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RESEARCH

Relative mtDNA copy number in embryo spent culture medium is not a reliable biomarker of human embryo aneuploidy

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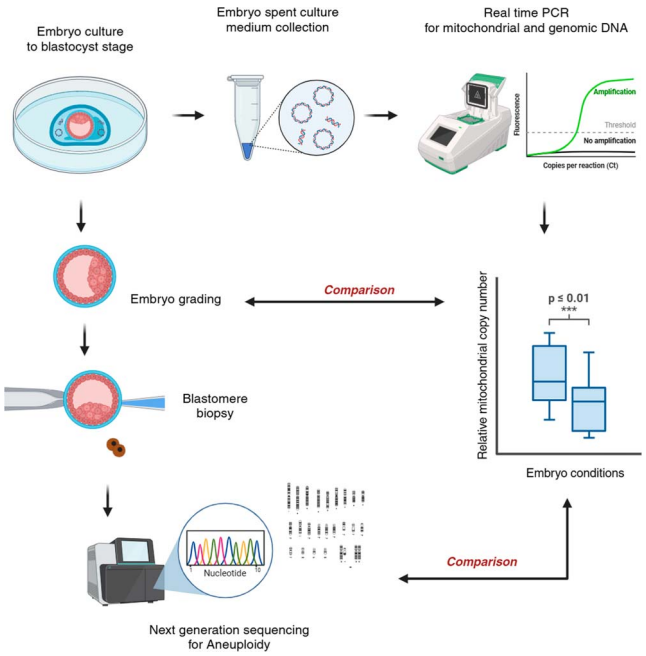
Graphical abstract



Abstract

Mitochondrial DNA (mtDNA) from embryonic cells is released into the spent culture medium (SCM) during cellular processes, providing a potential biomarker of embryo health. Analysing mtDNA levels in SCM enables a non-invasive

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evaluation of embryo quality and potential developmental abnormalities. In this retrospective study, we aimed to investigate the relationship between relative mtDNA copy number in embryo SCM and key factors, including embryo fragmentation, morphological quality and chromosomal abnormalities. Fertilised embryos produced through intracytoplasmic sperm injection were cultured to the blastocyst stage in an incubator. Embryo fragmentation was assessed on day 3 using the Istanbul criteria, while morphological grading was evaluated on day 5 using the Gardner criteria. On day 5, trophectoderm (TE) biopsies were performed for preimplantation genetic testing for aneuploidy, followed by embryo cryopreservation and collection of embryo SCM. The mtDNA was quantified using quantitative PCR. Statistical analyses using the Mann–Whitney U and Kruskal–Wallis tests (significance at $P < 0.05$) showed that relative mtDNA copy number did not significantly differ among embryos with fragmentation levels <10%, 10–25% and >25% ($P > 0.05$). For blastocyst grading, which evaluates the inner cell mass (ICM) and TE, no significant difference was observed in relative mtDNA copy number between grades B and C for ICM ($P = 0.190$) and TE ($P = 0.289$). Furthermore, a trend towards higher relative mtDNA levels was observed in aneuploid than in euploid embryos, although the difference was not statistically significant. Thus, relative mtDNA copy number in SCM may not accurately reflect embryo characteristics, such as fragmentation, morphological grading or chromosomal abnormalities.

Lay summary

This study examined whether the amount of mitochondrial DNA (mtDNA) in the fluid used to culture embryos in the laboratory could indicate embryo quality. We assessed various factors, including the appearance of the embryos, the presence of fragmented cells and the occurrence of chromosomal abnormalities. Fertilized eggs were cultured until they developed into blastocysts, and the amount of mtDNA in the culture fluid was measured using a machine that detects genetic material. The results revealed no clear association between mtDNA levels and embryo appearance or fragmentation. Although embryos with chromosomal abnormalities had slightly more mtDNA, the difference was not statistically significant. These findings suggest that mtDNA in the culture fluid may not be a reliable marker for assessing embryo quality or chromosomal status.

