EMBRYO BIOLOGY

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Blastulation timing is associated with differential mitochondrial content in euploid embryos

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Abstract

Purpose Preimplantation genetic screening (PGS) and assessment of mitochondrial content (MC) are current methods for selection of the best embryos for transfer. Studies suggest that time-lapse morphokinetics (TLM) may also be helpful for selecting embryos more likely to implant. In our study, we sought to examine the relationship between TLM parameters and MC to determine if they could be used adjunctively in embryo selection. We also examined the relationship between MC with ploidy and blastulation.

Methods Cryopreserved human embryos at the zygote stage were thawed and cultured in a time-lapse system. Blastomere and trophectoderm biopsies were performed on days 3 and 6. Biopsied cells and all whole embryos from day 6 were analyzed for MC (ratio of mitochondrial to nuclear DNA) and ploidy using next-generation sequencing.

Results In embryos, MC per cell declined between day 3 and day 6. While early cleavage parameters did not predict MC, embryos with longer blastulation timing had higher MC on day 6. Day 6 MC was lower in euploid vs. aneuploid embryos and lower in blastocysts vs. arrested embryos.

Conclusions A lower MC at the blastocyst stage was associated with euploid status and blastocyst formation, indicating better embryo quality compared to those with a higher MC. Higher MC in aneuploid and arrested embryos may be explained by slower cell division or degradation of genomic DNA over time. Blastulation timing may be helpful for selection of higher quality embryos. Combining blastulation timing and MC along with morphologic grading and euploid status may offer a new direction in embryo selection.

Keywords Time lapse · Mitochondrial DNA · Aneuploidy · Embryology

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Introduction

In the field of assisted reproductive technology (ART), clinical and laboratory practices have evolved to improve pregnancy success rates while minimizing the obstetric risks of multiples by transferring the least number and highest quality embryos possible. To date, there have been different strategies for achieving this. Elective single embryo transfer has been promoted, with clinical data to support the decrease in multiple gestations [1]. Some studies have demonstrated that transferring blastocyst-stage embryos results in higher clinical pregnancy rates than cleavage-stage embryos [2, 3]. More recently, the incorporation of preimplantation genetic screening (PGS) has led to a push for transferring single euploid embryos, which contributes, in some cases, to favorable ART and obstetrical outcomes [4]. In addition to PGS, some data have shown that a lower mitochondrial DNA copy number in

euploid embryos are more likely to lead to successful pregnancies [5, 6].

Data in both mice and humans suggests that mitochondrial organelles do not replicate during the early stages of embryo development [7, 8]. A typical oocyte carries a few hundred thousand mitochondria, ready to be randomly distributed among divided cells during the first 6 days of embryo development [9]. This is needed to ensure that new embryonic cells possess the energy source to carry on biological processes, including cell division [10]. A study by Fragouli et al. demonstrated that higher levels of mitochondrial DNA (mtDNA) were associated with decreased implantation in euploid embryos [6]. While they postulated that higher mtDNA in embryos was due to higher oxidative stress associated with aneuploidy [6], differential mitochondrial load may also be attributed to differences in kinetics of cell division during embryo development. mtDNA is measured as a ratio to nuclear DNA (nDNA), designated as mitochondrial content (MC), and abnormal or slow cell division may result in higher reads of mtDNA compared to normal cell division. To further investigate this, we sought to determine whether time-lapse morphokinetics (TLM) would provide additional information for determining which euploid embryos had high vs. low MC. Previous studies of TLM have shown that the duration of first mitosis, time between first and second mitosis, and time between second and third mitosis are predictive of blastocyst formation [11]. Other studies have shown a positive correlation with early mitotic division timing and implantation rates [12, 13]. Differences in cell cycle length, time to division, and interval between cell division from the two- to four-cell stage are different in euploid vs. aneuploid embryos [14]. No previous studies to our knowledge have examined the relationship between TLM and MC in human embryos. We sought for the first time to determine whether TLM parameters were correlated with MC, as they may provide additional information for optimized embryo selection. We also sought to determine if other factors, such as chromosomal status and blastocyst formation, were associated with different MC. Lastly, we examined whether time-lapse data correlate with euploid status or blastocyst formation, as these factors may serve as confounders for MC.

Materials and methods

We conducted a prospective study examining the relationship of cleavage timing and parameters on MC and chromosomal status in embryos. This study received IRB approval by the University of Southern California (HS-16-00249).

Patient selection

We selected 57 embryos from a total of five different patients that had previously undergone in vitro fertilization at the USC Fertility clinic. Embryos had been previously cryopreserved and subsequently donated to research after patients had successful pregnancies by fresh or frozen transfer, and did not wish to have more children. Consents were signed for donation of embryos to research.

