Peer Review File

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Reviewer A

This is an interesting manuscript that is easy to follow and that describes the effect of a second semen sample on DFI after 3-4 hours in infertile men.

Comment 1:
The authors may want to add some more information on the cutoff used and may want to clarify somewhat when a difference was in relative terms or on DFI as such, and consider to indicate both.

Reply 1: The manuscript has been revised according to each comment mentioned in the manuscript, including additional data regarding the 30% cutoff as well the comments on the percentage points and relative percentage change in DFI levels. Please see the attached PDF file that contains all comments and response to each comment with the referral to the revised text in the highlighted manuscript.
Changes in the text: Throughout the entire manuscript as mentioned in detail in the point-by-point response.

Specific questions are indicated in the attached file.
Reply: Please see attached PDF file with point by point reply to comments.
Point by point reply to comments appear in the attached PDF (the line number in the comment itself refers to the PDF fie, while the line numbers in the response refers to the lines in the R1 highlight version). We also copied the comments from the PDF file to this file for the the convenience of the editor and reviewer:

-Comment 1 (Line 94): Are there other views on whether a deterioration has occurred or not? What is the scientific consensus around this?

Reply 1: We agree there is still in an ongoing controversy. Some reports did not find a decline in semen quality over the last decades (Li WN, Jia MM, Peng YQ, Ding R, Fan LQ, Liu G. Semen quality pattern and age threshold: a retrospective cross-sectional study of 71,623 infertile men in China, between 2011 and 2017. Reprod Biol Endocrinol. 2019 Dec
9;17(1):107; Punjani N, Alawamlh OA, Kim SJ, Salter CA, Wald G, Feliciano M, Williams N, Dudley V, Goldstein M. Changes in Semen Analysis over Time: A Temporal Trend Analysis of 20 Years of Subfertile Non-Azoospermic Men. World J Mens Health. 2023 Apr;41(2):382-389), while others, including a recent meta-analysis and data from recent large study from Asia and Europe and a large combined analysis of semen analyses from over 100,000 men from the United States and Spain, suggested a deterioration in semen quality over the years, especially sperm concentration (Tiegs

AW, Landis J, Garrido N, Scott RT Jr, Hotaling JM. Total motile sperm count trend over time: evaluation of semen analyses from 119,972 men from subfertile couples. Urology 2019;132:109-16, Mishra P, Negi MPS, Srivastava M, Singh K, Rajender S. Decline in seminal quality in Indian men over the last 37 years. Reprod Biol Endocrinol 2018;16:103, Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update 2017;23(6):646-59, Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis of samples collected globally in the 20th and 21st centuries. Hum Reprod Update 2022 Nov 15:dmac035).
Therefore, in response to this comment, we highlighted this point and mentioned that there is still an ongoing debate on this matter.
Changes in the text: see page 5, line 96-99: "*An additional factor is the possible global deterioration in sperm quality over the last few decades reported by several studies and meta-analyses (4,5,6,7), although some studies have shown contrasting results (8,9) and this subject is still in debate*".

-Comment 2 (line 98): where in the guidelines is it written that it is recommended?
Reply 2:
We thank the reviewer for this comment. In the WHO 6th edition in the PDF version page 101/292 , section 3.2.1, at the end of the second paragraph - it is mentioned that: "is also known that sDF is prevalent among men with abnormal ejaculate parameters, and it has been proposed to be related to cases of infertility in normozoospermic individuals. Since sDF is only partially related to semen quality (164, 171), it could represent an important addition in the work-up of male infertility, becoming one of the most discussed and promising biomarkers in basic and clinical andrology".
We agree with the reviewer that it is not a clear recommendation and therefore revised this sentence accordingly.
Changes in the text: see page 5, lines 101-103: " *Sperm chromatin integrity is a promising biomarker in the evaluation of male infertility ,as recently mentioned in the 6th edition of the world health organization (WHO) guidelines (13)".*

-Comment 3 (line 114): Could the authors mention which outcomes that have been (repeatedly) associated with high DFI?

Reply 3:
Although, again, there is still controversy on this matter, the main outcomes negatively associated with elevated DFI are IVF pregnancy rates (*Henkel R, Hajimohammad M, Stalf T, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 2004;81(4):965–72 and Ribas-Maynou J, Yeste M, Becerra-Tomás N, Aston KI, James ER, Salas-Huetos A. Clinical implications of sperm DNA damage in IVF and ICSI: updated systematic review and meta-analysis. Biol Rev Camb Philos Soc 2021;96:1284-300, Osman A, Alsomait H, Seshadri S, et al. The effect of sperm DNA*

fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. Reprod Biomed Online 2015;30:120-7 and Jin J, Pan C, Fei Q, et al. Effect of sperm DNA fragmentation on the clinical outcomes for in vitro fertilization and intracytoplasmic sperm injection in women with different ovarian reserves. Fertil Steril 2015;103:910-6.) and IVF pregnancies miscarriage rates (*Simon L, Brunborg G, Stevenson M, et al. Clinical significance of sperm DNA damage in assisted reproduction outcome. Hum Reprod 2010;25:1594-608, Lin MH, Kuo-Kuang Lee R, Li SH, et al. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. Fertil Steril 2008;90:352-9, Robinson L, Gallos ID, Conner SJ, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 2012;27:2908-17, Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 2014;102:998-1005.e8*).

