Peer Review File

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Reviewer Comments

Overview and general recommendation:

For conventional slow freezing of human spermatozoa, the egg yolk cryopreservation media are actually widely used in ART, due to their important protective function of the plasma membrane, preventing thermal shock and improving sperm motility. Yet despite much promise, its use has been revised due to the biosafety aspects, with potential risk of transmitting infectious agents and variations in its composition. Because of this, the current study is on a topic of relevance and general interest to the readers of the journal. L-acetyl-carnitine mainly working on sperm metabolism, by modulating free CoA pools in the sperm with detoxification and anabolic properties, besides its antioxidant and antiapoptotic roles. PC based cryoprotectant obtained from soybean are related to its defined chemical composition, allowing to obtain good post-thaw quality. The current study attempts to use the synthetic cryoprotectant supplemented with L-α-phosphatidylcholine (PC) and L-acetyl-carnitine (ANTIOX-PC) to replace the standard egg-based TEST-yolk buffer (TYB) in preserving sperm motility and chromatin quality in cryopreserved human sperm. On the one hand, I found the introduction part of the paper to be overall well written and much of it to be well described. On the other hand, the discussion part to be overall general written and much of it should be described in point and logical. In the discussion part, the paper describes more data, however, those data were not found in the results part; In the results part, the paper uses a lot of descriptive sentences, not enough value descriptions. For the purpose of the paper, it should be explained more about the reason why it can replace the TYB buffer. The author explains exactly well about the L-α-phosphatidylcholine (PC) and L-acetyl-carnitine (ANTIOX-PC) in abstract. Therefore, I recommend that a major revision is warranted. I explain my concerns in more detail below.

Response: We thank the reviewer for their careful review of the manuscript and for their valuable comments. These comments are very constructive, and will help us to improve the manuscript, specifically in terms of clarifying our methodology and results of this paper. We address the reviewer's concerns in this letter, and corresponding changes will be made to improve the manuscript.

2.1. Major comments:

1. The first concern I have about the paper is with respect to the words using of Seminal, semen samples, and sperm. This study should distinguish the concepts of these words. Actually, this study cryopreserved human spermatozoa and evaluated the post-thaw quality.

Response: All the terms "seminal, semen, sperm and spermatozoa" were careful revised through the manuscript.

In the Abstract part, the author describes Seminal quality, semen and sperm separately, please make it clear and consistent with the following text. Because in your text, sometimes you write seminal samples, and then, spermatozoa (sometimes), or sperm samples, maybe next sentence, written semen.

Response: This section was re-written (Please, see lines 56-68) now we can read:

"METHODS: Prospective experimental study in which semen samples from 63 normospermic men and 58 asthenozoospermic men were included and analyzed both before and after cryopreservation using ANTIOX-PC or TYB freezing media. Sperm quality was evaluated by routine semen analysis and DNA fragmentation index using the Terminal deoxynucleotidyl transferase dUTP nick end labeling assay. RESULTS: Differences in the post-thaw progressive motility and DNA fragmentation index ($p>0.05$) were not detected between TYB and ANTIOX-PC cryoprotectants in both normal and low sperm motility groups. However, ANTIOX-PC medium retained higher non-progressive motility and lower percentage of immotile sperm when compared to TYB medium, resulting in a greater total motile sperm count ($p>0.05$), regardless baseline values of motility characteristic of the normospermic or asthenozoospermic samples.

2. The major concern I have about the paper is the method part. The description here is not very logical. Which method did you perform to collect spermatozoa? Before dividing into two aliquots and processing for cryopreservation, which medium you would use for collecting sperm? In page 8, line 195-196, "Semen and cryoprotectants were combined to a final 1:1." So which medium you performed to do 1:1 with cryoprotectants?

Response: We do not separate the sperm from the seminal plasma for cryopreservation. After seminal liquefaction, a routine semen analysis was performed using light microscopy to determine sperm concentration, vitality, morphology, and motility according to World Health Organization specifications. After that, the ejaculate was divided into two aliquots, and the fresh raw semen was pipetted into cryovials (Nalge Company, Rochester, NY) and mixed 1:1 (extended) with Antiox-PC or TYB.

In order to improve this section, the following sentence was re-written (lines 157-165):

"Ejaculates were obtained from 121 men from June 2015 to March 2018. All semen samples were obtained on-site by masturbation into sterile containers after at least 72 h of ejaculatory abstinence and left to liquefy at 37°C on a tube warmer for 30 minutes. Basic

semen analysis were performed in the andrology laboratory of HCFMRP/USP within 1 hour of collection and comprised the measurements of semen volume and sperm concentration, motility, vitality, and morphology. All the parameters were measured in accordance with WHO guidelines (32). The sperm morphology was evaluated according to Krüger's criteria (33) and the sperm vitality was assessed by using eosin-nigrosin test (32).

