebral ischemia *in vitro* and in focal cerebral ischemia *in vivo*. The purpose of this study is to show if Argon beneficially impacts brain contusion volume (BCV) as the primary outcome parameter as well as secondary outcome parameters such as brain edema, intracranial pressure (ICP), neurological outcome, and cerebral blood flow (CBF) in an *in vivo* model.

METHODS: Subjects were randomly assigned to either argon treatment or room air. After applying controlled cortical impact (CCI) onto the dura with 8 m/s (displacement 1 mm, impact duration 150 ms), treatment was administered by a recovery chamber with 25%, 50%, or 75% argon and the rest being oxygen for 4 h after trauma. Two control groups received room air for 15 min and 24 h, respectively. Neurological testing and ICP measurements were performed 24 h after trauma, and brains were removed to measure secondary brain damage.

RESULTS: The primary outcome parameter BCV and the secondary outcome parameter brain edema were not significantly reduced by argon treatment at any concentration, respectively. There was a highly significant decrease in ICP at 50% argon (p=0.001), and significant neurological improvement (beamwalk missteps) at 25% and 50% argon (p=0.01; p=0.049 respectively) compared to control.

CONCLUSIONS: Similar to prior *in vitro* studies argon exerts its best neuroprotective effects with regard to neurological outcome and ICP at a concentration of 50%. Furthermore, a significant improvement in neurological outcome was observed at an argon concentration of 25%. There was no significant reduction of BCV as the primary outcome parameter.

1 Background

Secondary brain damage emerges as a result of ischemia, excitotoxic events, brain edema, rising intracranial pressure (ICP), blood-brain barrier leakage, low systemic blood pressure, and hypoxia in the course of minutes to days after trauma [1, 2]. Today's therapeutic options focus on reducing the extent of secondary brain damage and thus improving neurological outcome. However, there is no established therapy that specifically targets preventing apoptosis of endangered cells in the penumbra [3].

Argon has shown several promising neuroprotective properties despite being chemically inert. Argon has shown significant reduction of neuronal cell death *in vitro* after mechanical trauma and glucose/oxygen deprivation [3, 4]. Moreover, *in vivo* studies showed protection against excitotoxic stress, i.e. NMDA-injections, and focal ischemia after middle cerebral artery occlusion (MCAO) in rats [4, 5]. The mechanism of neuroprotection mediated by argon remains unclear. Recently, a whole string of possible candidates is being discussed involving hydrophobic interactions at protein cavities leading to altered gene expression via the ERK (Extracellular Signal-Regulated Kinase) 1/2 pathway, modification of enzymes like tissue plasminogen activator (tPA) [6-9]. In contrast,other studies have found an increase in inflammatory mediators after argon treatment following *in vivo* ischemia in rats [10].

This study aims to examine argon in an *in vivo* model of TBI (controlled cortical impact, CCI) and at determining the optimal length, concentration, and timing of posttraumatic argon treatment. The three applied argon concentrations are based on the results of an *in vitro* TBI study [3]. Additionally, the systemic effects of argon were investigated.

The primary outcome parameter was brain contusion volume 24 h after TBI. Additionally, we measured intracranial pressure, neurological outcome and brain water content 24 h after trauma as secondary outcome parameters.

2 Material And Methods

2.1 Animals

Male C57Bl/6 mice (20-22 g) were obtained from a commercial breeder of laboratory animals (Charles River Laboratories, Sulzfeld, Germany). Animals were kept in Makrolon cages in a controlled environment (22.0°C / 55% humidity) and had free access to food and water before and during the experimental phase. Day/night cycles were kept constant with lighting from 8 a.m. to 8 p.m. After the experiments, all animals were anesthetized and sacrificed by cervical dislocation under deep anesthesia.

2.2 CCI

We used the widely established CCI [11, 12]. For analgesia i.p. injections of buprenorphine were applied (0.1 mg per kg bodyweight; mg/kg BW). Animals were put in short isoflurane anesthesia (4-5%vol for induction, 1-2% to maintain) with 30% O_2 and 70% N_2O via facemask. Craniotomy of the right temporal bone was performed under preservation of the dura above the motor cortex. The size of the craniotomy was approximately 5 x 5 mm. CCI (Mouse-Katjuscha 2000, L. Kopacz, University of Mainz, Germany)[13, 14] was as follows: diameter of the impact tip 3 mm; impact velocity 8 m/s; duration 150 ms. The bone flap was then placed back and affixed with histoacryl glue (Braun-Melsungen, Melsungen, Germany). The duration of the surgical procedure did not exceed 15 minutes.

2.3 Study Protocol

Blinding was performed by laboratory personnel who randomly presented the animals to the examiner for surgery and testing. Blinding was dissolved only after the complete assessment of the respective test series. Confounders were not controlled, as the procedure did not vary for each animal.

