Argon attenuates the emergence of secondary injury after traumatic brain injury within a 2-hour incubation period compared to desflurane: an in vitro study

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Abstract

Despite years of research, treatment of traumatic brain injury (TBI) remains challenging. Considerable data exists that some volatile anesthetics might be neuroprotective. However, several studies have also revealed a rather neurotoxic profile of anesthetics. In this study, we investigated the effects of argon 50%, desflurane 6% and their combination in an in vitro TBI model with incubation times similar to narcotic time slots in a daily clinical routine. Organotypic hippocampal brain slices of 5- to 7-day-old mice were cultivated for 14 days before TBI was performed. Slices were eventually incubated for 2 hours in an atmosphere containing no anesthetic gas, argon 50% or desflurane 6% or both. Trauma intensity was evaluated via fluorescent imagery. Our results show that neither argon 50% nor desflurane 6% nor their combination could significantly reduce the trauma intensity in comparison to the standard atmosphere. However, in comparison to desflurane 6%, argon 50% displayed a rather neuroprotective profile within the first 2 hours after a focal mechanical trauma ($P = 0.015$). A 2-hour incubation in an atmosphere containing both gases, argon 50% and desflurane 6%, did not result in significant effects in comparison to the argon 50% group or the desflurane 6% group. Our findings demonstrate that within a 2-hour incubation time neither argon nor desflurane could affect propidium iodide-detectable cell death in an in vitro TBI model in comparison to the standard atmosphere, although cell death was less with argon 50% than with desflurane 6%. The results show that within this short time period processes concerning the development of secondary injury are already taking place and may be manipulated by argon.

Key words: argon; desflurane; traumatic brain injury; neuroprotection; organotypic hippocampal brain slices; in vitro model; secondary injury; propidium iodide

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Introduction

It has been estimated that over 1,600,000 patients annually sustain a traumatic brain injury (TBI) in the European Union. Of these patients, 70,000 die and 100,000 suffer from disabilities. The majority of victims are children and young adults.1 Worldwide, TBI is the main cause of death and disability among the young.2 This also entails massive economic implications as demonstrated by Finkenstein et al.,3 who consider the lifetime costs of TBI in the United States to amount to $60 billion a year (total lifetime cost of TBI in 2000). Gustavsson et al.4 and Olesen et al.5 scrutinized the economic cost of brain disorders in Europe in 2010. Total annual cost for TBI was estimated to be €33 billion (direct health care costs, direct non-medical costs and indirect costs). Until now, treatment of TBI remains difficult due to the multitude of trauma mechanisms and
the limited knowledge of pathological pathways.\textsuperscript{6-8}

In the past few years, several studies have investigated the potentially protective and toxic effects of volatile anesthetics on the developing brain. On the one hand, they have revealed mounting evidence of potentially detrimental effects of inhaled anesthetics on the developing brain.\textsuperscript{9-13} On the other hand, considerable data exists that especially specific noble gases might act in a neuroprotective manner in models of hypoxic ischemia and TBI.\textsuperscript{6,9,14-17}

While argon 50\% has been shown to be effective in a TBI model involving incubation periods of 72 hours, desflurane 6\% displayed a rather neurotoxic profile in a similarly conducted study.\textsuperscript{6,18} However, the amount of data available regarding argon’s and desflurane’s effects on cells of the developing brain, which take incubation periods similar to narcotic time slots in a daily clinical routine into account, are sparse. Therefore, we tested the effects of argon in an \textit{in vitro} model of TBI in the developing brain after a clinically relevant incubation time of 2 hours and compared the results to those of desflurane. We also explored how argon’s potentially neuroprotective effects would interfere with desflurane’s rather neurotoxic impact.

**Materials and Methods**

**Ethics statement**

This study was approved by the local Institutional Ethical Review Committee and the animal protection representative at the Institute of Animal Research at the RWTH Aachen University Hospital according to the German animal protection law (TierSchG §4, III).

