Human Reproduction, Vol.32, No.4 pp. 954–962, 2017
Advanced Access publication on February 23, 2017 doi:10.1093/humrep/dex034

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

Levels of trophectoderm mitochondrial DNA do not predict the reproductive potential of sibling embryos

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Submitted on December 13, 2016; resubmitted on January 23, 2017; accepted on February 6, 2017

STUDY QUESTION: What is the predictive value of trophectoderm mitochondrial DNA (mtDNA) quantity for blastocyst reproductive potential?

SUMMARY ANSWER: This study demonstrates that, within a given cohort, mtDNA quantitation does not distinguish between embryos that implant and embryos that do not implant after double embryo transfer (DET).

WHAT IS ALREADY KNOWN: An association between implantation failure and increased quantities of mtDNA has been observed in two studies but not in a third.

STUDY DESIGN, SIZE AND DURATION: A total of 187 patients (nine who received donor oocytes) with DET of one male and one female euploid blastocyst were included in this retrospective study, with 69 singleton deliveries providing the primary dataset to evaluate the predictive value of mtDNA for reproductive potential between January 2010 and July 2016.

PARTICIPANTS/MATERIALS, SETTING AND METHOD: MtDNA was quantified in cell lines to validate the quantitative PCR assay on limited quantities of starting material and then applied to 374 blastocyst biopsies. Pregnancies resulting in a singleton outcome were analyzed and newborn gender was utilized as a means to identify the implanted embryo. MtDNA quantity was then compared between implanted and non-implanted embryos in order to define the predictive value of mtDNA content for reproductive potential in this subset of patients.

MAIN RESULTS AND THE ROLE OF CHANCE: An initial comparison of mtDNA levels between all successful and unsuccessful embryos revealed no significant differences. In order to control for patient-specific variables, gender was subsequently used to identify the implanted embryo in DETs resulting in a singleton ($n = 69$). No systematic difference in relative mtDNA quantity was detected between implanted and non-implanted embryos.

LIMITATIONS, REASONS FOR CAUTION: This study was conducted at a single center and did not evaluate the entire cohort of embryos from each patient to evaluate cohort specific variation in mtDNA quantity. Although the largest of its kind so far, the sample size of DETs leading to a singleton was relatively small.

WIDER IMPLICATIONS OF THE FINDINGS: These data highlight the importance of control over patient-specific variables when evaluating candidate biomarkers of reproductive potential. All current available data suggest that mtDNA quantification needs further study before its clinical use to augment embryo selection.

STUDY FUNDING/COMPETING INTERESTS: The authors have no potential conflict of interest to declare. No external funding was obtained for this study.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: mitochondrial DNA / IVF / comprehensive chromosome screening / embryo selection / blastocyst

Introduction

The role of mitochondrial function and proper energy production in the developing human embryo is thought to be critical to successful implantation and delivery (Van Blerkom *et al.*, 1995). Many aspects of the mitochondria may be important, including relative efficiency of energy production, the sequence integrity of the mitochondrial genome, the ability to undergo autophagy, biogenesis of new mitochondrion and the absolute quantity of mitochondria allocated to the original oocyte (Dumollard *et al.*, 2007, 2009; Harvey *et al.*, 2011; Van Blerkom, 2011). Great attention has been given to the ability to quantify mitochondrial DNA (mtDNA) content within the preimplantation embryo which initially demonstrated a correlation with the presence of chromosomal aneuploidy (Su *et al.*, 2010). More recently, Tan *et al.* (2014) confirmed that embryos with chromosomal aneuploidy possess significantly higher levels of mtDNA when compared to euploid embryos. As a result of these findings, many groups have begun to investigate the utility of quantifying mtDNA to predict reproductive potential amongst chromosomally normal embryos (Simpson *et al.*, 2015; Seli, 2016).

Fragouli *et al.* (2015) described a method for quantification, which demonstrated the ability to predict a negative outcome in 30% of euploid embryos by defining a threshold for abnormally high levels of mtDNA. Diez-Juan *et al.* (2015) subsequently developed a similar assay and demonstrated a similar inverse relationship between mtDNA quantity and reproductive potential. While these data support the conclusion that mtDNA may serve as a useful biomarker of reproductive potential, multiple questions still remain with regards to the clinical applicability of this technology. In addition, another group recently reported the lack of predictive value of mtDNA for reproductive potential using a modified methodology that improved normalization strategies (Victor *et al.*, 2016).

First, based on the available evidence, it is unclear whether higher levels of mtDNA are a marker for poor prognosis amongst patients or if embryos within a single cohort demonstrate significant variation in their levels of mtDNA. The former scenario would identify patients at high risk of failure but would provide no additional selection value amongst a patient's embryos. The latter scenario would permit prioritizing embryos for transfer within a single cohort. Comparing single embryo transfers (SETs) between patients limits the ability of investigators to differentiate between these two explanations. It is possible that a separate, unidentified confounding factor may influence both the mtDNA quantity of a given patient's embryos and thus impact the overall chance of success for that patient. This factor may be at play for all of a given patient's embryos and thus quantifying mtDNA may not provide additional information to aid in embryo selection.