All groups consisted of 10 animals. A total number of 210 animals were examined in all test series. In the further explanations, the number of animals included in the statistical analysis is shown in tabular form. Only animals that died in the course of the experiments or for which the evaluation was not possible due to technical difficulties were excluded.

Sample sizes in this orientational study were based on previous experiments with this damage model and argon treatment.

1. Test Series: Optimal Argon Concentration

Argon administration at defined concentrations 30 min prior to trauma and continued for 4 h after trauma. The purpose of this very first test series was to measure neuroprotection under optimal circumstances, i.e. administering argon before trauma to ensure optimal brain tissue concentrations although these conditions are unlikely in a clinical setting. All argon was supplied by a commercial producer with a purity level above 99.9% (SWF, Sauerstoffwerk Friedrichshafen, Friedrichshafen, Germany). Medical grade oxygen, nitrogen, and nitrous oxide were readily available in our laboratory.

Control group is exposed to room air for the entire duration of test series 1. All animals were placed in heated, semi-open boxes at a constant temperature of 26 degrees Celsius. For all test series, oxygen and argon levels were continuously monitored during treatment with an oxygen (Dräger Oxydig, Dräger, Lübeck, Germany) and argon meter (Servopro Monoexact, Servomex, Hamm, Deutschland). A continuous fresh gas flow of at least 0.1 l was established with a custom, calibrated flowmeter for oxygen, nitrogen, and argon, leading to a complete exchange of gas in the box within less than 15 minutes. The temperature in the boxes was fixed at 26° C.

Brain Contusion Volumes (BCV):

15-min survival: -no therapy (room air) n=10

24-h survival: -no therapy (room air) n=10

-25% Ar / 75% O_2 n=8

-50% Ar / 50% O_2 n=10

-75% Ar / 25% O_2 n=9

Brain Water Content (BWC) and ICP

24-h survival: -no therapy (room air) n=9

-25% Ar / 75% O_2 n=9

-50% Ar / 50% O_2 n=10

-75% Ar / 25% O_2 n=9

2. Test Series: Optimal Duration of Treatment

Utilizing the determined optimal argon concentration assessment of the optimal duration of argon application with two sub-test series follows. Start of argon 30 min before CCI. 50% argon / 50% oxygen in the therapeutic group corresponding to results of prior in vitro experiments and to our own findings in the first test series were used [3]. Control animals received room air. Moreover, an oxygen control group treated with 50% oxygen / 50% nitrogen was done.

BCV:

24-h survival: -no therapy (room air)   n=9

                     -oxygen / nitrogen 24 h treatment   n=9

                     -oxygen / argon 24h treatment   n=10

BWC and ICP

24-h survival: -no therapy (room air)   n=8

-oxygen / nitrogen 24 h treatment   n=8

                     -oxygen / argon 24h treatment   n=9

3. Test Series: Treatment at Physiological Oxygen Levels

This series was introduced to eliminate any interference that might occur because of elevated oxygen levels. Control animals received 20% oxygen and 80% nitrogen. The animals treated with argon received 20% oxygen, 50% argon, and 30% nitrogen. Start of therapy 30 min before and 24 h after CCI in both subgroups.

BCV:

24-h survival: -no therapy   n=7

                     -argon 24 h treatment n=9

BWC and ICP:

24-h survival: -no therapy   n=10

                     -argon 24 h treatment n=8

4. Test Series: Effects of Argon on MAP and CBF after CCI

After determining the optimal concentration of argon, we analyzed MAP and CBF 30 min before, until 2 h after CCI. Argon therapy or O_2/N_2 was administered at the same time period, when applicable.

BCV:

    2-h survival: -50% O_2 / 50% N_2 n=8

                                    -50% Ar / 50% O_2 n=10

2.4 Neuroscores and Beamwalk Testing

Neurological status was assessed 24 h after trauma after a modified Dixon's and Bederson's neuroscore (table 1) [15-17]. Mice underwent beamwalk testing, crossing a 40 cm long non-slip beam (diameter 1.5 cm) three times; missteps of the left leg were counted during that procedure.

2.5 Histology and BCV

After sacrificing the animals assigned for BCV measurement (after 24 h, exception: 15-min controls) through cervical dislocation in deep isoflurane anesthesia, brains were removed and frozen at -70°C. Coronal slices were prepared with a cryostat. 10 µm coronal slices were collected with an interslice thickness of 500 µm throughout the entire brain. Slices were H&E (hematoxylin and eosin)-stained and contusion areas a_n for each slice were measured, n being the number of slices, using a digital image analyzing software and microscope (Olympus Cell Sense, Olympus Lifescience, Hamburg, Germany). BCV was calculated using the formula

$V_{Contusion}/mm^3 = (a_1 + a_2 + a_3 + \cdots + a_n)*0.5$ mm.