And then, you describe "all cryotubes were immersed in cold water and refrigerated for 30 minutes…" So, the cryopreservation volume is 1 ml? and what's the temperature of cold water? please make it clear.

Response: Done as suggested. The cryopreservation volume was 1 ml. The extended semen aliquots were left for 30 minutes immersed in cold water at 4°C, and then frozen by static-phase vapor cooling—by this approach, the cryovials were suspended in liquid nitrogen vapor (10 cm above the level of liquid nitrogen; −80°C) for 10 minutes. The samples were then plunged into liquid nitrogen (196°C) and stored until required (Please, see lines 175-185).

And then, how about the concentration of sperm cryopreservation?

Response: Only samples showing sperm count of $\geq 15x10^6$/mL and minimum volume of 1 mL were included in this study.

In your tables, I found data about vitality, but there is no explanation in the method part. Please check your method part carefully!

Response: We included this information in the method section (Please, see lines 163-165).

3. For the results part, if it's possible, maybe you could try to divide the "Assessment of sperm functional features" into two parts or more? Please use a subtitle to classify this part. It's too long sentences to get your key point. It will be more clearly following your treatment groups to describe it. Like Normal motility group and Low group?

Response: This section was re-written to explain the results more clearly, according to the suggestions and comments of the reviewer. Please find below the new text (Pages 13-15; Lines 272-323).

Assessment of sperm functional features

The seminal characteristics before and after freezing into the two different TYB and ANTIOX-PC cryoprotectants were analyzed, and the baseline characteristics of the semen analysis according to the WHO criteria and all parameters are shown in Table 2.

Pre-freezing sperm analysis

Regarding the main outcomes of this study, in pre-freezing samples from normal and low motility groups, sperm progressive motility rates were 47.37 ± 9.55 and 17.88 ± 8.46, and the DFI were 7.71 ± 7.75 and 9.03 ± 7.69, respectively (Tables 2 and 3).

Post-thawing sperm analysis

In normal motility group, all routine semen measurements after thawing were significantly lower than the pre-freeze samples not exposed to cryoprotectants and cryogenic temperatures, despite the freezing medium investigated (Table 2; $p < 0.001$). In low motility group, the same analyzes pointed out the morphology as the only parameter unmodified after cryopreservation ($p > 0.05$). Regarding baseline characteristics in neat semen, the sperm progressive motility in normal and low motility groups were 47.4% and 17.8%, respectively. After cryopreservation, however, we observed a more pronounced drop in progressive motility for normal than low motility group (73.8% vs. 30.3%, respectively), despite the freezing medium investigated.

Impact of cryoprotectants on sperm motility

Regarding the motility parameters investigated in both normal and low motility groups, significant differences were observed in the present study between the two cryoprotectants. Sperm from normal motility samples cryopreserved in ANTIOX-PC displayed nonprogressive motility superior to TYB (26.94% vs, 22.92%; $p < 0.05$) and quite similar rates to those observed in fresh semen (30.56%; $p = 0.016$). On the other hand, a tendency to both decreased non-motile sperm rates (60.87% vs. 64.67%; $p = 0.043$) and slightly increased total motility rates (39.48% vs. 35.33%; $p = 0.028$) were observed in ANTIOX-PC compared to TYB medium, respectively.

By comparing post-thawing sperm kinetics in the low motility group (asthenozoospermic samples), the non-motile sperm rates were significantly decreased whereas both nonprogressive motility and overall motility rates were significantly increased in ANTIOX-PC compared to TYB medium ($p < 0.0001$).

Impact of cryoprotectants on sperm DNA fragmentation index

The DFI of sperm among the neat semen from normal and low motility samples, TYB, and ANTIOX-PC groups are presented in Tables 2 and 3. Sperm DNA fragmentation increased significantly after cryopreservation, despite of the baseline semen characteristics of normal and low motility samples included in this study. There was no statistically difference in the percentage of spermatozoa with fragmented DNA between TYB and ANTIOX-PC media (Tables 2 and 3). Regarding low motility samples, however, post-thawed sperm from ANTIOX-PC medium presented a slightly trend in DFI reduction ($p = .0742$). The percentage of DNA fragmentation were 15.8%, 13.3%, and 9% for TYB, ANTIOX-PC and fresh semen, respectively (Table 3) in these samples. Although the DFI differences between

the two freezing media evaluated were not significant, the DFI increased by about 75.8%, and 47.8% in spermatozoa recovered from TYB and ANTIOX-PC, respectively. Representative TUNEL images of post-thaw human spermatozoa from low motility samples cryopreserved in TYB and ANTIOX-PC media are shown in Figure 1.

