Mitochondria DNA Deletion and Copy Numbers of Cumulus Cells Associated with in Vitro Fertilization Outcomes

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OBJECTIVE: Mitochondria are important organelles in cell biology. We aimed to study the effects of mitochondrial DNA variations in cumulus cells (CCs) upon in vitro fertilization and embryo transfer (IVF-ET) outcomes.

STUDY DESIGN: A total of 51 women undergoing IVF-ET were recruited for the study. The CCs were collected during oocyte retrievals. Mitochondria DNA 4977-bp deletion (dmtDNA-Δ5Kb) and copy numbers (MCN) of CCs were analyzed by polymerase chain reaction. The relationships of dmtDNA-Δ5Kb and MCN with patients' age, embryo qualities and pregnancy rates (PRs) were detected and compared.

RESULTS: PRs were positively correlated with younger age, better transferred embryo qualities and lower dmtDNA-Δ5Kb ratios in CCs. The dmtDNA-Δ5Kb status was positively associated with older age and higher MCN but was not associated with embryo morphologic scoring. The dmtDNA-Δ5Kb ratios of transferred embryos in pregnancy and nonpregnancy groups were 0% and 10.4%, respectively. The dmtDNA-Δ5Kb in ≥34-year-old and <34-year-old groups were 6.9% and 3.2%, respectively.

Biochemical markers for predicting embryo qualities are essential to optimize embryo selection.

CONCLUSION: The dmtDNA-Δ5Kb and MCN statuses of CCs are negatively associated with PRs, which might be potential tools for oocyte evaluation and embryo selections during IVF-ET. (J Reprod Med 2010; 55:491–497)

Keywords: cumulus, DNA deletion, embryo, IVF, mitochondria.

Oocyte and embryo evaluations are crucial for embryo selection during in vitro fertilization and embryo transfer (IVF-ET). Traditionally, embryo selections are based on the pronuclear morphology, blastomere cleavage and fragmentation statuses.[1,2] However, the accuracy for such an embryo scoring system remains controversial and obscure. Clinicians must transfer 3–4 early-stage embryos or 2–3

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blastocysts to maintain favorable PRs. However, the more embryos transferred or more prolonged the embryo culture, the greater the risk of multiple gestations and the greater the economic burden.

Cumulus cells (CCs) or granulosa cells around the oocytes are essential for the microenvironmental requirements of follicle maturation, oocyte fertilization and embryo cleavage.[3] During follicle development, CCs undergo rearrangement and interactions. Complexly cooperative and intercellular metabolism exists between oocytes and CCs.

Mitochondria are major organelles that perform cellular respiration. Mitochondria play an important role in many metabolic tasks such as apoptosis-programmed cell death, glutamate-mediated excitotoxic neuronal injury, cellular proliferation, regulation of the cellular redox reaction, heme synthesis, steroid synthesis, etc.[4] Mitochondrial damage is associated with some mitochondria-related diseases. A defective mitochondrial respiratory chain might cause severe adenosine triphosphate (ATP) deficiency and augment reactive oxygen species (ROS) generation, which enhances some pathologic conditions.[5] Mitochondria are closely related to the maturation of mammalian oocytes. However, their functions upon embryo qualities have not been elucidated.

Various deletions of the mitochondrial gene have been reported. A 4977-bp deletion of mtDNA (dmtDNA-Δ5Kb), the most common deletion of mtDNA, is associated with numerous neoplasms.[6] The dmtDNA-Δ5Kb is associated with aging processes in oocytes.[7,8] Mitochondria defect or mutation might contribute to the pathogenesis of chronic degenerative illness such as coronary artery disease,[9] myositis,[10] sudden death[11] and hepatocellular carcinoma.[12] The incidence of dmtDNA-Δ5Kb was significantly higher in older women.[13] The statuses of mtDNA mutations are correlated with the aging processes in skeletal muscle fibers and heart tissue.[14]

