Author's response to reviews

**Title:** Bulky DNA adducts in human sperm associated with semen parameters and sperm DNA fragmentation in infertile men: a cross-sectional study

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

We highly appreciate the valuable comments of referees on our manuscript (MS: 4626119081026153), entitled "Bulky DNA adducts in human sperm associated with semen parameters and sperm DNA fragmentation in infertile men: a cross-sectional study". The suggestions are quite helpful for us, and these comments will greatly enhance the quality of this manuscript. We have modified the manuscript accordingly, and the detailed corrections are listed below point by point.

We deeply hope that our responses are satisfactory. If you have further questions, please let us know by E-mail. Thank you again for your consideration and we await a favorable response to the revision.

Thanks and Best Regards!

Yours Sincerely,

Guixiang Ji

2013-8-15
Responses to the quests:

Reviewer 1: Bernard Robaire

Reviewer's report:

The objective of this epidemiological, uncontrolled study was to explore the potential associations between PAH-DNA adducts, standard semen analysis parameters and sperm DNA integrity, assessed by the TUNEL assay. 433 infertile men were recruited for this study; stringent exclusion criteria were used. Sperm PAH-DNA adducts was measured using immunofluorescence and FACS; the subjects were divided into quartiles and the association between PAH-DNA adducts and (i) sperm quality endpoints - including sperm concentration, total sperm count, (ii) three CASA parameters sperm motility and curvilinear velocity, and linearity - assessed by computer assisted semen analysis; and (iii) sperm DNA fragmentation (TUNEL assay) was assessed. Potential associations were explored using linear regression models on ranked categories of sperm PAH-DNA adducts levels, and P values for linear trend as well as regression coefficients were adjusted for age, body mass index, abstinence time, smoking and alcohol drinking using multivariate models.

The authors identify a significant decrease in sperm concentration, count, motility, and curvilinear velocity in the higher quartile compared to the lower one. A significant increase in TUNEL positive sperm was also observed in the higher quartile. The data suggest an inverse association between sperm PAH-DNA adducts and sperm quality, and a positive association between PAH-DNA adducts and TUNEL positive. The study provides useful additional evidence that increased PAH-DNA adducts are associated with lower sperm quality, as measured by a number of different methods. There are a number of points the author may wish to consider:

Major Points

1. The lack of a control group renders the interpretation more limited, i.e., correlational within an infertile population. The authors should justify this approach.

Response:
Thanks for the reviewer’s comments. We only selected infertile men who may be more “susceptible” to PAHs and the results should only be applied to that sort of population. So the present findings may have implications for fertility on a population basis. We will try our best to extend our work in control group in our future project.

2. As the authors have identified in the discussion, immunofluorescence is not the most appropriate method for PAH-DNA adducts detection; this constitutes the major weakness of the study. It is essential that the authors provide data establishing the efficiency and concordance of flow cytometry and fluorescence microscopy, to support the “good correlation” mentioned on page 9. Having used a more appropriate assay, ideally a quantitative assay (e.g., GC-MS) would certainly have made this study stronger.
Response:

Thanks for the reviewer’s comments. Although the immunofluorescence method we used were not as specific or sensitive as mass spectrometry and provide a semiquantitative measure of DNA adduct level that cannot be directly translated into an adducts per nucleotide result, the monoclonal antibody 5D11 we used has been extensively validated for tissue-based quantification of relative DNA adduct levels [1-4]. Immunohistochemistry assay used 5D11 antibody, which in cell culture studies has been shown to produce strongly correlated staining levels (r = 0.99) with the treatment dose of benzo(a)pyrene diol epoxide [5]. The relationship between the immunofluorescence assay and qualitative assay (e.g., GC-MS) will be conducted in our future study.


3. Chromatin damage may be assessed by using a number of methods. The TUNEL assay simply reflects the number of open 3’OH end in DNA. Since the relationship is with PAH-DNA adducts, this positive correlation is intrinsically expected. Having another, or several other, markers of sperm chromatin damage would have been appropriate.

Response:

Thanks for the reviewer’s comments. Over the years, there have been an increasing number of tests developed to assess sperm DNA damage. The TUNEL assay is probably the least controversial because it measures the presence of free 3-OH terminus, which can be identified by terminal deoxynucleotidyl transferase (TdT). This assay measures both single- and double-strand DNA fragmentation. The other assays commonly used to detect DNA damage, the Comet and sperm chromatin structure assay (SCSA), reflect the ability of sperm chromatin to withstand exposure to highly alkaline or acid conditions. To assess the concordance of we applied these two methods in 20 human sperm samples. The neutral comet assay is performed according to the protocol as described previously [3]. As showed in Figure 1, a significant correlation between these two assays was also
observed \( (r = 0.848, P < 0.001) \). It also has been reported that the output of SCSA, TUNEL and Comet assays are all correlated to some degree [1-2], suggesting that whatever the nature of DNA damage in the male line, it is reflected in a variety of different assays and may suggest a common underlying mechanism.

![Graph showing correlation between Comet and TUNEL assays](image)

**Figure 1.** Correlation of results from Comet and TUNEL assays. A significant positive correlation was observed \( (r = 0.874, P < 0.001) \).


4. The rationale for the statistical methods used is poorly explained. This needs a more comprehensive discussion.

**Response:**

Many thanks for the reviewer’s nice suggestion. We have added some discussion about the reason we used this statistical methods in the “Statistical analysis” section.

