Mutation dependance of the mitochondrial DNA copy number in the first stages of human embryogenesis

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Mitochondrial DNA (mtDNA) content is thought to remain stable over the preimplantation period of human embryogenesis that is, therefore, suggested to be entirely dependent on ooplasm mtDNA capital. We have explored the impact of two disease-causing mutations [m.3243A>G myopathy, encephalopathy, lactic acidosis and stroke-like syndrome (MELAS) and m.8344A>G myoclonic epilepsy associated with ragged-red fibers (MERRF)] on mtDNA amounts in human oocytes and day 4–5 preimplantation embryos. The mtDNA amount was stable in MERRF and control materials, whereas gradually increasing from the germinal vesicle of oogenesis to the blastocyst stage of embryogenesis in MELAS cells, MELAS embryos carrying ~3-fold higher mtDNA amount than control embryos (P = 0.0003). A correlation between mtDNA copy numbers and mutant loads was observed in MELAS embryos (R2 = 0.42, P < 0.0013), suggestive of a compensation for the respiratory chain defect resulting from high mutation levels. These results suggest that mtDNA can replicate in early embryos and emphasize the need for sufficient amount of wild-type mtDNA to sustain embryonic development in humans.

INTRODUCTION

Mitochondrial DNA (mtDNA) is a maternally inherited small circular molecule located in the mitochondria. It is entirely dedicated to synthesis of 13 subunits of the enzymatic complexes of the respiratory chain. The mtDNA content has been shown to remain stable during the first days of embryonic development until mtDNA replication resumes after in utero implantation in many species. including frog (1), mouse (2–4) and cattle (5). In mice, some degree of mtDNA synthesis would potentially operate in the early embryo, compensating for the degradation of mtDNA molecules and, therefore, maintaining total mtDNA amount (6), feature playing a critical role in mouse early embryonic development (7).

The rare data available in humans also suggest that mtDNA amounts remain stable during the first 3 days of embryonic development. Indeed, despite strong interindividual variations, embryonic mtDNA content at the 8-cell stage is largely similar to that of unfertilized human oocytes (8–13). Early embryonic development is, therefore, thought to be entirely dependent on ooplasm mtDNA capital. In keeping with this, fertilization failures (9,12) and impaired post-implantation embryonic development (7) have been ascribed to qualitative or quantitative mtDNA defects in mouse oocytes.

On the other hand, mechanically and pharmacologically induced mtDNA depletion spontaneously corrected in cattle and pig early embryos, suggesting that an adaptive mechanism
could occur through activation of mtDNA replication (14,15). Yet, whether qualitative mtDNA defects impact mtDNA replication and early human embryonic development remains unknown.

In the context of preimplantation genetic diagnosis (PGD) procedure offered to couples at risk to transmit a mtDNA mutation, we have assessed the mtDNA content of normal human oocytes and embryos and compared it to mutant counterparts carrying either the \textit{MT-TL1} m.3243A > G mutation responsible for mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS syndrome, MIM 540000) or the \textit{MT-TK} m.8344A > G mutation causing myoclonic epilepsy associated with ragged-red fibers (MERRF syndrome, MIM 590060). Here, we show a significant increase in the mtDNA copy number in embryos carrying a mtDNA mutation versus controls, suggesting that human preimplantation embryos have the intrinsic ability to adapt their mtDNA content in response to impaired respiratory capacities triggered by high mtDNA mutant loads.

RESULTS

Mutant oocytes and embryos were retrieved from two unrelated individuals, heteroplasmic for the \textit{MT-TL1} m.3243A > G MELAS mutation (20% in blood, 30% in urinary tract and buccal cells, patient 1) and the \textit{MT-TK} m.8344A > G MERRF mutation, respectively (75% in blood, 85% in urinary tract cells and 90% in buccal cells, patient 2) (16,17). A large spectrum of mtDNA copy number was observed in control oocytes collected at various maturation stages, including germinal vesicle (GV) (n = 3), metaphase I (MI) (n = 7) and metaphase II (MII) (n = 9) oocytes (Fig. 1). The average mtDNA amount decreased as maturation stage proceeded (Fig. 2). The difference between the GV and MII stages was significant (P-value = 0.036, Wilcoxon test). In contrast, no difference in mtDNA amount between control oocytes and fertilized embryos was noted (P = 0.16). When embryos were ordered according to the developmental stage, no significant difference in mtDNA amount (P = 0.37) was observed between pre-blastocyst (mean $2.4 \times 10^5$, ranging from $10^4$ to $7.6 \times 10^5$) and blastocyst embryos (mean $2.2 \times 10^5$, ranging from $1.1 \times 10^5$ to $3.6 \times 10^5$).

