Author’s response to reviews

Title: Inhibition of adenosine A1 receptors abolished the nutritional ketosis-evoked delay in the onset of isoflurane-induced anesthesia in Wistar Albino Glaxo Rijswijk rats

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Version: 2 Date: 11 Jan 2020

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RESPONSE TO REVIEWERS

Zheng Xie (Reviewer 1)

Kovacs Z et al. reported that inhibition of adenosine A1 receptors abolished the nutritional ketosis-evoked delay in the onset of isoflurane-induced anesthesia in Wistar Albino Glaxo Rijswijk rats.

This group published the initial report on this topic in BMC anesthesiology in 2018, suggesting nutritional ketosis delays the onset of isoflurane induced anesthesia. In this article, the authors looked into the possible role of adenosine receptors in ketogenic diet induced delay onset of isoflurane induction in Wistar Albino Glaxo Rijswijk (WAG/Rij) rats, a model strain of human absence epilepsy.

The authors concluded that adenosine A1 receptors may be responsible for the ketosis-induced delay in the onset of isoflurane-induced anesthesia in this strain of rats. They also suggested that the delay effect correlated with blood ß-hydroxybutyrate (ßHB levels).

The finding of this study is a step forward from their previous publication with one of the possible mechanisms underlying the ketogenic diet induced delay of the induction of isoflurane anesthesia. The adenosine A1 receptor may play a significant role in this effect of ketogenic diets. The hypothesis and methods are reasonable.

Response 1 for Reviewer 1:
We appreciate the referee’s opinion that ‘The finding of this study is a step forward from their previous publication with one of the possible mechanisms underlying the ketogenic diet induced delay of the induction of isoflurane anesthesia. The adenosine A1 receptor may play a significant role in this effect of ketogenic diets. The hypothesis and methods are reasonable’.

There are a few main points which I would like the authors to address.

1. The authors used the term "immobility" to define the induction of anesthesia. If I understand it correctly, the authors defined the induction time as the start of 3% isoflurane to the animals stopped moving in the anesthesia chamber. In clinical anesthesia, immobility generally is referred as patients do not respond to noxious stimulation. Induction of anesthesia is referred as the induction of unconsciousness. In rodents, the induction of anesthesia is generally measured by the loss of righting reflex. Sometimes, the animals stop moving but still have righting reflex initially. The immobility is generally tested by tail clamping technique in rodents. The anesthesia level is in a deeper stage for immobility in both humans and rodents than the stage for the induction of unconsciousness. The sites (spinal cord vs brain) of actions and the molecular mechanisms for the induction of unconsciousness and immobility may be different. It is likely the "immobility" measured in the article is under the lighter phase of anesthesia than the phase of "loss of righting reflex". The authors should provide the explanation of their definition "immobility" is different from the one used in the field of anesthesia. This is particularly important when they discuss the mechanisms underlying the induction of anesthesia. The authors should be cautious to refer this finding is relevant to the surgical phase of anesthesia in their abstract.

Response 2 for Reviewer 1:

- Thank you for these suggestions. The text was modified as was suggested by Reviewer. For example, we defined the meaning of ‘immobility’ and conclusion section of the Abstract was also corrected (please, see e.g., the new Abstract of the corrected manuscript: line 43-44, 55-57).

2. Like humans, each individual rat in the same group may respond to the same concentration of isoflurane differently. Crossover design cannot be done in this study. Showing the means of different groups did not show the variability within the group. The author should show the individual data of each rats in the histograms (Fig 1). Using SD, not SE, will also be helpful for readers to see the dispersion.

Response 3 for Reviewer 1:

- We agree with the reviewer, thus part A of the Fig. 2 (partly new figure) as well as Fig. 3 (partly new figure) were changed as was suggested. As our recent study is an extension of our previous studies (as was described in the text of the manuscript), in order to compare responses, all results were expressed as means ± standard error of the mean (S.E.M.) similar to in our previous studies. Moreover, changes in the figures (part A of the Fig. 2 and Fig. 3) may be
enough for readers to see the data dispersion in relation to effects of isoflurane, KEKS and adenosine receptor antagonists on latency to immobility; thus we did not changed the SEM to SD. We hope our decision to keep things consistent with previous results is acceptable for the Reviewer (please, see Fig. 2A and Fig. 3).

