

Peer Review File

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Reviewer A

1. Figure 1-5 were not called in the manuscript. What information was provided and how did this data help to come up with the conclusion? The interpretation of these figures was missing.

Reply 1: we have modified our text as advised.

Changes in the text: we added text in paragraphs 3.2, 3.3.1 and 3.4. (see Page 6-8, line 224-228;237-243;249-254)

2. A complete list of metabolites with differential abundance should be provided, at least in the supplemental data. The false discovery rate should also be provided.

Reply 2: A complete list of metabolites with differential abundance were provided in the supplemental data.

3. Did the author try to perform correlation or mediation analysis on specific metabolite and sperm viability?

Reply 3: The next step is to focus on specific metabolite and sperm motility in our research.

4. Discussion on the findings was limited to choline and arachidonic acid metabolism. Is there any relationship between choline and arachidonic acid?

Reply 4: Arachidonic acid including compounds: C02165; C04577; C05957; C05960; C05965; C14717; C14732; C14748; C14772; C14773; C14775; C14794; C14795; C14807. there is any relationship between choline and arachidonic acid.

5. The legends for tables and figures require substantial clarification. For example, what is the meaning of MZ in table 2? What is the meaning of "The metabolite names are not shown if the number of metabolites exceeds 50" in figure 9? My understanding is that the names were shown, but were not readable due to the small size and limited resolution of the heat map.

Reply 5: we give the meaning of MZ in table 2.

Changes in the text: mz: mass-to-charge ratio (see Page 12, line 379)

The absence of metabolite names in the heat map is not a problem of resolution and size, but the name of each metabolite cannot be shown in the original image of the heat map because it shows all the different metabolites in a relatively large number.

6. Since necrozoospermia is a special form of asthenozoospermia, how would the findings in the present study compare to that obtained from asthenozoospermia e.g. Zhang et al. Clin Chim Acta 2015 and others?

Reply 6: We have modified our text as advised.

Changes in the text: But studies have found that the increased levels of choline containing in the seminal plasma of patients with asthenozoospermia[29], the conclusions diametrically opposed to ours, it may be related to the different patients. (see Page 14, line 458-460)

Reviewer B

1. The authors state that they used the WHO 5th edition for semen analysis. In lane 86 they mention “reference values”. These should be either given in the text or included in the respective table.

Reply 1: We added some data

Changes in the text: the reference values (sperm concentration $\geq 15 \times 10^6/\text{ml}$). (see Page 3, line 91)

2. The authors used a CASA machine for semen analysis. Information about the machine is missing and needs to be added to the text. Also give further information on the volume used for CASA.

Reply 2: we have modified our text as advised

Changes in the text: The volume was measured by weighing; The computer assisted semen analysis (CASA) system (Beijing, China, SAS-II), (see Page 3, line 101-102)

3. The authors should add information on their staining method eosin - aniline black.

Reply 3: we have modified our text as advised .

Changes in the text: mix the semen sample well, remove a 50- μl aliquot of semen, mix with an equal volume of eosin-aniline black suspension, and wait for 30 seconds, make a smear on a glass slide and allow it to dry in air, examine immediately after drying, or later after mounting with a permanent non-aqueous mounting medium, examine the slide with brightfield optics at $\times 1000$ magnification and oil immersion, tally

the number of stained (dead) or unstained (live) cells with the aid of a laboratory counter, evaluate at least 200 spermatozoa, to achieve an acceptably low sampling error.
(see Page 3, line 104-110)

4. A picture showing necrozoospermic cells is missing. Please provide a picture of the stained sperm cells.

Reply 4: we provide a picture of the stained sperm cells in the supplemental data.

5. There is no information on how the seminal plasma was obtained. Please add the protocol for semen separation.

Reply 5: we have modified our text as advised and add the protocol for semen separation.

Changes in the text: The semen sample remaining was centrifuged after semen analysis at 3000g for 15 minutes, (see Page 3, line 111-113).

6. Seminal plasma volume is not “about 1 ml” in all cases. Probably the authors mean that 1 ml of each seminal plasma was stored at -80°C. By the way, a semen volume of less than 1 ml + the volume needed for CASA analysis is also an exclusion criterion and needs to be added to the text.

Reply 6: we have modified our text as advised.

Changes in the text: a semen volume of less than 1 ml, (see Page 3, line 96).

