Human Reproduction Open, Vol.2023, No.1, hoac061, 2023
https://doi.org/10.1093/hropen/hoac061

human reproduction open

LETTER TO EDITOR

Technical specificities of the study of the mitochondrial genome

Sir,

We read with interest the article about mitochondrial DNA (mtDNA) content in cumulus cells (CCs) published in 2022 by Martínez-Moro *et al.* (2022). The aim of this study was to investigate the mtDNA content of CCs as a possible biomarker of embryonic development and implantation assessed in human and bovine samples.

In our experience, there are two pitfalls to be avoided in this type of study: one is related to the method of mtDNA quantification and the other to the existence of mitochondrial pseudogenes in the nucleus.

Quantification of mtDNA copy number in a sample should ideally be achieved by absolute quantification. This reference method was described by Li *et al.* (2021) and has been used to quantify mtDNA in CCs in several studies (Ogino *et al.*, 2016; Desquiret-Dumas *et al.*, 2017; Taugourdeau *et al.*, 2019; Liu *et al.*, 2021; Yang *et al.*, 2021). To summarize, absolute quantification involves performing two parallel qPCRs to amplify a mitochondrial and a nuclear gene. The Ct of each qPCR is converted to copy number using standard curves with known concentrations. Then, a copy number ratio between the mitochondrial and the nuclear genes is performed with a factor of 2, which reflects the number of mtDNA copies per diploid genome and thus provides the mtDNA content per cell.

In their study, Martínez-Moro *et al.* (2022) use a $2^{-\Delta Ct}$ relative quantification technique with a single normalization (Ct of the mitochondrial gene of interest versus the Ct of the reference nuclear gene) but without normalization of external conditions. These results are difficult to interpret and, above all, to compare with data from the literature obtained through absolute quantification. In addition, when describing this technique, they refer to the publication of Lamas-Toranzo *et al.* (2018), which in turn is based on a previous article (Bermejo-Alvarez *et al.*, 2008). However, these two publications use absolute quantification, which differs from the method used in this article.

Throughout evolution, mtDNA segments have been transferred to the cell nucleus and integrated into the nuclear genome. These portions of DNA, called pseudogenes or nuclear-encoded mitochondrial sequences (NUMTs), are thought to be nonfunctional (Puertas and González-Sánchez, 2020). The recent article by Wei *et al.* (2022) published in *Nature*, reinforces the knowledge on NUMTs and their importance in the study of the mitochondrial genome. These pseudogenes make the study of mtDNA complex since any amplification of the mitochondrial genome may be accompanied by artifactual amplifications in parallel from nuclear pseudogenes. Therefore, it is essential to verify that the primer pairs used to amplify mtDNA are totally specific for the mitochondrial genome, and that they do not amplify nuclear mitochondrial pseudogenes. This is usually done by checking the absence of amplification on cells lacking mtDNA (Rho0 cells) (Boguenet *et al.*, 2022). The « *Primer blast* » bioinformatics tool allowed us to see that the primer pair used in the Martínez-Moro *et al.* (2022) article for the human *MT-ND2* gene is not specific to mtDNA. Indeed, this primer pair would also amplify a NUMT sequence located in chromosome 1 with an identical amplification product size of 194 base pairs. The quantification bias is therefore potentially important since the authors do not mention that these primers were tested on Rho0 cells.

Quantification of mtDNA can be tricky and a source of many technical pitfalls. To compare results from one study to another and avoid biases such as those encountered with mtDNA and mitochondrial pseudogenes, it is important to establish quality standards that are shared by the scientific community.

Conflict of interest

The authors have no conflicts of interest to disclose.

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https://doi.org/10.1093/hropen/hoac061