Embryo culture

All embryos were previously cryopreserved using the slow-freeze technique with 1.5 M propylene glycol (PROH) and 0.1 M sucrose (Irvine Scientific, Santa Ana, CA). Embryos were thawed using Irvine Scientific Embryo Thaw Kit (Santa Ana, CA) by serial dilutions of cryoprotectant. After the thaw, individual embryos were placed in labeled wells corresponding to their study ID in the Miri Time-Lapse incubator (Esco Medical, Singapore). Fifty-seven embryos survived the thaw and were cultured in the Esco Miri Time-Lapse incubator (Esco Medical ApS, Denmark). All embryos were grown in continuous single culture media (CSC, Irvine Scientific, Santa Ana, CA) using TL micro-dish (CultureCoin, Esco Medical) overlay with LiteOil (Life Global) at 37 °C, with 6% oxygen and 6% carbon dioxide according to our standardized embryology laboratory protocol. Time-lapse images were collected every 5 minutes, while embryos were kept in the incubator. Embryos were removed from the incubator twice, once for blastomere biopsy (day 3) and the second time for trophectoderm biopsy of all blastocysts on day 6.

Morphologic assessment

During the cleavage stage, on day 3 of embryo development, a morphologic score was assigned based on a three-point grading system using morphological features including cell number, fragmentation, symmetry, and shape [15]. Embryos were given a score of good if they had ≥ 8 cells, < 10% fragmentation, and perfect symmetry. Fair embryos were defined as having four to eight cells, 11–25% fragmentation, and moderate asymmetry. Poor embryos were defined as having < 4 cells, > 25% fragmentation, and severe asymmetry.

At the blastocyst stage, morphologic score was based on expansion stage, quality of inner cell mass (ICM), and quality of trophectoderm (TE) [16]. Embryos were given a score of 1–4 based on their developmental stage: early blastocyst (1), blastocyst (2), expanded blastocyst (3), and hatched/hatching (4). The inner cell mass was given a grade of A–C based on their appearance of cellularity and how well they were adhered together. The trophectoderm was also given a score of A–C based on the number of cells and how adherent they were. Good embryos were classified as having and ICM and

TE scores AA and AB. Fair embryos had scores of BA, BB, or BC. Poor embryos were designated as CB or CC. One embryologist graded all embryos in order to maintain consistency of results.

Time-lapse parameters

Time-lapse images were recorded every 5 min. A single embryologist retrospectively analyzed data and marked relevant parameters. While we were unable to time events in hours post-injection, we set time 0 as pronuclear breakdown to standardize our analysis as the data for the number of hours post-retrieval was not available. We assumed that PN fading occurred at approximately 24 h of embryo life post-fertilization, based on previous studies [14]. The parameters included time in hours to reach two cells (t2), three cells (t3), four cells (t4), five cells (t5), six cells (t6), seven cells (t7), eight cells (t8), time to compaction (tc), time to morula formation (tm), time to early blast formation (tb_e), time to blast formation (tb), and time to expanded blast formation (tb_{ex}). Additionally, relevant kinetic parameters were calculated based on the cell division time parameters recorded. CC2 represents the timing of the second cell cycle or also the time it takes to get from a two-cell to three-cell embryo. CC3 represents the timing of the third cell cycle, or the time it takes to get from a three-cell to a five-cell embryo. S2 is the time of synchrony of the second cell cycle (time it takes to get from two-cell to four-cell stage). S3 is the time of synchrony of the third cell cycle (time it takes to get from a four-cell to eight-cell stage embryo) [17].

Embryo biopsy

On day 3, cleavage-stage embryos were removed from the incubator, and single blastomere biopsies were performed. Embryos were then returned to the incubator until day 6. On day 6, blastocysts underwent trophectoderm biopsy and three to five cells were cut using Lykos laser system (Hamilton Thorne, Beverly, MA). After blastocyst biopsy, the remainder of each blastocyst plus all arrested embryos were collected and stored at − 30 °C until all samples had been collected.

Preimplantation genetic screening and mitochondrial measurement

Day 3 blastomere biopsies, day 6 trophectoderm biopsies, and whole embryos (regardless of whether or not embryos were arrested or became blastocysts) were sent for next-generation sequencing (NGS). Samples were subject to cell lysis followed by whole genome amplification (WGA) using Picoplex (Rubicon, Inc). During this WGA process, mtDNA and nDNA were randomly and simultaneously amplified. All chromosomal analysis was performed using an NGS platform conducted by Progenesis Inc. (La Jolla, CA). WGA products

were quantified using Qubit dsDNA HS Assay (Life Technologies), and 300 ng of whole genome DNA was subject to fragmentation and adaptor ligation. The pool of libraries was quantified, and 26pM was used towards emulsion PCR for template enrichment on ion sphere using OT2 200 Kit (Life Technologies). The enriched library was then further purified to remove non-templated spheres, using Ion One Touch™ ES Enrichment. The final library was loaded on a 318 chips and sequenced using Ion PGM Sequencing 200 Kit v2 (Life Technologies).

