Creatine supplementation reduces the cerebral oxidative and metabolic stress responses to acute in utero hypoxia in the late-gestation fetal sheep

Nhi T Tran, Greg Kowalski, Anna M Muccini, Ilias Nitsos, Nadia Hale, Rod J Snow, David William Walker, and Stacey Ellery

DOI: 10.1113/JP282840

Corresponding author(s): Stacey Ellery (stacey.ellery@monash.edu)

The following individual(s) involved in review of this submission have agreed to reveal their identity: Barbara S. Stonestreet (Referee #2)

Review Timeline:
- Submission Date: 13-Jan-2022
- Editorial Decision: 04-Mar-2022
- Revision Received: 01-Apr-2022
- Accepted: 22-Apr-2022

Senior Editor: Laura Bennet
Reviewing Editor: Justin Dean

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Dear Dr Ellery,

Re: JP-RP-2022-282840 "Creatine supplementation reduces the cerebral oxidative and metabolic stress responses to acute in utero hypoxia in the late-gestation fetal sheep" by Nhi Tran, Greg Kowalski, Anna M Muccini, Ilias Nitsos, Nadia Hale, Rod J Snow, David William Walker, and Stacey J. Ellery

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

I hope you will find the comments helpful and have no difficulty returning revisions within 4 weeks.

If you need to check to make sure that your Methods section conforms to the principles of UK regulations, you may wish to refer to Grundy (2015):
Grundy (2015) J. Physiol. 2015 Jun 15;593(12):2547-9 https://doi.org/10.1113/JP270818

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available. This link is to the Corresponding Author's own account, if this will cause any problems when submitting the revised version please contact us.

The image files from the previous version are retained on the system. Please ensure you replace or remove any files that have been revised.

REVISION CHECKLIST:

- Summary data must be reported as mean {plus minus} SD or 95% confidence interval
- All table and figure legends with summary data must include the statistical test used in the table/figure and sample size
- Figures with summary data bars must include individual data points, or box whisker plots when n> 30.
- Article file, including any tables and figure legends, must be in an editable format (eg Word)
- Upload each figure as a separate high quality file
- Upload a full Response to Referees, including a response to any Senior and Reviewing Editor Comments;
- Upload a copy of the manuscript with the changes highlighted.

You may also upload:

- A potential 'Cover Art' file for consideration as the Issue's cover image;
- Appropriate Supporting Information (Video, audio or data set https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#supp).

To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

I look forward to receiving your revised submission.

If you have any queries please reply to this email and the Peer Review Coordinator will be pleased to advise.
If revision is not possible, or if you cannot respond to the requests for change, contact us by return email as soon as possible, giving reasons for the difficulties. Withdrawal of the manuscript may be necessary in these circumstances, and instruction will be given on how to proceed. Please note that a paper must be withdrawn before it can be submitted to another journal. If any issues remain unresolved please contact the Publications Office at jphysiol@physoc.org

If you would like help with English language editing, or other article preparation support, Wiley Editing Services offers expert help with English Language Editing, as well as translation, manuscript formatting, and figure formatting at www.wileyauthors.com/eeo/preparation. You can also check out our resources for Preparing Your Article for general guidance about writing and preparing your manuscript at www.wileyauthors.com/eeo/prepresources.

Yours sincerely,

Professor Laura Bennet
Senior Editor
The Journal of Physiology
https://jp.msubmit.net
http://jp.physoc.org
The Physiological Society
Hodgkin Huxley House
30 Farringdon Lane
London, EC1R 3AW
UK
http://www.physoc.org
http://journals.physoc.org

----------------

REQUIRED ITEMS:

-Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled with the revised version of the manuscript. See Information for Authors for further details.

-You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818. A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution’s animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

-Please upload separate high-quality figure files via the submission form.

-Your paper contains Supporting Information of a type that we no longer publish. Any information essential to an understanding of the paper must be included as part of the main manuscript and figures. The only Supporting Information that we publish are video and audio, 3D structures, program codes and large data files. Your revised paper will be returned to you if it does not adhere to our Supporting Information Guidelines

-A Statistical Summary Document, summarising the statistics presented in the manuscript, is required upon revision. It must be on the Journal's template, which can be downloaded from the link in the Statistical Summary Document section here: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics
In summary:

- If \( n \leq 30 \), all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

- If \( n > 30 \), then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- '\( n \) clearly defined (e.g. \( x \) cells from \( y \) slices in \( z \) animals) in the Methods. Authors should be mindful of pseudoreplication.

- All relevant '\( n \) values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

- Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

- Statistics Summary Document completed appropriately upon revision

---

EDITOR COMMENTS

Reviewing Editor:

Methods Details:
Please add details on the origin and source of animals, the animals access to food and water, and the anaesthetic protocols & monitoring.

Comments for Authors to ensure the paper complies with the Statistics Policy:
Please replace SEM with standard deviation in your manuscript. Please complete the statistical summary document.

Please also note the following: "If \( n \) (less than or equal to) 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (the latter two also preferably with data points included) are acceptable formats. Note: if each subject has numerous data points associated with it (e.g. time course data), we would treat 'n' as being each data point, not the number of subjects.