7. What are E-agents (heading of 2.4)?

Reply 7: we have change the wrong words

Changes in the text: Reagents, (see Page 3, line 131).

8. Lane 126: Were the samples air-dried? Probably not. This needs to be rewritten in order to make it understandable for the reader.

Reply 8: we have modified our text as advised.

Changes in the text: Accurately transfer an appropriate amount of the sample was transferred to a 2-mL centrifuge tube, which was added 400 µL methanol (stored at -20°C) and was vortexed at 1 min, followed by centrifugation for 10 min at 12,000 rpm at 4 °C. The supernatant was transferred to a new 2-mL centrifuge tube, which was concentrated and dried. The dried sample was added 150 µL of 2-chloro-1-phenylalanine (4 ppm) solution prepared with 80% methanol water (stored at 4°C) to re-dissolve the sample; the resulting supernatant was filtered through a 0.22-µm

membrane and transferred into the detection bottle for the LC-MS step. (see Page 4, line 141-148).

9. Lane 172: The reference is missing and needs to be added.

Reply 9: we have modified our text as advised.

Changes in the text: we delete the wrong sentence, (see Page 5, line 189-191).

10. The authors state that there were no significant differences between the age and the abstinence time of patients and controls. However, there are asterisks given in Table 1. An asterisk should only be given if there is a significant difference and not for a P value > 0.05 . Please give the exact P values to give the reader the chance to understand the differences.

Reply 10: we have modified our text as advised.

Changes in the text: give the exact P values in Table 1, (see Page 5-6, line 220-221).

11. The values of normal sperm morphology are far too low to be healthy or normal. According to the WHO criteria it should be $> 4\%$. Microscopic image sections of necrozoospermic samples and controls need to be added.

Reply 11: we have modified our text as advised.

Microscopic image sections of necrozoospermic samples were provided in the supplemental data.

12. There is no text in paragraphs 3.2, 3.3.1 and 3.4. What is the difference between Fig. 2 and Fig. 4?

Reply 12: we have modified our text as advised.

Changes in the text: we added text in paragraphs 3.2, 3.3.1 and 3.4. (see Page 6-8, line 224-228; 237-243; 249-254). The two pictures are different, one is QC, another is QA.

13. Some Figures are redundant for the main text and should only be shown as supplemental material. This applies for Fig. 1, Fig. 2(4), Fig. 3.

Reply 13: These figures show that our experiment is reliable.

14. Fig. 1, 2, 3, 4 and 5 are not mentioned in the text, probably because there is no text in the relevant paragraphs.

Reply 14: we have modified our text as advised.

Changes in the text: we added text in the relevant paragraphs, (see Page 6-8, line 224-226,236-240,246-249,263-269).

15. Fig. 5 and 9 are in a very bad quality and cannot be evaluated. However, as far as I can see, “con” and “test” do not cluster but are spreaded nearly equally across the plots.

Reply 15: The heat map represents a data matrix that visualizes the differences between data by using color gradients, scaling the data to retain larger differences while also highlighting smaller differences.

16. This was an untargeted analysis. I do not understand the meaning of telling the reader that you found 24,923 metabolites of which 7,523 were different (5,585 up, 1,938 down). What is the information for the reader out of that? In line 302 f. it is “The total number of metabolites was 24,923”, in line 324 it is “the total number of metabolites was 574”. What happened to the rest? How do you know that there were 24,923 metabolites? Did you get 24,923 signals that you could really assign to the respective molecule or were there just 24,923 peaks that could also arise to impurities, solvents etc.? You do not even name the metabolites. Furthermore, in the abstract you state that you found 194 differential metabolites.

Reply 16: Primary Analysis: The total number of metabolites was 24,923, of which 7,523 were differential metabolites (5,585 upregulated and 1,938 downregulated).

A search was conducted for differential metabolites from the list of primary substances in the sample, which were screened with the preset *P* value and VIP threshold in the statistical test. The total number of metabolites obtained was 574, of which 194 were differential metabolites (129 upregulated and 65 downregulated).

17. In the abstract you mention choline, benzaldehyde, 5-hydroxypyrazinamide, 5-aminoimidazole-4-carboxamide and dihydrothymine. But in table 2 there are “the top 10 differential metabolites”. These should not be sorted according to their *m/z* value but according to their fold change.

