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Reviewer A

This Manuscript aimed at evaluating the suitability of different devices to cryopreserve human sperm samples (cryopiece, mini- and micro-straws). Different post-thaw evaluation parameters (including MMP and DFI) were evaluated together with cooling, retrieval and recovery rates. The authors concluded that cryopiece, which showed higher recover rates, could be a good device to cryopreserve sperm samples

GENERAL COMMENTS

Although the Manuscript has some interest and the authors provide some proofs about the suitability of Cryopiece, the Manuscript has two shortcomings that do not allow me to recommend it for publication in this Journal:

1) Sperm motility, which is not shown in results section, was not evaluated using an objective, computer-assisted system

Reply: Sperm motility was shown in the result section titled "**The sperm cryopreserved using Cryopiece has the highest recovery rate and retrieval rate**" in the form of recovery rate, which indicated the ratio of the proportion of motile sperm after freezing-thawing to that before freezing. (See L267-L269).We agreed that CASA (computer-assisted semen analysis) would be an objective, repeatable method for semen examination. However, the sperm specimen evaluated in this work had a low sperm concentration and the total amount of sperm was extremely small, especially for that loaded on cryopiece, which was not suitable to be evaluated by CASA, so computer-assisted system was not employed in this work.

2) Sperm membrane integrity, which is the best way to evaluated sperm viability, was not determined. Without this parameter being evaluated, it is impossible to ascertain that Cryopiece was definitely the best device to use

Reply: Thank you for your suggestion. The reasons why we did not detect the sperm membrane integrity in our work were list below.

First, the basic standard of sperm selection for clinical embryologists is only based on sperm motility and morphology. Motile sperm indicated that the sperm was alive. So higher proportion of motile sperm observed in the specimen cryopreserved using cryopiece was sufficient to prove that cryopiece was better than the other two carriers

in sperm cryopreservation.

Second, in some cases, sperm membrane integrity was not necessary related with sperm viability. Sperm integrity test was performed after we received your comments. Eosin-nigrosine staining, which was widely used in clinical practice for sperm viability assay based on the testing of sperm membrane integrity, was performed to see the sperm membrane integrity before and after sperm freezing. It was surprising that though the recovery rate of the cryopreserved sperm was agreed to that reported in our article, which means that most sperm remained motile after freezing-thawing, almost all the sperm was staining in red, which indicated that the integrity of the sperm membrane was broken. We further checked whether the sperm membrane integrity was broken by freezing-thawing, or by the adding of CPA. It was determined that sperm specimen mixed with the commercially available CPA, which was widely used in clinical sperm cryopreservation all over the world, without further freezing, also turned out to be staining in red with a ratio over 95%. So it was the CPA that changed the integrity of sperm membrane, but it would not change the viability of these sperm.

In addition, the Manuscript contains several grammar/spelling mistakes, so that the English should be corrected by a native speaker or professional editing service, and stats should be revisited, since it is not clear whether ANOVA or t-test was run. Post-hoc tests other than LSD should have also been run.

Reply: Tukey test was included. And the normal distribution test and variances homogenous test had been performed as a packed function while using GraphPad for statistical analysis. Details were described in the section of "statistical analysis". (See L242-L248)

Finally, there are some methods that should be better detailed and sperm morphology should have been determined with TEM, since bright-field microscopy is not throughout enough to determine differences in cryoinjuries between devices.

Reply: Thank you for your suggestion. We'd like to perform TEM, while the amount of sperm loaded in/on these carriers, especially on cryopiece was too small to meet the requirement of TEM. It would be hard to find the targets after TEM slides were prepared using such few spermatozoa.

