Author’s response to reviews

Title: Parameters of ovarian reserve in relation to urinary concentrations of parabens.

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Version: 2 Date: 12 Jul 2019

Author’s response to reviews:

Associate Editor Comments:

Please work with a native English speaker and writer to revise your manuscript. In some places, the reviewers have struggled to understand your meaning, which makes reviewing for technical and scientific purposes very difficult. Please see the comments from the journal, at the end of this email message, with some suggestions for resources that may be helpful.

Answer: The English grammar and language has been corrected by the native speaker but also by professional service (http://www.englishprep.pl/korekta.html) which correct the text based on the standards of The Society for Editors and Proofreaders (http://www.sfep.org.uk/).

Please also follow the recommendations/requirements of the journal regarding formatting. For example, your citations are numbered in the bibliography, but listed by authors in the text, making it very difficult to reconcile references and the text. Please fix prior to resubmission.

Answer: The citations have been changed according to Journal requirements. The citations are numbered in the bibliography and in the text.
Other specific comments:

1) There is limited mechanistic evidence from rodents available on the effects of parabens on ovarian health, but the review provided of even these limited studies is not helpful. In what ways did paraben exposures alter histopathology of ovaries? In which direction was serum E2 or T4 affected? Which parabens were studied? Similarly, the authors note that only four prior epidemiology studies have been published - so it should be fairly easy to review these studies with more detail. Only 3 are discussed, and fairly superficially. Which parabens were studied in the Dodge study? If the authors wish to argue that their study increases statistical power over prior work, then the power/size of those prior studies, and other limitations, should be discussed. 

Answer: The part of Introduction about animal studies and the role of parabens has been rewritten. The epidemiological studies presented in the Introduction were more described and discussion presented.

2) Consider adding a table to summarize knowledge about the five parabens being evaluated. What is known about their mechanism of action individually? 

Answer: I have reviewed many papers about the possible mechanism of parabens. From the papers it can be concluded that the parabens have estrogenic effect and antiandrogenic effect. Depending of the studies some Authors found both effects and some only estrogenic effect or antiandrogenic, but in general the mechanism is the same. Only one differences in that the estrogenic activity of parabens increases with the length and branching of the alkyl chain. This information has been added to the paper.

3) As indicated by the reviewers, the authors must address how the study population (women at an infertility clinic) might influence interpretation of the data. The authors' data indicates that some of these women are fertile (38%), and it is their partners that are infertile. Can the data be separated for those women that are presumed to have normal fertility? 

Answer: The limitations of our study population (women from fertility clinic) have been discussed in the Discussion Section. Additionally we select only menstruating women who have confirmed ovulatory cycles without clinical co-existing chronic diseases that may reduce ovarian reserve (eg, adrenocortical insufficiency, abnormal karyotype, fragile X syndrome) were eligible for the study. Exclusion criteria included: spontaneous premature ovarian failure, previous surgical treatment of the ovaries, chemotherapy or pelvic radiotherapy (conditions that may lead to reduction of ovarian reserve), cysts in the ovaries including endometriates (excluding functional cysts) and conditions with no ovulatory cycles such as polycystic ovary syndrome, hypogonadotropic hypogonism, hyperprolactinemia. So we try to select women with no existing medical problem that may impact parameters of ovarian reserve. So in my opinion all the selected women were similar in medical history or condition to fertile ((38%) when their partners are infertile) in case of studying as an outcome ovarian reserve parameters.

4) Please carefully review the study details requested by the reviewers below. Additionally, information about the hormone measurement techniques is needed including the LOD, CV, number of replicates (duplicates/triplicates), etc. Please also indicate how the 120 women were
selected for a second urine sample, and demonstrate whether these women were representative of the larger group.

Answer: The revision of the article was based on Reviewers comments and all Reviewers’ suggestions have been included in the revised version of the manuscript. All changes in the manuscript have been done using the “track changes”. Information about measurement technique has been rewritten and changed.

A convenience urine sample was collected from each subject at the time of recruitment and from parts of study population (N=120) at subsequent visits during infertility treatment cycles within 3 months. As the most important problem is the epidemiological paper is exposure assessment based on one urine sample we try to overcome this problem by collecting second urine sample from the part of the study population. As the parabens are non-persistent chemicals we want to see if it is a habitual exposure or not.

Among 511 women, we were able to collected the second urine sample among 120 women (23%). Women with the second urine sample were not different (p>0.05) from the whole study population in terms of exposure to parabens, and characteristics (age, past diseases, alcohol consumption, duration of infertility that may be associated with parameters of ovarian reserve. This information has been added.