Changes in the text: see page 5, lines 118-120: "*there is now growing literature to support the association of high DFI with impaired IVF pregnancy and miscarriage rates.(18,24-28)*".

-Comment 4 (line 129): could the authors mention how much the DFI was improved in these all three studies? Did those studies use SCSA?

Reply 4:

In the study by Gosalvez– a 10% absolute reduction was reported (SCD). The SCD technique was also used in the studies by Dahan et al. (10.9% absolute decrease) and Kulkarni et al. 3.3% absolute reduction).

Changes in the text: see page 5, lines 132-139:"*Few studies have investigated this approach with the evaluation of two consecutive samples given in 1 to 4 hours apart (23-36) using sperm chromatin dispersion test. Gosálvez et al.(34) and Dahan et al.(35) focused on normozoosprmic or mostly normozoospermic men and showed an improvement in DFI (approximately 10%) after short abstinence. In the study by Kulkarni et al (36), seventeen men undergoing primary infertility treatment were evaluated for DFI change after short abstinence, with similar, though smaller, 3% improvement*".

-Comment 5 (line 147): did both conventional sperm parameters and DFI have to be abnormal for a man to be included or was it enough if either one or the other was abnormal?

Reply 5:

This sentence does need to be clarified. We only included infertile men referred to us due to infertility and that had abnormal sperm parameters and had a DFI measurement,

so that all men included had a semen analysis with abnormal sperm parameters. We revised this sentence to better clarify.
Changes in the text: see page 5, lines 160-161: "Infertile men with abnormal sperm parameters who had a DFI measurement were included in this study".

-Comment 6 (line 228):
do the authors mean percent or percentage points here and at the other places when a change in percent is mentioned?
Reply 6:
We thank the reviewer for this comment, allowing us to improve the clarity of the results reported. This data represents percentage points, meaning an absolute decrease of 5 and 10 percentage points (which represents higher relative percentage drop). We revised this sentence and the text throughout the manuscript, as suggested by the reviewer and clarified if we report percentage point (absolute DFI units) or relative percentage (relative reduction in DFI)

-Comment 7 (line 259): Don't the authors mean percentage points here and that the actual change in percent was even higher?
Reply 7:
Indeed, as mentioned, this does refer to percentage points, which represents 9.8% decrease. This clarification was added to the text.
Changes in the text: see page 5, lines 282-283: "*Our results demonstrate that this technique is associated with a decrease of nearly 4 percentage points (9.8% relative decrease) in DFI*".

-Comment 8 (line 265): How widely used is 30% as a cutoff, and what do reviews say about which cutoff may be the most common or clinically important?
Reply 8:
In addition to the 4 references mentioned in this paragraph, a thorough review ((Reference #14 in the manuscript (Agarwal A, Majzoub A, Baskaran S, Panner Selvam MK, Cho CL, Henkel R, Finelli R, Leisegang K, Sengupta P, Barbarosie C, Parekh N, Alves MG, Ko E, Arafa M, Tadros N, Ramasamy R, Kavoussi P, Ambar R, Kuchakulla M, Robert KA, Iovine C, Durairajanayagam D, Jindal S, Shah R. Sperm DNA Fragmentation: A New Guideline for Clinicians. World J Mens Health. 2020 Oct;38(4):412-471) and a guideline paper on Global Survey, Current Guidelines, and Expert Recommendations published in 2023 (Agarwal A, Farkouh A, Saleh R, et al. Controversy and Consensus on Indications for Sperm DNA Fragmentation Testing in Male Infertility: A Global Survey, Current Guidelines, and Expert Recommendations. World J Mens Health. 2023;10.5534/wjmh.220282), specified the cutoff of 30% in the context of DFI tested using SCSA as the commonly used and clinically relevant cutoff for elevated DFI.
Changes in the text: see page 12, lines 287-292: *"While this absolute decrease in DFI ,*

though significant, is relatively small, our study also shows that that almost two thirds (64%) of patients with high DFI within the 30-40% range will have DFI value of less than 30% on the second semen test, which is widely used as a cutoff for abnormal DFI levels and for which reproductive outcomes were reported to be better compared to DFI>30% (43-46,14)".

