Received: 12 October 2022 | Accepted: 23 November 2022

DOI: 10.1002/rmb2.12491

Reproductive Medicine and Biology

WILEY

REVIEW

Mitochondrial function of human embryo: Decline in their quality with maternal aging

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Funding information
the Japan Society for the Promotion of Science, Grant/Award Number: KAKENHI 20K09674

Abstract

Background: Female fertility declines with age, due to increased chromosomal aneuploidy and possible reduced mitochondrial function in the embryo.

Methods: This review outlines how mitochondrial function in human embryos, as predicted from oxygen consumption rate (OCR) measurements, changes in preimplantation stage, and what factors, particularly maternal age, affect mitochondrial function in embryos.

Main findings: The structure of the mitochondrial inner membrane and its respiratory function developed with embryo development, while the copy number of mitochondrial DNA per specimen was transiently reduced compared with that of the oocyte. The undifferentiated state of the inner cell mass cells appears to be associated with a low OCR. In contrast, the copy number of mitochondrial DNA increased in trophoblast cells and mitochondrial aerobic metabolism increased.

The OCRs at morulae stage decreased with maternal age, but there was no relationship between maternal age and the copy number of mitochondrial DNA at any stages. The higher oxygen spent at the morula stage; the shorter time was needed for development to the mid-stage blastocyst.

Conclusions: The mitochondrial respiratory function of human embryos developed along with embryonic growth. Mitochondrial function at morula stage declined with their maternal age and reduced mitochondrial function decreased the rate of development from morula to blastocyst.

KEYWORDS
human embryo, maternal age, mitochondrial function, morula, oxygen consumption rate

1 | INTRODUCTION

Women's fertility declines with age.[1] One of the main causes of low development of embryos obtained from older women is the rising proportion of chromosome abnormalities[2] provoked by premature separation of bivalent to univalent during meiosis.[3] Furthermore, age-related fall in mitochondrial function of oocytes has also been suggested as a cause of low embryonic developmental potential.[4–7]

Mitochondria produce ATP for cellular activity by oxidative phosphorylation. The tricarboxylic acid (TCA) cycle is characterized by a higher capacity to produce ATP per mole of substrate than the anaerobic glycolysis pathway. The ATP produced by mitochondria

plays an important role in the maturation of both the nucleus and cytoplasm of oocytes.[8–13] An investigation using mitochondrial transcription factor A (TFAM)-deficient mice, which is indispensable for mitochondrial DNA (mtDNA) replication, transcription, and maintenance, have shown that oocytes must have a threshold number of mtDNA to bolster their developmental capacity to develop viable fetuses.[14] In mice, the copy number of mtDNA in the whole embryo does not change during successive cell divisions,[14,15] inducing a gradual decrease in mtDNA in the dividing cells. By contrast, in bovine,[16] porcine,[17,18] and human,[19] it has been reported that the copy number of mtDNA decreases transiently after fertilization and then increases rapidly during blastocyst cavity formation. Thus, quantitative shift in mitochondria in the preimplantation embryos differ between animal species. Mitochondria are a source of large amounts of ATP, and as oxygen consumption is related to ATP production, mitochondrial oxygen consumption rate (OCR) may be a valid indicator of embryo quality. Several methods for assessing OCR have been described, including the Cartesian diver,[20] spectrophotometric,[21–23] and fluorescence[24–26] methods. However, these methods were not suitable for clinical application due to their low sensitivity, inability to measure the OCR of a single embryo, exposure to fluorescent dyes and the long time needed for assessment, which makes them highly invasive to the embryo.

Successful measurements of OCR in single embryos using scanning electrochemical microscopy (SCEM) based on self-reference microelectrode and nano-respirometer have been shown in mice,[27] cattle,[28–30] and human.[19,31–35] A modified SCEM system calculates the difference between oxygen concentrations near and far from the sample. (Figure 1).[27–30] Furthermore, it is also clear that for accurate measurement of oxygen consumption, it is important that an appropriate energy substrate is present.[27] It has also been suggested that human embryos with high oxygen consumption develop into blastocysts faster than embryos with low oxygen consumption.[22,34] It has also been shown that blastocyst cavity formation is dependent on Na/K-ATPase.[36] Therefore, the oxygen consumption of the blastocyst at 2–3 h after warming correlates with the subsequent re-formation of the blastocoel cavity and is an important predictor of embryonic development.[19]

This review outlines how the mitochondrial function of human embryos, as predicted by measurements of OCR, is changed in