5) I agree with the reviewers that the discussion section needs to be rewritten to provide a more accurate summary of the data presented in this manuscript. As written, the "parabens" are treated together rather than separating out those parabens that show associations with ovarian reserve (PP) compared to the rest that did not have these associations.

Answer: The Discussion Section has be rewritten according to Reviewers and Editor suggestions.

Reviewer reports:

Reviewer #1: This is an interesting study looking at parameters of ovarian reserve in relation to urinary concentration of parabens.

Answer: Thank you.

In general English grammar must be reviewed throughout the manuscript.

Answer: The English grammar and language has been corrected by the native speaker but also by professional service (http://www.englishprep.pl/korekta.html) which correct the text based on the standards of The Society for Editors and Proofreaders (http://www.sfep.org.uk/).

The Abstract must be reviewed as well, especially the conclusions. It is quite difficult to follow.

Answer: The Abstract has been reviewed and changed (also the Conclusions).

I would suggest adding to the statistical analyses 1) the sum of the parabens, and 2) explore non-linear associations using p-trends across quartiles of parabens and their sum (ancova/manova).
According to the Reviewer suggestions 1) the sum of the parabens, and 2) explore non-linear associations using p-trends across quartiles of parabens and their sum have been added to the Tables and text of the manuscript.

Where the dependent variables (AFC and hormones) normally distributed?, if so, please add it.

Answer: The AFC and reproductive hormones level were normally distributed. The information has been added to the text.

Discussion (lines 210-2): Please rewrite that sentence and add what paraben was significant (line 211).

Answer: The sentence has been rewritten and type of paraben has been added.

Reviewer #2: Environmental Health ENHE-D-19-00047R1
Title: Parameters of ovarian reserve in relation to urinary concentrations of parabens.

General comments:
Jurewicz et al., have examined the association of urinary paraben concentrations with markers of ovarian reserve in a large study of women seeking fertility treatment at several infertility clinics. The study explores an important and understudied question. Fortunately, there are a growing number of studies that aim to examine the impact of non-persistent endocrine pollutants exposure, such as parabens, on female reproductive health. In this work associations between urinary exposure to several parabens and ovarian reserve, used as variable to evaluate the woman's response to ovarian stimulation, were examined. Serum concentration of follicle-stimulating hormone (FSH), anti-Müllerian Hormone (AMH), and estradiol, as well as antral follicle count (AFC), were also assessed. Only one previous epidemiological study has explored a similar research aim.
I feel that this article may contribute to the literature on chemical exposure and female reproductive health but still need to improve several aspects, outlined below:

1. To improve the English (a native English speaker should review the manuscript for clarity).
   Answer: The English grammar and language has been corrected by the native speaker but also by professional service (http://www.englishprep.pl/korekta.html) which correct the text based on the standards of The Society for Editors and Proofreaders (http://www.sfep.org.uk/)

2. To include more information about the urinary assessment of the selected parabens
   Answer: More information has been added about urinary assessment of the selected parabens (please see Section: Urinary parabens measurements).

3. To expand the results and the discussion
   Answer: The Results Section and Discussion have been expanded according to all Reviewers and Editor suggestions.
Materials and Methods:

1. Participants.
   - Please include geographical study area, recruitment period, number of infertility clinics, and its public or private condition (it is not clear how many centers participated because among the strengths authors say that all study subjects were recruited in the same center).
   Answer: The information about geographical study area, recruitment period, number of infertility clinics has been added to the Methods Section. The women were recruited in one private infertility clinic in Poland (central region of Poland). This clinic is the biggest infertility clinic in Poland with the biggest reimbursement from Ministry of Health for IVF procedures.

   - Participant's age: the range is different in the abstract (20-39) than in the method section (25-39)
   Answer: Thank you. It was my mistake the age range should be 25-39. It has been corrected in Abstract and now is the same as in the Methods Section.

   - All the participants (511) gave one urine sample prior to the measurement of the markers of ovarian reserve, and a quarter of them (120) gave an additional urine sample during infertility treatment cycles. How was calculated the urinary paraben concentrations for each woman? How was computed the within-person concentration in those women with two urinary samples? Was this variation applied to all study population?
   Answer: A convenience urine sample was collected from each subject at the time of recruitment and from parts of study population (N=120) at subsequent visits during infertility treatment cycles within 3 months. As the most important problem is the epidemiological paper is exposure assessment based on one urine sample we try to overcome this problem by collecting second urine sample from the part of the study population. As the parabens are non-persistent chemicals we want to see if it is a habitual exposure or not. As we have observed the statistically significant correlations between the first and the second urine sample for MP, PP, EP only the first sample were analyzed as in the model. This is common practice in epidemiological paper. As the second sample was just informative about the habitual exposure to parabens.