-Comment 9 (line 273): Could the authors refer to studies or a review claimin 30% to be the most common cutoff?

Reply 9:

The paper by Agarwal et al **.**(Transl Androl Urol. 2016) mentioned this cutoff - in page 4, under "SCSA (6)" the authors mention that : "The clinical threshold is an SDF index of 30%...". Additionally, in page 8, under "SDF and natural pregnancy" the authors mention that "SDF has been found to be a valuable prognostic tool in assessing the chances of natural pregnancy in couples. The chances of natural pregnancy are reduced when the SDF index, measured by SCSA, is between 20–30% and is virtually nonexistent when the SDF index is higher than 30% (Evenson DP, Jost LK, Marshall D, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999;14:1039-49)".

A meta-analysis involving three studies and 616 couples demonstrated that this high SDF, determined by the SCSA test, was associated with failure to achieve natural pregnancy with an odds ratio (OR) of 7.01 (95% CI, 3.68– 13.36**) (**Zini A. Are sperm chromatin and DNA defects relevant in the clinic? Syst Biol Reprod Med 2011;57:78-85).

In a recent paper (Agarwal A, Farkouh A, Saleh R, et al. Controversy and Consensus on Indications for Sperm DNA Fragmentation Testing in Male Infertility: A Global Survey, Current Guidelines, and Expert Recommendations. World J Mens Health. 2023;10.5534/wjmh.220282), the cutoff of 30% is mentioned in the context of DFI tested using SCSA as the commonly used and clinically relevant cutoff **.**

However **,**as we agree that it is not definitive and choosing a specific cutoff level over the other is not clear, we revised this sentence to clarify that many studies have used this cutoff, rather than claiming that "most" studies have used this cutoff.

Changes in the text: see page 12, lines 298-299: "However, as many of the studies refer to this value" and page 14, lines 344-346: " Numerous studies have investigated the predictive value of DNA fragmentation levels on spontaneous abortion and ART outcomes – IVF and ICSI, with the use of the 30% cutoff value for abnormally high DFI (48,26,24)".

-Comment 10 (line 291): How much lower was the fragmentation?

Reply 10:

10 percentage points lower. This information was added to the introduction, as the reviewer suggested, in response to a previous comment.

Changes in the text: see page 6, lines 132-139:" *Few studies have investigated this*

approach with the evaluation of two consecutive samples given in 1 to 4 hours apart (23-36) using sperm chromatin dispersion test. Gosálvez et al.(34) and Dahan et al.(35) focused on normozoosprmic or mostly normozoospermic men and showed an improvement in DFI (approximately 10%) after short abstinence. In the study by Kulkarni et al (36), seventeen men undergoing primary infertility treatment were evaluated for DFI change after short abstinence, with similar, though smaller, 3% improvement".

Comment 11 (line 293): Was this truly a 3% percent change or a 3 pecentage points of change (which is more)?

Reply 11:

This was an absolute, 3.3 percentage points decrease. This clarification was added to this sentence.

Changes in the text: see page 13, lines 322-315: "A recent study, by Kulkarni et al.(36), evaluated the difference in DFI in 17 patients and showed a 3 percentage points (10.8%) decrease between tests".

-Comment 12 (line 299):

Reply 12:

The authors did not report the percentage points in their manuscript or tables but rather grouped all patients for which the decrease in DFI was of 30% or more.

Changes in the text: see page 13, lines 328-331**: "***They showed a significant decrease in DFI in the second sample and reported that the factors predictive of a relative 30% or more decrease in DFI levels are age and use of antioxidant formulation*".

-Comment 13 (line 314): how many studies have used other cutoffs? What is the often used clinical cutoff?

Reply 13: It is difficult to estimate the most used cutoff and indeed many studies have used other cutoffs, such while many as >25%, >35% or even 40% as their cutoff. Therefore, as we also responded to comment #9, we agree with the previous comments by the reviewer and revised the state in this paper claiming 30% is a widely used cutoff and mentioned only that it is used in many studies as the cutoff when assessing clinical outcomes.

-Comment 14 (line 315): What outcomes are the most important ones that were used for this cutoff? Chances of achieving pregnancy or other outcomes?

Reply 14:

IUI pregnancy and delivery rates, as shown in a study by Bungum et al. (Bungum M, Humaidan P, Axmon A, Spano M, Bungum L,Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod 2007;22:174-9) and IUI miscarriage rates (Yang H, Li G, Jin H, Guo Y, Sun Y. The effect of sperm DNA fragmentation index on assisted reproductive technology outcomes and its relationship with semen parameters and lifestyle. Transl Androl Urol.