If \( n > 30 \), data points do not need be plotted in the figure but the entire raw dataset must be uploaded either as 'Supporting Information for online publication' (which will be published online with the article) or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

Comments to the Author:
Both reviewers find the manuscript interesting and important, and have indicated that it should go back for revision. However, the reviewers have provided a number of comments on your manuscript. Please address these comments and revise your manuscript accordingly. The manuscript will then be sent back to the reviewers for assessment.
Kind regards

Senior Editor:

When revising the manuscript please review our updated statistics policy. If the number of data points per group per time point makes it too messy to plot individual data then mean and SD are okay, even if group n <= 30. Please also refer to our animal research reporting requirements.

-----------------

REFEREE COMMENTS

Referee #1:

This is a well written and well thought out study by Tran et al. It addresses the potential for a much-needed intervention against an acute hypoxic insult and cerebral damage in utero. Overall the manuscript is timely, it builds on well from their previously published work in the same model (cardiovascular physiology assessed). The cardiovascular parameters previously reported show no differences between saline and creatine groups- implying that any differences reported in the paper herein are not as a result of an altered cardiovascular response between groups. I suggest this manuscript be accepted after my comments below are adequately addressed.

1. Line 67: "many mins"- seems vague and mins has not been defined as minutes yet.

2. Line 67- perhaps give examples of how these in utero hypoxic episodes occur- ie UCO

3. Introduction- please be clear about what you mean by "prolonged hypoxia"- this term could also reference the chronic hypoxaemia experienced by IUGR fetuses.... I realise this is not what you referring to here but it is important to be clear.

4. Line 149: PMID: 32002263 should be referenced here.

5. Line 152: dose of maternal oxytetracycline and fetal ampicillin required.

6. Line 170: how much fluid was collected at each hour?

7. Please report blood gases during the pre UCO period, the UCO and in recovery, as well as the closest blood gas to tissue collection.

8. Table 1- Creatine group had lower pyruvate and glycerol levels during baseline. Did the creatine fetuses also have higher basal po2 and so2 and lower lactate as well? Beginning on page 26 you report the relationships between po2 and so2 during UCO with the percentage change in cerebral metabolites. A good step but if the po2 and So2 did not get as low in the creatine group than the controls- then the study could be confounded by the fact that the insult did not achieve the same level of hypoxaemia between groups. Could you also plot relationships of % change from baseline Po2/So2 against arterial creatine as well as the percentage change in cerebral metabolites during the UCO. This should tell us whether creatine concentrations are driving/inhibiting a change in po2/so2.

9. Line 377: no such thing as a trend. If the interaction was not significant then it was not significant. Change wording please.
10. Figure 2 legend. Please be more clear that the # and ## are referring to a time effect. No interaction. In fact please clearly state in the figure legend that there is no interaction between treatment and time.

11. Line 393- why after 9 minutes? Was it not a 10 min UCO?

12. Line 395: wording... incorrect to have negative correlation with decreased PO2- two negative. Just say negative correlation with PO2. Or - negative correlation with Po2 such that as Po2 decreased...etc

13. Line 414- no trends. If the p value isn’t significant then there is no relationship.

14. Line 484: “higher po2 and so2 during UCO” - is this just because they started higher?ie were basal po2 and so2 higher in the creatine treatment group.

15. Line 485: “and reduced cerebral pyruvate and lactate efflux following UCO”- but both started at lower levels during baseline... so with lower levels to begin how can you be sure that the predictive value does not lie solely in the pre UCO metabolite levels?

16. Line 493: Here it is important to discuss the durations and severity of the insult. Reference: PMID: 32088029

17. Line 502: “by reduced ROS production”- because the creatine fetuses did not get as hypoxaemic during their UCO as salines?

18. Line 651: ofcourse lower detriments in oxygenation during the UCO would result in more favourable energetics. I’m not sure you have fully teased out the driving force from creatine vs the decreased hypoxaemia during the UCO on these outcomes. I suggest a figure instead of a table to enforce this. Eg. Panel 1: Creatine concentrations prior to UCO vs the change in PO2 and So2 from baseline.

Panel 2: Change in PO2 and SO2 vs pyruvate/glycerol/lactate etc during UCO.

This will allow the reader to visualize the data and draw their own conclusions. Ie bring some of your supp graphs into the main body of the paper.

19. You have previously published fetal BP and heart rate in this cohort. Did you place flow probes on the carotid artery? This would allow you to calculate cerebral oxygen delivery and assess that against your metabolite parameters?

Referee #2:

General Comments

This study examines the effects of pre-treatment with intravenous creatine on microdialysis samples of hydroxyl radicals (•OH) and interstitial metabolites (lactate, pyruvate, glutamate, glycerol, glycine) after a brief (10 min) umbilical cord occlusion on from fetal sheep at 131 days of gestation. These are technically exceedingly difficult and complex studies to perform. The studies appear to have been carefully performed. There is very little data on direct microdialysate sampling
from the fetal brain in utero. The manuscript is well written and clear. The authors conclude that fetal creatine treatment decreased cerebral outflow of •OH, which was associated with an improvement in cerebral bioenergetics after acute hypoxia.