Each sample had an average of 150,000 to 200,000 reads and approximately 200 bp size per amplicon, totaling 30 million to 40 million base pairs per sample. All reads were filtered for polyclonals, aligned to the human genome database using Torrent Suite™ Software for sequencing data analysis. Quality reads were scored for aneuploidy using Ion Reporter™ software (Thermo Fisher Scientific). For quantification of mitochondrial copy number quantification, the ratio of mtDNA to nDNA is multiplied by the total sequence read number. There was an average of 100,000 sequence reads for mtDNA per sample. MtDNA quantification was performed according to the Thermo Fisher Scientific protocol and has been validated by this manufacturer using the reference mitochondrial sequence NC_012920 [18, 19].

MtDNA and nDNA sequences were aligned to the human genome and filtered for quality using Ion Torrent™ software. Chromosome copy and ratio of mitochondrial vs. nuclear sequences were calculated using Ion Reporter™ software (Thermo Fisher Scientific). The calculation of mitochondrial copy number takes into account autosome reads and not sex chromosomes; thus, a correction factor was implemented for any autosomal aneuploidy. We implemented the following correction for mitochondrial measurement to adjust for aneuploidy. All autosomal chromosome aneuploidies were weighted according to chromosome size, for example, chromosome 22 represents 2% of all chromosomes, so trisomy 22 or monosomy 22 will introduce 2% error. So in a sample with trisomy 22, the nDNA would increase by 2% and the ratio of mtDNA to nDNA would decrease by 2%. Since reads are randomly distributed across all chromosomes, we used the weight of each chromosome to estimate errors attributed to each autosomal monosomy and trisomy. If there was more than one aneuploidy, the sum of corrections for each was used to account for final mtDNA to nDNA ratio. (See Supplemental Table 1).

Statistical analysis

Mitochondrial content (MC) was defined as the ratio of mitochondrial to nuclear DNA as assessed by NGS, and treated as a continuous variable. We compared the median MC of embryos between day 3 blastomere biopsies and day 6 whole embryos using Wilcoxon matched pair signed-rank test. We

used day 6 whole embryos in lieu of trophectoderm biopsies as there were a few values from the trophectoderm biopsies that were not quantifiable (poor-quality DNA read). Additionally, there was no significant difference in median values between the two groups, suggesting a near perfect concordance (see "Results" section). We then evaluated the relationship between MC and each of the time-lapse morphokinetic parameters using linear regression. TLM parameters were treated as continuous variables (hours). In a subanalysis evaluating TLM parameters, we categorized MC values into quartiles and examined differences in TLM parameters between the 25th and 75th percentile categories. We used Kruskall-Wallis to determine the relationship between MC and chromosomal status. Chromosomal status was treated as a dichotomous variable defined as euploid or aneuploid. We defined chromosomal status based on the ploidy call using all day 6 whole embryos. We used the Kruskall-Wallis test to analyze the relationship between MC and blastocyst formation and morphology grading. Blastocyst formation was treated as a dichotomous variable of blastocyst formed vs. embryo arrested in the cleavage or morula stage. Morphologic grading was assessed on day 3 and day 6 of embryo life, and was made into a dichotomous variable of good/fair vs. poor. Wilcoxon rank sum was used to compare medians of each time-lapse parameter between dichotomous outcomes, including euploid vs. aneuploid embryos, good/fair vs. poor embryos, and blastocysts vs. arrested embryos. We performed an additional analysis to analyze the effect of blastocyst expansion (degree of cellularity) on mtDNA content. We used ANOVA, with mtDNA as the dependent variable. Covariates included morphology of the ICM and TE as well as the degree of cellularity in the blastocyst stage as defined by early blastocyst (1), blastocyst (2), expanded blastocyst (3), and hatched/hatching (4). We also performed a linear regression to measure the relationship between day 6 MC and cellularity in all blastocysts. ANOVA was also used to examine the relationship between maternal age and mitochondrial DNA content on day 3 and day 6. All p values were based on two-tailed tests, with statistical significance indicated by $p < 0.05$. STATA 13 (StataCorp, College Station, TX, USA) was used for all analyses.

Results

We thawed 57 embryos at pronuclear stage from five different patients. The median age of patients with available data was 39.1 ± 2.3 years (34–40 years). Embryos were cryopreserved for a median duration of 87.7 ± 28.4 months (51.5–111.2 months). Fifty out of 57 (88%) embryos survived the thaw process. Out of these 50 embryos, 32 (64%) progressed to the blastocyst stage. After analysis with NGS, 35/50 (70%)

embryos were determined to be euploid and 15/50 (30%) were aneuploid. Five of 15 (33.3%) aneuploid embryos had complex aneuploidy, meaning more than one chromosome was affected by monosomy and trisomy. Of note, there was a significant correlation between blast formation and euploid status, with 28/32 (87.5%) of blastocysts being euploid and 11/18 (61.1%) of arrested embryos being aneuploid ($p < 0.001$). (See Supplemental Table 2 showing classification of aneuploidies on day 3 and day 6).