Increased mitochondrial activities in CCs are correlated with increased apoptosis in oocytes.[15] Mitochondria in the ooplasm are essential for energy production, which is important for oocyte maturation, fertilization and embryo development. Mitochondrial reorganization and ATP levels are different between morphologically good and poor oocytes, which are responsible for their developmental capacities.[16] Mitochondrial DNA content is very important for embryo development.[17] Mitochondrial DNA deletion in CCs might influence the oocyte maturation and subsequently developmental capacity after fertilization. In this survey we tried to detect the dmtDNA-Δ5Kb and mitochondria copy numbers (MCNs) of CCs. We also evaluated their associations with embryo qualities and IVF outcomes. It might be useful to optimize the oocyte/embryo selection and decrease embryos transferred. To the best of our knowledge, this is among the first reports to qualify and quantify mitochondrial gene expressions in CCs as well as their relationship with IVF outcomes.

Materials and Methods

Taiwanese women who underwent controlled ovarian hyperstimulation (COH), IVF/intracytoplasma sperm injection (ICSI) and embryo transfer (ET) at the Changhua Christian Hospital were recruited. The CCs were collected during oocyte retrievals. This series was approved by the ethics committee and institutional review board of Changhua Christian Hospital. The COH protocol, oocyte insemination and ET were performed as previously described.[18] In brief, during menstrual days 2–7, younger patients (< 34 years) were administered 150–225 IU Gonal-F (Serono, Rome, Italy) daily. Older patients (≥ 34 years) were administered 225–300 IU Gonal-F daily. Ultrasound examination was performed on menstrual days 3, 6, 9 and 12. If the day 8 estrogen (E_2) was < 100 pg/mL, the daily gonadotropins were increased to 225 IU in younger patients and to 300 IU in older patients. Criteria for cancellation included lower E_2 level on menstrual day 8 (< 50 pg/mL) and poor follicle growth during the COH (no follicle growth > 8 mm).

Daily leuprorelin acetate (0.5 mg/day, subcutaneously) was administrated from day 23 of the previous menstrual cycle. Gonadotropin and leuprorelin acetate administration continued until two or more follicles ≥ 18 mm were obtained; then 10,000 IU human chorionic gonadotrophin (hCG) was administered. Serum LH and E_2 concentrations were tested on the day of hCG administration. Oocytes were retrieved transvaginally 34–36 hours later. Follicles were flushed with an HTF-HEPES solution containing 5 U/mL heparin. Oocytes were group cultured in a 900 µL IVF30 media (Vitrolife, Vitrolife Sweden, Göteborg, Sweden) in an organ culture dish covered with mineral oil immediately after oocyte retrieval. Cumulus sample of each oocyte was collected by pasture pipette. After washing with 100 µL human tubal fluid (HTF) and centrifuged at 300 g for 10 minutes, the pellets were im-

mediately stored in –20°C for the following surveys.

Depending on the sperm qualities, either intracellular sperm injection (ICSI) or IVF was chosen for insemination procedures. Around 17–19 hours after insemination, the oocytes were checked for survival and fertilization. On day 2, the morphology of embryos was assessed and scored by the Veek scoring criteria.[19] According to the proportion of fragments, cleaved embryos were assigned to one of the followed categories: Grade I, no fragmentation; Grade II, < 10% fragmentation; Grade III, 10–50% fragmentation; and Grade IV, > 50% fragmentation.

The ET was performed 48 hours after oocyte retrieval. A maximum of 4 embryos with the best qualities were selected and transferred. Luteal phase was supported with 2000 IU/d hCG administration on days 1, 4 and 7 post-ET and 400 mg/d progesterone beginning on day 1 post-ET. Chemical pregnancy was defined as elevated serum β-hCG (> 50 IU/L) 14 days after ET. Clinical pregnancy was determined with the visualization of gestational sac and fetal viability by ultrasound 4 weeks post-ET.

