BUKTI KORESPONDENSI PAPER

1. The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: A review

Gmail nining handayani <ninghandayani11@gmail.com>

Your Submission JARG-D-23-00024
1 pesan

Journal of Assisted Reproduction and Genetics <em@editorialmanager.com> 7 Maret 2023 pukul 22.10
Balas Ke: Journal of Assisted Reproduction and Genetics <markchristopher.ledesma@springernature.com>
Kepada: nining handayani <ninghandayani11@gmail.com>

You are being carbon copied ("cc:'d") on an e-mail "To" "Anom Bowolaksono" alaksono@sci.ui.ac.id
CC: "nining handayani" ninghandayani11@gmail.com, "Daniel Aubry" daniel.abidin.aubry@gmail.com, "Arief Boediono" arief.boediono@irsi-bunda.org, "Budi Wiweko" budi.wiweko@gmail.com, "Batara Sirait" batarasirait@gmail.com, "Ivan Sini" ivansini@irsi-bunda.org, "Arie Adrianus Polim" polim.arie@irsi-bunda.org, "Astari Dwiranti" astari.dwiranti@sci.ui.ac.id

Dear mr Bowolaksono,

I have read your manuscript, The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: A review, submitted to Journal of Assisted Reproduction and Genetics

Before it can be sent out for review, I would request that you carry out the corrections below.

While submitting, please check the filled in author data carefully and update them if applicable - they need to be complete and correct in order for the revision to be processed further.

Please submit your revised manuscript by accessing the following site:

https://www.editorialmanager.com/jarg/

Your username is: ********
If you forgot your password, you can click the 'Send Login Details' link on the EM Login page.

We are looking forward to receiving your revised manuscript before 21 Mar 2023.

With kind regards,
David F. Albertini
Editor in Chief
Journal of Assisted Reproduction and Genetics

Comments to the author (if any):
Reviewer #1: This review was undertaken to consider the value of assessing cell-free DNA (cfDNA) for determining ploidy of the human embryo. Specifically, the review focuses on 1) evaluating the evidence regarding the origin and possible mechanisms for the release of cfDNA from embryos into the culture medium; and 2) exploration of the possible mechanisms by which embryos may undergo self-correction by the release of cfDNA. The authors conclude that despite evidence for both the presence of aneuploid and euploid cells in the preimplantation embryo, and studies supporting the use of cfDNA for ploidy assessment, further research is required to prove which cell types shed the cfDNA into spent culture medium. Moreover, although apoptosis is a logical mechanism to account for the cfDNA, extracellular vesicles may also be involved.

COMMENTS
This is an excellent, unbiased review of the state of literature in this area with a considered appraisal of study results and knowledge gaps. While the manuscript is well-written, there are several places that would benefit from edits. Please see these below:

Page 5, l. 12: "…. this review is sought …". Please delete "is"
Page 5, l. 54: "cfDNA concentration in embryo-exposed SECM than in non-exposed SECM s" replace with "cfDNA concentration in embryo-exposed SECM compared with non-exposed SECM s"
Page 6, l. 45-46: "with the median number of embryonic DNA approximately only 8%". Insert "haplotypes" after DNA.

Page 7, l. 8: "SECM has yet to be recommended for PTM analysis". Define "PTM"

Page 10, l. 12: "during blastocyst maturation". Replace "maturation" with "development"

Page 11, l. 44: "cfDNA abundance in SECM varied remarkably". Replace "varied" with "varies".

Reply to the editor's and reviewers' comments

Reviewer Number	Original comments of the reviewer	Reply by the author(s)	Changes done on page number and line number