2019;8(4):356-365. doi:10.21037/tau.2019.06.22) are the most important outcomes assessed using this cutoff. Additionally, IVF pregnancy miscarriage rates (Braga DPAF, Setti A, Morishima C, Provenza RR, Iaconelli A Jr, Borges E Jr. The effect of sperm DNA fragmentation on ICSI outcomes depending on oocyte quality [published online ahead of print, 2023 Apr 2]. Andrology. 2023;10.1111/andr.13435. doi:10.1111/andr.13435) and livebirth rates (Repalle D, Saritha KV, Bhandari S. Sperm DNA fragmentation negatively influences the cumulative live birth rate in the intracytoplasmic sperm injection cycles of couples with unexplained infertility. Clin Exp Reprod Med. 2022;49(3):185-195.doi:10.5653/cerm.2021.05169) were also assessed using this cutoff.

This important clarification and additional references were added to the discussion.

Changes in the text: see Page 14, lines 338-342*:" Several studies and metanalyses on DFI and ART outcomes concluded that though it is still debatable, data suggests that high sperm DNA fragmentation in couples undergoing intra-uterine insemination (IUI) and IVF is associated with lower pregnancy rates and LBR as well as higher spontaneous abortion (43,49,50,22,48,24,26)".*

-Comment 15 (line 337): how clear is that?

Reply 15:

We accept this comment. We removed this sentence and elaborated on other possible mechanisms linking short abstinence and lower SDF. We have revised the text accordingly Changes in the text: see Page 15, lines 363-386: " *Several mechanisms on the link between short abstinence and improved SDF were hypothesized (38). It has been suggested that the main mechanism for SDF formation is the exposure of sperm to ROS(19), dead spermatozoa and leukocytes(51) during the transport of sperm from the testes to the epididymis and its storage there, resulting in sperm DNA breakage (30,31) The duration of transition through the epididymis varies and might be affected by the frequency of ejaculation, thus short abstinence might be associated with faster passage and decreased SDF. An additional theory focuses on the seminal plasma composition and its effect on SDF. Seminal plasma metabolites are secreted and accumulate with time and therefore short abstinence may result in an overall lower metabolites levels and subsequently lower fragmentation levels (52).*

A previous study by Shen et al. (53) investigated the mitochondrial plasma membrane potential (MMP), which correlates with sperm motility, in spermatozoa from samples given after several days vs. 1-3 hours of abstinence using proteomics technology. Their investigation demonstrated higher MMP after shorter abstinence, which represents another possible mechanism for improved sperm quality after short abstinence. Further research is required to identify these factors and enable prevention and treatment if needed.".

-Comment 16 (line 338): which could these substances be?
Reply 16: Please see our response to comment #15 and the revisions made in the text accordingly.

Reviewer B

Studies on men with infertility are scarce, and your study could be a contribution. However, this article was based on only one result.

Comment 1: Why were no further analyses performed with the samples (sperm and seminal plasma)?
Reply 1: The analysis for both samples was a CASA semen analysis and a sperm DFI assay. Other assays such as sperm aneuploidy testing, sperm penetration assays, acrosome reaction, sperm epigenetics etc. would be extremely useful and interesting. We do plan these for future studies.

Comment 2: There were very few exclusion factors. Why was the sample collection done over 5 years? It was very far.
Reply 2: We thank the reviewer for this comment. We were selecting patients based upon their interest and willingness to participate, and the availability of laboratory services. Therefore, this study took a long time to complete. We added this important clarification to the methods section.
Changes in the text: see page 7, lines 164-165: "*Patients were selected based upon their interest and willingness to participate, and the availability of laboratory services*".

Minor points

-Comment 1: Check page 5 line 96 “age6”.
Reply 1: Corrected. This was a typo and the number referred to reference number 6
Changes in the text: see page 5, line 95

-Comment 2: Check page 6 line 118 “levels9”.
Reply 2: Corrected.
Changes in the text: see page 6, line 124

-Comment 3: Page 6 – There were more recent studies with an abstinence period (short and 4 days) with analyzing DNA fragmentation. You need to update.
Reply 3: We thank the reviewer for this comment. We have added references of 2 recent studies - A study by Tvrdá et al. (*Tvrdá E, Ďuračka M, Benko F, et al. Ejaculatory Abstinence Affects the Sperm Quality in Normozoospermic Men-How Does the Seminal Bacteriome Respond?. Int J Mol Sci. 2023;24(4):3503. doi:10.3390/ijms24043503*) which compared bacterial profile, as well as sperm parameters, membrane integrity, membrane potential and DNA integrity in 51 men after 2 days and 2 hours abstinence