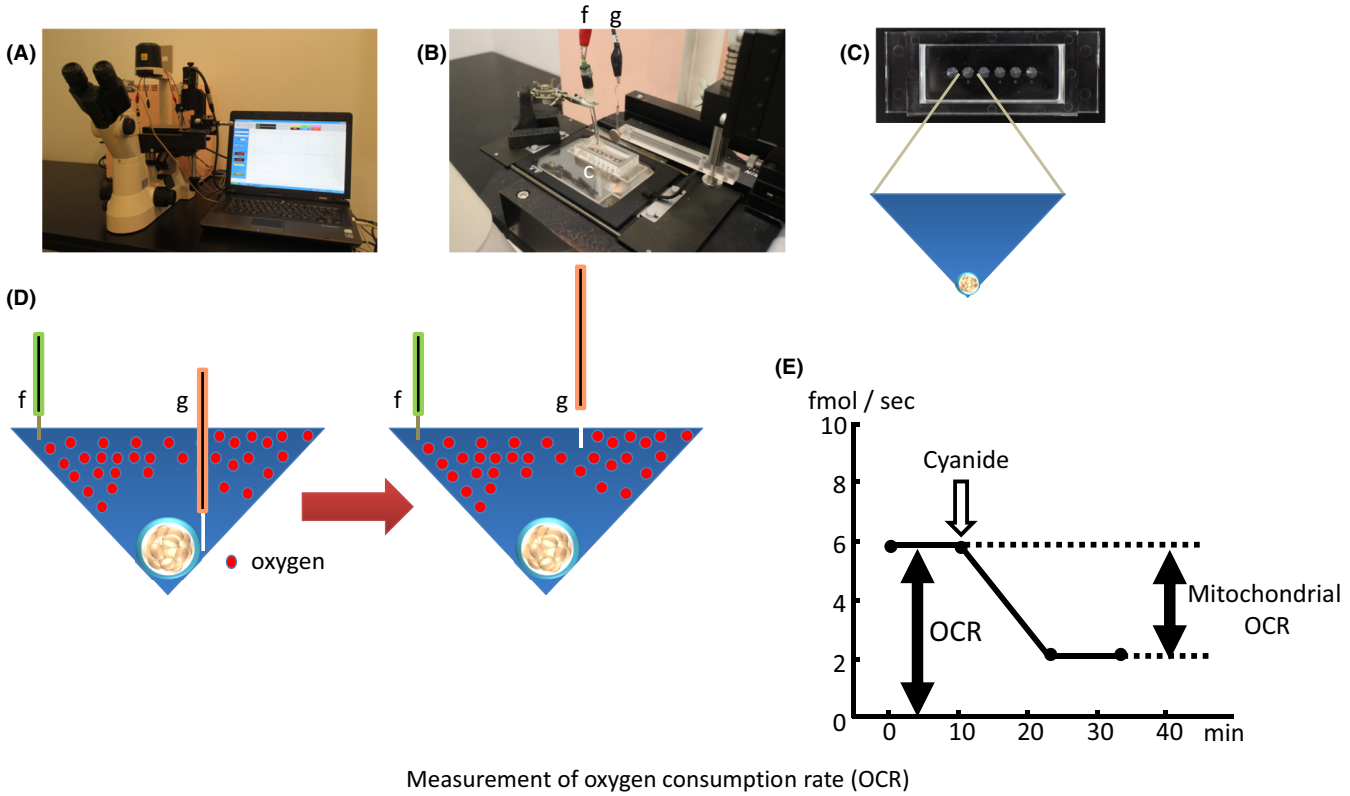

FIGURE 1 Oxygen consumption rate (OCR) measurement of mammalian embryo. (A) Scanning electrochemical microscope. (B) Cone-shaped micro well (c), reference electrocode (f), Microelectrocode (g). (C) Each embryo was transferred into a cone-shaped micro well. (D) The microelectrode scanned along the z-axis from the edge of morula, and OCR was calculated with custom software. Circle: Oxygen. (E) the OCR measured in buffer without mitotoxin represents the OCR of the whole embryo. By adding cyanide, a cytochrome c oxidase inhibitor, and measuring it, the OCR consumed outside the mitochondria can be obtained. The OCR in mitochondria (mtOCR) is calculated by subtracting the value obtained in the presence of cyanide from the value obtained without any mitotoxins. The figure is based on Morimoto et al.[35]

preimplantation human embryos and what factors affect mitochondrial function in early human embryos.

2 | QUANTITATIVE AND QUALITATIVE SHIFTS OF MITOCHONDRIA IN HUMAN EMBRYOS

2.1 | Copy number of mtDNA

The mtDNA copy number of embryos in the preimplantation period is unchanged in mice[14,15] but decreases after fertilization and increases during blastulation in cattle[16] and pigs.[17,18] Which trend does the human embryo show? To this end, we have examined the change in the copy number of mtDNA of human embryos.[19] The copy number of mtDNA per specimen in embryos at the 2-cell, 9-14-cell, and morula stages after fertilization was temporarily reduced compared with oocytes.[19] The copy number of mtDNA significantly increased ($p < 0.01$) in Day 5 blastocyst (expanded stage) compared with those in oocytes, 2–14 cell and morula. In this review, the data from previous studies were integrated[19,34] and reanalyzed, showing a rapid increase in mtDNA copy number at the time of blastulation ($p < 0.01$, Figure 2A). The mtDNA copy number changes described above were consistent with bovine[16] and porcine embryos,[17,18] but differed from murine embryos, where mtDNA copy number does not change in the pre-implantation period.[14,15] Therefore, although rodent embryos are often used as models in the construction of human embryo culture systems, given the significantly earlier activation of gene expression in embryo (two-cell stage) than in other animal species and the different patterns of mitochondrial DNA copy number changes, it is considered appropriate to use bovine and porcine embryos, whose conditions match those of human embryos, as models. The expression levels of transcription and replication factors of mtDNA prior to the morula stage were extremely low, even though these gene expressions could be detected during earlier period in several mammals, such as pig[18] and sheep.[37] The expression of these genes was thought to be downregulated in human embryos, as in other mammals, until the morula stage. In addition, the extremely low mitochondrial function in human embryos at cleavage stages prior to morula may have caused a transient reduction in the copy number of mtDNA, with no need to increase it.[38] The mtDNA copy number per cell decreased with progression of cell division (Figure 2B).