Reply 17: These sorted according to their *P* value.

18. Only the meaning of choline as a metabolite is discussed. There is not a word on the other metabolites. However, this would be the aspect that is most interesting for the reader. What is the connection between the differentially concentrated metabolites and necrozoospermia?

Reply 18: we added some data.

Changes in the text: 2-hydroxyglutarate (2-HG) is abundantly accumulated in the testis[30], which can be present in the form of as either a D- or L-enantiomers. 2-HG in the testis was an L-enantiomer. L-2-HG is generated from glutamine under hypoxic conditions[31], 2-HG was high in the seminal plasma of patients with necrozoospermia, which suggests that the occurrence of necrozoospermia may be related to hypoxia in the testis. And the severely impaired motility of sperm suggests a contribution of glutamate metabolism to energy production for sperm motility. (see Page 14, line 461-467).

Our next steps to carry out the connection between the differentially concentrated metabolites and necrozoospermia.

19. Benzaldehyde is a toxic substance and is not physiologic. It seems that benzaldehyde is decreased in necrozoospermic patients. If benzaldehyde is really stemming from the seminal plasma, what is the meaning of this chemical in semen?

Reply 19: Some studies have reported that benzaldehyde has been detected in urine, and may be the patients exposed to the enrolled population.

20. 5-Hydroxypyrazinamide has a molecular weight of 139.1 and its chemical formula is C₅H₅N₃O₂. The m/z value given in this study (m/z 123) might belong to pyrazinamide, which is a medication used to treat tuberculosis.

Reply 20: The m/z value given in this study (m/z 123) is pyrazinamide,

21. For 2-hydroxyglutarate and its appearance in semen this paper by Dodo et al. might be helpful: J Biochem. 2019 Apr 1;165(4):323-334. doi: 10.1093/jb/mvy108.

Reply 21: we have modified our text as advised.

Changes in the text: But studies have found that the increased levels of choline containing in the seminal plasma of patients with asthenozoospermia[29], the conclusions diametrically opposed to ours, it may be related to the different patients. (see Page 14, line 461-467).

22. Is it possible that there were some cancer patients included in this study? 5,6-dihydro-5-Fluorouracil is a hydrogenation product of 5-fluorouracil which is a cancer medication.

Reply 22: there are no cancer patients in our study.

23. It would be better to give the fold change of the metabolite not the log₂ fold change

of a differential metabolite. These changes seem to be only marginal. If I calculated correctly, these are between 1.73 for p-hydroxyphenylacetic acid and 0.57 for creatine.

Reply 23: Log₂(FC) is indicate whether the metabolites are up or down.

24. Tab. 3: What is a P value of 0.00E+00?

Reply 24: we have modified our text as advised.

Changes in the text: 5.80E-08 ; 1.09E-05 in table 3 (see Page 13, line 401-402).

Minor:

1. Abstract background: It should better be seminal plasma of necrozoospermic patients

Reply 1: we have modified our text as advised.

Changes in the text: seminal plasma of necrozoospermic patients, (see Page 1, line 15-16).

2. Lane 41: The causes of male infertility are complex and are brought about...

Reply 2: we have modified our text as advised.

Changes in the text: are complex and are brought about, (see Page2, line 43-44).

3. Lane 52: Describing seminal plasma as a chemical component of semen sounds strange.

Reply 3: we have modified our text as advised.

Changes in the text: The seminal plasma is particularly, (see Page2, line 55-56).

4. Lane 75 ff: This is not a sentence. Please rephrase. Furthermore, et al. should not be used in this context.

Reply 4: we have modified our text as advised.

Changes in the text: The routine parameters were acquired from all subjects, including age, sperm motility and duration of abstinence, (see Page2, line 80-81).

5. Lanes 81, 82: It must be 10⁶ /mL.

Reply 5: we have modified our text as advised.

Changes in the text: sperm concentration $\geq 15 \times 10^6$ /mL, percentage of forward motility $\geq 32\%$, semen white blood cells $\leq 1 \times 10^6$ /mL. (see Page2, line 85-87).

6. Lane 121: It should be “The frozen samples were prepared following published procedures.”

Reply 6: we have modified our text as advised.

Changes in the text: The frozen samples were then prepared following published procedures, (see Page4, line 139-140).

