Author's response to reviews

Title: A Randomized Trial of the Effects of the Noble Gases Helium and Argon on Neuroprotection in a Rodent Cardiac Arrest Model

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Author's response to reviews:

Reviewer #2: Xenon has profound neuroprotective effects after neurological injury and is currently undergoing phase 2 clinical trials in cardiac arrest patients. However, xenon is very costly, which might preclude its widespread use.

In this study, authors investigated helium and argon, which are more available, might also protect central nervous tissues and allow better functional recovery in a rodent model of global cerebral ischemia subjected to cardiac arrest.

They found that the replacement of air with either helium or argon in a 50:50 air/oxygen mixture for 24 hours did not improve histological or clinical outcome in rats subjected to 8 minutes of cardiac arrest.

This study is a meaningful exploration, though the result of this study is different with some published paper.

I focus here on main comments as below:

[Reviewer] The exposure to helium or argon in 50% oxygen for 24 hours did not ameliorate the extent of the damage. Did authors try other concentration of helium or argon mixed with oxygen? After all, others had found that rats treated with 70% argon in oxygen had better neurological outcomes than control rats. In addition, author may try shorter cardiac arrest time (4 min or 6 min) to compare current result to explore the role of helium or argon.

[Answer] The reviewer is right that he mentioned the success of argon neuroprotection with a 70% argon/oxygen mix, which we have mentioned in the background and more in detail in the discussion section. The rational for using a 50% mix was based on in-vitro experiments from H David's group and P Lötscher's group, both found a U-shaped concentration dependant neuroprotective effect of argon. The maximal effect could be detected with a 50%. We are not aware of in vivo experiments where a dose-response was tested. Unfortunately, we did not try different concentrations. But this is an interesting research question, which should be tested in the future. To clarify we have added in the discussion section the following sentence "The rational for administering 50% argon early after the insult in our protocol is based on the work by David et al [18] and Loetscher et al [17], both found a maximum neuroprotective effect with a 50% argon 25% oxygen 25% nitrogen gas mix. Increasing the argon concentration beyond 50% reduced the success of neuroprotection in their experiments." Regarding duration of cardiac arrest, our lab has started with sorter arrest times when we introduced the model before this experiment. With refinement and more experience we had to increase the arrest time to 8 minutes for this experiment because our 6 minutes cardiac arrest rats do not exhibit significant neuronal damage.

[R] There were no differences in the trends of temperature, weight, values of the aBGA (pH, pO2, pCO2), lactat, glucose and potassium (details Table 1). Please select simplify these figures and reserve physical index, such as pH, pO2, pCO2), lactat, glucose and potassium.

[A] We have rearranged the order in the table and in the text to improve readability, as the reviewer correctly points out.

[R] "Limitations of our study include the small numbers of animals in the groups, which decreases the potential to detect a small difference". Why not to try to increase the amount of animal?

[A] With our ambiguous results questioning previous studies e.g. by Brücken et al., the reviewer is right to increase the number of animals to limit the beta error and therefore increase the statistical power of a false negative study result. As we explained in the discussion, we have wide confidence intervals in our statistics, therefore by “simply” increasing the number of the study animals, we would theoretically need 114 animals per group (!). In our opinion, the next experimental steps should look for the differences between our negative trial and the successful ones to find the optimal concentrations, timing and administration durations for the noble gases, as we have said in the conclusions.

[R] Did authors plan to perform this study in vitro models?

[A] 2 of our co-authors (D Grandgirard and S Leib) have experience with organotypic hippocampal slice cultures in the setting of bacterial infections, and we have started to produce these OHC for oxygen and glucose deprivation. So the reviewer can expect more data from us in the future.

Reviewer #3: Synopsis

Zuercher et al. present a new study using their rat cardiac arrest (CA) model that aims to test whether the noble gases helium and argon are able to reduce CA-associated neurodegeneration. This work is similar in experimental design and analysis to their previous publication in BMC Anesthesiology (doi: 10.1186/1471-2253-15-2.) that assessed the effects of different anaesthesia regimes on neuroprotection in the CA model. In their latest work currently under review, the authors show that the noble gases tested provide no obvious clinical or histological benefits. Their results and conclusions are counter to previously published work from other groups, and possible reasons for these discrepancies are provided in the discussion.