**Organotypic hippocampal slices**

The organotypic hippocampal slices (OHBSs) were obtained from pups of 5- to 7-day-old mice (C57BL/6N, Charles River Laboratories, Sulzfeld, Germany) and cultured as previously described (Additional Table 1).\textsuperscript{6,7,18-22}

The cell cultures were incubated for 14 days with the growth medium (GM; Additional Table 1) being changed 24 hours after preparation and thereafter regularly every 3 days.

**TBI protocol**

After the incubation period, the GM was exchanged by experimental medium (EM; Additional Table 1) containing propidium iodide (PI). Eventually, baseline fluorescence imaging took place. Slices were then randomly assigned to four non-trauma and four trauma groups in which no intervention (standard atmosphere) and the three respective atmospheric interventions took place (Additional Table 2).

In the TBI groups, TBI was performed as described below and EM was exchanged followed by the incubation of the tissue cultures at 37°C in the respective atmosphere for 2 hours. In the non-trauma groups, EM was exchanged as well before the slices were incubated.

TBI was induced with an apparatus in a similar way to the procedure described by other researchers.\textsuperscript{6,7,18,23} A round metal stylus positioned 7 mm above the hippocampus slice was dropped onto the slice positioned at a marked spot. After the incubation period, final fluorescence images were taken.

**Microscopy and assessment of cell death**

PI, a dyeing agent that rapidly enters cells with damaged membranes and binds to their DNA, was used to assess the proportion of dead cells.\textsuperscript{24} Baseline and final fluorescence images were captured with a fluorescence microscope and MetaVue software (MetaVue, Molecular Devices, Sunnyvale, CA, USA).

The generated images were analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). A histogram of the red channel was generated for each image, showing the sum of all pixels sharing the same gray scale value from 0 to 255. As previous studies have shown, values below a threshold of 100 are caused by background fluorescence.\textsuperscript{6,21,22} That is why only the values of pixels above this threshold were summed up to evaluate the extent of cell injury. The extent of cell injury amounts to the integrated area under the histogram curve exceeding the threshold as demonstrated in \textbf{Figure 1}. The damage of a slice was thus represented in the sum of pixels of its image.
Statistical analysis

In each group of slices, the mean value ± standard error of the mean of the sum of pixels was calculated using SPSS 22.0 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA). The trauma no intervention (standard atmosphere) group served as a reference value, its mean value being normalized to unity.

One-way analysis of variance (ANOVA) was applied to investigate whether the administration of gases resulted in an effect in the trauma and non-trauma groups. In a next step, student’s *t*-test was used post hoc to determine the exact differences in between the different groups. *P* values < 0.05 were considered statistically significant.

In order to enhance comparability and reproducibility of our study we defined necessary checkpoints and developed specific inclusion and exclusion criteria which are presented and discussed in a different paper (unpublished). In short, pre-damaged slices were identified and restrained from entering into the experiment, and unrealistic outliers were detected and eventually excluded as they might distort the final outcome: First, if baseline image histograms already presented more than 1,000 pixels, they were sorted out as they were considered to be pre-damaged. We observed specific morphological peculiarities, such as “holes”, “frayed margins” or “emigrated cells”. As we did know why they occurred, we still included these images if the threshold was met. Some slices presented with a prominent margin. If a margin amounted to more than 250 pixels, this was regarded as a margin stemming from preparation errors and the pixels of the margin were not taken into consideration. In the non-trauma groups, if the number of pixels after incubation was lower than before, it was concluded that the picture taken was not sharp enough and the slices were excluded. Within the course of the experiment we observed that trauma intensities varied over time. Thus, we saw the necessity to define a scope of trauma strength. A boxplot analysis was performed for the trauma no intervention group (group 5; Additional Table 2) and extreme outliers were identified (n = 2) and excluded. The new mean value of this group plus 2× the standard deviation served as an upper threshold for all the other trauma groups. In non-trauma groups there was no reason for a set scope as no manual action was taken and no considerable differences between groups were expected. In these groups, a boxplot analysis was performed separately for each group and outliers were excluded. We subjectively excluded 11 slices that were too small to be considered as the hippocampus and 7 slices that were not clearly hit by the pin. 61% of all 1,459 slices met the proposed inclusion criteria.