Although the basic study design and results are clear, there are some concerns regarding the correlational analyses. It is not clear that all the many correlational analyses add that much to the paper. There is serious concern regarding the supplementary data 2 C. and D. in which the entire correlations depend upon one point in both the glycerol AUC and the Glutamate AUC. These correlations should not be included.

Additional comments are listed below.

Specific Comments

1. It appears to reduce duplication of some of the ewes in the study, animals were included from a series published in 1985. Although this is understandable because of the high cost and enormous effort that go into these studies, it remains possible that there could be a drift in the results over time that could increase the variability in the experiments.

2. It is not clear that the many correlational analyses add much to the studies. If you do enough correlations, something will show a significant correlation. What do the correlations mean?

3. Supplementary data 3. There appears to be 2 populations with the saline and creatine clustering separately. The 2 populations need to be adjusted with a "dummy coding" variable. It is suggested that the authors consult a statistician regarding the validity of these correlations.

4. Overall, the effects of creatine appear relatively modest. The most important data is Fig. 1.

5. The differences in Fig. 2 are very modest.

6. Line 501 "unreported observation". This should not be included. Include the data or the statement be omitted.

END OF COMMENTS
Dear Editorial Board of the Journal of Physiology,

We thank you and the reviewers for your thorough assessment of our manuscript (JP-RP-2022-282840) titled ‘Creatine supplementation reduces the cerebral oxidative and metabolic stress responses to acute in utero hypoxia in the late-gestation fetal sheep.’

Our response to all of the reviewers’ comments are outlined below. We have also made extensive changes to our manuscript accordingly. We have removed some of the correlation analyses following the suggestion of Reviewer 2, and we have reassessed some parameters at the suggestion of Reviewer 1. We believe these changes strengthen the results of this study and would like to highlight that these changes did not result in major changes to the reported outcomes of this study nor are there major changes to the discussion.

We trust that you now find our manuscript suitable for publication.

Yours sincerely,

Stacey Ellery

Dr Stacey Ellery, BBioMedSc (Hons), PhD
NHMRC Peter Doherty Early Career Research Fellow

The Ritchie Centre
Hudson Institute of Medical Research
Monash University
27 - 31 Wright Street, Clayton
Victoria, Australia 3800
ph: +61 (3) 8572 2870
www.hudson.org.au
Reviewer #1 comments:

This is a well written and well thought out study by Tran et al. It addresses the potential for a much-needed intervention against an acute hypoxic insult and cerebral damage in utero. Overall the manuscript is timely; it builds on well from their previously published work in the same model (cardiovascular physiology assessed). The cardiovascular parameters previously reported show no differences between saline and creatine groups- implying that any differences reported in the paper herein are not as a result of an altered cardiovascular response between groups. I suggest this manuscript be accepted after my comments below are adequately addressed.

We thank the reviewer for their close appraisal of our study and have addressed all comments below.

1. Line 67: “many mins”- seems vague and mins has not been defined as minutes yet.

2. Line 67- perhaps give examples of how these in utero hypoxic episodes occur- ie UCO

“many mins” was used to account for the fact that the exact duration of hypoxia cannot be accurately defined as it is highly dependent on a multitude of factors i.e., gestational age, adequate vs inadequate fetal growth, developmental maturation, mild vs severe, single or repeated, individual capacity to tolerate hypoxia etc. We have however clarified:

   Line 75-77: “Transient episodes of acute asphyxia such as those caused by nuchal cords, placental abruption or umbilical cord compression can range from <1 to 30 minutes or more…”

3. Introduction- please be clear about what you mean by "prolonged hypoxia"- this term could also reference the chronic hypoxaemia experienced by IUGR fetuses.... I realise this is not what you referring to here but it is important to be clear.

We have reworded for clarity.

   Line 80-81: “However, as the acute asphyxic event progresses, hypoxia and acidosis worsen…”

   Line 87: “At a cellular level, acute hypoxia causes a…”

4. Line 149: PMID: 32002263 should be referenced here.

We have now added this reference.

5. Line 152: dose of maternal oxytetracycline and fetal ampicillin required.

We have now included this information.

   Line 173-176: “…during which time antibiotics were administered daily (ewe: [50 mg in 5 mL] oxytetracycline, intravenous injection; fetus: ampicillin [100 mg in 1 mL] suspended in 25 IU.ml-1 Heparinised saline, intravenous injection; intra-amniotic ampicillin [400 mg in 4 mL] suspended in 25 IU.ml-1 Heparinised saline)”

6. Line 170: how much fluid was collected at each hour?

We have now included this information.

   Line 195-196: “…increase dialysate volume collection to 120 µL/h.”
7. Please report blood gases during the pre UCO period, the UCO and in recovery, as well as the closest blood gas to tissue collection.

We have now included these data as supplementary data 2; please also note these data have been reported in our previous manuscript (see Figure 2 in Tran et al., 2021).