Overall, the manuscript is relatively well written, the statistics are nicely performed and the study contains some provocative data given previous findings. However, I have a number suggestions

detailed below, which I believe will improve the flow and understanding of the manuscript, and the interpretation of the data. The majority, if not all, of my concerns can be remedied by improved explanations and further discussion. The points are largely described in sequential order.

Abstract

[Reviewer] 1 In addition to the NDS, TRT, and OFT, the vertical pole test is also used to analyse recovery from CA - this could be included in the Methods and Results section of the abstract.

[Answer] The reviewer is right, we have corrected this omission: “Secondary outcome was evaluation of neurobehavior by daily testing of a Neurodeficit Score (NDS), the Tape Removal Test (TRT), a simple vertical pole test (VPT) and the Open Field Test (OFT).

Background

[R] 2 I appreciate that references are provided later, but a citation or two (perhaps a review paper) to back up this first statement would be useful - "Although many substances affecting neuronal inflammation have neuroprotective properties in in-vitro and animal experiments…"

[A] We have added 2 review articles as reference: Weigl M, et al: A systematic review of currently available pharmacological neuroprotective agents as a sole intervention before anticipated or induced cardiac arrest. Resuscitation 2005, 65(1):21-39., and a more recent publication: Huang L, et al: A systematic review of neuroprotective strategies after cardiac arrest: from bench to bedside (part II-comprehensive protection). Med Gas Res 2014, 4:10.

[R] 3 The second part of the final sentence in the first paragraph, should be amended to say, "the only neuroprotective measure known to have a profound effect on survival and functional outcome after cardiac arrest in humans is temperature management."

[A] This has been done as the reviewer suggested.

[R] 4 Typo: change lessens to lessen.

[A] Has been done, thank you!

[R] 5 This whole section is a bit short and should be expanded to provide a better introduction to the field for the uninitiated reader - the neuronal pathology elicited by cardiac arrest should be elaborated upon; further information on the benefits of xenon and how it was first identified could be given in paragraph two; the results from references 21-24 could be expanded to highlight to the reader the past results showing positive effects of argon in rat and pig CA models.

[A] The reviewer is right in the sense that the first section is very short compared to the Methods or Results section. We have expanded the background section by introducing some cellular and molecular mechanisms which might be responsible for the neuroprotective properties of xenon, and described shortly the results from the successful argon trials in a rat and a swine model of cardiac arrest. The second paragraph now reads: The inert gas xenon is known for its anesthetic properties since 70 years [10], and it displays neuroprotective effects in vitro and in different animal models of neuronal injury [10-15]. The exact molecular mechanisms for neuroprotective properties of xenon are not fully understood and probably multifactorial; xenon modulates neuroinflammation and apoptosis, it induces hypoxia-inducible factor 1alpha (HIF-1alpha), it activates TREK-1 channels, and modulates adenosine triphosphate (ATP)-sensitive potassium channels (KATP channels) [10]. Unfortunately, xenon is not readily available. The more obtainable noble gases helium and argon have also revealed similar properties [16]. In vitro models demonstrated beneficial effects for helium regarding neuron survival in traumatic brain injury [17]. Similar effects are shown for argon in traumatic brain injury [14, 18] and in addition in hypoxia [18, 19] but not in stroke [20].

And:

[22]. Regarding argon, animals studies have demonstrated beneficial properties on neuroprotection in stroke [20, 23], in neonatal asphyxia [21] and in cardiac arrest in rats [24-26] and pigs [27].In the aforementioned rat experiments animals exposed to 40-70% argon after cardiac arrest demonstrated less neurologic dysfunction and less injury in histopathological analysis, even after delayed argon exposure. In the porcine experiments the 6 animals subjected

to a 4 hours treatment with 70% argon displayed significantly less neurological deficits, less increase in neuronen-specific enolase and less histological brain injury.