Second, given that the mitochondrial content in a given cell of a preimplantation embryo is dependent upon the number of cell divisions that preceded biopsy, it is useful to evaluate whether embryologic parameters also influence mtDNA quantity in embryo biopsy samples. It is possible that the higher mtDNA quantity observed in certain embryos in prior studies was a function of fewer cell divisions in that individual embryo prior to biopsy and thus less dilution of the mitochondria in the analyzed sample. Thus, the discrepancy in mtDNA quantity between successful and unsuccessful SETs may solely represent the confounding influence of differential mitogenic activity amongst preimplantation embryos in different patients. As a result, it would be useful to determine whether embryology parameters influenced mtDNA quantity values.

In order to determine whether mtDNA quantity could be a useful adjunct to embryo selection amongst euploid embryos, a two phase study would be optimal. An ideal first phase would involve characterizing mtDNA levels in sibling euploid embryos which were transferred together but led to a singleton pregnancy. If the phenomenon reported in prior studies is consistent within individual cohorts, embryos which implanted should possess less mtDNA than embryos which failed to implant. Such an outcome would indicate that mtDNA may be useful in embryo selection from among multiple embryos from the same cohort. This study design eliminates patient-specific variables, which may impact embryo-specific outcomes, since each patient serves as her own control. For example, factors such as maternal age, which have been demonstrated to be correlated with mtDNA content, would be controlled. If an association is observed in this phase, subsequent analysis of the impact on outcomes in a RCT would represent the next phase of evaluating mtDNA as a biomarker for embryo selection.

The present study provides data for the first phase of investigating the utility of mtDNA based embryo selection by evaluating the predictive value among sibling euploid embryos which led to a singleton delivery. As a secondary goal, this study also examined whether embryo specific data such as expansion grade, day of blastocyst biopsy and morphologic characteristics influenced mtDNA quantity.

Materials and Methods

Population

All double embryo transfers (DET) following comprehensive chromosomal screening where one male and one female embryo were selected for transfer between January 2010 and July 2016 were retrospectively identified for inclusion in the analysis. Each included patient had residual preamplification DNA from trophectoderm biopsy specimens stored in the onsite DNA repository for mtDNA quantification (see later). Specimens were stored with patient written informed consent for potential future use examining genetic associations with infertility. All patients were deemed to have a normal endometrial cavity, with the endometrium achieving at least 7 mm in thickness prior to transfer. Nine recipients of donated oocytes were included in the analysis. In all these cases both oocytes were retrieved from the same donor, and the donor's age is used for analysis.

DETs with transfer of one embryo from each gender from the same cohort were specifically evaluated. The high reproductive potential of