periods. This study showed improved DNA integrity, among other parameters, After short abstinence. We also mentioned the recent important metanalysis by Barbagallo et al. (*Barbagallo F, Cannarella R, Crafa A, et al. The Impact of a Very Short Abstinence Period on Conventional Sperm Parameters and Sperm DNA Fragmentation: A Systematic Review and Meta-Analysis. J Clin Med. 2022;11(24):7303. Published 2022 Dec 8. doi:10.3390/jcm11247303*). This meta-analysis investigated all sperm parameters and additionally demonstrated that very short abstinence improves SDF in patients with abnormal sperm parameters. This was added to the introduction, as suggested by the reviewer.
Changes in the text: see page 6, lines 139-144: "*A recent study by Tvrdá et al. (37) compared bacterial profile, as well as sperm parameters, membrane integrity and DNA integrity of 51 men after 2 days and 2 hours abstinence periods and showed improved DNA integrity, among other parameters, After short abstinence. A metanalysis by Barbagallo et al. (38) similarly showed that very short abstinence improves SDF in patients with abnormal sperm parameters*".

-Comment 4: Page 6 line 130 – "Seventeen men…"
Why was the collected period too long 2012 until 2016? Your excluded criteria was too small.
Reply 4: Please see our response to comment #2 in the major comments. We were selecting patients based upon their interest and willingness to participate, and the availability of laboratory services. This study took a long time to complete.
Changes in the text: see page 7, lines 164-165: "*Patients were selected based upon their interest and willingness to participate, and the availability of laboratory services*".

-Comment 5: Page 8 line 171
Please, correct the mistake in Makler chamber.
Reply 5: Corrected. We thank the reviewer for this comment.
Changes in the text: see page 8, line 187.

-Comment 6: How did you analyze acrosome membranes without PNA or PSA?
Reply 6:
We did not specifically analyze the acrosome reaction for this study. We have confirmed that this was not included in the report and clarified this point in the Methods section.
Changes in the text: see page 9, line 204-206: "*Other assays such as sperm aneuploidy testing, sperm penetration assays, acrosome reaction or sperm epigenetics were not performed for the purpose of this study".*

-Comment 7: How did you do the paired student's t-test? In the method, you did not insert this information.
Reply 7: We thank the reviewer for this comment. The reviewer is correct. The test used was the Wilcoxon signed-rank test and not the paired t-test, as some variables were

skewed in distribution. We revised the statistics section accordingly to clarify that.
Changes in the text: see pages 9-10, lines 215-217: "*Quantitative variables were presented as either mean± standard deviation (SD) or median. Differences in characteristics across samples were assessed using the Wilcoxon signed-rank test".*

-Comment 8: It is important to mention the period of ejaculatory abstinence. You are just emphatic for the second semen sample (3-4 hours); however, you used the first semen samples to compare, because of that I think you need to change the description for example in “Abstract” Page 3 line 63, “Methods” page 7 lime 162, “Results” Page 9 lines 208-210, etc.

Reply 8: We agree with the reviewer. In response to this comment, we revised the manuscript as suggested – abstract, methods and results.
Changes in the text:
Abstract – page 3, lines 62-64: "*Infertile men were instructed to provide two semen samples 3-4 hours apart (the first sample was given after 2-5 days of abstinence) to test the effect on DFI levels".*
Methods – page 7, lines 165-167: " *These men were instructed to provide two consecutive semen samples for analysis, the first sample was given after an abstinence of 2-5 days and the second sample was provided within the next 3-4 hours*", and lines 177-178:" *The baseline sample was provided following 2-5 days of abstinence and the second 3-4 hours later*".
Results – lines 229-232: "*During this study period we referred 52 patients to the andrology laboratory to perform a trial of "double ejaculation", giving two semen samples 3-4 hours apart (the first after a 2-5 day abstinence period), in order to evaluate the levels of DFI in each sample".*

Reviewer C

In this prospective cohort study, the authors compare 2 semen samples: one obtained after a WHO recommended abstinence period (2-5 days), and one collected after 3-4 hours.
Of the 52 men analyzed, DFI decreased about 3.8% on average ($p<0.001$), and most patients (40/52) demonstrated an absolute improvement.