Materials and Methods

[R] 6 It is stated throughout the manuscript that eight non-ischemic sham animals were included in the study, yet not incorporated in the randomisation process. In the Methods it is mentioned that this is for "internal laboratory purposes (quality of histology, natural history after anaesthesia only)." I think this reasoning should be expanded to at least a full sentence to increase understanding. It seems to me that it would have been very easy to include those extra eight rats in the randomisation process, and it seems odd to have excluded them. Nevertheless, I do appreciate that these animals were not used for the main statistical analyses, and that they are not central to the conclusions of the manuscript.

[A] From a pure scientific point of view the reviewer is right to point toward the sham-group, and especially to the point they were not randomized. From our previous experiments we knew that sham animals without cardiac arrest would have needed general anaesthesia to ventilate them for 4 hours. The factor “4 h deep anesthesia” would preclude comparisons with the other animals, so we forewent the postoperative ventilation (which is not simple per se and poses substantial risk to the animal). With this quite different postoperative period we resigned from randomisation and did the sham animals in a planned way. We expanded the paragraph to explain this and to clarify:

Eight non-ischemic sham animals were added to the study to overview histology and performance in the neurobehavioral tests after anesthesia and surgery only. These animals required a different postoperative care and were thus not randomized and were not included into the statistics. Results of these animals are presented but highlighted as not randomized.

[R] 7 Please provide a reference for this sentence, "All animals underwent the same anaesthesia and instrumentation procedures, and, except the nonischemic sham animals, experienced cardiac arrest/resuscitation with a standardized protocol." I know that there is more information later, but a reference would help here.

[A] We have included the references from our previous studies and from our “methods” paper

[R] 8 Were any quality controls in place to confirm the presence of helium or argon in the oxygen mixtures? i.e. what assurances/positive control can the authors provide that helium and argon were indeed present in the respective treatment groups?

[A] The oxygen concentration in the air-tight cages were measured continuously with the gas module of our S-5 Anesthesia monitor. We have now added:

The oxygen concentration in the delivered gas mix was monitored with the gas module of the Datex S-5 anesthesia monitor.

[R] 9 The sources of the equipment and reagents should be provided - this may help to clarify point 8.

[A] we have added: “Argon and helium was purchased in cylinders via hospital pharmacy (Carbagas Switzerland) and mixed with oxygen from the wall outlet.

[R] 10 Typo: surgically to surgical

[A] Done, Thank you!

[R] 11 How were the manual chest compressions delivered? By hand? How was the 220 bpm maintained if done by hand?

[A] We delivered manual chest compression with the index and middle finger, the rate was controlled by a metronome. We have added: “After 8 minutes resuscitation we started with manual chest compression (two finger approach using the index and middle finger) at a rate of 220 per minute guided by a metronome; and intravenous epinephrine and calcium.”

[R] 12 "sc" should be expanded.

[A] This is done

[R] 13 Typo: wounds closured to wounds closed

[A] Done, Thank you!

[R] 14 Is there any evidence (a reference) that the sevofluorane anaesthesia and fentanyl would have altered apoptosis in the sham-treated animals?

[A] We did not expect any apotosis in sham treated animals, in contrast to the cardiac arrest animals. As far as we know, sevoflurane or fentanyl should not induce apoptosis in adult brains. On the other hand, there is a large debate that volatile anesthetics could inhibit apotosis before and even after neuronal injury. In our opinion adding a large amount of sevoflurane in the postresuscitation care in one group only will create debate of the role of the volatile anesthetics and make comparisons difficult.

For the reader we have changed the sentence to clarify:

The rational for this deviation in the non-ischemic sham group is based on the assumption that the higher amount of sevoflurane might have a impact on apoptosis itself [30, 31], even though we would not expect any neuronal damage in sham operated animals.

[R] 15 Water and food consumption is likely to significantly affect recovery post CA; was it confirmed in any way (levels of remaining food/water) that the treatment groups were eating and drinking appropriately and at similar levels?

[A] After the 24 hours period the animals were kept in standard cages with unlimited access to food and water, we did not have the opportunity to weight remaining food/water. Animals unable to drink or feed themselves will rapidly lose weight, which could be seen in our animals (table), but we did not see differences between groups. Of note, even the sham animals lost weight initially.

[R] 16 Methods are best described in order that the results are presented, e.g. Fluor-Jade B staining before cresyl violet.