SPECIFIC COMMENTS

L29 'frozen'

Reply: The suggested word has been revised. (See L29)

LL38-39 Rewrite

Reply: The sentences have been rewritten. (See L38-L40)

LL48-54 Rewrite in present tense

Reply: The sentences have been rewritten in present tense. (See L49-L55)

L58 ‘very challenging’

Reply: The suggested phrase has been revised. (See L59)

L75 ‘a systemic evaluation of Cryopiece has not yet been conducted’

Reply: The sentence has been revised. (See L76-L77)

L91 ‘meaning unclear’

Reply: Sorry for the confusing descriptions. The basic operation of individual sperm cryopreservation is picking the selected sperm from the sperm specimen and loading it on the carrier, such as Cryopiece in this work, individually. Only the motile sperm would be picked in this procedure, so to make the work comparable, motile sperm should be used in all cryopreservation procedure, no matter what kind of carrier was used. And that’s why swim-up method was employed in the treatment of the semen specimens. A punctuation mistake was corrected so that it would be easier to be understood. (See L93-L95)

L93 More details on the swim-up procedure should be provided together with information on the evaluation of sperm quality before and after swim up. It should be described how sperm motility was determined and graded

Reply: Details on swim-up procedure were provided. (See L96-L104) The way determining and grading sperm motility was described in the methods, following the description of World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen (5th Edition). (See L88-L92 and L185-L186)

L126 at which temperature was the oil and for how long were sperm thawed in the bath?

Reply: The oil was pre-heated at 37 ℃, and these details have been added. (See L136-L140)

L146 the protocol followed for mini- and micro-straws should be briefly described

Reply: Detailed protocol followed for these carriers was described. (See L159-L173)

LL149-154 It is not clear to me why samples of 20 donors were involved, since 60 men were reported to participate in this research. Please clarify

Reply: The recovery rate and the retrieval rate of the sperm after cryopreservation were the most concerned in our work, since cryopiece was designed for the preservation of extremely rare sperm. To gain accurate recovery rate and retrieval rate, we performed the work strictly following to the clinical procedure, picking the sperm from the specimen and loading them onto the Cryopiece using a micro-manipulation system individually, and count all the sperm after freezing-thawing. Technological-repeatability was carried out 3 times for each specimen. It was a time-cost work, and would occupy the micro-manipulation system for a long time, which would inevitably interrupt the clinical work, so 20 specimens instead of all the 60 included specimens

were involved for biological repeatability. For the other examinations such as DFI detecting, morphology analysis, and MMP detecting, the limited sperm loaded on the carrier was far from enough. The procedure was then simplified by using up-swim screened motile sperm instead of micro-manipulation system picked sperm, and only a partial sperm in each specimen was analysis and counted after processing (for example, about 200 spermatozoa were counted in the DFI examination according to the instrument instead of all the spermatozoa mounted). To avoid the sampling errors, more samples (all the 60 specimens included in this work) were involved in these examinations.

L160 Was a computer assisted system used?

Reply: Computer assisted system was not employed in this work since the extremely low number of spermatozoa involved in this work could not be evaluated by a computer assisted system accurately. An experienced technician was better.

L169 Please give more details on the SCDt protocol, including how samples were stained

Reply: The details of SDC protocol for DFI detecting were provided. (See L196-L207)

L183 Was the evaluation with JC-1 combined with plasma membrane integrity?

Reply: No, it was not. As we mentioned previously, plasma membrane integrity was changed by CPA, which had no related with other measurement mentioned in this work.

L204 stats should be revisited. LSD is not a correct post-hoc test to run. Tukey or Sidak would be more appropriate. The authors should have also ensure that data followed a normal distribution and that variances were homogenous prior to running the linear model

Reply: Tukey test was included. And the normal distribution test and variances homogenous test had been performed as a packed function while using GraphPad for statistical analysis. Details were described in the section of "statistical analysis". (See L242-L248)

L207 Why aren't sperm motility data before and after freeze-thawing shown?

Reply: It was shown in the form of recovery rate in the results. (See L266-L269) And the way for calculate the recovery rate was list in the methods. (See L187-188)

Reviewer B

The work of Zhu et al assesses a device to freeze a low number of sperm and evaluate the effect on motility, morphology, DNA fragmentation and mitochondrial membrane potential.

The biggest problem is the experimental design, since when indicating the freezing of such a low number of sperm it is not possible to evaluate the percentage of morphology, since the sperm they choose is assumed to be normal, let alone determine DNA fragmentation and membrane potential mitochondrial, since when performing flow

cytometry they need at least 10,000 sperm.