This study adds to the growing body of evidence suggesting that a short ejaculatory abstinence period results in lower sperm DNA fragmentation levels. However, a few points need clarification:

-Comment 1: How was infertility defined for these men? Please add additional details.
Reply 1: Infertile men included in this study had either primary or secondary infertility and at least one abnormal semen parameter. Patients were selected based upon their interest and willingness to participate, and the availability of laboratory services.
Changes in the text: see page 7, line 158-165*: We included men who presented for a fertility evaluation in our male infertility clinic for primary or secondary infertility (at least 12 months or more of regular unprotected sexual intercourse). Infertile men with abnormal sperm parameters who had a DFI measurement were included in this study. Exclusion criteria were previous semen analysis showing azoospermia, previous exposure to chemo-radiotherapy, testicular cancer and surgical history of orchiectomy or Klinefelter syndrome. Patients were selected based upon their interest and willingness to participate, and the availability of laboratory services".*

-Comment 2: Line 180 = "DNA fragmentation was tested on a frozen semen sample"; do you think freezing sperm had an impact on DFI values for this cohort? Please include in discussion.
Reply 2: We thank the reviewer for this comment. The use of frozen samples for DFI is standardized and enables to standardize the testing of the samples. Previous studies (Lusignan MF, Li X, Herrero B, Delbes G, Chan PTK. Effects of different cryopreservation methods on DNA integrity and sperm chromatin quality in men. Andrology 2018; 6(6): 829-35) as well as our internal audit evaluation at Mount Sinai Hospital Andrology lab have confirmed that DFI assays performed on frozen or fresh sperm samples provide identical results. Therefore this is the standard in our center. We think that the use of frozen semen samples for DFI analysis does not affect the DFI measurement, especially as both samples are analyzed in the same technique. This important comment was added to the discussion section, as the reviewer suggested.
Changes in the text: see page 13, line 313-316: "*DFI assays were performed on frozen semen samples. As previously shown (47), DFI analysis done on fresh and frozen sperm samples provide identical results, and therefore the use of frozen semen samples for DFI analysis did not affect the DFI values*.

-Comment 3: Line 250-51 = "nearly reached statistical significance"; this seems misleading, as values are either statistically significant or not. Please revise.
Reply 3: We agree with this comment and as suggested, we have revised this paragraph and removed this sentence.
Changes in the text: see page 11, lines 270-273: "*A univariate and multivariate logistic regression models for the prediction of a relative 15% decrease in DFI between the first and consecutive semen sample (Table 4) did not identify a statistically significant independent predictive parameter*. "

-Comment 4: Line 271 = "...population of only infertile men, referred to our male infertility clinic" : again, how was infertility defined? Are there are particular referral criteria that would help to characterize this population?

Reply 4: Please see our response to comment #1 Abnormalities of semen parameters based upon the WHO definition and at least one year of infertility.

Changes in the text: see page 7, line 158-165: "W*e included men who presented for a fertility evaluation in our male infertility clinic for primary or secondary infertility (at least 12 months or more of regular unprotected sexual intercourse). Infertile men with abnormal sperm parameters who had a DFI measurement were included in this study. Exclusion criteria were previous semen analysis showing azoospermia, previous exposure to chemo-radiotherapy, testicular cancer and surgical history of orchiectomy or Klinefelter syndrome. Patients were selected based upon their interest and willingness to participate, and the availability of laboratory services*".

-Comment 5: Table 1 = what were the chronic diseases, medications used?

Reply 5: The chronic disease parameter included – cardiovascular disease or hypertension, IBD, diabetes, major depressive disorder, thyroid disease. Medication used included Levothyroxine for hypothyroidism, statins, SSRI and multi-vitamins and fertility supplements. This data was added to the footnotes of table 1.

Changes in the text: Table 1 footnotes (a and b)

-Comment 6: Overall, the change in DFI was about 3.8%. Does this seem clinically meaningful? Should this finding change current practice patterns? Were there any challenges to having these men provide a semen sample after a short abstinence interval that would make it difficult to incorporate into a male infertility practice?

Reply 6: We agree that the 3.8% percentage points decrease, which represents a 9.8% relative reduction in DFI, might be less clinically meaningful. However, the fact that almost two thirds (64%) of patients with high DFI within the 30-40% range had a DFI value of less than 30% on the second semen test, us clinically significant.

Changes in the text: see page 12, line 282-284: " *Our results demonstrate that this technique is associated with a decrease of nearly 4 percentage points (9.8% relative decrease) in DFI and that 42% of patients will have at least a 5 percentage points absolute reduction in DFI. Moreover, the group of patients with baseline DFI higher than 30% will have a larger decrease in DFI compared to those with DFI of 30% or less (-4.5 vs. -2.7 percentage points decrease in DFI). While this absolute decrease in DFI , though significant, is relatively small, our study also shows that that almost two thirds (64%) of patients with high DFI within the 30-40% range will have DFI value of less than 30% on the second semen test, which is widely used as a cutoff for abnormal DFI levels and for which reproductive outcomes were reported to be better compared to DFI>30% (43-36,14)".*

-Regarding the implications of our findings – we believe that these findings help, along

with previous papers, to establish the concept of improved DFI levels with short abstinence and serve as the basis for further evaluation that will correlate this technique with the IVF outcomes, preferably in prospective, randomized trials. We have mentioned this in the revised conclusion paragraph, as follows:
Changes in the text: page 16, lines 401-403: "This data may encourage further controlled trials that are required to determine if the second ejaculate provides improved reproductive outcomes for men with initially high DFI values".