MC in day 3 vs. day 6 embryos

When examining the difference in MC between whole day 3 and day 6 embryos, day 6 embryos had significantly lower MC, with a median ratio of 0.08 on day 3 vs. 0.001 on day 6, $p < 0.001$. Three embryos had an increase in MC from day 3 to day 6. They represented aneuploid embryos that arrested prior to compaction, so the higher MC on day 6 was likely due to degradation of nDNA. When excluding embryos that arrested prior to the blastocyst stage, there was still a significant difference in MC between day 3 and day 6 (0.009 vs. 0.0008, $p < 0.001$) (see Fig. 1 for box plot distribution of day 3 vs. day 6 MC by ploidy and blastulation status, Fig. 2 for line plot showing trend for MC values between day 3 and day 6 for individual embryos, and Supplemental Fig. 1 for histogram plot showing distribution of values for day 3 and day 6 MC.)

MC in trophectoderm biopsies vs. whole blastocysts

There was no significant difference in day 6 MC between trophectoderm biopsies and whole blastocysts. Median MC in trophectoderm biopsies vs. whole blastocysts was 0.00079 ± 0.001 vs. 0.0008 ± 0.0004, $p = 0.88$. Given that there was no significant difference, we used MC from whole blastocysts for any analysis using day 6 MC.
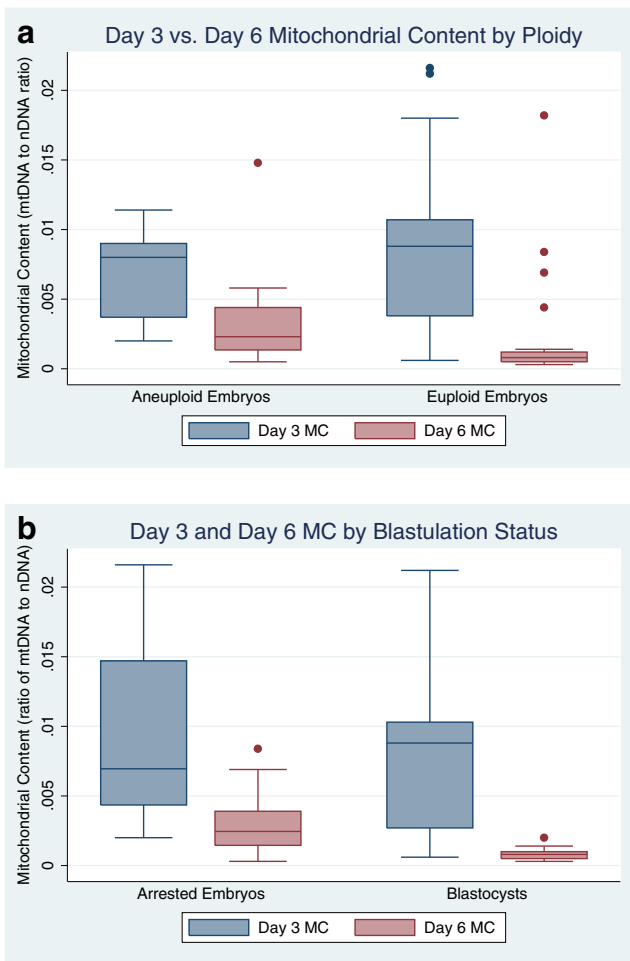
Euploid vs. aneuploid embryos

When comparing day 3 MC in whole euploid vs. aneuploid embryos, there was no significant difference (0.009 vs. 0.008, $p = 0.51$). However, there was a difference in day 6 MC in euploid vs. aneuploid embryos. In those reaching the blastocyst stage, euploid embryos had a significantly lower MC on day 6 (0.0008 vs. 0.002, $p = 0.003$). (See Table 1 for median values with interquartile ranges and Fig. 1 for visual distribution of MC on day 3 and day 6 by ploidy status).