1	This review was undertaken to consider the value of assessing cell-free DNA (cfDNA) for determining ploidy of the human embryo. Specifically, the review focuses on 1) evaluating the evidence regarding the origin and possible mechanisms for the release of cfDNA from embryos into the culture medium; and 2) exploration of the possible mechanisms by which embryos may undergo self-correction by the release of cfDNA. The authors conclude that despite evidence for both the presence of aneuploid and euploid cells in the preimplantation embryo, and studies supporting the use of cfDNA for ploidy assessment, further research is required to prove which cell types shed the cfDNA into spent culture medium. Moreover, although apoptosis is a logical mechanism to account for the cfDNA, extracellular vesicles may also be involved. This is an excellent, unbiased review of the state of literature in this area with a considered appraisal of study results and knowledge gaps. While the manuscript is well-written, there are several places that would benefit from edits. Please see these below:	Dear Reviewer 1 We would like to thank you for your useful recommendations. Following your suggestions, we have revised our manuscript accordingly.	
	Page 5, l. 12: "…. this review is sought …". Please delete "is"	Done	Page 3, line 10
	Page 5, l. 54: "cfDNA concentration in embryo-exposed SECM than in non-exposed SECM s" replace with "cfDNA concentration in embryo-exposed SECM compared with non-exposed SECM s"	Done	Page 4, line 4
	Page 6, l. 45-46: "with the median number of embryonic DNA approximately only 8%". Insert "haplotypes" after DNA.	Done	Page 4, line 28
	Page 7, l. 8: "SECM has yet to be recommended for PTM analysis". Define "PTM"	Sorry for the typo, we have revised the abbreviation	Page 5, line 7
	Page 10, l. 12: "during blastocyst maturation". Replace "maturation" with "development"	Done	Page 8, line 13
	Page 11, l. 44: "cfDNA abundance in SECM varied remarkably". Replace "varied" with "varies".	Done	Page 10, line 3
	Page 12, l. 46: "were budded from the embryonic plasma membrane to the". Replace "to" with "into".	Done	Page 11, line 7
	Page 12, l. 47: "the zona pellucida to propagate into the culture media". Replace with "through zona pellucida to accumulate into the culture media".	Done	Page 11, line 8
	Page 13. L. 53-55: "it is certainly permissible to isolate cfDNA from free-floated DNA or EVs in SECM." I think you mean "it is certainly possible to …."	Done	Page 12, line 6
	Page 15, l. 21: "it is clear that apoptosis eliminated aneuploid-cell could originate the cfDNA in SECM".	Done	Page 13, line 21

	Replace with "it is clear that the cfDNA in SECM may originate from apoptosis eliminated aneuploid-cells".		
	Page 15, l. 42: "Consequently, the genetic status between cfDNA and embryo could be…". Insert "the" before "embryo".	Done	Page 14, line 3
	Page 15, l. 54 "Even so, it is likely that other mechanisms …." Replace "other" with "additional".	Done	Page 14, line 9
2	The paper suffers from various minor, but still irritating, errors and needs editing. I have listed below some examples. I would be happy to recommend the paper for publication in the journal once the errors are corrected.	Dear Reviewer 2 We express our gratitude for the opportunity to make revisions to our manuscript. We found your suggestions very useful and have modified the manuscript accordingly.	
	1. Consistency and correct word usage. I list only several of many examples: p.3 line 47: "in-vitro" AND p.15 line 46: "in vitro" It must be written "in vitro" p.5 line 31: "NiPGT-A" AND p.15 line 46: "niPGTA" AND p.3 line 47: "niPGT-A" Non-invasive preimplantation genetic testing for aneuploidies is abbreviated as "niPGT-A" p.5 line 54: "Coworkers" AND p.5 line 58: "et al.," The use of "coworkers" (or "colleagues") is preferred to "et al.", however if the authors choose to use the Latin abbreviation they have to use it consistently throughout the text if this follows the publication rules p.4 line 44: "vs" AND p.5 line 60: "Vs" Versus is abbreviated as "vs" and not as "Vs" p. 4 line 13: "pre-implantation" AND p. 4 line 13: "preimplantation"	All suggestion has been accommodated in the revised version	Highlighted in red
	2. All the abbreviations have to be properly and correctly spelled out when they first used in the text of the article. Examples: p. 4 line 13: "pre-implantation genetic disease (PGD)" PGD is pre-implantation genetic diagnostics, not a "disease" Other abbreviations not spelled out include: IVF SECM cfDNA HAS ICSI	Errors have been corrected and list of abbreviations has been provided	Highlighted in red