-comment 7: Were there any challenges to having these men provide a semen sample after a short abstinence interval that would make it difficult to incorporate into a male infertility practice?
Reply 7: The patients included in this study that later returned to our clinic for follow up after providing 2 semen samples 3-4 hours apart did not mention any challenge or difficulty that may affect the incorporation of this technique to the male infertility practice.

Reviewer D

The manuscript investigated the impact of a very short abstinence period (3-4 hours) on sperm DNA fragmentation. Infertile patients with abnormal sperm parameters were asked to provide two semen samples 3-4 hours apart to test the effect on sperm DNA fragmentation index (DFI) levels. In the entire group, DFI decreased from 38.9±21.4% to 35.1± 21.6% in the second sample ($p<0.001$). Apart from semen volume, no statistical differences were found for the other conventional sperm parameters between the two consecutive ejaculations.

I have the following criticisms:
-Comment 1: The decline in sperm quality should be better discussed in the introduction. Please see: doi: 10.3390/jcm10050993.
Reply 1: We agree with this comment. We revised the introduction to further describe the debate regarding the possible deterioration in sperm quality in recent decades.
Changes in the text: see page 5, lines 96-99: "*An additional factor is the possible global deterioration in sperm quality over the last few decades reported by several studies and meta-analyses (6-9), although some studies have reported contrasting results (10-12) and this subject is still in debate".*

-Comment 2: Lines 97-100 should be reworded as sperm DNA fragmentation (SDF) assessment is not part of conventional semen analysis, although the latest version of the WHO manual recognizes its importance.
Reply 2: Again, we agree with the reviewer and revised this paragraph accordingly, to clarify that SDF in not part of the routine or conventional semen analysis.

Changes in the text: see page 5, lines 101-104: "*Sperm chromatin integrity is a promising biomarker in the evaluation of male infertility ,as recently mentioned in the 6th edition of the world health organization (WHO) guidelines* (13)".

-Comment 3: The Introduction should be improved. Indeed, a recent systematic review and meta-analysis have investigated the effects of a very short abstinence period on conventional sperm parameters and SDF (https://doi.org/10.3390/jcm11247303).
Reply 3: In response to this comment, we revised this paragraph and added a recent paper and the recent important first meta-analysis on short abstinence and their results regarding short abstinence and SDF.
Changes in the text: see page 6, lines 139-143: *" A recent study by Tvrdá et al. (37) compared bacterial profile, as well as sperm parameters, membrane integrity and DNA integrity of 51 men after 2 days and 2 hours abstinence periods and showed improved DNA integrity, among other parameters, After short abstinence. A metanalysis by Barbagallo et al. (38) similarly showed that very short abstinence improves SDF in patients with abnormal sperm parameters*".

-Comment 4: Previous studies have demonstrated a better improvement of semen quality in the second ejaculation of patients with abnormal sperm parameters compared to normozoospermic men. Was there a correlation between the improvement of SDF and the severity of the alteration of conventional sperm parameters of the first ejaculate?
Reply 4: This is an interesting point. Indeed, a recent meta-analysis by Barbagallo et al. (*Barbagallo F, Cannarella R, Crafa A, et al. The Impact of a Very Short Abstinence Period on Conventional Sperm Parameters and Sperm DNA Fragmentation: A Systematic Review and Meta-Analysis. J Clin Med. 2022;11(24):7303. Published 2022 Dec 8. doi:10.3390/jcm11247303*) demonstrated an improvement in sperm parameters in the second ejaculate. However, in our cohort we have performed two logistic regression analysis – one for prediction of a second DFI≤30% in men who had DFI>30% in their first semen analysis and a second for a 15% decrease in DFI. Both models did not find any conventional sperm parameter to be significantly associated with DFI improvement. We added this to the results
section when describing the results of the regression model for DFI improvement prediction.
Changes in the text: see page 11, line 270-273: *" A univariate and multivariate logistic regression models for the prediction of a relative 15% decrease in DFI between the first and consecutive semen sample (Table 4) did not identify statistically significant independent predictive parameters, including baseline sperm parameters*".

-Comment 5: Lines 257-258 and 346-347 should be rewritten. Indeed, previous studies have investigated the effects of a very short abstinence period on SDF.