### Blastocysts vs. arrested embryos

In embryos that reached the blastocyst stage, median day 3 MC was lower than that of embryos that eventually arrested, but this was not statistically significant (0.009 vs. 0.008, $p = 0.75$). There was a significantly lower MC on day 6 in

Fig. 1 Boxplots comparing MC on day 3 and day 6 by **a** ploidy status and **b** blastulation status. Note higher values in aneuploid and arrested embryos

blastocysts vs. arrested embryos (0.0008 vs. 0.004, $p < 0.001$). When comparing euploid and aneuploid blastocysts, euploid blastocysts had lower day 6 MC (0.008 vs. 0.0015), but this
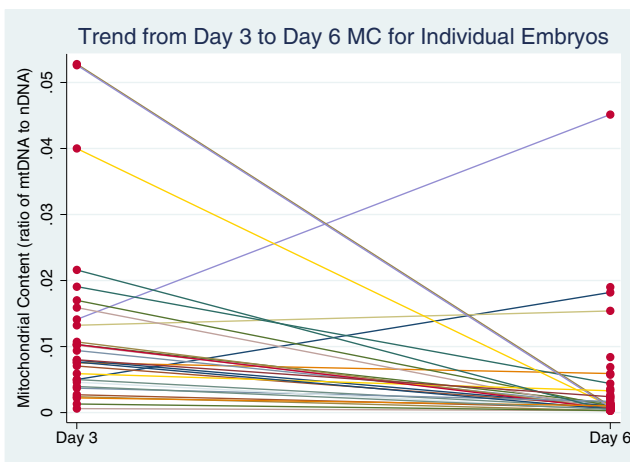
Fig. 2 Graph comparing day 3 and day 6 MC for individual embryos. Each line connects the day 3 MC value with the corresponding day 6 MC value for that particular embryo

was not significantly different ($p = 0.14$). We further stratified blastocysts and arrested embryos into euploid and aneuploid groups. In euploid blastocysts vs. euploid arrested embryos, day 6 MC was significantly lower in blastocysts ($p = 0.006$). There was no significant difference in day 6 MC when comparing aneuploid blastocysts and aneuploid-arrested embryos ($p = 0.12$). (See Table 1 for median values with interquartile ranges and Fig. 1 for visual distribution of MC on day 3 and day 6 by blastulation status).

Time-lapse parameters

When examining time-lapse parameters, individual parameters for cell division (t2 through t9) did not correlate with MC load on either day 3 or day 6. Of those that did make it to the blastocyst stage, we performed a subanalysis of time-lapse parameters with quartiles of day 6 MC. Day 6 MC values for the 25th, 50th, and 75th percentiles were 0.0005, 0.0008, and 0.001. Comparing time to blastulation between embryos with MC in the 25th and 75th percentile, those with 75th percentile MC took 12.45 hours longer to form blastocysts vs. those in the 25th percentile, beta = 12.45, $p < 0.001$. When examining mtDNA concentrations as a continuous variable in blastocysts, there was also a positive correlation between blastulation timing and higher MC on day 6 ($p < 0.001$). This relationship held true when looking at all embryos as well as only euploid embryos. See Fig. 3 for relationship between day 6 MC and time to blastulation in euploid embryos.

When examining cleavage events CC2, CC3, S2, and S3, no significant relationship was found with MC in day 3 or day 6.

Time-lapse and ploidy status

We also analyzed time-lapse parameters and their ability to predict euploid status. Individual time-lapse parameters, as well as early cleavage timing, did not predict euploid status (i.e., CC2, CC3, S2, or S3). However, we did note that cells that were aneuploid were more likely to arrest at the eight-cell stage or prior to compaction and euploid embryos were more likely to progress to blastocysts. (See Supplemental Table 3 for analysis of TLM parameters and chromosomal status).

Abnormal cleavage events

We had a subset of embryos with abnormal cleavage events during the time-lapse monitoring. The abnormal cleavage (AC) event was defined as the division from a mother blastomere cell to three daughter cells. AC events were seen in 15/50 (30%) of embryos. Eight of 15 (53.3%) embryos experienced an AC after the first cytokinesis. Seven of 15 (46.7%) embryos had ACs after the second cytokinesis. Aneuploid embryos had a significantly higher rate of AC events (73.3

Table 1   Comparison of day 3 and day 6 mitochondrial content (MC) by chromosomal and blastulation status

	Euploid embryos[a,b] ($n = 35$)	Aneuploid embryos[a,b] ($n = 13$)	p value[c]
Day 3 median MC (25th–75th %ile)	0.009 (0.005–0.17)	0.008 (0.004–0.013)	0.51
Day 6 median MC (25th–75th %ile)	0.0008 (0.0005–0.0012)	0.0024 (0.0017–0.0057)	0.003
	Blastocysts[a,b] ($n = 32$)	Arrested embryos[a,b] ($n = 18$)	p value[c]
Day 3 median MC (25th–75th %ile)	0.009 (0.004–0.01)	0.008 (0.005–0.14)	0.75
Day 6 median MC (25th–75th %ile)	0.0008 (0.005–0.001)	0.004 (0.002–0.008)	< 0.001
	Euploid blastocysts[a,b] ($n = 28$)	Aneuploid blastocysts[a,b] ($n = 4$)	p value[c]
Day 6 median MC	0.0008 (0.0005–0.001)	0.0015 (0.0007–0.0039)	0.14
	Euploid blastocysts[a,b] ($n = 28$)	Euploid-arrested embryos[a,b] ($n = 7$)	p value[c]
Day 6 median MC	0.0008 (0.0005–0.001)	0.0007 (0.0012–0.0182)	0.006
	Aneuploid blastocysts[a,b] ($n = 4$)	Aneuploid-arrested embryos[a,b] ($n = 9$)	p value[c]
Day 6 median MC	0.0015 (0.0007–0.0039)	0.0027 (0.002–0.006)	0.12