And please carefully check your P-value. In Page 13, line 295-297: "As shown in Tables 2 and 3, in both groups the concentration were not different as compared with … ($p < .05$)". "not different", why $P < 0.05$? In the abstract, the same as that.

Response: The P-value was careful checked and correctly in the text.

One important thing is the page 13, from line 309 to 313. It's better for you using data to describe your tendency. This is really a description of the site, not a result. Please move it to a more appropriate location in the manuscript. It is confusing when text is presented in the wrong section.

Response: This sentence was removed from the manuscript text.

4. Discussion part. It is very important for the reader to know how the L-α-phosphatidylcholine (PC) and L-acetyl-carnitine (ANTIOX-PC) working better to replace the standard egg-based TEST-yolk buffer (TYB) in preserving sperm quality in cryopreserved human spermatozoa.

Response: We have made this clear now by including the following text in the discussion section (lines 376-386):

Membrane lipid peroxidation and disrupted energy metabolism are major events leading to sperm cell death after cryopreservation. These cooperative pathways share as one common aspect the triggering of oxidative stress by free radical formation. L-carnitine is an antioxidant agent used in the treatment of men with low seminal quality removing the toxic excess of intracellular acetyl-CoA and protecting the spermatozoa from oxidative damage (27-29). Moreover, carnitines play a key role in energy metabolism, transferring free fatty acids from the cytosol to mitochondria, facilitating their oxidation and generation of adenosine triphosphate (27, 30). In cardiac cells, L-carnitine is essential for mitochondria function, to attenuate the membrane permeability transition, and to maintain the ultrastructure and membrane stabilization, in the presence of high fatty acid β-oxidation (37).

In this part, the author shows more data to describe the supplementation of PC or ANTIOX-PC is better than egg yolk, like page 15, line 344: "73.8% vs. 30.3%, respectively", the

author didn't mention that in results part. However, page14, lines 341-343, "were 47.4% and 17.8%, respectively", the author repeated the data of the results part. The author spends a lot of space to show these values, however, the author describes well about the functionality of PC and ANTIOX-PC in the introduction part. Perhaps the author should try to briefly describe PC and ANTIOX-PC in the introduction part, focus more details on the discussion section.

Response: Accordingly, we cited these data in the Results section, and as suggested, we described the functionality of PC and ANTIOX-PC in the Discussion section.

2.2. Minor comments:

9. Page 3, lines 65. "…index ($p<.05$) were not detected…". I feel it is not correct. I suggest re-checking this significant p. In Tables 2 and 3, $p >0.05$.

Response: Done as suggested. Please see line 64.

10. Page 3, Lines 60: "…Seminal quality…", Page 3, Lines 61 "…semen analysis..."; Page 3, Lines 66 "…semen samples..."; Page 3, Lines 70, "…human sperm...". Please make it clear using correct words.

Response: The terms were careful checked and correctly cited thought the text.

11. Page 8. Lines 178: "…and all oh them were analyzed". "oh", maybe it's "of".

Response: Done as suggested

12. Page 11, Lines 268: please add the detailed information of the software (SAS version 9.4 program13). Like company.

Response: Done as suggested.

14. Page 12, line 287: "…are shown in Table 2.". Maybe not only show in Table 2, but Table 3 is also the baseline characteristics of sperm analysis after freezing.

Response: This information was correct in the text. Please, see line 282.

15. Page 12, lines 289: "the mean sperm progressive motility...", please consider deleting the term "mean" in everywhere you used, because the author mentioned that in Statistical Analysis part, "all data are presented as the Mean ± SD".

Response: Done as suggested.

16. Page 13, Lines 297: I don't understand this. "were not different as compared with those of TYB medium ($p < .05$)..". If there were not different, why $p < 0.05$? I check the data in Table 2 and 3, for morphology and vitality of post-thawed, p=0.175/0.148 or p=0.875/ 0.1185 respectively. Please check it.

Response: Done as suggested.

17. Page 14, from lines 331-335: "…. above this threshold…". I don't understand this. Here you want to express your groups normal and lower progressive motility up to 32x106/mL or lower than? Or the post-thaw motility? Please check the long sentence.

Response: As suggested, this section was modified in the manuscript (lines 327-330):

"In this study we examined the progressive motility recovery rates and DNA integrity index of human spermatozoa from men with normospermia or asthenozoospermia, after freeze-thaw cycles using a synthetic soy-PC and L-acetyl-carnitine-based cryoprotectant (ANTIOX-PC) or the conventional egg yolk freezing medium (TYB)."

18. Page 16, lines 369: "…net semen…" Or neat?

Response: This spelling mistake was corrected.

19. Page 23: Figure 1: Please add the scale bar. And if it's possible, please use the arrow to mark the statistics that mentioned TUNEL-positive or negative spermatozoa.

Response: Done as suggested.