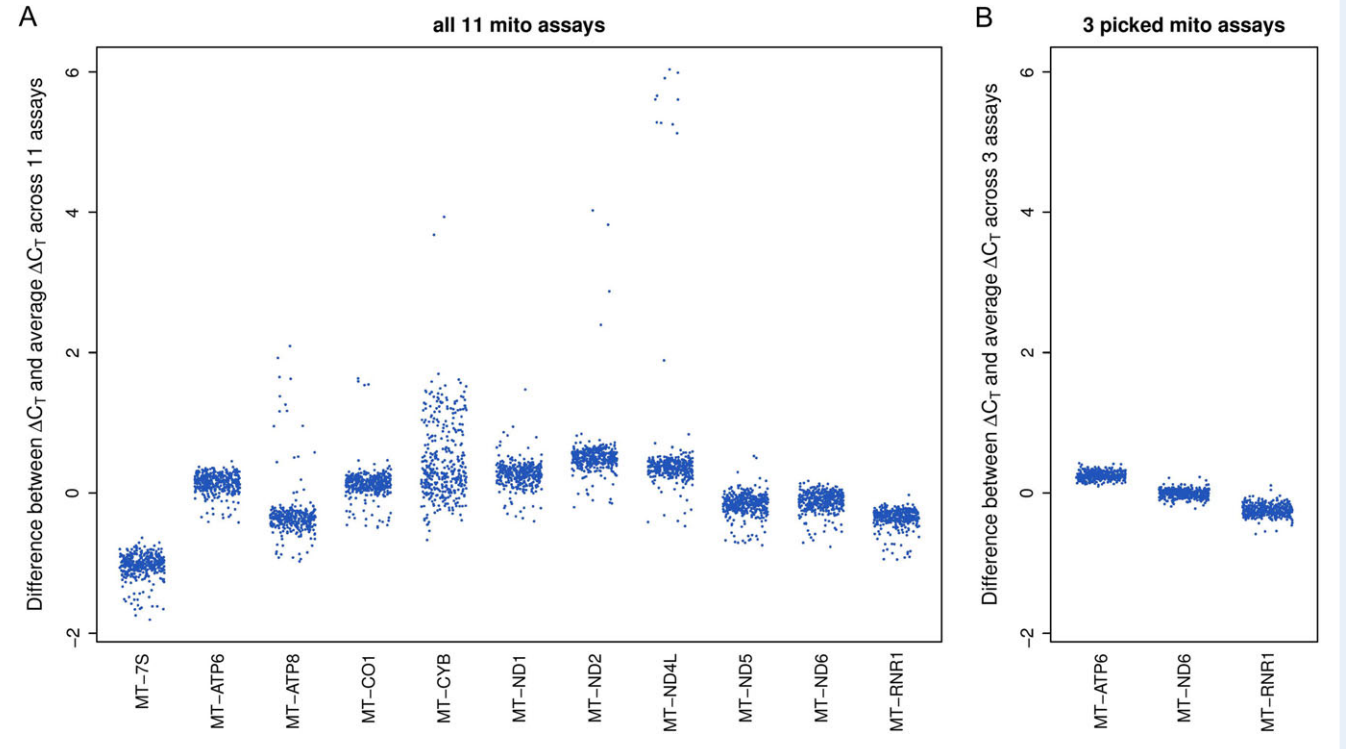

Figure 1 Selection of mitochondrial TaqMan assays. Some mitochondrial (mito) assays yield results that are sometimes inconsistent with other assays (**A**). Such assays are recursively eliminated until there is a set of three of the most robust assays for the quantitation of mtDNA level (**B**). The assays are named on the *x*-axis. ΔC_T = cycle threshold values.

euploid blastocysts made the availability of cases meeting this criteria relatively rare during the time period from which samples were screened for eligibility (i.e. double euploid blastocyst transfers). Pregnancies resulting in a singleton in this model allowed the opportunity to compare mtDNA quantity directly between one embryo that implanted and led to a delivery and one that failed to implant, while controlling for all patient-specific variables. Thus, variables, such as age, etiology of infertility, semen parameters, laboratory exposures and uterine environment were exactly equivalent for each embryo. Newborn phenotypic sex and comprehensive chromosome screening (CCS) results were used to confirm which of the two embryos implanted.

Embryos

All embryos were biopsied on Day 5 or 6. Only one of the 187 DET cases in this study involved two embryos biopsied on different days. Embryo transfers occurred either fresh on Day 6 or in a subsequent frozen embryo transfer cycle (Supplementary Table SI). Each embryo had previously undergone quantitative PCR (qPCR) based CCS using a previously described protocol with preclinical validation on control cell lines (Treff *et al.*, 2012), and two RCTs demonstrating improved outcomes (Forman *et al.*, 2012a,b; Treff *et al.*, 2012; Scott *et al.*, 2013a,b). In the process of qPCR based CCS testing, excess material (one half of the preamplification reaction) not used in the procedure was made available for analysis of the mtDNA content as described below.

Assays

A total of 11 different cataloged mtDNA-targeting TaqMan assays (Thermo Fisher Scientific, Foster City, CA, USA) and a previously described custom-made AluYa5 nuclear DNA assay (Treff *et al.*, 2011) were used to perform preamplification (multiplex PCR) (Fig. 1A). Each 100 ul reaction consisted of 25 ul of the leftover PCR reaction, 25 ul of 0.2× of each TaqMan assay and 50 ul of 2× TaqMan PreAmp Master Mix and was run for 18 cycles as recommended by the supplier (Thermo Fisher Scientific). Subsequent qPCR was performed with each assay run in quadruplicate 5ul reactions with 1ul of the mtDNA preamp reaction, 0.25 ul of each TaqMan assay (20×) and 2.5 ul of TaqMan Gene Expression Master Mix. Standard TaqMan PCR settings were used and real-time data wereas collected on an ABI 7900 Instrument (Thermo Fisher Scientific). Individual mtDNA assays that result in higher variation in the difference between its ΔC_T measurements (difference in cycle threshold values) as compared to the average ΔC_T of all assays across samples, reflecting inconsistent results of a particular assay as compared to all assays across many samples, were recursively eliminated until there was a set of three assays that had highly consistent results across hundreds of samples (Fig. 1B). These three most robust assays (ATP6, Hs02596862_g1; ND6, Hs02596879_g1; and RNR1, Hs02596859_g1) were used for measuring the mitochondrial level of samples in the current study. Each assay was carried out four times for each sample in final experiments.