[a] Values displayed are mitochondrial content (MC), defined as the ratio of mitochondrial to nuclear DNA

[b] Values depicted as medians (25th–75th percentile)

[c] Wilcoxon rank sum used for analysis, $p < 0.05$ considered significant

vs. 22.9%, $p < 0.001$). There was no difference in AC events between arrested embryos vs. those that progressed to the blastocyst stage. AC events did not correspond with cleavage-stage morphologic scoring, but were associated with poor morphology at the blastocyst stage vs. good/fair embryos (66.7 vs. 33.3%, $p = 0.03$). When examining TLM parameters, embryos with at least one AC took an average of 10.7 hours longer to develop from a morula to a fully expanded blastocyst compared to those without ACs (beta = 10.7, $p = 0.01$). Lastly, AC events did not correspond to MC on day 3 or day 6.

Morphology

On day 3, 1/50 (2%) embryos had a score of good, 40/50 (80%) of embryos had a score of fair, and 9/50 (18%) had a
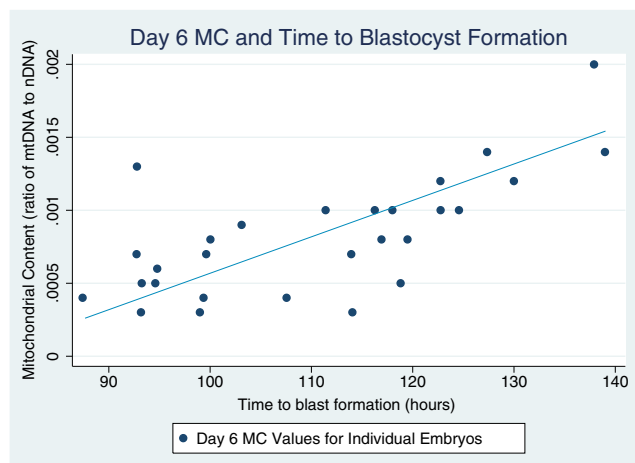
Fig. 3 Scatterplot of day 6 MC values plotted by time to blast formation. Mitochondrial content (MC) increases as the number of hours needed to reach blastocyst stage increases. Each circle represents an individual embryo's MC ratio. A line has been superimposed to demonstrate the trend in MC value as it relates with the number of hours taken to reach the blastocyst stage. $R^2 = 0.49$, $p < 0.001$

score of poor. Morphologic grading scores were not correlated with MC on either day 3.

On day 6, 18/50 (36%) embryos were arrested and 32/50 (64%) of embryos reached the blastocyst stage. None of the 32 (0%) embryos had a score of good. Eighteen of 32 (56.3%) had a score of fair, and 14/32 (43.7%) of embryos had a score of poor. There was no association between day 6 MC and morphologic score when defined dichotomously and good/fair vs. poor in embryos reaching the blastocyst stage.

In the model used to examine the degree of cellularity as well as quality of ICM and TE, there was no significant association with these parameters and day 6 MC. When performing linear regression to examine the relationship between day 6 MC and cellularity alone, we found that a higher degree of cellularity corresponded with a lower concentration of day 6 MC, which trended towards significance (beta = $-$ 0.0002, $p = 0.06$). Median values of day 6 MC for early blastocysts, blastocysts, and expanded blastocysts were 0.001, 0.0007, and 0.0005. See Fig. 4 demonstrating day 6 MC levels by degree of blastocyst expansion.

Age and mitochondrial DNA

We had data on maternal age for 28 embryos (56%). Maternal ages for these donated embryos were 34, 38, and 40. Using one-way ANOVA, we found no relationship between maternal age and day 3 mitochondrial content ($p = 0.41$) or day 6 mitochondrial content ($p = 0.98$).

