

Peer Review File

Article information: <http://dx.doi.org/10.21037/atm-20-397>.

Reviewer:

This manuscript entitled “Peroxiredoxin 4, a new oxidative stress marker in follicular fluid, may predict in vitro fertilization and embryo transfer outcomes (Manuscript ID: ATM-20-397)” provides data on the quantification of Prdx4 in the follicular fluid of women undergoing IVF/ICSI and evaluates its value to predict the chance of clinical pregnancy after embryo transfer.

I have major comments.

A) Abstract:

1. Please modify the abstract according to the corrections required for the revision.

Reply: Thank you. The revised manuscript has been modified accordingly.

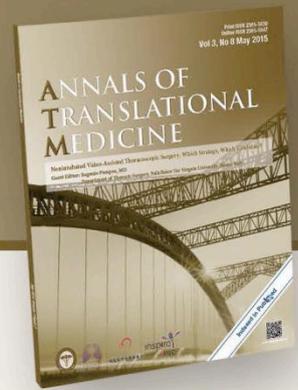
2. Page 2, line 12: It would be more accurate to say that Prdx4 quantification would not add to the risk/time of retrieval, rather than claiming obtaining follicular fluid is non-invasive.

Reply: Thank you for the reminder, the sentence has been revised accordingly (see Page 3, Line 36-37) .

B) Introduction:

The first objective and secondary objective(s) of this study should be clearly specified at the end of the Introduction. What is the primary objective? The other ones should be secondary objectives.

Reply: Thank you. We changed this text into “The aim of this study was to investigate the relationship between Prdx4 levels in FF of infertility patients and the subsequent oocyte quality



and IVF-ET outcomes, especially the clinical pregnancy rate. These results may provide clinically relevant information for predicting IVF outcomes and reveal the mechanism of Prdx4 activity in oocyte development.” (see Page 6, Line 83-87)

C) Materials and methods:

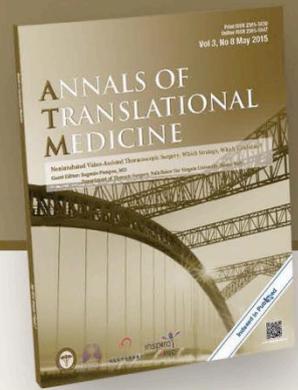
1. My main concern is the lack of information about the calculation of the sample size necessary to achieve a correct statistical power regarding the primary objective (which is I think the value of FF Prdx4 concentration to predict clinical pregnancy?). This gives the impression that the number of patients enrolled in this study has been chosen arbitrarily. The sample size does not seem to have been calculated before the start of the study. More details are needed.

Reply: Thank you for your consideration. We have recalculated the sample size using the STATA program and the result showed at least 88 samples should be enrolled. Our study included over a hundred patients, therefore our results are convincing.

2. Page 6, lines 88-89: Please explain why only follicles with diameters in the range of 17-20 mm have been retrieved, as it seems very strict and unusual (for example, see Bedient et al., 2019, “The optimal size of ovarian follicles at oocyte collection”).

Reply: Thank you for pointing out this issue. We have revised the manuscript accordingly (see Page 7, Line 111-114): “FF from optimal size follicles (diameters in the range of 16–20 mm) of one patient during oocyte retrieval was carefully aspirated and collected together.” (referred from Renato Fanchin et al.2005, Fertility and Sterility).

3. The concentration of Prdx4 is reported as a predictive marker of clinical pregnancy by the authors. Hence, all factors that could influence or bias its concentration should be carefully controlled. Was follicular flushing (with flushing medium® or another medium) performed during ovarian puncture? If so, how did the authors control the volume of the flushing medium added to the FF? Moreover, was there a small volume of medium in the collection tubes of FF? Indeed, it is recommended to add 1 cc of medium (such as heparinized modified HTF medium®



for example) in the collection tubes to protect the cumulus-oocyte complexes during ovarian puncture. However, this could have been associated with dilution bias. Furthermore, if a medium has been added (in the collection tubes or for follicular flushing), the authors should check the presence of Prdx4 by assaying this medium with their quantification kit (which can also cross with other molecules present in this medium) and report data or information about this. More details are needed.