Line 337-338: “Raw arterial blood gases and metabolites (-30 min, +9 min and +72 hours relative to UCO) are reported in Supplementary data 2.”

8. Table 1 - Creatine group had lower pyruvate and glycerol levels during baseline. Did the creatine fetuses also have higher basal po2 and so2 and lower lactate as well?

The creatine fetuses did not have a higher basal pO2 and sO2 or lower lactate over baseline time period as has been reported in our previous manuscript (Tran et al., 2021), but when assessing using a t-test only at -30 min we found creatine fetuses had significantly lower lactate – this therefore supports presenting the relationships as % change from baseline. This data is reported in supplementary data 2 as suggested by the reviewer to be included.

Beginning on page 26 you report the relationships between po2 and so2 during UCO with the percentage change in cerebral metabolites. A good step but if the po2 and So2 did not get as low in the creatine group than the controls- then the study could be confounded by the fact that the insult did not achieve the same level of hypoxaemia between groups. Could you also plot relationships of % change from baseline Po2/So2 against arterial creatine as well as the percentage change in cerebral metabolites during the UCO. This should tell us whether creatine concentrations are driving/inhibiting a change in po2/so2.

We thank the reviewer for their suggestion and agree that presenting the relationships as % change from baseline is a better indication of the level of hypoxaemia, hypercapnia and lactic acidosis between groups as was intended.

Line 333-336: “The percentage change from baseline of blood gas parameters was calculated from the value obtained -30 min prior to the start of the UCO and the value at the 9 min timepoint of the 10 min duration of UCO, and was used to establish the degree of hypoxaemia, hypercapnia and lactic acidosis each fetus experienced."

Accordingly, the relevant result section has been rewritten to reflect the new outcome of these analyses and to address the reviewer 2’s concerns of presenting a multitude of correlational data. We have now focused on presenting the multivariate regression analysis (% change of blood gas vs. arterial creatine concentrations vs. % change of cerebral metabolites, Lines 405-440 of the original submission which included Table 2 and Table 3 and supplementary data 3 and 4; and discussion Lines 572-587 have now been removed).

Line 438-453: “Potential relationships between the circulating arterial creatine concentrations prior to UCO, the degree of hypoxaemia, hypercapnia and lactic acidosis experienced by each fetus, and changes in cerebral 2-OH-TA and metabolites following UCO were then assessed with multivariate regression analyses. Overall, there were significant associations between fetal arterial creatine concentrations, arterial pO2 and sO2, and the accumulative percentage change of cerebral interstitial pyruvate, lactate and 2-OH-TA (Figure 3; Supplementary data 3). The level of arterial creatine was positively correlated with the percentage change of pO2 and sO2, which were then negatively associated with percentage change in cerebral pyruvate accumulation, suggesting that higher levels of arterial creatine and reduced changes in arterial pO2 and sO2 during UCO resulted in lower levels of pyruvate accumulation (P=0.006 and P<0.0001 respectively; Figure 3; Supplementary data 3). A similar relationship between higher levels of creatine and reduced changes in arterial sO2 during UCO resulting in lower levels of cerebral lactate
accumulation was also found (P=0.017; Figure 3; Supplementary data 3). In addition, a significant relationship between arterial creatine, arterial pO2 and cerebral 2-OH-TA was observed, in which higher arterial creatine and reduced changes in sO2 during UCO was associated with lower cerebral 2-OH-TA levels (P=0.050; Figure 3; Supplementary data 3).

The new results are now discussed further within the Discussion section as follows:

Line 494-502: “Further, multivariate regression analysis revealed that fetuses with higher circulating creatine levels were associated with reduced 2-OH-TA and a reduced degree of hypoxaemia during the UCO. We hypothesise that this relationship results from a decrease in fetal tissue aerobic metabolism due to the increased creatine levels maintaining ATP turnover for longer under hypoxic conditions, thereby reducing the need for oxidative phosphorylation (48). The relationship between reduced hypoxaemia during UCO and the smaller changes of cerebral pyruvate and lactate (discussed further below) also supports the hypothesis that there is a reduced reliance on mitochondrial bioenergetics during UCO conferred by increased creatine levels (49).”

Line 504-508: “While the relationship between oxygen deprivation and ROS production in the brain is well established (51, 52), our results suggest there is now a need to ascertain in real time if creatine preserves mitochondrial bioenergetics during acute hypoxia and investigate the actual cellular and mitochondrial oxygen flux and subsequent ROS rates (42).”

While we agree with the reviewer about the usefulness of % change from baseline and have updated the results accordingly, we contend that absolute values of PO2 and SO2 remain important in a clinical setting as it is only such values that will be available through routine fetal monitoring during labour. We have added a brief discussion of this within the section on “methodological considerations”

Line 641-647: “Furthermore, the multivariate analysis revealed that relative changes of blood gases, rather than the absolute values denoting the degree of hypoxaemia, were predictive of cerebral metabolic changes that followed the UCO. Of course, a clinical measurement of such changes is unlikely to be feasible”.

9. Line 377: no such thing as a trend. If the interaction was not significant then it was not significant. Change wording please.