Discussion

In our study, we evaluated whether or not TLM parameters would be helpful in prediction of differences in MC among
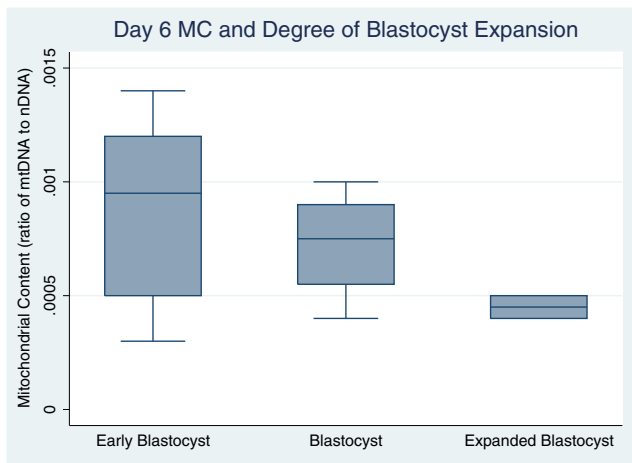
Fig. 4 Boxplot showing distribution of day 6 MC by degree of blastocyst expansion

embryos. Our results showed that MC was approximately 10-fold lower on day 6 as compared to day 3 in embryos reaching the blastocyst stage. This difference was less dramatic in more slowly developing embryos, as arrested embryos and those with delayed blastulation had higher MC.

During early stages of embryogenesis, new mitochondrial units are not typically synthesized. Therefore, the number of mitochondrial units per cell actually decreases during mitochondrial segregation that occurs with cell division [7, 8]. Since nuclear DNA continues to replicate during preimplantation embryonic mitosis, the result is a decline in the mitochondrial to nuclear DNA ratio per cell over time. In developmentally delayed embryos, cells may divide more slowly while the cell undergoes repair of nuclear or mitochondrial DNA mutations. Campbell et al. demonstrated this when they reported delayed rates of blastulation in aneuploid embryos [14]. They postulated that temporal delay seen in aneuploid embryos might be due to the activation of error detection and DNA repair mechanisms for mitotic errors, which may then delay cytokinesis [14]. Similarly, our study found that embryos that took longer to become blastocysts had higher MC on day 6. Additionally, in our cohort of embryos reaching the blastocyst stage, aneuploid vs. euploid embryos took a median time of 118.8 vs. 107.8 hours to become blastocysts. The combination of delayed blastulation timing corresponding to higher MC on day 6 is consistent with our theory that slow nuclear DNA replication leads to a higher ratio of mtDNA to nDNA in more slowly growing embryos. It is reasonable to extrapolate that these blastocysts are of lower quality. Thus, lower MC at the blastocyst stage in combination with shorter blastulation timing may serve as indicators of better embryo quality.

We also examined early cleavage parameters CC2, CC3, S2, and S3 in addition to standard morphokinetic parameters and their relationship with ploidy status, blastulation, and MC. A study by Chavez et al. showed differences in parameters between euploid and aneuploid embryos, including time to division, length of cell cycle, and interval between cell division from the two- to four-cell stage [20]. Data suggests that the embryonic nuclear genome is activated after the eight-cell stage and that early cleavage patterns may predict blastocyst potential and euploid status, both of which are markers of better embryo quality [20, 21]. In our study, early cleavage parameters CC2, CC3, S2, and S3 did not correlate with euploid or aneuploid status, and did not reliably predict which embryos progressed to the blastocyst stage. This may have been due to the variability in cell division parameters between embryos or a small sample size. Other than time to blastocyst formation, there was no relationship between parameters and MC at the cleavage or blastocyst stage.

Changes in MC over time have been studied in growing oocytes of other mammalian species, but there are limited longitudinal data in human embryos. The primary follicle contains 10,000 copies of mitochondria, which increases to 50,000–1,500,000 in a mature oocyte [22, 23]. After fertilization, mtDNA replication is suppressed until the blastocyst stage, when mitochondria production increases in the trophoblast cells [24]. Nuclear gene expression for mitochondrial transcription factors is upregulated in morula and blastocyst embryos thus enabling mitochondrial replication to prepare for implantation [25]. While all mtDNA required for energetic processes is typically derived from the ooplasm, embryos with significant oxidative stress or severe respiratory chain defects serve as an exception. In a study by Sonnet et al., embryos with mtDNA mutations had significantly higher mtDNA compared with controls and this continued throughout embryo development to the blastocyst stage [26]. They also found a correlation between mtDNA mutation load and mtDNA copy number. The authors suggest that this indicates that embryos possess the ability to replicate mtDNA in situations of embryonic distress.