Therefore, this should be made explicit in the methodology that sperm concentration was used to carry out the evaluation of morphology, DNA fragmentation and mitochondrial membrane potential.

Reply: The recovery rate and the retrieval rate of the sperm after cryopreservation were the most concerned in our work, since cryopiece was designed for the preservation of extremely rare sperm. To gain accurate recovery rate and retrieval rate, we performed the work strictly following to the clinical procedure, picking the sperm from the specimen and loading them onto the carrier using a micro-manipulation system individually, and count all the sperm after freezing-thawing. Technological-repeatability was carried out 3 times for each specimen. It was a time-cost work, and would occupy the micro-manipulation system for a long time, which would inevitably interrupt the clinical work, so 20 specimens instead of all the 60 included specimens were involved for biological repeatability. For the other examinations such as DFI detecting, morphology analysis, and MMP detecting, the limited sperm loaded on the carrier was far from enough. The procedure was then simplified by using up-swim screened motile sperm instead of micro-manipulation system picked sperm, and only a partial sperm in each specimen was analysis and counted after processing (for example, about 200 spermatozoa were counted in the DFI examination according to the instrument instead of all the spermatozoa mounted). To avoid the sampling errors, more samples (all the 60 specimens included in this work) were involved in these examinations. The concentration of sperm specimens after swim-up screening was indicated in the method, the total number of screened spermatozoa was sufficient for these examinations.(See L102-L103)

Since the damage is also associated with the concentration of sperm used and it should be noted that these are values that could occur in the sperm that were selected and frozen in the cryopiece.

Reply: Thank you for reminding us about the relationship between concentration of sperm and sperm damage. The sperm concentration was different only in the assessment of sperm recovery rate and retrieval rate, which seems that Cryopiece performed better than the other two carriers, and Cryopiece was designed for the cryopreservation of such few spermatozoa. In the later examinations such as DFI testing, morphology assessment, and mitochondrial membrane potential detecting, the sperm concentrations in different groups were the same, as we using swim-up screened sperm directly. The reason why we did so has been explained. As a result, the only differences between different groups were the carrier used and the volume of the specimen, the concentration of sperm would not cause differences in the results.

Nor in the discussion do they refer to simpler and cheaper methods and with better results than the freezing carried out in this protocol, which is vitrification with a small

number of sperm (Spis, M, Bushkovskaia A. Isachenko E, Todorov P, Sanchez R, Skopets V, Isachenko V. Conventional freezing vs. cryoprotectant-free vitrification of epididymal (MESA) and testicular (TESE) spermatozoa: three live births. Cryobiology 90:100-102, 2019).

Reply: Thank you for providing the reference. The work you mentioned was referred in the revised version of our manuscript. (See L357-363) To be honest, the number of sperm to be cryopreservation in our clinical work is far rarer than that described in the quoted article. Usually, no more than 100 spermatozoa could be retrieved in the whole sample. Sometimes even only 1 spermatozoon could be retrieved. These spermatozoa would be lost in the procedure of cryopreservation. Cryopiece was designed to avoid the loss of available sperm during cryopreservation, and it was proved that it had a better sperm retrieved rate and recovery rate in our work. The method in the quoted article could provide a better recovery rate, but since different source of sperm was used in these two works, the results cannot be compared. Besides, no retrieval rate after sperm freezing-thawing was provided in the quoted article. Could sperm still be retrieved after freezing-thawing when no more than 10 spermatozoa were cryopreserved following this protocol? Anyway, thank you for provide a valuable method for us. We'd like to try this protocol, combining with the use of cryopiece in our future work.

Minors

Line 93 Swimming-up change for swim-up, more commonly used in scientific articles.

Reply: Swimming-up had been changed for swim-up. (See L95)

Line 93 Fumigated is confusing, better use vapors of liquid nitrogen

Reply: Vapors of liquid nitrogen had been used. (See L134)

The figures must have the reading of the evaluation that is being carried out

Reply: The reading had been added in the revised figures. (See Figure 3-6)