Keywords: embryo spent culture medium; mitochondrial DNA copy number; chromosome abnormality; fragmentation

Introduction

Cell-free DNA (cfDNA) refers to DNA fragments released into the bloodstream or bodily fluids, such as cerebrospinal fluid, pleural fluid, urine and saliva, during cell death. It can serve as an indicator of various abnormalities in the body, including autoimmune diseases, inflammation and cancer, and is a valuable diagnostic test in obstetrics. cfDNA-based approach is non-invasive and does not disturb cells or cause cellular damage; it is therefore becoming popular as a promising biomarker. Moreover, it is a simple, repeatable and effective method for monitoring disease progression (Yan *et al.* 2021, Chen *et al.* 2022). In obstetrics, one of the most widely adopted applications of cfDNA is non-invasive prenatal testing (NIPT), which screens for foetal genetic abnormalities. This approach involves analysing foetal cfDNA, which is derived from placental cells and circulates in the maternal bloodstream. NIPT enables the detection of foetal chromosomal abnormalities without the need for invasive procedures that may pose a risk to the foetus (Suciu *et al.* 2019), and is highly beneficial in the field of assisted reproductive technology, particularly for assessing the cfDNA present in embryo spent culture medium (SCM). Preimplantation genetic testing for aneuploidy (PGT-A) is a technique used to detect chromosomal abnormalities in embryos before implantation and enables the selection of viable embryos by identifying embryos with normal chromosomal configurations, thereby increasing the likelihood of successful implantation and reducing the risk of miscarriage (Fodina *et al.* 2021, Kasaven *et al.* 2023). This procedure involves embryo biopsy, typically performed on trophectoderm (TE) cells, which are the outer layer of the blastocyst that later forms the placenta (Pan *et al.* 2020, Tocci 2020). The biopsied cells are then analysed for chromosomal abnormalities, providing critical information to support informed decision-making during assisted reproductive procedures. This approach facilitates the selection of genetically healthier embryos, thereby reducing the need for multiple embryo transfer (ET) attempts and improving the overall efficiency of assisted reproductive treatments (Basille *et al.* 2009, Fesahat *et al.* 2020). Biopsy is a widely used but invasive technique for PGT-A, involving the removal of a portion of the embryo, which can compromise

its viability, and requires highly skilled embryologists. To address these limitations, non-invasive methods for assessing embryo abnormalities have been developed. Current research suggests that cfDNA in embryo SCM originates from the breakdown of embryonic cells during cell division, particularly due to cellular fragmentation (Stigliani *et al.* 2013, Brouillet *et al.* 2020, Shitara *et al.* 2021). This fragmentation, often associated with genetic abnormalities, such as aneuploidy and mosaicism, may reflect defective embryonic processes and impair subsequent development. Severe fragmentation has also been linked to reduced mitochondrial DNA (mtDNA) levels and altered mitochondrial structures, which can diminish ATP production and impede embryonic growth. The utilization of mtDNA from embryo SCM to evaluate embryo quality and detect chromosomal abnormalities remains a topic of debate among embryologists and researchers. Although it offers a non-invasive alternative, its reliability and clinical significance are still under investigation (Huang *et al.* 2019, Zhang *et al.* 2019*b*, Kobayashi *et al.* 2020, Sheaba *et al.* 2022). Some studies suggest a correlation between mtDNA levels and embryo quality, highlighting its potential as a non-invasive alternative to PGT-A (Stigliani *et al.* 2013, Shamonki *et al.* 2016), whereas others argue that mtDNA levels may not reliably predict *in vitro* fertilization (IVF) outcomes and may reflect physiological stress rather than genetic health, potentially leading to misclassification of embryos (Winstanley *et al.* 2024). Furthermore, the techniques used to extract and analyse cfDNA from embryo SCM are technically challenging and prone to inter-laboratory variability, which could affect the reproducibility and accuracy of the results (Peng *et al.* 2024). The potential discarding of viable embryos based on genetic indicators that may not definitively determine their developmental potential raises ethical issues (Kaye 2023). The clinical application of mtDNA analysis requires further validation through rigorous, large-scale studies to establish its efficacy and address the existing challenges. Consequently, in the present study, we aimed to investigate the correlation between the relative mtDNA copy number in embryo SCM and embryo quality and chromosomal abnormalities to provide further insights into its potential as a non-invasive biomarker.

Methods

Ethical approval

The protocol for the present study was approved by the Institutional Review Board under approval number SWUEC-M-079/2565E. Informed consent was obtained from all participants before their inclusion in the study. Embryo SCM was collected and analysed.

Enrolment of participants and ovarian stimulation

Eleven infertile women aged 25–45 years underwent intracytoplasmic sperm injection (ICSI) at the Paragon Fertility Clinic, Thailand. The participants were carefully screened to exclude individuals with underlying medical conditions, including diabetes, endocrine disorders, immunological diseases or haematological abnormalities. Controlled ovarian stimulation was performed using human chorionic gonadotropin (hCG) administration to promote follicular development and facilitate oocyte retrieval for IVF using the ICSI technique. Once the follicles reached maturation, ovulation was triggered by injecting hCG. Mature metaphase II oocytes were retrieved and cumulus cells were enzymatically removed using hyaluronidase to ensure optimal preparation for fertilisation via ICSI.