[A] We have rearranged the order of methods, results and tables, this is now in line.

[R] 17 A sentence elaboration on each test - NDS, VPT, and TRT- would be very useful here.

[A] We have expanded the paragraph as following: "As secondary outcome we assessed general health and neurological function daily from baseline (pre-arrest) until day 5 using several tests: NDS [33] assessing general behaviour deficits (consciousness, respiration) as well as cranial nerve, motor, sensory and coordination function deficits. In addition, a simple VPT [34, 35] was performed, assessing forelimb strength, ability to grasp and balance as well as the TRT [36], which is considered a sensitive test evaluating sensorimotor integration, originally used to lateralize sensorimotor deficits and adopted and modified to record the time period until removal of both tapes for quantifying neuronal damage. "

Results, Figures, and Tables

[R] 18 More details should be provided on the baseline characteristics at this point - e.g. what haemodynamic analyses were performed, what respiratory variables are being alluded to?

[A] We have expanded and rearranged the presentation of the baseline characteristics to clarify and to bring them in line with the methods section and the details presented in the tables. It reds now: "Baseline characteristics of all animals in terms of haemodynamics (heart rate, mean arterial pressure (MAP)), metabolics/respiration (glucose, lactate, pH, pO2, pCO2), temperature and weight did not differ between groups (Tables 1 and 2)."

[R] 19 I think that the data in tables 1 and 2 should be presented and described before the histologic results, as the latter are presented in Table 3. Please rearrange order to reflect presentation sequence.

[A] This has been done as proposed

[R] 20 For clarity, I would add, ", as assessed by pyknotic cell counts." At the end of this sentence, "In the cresy violet stained slides, no cells with signs of ischemic damage could be seen in the non-randomized nonischemic sham animals."

[A] This has been done as proposed

[R] 21 Typo: cresy to cresyl

[A] Done, Thank you!

[R] 22 Rationale for looking at the CA1 region of the hippocampus should be provided either in the introduction or here in the results, rather than just in the discussion.

[A] We have added the following sentence to give the rational for analysing the CA1 segment of the hippocampus, and refer to our figure 2, where only very few damaged neurons can be found. “Only few injured neurons could be seen in the cortex in the FJB (picture 2 E and F). This due to the uneven distribution of neuronal injury after hypoxia, where the neurons of the CA1 layer are considered as the most vulnerable neurons throughout the brain [38, 39].

[R] 23 Typo: lactat to lactate.

[A] Done, Thank you!

[R] 24 Similar to the introduction, I feel that the results section can be expanded to aid the reader's understanding. For example, a single sentence covers the TRT, NDS and VPT analyses. This is nice and concise; nonetheless, the tables provided are very detailed, and a better description of the analyses in the results, would really ease reader digestion of the data.

[A] We have added a short summary of the tables without providing detailed data: “There were no differences in the trends of haemodynamics, metabolics/respiration, temperature or weight between goups. The animals developed a significant lactic acidosis, but without differences between the groups. MAP increased after resuscitation, at 15 minutes the blood pressure reached a minimum and then started to increase towards baseline pressure again.”

And

“In these tests the animals demonstrate significant impairment on day one, and start to recove until they reach baseline levels on day 4-5 (TRT), or day 2 (NDS and VPT).”

[R] 25 Including the words "degenerating neurons" in Figure 2 title would be useful.

[A] This has been done as proposed. In line with the rearrangement of the results section, the figure legend was updated and is now: Fig. 2: Examples of Fluoro-Jade B staining of the hippocampus. Overview of the hippocampus (A and C) and close-up view of the CA1 region (B) and (D) of a sham animal without cardiac arrest (A and B) and of an animal with cardiac arrest (C and D). Compared to the animal with surgery only, FJB staining (indicating degenerating neurons, white arrowheads) was prominently present in the CA1 region, with occasionally positive cells scattered throughout the hilus region. A few dispersed cells (white arrowheads) are found in the cortex of animals with cardiac arrest stain positive for FJ (E, overview and F, close-up view).