The CC samples were dissolved by adding proteinase K (20 mM, 0.05 mg/mL) at 56° for 1 hour and heat inactivation at 95° for 10 minutes. The dmtDNA-Δ5Kb fragment was amplified in a 20 μL reaction mixture, which contains distilled water 5.8 μL, DMSO 1 μL, 2.5 mM dNTP 5 μL, 20 pmol of each primer 2 μL, 10× buffer 2 μL, 5 IU FastStart Taq (Roche Diagnostics Corporation, Indianapolis, Indiana) 0.2 μL and DNA template 2 μL. The primer sequences and PCR conditions for MITIN and MITOUT are listed in Table I.

The DNA extraction of CCs was performed by M48 reagent (QIAGEN, MagAttract, Valencia, California) and BioRobot M48 machine. To detect the MCN, real-time quantitative PCR (Q-PCR) was performed using Roche LightCycler. We also used 10 ng/1 μL human blood cancer cell K562 DNA (Promega) and β-actin (540 bp) as internal controls. The 20 μL PCR mixture contains 12.6 μL of distilled water, 2.4 μL of MgCL2, 1 μL each of 10 μM primer, 0.5 μL of SYBR green I dye, 0.5 μL of Master Mix and 2 μL of template. The PCR primers and conditions are listed in Table I. The PCR products were examined by 2.5% agarose gel electrophoresis and stained with ethidium bromide (Figure 1).

The position of nuclear DNA in the sample presented at 268 bp; the position of mtDNA presented at 153 bp. PCR products were confirmed by DNA sequencing. For mtDNA 4977 deletion quantitative assay, the 20 μL PCR mixture contains 7.6 μL of distilled water, 2.4 μL of MgCL2, 1 μL each of 10 μM primer, 2 μL of Master Mix, 0.5 μL of probe (4 μM), 5% DMSO and 4 μL of template. The PCR conditions and electrophoresis are presented in Table I and Figure 2.

Personal data (age, body weight, body mass index [BMI], cause of infertility), oocyte/embryo numbers, morphologies and clinical outcomes were collected. The roles of dmtDNA-Δ5Kb and MCN upon embryo qualities and clinical PRs were evaluated. The statistic analyses were performed using the SAS statistic package (Version 8.1, SAS Institute Inc., Cary, North Carolina) with χ^2 and *t* test. A p value < 0.05 was considered statistically significant.

Results

A total of 51 individuals with 504 embryos and 159 transferred embryos were recruited in this survey. The mean ages and BMI were 34 ± 3.5 years and 21.3 ± 3.2, respectively. The clinical PRs were posi-

Table I *Primers and PCR Conditions for MITIN, MITOUT, β-globin, L1-3243 and L4-H2*

Genes	Primers sequences (5′–> 3′)	Denature	Annealing	Extension	DNA fragment size(bp)
MITIN (non-dmtDNA-Δ5Kb)	MITIN F (9518–9538 nt)–CTGAGCCTTTTACCACTCCAG MITIN R (9644–9665 nt)–GGTGATTGATACTCCTGATGCG	94°C/30s	58°C/30s	72°C/30s	142
MITOUT (dmtDNA-Δ5Kb)	MITOUT-F (8432–8500 nt)–CCCAACTAAATACTACCGTATGG MITOUT-R (13477–13590 nt)–GGCTCAGGCGTTTGTGTATGAT	94°C/30s	58°C/30s	72°C/30s	214
β-globin	F-GAAGAGCCAAGGACAGGTAC R-CAACTTCATCCACGTTCACC	95°C/10s	58°C/5s	72°C/14s	540
L1-3243 (non-dmtDNA-Δ5Kb)	L1-F (3304–3322 nt)–AACATACCCATGGCCAACCT 3243-R (3435–3455 nt)–AGCGAAGGGTTGTAGTAGCCC	95°C/10s	58°C/5s	72°C/14s	153
L4-H2 (dmtDNA-Δ5Kb)	L4-F (8343–8362 nt)–ACCAACACCTCTTTACAGTG H2-R (13630–13649 nt)–GGGGAAGCGAGGTTGACCTG	95°C/10s	56°C/10s	72°C/20s	330