2.6 BWC and ICP Measurement

All animals assigned for ICP and BWC were put in short inhalative anesthesia 24 h after CCI. Contralateral to the trauma side, the temporal muscle was mobilized by blunt preparation and a small trephination was performed in the lateral temporal bone. An ICP microsensor (CP Express, Codman Neuro, Raynham, MA, USA) was inserted in the epidural space of the contralateral hemisphere after being calibrated in distilled water. After 5 min the ICP value was registered. Brains were promptly removed, cerebellum and olfactory

bulbs removed and hemispheres divided. They were then measured wet (W_{Wet}) and again after drying for 24 h at 90° C (W_{Dry}). BWC of the hemispheres was then determined via:

$$P_{Water} = (W_{Wet} - W_{Dry}) / W_{Wet}$$

2.7 MAP and CBF measurement

For this study 18 mice were anesthetized with intraperitoneal injections of medetomidine, midazolam, and fentanyl. As the experiments had to last at least 2.5 hours mice were intubated and mechanically ventilated (MiniVent 845, Hugo Sachs Elektronik, March-Hugstetten, Germany). Gas concentrations were 50% oxygen and 50% nitrogen for all animals prior to CCI. In order to ensure appropriate ventilation, arterial blood gas analyses were taken at regular intervals (after 0 , 30, 90, 150 min).

In order to monitor MAP the femoral arteries were dissected and a fluid filled catheter inserted into the femoral artery and the pressure measured by a manometer (Pressure Monitor BP-1, World Precision Instruments, Sarasota, Florida, USA). To control the cerebral blood flow the right temporal muscle was detached from the skull with a scalpel. The laser Doppler probe (Periflux 5000, Perimed, Järfälla, Sweden) was then fixated directly on the skull. All data were automatically recorded and saved at 1 Hertz via computer interfaces (Labscribe 2, iWorx, Dover, NH, USA). CCI was performed as stated above.

MAP and cerebral blood flow were recorded for 30 minutes prior to CCI in order to have baseline values for all animals. After CCI, 8 control-group animals continued to receive 50% oxygen and 50% nitrogen, whereas 10 argon-treated mice received 50% argon and 50% oxygen. Treatment was performed for two hours with continuous monitoring of MAP and CBF. Afterwards the mice were sacrificed. We refrained from assessing these animals neurologically due to limited validity after long anesthesia.

2.8 Statistical Analysis

Statistical work was performed with SPSS 22 (IBM, Ehningen, Germany). ANOVA with an unpaired t-test analysis was used for ICP, BWC, BCV, and beamwalk testing. For neuroscores a Mann-Whitney-U test was used. All results are displayed as mean value ± standard deviation (SD).

3 Results

3.1 1st Test Series: Optimal Argon Concentration

No animals died during treatment.

Primary Brain Damage

BCV in the 15-min control group, representing primary damage after CCI were 10.5 ± 1.1 mm³. Compared with the 24 h control group (19.4 ± 3.2 mm³), there was a mean growth of BCV by 8.9 mm³ (85% of primary BCV) (p<0.001) due to secondary brain damage.

Effect of Argon on BCV

There were no significant differences in BCV in all groups (Figure 1).

Effect of Argon on ICP and BWC

ICP directly before removal of the brains showed mean ICP values of 44.9 ± 6.4 mmHg (24-h control), 42.3 ± 3.6 mmHg (25% argon, p=0.31), 29.5 ± 6.2 mmHg (50% argon, p<0.001), and 39.2 ± 8.9 mmHg (75% argon, p=0.13) (Figure 1).

Mean BWC in the ipsilateral hemispheres were as follows: 80.72 ± 0.98% (24 h control), 81.27 ± 0.35% (25% argon, p = 0.13), 81.03 ± 0.53% (50% argon, p = 0.40), and 81.40 ± 0.47% (75% argon, p = 0.65). There was significant increase in BWC due to trauma in all groups in the ipsilateral hemispheres (p<0.001) compared to the contralateral hemispheres (Figure 1). Mean BWC in the contralateral hemispheres did not differ significantly (Figure 1).

Neurological Outcome and Beamwalk Test

Beamwalk was assessed 24 h after CCI for all animals except the 15-min control. Cumulative numbers of missteps in 3 walks were 5.8 ± 3.1 (24 h control), 5.2 ± 3.8 (25% argon, p=0.56), 2.8 ± 2.2 (50% argon, p=0.001), and 4.2 ± 3.3 (75% argon, p=0.14). There was a significant reduction of missteps in the group treated with 50% argon (p=0.001) compared to the control group (Figure 2).