Reply 5: We agree with this comment and revised this paragraphs accordingly.
Changes in the text: page 12, line 280-281:" *This study demonstrates a reduction in DFI in the population of interest – men with male factor infertility*."
see page 15, line 397-398: " *To conclude, this study demonstrates significant improvements in DFI in the second sample given within 3-4 hours from the first for men with infertility."*

-Comment 6: The possible reasons for the improvement in sperm DNA fragmentation in the second ejaculation should be better discussed.
Reply 6: In response to this comment, we elaborated on possible mechanisms by which short abstinence is related to improved DNA fragmentation.
This paragraph was added to the discussion section.
Changes in the text: see page 15, lines 363-386:" *Several mechanisms on the link between short abstinence and improved SDF were hypothesized (38). It has been suggested that the main mechanism for SDF formation is the exposure of sperm to ROS(19), dead spermatozoa and leukocytes(51) during the transport of sperm from the testes to the epididymis and its storage there, resulting in sperm DNA breakage (30,31) The duration of transition through the epididymis varies and might be affected by the frequency of ejaculation, thus short abstinence might be associated with faster passage and decreased SDF. An additional theory focuses on the seminal plasma composition and its effect on SDF. Seminal plasma metabolites are secreted and accumulate with time and therefore short abstinence may result in an overall lower metabolites levels and subsequently lower fragmentation levels (52).*

A previous study by Shen et al. (53) investigated the mitochondrial plasma membrane potential (MMP), which correlates with sperm motility, in spermatozoa from samples given after several days vs. 1-3 hours of abstinence using proteomics technology. Their investigation demonstrated higher MMP after shorter abstinence, which represents another possible mechanism for improved sperm quality after short abstinence. Further research is required to identify these factors and enable prevention and treatment if needed".

-Comment 7: The possible effects of a very short abstinence period on assisted reproductive technologies outcomes should be better discussed. Please see: doi: 10.3390/antiox12030752.

Reply 7: We thank the reviewer for this important comment. This recent systematic review and meta-analysis by Barbagallo et al. is the first to explore the impact of very short abstinence on ART outcomes. The authors investigated the ART outcomes of fertilization and implantation rates, miscarriage rate, clinical pregnancy rates and live birth rate in this regard. They were able to show improved implantation rates, clinical

pregnancy rates and live birth rates following very short abstinence. This study shows that in men with altered sperm parameters, short abstinence may some ART outcomes. We added a paragraph focusing on this investigation to the discussion section.
Changes in the text: see page 16, lines 390-394:" *The association between very short abstinence and ART outcomes has been investigated recently in a systematic review and meta-analysis by Barbagallo et al. (54). The authors demonstrated significantly improved ART outcomes – implantation, clinical pregnancy, and live birth rates - following very short abstinence, compared to standard, 2 days or more, abstinence period in men with altered sperm parameters".*

Comment 8: Formatting errors should be revised (please see lines 96, 118, and 130)
Reply 8: We thank the reviewer for his careful review. These errors were corrected throughout the text.
Changes in the text: see page 5, line 95; page 6, line 123 and page 6, line 136.

Reviewer E

-Comment 1: The authors did not include a control in the study and this creates a major flaw in the study design.
Reply 1: We agree that ideally a prospective study design with control group would provide stronger evidence. However, as DFI is self-funded in Canada, we only referred infertile men to provide two consecutive semen samples. Moreover, one of the advantages of such study design is that each man acted as his own control – comparing his first , baseline, semen analysis (after 2-5 days abstinence) to his second, following a very short abstinence period. As we agree with the reviewer, we added this limitation to the limitation paragraph in the discussion.
Changes in the text: see page 13, line 302-305:" *This study has several limitations, the main being the relatively small sample size and the lack of a control group and data on fertility outcomes with the second sample, which does not allow us to draw conclusions regarding its beneficial effect on IUI or IVF outcomes*".

-Comment 2: Secondly, the authors need to provide a rational for using frozen samples for the DNA fragmentation test.

Reply 2: We thank the reviewer for this comment. Use of frozen samples for DFI is standardized and enables to standardize the testing of the samples. Previous studies (Lusignan MF, Li X, Herrero B, Delbes G, Chan PTK. Effects of different cryopreservation methods on DNA integrity and sperm chromatin quality in men. Andrology 2018; 6(6): 829-35) as well as our internal control evaluation at Mount Sinai

Hospital Andrology lab have confirmed that DFI assays performed on frozen or fresh sperm samples provide identical results. Therefore this is the standard in our center. We think that the use of frozen semen samples for DFI analysis does not affect the DFI measurement, especially as both samples are analyzed in the same technique. This important comment was added to the discussion section, as the reviewer suggested.

Changes in the text: see page 13, line 313-316: "*DFI assays were performed on frozen semen samples. As previously shown (47), DFI analysis done on fresh and frozen sperm samples provide identical results, and therefore the use of frozen semen samples for DFI analysis did not affect the DFI values".*