Results

Our results demonstrate that the trauma induced significant PI-detectable cell death (*P* < 0.001, no-intervention trauma vs. no-intervention non-trauma group; Figure 2). We analyzed a total of 93 slices in the trauma standard atmosphere group, 136 slices in the trauma argon 50% group, 60 slices in the trauma argon 50%/desflurane 6% group and 52 slices in the trauma desflurane 6% group.

In the non-trauma groups analogously, 171 slices in the standard atmosphere group, 166 slices in the argon 50% group, 92 slices in the argon 50%/desflurane 6% group and 118 slices in the desflurane 6% group were taken into consideration.

Within the four trauma groups, one-way ANOVA did not show a significant effect (*P* = 0.07). Student’s *t*-test revealed no differences of PI-detectable cell death except for the trauma desflurane 6% group vs. the trauma argon 50% group (*P* = 0.015). The difference between the trauma desflurane 6% group and the trauma standard atmosphere group was not statistically significant (*P* = 0.058; Figure 3). A 2-hour incubation in an atmosphere containing argon 50% and desflurane 6% did not result in a significant effect.
in comparison to the other groups (argon 50%/desflurane 6% vs. standard atmosphere: \( P = 0.324 \); argon 50%/desflurane 6% vs. argon 50%: \( P = 0.144 \); argon 50%/desflurane 6% vs. desflurane 6%: \( P = 0.304 \)).

Within the four non-trauma groups, one-way ANOVA showed no significant effect either (\( P = 0.056 \)). Differences determined by student’s \( t \)-test showed a slightly significant difference between the non-trauma argon 50% group and the non-trauma desflurane 6% group (\( P = 0.047 \)). The difference between the non-trauma desflurane 6% group and the non-trauma standard atmosphere group was statistically not significant (\( P = 0.075 \); Figure 4). In the non-trauma groups an incubation in an atmosphere containing argon 50% and desflurane 6% did not result in a significant effect in comparison to the other groups (argon 50%/desflurane 6% vs. standard atmosphere: \( P = 0.229 \); argon 50%/desflurane 6% vs. argon 50%: \( P = 0.139 \); argon 50%/desflurane 6% vs. desflurane 6%: \( P = 0.372 \)).

**DISCUSSION**

We investigated the effects of a 2-hour exposure of argon and desflurane alone as well as combined in an *in vitro* model of TBI on hippocampus brain slices. Our results show that there is no difference in trauma intensity when comparing groups of different gas administrations to the standard atmosphere group. However, when comparing argon 50% to desflurane 6% administration, a neuroprotective effect of argon 50% could be demonstrated. The combination of argon 50% and desflurane 6% did not result in significant differences in comparison to the argon 50% group or the desflurane 6% group.

Previously, *in vitro* studies were conducted with incubation periods of up to 72 hours assuming that the neuroprotective effect of gases unfolds when the secondary injury emerges: Loetscher et al. found that argon significantly reduces cell damage in an *in vitro* model of TBI or oxygen-glucose deprivation (OGD) when applying argon 25%, 50% and 74% for 72 hours after inducing a trauma. Within the TBI set-up, the largest impact was seen in the group exposed to argon 50%. Harris et al. confirmed a neuroprotective effect of argon 50% in a similar TBI model. At 24, 48, and 72 hours, argon 50% reduced the advancement of secondary injury. It was also shown that the injury followed by a mechanical trauma on mice hippocampal slices developed to 32 ± 4% of total injury by one hour and to 51 ± 8% by 6 hours (total injury present at 72 hours). This underlines the importance of the initial time slot right after a trauma with regards to the development of secondary injury.

Surprisingly, there is hardly any data to be found on *in vitro* studies investigating the effect of gases in combination with a shorter exposure time in the first critical hours after a mechanical trauma takes place.