Validation of methodology for mtDNA quantification

To validate the methodology of quantifying limited amounts of mitochondria from the number of cells typically obtained from a trophectoderm biopsy, the lymphocyte cell line (GM13118) was used to create 5-cell samples. The cell line was cultured and passaged as recommended by the supplier (Coriell Cell Repository, Camden, NJ, USA). Cells were exposed to 25 ng/ml of ethidium

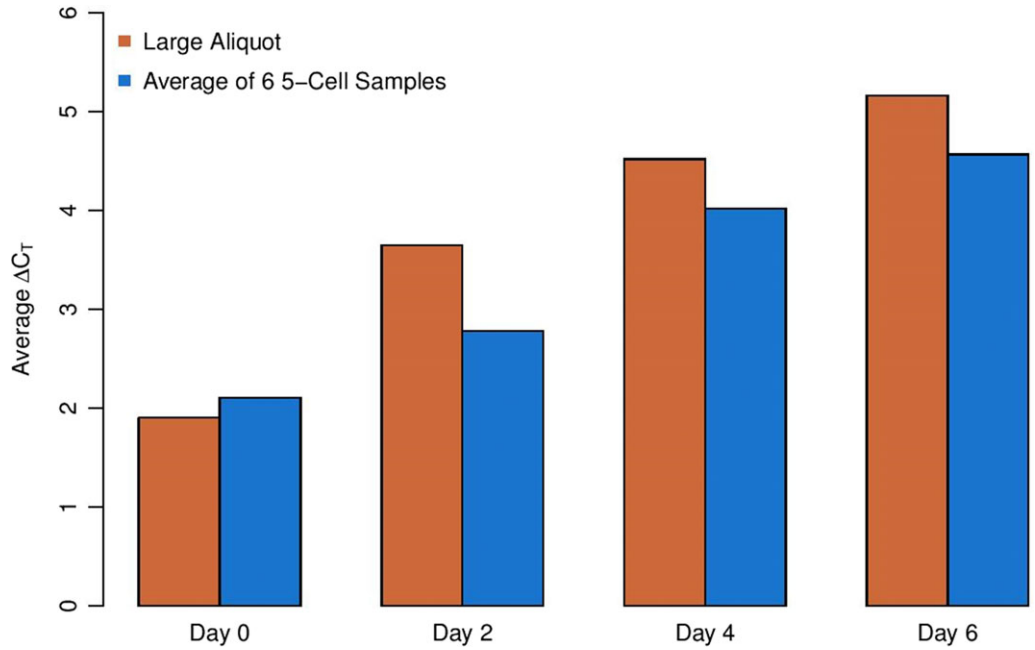

Figure 2 Comparison of average ΔC_T values for either limited quantities of cells (5-cells from cell lines) or large quantities (aliquot of same cell line) using Coriell Cell Repository lymphocyte cell line GM13118. Cells were exposed to ethidium bromide for 0, 2, 4 and 6 days. Relative mtDNA quantity was defined as the additive inverse of the average ΔC_T for the three mtDNA assays. Results demonstrate similar mtDNA quantitation with numbers of cells typically obtained from a trophectoderm biopsy compared to large quantities that do not require additional amplification.

bromide in the culture medium for 6 days in order to reduce mtDNA content and confirm quantification (Kao *et al.*, 2012). Cells were obtained prior to exposure, as well as on Days 2, 4 and 6 after treatment and stored in PCR tubes. In parallel, a large aliquot (>1 million cells) was obtained at the same time points for conventional DNA isolation with QIAgen columns (QIAgen Inc., Germantown, MD, USA). Multiple replicates using 5-cell samples were processed and compared with the large aliquot of cells (>1 million cells) in order to validate the assay for use on limited quantities of mitochondria. Subsequently, ΔC_T values from replicates were averaged and compared with ΔC_T values from the large aliquot at each time point.

For relative quantitation of mtDNA in a sample, the median C_T value for the four measurements for each individual assay was calculated first, followed by the calculation of a ΔC_T between each mtDNA assay and the reference AluY5a assay. Relative mtDNA quantity was defined as the additive inverse of the average ΔC_T for the three mtDNA assays.

Accurate quantitation of mtDNA was achieved with qPCR using three mitochondrial-specific primers (ATP6, Hs02596862_g1; ND6, Hs02596879_g1; and RNR1, Hs02596859_g1) (Thermo Fisher Scientific) and an AluY5a assay (Treff *et al.*, 2011) for normalization. As expected, ΔC_T values from the large aliquots increased from Day 0 to 6 indicating a decreasing mitochondrial copy number (Fig. 2). In order to evaluate the assay's performance using limited amounts of DNA resembling excess material available from clinical specimens, 5-cell samples were subsequently evaluated in a similar fashion. Samples processed in replicates of six resulted in consistent results and likewise had increasing ΔC_T values signifying decreasing copies of mtDNA (Fig. 2).

Data analysis

Embryo relative mtDNA quantity was evaluated in order to assess putative associations with reproductive success. First, a likelihood ratio test based on nested logistic regression models was utilized to test if relative mtDNA quantity values predicted overall outcome among all embryos transferred (implanted and delivered versus failed). The null hypothesis was that there is no association between relative mtDNA quantity and embryo success status. The test statistic should follow a chi-squared distribution with one degree of freedom when the null hypothesis is true. Maternal age at time of oocyte retrieval was used as a covariate in this model.

