Author's response to reviews

Title: Effects of chronic carbon monoxide exposure on fetal growth and development in mice.

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Author's response to reviews: see over
To: BMC Pregnancy and Childbirth  
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Re: Submission of revised version of full length research article for publication in BMC Pregnancy and Childbirth.

Corresponding Author: Dr. Graeme N. Smith. MD, PhD, Queen’s University, Kingston, Ontario, Canada

Dear BMC Pregnancy and Childbirth Editorial Team,

Thank you for reviewing our manuscript entitled “Effects of Chronic Carbon Monoxide Exposure on Fetal Growth and Development in Mice”.

Please find below our answers to each of the reviewer comments, as well as the edits requested from the editor. We previously uploaded a revised version of the manuscript, with all revisions highlighted. We have now uploaded a revised version without highlights, as the formatting of the manuscript was altered with the highlights included. We have also included a “conclusions” section in this manuscript.

Editor Comments:
Abstract: Keywords are not required for BMC series journals.
We have removed these words from the manuscript.

Competing interests: Please include a 'Competing interests' section after the Conclusions. If there are none to declare, please write 'The authors declare that they have no competing interests'. Please check the instructions for authors on the journal website for a full list of questions to consider when writing your competing interests statement.
We have added this section following the discussion.

Tables: Please note that we are unable to display vertical lines or text within tables, no display merged cells: please re-layout your table without these elements. Tables should be formatted using the Table tool in your word processor. Please ensure the table title is above the table and the legend is below the table. For more information, see the instructions for authors on the journal website.
We have removed all vertical lines from our tables. All tables are formatted using the Table tool in word, and made to fit on portrait paper.

REVIEWER 1
No comments to respond to.

REVIEWER 2
Major compulsory revisions:

1. COHb dissociation curves are sigmoidal in shape and not linear. Is a linear regression analyses of maternal/regal %COHb and Hb therefore appropriate?
The reviewer is accurate in referencing a sigmoidal curve for the oxygen dissociation curve in the presence of carbon monoxide (CO). Our study is evaluating CO levels at one distinct time point for each mouse analyzed, and comparing each CO level based on the dose exposure. Thus, a linear regression analysis is necessary, in order to compare our dose response effects observed. We feel that the COHb and Hb figures should remain as linear regression analyses for this reason.

2. Figures 3 and 4 indicate that up to 15 litters were exposed to between 0 and 60 ppm CO but only 6-7 litters were exposed to CO concentrations >100 ppm. Why were twice as many litters studied at the lower CO
concentrations than the higher CO concentrations? Was this due to a decrease in successful maintenance of pregnancy (i.e., presence of seminal plug at mating but no later indication of a successful pregnancy)? If so, this information should be included.

The reviewer is accurate to identify that we have more litters in the CO exposure groups of 0, 25 and 60 ppm CO, compared to the other groups. Shortly after we began the experiment, we determined a method to collect and therefore measure fetal blood CO. This meant that we needed to obtain these measurements from the first CO exposures, 0, 25 and 60 ppm CO, and thus we have more litter values at these CO doses. We have included this in our methods section of the paper.

3. Increasing the number of litters studied at 400 ppm may reduce the error associated with CO concentrations in maternal organs, especially when the error (SD?) seen for the liver and lung data at 400 ppm is almost as large as the mean. This casts doubt on whether CO concentrations in these organs at 400 ppm is actually significantly different to control.

The tissues analyzed for CO levels were those of the maternal mice, thus increasing litters would not be of benefit. While increasing the number of tissue samples (more maternal mice, may decrease the standard deviation (SD) observed in the liver and lung tissue at 400 ppm, we believe that the scope of the figure was achieved with our data; that being a rise in tissue CO levels was demonstrated with increasing maternal CO exposure.

4. While I agree that the number of live fetuses per litter at 400 ppm CO may be significantly reduced compared to control, what evidence is there that “Total live fetuses/litter were negatively correlated with CO concentrations”? Looking at the values in Table 2, I doubt that a linear regression analysis would indicate this to be true (control = 12.6, 150 ppm = 13.5, 250 ppm = 12.4, 400 ppm = 8.2 live fetuses per litter). I would also suggest the correlation between EGD/LGD and maternal CO exposure would be weak. Were these three correlations significant?

As per the reviewer’s questions, we have now run linear regression analyses on each of the following for CO exposures: litter size, EGD and LGD. We found no statistical significance for litter size, but a statistical significance for both EGD and LGD compared to maternal CO exposure. We have not included this data in the manuscript, as we feel that the relative risk data we have shown is sufficient for the reader.

5. Analyses indicated in the ‘Statistical Analysis’ section (‘The EGD and LGD data was analysed with a chi squared test’) do not match that indicated in the ‘Results’ section (implication that a linear regression analyses were performed for EGD and LGD data). Please clarify which form of analyses were actually used.

We acknowledge the error in reporting live litter numbers per CO concentration, as well as EGD and LGD results using correlation terms. We have changed our results section to make these findings more clear and to refer to our chi squared results.

6. For 4% PFA perfused fetuses, were the six embryos used for each CO concentration from different litters?

A minimum of 2 mice per experiment were used for PFA perfusion, and subsequently, 3 embryos were removed from each mouse’s uterus. Therefore, they were from a minimum of 2 litters. We have made this more clear in the methods section of the manuscript.

