Author's response to reviews

Title: Relationships between heavy metal concentrations in three different body fluids and male reproductive parameters: A pilot study.

Authors:

Jaime Mendiola (jaime.mendiola@um.es)
Jose M Moreno (sele.moreno@upct.es)
Manuela Roca (manoliroca@yahoo.es)
Nuria Vergara-Juarez (Nuria.vergara@upct.es)
Maria J Martínez-García (Mariaj.martinez@upct.es)
Antonio Garcia-Sanchez (antonio.garcia@upct.es)
Belen Elvira-Rendueles (Belen.elvira@upct.es)
Stella Moreno-Grau (stella.moreno@upct.es)
Jose J Lopez-Espin (jlopez@umh.es)
Jorge Ten (jten@institutobernabeu.com)
Rafael Bernabeu (rbernaeu@institutobernabeu.com)
Alberto M Torres-Cantero (amtorres@um.es)

Version: 3 Date: 23 December 2010

Author's response to reviews: see over
Dear Editors,

Ref: MS: 5834987274578629. Relationships between heavy metal concentrations in three different body fluids and male reproductive parameters: A pilot study.

First of all, we would like to thank the thoughtful and useful comments and suggestions that we believed have been addressed in the current form of the paper.

The revised copy of the document is submitted with all the changes tracked in order to facilitate review.

As requested, and in order to facilitate the review, we address specific reviewers’ comments one by one. A detailed explanation of all the changes introduced in the paper is also provided.

To Whom It May Concern,

1. Our manuscript has been submitted solely to Environmental Health and is not published, in press, or submitted elsewhere.

2. All authors have read the manuscript, agree that the work is ready for submission to Environmental Health, and accept responsibility for the manuscript's contents.

Please, do not hesitate to contact me for any further clarifications that you or the Reviewers may find appropriate.

Looking forward to hear from you,

Sincerely,

Jaime Mendiola, PhD
Preventive Medicine and Public Health
School of Medicine
University of Murcia, Spain
AUTHORS RESPONSE TO THE REVIEWERS

Ref. MS: 5834987274578629. Relationships between heavy metal concentrations in three different body fluids and male reproductive parameters: A pilot study.

REVIEWER #1.

1. A more extensive description of the demographics for the participants is needed, including variables for education, alcohol consumption and race, as they have been reported to affect semen quality. The characteristics should be provided for cases and controls separately.
   
   **Response:**
   
   A new table 1 has been added to the manuscript stating the variables above-mentioned for cases and controls separately. The subjects were all white Caucasian and therefore there was no ethnic variability.

2. I suggest using a common measure units for the metals (µg/L).
   
   **Response:**
   
   All the metal units have been converted to µg/L.

3. The species of mercury - methy or inorganic - should be specified.
   
   **Response:**
   
   We completely agree with the reviewer, and there is an explanation why we could not do the speciation. We had a very short semen sample (quantity) to do all the metals, so we had to select what to do. At that time we thought that total Hg could be a good indication in this preliminary study. To be consistent, we did the same procedure with blood plasma and whole blood. For sure in future studies the speciation of mercury will be accomplished.

   The following sentence has been changed, Page 5, line 125: “Determination of total Hg was carried out …”

4. I’m not familiar with anodic stripping voltammetry, so cannot comment on its utility for measuring mercury.
   
   **Response:**
   
   Pb and Cd were measured using anodic stripping voltammetry and Hg using atomic absorption spectrophotometry. Anodic stripping voltammetry is being used widely in chemical studies for the detection of lead and other toxic heavy metals (please see: Yantasee W, et al. Electrochemical sensors for the detection of lead and other toxic heavy metals: the next generation of personal exposure biomonitors. Environ Health Perspect. 2007 115:1683-90). We would be very pleased to provide you more information about it.

5. The authors say the participants provided least 2 semen samples. How were the semen values in the manuscript derived? Are they means of the multiple samples? If so how many? The SD of the means needs to be included.
   
   **Response:**
Thank you for the comment. The following paragraph has been changed, Page 4, lines 97-100: “Subjects provided two semen samples and were requested to observe a 3- to 5-day abstinence period. The importance of the abstinence period was stressed on the interviews with the participants (21). The average of the two samples was used in our statistical analysis (Table 1).”

The information requested by the Reviewer has already been included in a new Table 1.

6. The major comparison made was between cases (low for all three sperm parameters –oligo-asthenoteratospermia) and the normospermic men attending the same clinics. This definition of case may be too restrictive as patients with OAT are usually regarded as more severely affected than men with only one or two abnormal sperm parameters.

Response:
We concur with the Reviewer. However, we wanted to make sure that they were two clearly different groups of participants in terms of semen quality to compare. If there were any associations between heavy metal concentrations and semen quality, one would expect that the differences would be more apparent between those two groups.

7. Although their major finding is not new, the comparisons of metal levels in the 3 compartments is helpful but would be much more important if found in a larger sample.

Response:
We agree with the Reviewer. There are a few published studies on that topic and we believe that, even if small, our study can provide helpful information to our current knowledge. In fact, as we stated in our discussion, one of our limitations is the relatively low number of participants. We are initiating a larger prospective study and results should be available in two years.

Minor points
All the minor points listed below have been fixed.