2. Measurement of urinary paraben concentrations
   - Add: Time and conditions (fasting, first of the morning) of the urine sample collection
   Answer: A convenience urine sample was collected in sterile polypropylene cup from each subject at the time of recruitment and from parts of study population (N=120) at subsequent visits during infertility treatment cycles. This information has been added to the Method Section.

   - Add: Strict control and validation procedures: Methods for preparation of samples, standard solutions and quality controls as well as the instrumental analysis and measures taken to avoid external contamination of samples during collection and processing, is needed
   Answer: All the information regarding: methods for preparation of samples, standard solutions and quality controls as well as the instrumental analysis has been added to the Method Section - Urinary parabens measurements.
3. Statistical analysis:
- Please clarify why the multivariate model only included three covariates for adjustment (age, smoking habit and BMI). In this large study, female varied on the main cause of infertility diagnosis (male, idiopathic, female factor). This covariate should also be explored/included in the multivariate statistical model, for example in a sensitivity analysis. Please, assess whether cause of infertility had further influence on the effect estimates.
Answer: Inclusion of covariates in the multivariable regression models was based on biological and statistical consideration. The following covariates were considered as potential confounders: age (years), smoking (yes/no), BMI (kg/m2)
Additionally we select only menstruating women who have confirmed ovulatory cycles without clinical co-existing chronic diseases that may reduce ovarian reserve (eg, adrenocortical insufficiency, abnormal karyotype, fragile X syndrome) were eligible for the study. Exclusion criteria included: spontaneous premature ovarian failure, previous surgical treatment of the ovaries, chemotherapy or pelvic radiotherapy (conditions that may lead to reduction of ovarian reserve), cysts in the ovaries including endometriates (excluding functional cysts) and conditions with no ovulatory cycles such as polycystic ovary syndrome, hypogonadotropic hypogonism, hyperprolactinemia. So in this case the “infertility diagnosis” have no impact on ovarian reserve. It was also checked during analysis and in current study “infertility diagnosis” was not a confounder.

- Because general population is exposure to a large number of xenoestrogens, we suggest taking also into account the potential effect of mixtures and at least consider exploring the association between Σ parabens with the outcome.
Answer: According to Reviewer suggestions the Σ of parabens have been analyzed and the association of the Σ of parabens on parameters of ovarian reserve have been performed. No statistically significant associations were found between Σ of parabens and parameters of ovarian reserve (please see Table 6).

Results
- There were great differences between the urinary parabens concentrations in the first and second set of urine samples; please explain the biological reasons. Could the infertility treatment cycles affect paraben levels in the second set of urinary samples (n=120)?
Answer: It a difference when we have a look on the concentration of parabens among 511 women in first and 120 women in the second urine sample, but when we look at the same 120 women with the first and the second urine samples the concentration of parabens were correlated and statistically significant (please see Table 5).
Additionally, the antral follicle count and hormone analysis because of the insurance coverage are performed once a year. In special cases the measurement may be performed more frequently but not often than once in each half of the year. After one month after stimulation of ovulation the situation (the levels of hormone and antral follicle count) going back to normal. So the prior treatment does not impact on ovarian reserve.

- Table 1: Translate Wykształcenie
Answer: Thank you. It was my mistake Wykształcenie- Education
Table 3: In the previous similar epidemiologic they found that nearly 100% of the women included had detectable urinary concentrations of MP and PP, and 75% of BP concentrations. However, Jurewicz et al have found lower detectable level (mainly for BP) in this study population. Some discussion should be added in this regards (detection levels showed in the text differ, lines 136, 137, from the levels included in table 3)

Answer: In our previous study (Jurewicz J, Radwan M, Wielgomas B, Dziewirska E, Karwacka A, Klimowska A, Kahužny P, Radwan P, Bochenek M, Hanke W. Semen quality, sperm DNA damage, and the level of reproductive hormones in relation to urinary concentrations of parabens. J Occup Environ Med. 2017;59(11):1034-40) the study population consist of men attending the infertility clinics and they recruited earlier (2008-2011). So in my opinion it is difficult to compare their level of exposure to current study among women. This reference was used to confirm that in both studies the same methods of parabens exposure was used.