Reason for infertility did not affect the copy number of mtDNA in 2-cell (endometriosis vs. male factor) and in 3–4 cell embryos (endometriosis vs. male factor). On the other hand, there are also reports that do not always agree with this, as follows; the copy numbers of mtDNA in oocytes or embryos have been demonstrated to be related to reason for infertility (ovarian insufficiency,[39] endometriosis[40]), mutations of mtDNA,[41] female age[42] and aneuploidy of embryos.[42,43] On the other hand, it has been indicated that overall number of mtDNA is mostly similar between blastocysts stratified by ploidy, maternal age, or implantation potential.[44] Thus, the relationship between infertility causes and the copy number of mtDNA in human embryos should be investigated further in the future.

2.2 | Threshold for mtDNA copy number in oocytes

Sufficient mtDNA copy number in mature oocytes has been proposed to be a genetic reservoir designed to share out mitochondria and mtDNA to cells in the early post-implantation embryo before mitochondrial biosynthesis and mtDNA replication resume in mice.[14] Wai et al. showed that the mtDNA content in murine oocyte less than approximately 50000 copies is unlikely to normally develop through postimplantation stage, since the mtDNA copy number does not change before implantation in mice.[14] However, mtDNA copy number increases rapidly during blastocyst formation in bovine,[16] porcine[17,18] and human embryos.[19] In species where mtDNA copy number increases during blastocyst formation, such as bovine, porcine, and human, it is not certain whether there is a copy number of mtDNA in oocytes that restricts postimplantation development. It is also possible that too high-mtDNA copy number may even inhibit development. On the other hand, it is not well understood how the copy number of mtDNA in the oocyte affects the development of embryos, as bovine parthenogenic embryos developed into blastocysts even when the mtDNA copy number was artificially reduced,[45] and mtDNA copy number did not affect pre-implantation development in mouse embryos.[14]

2.3 | Mitochondrial function

Is mitochondrial function in embryo correlated with mtDNA copy number? To address this, changes in mitochondrial function in human embryos prior to implantation were investigated.[19] No difference in mtOCR was observed from oocyte to 8-cell stage (Figure 2C). However, mtOCR increased more rapidly at morula stage than up to the 8-cell stage ($p < 0.01$). Furthermore, mtOCR in the expanded blastocyst stage was even higher than in morula stage ($p < 0.01$). OCRs intensified toward the morula stage in advance of an increase in mtDNA copy number at blastulation. The contribution of mitochondrial respiration to energy requirements during embryogenesis is estimated to increase from about 10% of available glucose metabolized by aerobic respiration in early development to 85% in the blastocyst stage,[46] coinciding with a period of increased OCR in mice[24,27,47] and cattle.[25] A study of early human embryos has shown that mtOCR and the activity of cytochrome c oxidase (CCO) in human pre-implantation embryos increases with development, while the copy number of mtDNA transiently decreases.[19] The mtOCR of the normally developed embryos on Day 4 was larger than that of the delayed embryos, and the mtOCR/mtDNA ratio increased accordingly.[19] This may act as a marker for surviving embryos after Day 4, as the OCR increases with embryo development and the formation