[R] 26 In my opinion, the ordering of information within figures 2 and 3 is a bit clumsy, i.e. Fig. 2B and D are mentioned before A and C. I think a rearrangement would be useful so that the overview panels are provided before the zoomed in panels.

[A] We acknowledge the reviewer opinion and re-organized the arrangement of figures 2 and 3, so that they follow the order by which they are described in the figure legend. For figure 2, all overviews have been now placed on the left side, and for figure 3 on the upper positions.

[R] 27 Figure 2: arrows pointing to regions of staining would be helpful (similar to Figure 3), i.e. in the CA1 and hilus regions.

[A] We followed the suggestion of the reviewer and added arrowheads pointing to FluoroJade positive cells.

[R] 28 Figure 2 typo: FJ to FJB?

[A] We have changed to FJB, and checked the manuscript for further mishaps

[R] 29 I would make sure that there is uniformity in scale size and lettering in Figures 2 and 3.

[A] We agree with the reviewer that uniformity in scale size/lettering would have been better. However, scaling and lettering have been performed with the original software during the capture of immunofluorescence pictures. The observed differences actually reflect resizing necessary for the arrangement of the different panels in the present figures and cannot be modified.

[R] 30 To improve immediate understanding of a figure, I often think that having labels such as, "Sham" and "Cardiac Arrest" somewhere on the figure is useful. I appreciate that this is preference, so will leave this to the authors.

[A] We followed the suggestion of the reviewer and added labels in every panel of figures 2 and 3.

[R] 31 Table 1: MAP is not defined

[A] This has been corrected as proposed

[R] 32 I think emboldening or in some other way highlighting the linear regression P value would make table interpretation easier, i.e. by identifying the headline result of the table section.

[A] This has been corrected as proposed

[R] 33 It is stated in the methods, but a re-statement in table legends of what the Friedman and Wilcoxon tests are comparing would be useful.

[A] We have followed the suggestion of the reviewer and added to clarify in the table legends: “The post-hoc Kruskall Wallis and Friedman tests were performed to compare groups at baseline and the time effect within the groups, but should be interpreted

[R] 34 My preference, again, but I really think that presenting the data included in tables 1-3 in graphical form would drastically improve visualisation and interpretation of the information for the majority of readers. This could perhaps be incorporated into supplementary figures.

[A] We are aware that the large tables are not suitable for a quick overview. The problem is redundancy of the information presented once in the text, then in figures, and then in a detailed table. We are prepared to present the data in figures, but leave this decision to the reviewer and editor. Moving the figures into an electronic supplement is a good proposal; or the tables could be moved into an electronic supplement file.

[R] 35 FJB staining is observed in the CA1 region of the hippocampus and the cortex, while pyknotic cells are only alluded to in the hippocampus; did the authors look for these cells in the cortex also?

[A] With Fluoro Jade B staining the examiner is directed towards to fluorescent neurons, so the very few degenerated cells in the cortex can be identified (see Figure 2 E). It is nearly impossible to find these few cells in the cortex in the CV staining.

Discussion

[R] 36. Please reference the figures/tables within the text so that the reader can easily see to which data you are referring.

[A] This has been done as proposed by the reviewer.

[R] 37. Can the authors speculate on discrepancy between FJB and cresyl violet staining results?

[A[With CV staining there is more interrater variability than with FJB because the cells are counted on their appearance. The discrepancy between the methods is based on that. There is no discrepancy in the results:in CV, there is a "trend" towards more damage in the control group which cannot be seen in the FJB staining, but the interquartile ranges are wide. Regarding the discrepancy between the CV cell count in the noble gas groups and cell layer breadth, we have rewritten and added in the first paragraph of the Discussions: "In the CV staining, the histological analysis tends towards less damaged cells in the CA1 segment of the hippocampus in the noble gas groups (helium better than argon), resulting in a more preserved cell layer of the CA1 segment. But the in the cell layer breadth, argon treated animals have more preserved cells than the rats in the helium group, which is unexpected, regarding the cell count in the CV staining. We believe this difference is based on the difficulties to delineate the borders of the curved CA1 segment exactly in plane microscopy pictures."

[R] 38. This sentence should be clarified: "The pigs used in the Ristagno's experiment had a combined no-flow and low-flow time of 817 seconds."