Additionally the detection frequency in Jurewicz et al., 2017 were (MP - 98.7%; EP - 42.2%; PP - 89.2%; BP - 10.5% and iBuP - 15.9%) where as in current study were similar (MP - 93.92%; EP - 84.31%; PP - 84.11%; BP - 64.12%; iBuP - 10.6%).

According to EU regulation No 358/2015 products placed on the market after 30 October 2014 should be free from isopropylparaben, isobutylparaben, phenylparaben, benzylparaben and pentylparaben. That is why in the second urine sample in current study the iBuP was not detected.

It was mistake in the text regarding the detection levels now in Table 3 and text the values are the same.

Table 6 and 7: Which urinary paraben concentrations were used in this statistical assessment for each woman? Please add appropriate information in the table legend explaining the statistical model used in addition to the adjusted covariates

Answer: A convenience urine sample was collected from each subject at the time of recruitment and from parts of study population (N=120) at subsequent visits during infertility treatment cycles within 3 months. As the most important problem is the epidemiological paper is exposure assessment based on one urine sample we try to overcome this problem by collecting second urine sample from the part of the study population.

As we have observed the statistically significant correlations between the first and the second urine sample for MP, PP, EP only the first sample were analyzed as in the model. This is common practice in epidemiological paper. As the second sample was just informative about the habitual exposure to parabens.

In the table 6 and 7 the legends have been added.

Discussion

Lately analytical method based on sample treatment using dispersive liquid-liquid microextraction (DLLME) followed by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis are the proposed methodology to assess human exposure to non-persistent endocrine disruptors such as the selected parabens instead Gas chromatography. Please discuss it.

Answer: We are fully aware that a significant number of analytical methods for determining parabens in urine exist. The method we use has been developed in our laboratory and to date covers a range of more than 30 urinary biomarkers of exposure including BPA and its 6
analogues, benzophenone-3, triclosan, chlorophenols and several metabolites of PAHs and pesticides. Both internal and external quality control procedures were implemented during laboratory measurements. Internal quality control was realized by in-house preparation of appropriate quality control (QC) materials. This information has been added.

The data is discussed within the context of these existing studies as well as within the context of the epidemiological literature (there are some important missing articles). For example, authors compared urinary concentrations of parabens found with those reported among USA populations. Several studies have also been conducted among European population (see Jiménez-Díaz et al. Urinary levels of bisphenol A, benzophenones and parabens in Tunisian women: A pilot study. Sci Total Environ. 2016;562:81-88). Answer: Thank you. The reference has been added.

Results should interpret with caution because, for example some limitations have not been considered: the cross-sectional design of this study does not allow the inference of causal relationships and, consequently, reverse causality issues cannot be ruled out. A further limitation is that you did not collect the use of cosmetic product before urine sampling. Additionally, you only considered paraben exposure, and women are exposed to a wide range of environmental chemicals that may contribute to the cocktail effect. Answer: The limitations of the study have been added to the Discussion Section (cross-sectional design, mixture effect) regarding the use of cosmetic product before urine sampling we have collected such information.

References
The references are cited by numbers in the reference list at the end of the article but the reference style inside the text is different. Please clarify Answer: The numbers have been added to the text.

Minor:
Line 58: the reference is lacking
Answer: The reference has been added.

Line 83: I do not agree with this sentence: "This study adds to the previous human studies of parabens exposure more statistical power, by including additional parabens (five parabens- ethyl paraben (EP), butyl paraben (BP), methyl paraben (MP), iso-butyl paraben (iBu-P)"
Answer: The sentence has been changed.

Reviewer #3: This is an interesting study since parabens are preservatives commonly used in personal care products, pharmaceuticals and foods. In this area, there are few epidemiological studies that relate the use of parabens with fertility problems. Fertility problems are unfortunately increasingly. Therefore it is very relevant to know the effects of these products on the health of women. However, this work only includes women with fertility problems. It would have been convenient to include women without fertility problems to be able to better compare the results. Answer: The women from general population do not have routinely measurement of the ovarian reserve. Only when we have some problem with fertility (we are waiting for a long time) the
measure of ovarian reserve is performed. Nowadays more women is checking their fertility and sometime measure the parameters of ovarian reserve to see if she can be later in pregnancy, but it is not a common practice. So it will be difficult to perform the study among women from general population because we do not have routinely measurement of ovarian reserve. As in case of some parameters of ovarian reserve is not a problem (to measure the hormone levels: AMH, FSH, estradiol), but in case of antral follicle count (performed by USG) it can be a problem.