Neuroscores were obtained at the same time as the beamwalking test. The scores were 2.1 ± 0.7 (24 h control), 1.47 ± 0.6 (25% argon, p=0.01), 1.7± 0.8 (50% argon, p=0.049), and 1.8 ± 0.6 (75% argon, p=0.21). There was a significant improvement in neuroscores in animals treated with 25% (p=0.01) and 50% argon (p=0.049) when compared to controls (Figure 2).

3.2   2nd Test Series: Optimal Duration of Treatment

Each group contained 19 animals for analysis. One animal of the room air control group and one of the argon-treated group died during treatment, approximately 30 min and 6 h after trauma, respectively. One animal of the oxygen control group died before trauma during preparation of the craniotomy.

Effect of Argon on BCV

BCV was determined as above mentioned after 24 h. There were three groups. Mean BCV were 21.6 ± 2.4 mm³ (room air control), 21.7 ± 4.0 mm³ (control group, oxygen), and 19.7 ± 3.4 mm³ (argon treatment), respectively (n.s.).

Effect of Argon on ICP and BWC

ICP measurements after 24 h were as follows: 45.4 ± 9.3 mmHg (room air control group), 47.4 ± 5.7 mmHg (oxygen control group), and 42.4 ± 4.4 mmHg (argon treatment) (n.s.). BWC in the ipsilateral hemispheres after 24 h showed percentages of 81.0 ± 0.7% (room air control group), 81.1 ± 0.4% (oxygen control group), and 80.8 ± 0.6% (argon treatment) (n.s.). BWC in the contralateral hemispheres were 79.0 ± 0.4% (room air control group), 78.9 ± 0.4% (oxygen control group), and 78.8 ± 0.4% (argon treatment), respectively (n.s.).. There was a highly significant (p<0.001) difference in BWC when comparing ipsilateral and contralateral hemispheres in all groups.

Neurological Outcome and Beamwalk Test

The number of missteps in beamwalk tests did not show any significant difference when comparing argon treatment (3.0 ± 4.3) with room air control (4.0 ± 2.2) (p=0.36) and oxygen control (5.16 ± 2.9) (p=0.08). The neuroscores of argon treated mice (1.7 ± 0.6) improved significantly when compared to oxygen control animals (2.5 ± 0.7) (p=0.001), while there was no significant change when compared to room air controls (1.9 ± 0.7) (p=0.37).

3.3   3rd Test Series: Treatment at Physiological Oxygen Levels

The introduction of an oxygen control group has shown us worse outcomes for animals that received 50% oxygen and 50% nitrogen. Although not significant, the difference between oxygen control and argon treatment prompted us to introduce a test series in which we set oxygen at 20%. 17 animals were included in the analysis in each group. 6 subjects were excluded because of methodical reasons of failed recording. No animal died.

Effect of Argon on BCV

There was no significant difference in mean BCV after 24 h when comparing argon treated mice (25.6 ± 3.1 mm³) with control mice (27.3 ± 1.5 mm³) (p=0.19).

Effect of Argon on ICP and BWC

There was no significant difference in mean ICP after 24 h when comparing argon treated animals (43.7 ± 8.3 mmHg) with the control group (47.6 ± 8.0 mmHg) (p=0.30). In the ipsilateral hemispheres BWC after 24 h was 81.3 ± 0.5% for argon treatment and 81.3 ± 0.4% for controls (n.s.) BWC in the contralateral hemispheres showed no significant difference between argon treatment (79.0 ± 0.4%) and control (79.0 ± 0.3%) (p=0.78).

There was a highly significant (p<0.001) difference between ipsilateral and contralateral hemispheres in both groups in BWC.

Neurological Outcome and Beamwalk Test

There was no significant difference when comparing neuroscores of argon treatment (2.4 ± 0.8) and control (2.1 ± 0.9) (p=0.40). Moreover, there was no significant difference between argon treatment (6.2 ± 3.6) and control (5.3 ± 3.2) (p=0.41).

3.4   4th Test Series: Effects of argon on MAP and CBF after CCI

When comparing the mean values between argon treatment and controls, there was no significant difference in mean CBF (all p-values at p=0.53 or above) and MAP (all p-values at p=0.46 or above) during each interval for the entire recorded period of time (Figures 3 & 4).

4 Discussion

The results suggest a certain neuroprotective effect, namely at a functional level and at lowering ICP. However, these results have to be considered with caution.