	In addition, I would recommend including the list of abbreviations at the end of the article		
	3. Unnecessary hyphenation p. 13: "Cell-death" AND "aneuploid-cell" These words are not hyphenated	Done	Highlighted in red
	4. References are not formatted as required by Submission Guidelines. DOI is missing from many papers referred to. When the DOI is present the URL is incorrect. It must be "https://" and not "http://" Often, the authors provide a URL from the search engine where the referred paper is available however, they must provide the proper reference and DOI unless the paper is only available from the resource they refer to. In such a case they must include the data of accessing the URL. Here is an example of incorrectly formatted reference: Stigliani S, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. Hum Reprod [Internet]. 2013;28(10):2652-60. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84885133553&doi=10.1093%2Fhumrep%2Fdet314&partnerID=40&md5=e3aa548b66b6843f2bf9085cc99e9f81	DOI of each reference has been completed	References
	5. URL's for the search engines are missing The authors must provide URLs for the search engines they used and which are described in Methods	Done	Page 3, lines 15-16
	6. Tables and the figure are not referred to in the text. The manuscript has 3 tables and 1 figure however authors never refer to the tables and the figure in the text. The tables and the figure either must be removed, or they have to be referred to and discussed in the text.	Errors have been corrected	Highlighted in red
	7. In the Introduction the authors mention that PGT-A can possibly induce trauma to the embryo. They must provide a reference to a scientific publication dealing with this issue. Otherwise this statement is misleading and must be removed	Done	Page 2, line 12
	8. The title of the column "Time of sample collection" in Table 1 should be changed to "Day of sample collection" as the data in the column are given in full days	Done	Table 1 (highlighted in red)
	9. Table 3 precedes Table 2 in the text. The order of tables or the numbering must be changed	Done. Thank you very much for the correction.	Highlighted in red

3	'The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: a review' is a comprehensive literature summary. The manuscript summarizes the cellular origin of cfDNA in SECM and possible contamination sources. It also discusses the possible mechanisms of cfDNA release from embryos of differing ploidy states and concordance with ICM/TE analysis. The subject is a highly relevant topic in reproductive medicine, with niPGT advancing clinically before the underlying mechanisms of cfDNA release are fully understood. However, some additional clarification and revision would benefit the manuscript.	Dear reviewer 3 We appreciate the time and effort that you have dedicated to providing your valuable feedback on our manuscript. Here are our responses point-to-point to your suggestions.	
	In the introduction, a short description of the role of TE and ICM for successful implantation is needed. Defining euploid, aneuploid, and mosaic embryos would be beneficial early on. The sentence indicating that pgt-a in high volume IVF clinics is ineffective is incorrect, many high volume ivf labs perform a high volume of pgt-a successfully. niPGT media collection is only slightly less time consuming but eliminates the possible embryo damage aspect.	We have incorporated the changes in this revised version and have included the definition of euploid, mosaic, and aneuploid embryos. We have rewritten the sentence.	Page 2, 12, 14-16 Page 7, lines 5-9 Page 2, line 12
	The PGD, PGS, and PGT-A introduction sentence is confusing… a description of PGT-a, PGT-m, and PGT-sr would be more informative as pgd and pgs have been out of use for many years and no longer used. This review focuses on the origin of cfDNA in SECM as an alternative to PGT-a. Can SECM be used for PGT-M/SR (table 1 capalbo et.al. was for a targeted disorder -was this PGT-M?).	We have rewritten the sentences regarding the introduction of PGD and PGS. Capalbo and Colleagues (table 1) have tested the diagnostic efficacy of PGT-M as well as PGT-A protocol on the cfDNA in SECM as well as blastocoel fluid. For PGT-M, samples were generated from 14 couples who were referred to the clinic as carriers of an inheritable genetic condition. Using such kind of inheritable genetic condition information, the research group has genotyped the embryonic DNA.	Page 2, lines 5-10 Page 5, line 7
	Some discussion about our increased understanding of mosaicism in recent years and how TE biopsies represent a small number of cells that might not be representative of all cells is needed. Similarly, cfDNA analysis may be reflective of all cells of the embryo, even those excluded from further development. Mosaic implantation rates can be better used to argue the self-correction model proposed. Addressing the reliability/accuracy limitations of current PGT-a practice is relevant for comparing the accuracy/reliability using SECM analysis. Both strategies have to contend with contamination, mosaicism, and concordance between ICM and TE.	We have incorporated the changes in this revised version.	Page 2, lines 13-15 Page 4, lines 12-15 Page 7, lines 10-13
	Results/methods: Do you have any numbers for the search results, how many were eliminated from the review?	EMBASE: 12.570 documents PUBMED:1 document Scopus: 8 documents In total, 12.579 documents were retrieved. The screening was conducted after duplication removal. Screening for eligible papers started with reading the title and abstract. If suitable, the full text was downloaded and read. CSV file was downloaded from 3 databases.	-
	Further questions: Does cfDNA degrade in SECM? It was mentioned that the EV membrane may protect from this, but	 There are several reviews in the current literature discussing the percentage of SECM cfDNA samples that	