In a study similar to ours, Krings et al. showed that incubation for 2 hours with desflurane 2% and 4% and 6% after performing a trauma resulted in no difference between the groups. However, an incubation time of 24 hours showed a significant increase of trauma intensity in the desflurane 4% and 6% groups compared to the control group. This development was preeminent in the desflurane 4% and 6% groups compared to the control group. This development was preeminent in the desflurane 4% and 6% groups compared to the control group.
hours. Thus, we aimed at investigating cell injury in the early and critical time period after a trauma. Furthermore, this study design provided a more adequate simulation of the clinical setting. To date, it is not clear how even short periods of anesthesia affect the developing brain of the young. Jevtovi-Todoro\textsuperscript{25} pointed to a “therapeutic dilemma” explaining that anesthetics on the one hand have displayed a neuroprotective profile during focal and global brain ischemia but on the other hand also have induced developmental neuroapoptosis. It is of far reaching interest to explore potential neuroprotective and injurious side effects of anesthetics.\textsuperscript{9,10,13}

Our results suggest that within an incubation period of 2 hours neither argon nor desflurane could display a definite neuroprotective or neuro-harming profile in a hippocampal TBI model compared to the standard atmosphere. However, when comparing argon 50% to desflurane 6%, a tendency towards neuro-protectivity related to argon was observed suggesting that argon’s neuroprotective impact as demonstrated by Loetscher and Harris,\textsuperscript{6,17} may start to take place within the first 2 hours. Our findings regarding the effect of desflurane are consistent with Krings et al.\textsuperscript{5,18} result that a 2-hour incubation period does not significantly affect the development of secondary injury in comparison to the control group.

Loetscher et al.\textsuperscript{6} confirmed a neuroprotective effect even if argon administration set for a total of 72 hours was delayed by 2 or 3 hours. Trauma intensity that developed within the third hour was significantly higher in comparison to a 0 and 2 hours delay.\textsuperscript{4} This could hint at the importance of the third hour after a mechanical trauma took place or at an exponential development of emerging secondary injury. Considering our results and Harris et al.'s\textsuperscript{1,17} findings, it would be appropriate to test argon’s and desflurane’s impact for 3–6 hours.

Hypothesizing that there were differences in the two gases’ neuroprotective impact, our study also aimed at scrutinizing the effect when administering argon and desflurane together. Neither argon nor desflurane seemed to dominate the overall effect as no significant differences were seen among the groups.

A lot of information on \textit{in vitro} studies originates from OGD models, \textit{e.g.}, it was shown that “postischemic” argon reduces lactic dehydrogenase levels in such a set-up.\textsuperscript{26} As for desflurane, it was found that preconditioning generated a neuroprotective effect.\textsuperscript{27} A protocol on cortical cells demonstrated a neuroprotective effect when applying desflurane before and throughout OGD.\textsuperscript{28}

Up until now argon’s and desflurane’s mechanisms of action are not fully understood.\textsuperscript{14,26-31} Action at GABAA receptors is discussed to be a possible neuroprotective component; however, it remains unclear whether argon’s neuro-protectivity evolves along these lines.\textsuperscript{14,29,32-34} In a systematic review on argon and its effects as a neuroprotectant, it is summarized that impact on further signaling as Bcl-2 involvement, ERK 1/2-signaling and argon’s oxygen-like properties might play a decisive role whereas N-methyl-D-aspartic acid (NMDA)-receptor signaling or action at potassium channels seem to be less relevant.\textsuperscript{14,17,26,35-39}

Although we could not confirm a neuroprotective effect of desflurane in our TBI-model, several researchers could demonstrate a positive impact. Wang et al.\textsuperscript{27} suggested that involvement of excitatory amino acid transporters may be one of the mechanisms responsible for the positive preconditioning effect of desflurane. Recently, it was also shown that desflurane caused an increase of excitatory amino-acid carrier 1 activity in Xenopus oocytes suggesting that this may be a mechanism for the potential neuroprotective effect.\textsuperscript{40} Schallner et al.\textsuperscript{41} could show that whereas isoflurane postexposure aggravated neuronal cell injury in an \textit{in vitro} model in pre-injured SH-SY5Y cells and in an \textit{in vivo} retina ischemia-reperfusion injury model through activation of p75\textsuperscript{NTR} and NF-κB, desflurane postexposure, which was tested \textit{in vitro}, did not increase neuronal cell injury. Desflurane postexposure reduced NF-κB DNA-binding activity and was not associated with an increase in NF-κB-dependent gene expression.\textsuperscript{41} In their review, Deng et al.\textsuperscript{28,42-44} explained that possible mechanisms besides an improved cerebral blood flow and oxygen supply might implicate reduction of sympathetic activity, inhibition of neuronal apoptosis and preservation of mitochondrial function.