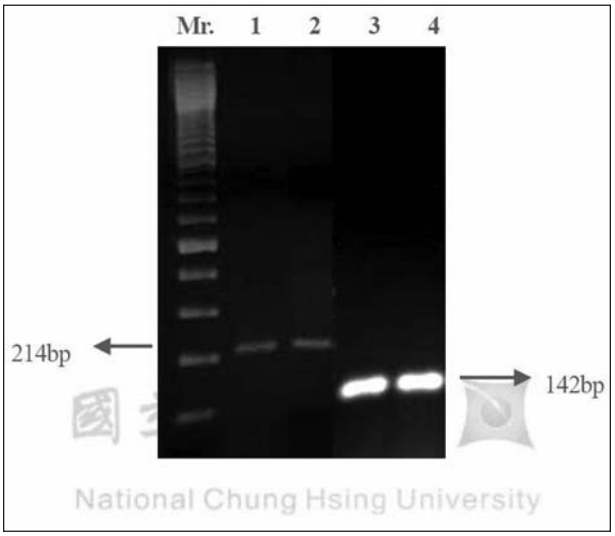

Figure 1 Electrophoresis of PCR products for MITOUT (dmtDNA-Δ5Kb, 214 bp, lanes 1 and 2) and MITIN (non-dmtDNA-Δ5Kb, 142 bp, lanes 3 and 4).

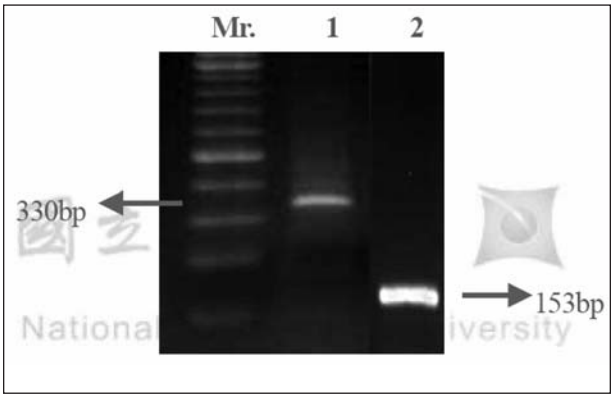

Figure 2 Electrophoresis of PCR products for L4-F/H2-R (dmtDNA-Δ5Kb, 330 bp, lane 1) and L1-F/3243-R (non-dmtDNA-Δ5Kb, 153 bp, lane 2).

tively correlated with younger age, better embryo qualities, and lower dmtDNA-Δ5Kb ratios of transferred embryos (Table II). The clinical PRs in the ≥34-year-old and <34-year-old groups were 12.5 and 40%, respectively (p value <0.05). The E_2 changes were nonassociated with PRs. The E_2 changes between basal and hCG day (Log) were 6.98 ± 0.65 and 7.55 ± 0.67, respectively (Table II, not significantly different). The Gr I/II and Gr III/IV of transferred embryo qualities in clinical pregnancy and nonpregnancy groups were 81.3/18.7% and 67.4/32.6%, respectively (Table II, p value < 0.05). The dmtDNA-Δ5Kb ratios in clinical pregnancy and nonpregnancy groups were 0% and 10.4%, respectively (p value < 0.05).