[A] We have reformulated the sentence for clarity: “As for the pigs used in the Ristagno’s experiment, the time period until return of a stable circulation was even longer, with a combined no-flow and low-flow time of 817 seconds.

[R] 39. This sentence is clumsy and should be ammended: "The duration of administration also differed significantly between our model and the above cited ones, we had a longer administration time (24 hours), compared to 1, respective 4 hours."

[A] We have reformulated the sentence for clarity: “Another significant difference between these above mentioned studies affects the duration of gas administration. In our experiment we had a long exposure for 24 hours compared to 1 hour (Brücken), respective 4 hours (Ristagno).

[R] 40. Reference to "her" air and argon groups should be amended to be more scientific.

[A] We have rewritten these 2 sentences: “As expected, in the Brücken experiments most damage could be found in this specific region, with a neuronal damage index of about 3 (from 0 to 11; 0 indicating no damage, 11 indicating complete destruction) in both (air and argon) groups. The statistically significant difference in neuronal damage between the animals in the air and argon groups were discovered in the less vulnerable neocortex areas and in the CA3/4 segment of the hippocampus with a neuronal damage index of about 1.0 in the air group and 0.7 in the argon group, but there were no significant differences within the CA1 segment between the groups.”

[R] 41. Rationale for not looking at the CA3/4 regions, which were shown to be improved in a previous model, should be provided. If this was performed in details, allusion to this should be made in the results.

[A] We have not looked at the CA3 and CA 4 regions, and therefore cannot provide results. Dr Anne Brücken in Aachen/Germany reported histologic results from different brain regions including basal ganglia/striatum and neocortex in her 2013 BJA paper. In the experiments after this manuscript, she focus on the CA3/4 region and neocortex only, and does not even mention CA1. We have stated this in the Discussion section. The rational for not looking at other brain regions or CA3/4 is that we expect the significant results in the most affected area, which is the

CA1 segment. This is in line with manuscripts published by other groups working with a rat cardiac arrest model (B. Böttiger in Heidelberg/Germany or Xu/LaManna from Cleveland).

[R] 42. MCAO should be defined.

[A] This has been done as proposed

[R] 43. Please change CV to cresyl violet, or alternatively define and use the acronym throughout.

[A] We use cresy violet and removed all acronyma

[R] 44. Beginning sentences with, "But" and "And" is a bit clumsy.

[A] We have tried to improve the readability with some language editing.

I really thank the reviewer for the thoughtful comments, and also for the “courage” of pointing towards minor typos andlanguage problems. This indicates a deep interest on the manuscript and a wish to improve it!

Reviewer #4: This manuscript reports several negative data on use of either argon or helium administration in a rat model of cardiac arrest. The topic is of interest due to the recent attempt of using helium ventilation in human subjects after cardiac arrest. The manuscript appears well written and the methodological work is well documented. Compared to previous experimental works using the same gas even though in different conditions, the findings of this study didn't reach any statistical significance considering the use of non-parametric tests that could have undermined the statistical power. However, the authors have listed a number of limitations of this study including the sample size, the lack of specific assays for neuronal apoptosis as well as dose and duration of gas administration. Although lacking of positive findings, this work may add to this issue.

[Answer] Thank you very much

Reviewer #5: The focus of paper concerns the effects on neuronal system of the administration, 24 hours after resuscitation from cardiac arrest, of gases helium and argon. The results obtained from Zurcher P et al are interstings, although the number of animals used is low. Moreover, there are any results showing the effects on biochemical pathways involved in cell death, even if the authors reported in the discussion that signs of apoptosis is not found within 2 days. However, the authors could investigate others pathways involved in stress conditions as the level of Erks or p38 phosphorylation. Also the brain aerea investigated by authors seems to be restricted. May be the authors could investigate if specifc neurons are affected by treatments performing immunofluorescences against differents neuroreceptors.

[Answer] The reviewer is right with his critics that the number of animals is low, and with the limitations we have described in the discussion section. The proposal to look into specific pathways of delayed cell death like Erks, p38, p52 and others is very interesting. And we will continue. On the other hand, regarding argon, we think (and we mentioned it in the conclusions) that we should focus on better timing/duration and optimal concentration of argon administration in animal experiments to advance from the bench towards the clinics. Our groups is now preparing Organotypic Hippocampal Slice Cultures to advance with respect in the basic science part the reviewer mentions.