5. The lack of graphical representation of correlational analysis between PAH-DNA adducts and any of the endpoints deemed to be positively associated renders interpretation of the data difficult. Inclusion of figures that clearly demonstrate this correlation would strengthen the manuscript.

**Response:**

Thanks for the reviewer’s advice. According to your suggestion, we have added the figures (fig.2.-fig.5.) represent the relation between PAH-DNA adducts levels and the semen quality.
Fig. 2. Association between logarithm of the sperm concentration and sperm PAH-DNA adduct. Adjusted for age, smoking, alcohol drinking, BMI and abstinence time, the PAH-DNA adduct levels were significantly associated with sperm concentration using a linear regression: beta coefficients = -0.632, P < 0.001.

Fig. 3. Association between logarithm of the sperm count and sperm PAH-DNA adduct. Adjusted for age, smoking, alcohol drinking, BMI and abstinence time, the PAH-DNA adduct levels were significantly associated with sperm count using a linear regression: beta coefficients = -0.632, P < 0.001.

Fig. 4. Association between the sperm motility and sperm PAH-DNA adduct. Adjusted for age, smoking, alcohol drinking, BMI and abstinence time, the PAH-DNA adduct levels were significantly associated with sperm motility using a linear regression: beta coefficients = -9.647, P = 0.012.
Fig. 5. Association between logarithm of the sperm DNA fragmentation and sperm PAH-DNA adduct. Adjusted for age, smoking, alcohol drinking, BMI and abstinence time, the PAH-DNA adduct levels were significantly associated with sperm DNA fragmentation using a linear regression: beta coefficients = 0.130, P < 0.001.

6. The discussion is overly long for the data presented in the manuscript. It should be reduced by about 30%.

Response:
Many thanks for the wonderful suggestion. The discussion section has been reduced by about 30%.

Minor Point

1. Additional references are needed to better establish their reasoning (e.g. references [5-8], page 1 and [18], page 7).

Response:
We appreciate the correction from the reviewer. All these references have been replaced in our revised manuscript.

2. All abbreviation must be defined the first time they are used.

Response:
Many thanks for the reviewer’s correction. These mistakes have been corrected in our revised manuscript.

3. Several of the sentences are awkward. The text would benefit from a further reading from someone fluent in scientific English.

Response:
Many thanks for the reviewer’s suggestion, we have invited Professor B. Jiang from Mary Babb Randolph Cancer Center, and Department of Microbiology, Immunology and Cell Biology, West Virginia University to help us to check for English and correct mistakes in our manuscript. All the revisions were shown in the revised manuscript.
Level of interest: An article of limited interest

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

'I declare that I have no competing interests
Reviewer 2: Radim J Sram

**Reviewer's report:**

**Major Compulsory Revisions:**

This ms. presents a very nice study, in which was obtained a large number of samples.

To improve the quality of text, authors should still discuss or analyze following items:

p. 1 – “…However, very limited epidemiologic data exist on the potential effects of PAHs exposure on human reproductive functions…” It seems to be peculiar, they do not mention papers by Rubes et al. (Mutat Res 625, 2007:20-28, Mutat Res 683, 2010:9-15), as both papers analyze the relationship between PAHs exposure and sperm DNA damage.

**Response:**

Thanks for the reviewer’s help and good suggestion. We have cited these two papers in our manuscript. In the page 2, paragraph 2, we added “In humans, several studies suggested the relationship between air pollution exposure to PAHs and increased sperm DNA damage [Rubes et al., 2007, 2010].”


According to the ref. 28 Horak et al. bulky DNA adducts in human sperm were associated with smoking. Therefore it should be preferable to present data for smokers and nonsmokers separately in Tables 2 and 3.

**Response:**

Thanks for the reviewer’s comments. Because no significant association between cigarette smoking and sperm PAH-DNA adducts in our study population was found [Ji et al., 2010], we did not present data for smokers and nonsmokers separately in Tables 2 and 3.


p. 9 – “…We only selected infertile men who may be more “susceptible” to PAHs…” It could be useful to add any information about air pollution in the Nanjing Region, e.g. c-PAHs exposure, life style – diet. As Xia et al. (Environ Sci Technol 43, 2009: 4567-4573)
put forward, 1-OH concentration in the urine of Chinese men is 16-fold higher than in U.S. populations.

**Response:**

We deeply appreciate the reviewer for the wonderful comments. In page 2, paragraph 1, we added “In China, due to the conventional eating habits that involve heavily fried, roasted, or grilled foods and the rapid increase of automobile and industrial production, the general population has more opportunities to be exposed to PAHs from multiple sources and routes compared to other nations. It has been reported that the creatinine-adjusted 1-OHP metabolites levels in China in Nanjing region is 16-fold higher than in U.S. populations [Xia et al., 2009]. This result suggests Chinese adult males are highly exposed to PAHs in the environment, so the potential health hazard of PAHs deserve more attention in China.”


Minor Essential Revisions:

Fig. 1 – axis y Frequency – there is something wrong with the units – total should be 100%.

**Response:**

Thanks for the reviewer’s comments. The axis y represents the number of people, and the total number is 433.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests.

We deeply hope our responses are satisfactory. If you have further questions, please let us know by E-mail. Thank you again for your consideration and we await a favorable response to the revision.