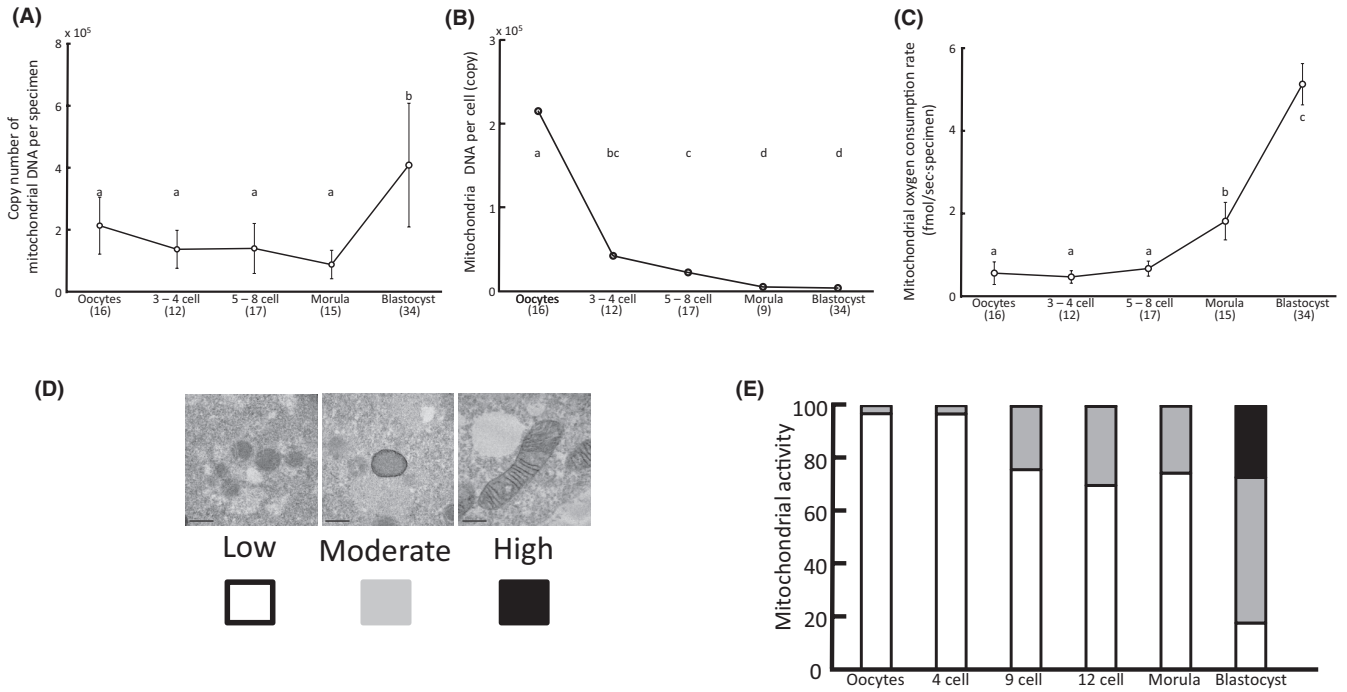

FIGURE 2 Quantitative and qualitative changes of mitochondria in human preimplantation embryos. (A) Changes in mitochondrial DNA (mtDNA) copy number per specimen (mean), (B) changes in mtDNA copy number per cell and (C) mtOCR (mean) during preimplantation development of human embryos. The mtOCR was calculated by subtracting the value obtained in the presence of cyanide from the value obtained without any mitotoxins (Figure 1). The numbers of oocytes or embryos examined are shown in parentheses. [a-c]Different superscript letters indicate significant differences ($p < 0.01$) by Tukey–Kramer test following ANOVA. (D), (E) changes of cytochrome c oxidase (CCO) activity in human embryos during preimplantation development. DAB tetrahydrochloride is easily incorporated into mitochondria, and oxidative polymerization occurs by high-voltage potential. Accordingly, mitochondria with CCO activity were stained for transmission electron microscopy. (D) TEM image of blastocyst stained with active mitochondria (left). High: Mitochondria with high CCO activity (black) showed well-developed cristae structures and deeply stained membrane. Moderate: Mitochondria with deeply stained membrane and without cristae structures were categorized as having moderate CCO activity (gray). Low: Mitochondria with poorly stained membranes and few cristae structures were categorized as having low CCO activity (white). Original magnification × 50 000; Bar = 0.5 μm. (E) The proportions of mitochondria with high, moderate, or low CCO activity in mature oocytes at meiosis II (MII) through to blastocysts (Day 5) are shown. Data for CCO activity were compared by Cochran–Armitage test among five groups. Three embryos were used at each developmental stage. The figure is based on Hashimoto et al.[18]

of the embryonic cavity relies on intracellular Na/K-ATPase activity.[36] Moreover, mtOCRs rose remarkably from morula to blastocyst stage earlier than a surge of mtDNA at the time of blastulation.[19]

Next, to directly determine whether mitochondria in the cells of human embryos are activated, the presence and percentage of active mitochondria with CCO activity were examined by direct staining of activated mitochondria. The ratio of highly active mitochondria has increased in blastocysts compared to mature oocytes, 4-, 9-, 12-, and morula ($p < 0.0001$; Figure 2D,E). The percentage of mitochondria with moderate activity also grew in blastocysts compared with mature oocytes, 4-, 9-, 12-, and morulae ($p < 0.01$). Taken together, mitochondria activity grew with embryo development and was significantly elevated in blastocysts. The mtOCR/mtDNA ratio increased toward the morula and blastocyst stages ($p < 0.01$) compared with the oocyte-to-8-cell stage.[19] The mtOCR per mtDNA has been found to increase with embryonic development up to the morula stage.

The development of continuous culture media designed to meet the changing requirements during embryo development has been revealed to produce blastocysts with high potential.[48–50] Efficiently supplying the required energy source in response to changes in mitochondrial function may provide a less stressful environment for the embryo.

3 | MITOCHONDRIAL FUNCTION OF HUMAN BLASTOCYST

3.1 | Mitochondrial function of ICM and TE

Mitochondrial function in blastocysts increases before the formation of the blastocoel cavity.[19] So, does mitochondrial function of ICM also increase with blastulation? To address this, we measured the oxygen consumption of TE far from the ICM (TE side) and TE adjacent to the ICM (ICM side).[32] No differences were observed in the oxygen consumption between the TE and ICM sides of human blastocysts at any measurement times (Figure 3A). In addition, the presence and percentage of active mitochondria with CCO activity were examined by

(A) The mean oxygen consumption of their trophectderm (TE) adjacent to and far from the inner cell mass (ICM).

	Hours after warming						
	1.5	3	4.5	6	7.5	9	24
TE adjacent to ICM	3.9 (29)	4.7 (36)	5.6 (38)	6.3 (31)	7.1 (28)	7.8 (28)	9.7 (25)
TE far from ICM	4.1 (29)	4.9 (36)	5.8 (38)	6.7 (31)	7.3 (28)	7.9 (28)	10.2 (25)

Oxygen consumption is expressed as fmol/s/blastocyst (no. of blastocysts)
If the ICM was clearly discernible, the oxygen consumptions of the TE adjacent to and far from the ICM were measured. There were no significant differences in oxygen consumption between the points near and far from the ICM.