To our knowledge, there is no data to be found on argon or desflurane in an \textit{in vivo} TBI model. However, neuro-protection by argon could be confirmed in several other \textit{in vivo} models warranting further investigation in a TBI protocol. Interestingly, argon administration in \textit{in vivo} models already seems to result in significantly positive effects in a shorter time period: Brücken et al.\textsuperscript{45} showed that a single 1-hour application of argon 70% in rodents subjected to cardiac arrest led to significantly better results in the neurological deficit score and a significant reduction in the neuronal damage index in the CA3/4 region of the hippocampus and in the neocortex. In an \textit{in vivo} rat model of transient middle cerebral artery occlusion, treatment with argon 50% for 60 minutes resulted in a significant reduction in infarct size and composite adverse outcomes.\textsuperscript{46} Recently, it was also shown that argon improved mortality and quantity of vital hippocampal neurons in rats after experimental subarachnoid hemorrhage.\textsuperscript{47}

As for desflurane, the neuroprotective influence of preconditioning demonstrated in \textit{in vitro} experiments could be transferred to an \textit{in vivo} setting. 9-day-old mice undergoing
3 hours of preconditioning with desflurane, isoflurane or sevoflurane 24 hours before being exposed to 60 minutes of hypoxia-ischemia performed better in certain behavioral tests. Histologically, preconditioning did not significantly affect injury scores.\(^{48}\) Moreover, post-ischemic desflurane administration in rats subjected to incomplete cerebral ischemia was investigated and it was found that, when evaluating injury in the early recovery period, histopathological injury and ischemic neurons could thus be reduced.\(^ {49}\) On the other hand, a study comparing apoptotic cell death in neocortical neurons of neonatal mice exposed to desflurane, isoflurane or sevoflurane for 6 hours, revealed a similar neurotoxic profile for all three anesthetics.\(^ {50}\)

**Limitations**

In general, in vitro experiments can only to some extent imitate the in-vivo conditions. Vital parameters such as blood pressure or oxygenation cannot be considered. Furthermore, it is impossible to evaluate whether effects seen histologically in one area of the brain are of relevance to the living organism, i.e., with regards to performance of cognition. However, OHBSs provide a useful tool to simulate the in vivo state enabling investigations into mechanisms of brain synapses.\(^ {51,56}\) The model we worked with has successfully been used in different settings to testing other gases or substances and incubation times.\(^ {6,7,18,19,21,22}\) As OHBSs are very sensitive and can be influenced by many factors, variation in an OHBS TBI model should be taken into consideration. To our knowledge, other researchers have not described how exactly they dealt with i.e., pre-damage. The eligibility criteria mentioned above are discussed in detail in a different paper (unpublished). On the one hand, the criteria present a first approach to cope with the variation in such a TBI model as we could objectively set the same requirements for all groups. On the other hand, several slices were sorted out as they did not meet the inclusion criteria. In order to avoid subjective choices, we recommend setting a defined size prior to the experiment that has to be met concerning the hippocampi and the area of TBI. The morphological peculiarities should be scrutinized to determine whether electrophysiological consequences are entailed.

**Conclusion**

Our findings demonstrate that within a 2-hour incubation time neither argon nor desflurane could affect PI-detectable cell death in an in vitro TBI model in comparison to the standard atmosphere, although cell death was less with argon than with desflurane. These results demonstrate that within this short time period processes concerning the development of secondary injury have already taken place and may be manipulated by argon. Taking into consideration that previous in vivo studies investigating other diseases than TBI have shown that shorter exposure periods result in significantly different outcomes, further investigation into the first hours after TBI is warranted.