Interestingly, mtDNA copy number in m.3243A > G oocytes (n = 12, Supplementary Material, Table S1) was significantly lower than in control oocytes (P = 0.016), whereas m.3243A > G embryos (n = 12, Supplementary Material, Table S1) carried significantly more mtDNA copy number.

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Mean copy number</th>
<th>SD</th>
<th>Median copy number</th>
</tr>
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<td>Control oocytes</td>
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<td>MELAS oocytes</td>
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<td>127061</td>
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<td>MERRF oocytes</td>
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<tr>
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<td>12</td>
<td>220381</td>
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</tbody>
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than controls (2.7-fold higher, $P = 0.0003$, Fig. 1). The average mtDNA copy number was, therefore, 4-fold higher in m.3243A$\rightarrow$G embryos than in m.3243A$\rightarrow$G oocytes ($P = 0.0001$). To investigate the time course of mtDNA copy number variation following fertilization, m.3243A$\rightarrow$G oocytes and embryos were ordered according to their developmental stages (Fig. 2). A gradual increase in mtDNA amount from the GV to the blastocyst stage occurred during mental stages (Fig. 2). A gradual increase in mtDNA amount, from the GV to the blastocyst stage occurred during mental stages (Fig. 2). The mean value for each stage is indicated by a dash.

DISCUSSION

Here, we report on similar mtDNA amounts in control oocytes and embryos. To our knowledge, this is the first report simultaneously quantifying mtDNA amount in human oocytes and embryos. A large spread of copy number in control oocytes and embryos was observed, similar to the ranges reported in previous studies focusing on either human oocytes (50 000–1 500 000) (8,9,11–13) or embryos (170 000–1 500 000) (10,18). In our series, similarity of the mtDNA content between control oocytes and fertilized embryos does not advocate any relationship between the mtDNA copy number and the capacity of an oocyte to fertilize or not and of an embryo to develop until day 5 or not (Fig. 2). Most interestingly, we show that the m.3243A$\rightarrow$G MELAS mutation resulted in a gradual increase in mtDNA amount, from the GV to the blastocyst stage (Fig. 2).

The increase in mtDNA amount in m.3243A$\rightarrow$G embryo could be regarded as an adaptive mechanism to the respiratory deficiency induced by high mutant levels. Consistently, we found a significant correlation between the mutant load and the mtDNA amount in oocytes and embryos. A significant correlation was found between these two parameters in m.3243A$\rightarrow$G embryos plus control embryos ($R^2 = 0.47$ for a linear fit, $P < 0.0001$, Fig. 4), whereas no significant correlation was observed in m.8344A$\rightarrow$G embryos ($P = 0.99$, Fig. 4), m.3243A$\rightarrow$G oocytes ($P = 0.18$, Data not shown) or m.8344A$\rightarrow$G oocytes ($P = 0.37$, Data not shown).

![Figure 2](image-url)  
**Figure 2.** mtDNA amount during late oogenesis and preimplantation development in MELAS m.3243A$\rightarrow$G oocytes and embryos. The mtDNA contents of m.3243A$\rightarrow$G (red) oocytes and embryos and in control oocytes and embryos (black) are plotted against corresponding stages of development. GV, germinal vesicles; MI, metaphase I; MII, metaphase II; PreBlast, pre-blastocyst; Blast, blastocyst. The mean value for each stage is indicated by a dash.

![Figure 3](image-url)  
**Figure 3.** mtDNA mutant loads during late oogenesis and preimplantation development in MELAS m.3243A$\rightarrow$G and MERRF m.8344A$\rightarrow$G oocytes and embryos. The mtDNA mutant loads in m.3243A$\rightarrow$G (black symbols) and m.8344A$\rightarrow$G oocytes and embryos (green symbols) are plotted against corresponding stages of development. Uterine transferred embryos ($n = 6$) are not shown. GV, germinal vesicles; MI, metaphase I; MII, metaphase II; PreBlast, pre-blastocyst; Blast, blastocyst. The mean value for each stage is indicated by a dash.
shows a correlation coefficient between mtDNA copy number and heteroplasmy level in MELAS m.3243A>G and MERRF m.8344A>G embryos. Total (A) and wild-type (B) mtDNA copy numbers are plotted against mutant levels in control (black), m.3243A>G (red) and m.8344A>G (green) preimplantation embryos. The regression line (A) fitting both m.3243A>G and control data shows a correlation coefficient $R^2$ of 0.47 ($P < 0.0001$). Uterine transferred embryos ($n = 6$) were not available for mtDNA copy number assessment and are, therefore, not shown in the figures.