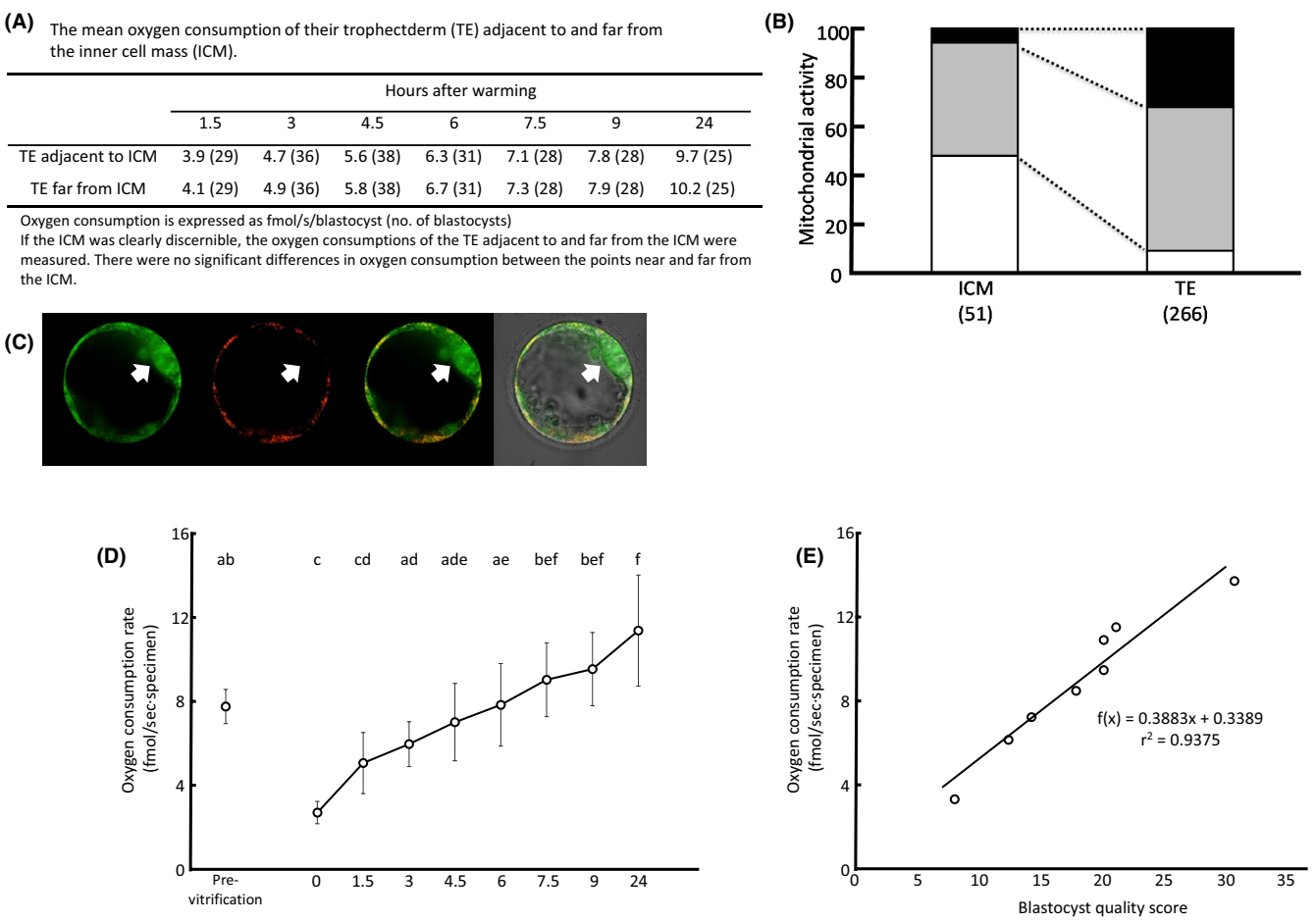

FIGURE 3 Mitochondrial function of human blastocyst. (A) The mean oxygen consumption of their trophectoderm (TE) adjacent to and far from the inner cell mass (ICM). (B) The proportions of mitochondria with these CCO activities in the inner cell mass (ICM) and trophectoderm (TE) of three blastocysts are shown. The numbers of mitochondria in oocytes or embryos examined are shown in parentheses. Data for CCO activity were compared by Fisher's exact test between two groups. (C) Representative images of human expanded blastocyst stained with JC10. JC10 monomers (low membrane potential) were detected using a confocal microscope (excitation 488 nm, emission BP525/50, CellVoyager CV1000; Yokogawa electronic, Tokyo, Japan), and JC10 aggregates (high membrane potential) were detected (excitation 561 nm, emission BP617/73). From left to right: JC10 monomer, dimer, monomer, and dimer, and further brightfield images overlaid. White arrows indicate ICM. Active mitochondria have been observed only in TE. (D) Change in mean oxygen consumption (x 10–14 fmol/s) of vitrified-warmed blastocysts over time. Values with different superscripts are significantly different ($^{abc}p < 0.05$, by Tukey–Kramer test following ANOVA). (E) The relationship between OCRs of blastocyst and their blastocyst quality score. There were extremely high correlations between OCR and BQS ($r^2 = 0.9375$). Eight blastocysts were used. The figure is based on Yamanaka et al.[32] and Hashimoto et al.[19]

direct staining of activated mitochondria (Figure 2D). The percentage of mitochondria with high CCO activity was much lower in ICM cells than in TE cells ($p < 0.01$; Figure 3B). In addition, active mitochondria have been observed only in TE (Figure 3C). These results are consistent with data from animal embryos. TE used significantly more oxygen, generated more ATP, and had more mitochondria than ICM in mice.[24] Metabolic efficiency of amino acids was also much higher in TE than in ICM. TEs are thought to produce about 80% of their ATP in the mitochondria.[51] Furthermore, in bovine blastocysts, TE consumed more pyruvate and less glucose than ICM.[52] In summary, the ICM of human blastocysts is considered to have relatively lower mitochondrial function than the TE. As a result, oxygen consumption in TEs adjacent to the ICM may be similar to that in TEs away from the ICM.