Subsequently, only the 69 DETs where one of the two sibling embryos successfully implanted and was delivered were analyzed. A binomial test was carried out to determine whether the successful embryo tended to have higher or lower relative mtDNA level compared to the unsuccessful one. Specifically, the null hypothesis was that the relative mtDNA quantity of the successful embryo was equally likely to be increased or decreased when compared to the unsuccessful embryo. The type I error deemed to be statistically significant in either direction (successful embryo with increased relative mtDNA or decreased relative mtDNA) was set with a $P < 0.05$.

Lastly, utilizing simple linear models and standard ANOVA, other patient and embryo specific parameters, such as oocyte maternal age, embryo biopsy day (Day 5 or 6) and embryo morphology at time of biopsy, were tested for correlations with mtDNA quantity. More specifically, blastocyst expansion grade (1–6), inner cell mass (ICM) score (A, B or C) and trophectoderm score (A, B or C) were evaluated using R, version 3.2.5 (https://www.r-project.org/).

Power analysis

A post hoc power analysis was performed based on the observed SD (0.679) of the difference between the two sets of average ΔC_T values for the 69 pairs of embryos with singleton births. Using a significant threshold of 0.05, the power for detecting a difference of 0.242 in average ΔC_T

values (corresponding mitochondrial level fold change of 1.18) for the one-sided paired *t*-test was 90%.

All data collection and analysis was approved by the Western IRB, protocol number 20031397.

Results

A total of 374 embryos utilized in 187 DETs were available for analysis. The average maternal age at time of oocyte retrieval was 34.6 years (range: 22.7–42.8). Of these, 69 (36.9%) resulted in the live birth of a singleton, 84 (44.9%) resulted in multiple births and 34 (18.2%) failed to result in any birth. The overall implantation rate was 63.4% per embryo. Analysis of the entire cohort of 374 embryos did not identify a significant association between relative mtDNA quantity and the likelihood of an embryo leading to delivery ($P = 0.556$) (Fig. 3A).

The 69 DETs resulting in singleton delivery were subsequently analyzed separately. Since a male and a female embryo were always transferred together, offspring gender was used to distinguish the implanted embryo from the non-implanted one. Relative mtDNA quantity from embryos that implanted and delivered versus those that did not were compared (Fig. 3B–D). Data plots showed no obvious signs of any systematic difference between the mtDNA level of the successful embryo and the unsuccessful one across the 69 pairs. The binomial test for evaluating whether the successful embryo tended to have a higher or lower relative mtDNA quantity produced $P = 0.81$ (Fig. 3D). Therefore, the data did not support the alternative hypothesis that the difference in relative mtDNA quantity between the 69 embryo pairs was more likely to be increased or decreased.

Additional clinical and embryological parameters were evaluated for associations with mitochondrial copy number in all 374 embryos (Fig. 4). Mitchondrial DNA copy number was negatively correlated with oocyte age ($P < 0.001$). Embryos biopsied on Day 5 were more likely to have higher quantities of mtDNA compared with those biopsied on Day 6 ($P < 0.001$). Greater blastocyst expansion scores were correlated with lower mitochondrial content ($P < 0.001$). Higher quality trophoblast and ICM were similarly correlated with lower mtDNA quantity ($P < 0.001$ and $P = 0.0010$, respectively). A comparison of the average mtDNA quantity for each of these parameters is included in Supplementary Table SII. Please note that these results are reported for the sake of completeness. Since embryos were selected for DET with consideration of morphological criteria, care needs to be exercised before drawing general conclusions.

The raw data for each of these parameters as well as additional embryo specific and patient-specific variables, such as embryo karyotype, oocyte donation status, maternal age, fresh or frozen status, day of biopsy, day of embryo transfer, morphological grades on day of biopsy and day of embryo transfer, embryo success status and relative mtDNA quantity data are also available for all 374 embryos in Supplementary Table SI. Notably, somewhat more biopsies took place on Day 6 than Day 5 (57.4 versus 42.6%). The majority of transfers were frozen embryo transfers (64.2 versus 35.8%). Fresh embryo transfers all were performed after Day 5 biopsy. Not surprisingly, the average mtDNA quantity for these transfers was higher than frozen embryo transfers (which included both Day 5 and Day 6 embryo biopsies).

Discussion

Over the past decade, the search to identify markers of embryonic reproductive competence has primarily focused on ploidy status based on the observation that aneuploidy represents the primary genetic cause of failed pregnancies (Hassold and Hunt, 2009). However, there has been a recent shift in the direction of research to identify factors predictive of reproductive success outside of chromosomal screening. Among the many novel avenues currently being investigated, one area of interest has been usefulness of mtDNA content as an independent measure of embryonic potential (Fragouli *et al.*, 2015).