We have now removed all non-significant data

We thank the reviewer for their comment, and strictly speaking, they are correct. However, a major limitation of large animal studies such as this is the relatively low number of animals available per group. Given that setting significance at P equal to or less than 0.05 is entirely arbitrary, we argue that P values between 0.05 and 0.07 can be interpreted as being biological relevant, particularly if the results would have been significant for a group size of 10 or more. We believe we have not overstated non-significant data but have taken the opportunity to discuss the potential relevance of the results in a broader context. We have reworded the text accordingly, and also removed any comment on data with P> 0.07 to ensure clarity.

Line 394-395: “...though there was a near-to-significant interaction between time and creatine treatment...”

10. Figure 2 legend. Please be more clear that the # and ## are referring to a time effect. No interaction. In fact, please clearly state in the figure legend that there is no interaction between treatment and time.

We have now included necessary information.
Line 951-953: “...There were no significant interaction effects of time and treatment. \#P≤0.05, ##P≤0.01 refers to a time main effect and indicates statistically significant differences compared to baseline in both saline and creatine treated fetuses.”

11. Line 393- why after 9 minutes? Was it not a 10 min UCO?

As reported in our previous study (Tran et al., 2021) we took blood gases at 2, 5 and 9 mins into UCO, and blood samples and the blood pressure/heart rate recordings were taken from the same arterial line, so that taking a blood sample temporarily disrupts the blood pressure and heart rate recording. Therefore, it was decided a priori, that a 10 min blood sample would not be taken so we could record the level of hypotension and bradycardia at the end of 10 min UCO instead.

12. Line 395: wording... incorrect to have negative correlation with decreased PO2- two negative. Just say negative correlation with PO2. Or - negative correlation with Po2 such that as Po2 decreased...etc

This text has since been removed according to the changes outlined in comment 8

13. Line 414- no trends. If the p value isn't significant then there is no relationship.

These results have been since removed according to the changes outlined in comment 8

14. Line 484: "higher po2 and so2 during UCO" - is this just because they started higher?ie were basal po2 and so2 higher in the creatine treatment group.

Please refer to comment no.8. reply; basal po2 and so2 were not higher in the creatine treatment group.

15. Line 487: “and reduced cerebral pyruvate and lactate efflux following UCO”- but both started at lower levels during baseline... so with lower levels to begin how can you be sure that the predictive value does not lie solely in the pre UCO metabolite levels?

We have stated in the methods that these measurements refer to the percentage change and therefore are independent of the exact baseline values present before the UCO; viz., Lines 337-341 now states: “The oxidative and metabolic cerebral metabolite changes were calculated as the area under the curve (AUC) of the relative (percentage) change from average basal levels from 0 to 72 h after UCO. This approach was taken to represent the total impact of the hypoxia on the specific metabolite as a result of UCO.”

16. Line 493: Here it is important to discuss the durations and severity of the insult. Reference: PMID: 32088029

We thank the reviewer for this reference. However, this reference is not specifically relevant as it focusses on intrauterine growth restriction (IUGR) in which a chronic hypoxic insult underlies the etiology of the pathology, whilst the present study imposed an acute, transient hypoxic insult.

17. Line 502: "by reduced ROS production"- because the creatine fetuses did not get as hypoxaemic during their UCO as salines?

The reviewer is correct; upon analyzing the new data following the reviewer’s suggestion for assessing percentage change of blood gases rather than absolute values, it was revealed that the reduced ROS production was indeed associated with reduced hypoxaemia during UCO, which was also driven by increased levels of arterial creatine. This result has now been included within the results (Line 450-453; also see reply to reviewer comment 8).
The exact underlying cause for reduced ROS production due to reduced hypoxaemia in creatine fetuses cannot be deduced from the assessments we have conducted in this study. We have however suggested three mechanisms in which this may have occurred: 1) improved mitochondrial energy, 2) direct ROS scavenging 3) vasodilation. Indeed, further studies are required to elucidate the exact mechanisms in which creatine reduces hypoxaemia during UCO and therefore ROS production within the fetal brain.

18. Line 651: ofcourse lower detriments in oxygenation during the UCO would result in more favourable energetics. I'm not sure you have fully teased out the driving force from creatine vs the decreased hypoxaemia during the UCO on these outcomes. I suggest a figure instead of a table to enforce this. Eg. Panel 1: Creatine concentrations prior to UCO vs the change in PO2 and So2 from baseline. Panel 2: Change in PO2 and SO2 vs pyruvate/glycerol/lactate etc during UCO. This will allow the reader to visualize the data and draw their own conclusions. Ie bring some of your supp graphs into the main body of the paper.

We have replaced the table with figures highlighting the multivariate relationship (Figure 3). The table is now supplementary data 3. We have also now included a graphical abstract which better summarizes the results and have taken into account the reviewer’s suggestions.

19. You have previously published fetal BP and heart rate in this cohort. Did you place flow probes on the carotid artery? This would allow you to calculate cerebral oxygen delivery and assess that against your metabolite parameters?