7. The sentence in lane 123 ff needs to be rephrased.

8. Lane 128: What is methanolwater?

Reply 8: The right words are methanol water. (see Page3 line 146).

9. Chemicals and reagents: Batch numbers and catalog numbers are just necessary for antibodies and cell culture media but not for solvents.

Reply 9: We didn't change it.

10. Either write p value or P value (in italics) throughout the text.

Reply 10: we have modified our text as advised.

Changes in the text: we wrote p value or P value (in italics) throughout the text.

11. Lane 184: Kolmogorov–Smirnov test

Reply 11: we have modified our text as advised.

Changes in the text: Kolmogorov–Smirnov test (see Page5, line 203).

12. Lane 219: “the data is good” needs to be rephrased.

Reply 12: we have modified our text as advised.

Changes in the text: the data are good, (see Page7 line 256).

13. Lane 282: “the the”

Reply 13: we have modified our text as advised.

Changes in the text: the model is simplified, (see Page11, line 322).

14. Lane 283: “allowing optimal”

Changes in the text: thus allowing optimal, (see Page11, line 333).

15. Lane 289: What in “Ehen VIP”?

Changes in the text: then VIP is > 1 for a variable, (see Page11, line 340).

16. Ref. 22: Did you really

We read the abstract of the article.

Reviewer C

1-In the introduction, the authors state that necrozoospermia is a special type of asthenozoospermia. However, in necrozoospermia the spermatozoa are mostly dead and not immotile, thus, these two abnormalities have different pathogenesis. The authors should better clarify this sentence.

Reply 1: we have modified our text as advised

Changes in the text: the sperms in necrozoospermia are mostly dead and not immotile.
(see Page 2, line 48)

2 - On page 4 lines 166 and 167 the abbreviations OPLS and PLS are switched

Reply 2: we have modified our text as advised

Changes in the text: orthogonal partial least-square discriminant analysis (OPLS-DA) and partial least-square discriminant analysis (PLS-DA). (see Page 4, line 185).

3 - Table 1: units of measure as well as the type of measure of dispersion used are missing

Reply 3: we added some data:units of measure

Changes in the text: Semen volume(ml),Sperm concentration(10^6 per ml),Forward motility percentage(%),Sperm viability(%),Seminal plasma neutral α - glucosidase(μ mol per ejaculate),Seminal plasma zinc(μ mol per ejaculate).(see Page 5, line 220-221).

4 - Figure 1: Please identify the two chromatograms

Reply 4: we have modified our text as advised

Changes in the text: two images A :atypical positive ion (BPC) ;B: typical negative ion (BPC),(see Page 6, line 228-233).

5- Figure 4: Please clarify each abbreviation. What do CON, PRE and test mean?

Reply 5: we added some data:units of measure

Changes in the text: con:control group;test:test group;PRE:preliminary experiment
(see Page 8, line 266).

6 - Figure 6: Please clarify what Con and test mean

Reply 6: we have modified our text as advised

Changes in the text: con:control group;test:test group.(see Page 10, line 303)

7 - Tables 2 and 3: P values are incorrect. The authors also should identify which metabolites are upregulated or downregulated in the necrozoospermia group

Reply 7: we have modified our text as advised

Changes in the text: *P* values were modified, we have mark which the metabolites are upregulated or downregulated in the necrozoospermia group in Table 2.(see Page 12, line 378-379)

8 - Table 3: FDR values are incorrect

Reply 8: The FDR values are represented by scientific counting.

9 - Table 3: what does bile secret mean?

Reply 9: we have change the wrong words

Changes in the text: bile secret → Bile secretion.(see Page 13, Table 3)

10-The discussion regarding the importance of the main metabolites and metabolic pathways identified is too short. Authors need to deepen more on the possible relationship of these findings with necrozoospermia. Only choline and AA metabolism were discussed.

Reply 10: we added some data.

Changes in the text: 2-hydroxyglutarate (2-HG) is abundantly accumulated in the testis[30], which can be present in the form of as either a D- or L-enantiomers. 2-HG in the testis was an L-enantiomer. L-2-HG is generated from glutamine under hypoxic conditions[31], 2-HG was high in the seminal plasma of patients with necrospermia, which suggests that the occurrence of necrozoospermia may be related to hypoxic in testis. And the severely impaired motility of sperm suggests a contribution of glutamate metabolism to energy production for sperm motility.(see Page 14, line 461-467).