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**Author contributions**

LG conducted the experimental laboratory work, analysed and interpreted the data and wrote the manuscript. RB, MK, BK contributed to the laboratory work and assisted in drafting the manuscript. RR participated in the study coordination and helped to draft the manuscript. AH helped to draft the manuscript. MC originated the study, participated in its study design and coordination and helped to draft the manuscript. LG and MC are designated as “guarantors”. All authors read and approved the final text. The requirements for authorship are met and each author believes that the manuscript represents honest work.

**Conflicts of interest**

None declared.

**Research ethics**

The study protocol was approved by the local Institutional Ethical Review Committee at Aachen, Germany. The experimental procedure followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and “Consensus Author Guidelines on Animal Ethics and Welfare” produced by the International Association for Veterinary Editors (IAVE). All efforts were made to minimize the number and suffering of the animals used in the experiments. The paper was prepared in accordance with the “Animal Research: Reporting of In Vivo Experiments Guidelines”(ARRIVE Guidelines).

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Additional file
Additional Table 1: Different media used for culturing of the slices.
Additional Table 2: Groups according to the protocol.

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**Table 1: Different media used for culturing the slices**

| Content                                                                 | PM  | GM  | EM  |
|-------------------------------------------------------------------------|-----|-----|-----|
| Gey’s balanced salt solution (Sigma-Aldrich, Munich, Germany)           | 100%|     |     |
| Eagle minimal essential medium with Earle’s salts (Sigma-Aldrich)      | 50% | 75% |     |
| Hank’s balanced salt solution (Sigma-Aldrich)                          | 25% | 25% |     |
| Heat inactive horse serum (Sigma-Aldrich)                              |     |     | 25% |
| D-(-)Glucose (Roth, Karlsruhe, Germany)                                 |     |     | 25% |
| 1 Vol.% antibiotic/antimycotic solution: penicillin G GIBCO™           |     | x   | x   |
| 10,000 units/mL, streptomycin sulphate 10 mg/mL, amphotericin B         |     |     |     |
| 25 µg/mL (Thermo Fisher Scientific, MA, USA)                            |     |     |     |
| L-Glutamine solution (Sigma-Aldrich)                                   |     | x   | x   |
| HEPES buffer solution (Sigma-Aldrich)                                  |     | x   | x   |
| Propidium iodide (Sigma Aldrich)                                        |     |     | 3 µL/mL |

Note: Three different kinds of media were used in the experiment. First, the pups’ heads were cut off with scissors, followed by the rapid removal of the brains and the direct immersion into an ice-cold preparation medium (PM). Further preparation was performed before the hippocampal slices were arranged onto the membrane of a MilliCell tissue culture insert (MilliCell-CM, Millipore Corporation, Billerica, MA, USA). The inserts were then placed in tissue culture plates and a growth medium (GM) was placed underneath the tissue culture insert. For the experiment, an experimental medium (EM) containing propidium iodide was used.
**Additional Table 2: Groups according to the protocol**

| Group  | Incubation time | Incubator atmosphere                                                                 | Traumatic brain injury |
|--------|-----------------|--------------------------------------------------------------------------------------|-----------------------|
| Group 1| 2 hours         | Standard atmosphere, no atmospheric intervention                                      |                       |
| Group 2| 2 hours         | Argon 50%, CO\(_2\) 5%, O\(_2\) 21%, N\(_2\) 24%                                 |                       |
| Group 3| 2 hours         | Desflurane 6% in Argon 50%, CO\(_2\) 5%, O\(_2\) 21%, N\(_2\) 24%                  |                       |
| Group 4| 2 hours         | Desflurane 6% in CO\(_2\) 5%, O\(_2\) 21%, N\(_2\) 74%                            |                       |
| Group 5| 2 hours         | Standard atmosphere, no atmospheric intervention                                      | x                     |
| Group 6| 2 hours         | Argon 50%, CO\(_2\) 5%, O\(_2\) 21%, N\(_2\) 24%                                 | x                     |
| Group 7| 2 hours         | Desflurane 6% in Argon 50%, CO\(_2\) 5%, O\(_2\) 21%, N\(_2\) 24%                  | x                     |
| Group 8| 2 hours         | Desflurane 6% in CO\(_2\) 5%, O\(_2\) 21%, N\(_2\) 74%                            | x                     |