Figure 4. Relationship between mtDNA copy number and heteroplasmy level in MELAS m.3243A>G and MERRF m.8344A>G embryos. Total (A) and wild-type (B) mtDNA copy numbers are plotted against mutant levels in control (black), m.3243A>G (red) and m.8344A>G (green) preimplantation embryos. The regression line (A) fitting both m.3243A>G and control data shows a correlation coefficient $R^2$ of 0.47 ($P < 0.0001$). Uterine transferred embryos ($n = 6$) were not available for mtDNA copy number assessment and are, therefore, not shown in the figures.

was found to be comparable to the copy number in controls (mean 241 963), irrespective of the mutant loads (Fig. 4B). Taking into account these data, one can hypothesize that a sufficient amount of wild-type mtDNA is necessary to rescue from energy deficiency induced by the m.3243A>G MELAS mutation in the developing embryo. If this were the case, why would a relatively lower copy number be consistent with normal oocyte maturation? A likely explanation is that energy demand in oocytes is markedly lower than in embryos and that fertilization triggers the transition from a relatively quiescent cell into an actively dividing embryo (19). Alternatively, the increased mtDNA amount observed in m.3243A>G embryos could be related to the exact nature of the molecular defect, rather than a general problem with aerobic energy metabolism because before implantation, the embryo would find itself in a relatively hypoxic environment and may not rely to a great degree on oxidative phosphorylation.

Whereas the mutant loads were higher in m.8344A>G (mean 70%) than in m.3243A>G embryos, m.8344A>G did not apparently impact the mtDNA amount, suggesting that the m.8344A>G mutation is less detrimental than m.3243A>G mutation for early embryonic development. In keeping with this, it is worth remembering that the mutation load in the muscle of MERRF patients is higher (>80%) than in MELAS patients (>40%) (20), suggesting that the mutant load above which mitochondrial dysfunction appeared (i.e. critical threshold) is lower in m.3243A>G than in m.8344A>G (21,22), even if the difference between the thresholds of m.3243A>G and m.8344A>G is less marked in single-cell studies in vitro (23–25).

Interpretation of this observation has to take into account that the embryos analyzed are different from the oocytes because we did not perform sequential analyses. A first hypothesis is that for m.3243A>G mutation, only oocytes with a high copy number are going to be successfully fertilized and to develop until day 4 or 5. A second possibility is that m.3243A>G activates mtDNA replication as a compensatory mechanism. Should this prove to be the case, the shift toward high mtDNA copy number in m.3243A>G embryos was not associated with a change in the level of heteroplasmy (Fig. 3), suggesting that upregulation of mtDNA replication equally impacted wild-type and mutant molecules. To our knowledge, the ability to promote mtDNA replication during the first embryonic cleavages has never been reported to date in humans. Ongoing transcriptomic experiments and in vivo incorporation of 5-bromo-2′-deoxyuridine on m.3243A>G oocytes and embryos will hopefully help addressing this question, of particular relevance for innovative therapeutic approaches in MELAS and mitochondrial diseases.

In conclusion, in the course of our PGD service to couples at risk to transmit mtDNA mutations, we have observed that human embryos would have the intrinsic ability to regulate their mtDNA content in response to impaired respiratory capacities. Even if these data are from single patients and should, therefore, be interpreted cautiously, this observation emphasizes the critical role of mitochondria during human early development and may inspire novel researches aimed at improving the condition of MELAS patients and respiratory chain enzyme deficiency.

**MATERIALS AND METHODS**

**Patients and materials**

This study was approved by the National Ethics Comittee from l’Agence de Biomedecine (project RE06-015R), and all couples gave their informed and written consent to DNA analyses for themselves and their embryos.

Patients 1 and 2 underwent three and one PGD cycles, respectively. A total of 17 oocytes collected by transvaginal ultrasound-guided aspiration after a standard ovarian stimulation protocol and at risk of carrying the m.3243A>G ($n = 12$) or the m.8344A>G mutation ($n = 5$) were included in the study (Supplementary Material, Table S1). These cells were discarded at various maturation stages, including GV ($n = 9$) and MI ($n = 3$) and oocytes unfertilized following intracytoplasmic sperm injection (ICSI, MII, $n = 5$). A total of 30 embryos were collected at days 4–5 (Supplementary Material, Table S1). Embryos with less than six cells at day 3 ($n = 10/30$) were considered arrested and collected in toto.
(5 m.3243A>G and 5 m.8344A>G). The remaining 20 out of 30 embryos were submitted to a 2-blastomere biopsy at day 3 post-ICSI for mutant load assessment only. Embryos that carried low mutant loads were transferred to maternal uterus and not available for mtDNA copy number quantification (n = 6 out of 20; 4 m.3243A>G and 2 m.8344A>G). The remaining embryos (14 out of 30; 7 m.3243A>G and 7 m.8344A>G) were either of bad quality or carried high mutant loads and were not transferred. Both couples donated their discarded embryos for research. Control oocytes (n = 19; 3 GV, 7 MI and 9 MII) and embryos were donated for research by couples undergoing PGD for prevention of a non-mitochondrial genetic disorder (17 embryos: 7 whole arrested embryos and 10 sampled embryos affected by a non-mitochondrial disorder, Supplementary Material, Table S1).