3.2 | Relationship between blastocyst after cryopreservation and its mitochondrial function

Is there a correlation between blastocyst after cryopreservation and its mitochondrial function? To address this, we examined how human-vitrified blastocysts alter their mitochondrial function after warming. The oxygen consumption rates of all vitrified-warmed blastocysts immediately after warming was found to be significantly smaller than pre-vitrified blastocysts ($p < 0.05$, Figure 3D). The oxygen consumption rate of surviving blastocysts after thawing increased over time. Mitochondria with CCO activity (Figure 2D) were observed in blastocysts at 24 h after warming.[32] Cellular activity is considered to have arrested at cryogenic temperature and

mitochondrial function has not yet been restored immediately after warming and gradually recovers.

3.3 | Relationship between mitochondrial function in blastocyst and its morphology

Does mitochondrial function in blastocysts predict morphological change or morphology itself? To find an answer to this question, the correlation between blastocyst morphology and its mitochondrial function was investigated after numerical conversion of the Gardner classification to Blastocyst quality score (BQS).[53] OCR and BQS showed extremely high correlations (Figure 3E). Next, the relationship between the OCR of blastocysts with identical morphological evaluation from women in the same age group and the implantation rate after single blastocyst transfer was examined, but the results showed that embryos with higher OCR were not necessarily more likely to implant.[54] These suggest that the OCR of blastocyst may reflect its morphology and not predict its subsequent viability such as implantation. Large-scale research studies are required to examine whether OCR is predictive of blastocyst potential, as implantation is affected by various factors, including maternal conditions.

4 | DOES MATERNAL AGE AFFECT THE MITOCHONDRIAL FUNCTION IN THEIR EMBRYOS?

4.1 | Oxygen consumption rate in mitochondria of human morulae decreases with increasing maternal age

Maternal aging is associated with a decline of the copy number of mtDNA in oocyte[55,56] or mitochondrial function.[57] Do these reductions in mtDNA copy number or mitochondrial function occur in the embryo? To address this, we examined the relationship between maternal age and mtDNA copy number or mitochondrial function.[34] The mtOCRs of morulae on Day 4 decreased with maternal age ($p < 0.05$, $r^2 = 0.4834$, Figure 4A).[34] However, any

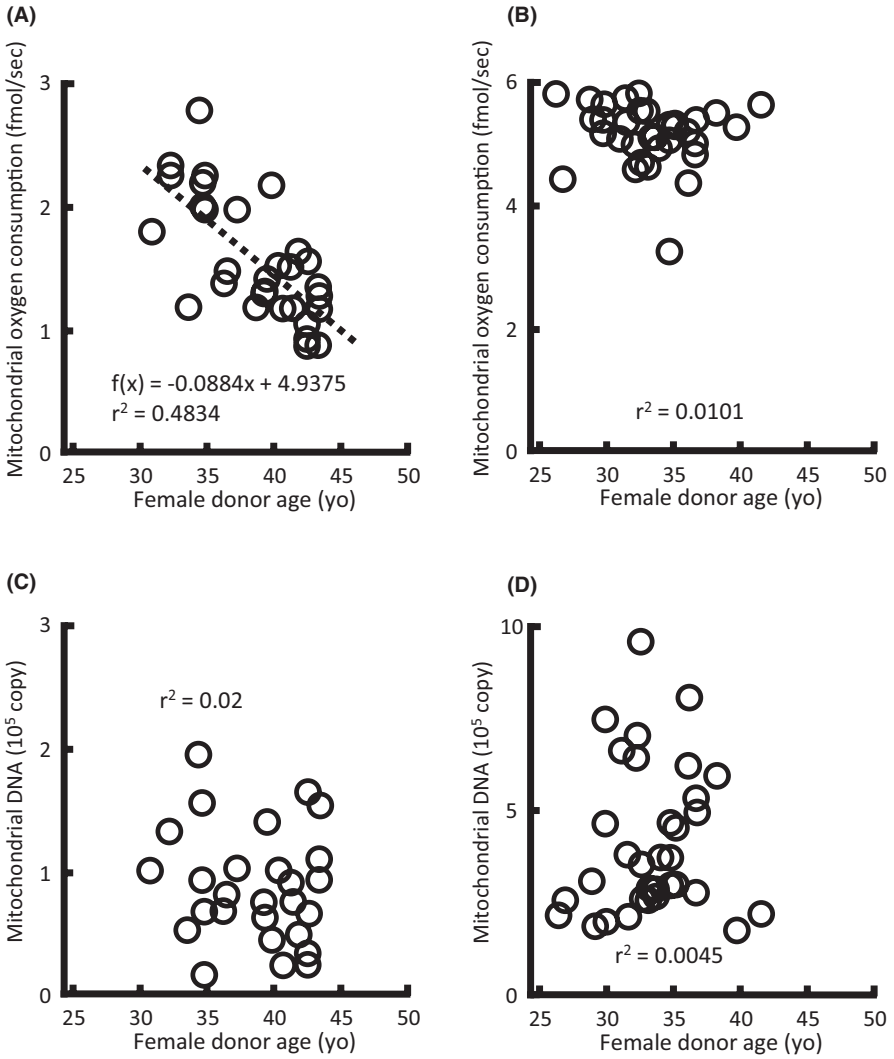

FIGURE 4 Relationship between maternal age and mitochondrial oxygen consumption rates (mtOCRs) or mitochondrial DNA (mtDNA) numbers of embryos. (A) Relationships between maternal age and mtOCRs of morulae on Day 4 ($n = 30$); and (B) expanded blastocysts on Day 5 ($n = 34$). (C) Relationships between maternal age and mtDNA copy number of their morulae on Day 4 ($n = 28$); and (D) expanded blastocysts on Day 5 ($n = 34$) are shown. The mtOCRs of morulae on Day 4 after ICSI decreased with maternal age ($p < 0.05$, $r^2 = 0.4834$) only embryos inseminated by ICSI were used to avoid contamination with mtDNA from spermatozoa on the zona pellucida. The figure is based on Morimoto et al.[34]