Mitochondria are among the most abundant and important organelles in mammalian cells. They have long been known to be the primary producers of adenosine triphosphate and they are also involved in a diverse range of signaling and intracellular functions (Ernster and Schatz, 1981). Specifically, mitochondria sequester and release calcium ions, produce reactive oxygen species, regulate apoptosis and have pivotal roles during fertilization (Van Blerkom *et al.*, 1995; Duchen, 2000; Dumollard *et al.*, 2007, 2009). Disproportionate mitochondrial inheritance between human blastomeres as early as the first cleavage division has also been demonstrated, potentially leading to differences in mtDNA content within embryos (Van Blerkom *et al.*, 2000). Although proper mitochondrial function is essential for embryo development and viability, new modalities which allow quantitation of mtDNA have led researchers to investigate the question of whether quantity is important. In fact, some suggest that measurement of mtDNA quantity should be used as a tool to allow better selection of IVF embryos for transfer (Fragouli and Wells, 2015).

In the present study, a stepwise approach was used to preform accurate mtDNA quantification on embryo biopsy material. The focus of this study was different than prior attempts to describe the association between mtDNA content and embryo viability. We sought to specifically address whether mtDNA quantitation could be used as an adjunct selection tool to identify embryos with increased reproductive competence within the same cohort. Thus, the methodology of this study differed from prior attempts at correlating mtDNA quantity and pregnancy outcome in that DETs were utilized. Paired comparisons of mtDNA content on embryos developed in the same cohort allows significant advantages. This methodology eliminates patient-specific variables leading to a well controlled investigation of the predictive value of mtDNA for reproductive outcome in a single cohort. While prior studies observed a relationship between mtDNA content and outcomes in SETs, it is unclear whether this relationship only distinguished poor prognosis patients from good prognosis patients or identified poor reproductive competence of individual embryos in a cohort. However, the data presented here suggest that mtDNA quantification does not significantly augment selection beyond confirmation of euploidy and morphology within a given patient's cohort.

The only other comparison of DETs of euploid embryos in the literature to date comes from the paper of Diez-Juan *et al.* (2015). In this investigation, 10 DETs involving one male and one female euploid embryo were compared as a subset of the larger study. Predefined thresholds of relative mtDNA content were applied to compare embryos that implanted and failed to implant. In these data, 4 of 10 implanted embryos had either equivalent relative mtDNA content or higher relative mtDNA content than the failed embryos. These findings are consistent with the data presented here, in that relative mtDNA quantity does not provide