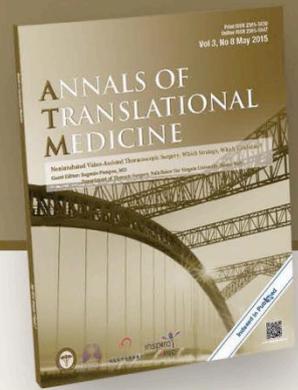
Reply: Thank you for this advice. We have added the information and revised the manuscript as follows: “The FF was aspirated without contamination with the flushing medium or culture medium.” (see Page 7, Line 114-115) and “FF contaminated with culture medium or blood was discarded.” (see Page 8, Line 117).

4. Page 6, lines 91-92: “FF contaminated with culture medium or blood was discarded”

- a. Is culture medium a synonym for flushing medium? On what criteria did the physicians use medium or not in the FF?
- b. The definition of ‘blood’ is needed. Almost all FF have some erythrocyte presence. What was the objective criterion to diagnose a FF as contaminated by blood?
- c. The proportion of discarded FF per patient and in both groups, should be notified as this could lead to biased results.

Reply: Thank you. Firstly, we have revised the manuscript as follows (see Page 7, Line 114-115): “The FF was aspirated without contamination with the flushing medium or culture medium” Secondly, when the follicular fluid have macroscopically exhibited a red color, the sample was discarded. Follicular fluid contaminated with significant quantities of blood cells was not used for analysis. Moreover, the FF was centrifuged at 2,000 rpm for 10 min to remove the red blood cells.

4. Regarding the Prdx4 ELISA assay, does it cross with other Prdx family members? More details or data are needed. Moreover, what is the intra (within the same plate) and inter (between



2 different plates) variability of the same sample? This information should be mentioned in the Materials and Methods.

Reply: Thank you for your concern. We have rechecked and revised this text as follows (see Page 8, Line 120-122): “Prdx4 levels in FF were assessed using an enzyme-linked immunosorbent assay (ELISA) (Abnova, Taipei, Taiwan) according to the manufacturer’s protocol as previously described.” (referred from Nawata A, et al.2016, PLoS one). According to the instrument of Prdx4 elisa kit: “No significant cross-reactivity or interference between Peroxiredoxin 4 (PRDX4) and analogues was observed.”

5. Page 7, line 97: the authors mentioned that standard solutions vary from 2 to 32 ng/l (i.e., per liter). But the results mention that Prdx4 is around 20 ng/ml (i.e., per ml) (Table 2). Is it a mistake or the concentration of Prdx4 reported in the results is not within the limits of the range of the ELISA Assay?

Reply: Sorry for the mistake, we have corrected it.

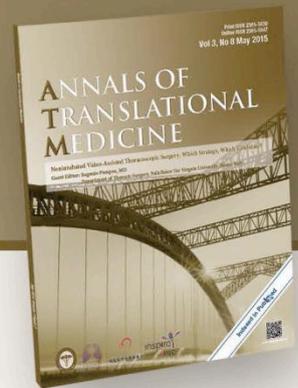
6. Page 8, lines 126-127: “at least 7 cells and 10 % fragmentation”

I think that the authors meant: “and less than or equal to 10% fragmentation”?

Reply: Sorry for the misunderstanding, we have corrected the text. (see Page 9, Line 144)

7. Please, add information about the characteristic of embryo transfer. Have all the transfers been made on day 3? What were the morphokinetic parameters of the transferred embryos? I assume that not only “good-quality embryos” have been transferred. Please, add a description of the transferred embryos with categories such as high, moderate, and low implantation potential based on morphokinetic parameters.

Reply: Sorry for the misunderstanding, we have corrected this issue by revising the text as follows (see Page 9, Line 145-146): “One or two embryos were transferred 3 days later after oocytes retrieval. ” Moreover, we calculated the transferred embryo number and good quality embryo number per transferred cycle, and the results showed that no significant difference existed between the groups (Table 4).



8. The authors chose to assess the predictive value of Prdx4 in relation to the occurrence of clinical pregnancy. However, the best proof of oocyte competence is live birth. As the patients have been enrolled from September 2017 to December 2018, live birth rates are already known and the authors should also present these data.

Reply: Thank you for the advice. We have added this data to Table 4.