relationships between maternal age and mtOCRs of their blastocysts on Day 5 were not observed (Figure 4B). Similarly, the mtDNA copy number of developing embryos on Days 4 and 5 post-ICSI is found to be uncorrelated with maternal age (Figure 4C,D). Accordingly, it was found that the OCR of morula decreases with increasing maternal age.

Interestingly, the variation in OCR of blastocysts (5.18±0.5 fmol/s) was very small compared with the variation in their mtDNA copy number (416200±202055 copy), which was almost constant with no effect of maternal age (Figure 4B,D). The blastocysts used for the measurements were almost identical in morphology, as their developmental stages were mid-stage to expanded (3 and 4 in the Gardner classification[58]) and all ICM grades were B in the Gardner classification. These indicated that the OCR in the blastocysts reflected their shape.

4.2 | Are morulae with high OCRs developmentally competent?

What does it mean that the OCR of morulae decreases with increasing their maternal age? To elucidate this question, following OCR measurements of the morula without mitotoxins, time-lapse images were taken from the morula to the blastocyst stage. (Figure 5A).[34] In the morula stage, the higher the OCR, the shorter time needed for development to mid-blastocyst. ($r^2 = 0.236$, $p < 0.05$; Figure 5A,B).[34] Blastocoel formation requires a large amount of energy, as fluid must be actively transported into the blastocoel cavity.[26] Therefore, it is thought that morulae with higher OCR quickly formed the blastocoel cavity and reached the blastocyst stage in a short time.

To examine how maternal age affects morphogenetic change in human embryos, the blastulation and morphologically good