The clinical data for the utility of mitochondrial DNA in embryo selection is contradictory. Diez-Juan et al. report a correlation between lower MC in euploid embryos and implantation potential, and have cited a threshold above which embryos fail to implant [6]. Fragouli et al. also discovered a relationship between MC and implantation rates, reporting that embryos with lower MC are more likely to implant. They also reported higher MC in older women and aneuploid in comparison to euploid embryos [5]. The theory for the relationship between MC and implantation potential was that embryos with a higher MC were likely in a state of respiratory stress, leading to increased synthesis of mitochondria in early embryo development [6]. In contrast to these studies, Victor et al. reported no relationship between mitochondrial DNA with maternal age, euploid status, or implantation potential, citing that there is variability in the measurement of MC when considering aneuploidy and embryo sex [27]. After using a correction factor to control for these two factors, the MC was

not found to be predictive of embryo implantation. We have implemented a correction factor to correct for autosomal aneuploidy, and even after correction, our results still suggest that higher quality embryos have lower MC at the blastocyst stage. Additionally, Treff et al. published a study on mtDNA and its predictive value for reproductive potential in a sibling embryo model [28]. They found that there was no difference in mtDNA when comparing embryos that implanted vs. those that did not implant [28]. They concluded that mtDNA quantification did not improve embryo selection much more than what using euploid status and morphology status for any given patient. Overall, blastocyst stage morphology has been found to be predictive of implantation and live birth rate in the literature [29]. When examining morphology in our cohort of embryos, we did not find a correlation between MC and morphologic grading in the cleavage stage, consistent with findings from Lin et al.'s study [30]. Additionally, our data did not show a relationship between day 6 MC and blastocyst morphologic grade when defined dichotomously as fair/poor. When we examined the relationship between day 6 MC and relative cellularity as determined by degree of blastocyst expansion, we found a lower MC in blastocysts with higher cellularity, which is consistent with the hypothesis that more cell division events lead to a dilutional effect on relative mtDNA in a sample. Treff et al. found significant differences in mtDNA by blastocyst morphology and expansion [28], which is consistent with this theory. We believe that our findings may not show statistical significance due to the small sample size of 32 blastocysts. While blastocyst expansion and morphology are highly useful for embryo selection, having information regarding MC and time to blastulation may still serve as helpful methods to differentiate between blastocysts that may be of similar quality based on morphology.

In our study, there was no difference in MC when comparing cleavage-stage euploid and aneuploid embryos. There was also no difference in day 3 MC between embryos that reached blastocyst stage and those that arrested. There was however a significant difference in day 6 MC for euploid vs. aneuploid embryos and blastocysts vs. arrested embryos. Given that differences in MC are not seen until day 6, it is unlikely that mitochondrial replication increased in response to increased embryonic stress seen in aneuploidy. While our study does not address implantation potential of embryos based on MC, we have found that cell division timing, particularly blastulation timing in euploid embryos, contributes to differences seen in mitochondrial content between embryos. While euploid status and morphology may be the current methods for selecting the best embryos for transfer, utilizing other factors such as cell division timing (time to blastulation) and mtDNA may serve as adjunctive tools for selecting a single embryo for transfer while improving ART outcomes. Further clinical studies are needed to validate this model as an embryo selection tool.

Strengths and limitations

To our knowledge, this is the first study to establish MC load in the same embryo in the cleavage and blastocyst stage. We were able to correlate these with time-lapse morphokinetic parameters and chromosomal status. Limitations of this study include a small sample size and the use of cryopreserved embryos, limiting the generalizability of our findings. We also did not have data for maternal age for 22 donated embryos; however, analysis of other embryos revealed no differences in MC on day 3 or day 6 by maternal age. Given that these were research embryos, we were unable to correlate our findings with clinical outcomes such as implantation, clinical pregnancies, or live births. Additionally, time-lapse incubators are not universally used in IVF centers, limiting the findings to only those with the capability of recording TLM parameters. With respect to mtDNA measurement, we did not use the same correction factor published by Victor et al., as their mtDNA quantification method incorporated sex chromosome into the calculation, whereas our method utilizes autosomal chromosomes for calculation of MC [27]. Thus, sex would not factor in calculating ratio of mtDNA to nDNA for quantification of mitochondrial copy number when using the method as described earlier in this paper [18].

Conclusions

Based on our study, a lower MC on day 6 reflects better embryo quality, as it is associated with euploid status and time to blastocyst formation, which is consistent with previous studies. Euploid embryos that take longer to form blastocysts may be of lower quality given their higher day 6 MC. Higher MC in aneuploid and arrested embryos may be explained by slower cell division or degradation of genomic DNA over time. TLM parameters, particularly time to blastulation, may be helpful for selection of higher quality embryos. These findings suggest that aneuploid and poor-quality embryos may have defects in cell division, leading to a higher mtDNA to nDNA ratio. Slower cell division may be attributed to cell mechanisms devoted towards meiotic or post-mitotic errors, which remains to be explored in future studies.

In patients over 35 years old, live birth rates per cycle for women over 35 in the USA range from 3.9 to 42% [31]. The selection of an embryo with the highest implantation potential is necessary and may require a multidimensional approach. The current work is the first attempt to combine time lapse, mitochondrial measurement, aneuploidy screening, and embryo grading system as a first step towards a multidimensional assessment of embryo implantation potential.

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