Oocytes and embryos were transferred to alkaline buffer (26) and lyzed by 10 min heating at 65°C. The volume was first adjusted to 18 µl using double-distilled water and split into two parts to quantify the mutant load and the mtDNA copy number. Specific assays were devised to enable the simultaneous assessment of mtDNA copy number and mutant load.

Assessment of the mutant load

Mutant load was quantified using a semi-quantitative fluorescent PCR restriction test (16,17). Such tests have been shown suitable for mutant load assessment over a wide range of mtDNA copy number templates (from 10^2 to 10^8 molecules), with a good repeatability (±2%). Results were analyzed with the Genescan and Genotyper softwares (Applied Biosystems, Courtaboeuf, France). The mutant load was calculated by dividing the mutant peak area by the sum of the mutant and wild-type peak areas. The mutation level and the normalized variances in oocytes and embryos were assessed using the following formula:

\[ V_{\text{normalized}} = \frac{V}{m(1-m)} \]

where \( V \) is the variance of the set of measured mutation levels and \( m \) is the average mutation level (in the range 0–1). This normalization is based on the random drift theory to remove the dependence of variance \( V \) on the mutation level \( m \) (27).

Quantification of the mtDNA copy number

The quantification of mtDNA copy number in mutants (17 oocytes and 24 embryos) and controls (19 oocytes and 17 embryos) was performed by real-time PCR using Sybr Green (Qiagen, Courtaboeuf, France) in a 7300 Real Time PCR System (Applied Biosystems). Absolute quantification was obtained by the standard curve method. The external standard was a plasmid encompassing nt109-nt1714 of the mitochondrial 12S RNA gene (MT-RNR1). The plasmid was phenol–chloroform purified, ethanol precipitated, diluted in water and quantified by UV absorbance at 260 nm. One nanogram of plasmid was assumed to contain 1.96 × 10^8 molecules of double-stranded DNA. Standard curves were generated using 10-fold serial dilutions of the plasmid. Cross hybridization of oligonucleotide primers to genomic DNA was ruled out by PCR amplification on mtDNA-less Rho0 cells (28). Amplification of oocyte/embryo mtDNA (4 µl) and external standard mtDNA plasmid (4 µl) was carried out in a 20 µl reaction volume containing 0.5 µM of each primer (12SF 5’-taggga gcttgttggatca-3’ and 12SR 5’-gegettaattgtgacctcat-3’, 111 bp) and 10 µl of QuantiTect SYBR Green PCR Kit 2X (Qiagen). PCR program consisted of 15 min of denaturation at 95°C, followed by 40 PCR cycles including 15 s at 95°C, 20 s at 60°C and 30 s at 72°C. All PCR amplifications were performed in triplicate. The dissociation curves were systemically analyzed to check for the amplification specificity. Because the quantification was performed on 4 out of 18 µl of the oocyte/embryo DNA, the raw data were increased 4.5-fold to achieve the overall oocyte/embryo mtDNA copy number.

Because two cells were removed for genetic testing in the sampled embryos, a correction of the mtDNA copy number was necessary to get the total amount of whole embryo mtDNA. We postulated that mtDNA molecules from a given embryo were homogeneously distributed among cells. A correction procedure was, therefore, applied to the 24 sampled embryos (14 mutants and 10 controls) by multiplying the measured copy number by n/(n–2), where n is the total number of cells at the time of the biopsy. Finally, according to their developmental stage, embryos were split into two groups, according to ability (pre-blastocyst embryos) or inability to count the blastomeres (blastocyst embryos, i.e. morula and blastocyst embryos).

Statistical tests

The sets of mtDNA copy numbers were compared between two groups using non-parametric Wilcoxon’s rank sum test. The test of mtDNA copy number versus the embryo development stage was done using Kendall’s rank correlation tau test. All tests were carried out in the statistical software R.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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REFERENCES