	that EVs might not be the only source of cfDNA. Does degraded cfDNA yield results that could impact ploidy determination or would it be overshadowed by intact DNA amplification? Similarly, are these studies using cfDNA accumulation over several days or a defined culture period (24 hours of culture?). Does the time in culture increase cfDNA with errors, possibly due to degradation or more cells undergoing apoptosis? Is the cfDNA quantity and quality variable depending on the day of collection and the amount of time in culture? Table 1 has time of sample collection - but how long was the blastocyst cultured in the SECM? Current practices in clinical embryo culture vary, with some labs using continuous culture from day 1 to day 6 and some refreshing culture media at regular intervals. NiPGT protocols are very specific about timing of culture media collection.	failed to be amplified due to the low quality of cfDNA (probably degraded in SECM) and also the duration of embryo culture prior to sample collection (Brouillet et al. (2020), Navarro-Sanchez et al. (2020)). Therefore, we opted not to discuss it again in the present review.	
	Rubio and coworkers suggested ICM and TE have similar contribution to cfDNA in SECM. Was the proportion of ICM to TE cells in a blastocyst discussed and is the amount of cfDNA from a blastocyst proportional to the ratio and number of TE and ICM? From the wording, it seems the TE and ICM contribution to SECM were equivalent…indicating ICM cells may be more active in contributing to SECM relative to low cell number.	Rubio and Coworkers state that the concordance of both TE and ICM and cfDNA are similar. None of the experiments was undertaken to prove the proportion of ICM and TE with cfDNA quantity. Therefore, we realize that our sentence is less accurate to express that study results. We have revised the sentence properly. Thank you.	Page 4, lines 12-15
	There should be some mention of the ongoing use of ni-PGT by commercial reference laboratories, either as a supplement to biopsy, as a re-biopsy alternative when results are inconclusive, or as a stand-alone screening tool.	Done, thank you for your suggestion.	Page 13, lines 12-14
	In the EV section, EVs have plasma membranes that are reflective of their cell of origin. Is it possible to sort EVs to remove maternal contamination or just look at ICM originating EV for DNA analysis?	Thank you immensely for pointing out this intriguing question. As we understand, the investigation of embryo-released EVs in human reproduction is just at the beginning. Since the quantification technique of EVs remains lacks standardization, we are not sure about the possible use of EVs for avoiding maternal contamination as a bottleneck for wider clinical application of cfDNA SECM. But scientifically, it is possible to use ICM-originating EVs for chromosomal analysis once specific marker(s) of ICM-released EVs can be specified.	-
	A more thorough review of grammar, sentence structure, and errors should be undertaken (example: p7 line 13 -euploid, mosaicism, and euploid…should be aneuploid). Author should be more consistent with introducing abbreviations before use and then using the defined abbreviation (ex: p5 line 8 - PTM analysis, what is PTM?). Tables should be referenced in text and in the correct order. P8 line48, confusing sentence…unlikely logical?	Done	Corrections were highlighted in red
4	The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: A review My Comnets and Questions: 1. Define genetic constituent on line 6. 2. In line 33, the authors should consider adding the paper from Tobler et al published in Fert & Stert	Dear reviewer 4 We respect your opinion regarding our review while at the same time also receiving some benefits from your comments and perceptions. Here are our responses to your comments: 1. The sentence has been rewritten 2. We have included the suggested reference	Page 12-13, line 262-267