D) Results

1. I recommend incorporating a flow chart to visually demonstrate study design.

Reply: Thank you, we have added a flow chart as Figure 1.

2. Page 9, line 143: “were included”. Is it not “excluded”?

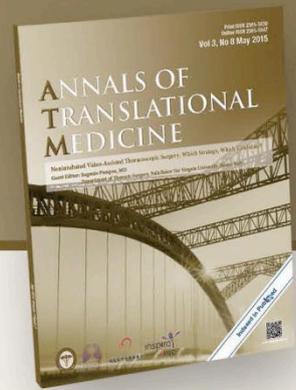
Reply: Sorry for the mistake, we have corrected the text.

3. Table 1: a lot of information are missing.

a. The authors do not report any demographic or clinical data on the men enrolled in this study to conceive the embryos, which could potentially confound the results.

b. The authors should at least add the following information with the appropriate statistical analysis: paternal age, BMI, toxic consumption (tobacco and drugs), sperm parameters.

c. Some demographic or clinical data about women and some characteristics of IVF/ICSI attempts are also missing. Please, add at least the following information with the appropriate statistical analysis: AMH concentration, toxic consumption (tobacco and drugs), the rank of IVF/ICSI attempt, Gonadotropin dose used for ovarian stimulation, number of days of ovarian stimulation, number of retrieved follicles, mean size of retrieved follicles (see comment “c” below), proportion of cumulus-oocyte complexes per retrieved follicles (see comment “d” below), proportion of "empty" follicles, mean volume of FF, number of mature oocytes (defined as cumulus-oocyte complexes put in fertilization in conventional IVF attempts and metaphase II oocytes in ICSI attempts), proportion of mature oocytes per retrieved cumulus-oocyte



complexes, proportion of conventional IVF compared to ICSI, endometrium thickness on the day of embryo transfer.

d. What was the mean size of the retrieved follicles in both groups? Please, add this information with the appropriate statistical analysis. Indeed, the authors mentioned in the Introduction (Pages 4-5, lines 54-55): “The expression of Prdx4 in the GCs of mature follicles was higher than that in the GCs of immature follicles”. This suggests that the size of follicles could influence the concentration of Prdx4 in FF.

e. No measure of ‘efficiency’ of FF collection is provided. How many follicles retrieved per women? How many oocyte cumulus complexes retrieved per retrieved follicles? What is the proportion of “empty” follicles in both groups?

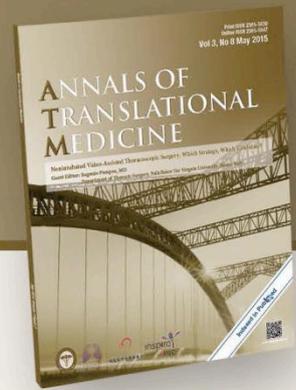
Reply: Thank you for pointing out these issues. We have added all the information and revised the manuscript accordingly (see Table 1).

4. Page 10, line 157, Table 2: it is not a correlation. The authors should mention a higher concentration in the pregnant group but not a correlation. Please, correct the sentence.

Reply: We have corrected it (see Page 11, Line 178-190).

5. The cut-offs used for Figure 1 (“low”, “middle” and “high”) seem arbitrary. This leads to the question whether these groupings were chosen after the results to show a difference between groups. Moreover, the authors could not claim that there is a correlation as no correlation test has been performed. Moreover, there is no statistical analysis to assess if the differences between these 3 arbitrary groups are significant.

Reply: Thank you for your kindly suggestion. We have revised and recalculated the data according to Prdx4 concentration quartiles as follows (see Page 12, Line 204-210): “To further investigate the association between the Prdx4 level and oocyte quality, we divided all participants into four groups by quartering the levels of Prdx4 (<13.38 ng/mL, 13.83-16.93 ng/mL, 16.93-22.93 ng/mL, >22.93 ng/mL). Then the oocyte quality in these four groups was analyzed, and the results are shown in Table 4. The fertilization rates, clinical pregnancy rates



and live pregnancy rates all significantly higher in the highest Prdx4 quartile group in comparison with those in the lowest quartile. ($p < 0.01$).”