We thank the reviewer for this comment and agree that it would be interesting to know what happened to carotid blood flow. While this is sometimes used as a surrogate measure of global cerebral blood flow, it cannot be safely assumed that it reflects changes in intra-cortical blood flow; i.e., in the tissue close to the position of the microdialysis probe. Also, it should be appreciated that microdialysis sampling was already technically difficult and we did not want to increase the risk of over-instrumenting the fetuses by inserting flow probes that occasionally can compromise blood flow to the fetal head.
Reviewer #2 comments:

This study examines the effects of pre-treatment with intravenous creatine on microdialysis samples of hydroxyl radicals (•OH) and interstitial metabolites (lactate, pyruvate, glutamate, glycerol, glycine) after a brief (10 min) umbilical cord occlusion on from fetal sheep at 131 days of gestation. These are technically exceedingly difficult and complex studies to perform. The studies appear to have been carefully performed. There is very little data on direct microdialysate sampling from the fetal brain in utero. The manuscript is well written and clear. The authors conclude that fetal creatine treatment decreased cerebral outflow of •OH, which was associated with an improvement in cerebral bioenergetics after acute hypoxia.

Although the basic study design and results are clear, there are some concerns regarding the correlational analyses. It is not clear that all the many correlational analyses add that much to the paper. There is serious concern regarding the supplementary data 2 C. and D. in which the entire correlations depend upon one point in both the glycerol AUC and the Glutamate AUC. These correlations should not be included.

We thank the reviewer for their close appraisal of our study and have addressed all comments below. Upon reassessment of the data we agree that the inclusion of the many correlation analyses was unnecessary. As the results of the bivariate correlations are reflected in the multivariate analyses we have chosen to just present the latter in the revised version of the manuscript.

We have therefore removed Lines 405-440 of the original submission which included Table 2 and Table 3 and supplementary data 3 and 4 (Note we have added additional supplementary data as suggested by reviewer 1) and discussion Lines 572-587. The removal of these results did not result in major changes to the reported outcome of the study and there are no major changes to the discussion. The reviewer can view omitted results and discussion text within the manuscript document with tracked changes, if they wish. We also summarise these changes in detail in response to reviewer comment 2 (please see below).

In addition, as suggested by reviewer 1 we have altered our assessments of blood gas and metabolite data by calculating percentage change from baseline (baseline = 30 min pre-UCO; to highlight the degree of hypoxaemia, hypercapnia and lactic acidosis each fetus experienced (Line 333-336)). This has subsequently eliminated the concern of the one data point that looked like an outlier within the previous analyses.

1. It appears to reduce duplication of some of the ewes in the study, animals were included from a series published in 1985. Although this is understandable because of the high cost and enormous effort that go into these studies, it remains possible that there could be a drift in the results over time that could increase the variability in the experiments.

We would like to highlight the (1985) refers to the journal number and not the year of the study. In fact this study was funded by National Health & Medical Research Council of Australia (1124493) awarded in 2017 and published in 2021.

86. Tran NT, Muccini AM, Snow RJ, Nitsos I, Hale N, Walker DW, and Ellery SJ. The physiological effects of creatine supplementation in fetal sheep before, during, and after umbilical cord occlusion and global hypoxia. J Appl Physiol (1985) 131: 1088-1099, 2021.

We have however reworded to clearly indicate how the animals were used in the previous publication:

Line 150-153: “Ewes and fetuses in this study were used in a previously published study detailing the experimental model and physiological outcomes of the creatine and cord occlusion protocols (Tran et al., 2021). Experimental protocols unique to this study are reported in detail below.”
2. It is not clear that the many correlational analyses add much to the studies. If you do enough correlations, something will show a significant correlation. What do the correlations mean?

As addressed above, we agree that the many correlational analyses were unnecessary. We have removed all bivariate correlations however maintain that the analysis of “Relationship between arterial creatine concentration and blood gas measurements during UCO on metabolic changes within the fetal brain” (Line 438-453) remains relevant – we have now reiterated the reason for the multivariate regressions more clearly within the manuscript, both in the results and in “methodological considerations”

Line 438-441: “Potential relationships between the circulating arterial creatine concentrations prior to UCO, the degree of hypoxaemia, hypercapnia and lactic acidosis experienced by each fetus, and changes in cerebral 2-OH-TA and metabolites following UCO were then assessed with multivariate regression analyses.

We believe the multivariate linear regression analyses was useful to explore and identify relationships worthy of future investigations. Our intention was to examine for the first time the question: does the level of creatine in the fetal circulation affect the severity of a transient hypoxic event on the multiple parameters of cerebral metabolism that we were able to measure by microdialysis. Thus, we have used regression analyses to identify relationships between 3 main sets of parameters: circulating arterial creatine, blood gas and pH changes during UCO, and the resulting changes in cerebral metabolism as detected by microdialysis.

Line 641-647: “Thus, a strength of conducting multiple variable regression modelling accounts for subject variability, such as that often observed clinically. Furthermore, the multivariate analysis revealed that denoting the degree of hypoxaemia by the relative changes of blood gases, rather than from the absolute values, were predictive of cerebral metabolic changes that followed the UCO. While measurement of relative or absolute changes of fetal oxygenation is unlikely to be clinically feasible, the potential relationships we have identified warrant further investigation.”