Reviewer #6: The authors studied the effects of noble gases helium and argon on neurological and histological outcome after resuscitation from cardiac arrest in animal model. The topic of this research is very important and actual (recently was published study (Brücken, A. et al. Delayed argon administration provides robust protection against cardiac arrest-induced neurological damage. Neurocrit Care 22, 112-120 (2015) which had a similar design and the authors of presented study compare results in discussion).

Critical issue: Ambiguous results

Both the results of neurological assessment and histology are ambiguous. There was almost none neurological deficit in the study group. The neurological deficit was detectable only on day one after resuscitation, but not beyond this time point. This may suggest that brain ischemia was too mild and thus this study group possibly does not represent clinically relevant situation. The results of histology are also ambiguous. The neuronal damage was detected in the hippocampus and there were no significant differences between "non-treated" and "treated" groups. There was, however tendency to lower numbers of pycnotic cells in "treated" groups. We may again speculate whether the more severe brain ischemia could be associated with the higher contrast between "non-treated" and "treated" groups or not. The amount of used animals is another issue, but this was discussed in the article by the authors themselves.

I am afraid that presented results are not sufficient to conclude whether the use of helium/argon after resuscitation from cardiac arrest may be neuroprotective or not.

[Answer]: We agree with the reviewers comments regarding the low numbers of animals which rises the risk of a type 2 error. We have mentioned this in the discussion section, and brought up an example of a power analysis we performed on the base of these results. If stained with FJB only, the group size would be 114 per group, with CV staining 44 animals per group. Looking at the confidence intervals in the regression analysis, a simple increase in number of animals is unlikely that the study would render “positive”.

The reviewer mentions Dr Brücken’s experiments, where she was able to show significant results with less animals. We refer to her manuscripts in the text and explain why, in our opinion, the results are different. We do not discuss her experiments in detail in our manuscript because we can only interpret the results she has presented in her work, but I am not sure that we have contradictory results. In the 2013 BJA manuscript the significant neuropathologic results were in the less damaged brain regions, where on a scale from 0-11 (11 indicating the most severe damage) the difference was 1.0 vs 0.7. In the more affected regions (CA1 segment, with a score of 3) argon did not make a difference. I fully agree with the reviewer that more severe brain damage should demonstrate a greater effect, but this assumption cannot be based on Dr Brücken’s work. In accordance with the reviewers suggestion, we have started a 10 minutes cardiac arrest model to increase severity (but this does not affect this manuscript).

The reviewer has also remarked that in the CV staining both noble gas treated animals have less pyknotic appearing cells than the control animals, but this did not reach statistical significance. If we would have made a “helium-only” experiment, we would have found a positive result in the CV cell count (but not in FJB or cell layer breadth). The inverse is true for a “argon-only” experiment: FJB and CV cell count not significant, cell layer breath positive. We explain this with methodological difficulties with delineation borders of the curved hippocampus on a plane picture. This was also mentioned by another reviewer, and we have added I the discussion section:” The exposure to helium or argon in 50% oxygen for 24 hours did not ameliorate the extent of the damage: we did not find any significant statistical differences in the neurobehavioral tests and in Fluoro-Jade B staining. In the cresyl violet staining, the histological analysis tends towards less damaged cells in the CA1 segment of the hippocampus in the noble gas groups (helium better than argon), resulting in a more preserved cell layer of the CA1 segment. But the in the cell layer breadth, argon treated animals have more preserved cells than the rats in the helium group, which is unexpected, regarding the cell count in the resyl violet staining. We believe this difference is based on the difficulties to delineate the borders of the curved CA1 segment exactly in plane microscopy pictures”.

We still believe that exposure to argon or helium in the way we have done (50% in oxygen) for 24 hours, starting 15 minutes after ROSC will not have beneficial effects. Future studies should include other timing (intra-arrest argon ventilation?) or higher concentrations, and maybe a shorter exposure.