Embryo culture and medium collection

Cumulus cell removal was performed to reduce maternal DNA contamination in SCM. After oocyte retrieval, cumulus cells and corona radiata were removed using ICSI Cumulase® (ORIGIO, Denmark), followed by mechanical pipetting and multiple washes in fresh medium. During ICSI, the embryologist re-examined oocytes to ensure complete removal of cumulus cells. A total of 40 MII oocytes were fertilised using ICSI within an IVF working chamber maintained at 37°C with 5% CO_2 and 5% O_2 (Pulse Science, Denmark). The resulting zygotes were cultured individually in 20 μL drops of continuous medium (Life Global, Denmark) until the blastocyst stage, which typically occurred over 5–6 days in an incubator. Embryonic development was monitored using an Olympus IX73 inverted microscope. Embryo grading was performed at two key time points. On day 3 post-ICSI, cleavage-stage embryos were evaluated for fragmentation based on the Istanbul criteria (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology 2011): <10% fragmentation, 10–25% fragmentation and >25% fragmentation. On day 5 post-ICSI, blastocyst stage embryos were assessed according to the Gardner's criteria (Gardner *et al.* 2000), focussing on the quality of the inner cell mass (ICM) and TE, which were graded as A, B or C. At the blastocyst stage 3–5, TE cells were extracted via biopsy, collected in 1× phosphate buffered saline and sent to the laboratory for PGT-A using next-generation sequencing techniques. The remaining embryos were cryopreserved for future uterine transfer. In addition, 20 μL SCM from each embryo (n = 40) was carefully aliquoted into DNase- and RNase-free vials and immediately frozen at −80°C for subsequent DNA extraction.

PGT-A

PGT-A was performed by scientists at Next-Generation Genomic Co., Ltd (NGG, Thailand) using 3–5 TE cell

samples from each embryo. Library preparation was performed using the EmbryoMap Sample Prep Kit (Vitrolife, Sweden), following the manufacturer's protocol, and sequencing was performed on a MiSeq platform. The results were reported as copy number variations, providing detailed insights into chromosomal integrity by identifying insertions or deletions across the 23 pairs of chromosomes. These data were used to assess the chromosomal status of the embryos for potential abnormalities.

DNA extraction, amplification and determination of relative mtDNA copy number from SCM

Genomic DNA (gDNA) was extracted from the embryo SCM using a DNeasy Blood and Tissue Kit (Qiagen, Germany). DNA amplification was performed with the GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, USA), following the manufacturer's protocol. Fresh embryo SCM (Life Global, Denmark) was used as a negative control. mtDNA was subsequently amplified from the total DNA obtained during the extraction using a REPLI-g Mitochondrial DNA Kit (Qiagen, Germany), according to the manufacturer's instructions. Amplification was conducted using an Eppendorf MasterCycler Nexus Gradient Thermal Cycler (GMI, USA). After extraction and amplification, gDNA and mtDNA were quantified using a real-time PCR. DNA quantification was performed by amplifying the genetic material with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA). Real-time PCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Primers specific to the *MT-ND1* gene (forward: CAGACGAGGTCAACGATCCC, reverse: GTCGTGTAGCGGTGAAAGTG) were used to target mtDNA, whereas primers for the *B2M* gene (forward: GCTTCCTCTAGCTTTTGTGGC, reverse: TAGAGCTACCCAGCAGGAACA) served as a reference for nuclear DNA (Rooney *et al.* 2015). The mitochondrial copy number was calculated using the following formula:

$$\Delta CT = (CT\,gDNA - CT\,mtDNA)$$

The relative mtDNA copy number was then determined using the equation:

$$\text{Relative mtDNA copy number} = 2 \times 2^{\Delta CT}$$

Statistical analysis

The relative mtDNA copy number in fresh medium and embryo SCM was expressed as the median and interquartile range (IQR) and compared using the Mann–Whitney U test performed with the GraphPad Prism (version 10.1). The median of mtDNA copy number of day 5 and 6 blastocysts was compared. The Kruskal–Wallis test was conducted to evaluate the relationship between relative mtDNA copy number and embryo fragmentation. In addition, the association between the relative mtDNA copy number in embryo SCM and blastocyst grade, and chromosomal abnormalities, was assessed using the Mann–Whitney U test. Statistical significance was set at P-value <0.05.