There has been no significant reduction of BCV. If at all, we could observe a slight increase of BCV in all groups treated with argon compared to controls. Moreover, BWC of both hemispheres were at almost identical levels in all groups. Somewhat contradictory to these results is the observed reduction of ICP in mice treated with 50% argon. Likewise, the improved functional outcome in mice treated with 25% and 50% argon seem to have to no correlate in the other findings. We suspect that an inhibition of apoptosis leads to an increased number of neurons within the contused tissue and therefore to a better outcome. This could be shown in Loetscher's *in vitro* model of TBI and OGD where propidium iodide staining was used to identify necrotic cells [3]. The most effective concentration was 50% in TBI. However, slices were incubated for 72 h as opposed to the 4 h we used. A similar study has also shown *in vitro* neuroprotection of argon in OGD after 24 h of incubation [18].

Several studies suggest a mechanism of action not involving ion channels. Argon is capable of inducing heat shock proteins via activation of ERK1/2, increasing Bcl-2 expression, increasing expression of inflammatory markers, inhibiting apoptotic proteins like caspase-3, and modifying tPA activity [7-10, 19, 20].

The ERK1/2 pathway is essential in regulation of cell cycle and apoptosis. It has significant importance in oncological studies as defects might lead to uncontrolled activation and therefore uncontrolled growth of tumors.

The antiapoptotic and neuroprotective effect by argon shown via ERK1/2 itself is mediated by decrease of density and expression of TLR (toll-like-receptor) 2 and 4 after rotenone induced cell damage.

Moreover, argon's effect can be attenuated by administering inhibitors of the ERK1/2 and TLR pathway [21].

In an *in vivo* models of stroke, i.e. middle cerebral artery occlusion (MCAO), 50% argon has turned out to reduce total infarct volume and functional outcome after 2 h of ischemic trauma in rats [5]. In this study, rats were administered 50% argon and 50% oxygen via facemask for 1 h after middle cerebral artery occlusion, i.e. intraischemic.

Another study found neuroprotective properties of argon when given *in vitro* after OGD [4]. The same study has shown neuroprotection after intra-striatal injection of NMDA *in vivo* in rats.

NMDA receptors are an important mediator in the development of secondary brain damage, leading to higher intracellular ion influx after massive glutamate release due to widespread depolarization of neurons and consecutive cellular swelling and cell death, hence excitotoxicity [22]. Xenon, another noble gas, has already shown neuroprotection by modifying the glycin-receptor site of NMDA-channels. However, argon does not seem to work through the same mechanism of action [23]. ATP-dependent potassium channels have also been ruled out in another *in vivo* study [24]. A potentiation of GABA receptors due to argon is under discussion [25].

Summarizing all findings of this and former studies, we can assume neuroprotective properties of argon when administered *in vivo* after CCI in mice through inhibition of apoptosis. However, the lack of reduction of BCV and BWC require further studies. This does particularly involve extension of treatment durations as argon was applied for 72 h in the only study that has shown neuroprotection after (*in vitro*) TBI. Additionally, as latest research suggests that argon does not target ion channels but modifies gene expression, a longer treatment duration might enhance these mechanisms and improve results. If the ERK1/2 pathway is indeed the main course of action supplementary drug intervention may further facilitate and enhance antiapoptotic effects [10, 21, 23, 26].

5 Conclusions

In summary, argon exerts neuroprotection mainly regarding functional outcome and to a lesser extent ICP. There is no effect on BCV, BWC, MAP, and CBF. Being efficient in improving functional outcome, argon has the potential to be used as an additional option besides established anti-edematous therapies. However, it has to be taken into consideration that the control group in the first test series received different oxygen levels weakening the informative value of the significant results in this series. More experiments regarding the involved molecular pathways are needed to confirm inhibition of apoptosis and the underlying mechanisms.

6 List Of Abbreviations

BCV Brain Contusion Volume

BWC Brain Water Content

CBF Cerebral Blood Flow

CCI Controlled Cortical Impact

ICP Intracranial Pressure

MAP Mean Arterial Pressure

MCAO Middle Cerebral Artery Occlusion

TBI Traumatic Brain Injury

7 Declarations

7.1 Ethics approval and consent to participate

All animal experiments were registered and conducted with the approval of the Government of Upper Bavaria.

7.2 Consent for publication

Not applicable

7.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

7.4 Competing interests

The authors declare that they have no competing interests.

7.5 Funding

The study was funded by the department for neurosurgery at the university hospital Klinikum Rechts der Isar in Munich of the Technical University Munich.

7.6 Authors' contributions

FS performed all experimental procedures including surgery, treatment, and analysis as described above and was a major contributor to the manuscript. The other authors contributed to the planning and practical implementation of the experiments.