7. What is meant by ‘in each case the abnormal fetus number was compared to implantation total subtract EGD or LGD’?

We are referring to the number of EGD or LGD per CO concentration and the method by which we displayed these values. Due to the different litter sizes, we displayed the EGD and LGD as percentages of total implantation sites. We have written this more clearly in the statistical analysis section of the methods portion of the paper.

8. Was fetal to placental size altered by maternal CO exposure? This parameter indicates whether the placenta is able to appropriately transfer nutrients to the fetus for growth and development.

As per the reviewer’s request, we have now analyzed fetal to placental size, and this parameter was not altered by maternal exposure. We have not included this in the manuscript, but we would be happy to, if it is so desired.

Minor Essential Revisions
1. Page 7, line 12: a word (existed?) is missing between the words ‘distinction’ and ‘between’.

This has been corrected.
2. When embryos were sectioned, how many sections were collected from each embryo? How thick were the sections? Where were the sections taken from within the embryo? Were the placentas that were used for histological analyses taken from the same embryos that were used for histological analyses? That is, were they embryo and placental pairs? If the placentas were photographed whole, how were the images taken randomly? What was meant by ‘random pictures’?

While the matching placentas were imaged with each embryo, separate placentas were imaged for the specific placental analyses. The confusion with the word “random” has been rectified in our manuscript. This referred to the selection of five slides for staining from the 10 slides prepared.

3. Page 8, line 12: Analysis is singular; please change to analyses.
This has been corrected.

4. Page 9, last sentence: please change to ‘these organs are shown separately as they contained…’
This has been corrected.

5. Table 1 is of little value and should be removed.
We feel that this table is of importance to the manuscript, as it provides a reference for readers to relate our experimental CO levels. We believe the table will help readers to better understand our results and should be kept in the manuscript.

6. Table 2 includes a CO concentration of 330ppm. Such a group is not included anywhere else in the manuscript. Is this an error? Should this not be 300ppm?
This was in fact an error and has been changed to 300ppm.

7. Do the errors in the figures represent SD or SEM?
All errors in figures represent SD and we have written this in the methods section, statistical analyses.

8. Figure 1: While all panels show a scale bar, the images are of different sizes. Please make them all the same size so the structures in images A and B can be seen more clearly.
We have fixed figure 1 to make the scale bar the same size between images. A and B should now be more clearly visible.

9. Figure 4 provides the same information as that in Table 2 (Total live fetuses/litter column) and therefore is unnecessary.
We have removed this figure from the manuscript, however we have left this information in a brief sentence in the results section of the paper. The results are shown in Table 2, and have been changed to reflect the significance that was shown in the Figure 1.
We have corrected references to Figure 5 and 6 to reflect the figure numbering changes.

10. Does figure 5 represent CO concentrations in the maternal or fetal spleen?
Figure 5 represents CO concentrations in the maternal spleen.

11. Page 14, line 13-14: ‘…fetal/placental abnormalities were insignificantly increased.’ What abnormalities were these? The results indicate that placental size, dimensions and histomorphology were not different between groups and apart from fetal weight and survival, no other fetal measurements were reported.
In the manuscript, we had written, “…fetal/placental abnormalities were significantly increased.” We have fixed this term “abnormality”, to reflect the results concerning the LGD and EGD values.

12. The placenta is not a maternal tissue. Therefore all references to the ‘maternal placenta’ should be replaced with ‘placenta’.
This has been corrected in the manuscript.
Discretionary Revisions
1. A brief statement/introduction as to why CO may be therapeutically administered to the pregnant women (as given in the last paragraph of the discussion) would be of value.
   The objective of our manuscript was to determine at what maternal CO exposure fetal toxic effects are observed, therefore elucidating a toxicity experiment. We therefore did not feel that including therapeutic uses of CO in the introduction was of much benefit. We have included a small section in the discussion as a potential use for our data in current research projects. We would be happy to remove this section, if it is not believed to be warranted.

2. A stronger link between maternal cigarette smoking, and resulting maternal CO concentrations, and the developmental effects fetal hypoxia would also be of value.
   To our knowledge, this data is extremely difficult to measure and thus to report on. Maternal cigarette smoking varies, as cigarette brands, smoking habits and puff patterns differ between individuals. It is therefore impossible to relate a cigarette number smoked, to a certain CO level. In the introduction section of our manuscript we referred to the %COHb level measured in smokers versus non-smokers.

   Measuring fetal hypoxia in relation to cigarette smoking is not possible. Potential hypotheses and extrapolation of data have reported on markers of oxygen levels or molecular proteins in placenta/blood samples following birth. This data offers a broad conclusion that cigarette smoking increases %COHb levels and thus might cause a hypoxic fetal environment. We have already referred to the unknown hypoxic fetal effect in our introduction section.

3. What is the relevance of a chronic exposure model to humans?
   We sought to evaluate the maximal effects of CO exposure on pregnant mice. We felt that in this way we were subjecting mice to the worst possible effects at each CO dose. The chronic exposure model is relevant to humans, as it allows for knowledge of the worst toxicities observed at each of the CO exposure levels.

   We hope that you will find our revisions suitable. We look forward to hearing from you.

Sincerely,

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