Results

Background of the patient and embryo development

The participants' mean age was 32.8 ± 5.6 years. The body mass index was 18.9 ± 1.6 kg/m^2. The baseline AMH was 4.07 ± 2.89. The average number of OPU cycles was 1.63 ± 0.9, and the cumulative ET was 0.91 ± 0.83. The average number of MII oocyte underwent ICSI was 18.63 ± 7.3 and fertilization rate was 80.7 ± 11.3%. The individual patient's background is presented in Supplementary Table 1 (see section on Supplementary materials given at the end of the article). Embryo development was assessed on day 3 of culture using inverted microscopy, with fragmentation grading conducted in accordance with the Istanbul criteria. The findings indicated that all embryos had reached the cleavage stage, each exhibiting a varying number of cells, as presented in Supplementary Table 2.

Quantification of relative mtDNA copy number in embryo SCM

Fresh medium not exposed to the embryos (n = 3) that served as the control group. No mtDNA copy numbers were detected in the control samples, whereas variations in relative mtDNA copy numbers were observed in the 40 embryo SCM samples, as presented in the Supplementary Table 3. The relative mtDNA copy number in embryo SCM was significantly higher than that in the fresh medium (P < 0.05), as illustrated in Fig. 2A. Although the number of embryos in the present study was limited, the median of relative mtDNA copy number of day 5 and 6 blastocyst was compared. The results showed no statistically significant difference in mtDNA copy number between the medium collected on day 5 and 6 blastocysts (57.4 (IQR: 110.03) and 41.62 (IQR: 116.46), respectively, (P = 0.770)).

Relative mtDNA copy number and embryo fragmentation

Fragmentation analysis was performed using inverted microscopy (Fig. 1A), with the fragmentation grade of each embryo presented in Supplementary Table 2, and

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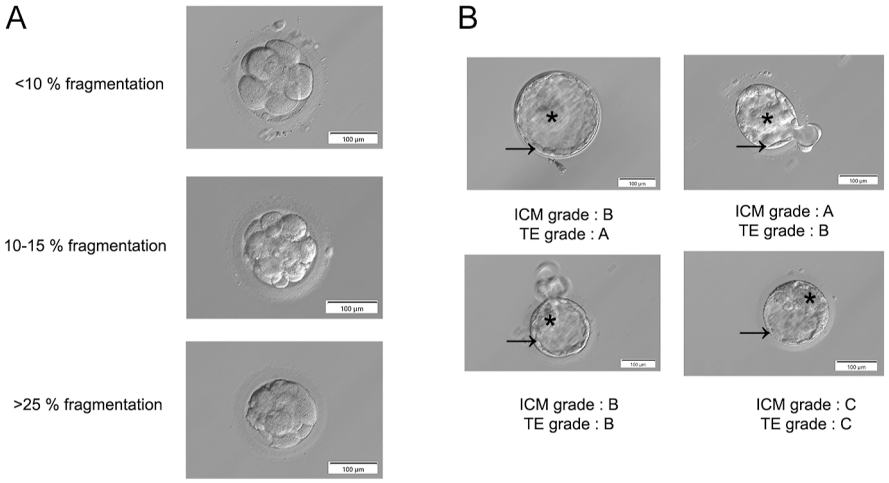

Figure 1

Embryo morphology on day 3 and day 5. (A) The degree of embryo fragmentation was assessed using inverted microscopy and categorised into three groups. Scale bar = 100 μm. (B) Blastocyst grading was performed using inverted microscopy. The ICM is indicated by a star, while the TE is marked by a black arrow. Scale bar = 100 μm.

revealed that the median relative mtDNA copy numbers were 40.25 (IQR: 117.5) for embryos with <10% fragmentation (n = 11), 40.41 (IQR: 203.31) for embryos with 10–25% fragmentation (n = 19) and 80.33 (IQR: 128.8) for embryos with >25% fragmentation (n = 10). Statistical analysis showed no significant differences in the mtDNA copy numbers across these fragmentation categories (P = 0.704), as illustrated in Fig. 2B.