7.7 Acknowledgements

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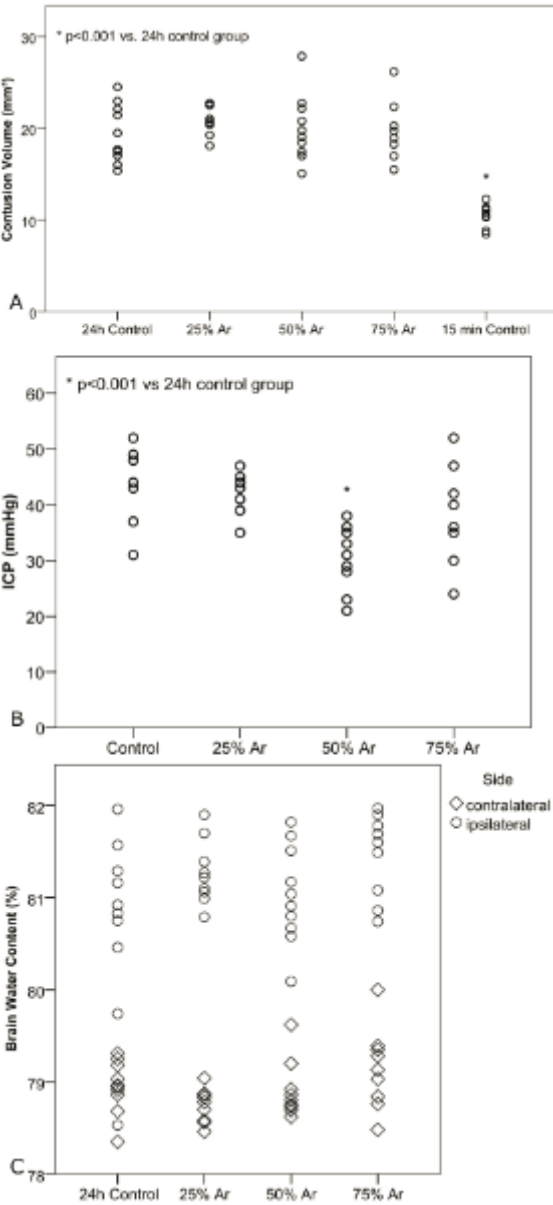
Figures