3. In table 3, the largest difference (44.25 sec) in mean between groups is group 8 and group 1. It is about 31% delay in the induction of “immobility”. The results are statistically different. But it is relatively insignificant clinically. The study would be better if it includes the effects on the righting reflex and the emergence from anesthesia. In addition, the authors used in Wistar Albino Glaxo Rijswijk (WAG/Rij) rats in this study. The data would be stronger if similar experiments were also done in a normal strain, Sprague-Dawley rats which were used in their initial study. I understand the authors tried to explain the reason to use WAG/Rij in this study in the introduction. I do not mean to ask the authors to perform all these studies for this publication. However, it is important to discuss the limitations and future directions.

Response 4 for Reviewer 1:

- Thank you very much for suggestions. We agree with the reviewer, thus the last part of the Discussion section was completed (please, see last paragraph of Discussion section, p. 15-16).

4. In table 1, the levels of blood βHB were higher with the ketone supplement groups. The data suggested some positive correlation. However, the R value is relatively weak. It is correct for the author to claim some correlation, but not causation in their discussion.

Response 5 for Reviewer 1:

- Indeed, in spite of that R values were strong in our previous study, in the recent study R values were relatively weak. Thus, new studies are needed to find the optimal ketone supplement(s) and the proper dose(s) of these agents for future studies. However, in regards to the weak correlation, the text was modified and we used the word ‘ketosis’ by greater caution. For example, the Conclusion section of both Abstract and the Discussion section was edited and (in relation to our new results and their conclusions) the word ‘ketosis’ was parenthesized in the Discussion section (please, see e.g., last rows of the Abstract and line 370-373 of Discussion section, p. 16).

5. The main finding of this article is the involvement of adenosinergic system in the induction of anesthesia. The authors suggested that exogenous ketone diets may affect adenosinergic system, which is subsequently responsible for the delay of the onset of isoflurane-induced anesthesia in WAG/Rij rats. The authors tested A1 receptors and A2A receptors in their experiments. They showed that the effect of ketosis diets on the induction of anesthesia was completely abolished by DPCPX, an A1 receptor antagonist, but was affected by SCH 58261, an A2A receptor antagonist. While these antagonists were believed as selective antagonists, the concentrations
used in this study were high and might not be selective. Was the same amount of DMSO given to both groups? In addition, only one concentration of each antagonist was administrated by IP. IP was not the best way to deliver the drugs accurately to determine the selectivity. With intravenous injection, the drugs may be delivered more efficiently and reliably with lower concentrations. In the literatures, both A1 and A2A receptors have been reported to be targets of anesthetics. The authors should explain about discrepancies and their conclusion in the discussion. Again, I would like to ask the authors should show the individual data in their histograms to show the variability within the group.

Response 6 for Reviewer 1:

- Selectivity and effective doses of these antagonists were demonstrated previously, thus, investigation of effectiveness/selectivity of these drugs was not the aim of our study. Moreover, tolerability and effectiveness of the used concentration of drugs were also demonstrated. So, we administered these doses of drugs in our recent studies. However, of course, other doses of drugs will also be tested in our future studies as was suggested in the revised version of the manuscript (please, see line 363-367 of Discussion section, page 16).

- We agree with the Reviewer that i.v. administration of different drugs may be more effective and lower doses may be enough for triggering of their effects, but our recent study is an extension of our previous studies (as was described in the text of the manuscript and above (Response 3 for Reviewer 1). Thus, we used these drugs i.p. in the current study as a comparison to be consistent with previous work with these agents.

- Yes, same amount of DMSO was given to both groups. To be more precise we revised the description of the method; Method section was corrected (please, see Methods section, line 173-184, page 8).

- In relation to ‘… both A1 and A2A receptors have been reported to be targets of anesthetics.’ and ‘The authors should explain about discrepancies and their conclusion in the discussion’ the text was completed (please, see the 4th and 5th paragraph of Discussion section, page 13-15).

- The individual data were shown on the partly new figures, as was described above (please, see Response 3 for Reviewer 1).

6. Can the authors measure adenosine levels in the brains of these rats after their behavioral study? If the ketogenic diets affect the adenosinergic system, do the authors have any plan to test other neurotransmitter systems which may play roles in the induction of anesthesia in animals fed with ketogenic diets? The recent review article by Kelz, M at al. in Anesthesia and Analgesia (128: 726-36, April, 2019) summarized the current findings in adenosinergic and other systems which may play important roles in the anesthetic-induced unconsciousness (probably both in the induction unconsciousness and the emergence from unconsciousness).
Response 7 for Reviewer 1:

- Yes, we can measure level of adenosine and others neuromodulators/neurotransmitters (e.g., adenosine, glutamate and GABA) in the brain in our later studies by microdialysis. Thank you for these suggestions, these were incorporated into the revised text and the suggested paper was cited (please see line 358-363 of Discussion section, p. 16)

Minor points:

1. Line 158 on page 7, should the unpublished and preliminary results be disclosed? The full name of SWD should be written.