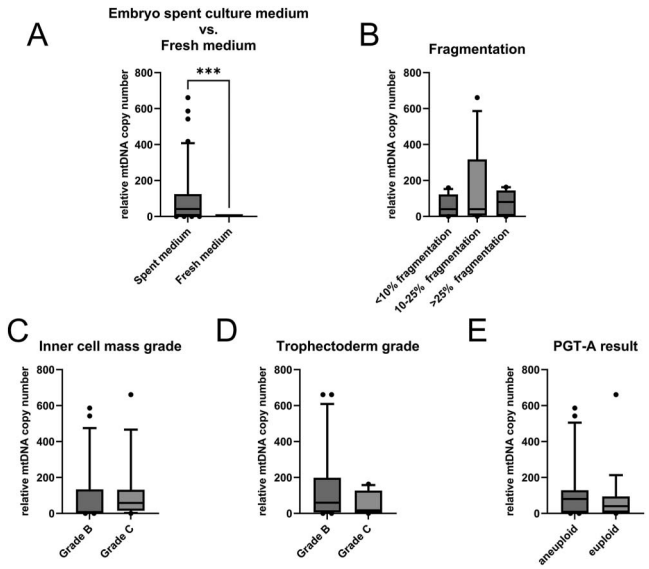

Figure 2

Box plots of relative mtDNA copy number and various embryo quality factors. (A) The relative mtDNA copy number in embryo SCM was significantly higher than that in the fresh medium (*P < 0.05). (B) The relative mtDNA copy number across the three fragmentation categories shows no significant differences. The relative mtDNA copy number with both the blastocyst ICM (C) and TE grades (D) shows no significant differences. (E) The relative mtDNA copy number with preimplantation genetic testing for aneuploidy revealed no significant differences.

Relative mtDNA copy number and blastocyst grading

The present study utilised inverted microscopy to assess blastocyst quality, focussing on two key components: the ICM and TE, as illustrated in Fig. 1B. Due to the random selection of samples, there were insufficient embryos with grade A in both the ICM and TE grading categories (n = 1) for statistical analysis. Grade A embryos were excluded and statistical comparisons were conducted only between grade B and C embryos. For ICM, the median relative mtDNA copy number was 7.9 (IQR: 122.51) for grade B embryos (n = 22) and 58.62 (IQR: 100.94) for grade C embryos (n = 17). For TE, the median relative mtDNA copy number was 58.62 (IQR: 119.38) for grade B embryos (n = 25) and 17.32 (IQR: 115.98) for grade C embryos (n = 14). Statistical analysis indicated no significant differences in the relative mtDNA abundance in the embryo SCM between the morphological quality grades of blastocysts, specifically for ICM (P = 0.190) and TE (P = 0.289), as shown in Fig. 2C and D.

Relative mtDNA copy number and PGT-A

The PGT-A results for each embryo are summarised in Supplementary Table 3. The median relative mtDNA copy number was 40.33 (IQR: 65.49) for euploid embryos (n = 18) and 80.24 (IQR: 120.86) for aneuploid embryos (n = 22). Although statistical analysis indicated a trend towards higher relative mtDNA levels in aneuploid embryos than in euploid embryos, this difference was not statistically significant, as illustrated in Fig. 2E.

Discussion

The use of embryo SCM to evaluate embryo abnormalities offers a non-invasive alternative to traditional embryo biopsy techniques. This approach was developed to

minimise the potential damage associated with biopsies, which can impair embryonic growth or result in other abnormalities. Embryo SCM contains several components, including cfDNA, RNA, microRNA and various metabolites, which may provide valuable insights into embryo quality, implantation potential and chromosomal abnormalities. This study focussed on cfDNA, which comprises both gDNA and mtDNA. Notably, the amount of mtDNA is higher than that of gDNA, highlighting its potential as a key marker for assessing embryo characteristics (Hammond *et al.* 2017). Scientists hypothesise that elevated mtDNA levels in embryo SCM may result from cellular breakage during division, which leads to the release of cellular organelles, including mitochondria. This hypothesis aligns with the findings of Stigliani *et al.* (2013) who reported a correlation between high mtDNA copy numbers in embryo SCM and severe fragmentation. Fragmentation occurs during the cleavage stage of embryonic development due to irregular cell division, resulting in portions of the cytoplasm pinching off. This process can lead to the loss of critical organelles, such as mitochondria, which are essential for cellular energy production, and may negatively affect embryonic development. However, in the present study, no significant association was found between mtDNA copy number and fragmentation. This discrepancy may be attributed to differences in the timing of embryo SCM collection; in our study, it was collected on day 5 after the embryos were cultured, further following fragmentation grading on day 3. This delay may have allowed additional developmental processes to occur, potentially altering the mtDNA levels through secretion or reabsorption by blastomeres. In contrast, Stigliani *et al.* (2013) collected embryo SCM immediately after fragmentation grading on day 3, likely capturing a more accurate relationship between mtDNA levels and fragmentation status. This discrepancy highlights the importance of standardising sample collection protocols for mtDNA analysis in future research (Stigliani *et al.* 2013). Therefore, the timing of medium collection modulates DNA concentration in SCM governed by embryonic developmental stage, cellular turnover and embryo quality. Advanced-stage embryos, such as day 6 blastocysts, may exhibit increased DNA release, while compromised embryos may shed more due to cellular fragmentation. However, this trend was not observed in our study, consistent with findings by Hammond *et al.* (2017) and Kobayashi *et al.* (2020).