Figure 1

Treatment was administered 30 min before trauma until 4 h after trauma A: Contusion volumes for the 15 min and 24 h control groups and the animals treated with argon. B: Intracranial pressure (ICP) values 24 h after TBI. C: Brain water content (BWC) values of each hemisphere 24 h after TBI, divided by groups.
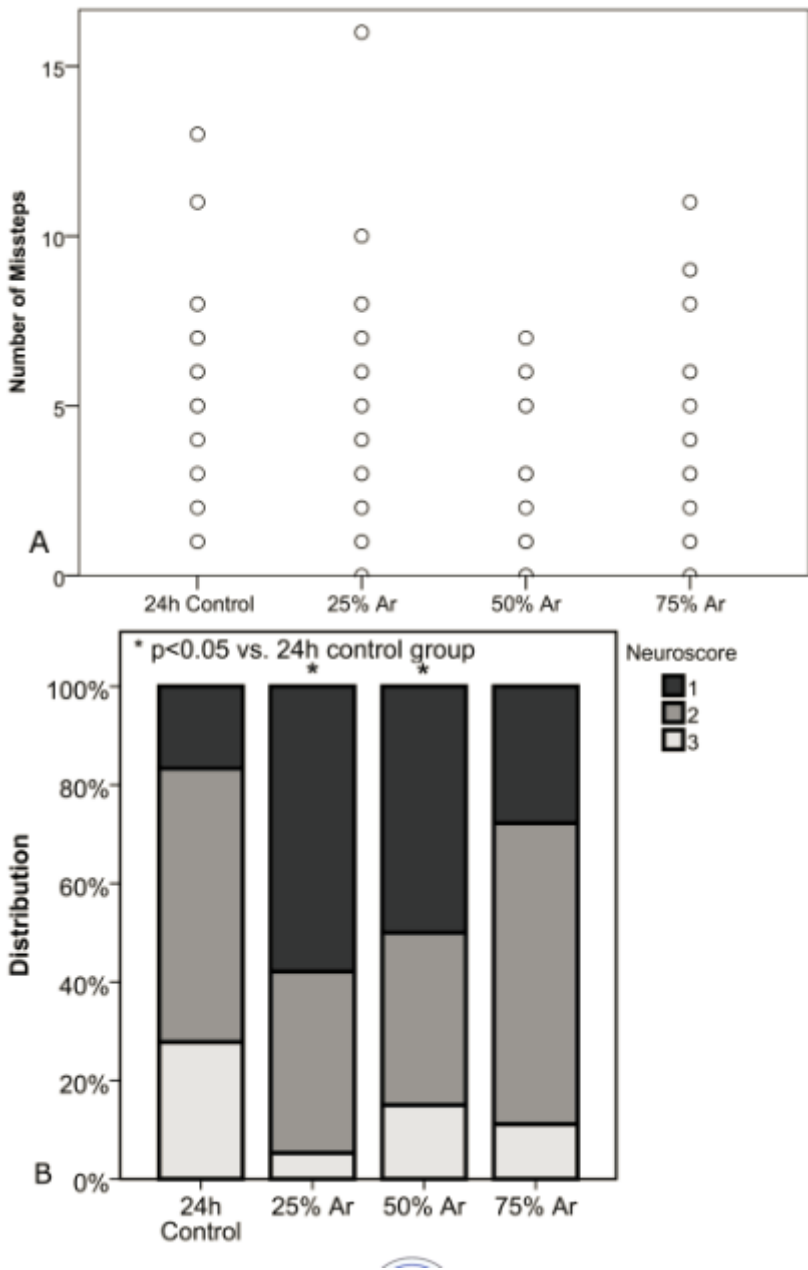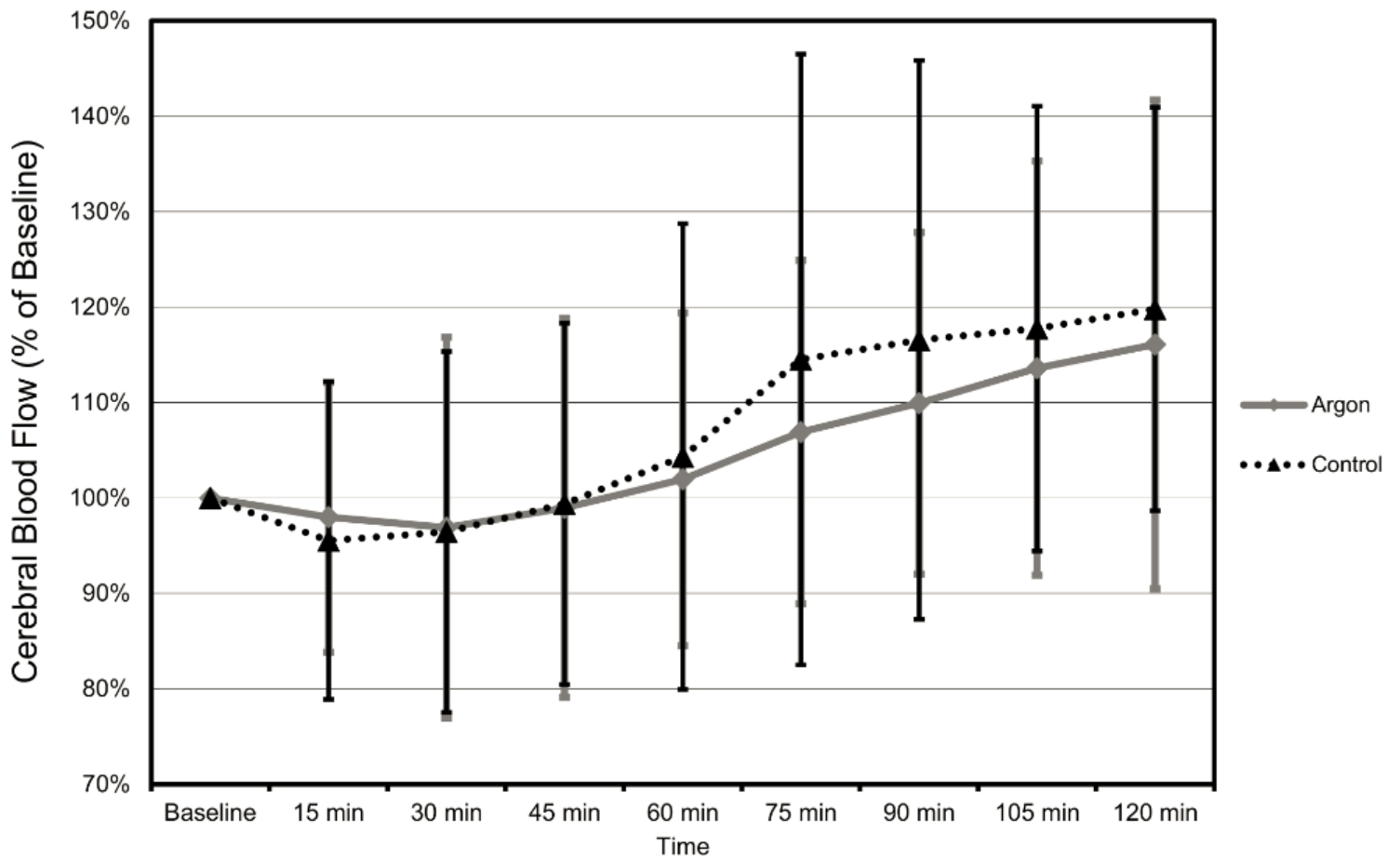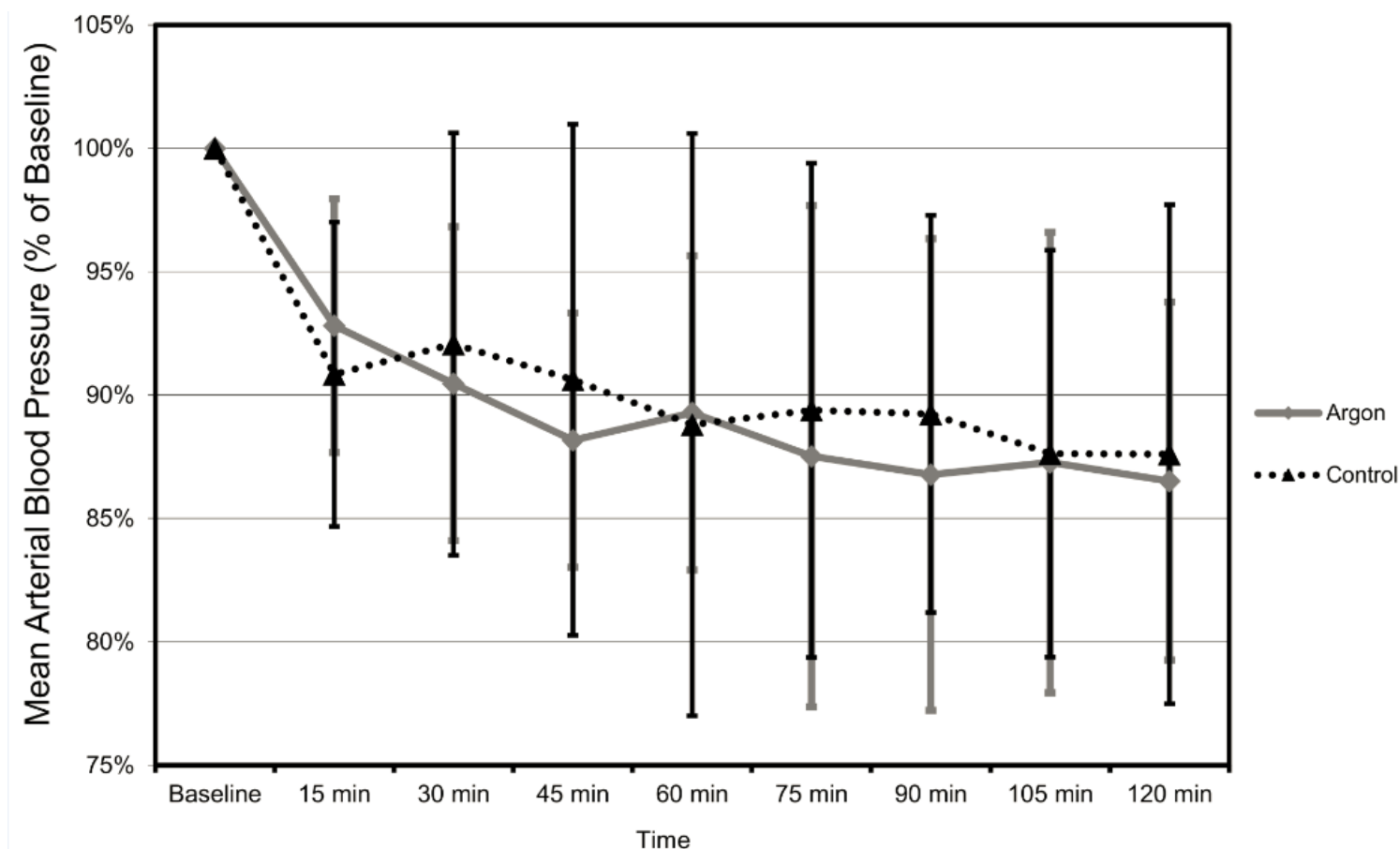

Figure 2

Treatment was administered 30 min before trauma until 4 h after trauma A: Total number of beamwalk missteps 24 h after trauma. B: Dixon's and Bederson's neuroscores 24 h after trauma.

Figure 3

CBF values in time course 2 h after trauma in 15 min intervals. All values in relation to baseline 30 min prior to trauma. Therapy was administered 2 h after trauma.

Figure 4

Mean arterial blood pressure (MAP) values in time course 2 h after trauma in 15 min intervals. All values in relation to baseline 30 min prior to trauma. Therapy was administered 2 h after trauma.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- BMCAuthorChecklistFull.pdf