Response 8 for Reviewer 1:

- The text was modified (please see Methods section, line 156-161, p. 7)

2. Please explain why gavage for 7 days was chosen in this study and the previous paper. Any evidence to suggest adenosine levels will go up after gavage for 7 days?

Response 9 for Reviewer 1:

- We have no data on level of adenosine after 7 days gavage, but we demonstrated previously that 7 days administration of ketone supplements evoked not only significant increase in βHB levels but also anxiolytic effects and suppression of epileptic activity. Thus, 7 days gavage may be enough for alteration/modulation of the biochemical/signaling processes in the brain (such as purine metabolism/signaling), which may be implicated in beneficial influences of ketone supplements. Moreover, as was also described in the revised manuscript, ‘These types and dose of ketone supplements introduced by oral gavage once per day for 7 days effectively induced and maintained ketosis in our previous studies without causing any observable side effects.’ (please, see Methods section, line 147-149, p. 7)

3. Can the authors explain why water gavage for 5 days (adaptation period) was used? It is good to have an adaptation period. Gavage alone is a stressful stimulant to animals. Stressful stimulation alone may change the sensitivity of these animals to anesthetics (Wang, L et al March 2019, PLOS one, https://doi.org/10.1371/journal.pone.0214093)

Response 10 for Reviewer 1:

- Thank you for this suggestion. The text was modified and the suggested paper was cited (please, see Methods section, line 162-163, p. 7).
Thank you very much for the suggestions, helpful criticism and pointing out these limitations above. We believe that the manuscript was greatly improved by the revision based on the feedback of the reviewer. We hope that our responses and revisions, which were carried out in the manuscript, are acceptable for the Reviewer.

Li Ma, MD., PhD. (Reviewer 2)

The authors' previous study has shown that supplementation of nutritional ketones delays the onset of isoflurane-induced anesthesia in rats. In the current study, the authors expanded their study and demonstrated that the delaying effect of ketosis was abolished by blocking of adenosine A1 but not A2A receptors. The study is straightforward and very interesting. However, there are some concerns regarding the experimental design and manuscript discussion.

Response 1 for Reviewer 2:

- We appreciate the referee’s opinion that ‘The study is straightforward and very interesting’.

1. According to the manuscript, ketosis increases adenosine level [14] in the brain tissues and adenosine is closely associated with sleep and sleep-like state, including anesthesia. It is plausible that the authors investigate whether adenosine is involved in ketone-evoked delay of isoflurane-mediated anesthesia. Adenosine A1Rs are inhibitory and A2ARs are excitatory in the ventrolateral/lateral preoptic and basal forebrain, which areas are implicated in the generation of sleep and sleep-like effects [28]. It is easy to understand that stimulation of A1R (inhibitory) is one of the mechanisms by which isoflurane produces anesthesia [49] and A1R specific agonist causes an increase in recovering time from anesthesia [48]. However, it is difficult to comprehend that inhibition of A1Rs (inhibition of inhibitory signaling which is supposed to help to stay awake) could abolish the delay of isoflurane-mediated anesthesia by ketosis. Before moving any further, I think the authors should include an experiment using animals that are not fed with ketones to see whether these agents themselves have any effects on the induction of isoflurane-induced anesthesia. [E.g, three groups: 1. standard diet (SD) alone; 2. SD + A1R antagonist; 3. SD + A2AR antagonist].

Response 2 for Reviewer 2:

We are thankful to the Reviewer for pointing out this shortcoming and suggestion. New experimental groups were included (group 7: SD+DPCPX; group 8: SD+SCH58261) and the text was completed. Please see Figure 3 and Table 3 (partly new figure and table) and: Background section (line 98-102, p. 5); Methods section (line 173-177, p. 8), Results section (line 236-239, p. 11).
2. If these agents themselves do not have any effects on isoflurane-induced anesthesia but only abolish the ketosis-mediated delay of induction of anesthesia, more discussions on the interrelationships among ketones, adenosine A1Rs /A2ARs and anesthesia, as well as interpretations of the discrepancy and possible mechanisms should be included in the manuscript.

Response 3 for Reviewer 2:

The Discussion section was completed (please, see the 4th and 5th paragraph of Discussion section, page 13-15; last 10 lines of Discussion section, p. 16).

We believe that the manuscript was greatly improved by the revision based on the insightful suggestions of the reviewer. We hope that our responses and revisions, which were carried out in the manuscript, are acceptable for the Reviewer.