In this study, we found that the relative mtDNA copy number was not associated with fragmentation grade, morphology grade or chromosomal abnormalities in embryos. This may be attributed to the mechanism of mtDNA replication during embryonic development. Before fertilisation, oocytes accumulate large numbers of mitochondria and mtDNA copies to provide sufficient energy for early development. After fertilisation, mtDNA levels in the embryo decrease until the blastocyst stage (Piko & Taylor 1987). At the blastocyst stage, mtDNA replication factors, which reactivate mtDNA replication, are upregulated. However, replication occurs primarily in TE cells, which form the placenta and mediate implantation for further embryonic development (St John *et al.* 2010). This finding aligns with that of Wang *et al.* (2021) who observed an association between mtDNA content and TE quality but not with ICM. Similarly, Klimczak *et al.* (2018) noted a relationship between mtDNA content and embryo morphological grade using TE biopsy. However, in our study, no association was found between mtDNA content and embryo morphology grades, which was consistent with other studies (Zhang *et al.* 2019*a*, Kobayashi *et al.* 2020, Ritu *et al.* 2022), but contradicted the findings of Wang *et al.* (2021) and Klimczak *et al.* (2018). This discrepancy may have occurred as we included only moderate- and poor-quality embryos. Consequently, the differences in mtDNA quantities across the groups were not statistically significant.

Regarding chromosomal abnormalities, previous studies have reported a correlation between mtDNA copy number and aneuploidy using blastomere biopsies (Tao *et al.* 2017, Ritu *et al.* 2022). This supports the 'quiet embryo hypothesis', which posits that embryos under stress or with limited energy reserves upregulate mitochondrial replication early to meet increased energy demands at the blastocyst stage (Cecchino & Garcia-Velasco 2019). Although this study found a trend towards higher mtDNA copy numbers in the aneuploid group, this difference was not statistically significant. These findings were consistent with those reported by Victor *et al.* (2017) and Zhang *et al.* (2019*a*). However, Fragouli *et al.* (2015) reported a significant association between mtDNA copy number and chromosomal abnormalities. This inconsistency may be due to the small sample size of this study (n = 40), which limited the statistical power to detect significant differences. Expanding the sample size in future studies may yield more conclusive results. Another potential factor contributing to the lack of correlation between mtDNA copy number in the embryo SCM and the embryo itself is contamination by maternal DNA (Hammond *et al.* 2017). Maternal DNA from incompletely removed cumulus cells may confound mtDNA measurements. Enhanced denuding techniques to ensure the complete removal of cumulus cells could improve the accuracy of the results. In addition, the sensitivity of the real-time PCR technique used for mtDNA detection in this study may have been insufficient. Employing digital PCR, which is a more precise and sensitive technique, could improve the detection of mtDNA in embryo SCM.

Conclusion

Based on the findings of this study, the relative mtDNA copy number in embryo SCM may not serve as a reliable biomarker for assessing blastocyst morphology or

chromosomal abnormalities. Although a trend towards higher mtDNA levels was observed in the aneuploid group, its clinical utility remains uncertain. Factors, such as maternal DNA contamination, small sample sizes and limitations in mtDNA quantification techniques, may have contributed to these results. Future studies should aim to increase the sample size, minimise maternal DNA contamination through improved cumulus cell removal techniques and adopt more sensitive methods, such as digital PCR, to enhance the reliability and accuracy of mtDNA analysis in embryo SCM.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-25-0001.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The study was conceptualized and designed by ST, AN and RR, who also contributed to data analysis and interpretation. Data collection and experiments were conducted by ST, KW, NN, KW, PB and PK, with PA handling sample preparation. ST, KW, NN, KW, PB, PK, AN, SP and RR contributed to data analysis, interpretation and manuscript preparation.

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