The dmtDNA-Δ5Kb statuses of CCs were positively associated with maternal age and MCN but were nonassociated with embryo qualities. The percentages of dmtDNA-Δ5Kb in the ≥34-year and <34-year groups were 6.9 and 3.2%, respectively (p value < 0.05). In contrast, the embryo qualities were nonassociated with dmtDNA-Δ5Kb. The dmtDNA-Δ5Kb in Gr I/II and Gr III/IV embryos were 6.5% and 3.1%, respectively (Table III, not significantly different). The dmtDNA-Δ5Kb in transferred Gr I/II and Gr III/IV embryos were 6.9% and 4.6%, respectively (not significantly different). MCNs (Log) in dmtDNA-Δ5Kb and non-dmtDNA-Δ5Kb groups were 5.77 ± 1.04 and 4.99 ± 1.12, respectively (p value < 0.05).

Table II *Age, E_2 Changes, Transferred Embryo Qualities, and dmtDNA-Δ5Kb Statuses in Women With and Without Pregnancy*

	Clinical pregnancy (n = 16)	Nonpregnancy (n = 35)	p Value
Age (yr) (n = 51)			< 0.05
≥34	2 (12.5%)	14 (87.5%)	
< 34	14 (40%)	21 (60%)	
E_2 changes between basal and hCG day (Log)	7.55 ± 0.67	6.98 ± 0.65	NS
Transferred embryo qualities (n = 159)			< 0.05
Gr I/II	52 (81.3%)	64 (67.4%)	
Gr III/IV	12 (18.7%)	31 (32.6%)	
mtDNA-Δ5Kb (n = 159)			< 0.05
Deletion	0 (0%)	10 (10.4%)	
Nondeletion	63 (100%)	86 (89.6%)	

NS = not significant.

Table III *Age, Embryo Qualities, Transferred Embryo Qualities, and Mitochondria Copy Numbers Statuses in Embryos With or Without dmtDNA-Δ5Kb*

Embryos	dmtDNA-Δ5Kb	Non-dmtDNA-Δ5Kb	p Value
Age (yr) (n = 504)			< 0.05
≥ 34	9 (6.9%)	121 (93.1%)	
< 34	12 (3.2%)	362 (96.8%)	
Embryo qualities (n = 504)			
Gr I/II (%)	10 (6.5%)	143 (93.5%)	NS
Gr III/IV (%)	11 (3.1%)	340 (96.9%)	
Transferred embryo qualities (n = 159)			
Gr I/II (%)	8 (6.9%)	108 (93.1%)	NS
Gr III/IV (%)	2 (4.6%)	41 (95.4%)	
Mitochondria copy numbers (Log)	5.77 ± 1.04	4.99 ± 1.12	< 0.05

NS = not significant.

Discussion

In an IVF-ET program, embryo selections are critical for physicians and embryologists. Traditional Veek scoring system is not accurate to predict the qualities of embryos. Around 40% of embryos with normal morphology present with abnormal chromosomes.[20] Therefore, biochemical markers for predicting embryo qualities are essential to optimize embryo selection. Applications of molecular parameter for embryo selections might allow fewer transferred embryos numbers without decreasing PRs.

CCs are imperative for oocyte development and embryo implantation.[19] CCs could secrete hyaluronic acid–rich matrix, which binds the oocyte and CCs together. CCs also facilitate follicular extrusion, oviductal fimbrial capture, sperm penetration and fertilization.[21] CCs are mediators and regulators for oocyte development and fertilization.[22] The presence of CCs during IVM functionally affected the ooplasmic mitochondria distributions and ATP contents, which might affect the calcium release pattern and developmental competence of oocytes.[23] CCs play essential roles in oocyte growth and metabolism by providing nutrients, amino acids and energy.[24,25] CCs are also associated with the biological mechanisms of calcium signals and ATP response toward oocytes.[26]

Mitochondria are maternally inherited organelles that contain oxidative phosphorylation system for ATP supply. Mitochondria are the most abundant organelles in mammalian oocytes and early embryos. Large levels of ATP are essential for gamete development. ATP production is associated with the developmental competences of the oocytes and embryos.[27] The activities and organization of mitochondria are necessary for the maturation and diverse events for oocytes.[28] Transfers of mitochondria into oocytes might be beneficial for oocyte development and embryo implantation.[29] Defects in sperm mitochondria might lead to asthenozoospermia and male infertility.[30]