3. Supplementary data 3. There appears to be 2 populations with the saline and creatine clustering separately. The 2 populations need to be adjusted with a "dummy coding" variable. It is suggested that the authors consult a statistician regarding the validity of these correlations.

We have now removed supplementary data 3. Though to address the reviewer’s comment: the reviewer is correct, there are indeed two populations that cluster – the saline and the creatine group – this is due to the x-axis referring to arterial creatine levels; the “creatine-treated” group is therefore expected to have higher creatine levels. The fact that there is one saline fetus with creatine levels similar to two creatine-treated fetuses highlights the importance of these correlation analyses that aimed to investigate the effects of creatine levels irrespective of whether creatine was administered or not (Line 343-344: “Within this analysis, the saline UCO and creatine UCO groups were combined to assess the effect of arterial creatine concentrations alone, irrespective of treatment group”).

4. Overall, the effects of creatine appear relatively modest. The most important data is Fig. 1.

We thank the reviewer for their comments and we agree that the effects of creatine appear relatively modest – we do not hypothesize that creatine treatment would prevent severe brain injury or death from hypoxic insult, but rather it would work to enhance the already physiologically available creatine kinase energy buffering system for more mild-to-moderate cases of hypoxia. Indeed, these modest effect sizes for a treatment administered prophylactically in a small number of animal subjects could potentially have a significant effect on a population scale.
5. The differences in Fig. 2 are very modest.

We agree the changes in metabolites are modest, however it should be noted that microdialysis takes a sample from a small, localized area within the brain – there could be significant changes in other regions not sampled within the brain such as the deep grey matter and/or hippocampus. Future studies investigating the oxidative and metabolic stress responses within other regions are warranted. Furthermore, despite these modest changes, there is evidence that brain injury after even mild-moderate hypoxia or HI can evolve over many weeks and may result in neurological deficits seen only later in postnatal life; i.e., during early childhood and adolescence (Nagy et al., 2005, Perez et al., 2013, Finder et al., 2020). Thus, these mild pathologies and cerebral metabolism changes should not be ignored when assessing the importance of developmental origins of health and disease.

6. Line 501 "unreported observation". This should not be included. Include the data or the statement be omitted.

We have removed this from the manuscript.
Reviewing editor comments:

Please add details on the origin and source of animals, the animals access to food and water, and the anaesthetic protocols & monitoring.

Animal protocols have now been added in detail.

Line 134-145: “Twenty-eight pregnant Border-Leicester/Merino cross ewes ... were placed separately in individual pens at the Monash Medical Centre animal house holding facility, always in the company of other sheep, and allowed to acclimatize for 6 days prior to surgery, and during this time they were maintained in a 12 h light/dark cycle (8am to 8pm) at an ambient temperature of ~20°C. Ewes were fed twice daily with a lucerne chaff mixture with access to water ab libitum. The well-being and food and water intake of each ewe was monitored daily throughout the entire experiment.”

Line 1554-157: “Briefly, at 118 days gestation (dGA), after withdrawal of food for at least 18 hours, each ewe was anaesthetized with intravenous sodium thiopentone (20 mg.kg⁻¹), intubated, and the anesthesia then maintained by mechanical ventilation and inhalation of 1.5-3.5% isoflurane in oxygen:air (70:30).”

Please replace SEM with standard deviation in your manuscript. Please complete the statistical summary document.

We have replotted each graph figure and reported all data with mean and SD. Statistical summary documents have been completed.

Please also note the following: "If n (less than or equal to)30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (the latter two also preferably with data points included) are acceptable formats. Note: if each subject has numerous data points associated with it (e.g. time course data), we would treat ‘n’ as being each data point, not the number of subjects. If n>30, data points do not need be plotted in the figure but the entire raw dataset must be uploaded either as ‘Supporting Information for online publication’ (which will be published online with the article) or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

We have followed the senior editor’s suggestion of plotting all over time data as mean and SD.

Summary data must be reported as mean {plus minus} SD or 95% confidence interval

This has now been completed.

All table and figure legends with summary data must include the statistical test used in the table/figure and sample size

All figure and table legends now include statistical tests and sample sizes.

Figures with summary data bars must include individual data points, or box whisker plots when n> 30.

We have followed the senior editor’s suggestion of plotting all over time data as mean and SD.

Article file, including any tables and figure legends, must be in an editable format (eg Word)

This has now been completed.

You must start the Methods section with a paragraph headed Ethical Approval.
This has now been included Line 133

Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph.

This has now been completed.

'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

This has now been completed; Lines 148-149; 226; 244.

Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file.

This has now been completed. Abstract figure legend is provided in main article file Line 24-31.
Senior editor comments:

When revising the manuscript please review our updated statistics policy. If the number of data points per group per time point makes it too messy to plot individual data then mean and SD are okay, even if group n =< 30. Please also refer to our animal research reporting requirements.