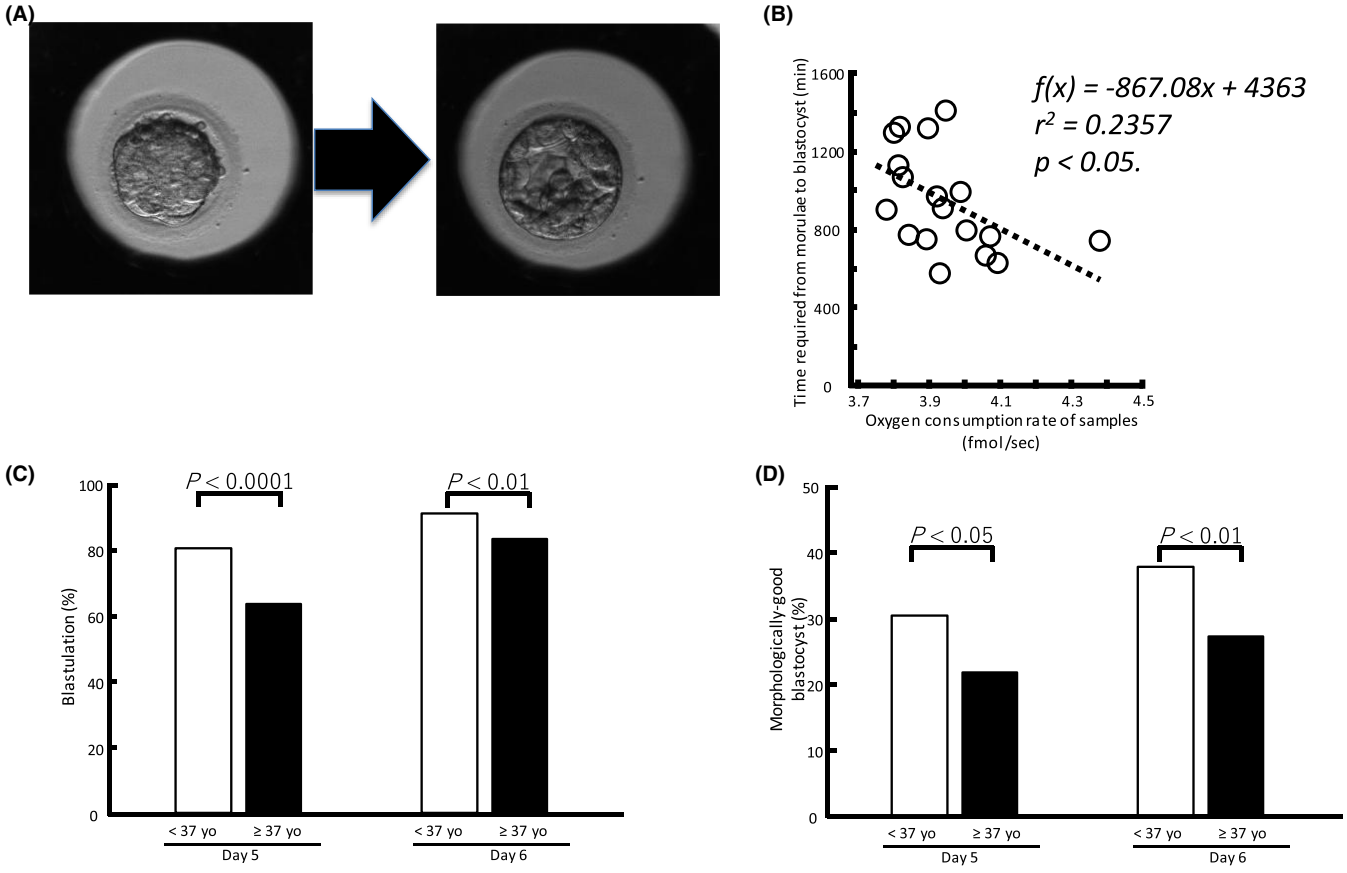

FIGURE 5 Oxygen consumption rate indicates the ability to develop into blastocysts and the ability to develop from morula to blastocyst decreases with maternal aging. (A) The left image shows a representative morula. The right image shows a representative mid-stage blastocyst. Mid-stage was defined as when the blastocoel reached half the size of the blastocyst. (B) Relationship between OCRs of morulae and duration required to develop from morulae to mid-stage blastocysts. Morphological changes from morulae to blastocysts following measurement of OCRs of morulae cultured without mitotoxins were recorded every 10 min. To avoid the effect of maternal age, 6–8-cell stage embryos on Day 3 were used from women aged 34–36 year at OPU. Eighteen embryos were donated from 10 couples. The more oxygen morulae consumed, the shorter was the duration required for embryo development between OCR measurement and mid-stage blastocysts ($r^2 = 0.236$, $p < 0.05$). Retrospective analysis of developmental competence from morulae to blastocysts in the clinical dataset. The rates of development from morulae to blastocysts (C) and to form morphologically good blastocysts (D) were compared retrospectively between two maternal age groups (<37 year; $n = 280$; vs. ≥37 year, $n = 251$). Retrospective analysis revealed that the rates of development from morulae to blastocysts and to morphologically good blastocysts on Day 5 after insemination decreased with maternal age. The rates of development from morulae to blastocysts and to morphologically good blastocysts on Day 6 also decreased with maternal age. The figure is based on Morimoto et al.[34]

blastocysts rates from morulae were retrospectively examined between women in the two age groups (<37 year: younger group, vs. ≥37 year: older group).[34] Retrospective analysis demonstrated that these developmental rates on Day 5 after insemination declined significantly with maternal age, 81% in the younger group compared with 64% in older group ($p < 0.01$), and the morphologically good blastocyst rate was 31% in younger group compared to 22% in older group ($p < 0.05$; Figure 5C,D). The rates of blastocyst and morphologically good blastocyst on Day 6 also decreased significantly with maternal age, with a blastocyst formation rate of 92% in younger group versus 84% in older group ($p < 0.01$) and a morphologically good blastocyst formation rate of 38% in younger group versus 28% in older group ($p < 0.01$). From the above, it is clear that an increase in maternal age decreases the OCR of morula, resulting in a lower energy supply and a lower development rate from morula to blastocyst.

4.3 | How to improve mitochondrial function in morulae?

Is it possible to improve mitochondrial function in morulae that have declined with maternal aging or for other reasons?

Laevo (L)-carnitine serves an important function in reducing the cytotoxic effects of free fatty acids by stimulating beta-oxidation,[59,60] mitigating of cellular injury. It is present ubiquitously in mammalian plasma and tissues, particularly in muscles, and curbs mitochondrial damage and mitochondrially triggered apoptosis.[61] It has been reported that L-carnitine also perform a crucial function for oocyte growth,[62,63] oocyte maturation,[64] and embryo development[65,66] and that increases ATP content in murine morulae.[35] We investigated whether such beneficial effects of L-carnitine boost the mitochondrial function in human morulae.[35] An addition of L-carnitine boosted mtOCRs of morulae and the development to morphologically good blastocysts on Day 5. Thus, reagents that enhance mitochondrial function such as L-carnitine would be a prospective medium supplement that can help bring back the lowered development potential associated with maternal age.

5 | CONCLUSION

Mitochondrial functions advanced with embryo development while the mtDNA copy numbers declined transiently after fertilization and skyrocketed at blastulation.

Mitochondrial function at morula stage of human embryos reduced with maternal age and a decline of mitochondrial function slowed embryo development and diminished developmental rate from morulae to blastocysts. This reduced developmental capacity may be overcome by the use of compounds that improve mitochondrial function, such as L-carnitine.

ACKNOWLEDGMENTS

The authors thank Mr. M Yamanaka and members of Reproductive Science lab, Graduate School of Medicine, Osaka Metropolitan University for their helpful comments.

FUNDING INFORMATION

Part of this work was supported by a grant from the Japan Society for the Promotion of Science (KAKENHI 20K09674 to S.H.).

CONFLICT OF INTEREST

The authors declare no conflict of interest. The studies (reference number: 19 and 32–35) we have mainly referred to were performed in accordance with the Declaration of Helsinki protocols 7th version. Prior to inclusion in the study, all donating couples gave their written informed consent. These studies were approved by the local ethics Institutional Review Board.

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How to cite this article: Hashimoto S, Morimoto Y. Mitochondrial function of human embryo: Decline in their quality with maternal aging. *Reprod Med Biol*. 2022;21:e12491. doi:10.1002/rmb2.12491