In my opinion, I think it should review certain points listed below. The manuscript should be carefully reviewed so that it can be published.

Abstract
1) Objective. Specify that the sample corresponds to women with fertility problems.
Answer: The information has been added.
2) Methods. When the study was conducted? (Duration, year of the study)
Answer: The information has been added.
3) Results needs explain more.
Answer: The information has been added.
4) Conclusions. What kind of association has been found? Specify.
Answer: The information has been added.

5) Keywords. Some words do not match to MeSH terms (Review)
Answer: The keywords have been corrected.

Manuscript
Background
6) Lines 55-62: Improve the wording and structure of the text (Citation: the same structure of all document).
Answer: The wording and structure of the text have been corrected.

7) Lines 88-89: The aim should specify that the sample corresponds to women with fertility problems.
Answer: The information has been added.

Methods
8) Lines 93-94: Reference is needed to justify the infertility criterion.
Answer: The reference has been added.

9) Lines 106-107: It would be interesting to list issues that were collected in the interview. Explain more.
Answer: The information about issues that were collected in the interview has been added.

10) Line 113: "...of Broekmans et al., 2010...". Bibliographical citations should adopt the appropriate format. Check here and in the rest of the document.
Answer: The format of the reference has been changed.
11) Lines 115-117: "Antral follicles with dimensions of 2 to 10 mm was considered for the assessment. The sum of antral follicles from the left and right ovaries was used for the analysis." Reference is needed.
Answer: The reference has been added.

12) Line 118: "The intravenous blood sample was drawn in the morning......". Why in the morning? At what time? What protocol or procedure is it based on? Explain and reference.
Answer: The sentence has been changed.

13) Lines 128-130: Clarify why sample is 120 in this point. Justify. I don't understand. Why two urine samples are collected? Explain.
Answer: A convenience urine sample was collected from each subject at the time of recruitment and from parts of study population (N=120) at subsequent visits during infertility treatment cycles within 3 months. As the most important problem is the epidemiological paper is exposure assessment based on one urine sample we try to overcome this problem by collecting second urine sample from the part of the study population. As the parabens are non-persistent chemicals we want to see if it is a habitual exposure or not. The information has been added.

14) Line 135: "The limit of detection was ...". Reference is needed.
Answer: The reference has been added.

15) Lines 128-137: The methods and reference values used must be justified.
Answer: The whole section Urinary parabens measurements has been rewritten and changed.

Results
16) Table 1 isn't necessary because the information can explain in the text.
Answer: In my opinion it is more visible to have the data also in the Table.

17) Table 2 has a lot of information (min, Q25, median, Q75, Q95 and max). Is necessary? Clarify.
Answer: In my opinion yes this is the statistical variables presenting the outcome.

18) Lines 180-185: Respect to comments of first and second urine sample, the author hasn't explained well this point. Explain.
Answer: A convenience urine sample was collected from each subject at the time of recruitment and from parts of study population (N=120) at subsequent visits during infertility treatment cycles within 3 months. As the most important problem is the epidemiological paper is exposure assessment based on one urine sample we try to overcome this problem by collecting second urine sample from the part of the study population. As the parabens are non-persistent chemicals we want to see if it is a habitual exposure or not. The information has been added.

19) Line 186: In the text, the value MP doesn't match Table 3. Fix it.
Answer: The values have been corrected.

20) Table 3 has a lot of information. Clarify (to synthesize the information or change the format).
Answer: In my opinion all the information in Table 3 are important because shows the range of exposure. Additionally the quartiles are used in the final model so in my opinion it is important to see the real values of the quartiles.

21) Table 4: Review format and convenience. The relevant information can be written in the text.
   Answer: The format has been changed.

Discussion
22) Lines 209-212: Compare with published studies.
   Answer: The results have been compared with published studies.

23) Lines 213-214: Reference is needed.
   Answer: The reference has been added.

24) Review all the section. Arguments are needed.
   Answer: All the sections have been changed.

25) Lines 261-264: This text could be placed in the Methods section.
   Answer: This information has been included in Methods section.

26) Missing the conclusions of the study. They must include.
   Answer: The Conclusions have been added.

References

27) References should be in the format of the journal. Check all.
   Answer: The references have been corrected.