We have reported all data with mean and SD. We have headed the senior editor’s advice of plotting data as mean and SD.
References:

FINDER, M., BOYLAN, G. B., TWOMÉY, D., AHEARNE, C., MURRAY, D. M. & HALLBERG, B. 2020. Two-Year Neurodevelopmental Outcomes After Mild Hypoxic Ischemic Encephalopathy in the Era of Therapeutic Hypothermia. *JAMA Pediatr*, 174, 48-55.

NAGY, Z., LINDSTRÖM, K., WESTERBERG, H., SKARE, S., ANDERSSON, J., HALLBERG, B., LILJA, A., FLODMARK, O., LAGERCRANTZ, H., KLINGBERG, T. & FERNELL, E. 2005. Diffusion Tensor Imaging on Teenagers, Born at Term With Moderate Hypoxic-ischemic Encephalopathy. *Pediatric Research*, 58, 936-940.

PEREZ, A., RITTER, S., BROTSCHI, B., WERNER, H., CAFLISCH, J., MARTIN, E. & LATAL, B. 2013. Long-term neurodevelopmental outcome with hypoxic-ischemic encephalopathy. *J Pediatr*, 163, 454-9.

TRAN, N. T., MUCCINI, A. M., SNOW, R. J., NITSOS, I., HALE, N., WALKER, D. W. & ELLERY, S. J. 2021. The physiological effects of creatine supplementation in fetal sheep before, during, and after umbilical cord occlusion and global hypoxia. *J Appl Physiol (1985)*, 131, 1088-1099.
Dear Dr Ellery,

Re: JP-RP-2022-282840R1 “Creatine supplementation reduces the cerebral oxidative and metabolic stress responses to acute in utero hypoxia in the late-gestation fetal sheep” by Nhi T Tran, Greg Kowalski, Anna M Muccini, Ilias Nitsos, Nadia Hale, Rod J Snow, David William Walker, and Stacey Ellery

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors’ comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

The last Word version of the paper submitted will be used by the Production Editors to prepare your proof. When this is ready you will receive an email containing a link to Wiley’s Online Proofing System. The proof should be checked and corrected as quickly as possible.

Authors should note that it is too late at this point to offer corrections prior to proofing. The accepted version will be published online, ahead of the copy edited and typeset version being made available. Major corrections at proof stage, such as changes to figures, will be referred to the Reviewing Editor for approval before they can be incorporated. Only minor changes, such as to style and consistency, should be made at proof stage. Changes that need to be made after proof stage will usually require a formal correction notice.

All queries at proof stage should be sent to TJP@wiley.com

Are you on Twitter? Once your paper is online, why not share your achievement with your followers. Please tag The Journal (@jphysiol) in any tweets and we will share your accepted paper with our 23,000+ followers!

Yours sincerely,

Professor Laura Bennet
Senior Editor
The Journal of Physiology
https://jp.msubmit.net
http://jp.physoc.org
The Physiological Society
Hodgkin Huxley House
30 Farringdon Lane
London, EC1R 3AW
UK
http://www.physoc.org
http://journals.physoc.org

P.S. - You can help your research get the attention it deserves! Check out Wiley’s free Promotion Guide for best-practice recommendations for promoting your work at www.wileyauthors.com/eeo/guide. And learn more about Wiley Editing Services which offers professional video, design, and writing services to create shareable video abstracts, infographics, conference posters, lay summaries, and research news stories for your research at www.wileyauthors.com/eeo/promotion.

* IMPORTANT NOTICE ABOUT OPEN ACCESS *

Information about Open Access policies can be found here https://physoc.onlinelibrary.wiley.com/hub/access-policies

To assist authors whose funding agencies mandate public access to published research findings sooner than 12 months after publication The Journal of Physiology allows authors to pay an open access (OA) fee to have their papers made freely available immediately on publication.

You will receive an email from Wiley with details on how to register or log-in to Wiley Authors Services where you will be able to place an OnlineOpen order.

You can check if you funder or institution has a Wiley Open Access Account here https://authorservices.wiley.com/author-resources/Journal-Authors/licensing-and-open-access/open-access/author-compliance-tool.html
Your article will be made Open Access upon publication, or as soon as payment is received.

If you wish to put your paper on an OA website such as PMC or UKPMC or your institutional repository within 12 months of publication you must pay the open access fee, which covers the cost of publication.

OnlineOpen articles are deposited in PubMed Central (PMC) and PMC mirror sites. Authors of OnlineOpen articles are permitted to post the final, published PDF of their article on a website, institutional repository, or other free public server, immediately on publication.

Note to NIH-funded authors: The Journal of Physiology is published on PMC 12 months after publication, NIH-funded authors DO NOT NEED to pay to publish and DO NOT NEED to post their accepted papers on PMC.

----------------

REVIEWING EDITOR COMMENTS

Thank you for your submission. Both reviewers are satisfied with your responses and the revised manuscript.

----------------

REFEREE COMMENTS

Referee #1:

Thank you for adequately addressing my comments. Well done on a good body of work.

*********************

1st Confidential Review 01-Apr-2022