6. The predictive value of Prdx4 should be determined statistically using a ROC curve, with also the sensitivity, specificity, positive and negative predictive values of this threshold. The area under the curve and the confidence interval must imperatively be presented as it is expected that the small sample size would result in a broad CI. The level of significance of the ROC curve must also be presented.

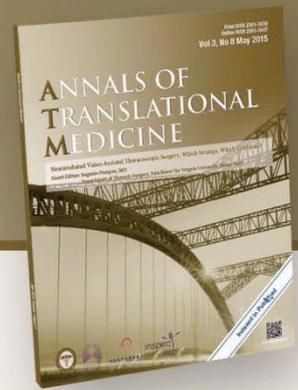
Reply: Thank you. We have added the ROC curve of Prdx4 in the new manuscript as follows (see Page 13, Line 215-219): “The predictive abilities of Prdx4 for clinical pregnancy were further analyzed by ROC curve (Figure 3). Prdx4 showed a high accuracy for the prediction of clinical pregnancy with an AUC of 0.754 (95% CI: 0.659–0.849). The Prdx4 cutoff value for clinical pregnancy prediction was 22.30 ng/mL with a sensitivity of 65.0% and a specificity of 81.1%.”

7. Table 2: is there a correlation between Prdx4, GSH-Px and SOD?

Reply: Thank you for your question. We analyzed the association between GSH-Px, SOD and Prdx4 as follows. We found the Prdx4 had no correlation with GSH-Px and SOD [$(r = 0.016; p = 0.906)$, $(r = 0.082; p = 0.532)$].

8. What is the concentration of Prdx4 in FF from women “who did not have oocyte or embryo of ideal quality” (i.e., excluded patients) (page 9, lines 143-144)? These data should be presented and adequately discussed within the manuscript, as the authors would argue the idea that FF Prdx4 concentration is a marker of oocyte competence.

Reply: Sorry for the missing information. We did not detect Prdx4 levels in excluded patients. Thank you for pointing out this issue. In a future study, we plan to continue exploring the expression of Prdx4 in patients from whom no oocytes were retrieved or no ideal quality embryos formed.



9. What is the inter-individual variation of Prdx4 concentrations? A graph or figure could be helpful.

Reply: Sorry, we only measured the Prdx4 concentration in each patient. We could add this information in further experiments.

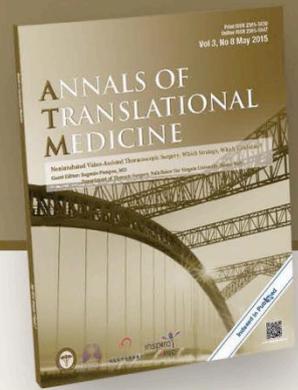
E) Discussion

1) The discussion should be rewritten to take into account all the corrections made in the manuscript after revision.

Reply: Thank you. We have revised the discussion section accordingly.

2) It is unclear how Prdx4 would be used as a biomarker. The authors state these biomarkers have the potential to improve outcomes, but do not state how they believe this will be possible. Would concentration be measured in follicular fluid aspirate with the idea that this would help embryo transfer order? Pooling of FF from different follicles leads to a “per patient basis” approach. Hence, this approach could not help the physicians to select the embryo(s) to transfer. Please, add information about the potential impact of your work on the management of IVF patients.

Reply: Thank you for your patience. We have revised the text in the new manuscript as follows (see Page 16, Line 280-287): “Previous studies have shown that during the oocyte IVM process, exogenous addition of the antioxidants, such as β -cryptoxanthin, metformin, and CoQ10, can significantly reduce oxidative stress levels and improve oocyte quality and developmental potential. Based on this study results, we supposed that exogenous addition of Prdx4 protein into the oocyte IVM culture medium may promote oocyte quality. In addition, when a patient shows a high level of Prdx4 in FF and she has extra oocytes for donation, we may suppose a better ongoing pregnancy outcome of the oocytes and might tend to use her oocytes priorly.”



For editor:

There are two major critical points in this work.

1) What are the quality controls carried out by the authors concerning the collection of the FF?

A lack in these quality controls could lead to major biases.

2) The authors should perform a ROC analysis to evaluate the predictive value of their marker.

The lack of data on these two critical points makes the assessment of the quality and relevance of their work very difficult.