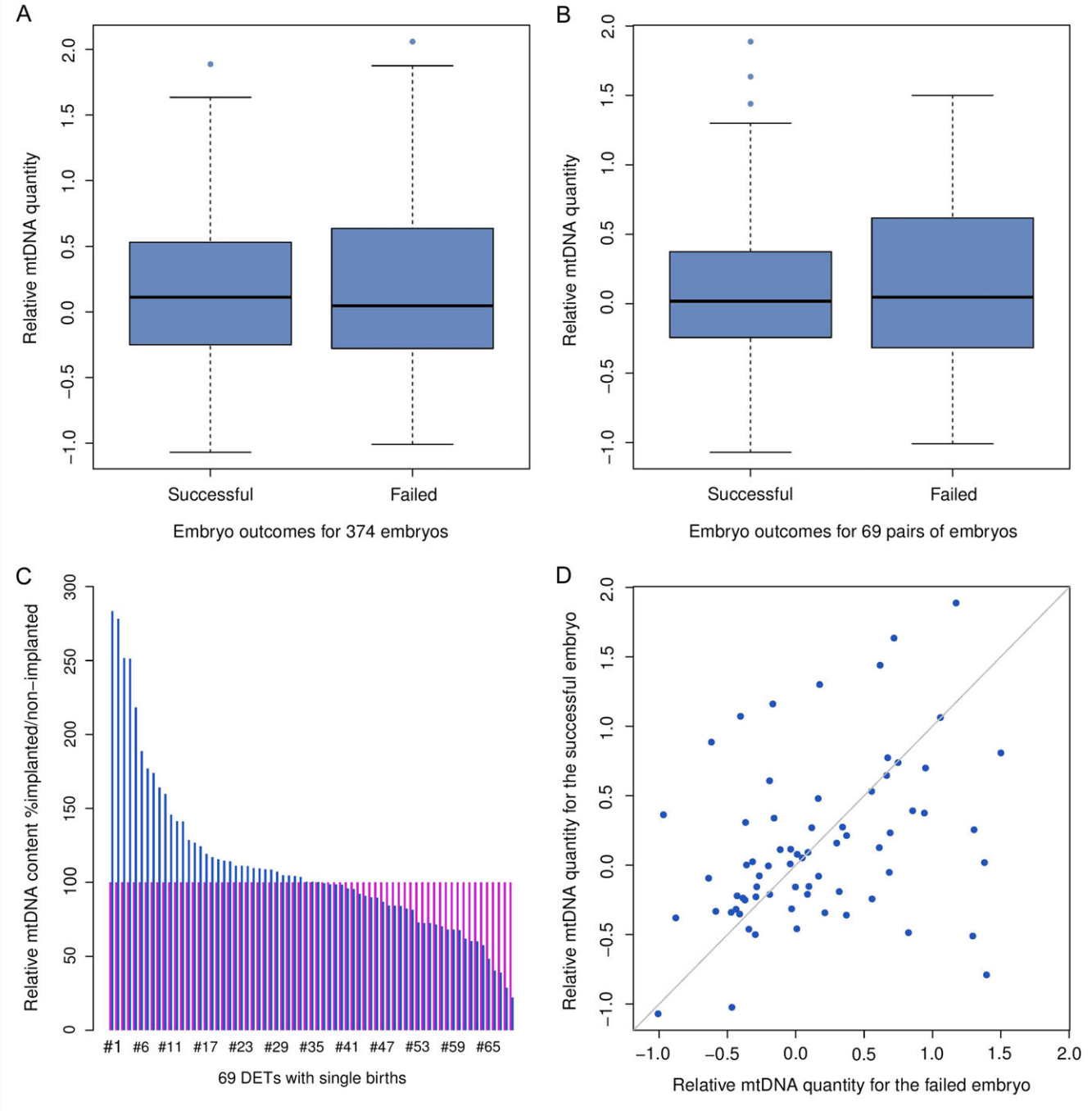

Figure 3 Relative mtDNA quantity versus pregnancy success in women. (**A**) Relative mtDNA quantity stratified by embryo pregnancy success status (delivered or not delivered) for all 374 embryos in the current study. (**B**) Relative mtDNA quantity stratified by embryo pregnancy success status for the 69 pairs of embryos used in double embryo transfer (DET) that resulted in single births. (**C**) Relative mtDNA quantity ratio between each of the 69 pairs of embryos used in DET that resulted in single births. Blue bars indicate the ratios between two embryos within each pair. (**D**) Comparison of the relative mtDNA quantity for the successful versus failed embryo for each of the 69 pairs. The equivalence line is shown in gray. For each box and whisker plot, the thick horizontal line shows the median, the box shows the interquartile range, the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box, and outliers are indicated by points.

strong discriminatory value that prognosticates success within a single cohort. The data presented here comes from a larger sample size, including 69 DETs. While these findings provide the largest experience to date with this experimental paradigm, a larger sample size of euploid DETs would address this question in a more robust fashion. Furthermore, these data require further study in other settings.