Line 71: make associations plural
Line 72: remove the period after both
Line 71: combine the sentences from 71 through 81 into one paragraph
Line 92: change seminal to semen
Line 165: Remove A from the end of the line
Line 166: Capitalize the s in summary
Line 168: the first sentence is redundant – remove
Line 184: Around is imprecise- give the exact percentages.
REVIEWER #2:

The present study analyzed the relationship between lead, cadmium and mercury in seminal plasma, blood plasma and whole blood and levels of reproductive hormones as well as some conventional semen parameters among 61 men under infertility assessment. There are some major points I want to address to the authors.

1. The number of cases is very low.
   **Response:**
   We concur with the Reviewer. However, there are a few published studies on that issue and we believe that, even if small, our study can provide helpful information to our current knowledge. In fact, as we stated in our discussion, one of our limitations is the relatively low number of participants.

2. Length of sexual abstinence was not included as a covariant.
   **Response:**
   That is correct, but men were requested to observe a 3- to 5-day abstinence period, and the importance of this issue was stressed.
   The following paragraph has been changed, Page 4, lines 97-100: “Subjects provided two semen samples and were requested to observe a 3- to 5-day abstinence period. The importance of the abstinence period was stressed on the interviews with the participants (21). The average of the two samples was used in our statistical analysis (Table 1).”

3. Control group is unrepresentative.
   **Response:**
   We agree with the Reviewer; they do not represent the general population. However, as a case-control study, by choosing these controls we ensure that both groups are comparable. We selected our control group following the criteria and guidelines for case-control studies outlined by Schlesselman (1982) (Schlesselman JJ. Case-control studies: design, conduct, analysis. New York: Oxford University Press, 1982).

4. Why plasma blood and whole blood?
   **Response:**
   Thank you for the comment. Several papers have studied the relationship between metal concentrations in those two fluids separately and male function but, to our knowledge, no one has explored metal concentrations in those two fluids in relationship to male reproductive parameters on the same subjects. That was the main reason why we decided to include both.

5. Why pilot study?
   **Response:**
   We conducted this study to explore our main research hypothesis and serve as the base of a larger prospective study. We are initiating that larger study and results should be available in two years.
REVIEWER #3:

However, the shortcomings in the study are:
1. too small number of subjects
   **Response:**
   We agree with the Reviewer. However, there are a few published studies on that topic and we believe that, even if small, our study can provide helpful information to our current knowledge. In fact, as we stated in our discussion, one of our limitations is the relatively low number of participants.

2. an insufficient control of possible influence of smoking (as smoking intensity and duration, expressed as the number of cigarettes per day and/or cigarette-years, rather than a number of smokers, affects the levels of measured metals).
   **Response:**
   We concur with the Reviewer. We have rerun our statistical models including now a new variable called: number of cigarettes per day (instead of smoking: yes/no) and have gotten very similar results. Tables 5 and 6 have been updated and the name of the variable has been changed throughout the paper.

Major Compulsory Comments:

1. Methods: In the description of the metals measurements in body fluids on page 5, authors should provide quality control data for the metal analysis (e.g. the use of certain quality control reference samples; participation in external quality assessment scheme) to demonstrate the accuracy of their results.
   **Response:**
   We agree with the Reviewer. We used reference blood samples to check our methods out. The reference samples we used were Seronorm™ Trace Elements Whole Blood L-3 LOT 0512627. We are attaching a copy of the data from this reference material for your consideration. However, in anodic stripping voltammetry the addition sampling technique is used. This means that to each sample a known amount of the metal is added to get a given signal that is used to calculate the unknown sample concentration. This procedure is repeated with 3 different known concentrations to get a calibration line.

   The following paragraph has been added, Page 6, line 135: “To guarantee the accuracy and precision of the applied technique regarding heavy metals, whole blood reference materials (Seronorm™ Trace Elements Whole Blood, SERO AS, Billingstad, Norway) were employed.”

2. Table 1: Reported value for blood plasma lead concentration (2.7-3.3 microg/dL) seems to be overly high, especially with regard to relatively low whole blood lead concentration (7.5-13.3 microg/dL) – sample contamination or use of EDTA plasma (it is known that EDTA may cause a release of Pb from RBC into plasma yielding a higher plasma lead concentration)!
   **Response:**
   Thank you for the comment. We did not use EDTA tubes in the collection of blood for our study. Regarding whole blood, the samples were poured directly into borosilicate glasses. As to blood plasma, GEL tubes were used. Inside the tube there is a barrier gel present at the bottom. After centrifugation, the gel barrier can effectively separate the plasma from
fibrin and cells. Then, the plasma was poured directly into borosilicate glasses. Those tubes were pre-tested for heavy metals (Pb, Cd and Hg). We think therefore that sample contamination is highly unlikely. We would appreciate any further considerations on this issue if you believe it is necessary.

Minor Essential Revisions:
**All the minor points listed below have been fixed.**

1. Decimal symbol should be point instead of comma (pg 5, line 125; pg 6, line 139).
2. Table 4, results of multivariate analysis for T and Cd S.P.: in the column for 95% CI, "10.5" should be corrected.
3. Table 5, results of multivariate analysis for Morphologically Normal Sperm and Mercury B.P.: in the column for 95% CI, "1.3" should be corrected.

Discretionary Revisions:

1. Methods, Study population, design and semen analysis, p.4: If possible, please provide data on smoking habits of study subjects (expressed as the number of cigarettes per day and/or cigarette-years).
   
   **Response:**
   This data has been added to Table 1

2. Please provide data on semen parameters (Table 1)
   
   **Response:**
   This data has been added to Table 1