The mtDNA point mutations or deletions are correlated with dysfunctional mitochondria.[31] When the mutant mtDNA accumulates to a significant level, a reduction in oxidative phosphorylation might occur.[32] Accumulation of mtDNA deletions might contribute to mitochondrial dysfunction and impaired ATP production. Compromised mtDNA expressions are associated with arrested embryos.[25]

Mitochondria-related diseases are associated with impaired mitochondrial electron transport chain (ETC) and intracellular oxidative stress.[34] More reactive oxygen species (ROSs) are generated from mitochondria in ETC-inhibited and mtDNA-damaged cells, which further impair ETC.[34] Oxidative stress, ionizing radiation, superoxides and other ROSs would increase the risks of mitochondria mutation.[35] Dysfunctional mitochondria expose the cell suffering from oxidative phosphorylation and ROS. The mtDNA point mutations or deletion might contribute to the poor bioenergetic statuses in mitochondria. The dmtDNA-Δ5Kb increases the susceptibility of human cells to UV-induced apoptosis in a quantitative manner.[36] However, information about the association between dmtDNA-Δ5Kb and mitochondrial ROS generation is insufficient. The dmtDNA-Δ5Kb disrupts the mitochondrial complex I, IV and V on the electron transport chain (ETC).[34]

A large score of mtDNA mutations in neoplasm suggests the contributions of mitochondrial defect

upon tumorogenesis.[37] Total mitochondrial mass and MCN determines the functional competency of cells.[38] Reduced MCN, impaired mitochondrial biogenesis and somatic mutations in mtDNA are important events during carcinogenesis.[39] MCN levels are different in individual tissues; the highest MCNs appeared in the cardiac muscle, while the lowest MCNs presented in the cortex cerebellum.

In this study we observed that the older age group was associated with a higher percentage of dmtDNA-Δ5Kb in CCs and lower PRs. The dmtDNA-Δ5Kb statuses of CCs were negatively associated with PRs during IVF-ET. The MCN was positively associated with dmtDNA-Δ5Kb, whereas negatively associated with PRs. Our findings were compatible with the reports of Gibson et al[39] and Hsieh et al.[33] Gibson et al[39] demonstrated that accumulation of dmtDNA-Δ5Kb might contribute to mitochondrial dysfunction and impaired ATP production in oocytes. Hsieh et al[33] demonstrated the decreased mitochondrial gene expressions in compromised oocytes and embryos. Defective mtDNA might compromise the oxidative phosphorylation and mitochondrial RNA, which further affects the oocyte and embryo qualities.[33]

In our survey we observed the noncorrelation between the dmtDNA-Δ5Kb statuses and embryo morphology scoring. Their noncorrelation might be due to the low accuracy of Veek's assessment system upon the evaluation of embryo genetic statuses. It also suggested that assessment of gamete/embryo qualities could not be made simply by traditional morphology scoring systems. Our findings indicated that mitochondria statuses of CCs might influence the oxidative phosphorylation as well as oocyte activation, maturation, fertilization and embryo development.

In conclusion, dmtDNA-Δ5Kb and MCN in CCs are potential tools for oocyte evaluation and embryo selection during IVF-ET. The surveys for dmtDNA-Δ5Kb and MCN of CCs provide direct assessments of the biological statuses of oocytes and embryos without compromising their integrity. Lower levels of dmtDNA-Δ5Kb and MCN in CCs are associated with higher implantation rates of embryos. The dmtDNA-Δ5Kb statuses are not closely associated with embryo scoring qualities. Our findings provide a valuable database for the future surveys of mitochondrial expressions in ART. However, the real roles of mtDNA variations upon the oocyte and embryo developments remain to be clarified. The underlying molecular and biological mechanisms as well as other mitochondrial genetic variations upon gamete and embryo development merit further surveys.

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