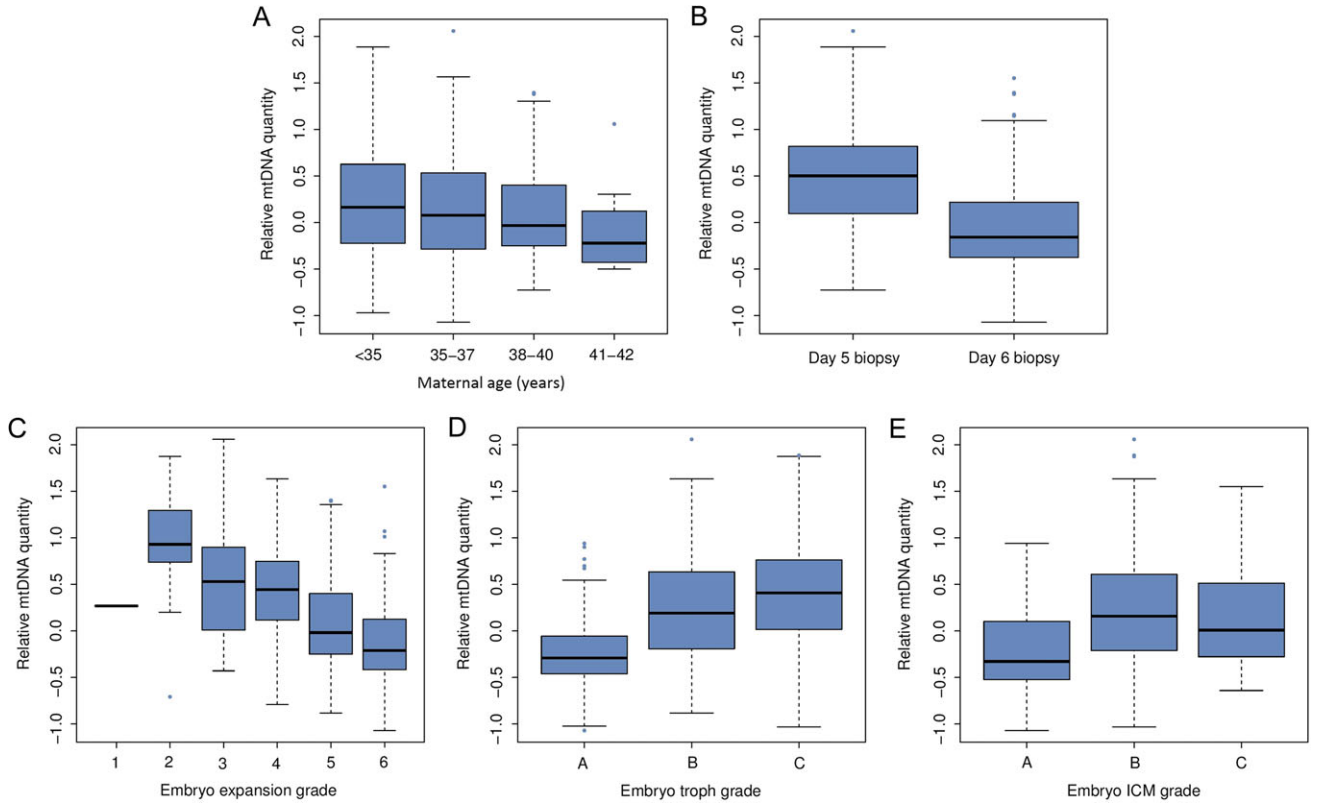

Figure 4 Association of secondary clinical parameters with mitochondrial quantity among 374 embryos (**A**). Relative mtDNA quantity stratified by maternal age group. (**B**) Mitochondrial quantity in biopsy samples stratified by biopsy day. (**C**–**E**) Mitochondrial quantity in biopsy samples stratified by embryo expansion stage, trophectoderm (troph) grading and inner call mass (ICM) grading, respectively. For each box and whisker plot, the thick horizontal line shows the median, the box shows the interquartile range, the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box, and outliers are indicated by points.

It is possible that mtDNA quantity provides useful information to aid in selecting embryos for transfer only in the case of extremely elevated levels. One prior study found that the SET of embryos carrying mtDNA quantities in the 90th percentile produced no implantations in 22 transfers (Fragouli *et al.*, 2015). Our study was not powered to specifically address embryos with mtDNA levels in the extremely high range. However, while a slight reduction in the implantation rate of embryos with mtDNA quantity in >90th percentile was noted, the implantation rate was much higher than previously reported at 48.6% (18/37). Further studies are needed to address whether or not significant variation is seen within individual cohorts and embryos with extremely high mtDNA quantities can be deprioritized or if significantly elevated mtDNA levels are found more commonly in specific patients.

This study investigated the relationships between mtDNA quantity and multiple clinical parameters available during IVF cycles. A significant association was found between mitochondrial quantity and maternal age providing evidence that preimplantation blastocysts developed from older patients have lower levels of mtDNA compared with those from younger ones in our ascertained patient population. The association of age and mtDNA content has been studied previously in both oocytes and embryos. Studies evaluating oocyte mitochondrial levels appear to be in agreement with the present study, showing decreasing levels in older patients(Chan *et al.*, 2005). However, the opposite effect was recently shown in a study evaluating blastocyst levels, suggesting that blastocysts from older women may have more demanding energy requirements due to a continuously declining organelle function over time (Fragouli *et al.*, 2015). Additionally, a comparison of embryos biopsied on Day 5 versus Day 6 identified that mitochondrial content per cell may decline during blastocyst development. Evaluation of blastocyst morphology also revealed significant associations. Embryos identified to have superior morphological qualities were found to contain lower quantities of mtDNA. These associations may reflect that mtDNA content is impacted by the degree of expansion and mitogenic activity of the blastocyst. A more expanded, more cellular blastocyst that has experienced more cell divisions would be expected to have less mtDNA per cell due to greater partitioning of available mitochondria at each division.

Of particular clinical importance is the potential use of mtDNA quantity as a modality to predict embryo success. Proper evaluation of a marker for its ability to predict outcomes among sibling embryos is challenging, but it can be accomplished using a variety of study designs that allow for control over known and unknown

patient-specific variables. By analyzing only those patients undergoing DET's and identifying the implanted embryo, the predictive values of a marker for reproductive potential can be better isolated. Identification can be achieved using gender if sibling embryos are of different sex. Alternatively, fingerprinting analysis on the delivered offspring can be utilized regardless of gender. This approach is more complicated and costly, but the technology has been available and used in prior studies successfully (Forman *et al.*, 2012a,b; Scott *et al.*, 2012, 2013a,b; Werner *et al.*, 2016).

To the authors' knowledge, this is the largest study to date evaluating the predictive value of mtDNA content using a sibling embryo study design. We identified 69 cases where both a euploid male and female embryo were transferred simultaneously and resulted in a singleton outcome. A paired analysis showed that relative mtDNA levels did not distinguish between embryos that implanted and those that failed to implant. Based on these results, the current available data suggest that mtDNA quantification needs further study before its clinical use to augment selection in addition to more validated techniques.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

N.R.T. and R.T.S. designed the experiment, X.T., M.O., M.H., J.R. and L.M. performed the experiments and X.T., S.M., M.O. and Y.Z. performed data analysis. All authors approved the content and contributed to drafting and revising the article.

Funding

No external funding was obtained for this study.

Conflict of interest

None declared.

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