	3. In line 42, the authors cite a paper showing an "impressive concordance" between whole embryonic genomes and cfDNA. Please clarify how a whole genome analysis can be accurately compared to sequencing of TE cells. 4. Please address my concerns in the next reference. 5. For both my comments in #3 and #4 above, the important point is how things correlate with the ICM, not diluted by analyzing whole blastocysts. These studies did not prove convincing evidence of the use of cfDNA for PGT-A analyses. 6. The authors state that " Cellular fragmentation during embryo development has been presumed to cause the release of embryonic cfDNA into the SECM, which therefore was thought to correlate positively with embryo fragmentation rate [4]. Unfortunately, several studies have observed that cfDNA was detected even in the negative control culture droplet which had no contact with any embryos, suggesting that the commercial culture media might carry DNA contaminations". These comments are speculative at best. 7. The authors state " To bridge the gap of knowledge between the good concordance rate of cfDNA for embryo ploidy and the unclear scientific rationale of cfDNA release in SECM, this review is sought to evaluate current literature which elucidates the source of embryonic cfDNA and the possible mechanism for its release in SECM" The authors assume that cfDNA and embryo ploidy have a good concordance rate. Concordance to what? The TE, the ICM or nuclei within the blastocoel fluid? 8. The authors failed to discuss a paper from Griffin et, al, published in Hum Reprod, demonstrating the movement of aneuploid cells away from the ICM during development. This review is incomplete and flawed including such important data.	3. Thank you for pointing this out. We agree that the golden standard for PGT-A analysis should be the ICM of embryos. However, we think it is rational to evaluate which type of samples are more representative of the sequencing result of the whole embryo. Especially if we think that IVF-generated mosaic blastocysts are quite high. 4. Done (page 4, line 22) 5. We agree that the golden standard for PGT-A analysis should be the ICM of embryos. 6. We have cited the references for that statement properly. 7. Sentences have been rewritten 8. Suggested reference has been added	
	My overall opinion - The authors made a critical mistake in their compilation of references and data that only included support for the strong correlation between cfDNA and the ICM. They should have been openminded and reviewed all references and data and let the results speak for themselves.	While we respect your opinion, this review has included both positive and negative results of the current study pertaining to cfDNA. For instance, we have raised the study results from Vera-Rodriquez and Colleagues which states that cfDNA does not represent the embryonic genetic materials (only containing 8% of embryonic material).	

Gmail nining handayani <ninghandayani11@gmail.com>

Fwd: JARG: Your manuscript entitled The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: A review - [EMID:1358c21bb7ec5071]

1 pesan

Anom Bowolaksono <alaksono@sci.ui.ac.id> 21 April 2023 pukul 17.19
Kepada: nining handayani <ninghandayani11@gmail.com>

---------- Forwarded message ---------
Dari: **David F. Albertini** <em@editorialmanager.com>
Date: Jum, 21 Apr 2023 17.17
Subject: JARG: Your manuscript entitled The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: A review - [EMID:1358c21bb7ec5071]
To: Anom Bowolaksono <alaksono@sci.ui.ac.id>

CC: "nining handayani" ninghandayani11@gmail.com, "Daniel Aubry" daniel.abidin.aubry@gmail.com, "Arief Boediono" arief.boediono@irsi-bunda.org, "Budi Wiweko" budi.wiweko@gmail.com, "Batara Sirait" batarasirait@gmail.com, "Ivan Sini" ivansini@irsi-bunda.org, "Arie Adrianus Polim" polim.arie@irsi-bunda.org, "Astari Dwiranti" astari.dwiranti@sci.ui.ac.id

Ref.:
Ms. No. JARG-D-23-00024R1
The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: A review
Journal of Assisted Reproduction and Genetics

Dear mr Bowolaksono,

I am pleased to tell you that your work has now been accepted for publication in Journal of Assisted Reproduction and Genetics.

Thank you for submitting your work to this journal.

With kind regards,

David F. Albertini
Editor in Chief
Journal of Assisted Reproduction and Genetics

Reviewer #1: Thank you for responding to my original comments and for editing your manuscript accordingly.

Reviewer #3: The revised manuscript provides a much clearer presentation of the reviewed material. The authors answered most of the reviewers concerns and the manuscript is a comprehensive review of the current science. Although a clear consensus cannot yet be reach regarding the origin, underlying mechanisms, and utility of cfDNA in SECM, this manuscript can be used when considering and directing future research on the topic.

Please note that this journal is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted.

Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. If your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible.