FERTILITY AND STERILITY®
VOL. 81, SUPPL. 1, MARCH 2004

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Printed on acid-free paper in U.S.A.

Decreased expression of mitochondrial genes in human unfertilized oocytes and arrested embryos

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Objective: To evaluate the relationship between mitochondrial gene expression of oocytes/embryos and their fertilizability in unfertilized oocytes, arrested embryos, and tripronucleate zygotes, because both nuclear and cytoplasmic factors contribute to oocyte activation, fertilization, and subsequent development.

Design: Prospective laboratory research.

Setting: In vitro fertilization (IVF) laboratory in a university hospital.

Patient(s): Seventy-five unfertilized oocytes, 45 arrested embryos, and 24 tripronucleate (3PN) embryos from 45 female patients undergoing IVF.

Intervention(s): Analysis of mitochondrial gene expression by semiquantitative reverse transcription polymerase chain reaction (RT-PCR).

Main Outcome Measure(s): Comparison of the expression levels of mitochondrial genes including ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, and Cyt b in three groups.

Result(s): Significantly decreased transcription levels were expressed in unfertilized oocytes and arrested embryos. The average expression levels of the eight determined genes compared with the control (GAPDH) was 4.4 ± 0.7, 6.4 ± 1.1, and 13.2 ± 1.1 in unfertilized oocytes, arrested embryos, and 3PN embryos, respectively. Significantly decreased expressions of the ATPase 6, CO III, and ND3 genes were detected from samples with 4977-bp common deletion in the mitochondrial DNA (mtDNA) compared with the non-deletion group.

Conclusion(s): The present study is the first report to present globally decreased mitochondrial gene expression levels in human compromised oocytes and embryos. These data support the notion that the down-regulation of mitochondrial RNA by defective oxidative phosphorylation genes possibly affects oocyte quality including fertilization and further embryo development. (Fertil Steril® 2004;81(Suppl 1):912–18. © 2004 by American Society for Reproductive Medicine.)

Key Words: Embryo, mitochondrion, oocyte, RT-PCR

Received July 28, 2003; revised and accepted November 18, 2003.

Supported by research grants from the National Science Council of the R.O.C. (NSC 91-2320-B-038-026) and Taipei Medical University (TMU 90-Y05-A105 and TMU 91-Y05-A115).

Presented at the 57th Annual Meeting of the American Society for Reproductive Medicine, Orlando, Florida, October 20–25, 2001 (Program: O21).

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0015-0282/04/$30.00
doi:10.1016/j.fertnstert.2003.11.013

In eukaryotic cells, mitochondria are special organelles that are responsible for the synthesis of adenosine triphosphate (ATP). Two distinct genomes exist in all eukaryotic cells. One is located in the nucleus and is transmitted in the mendelian fashion, and the other is located within the mitochondria and is transmitted through maternal lineage. The respiratory chain comprises five enzyme complexes located on the inner mitochondrial membrane. Complex I is the largest of these proteins with at least 26 polypeptides. The NAD-linked substrates feed reducing equivalents into the chain via complex I, which passes electrons down the chain to ubiquinone.

Complex II accepts reducing equivalents from succinate and subsequently passes electrons to ubiquinone. From ubiquinone, electrons pass to complex III, to cytochrome c, and then via cytochrome oxidase to oxygen. The electrochemical proton gradient that supplies the energy for complex V to generate ATP is produced by complexes I, III, and IV (1–3). Present in one or more copies in every mitochondrion, mtDNA comprises a circular, histone-free molecule composed of 16.6 kb of DNA.

There are 13 protein subunits: NADH:ubiquinone oxidoreductase subunit 1 (ND1), ND2, ND3, ND4, ND4L, ND5, and ND6 of complex I; cytochrome b of complex III; cytochrome c oxidase subunit 1 (CO I), CO II, and CO III of complex IV; and subunits 6 and 8 of

ATPase of complex V. They are required for oxidative phosphorylation of a total of about 83 subunits, with the remainder of 70 subunits being encoded by nuclear genes and imported into the mitochondrion. Also, mtDNA contains two ribosome subunits and 22 transfer RNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes; mtDNA encodes 13 proteins that are all components of the respiratory chain, whereas nuclear DNA encodes the majority of the respiratory chain proteins, all proteins that regulate replication and transcription of mtDNA, as well as proteins necessary for the biogenesis of mitochondria (2).

The mitochondria in an oocyte must have produced and stored all the energy required for the resumption of meiosis II, fertilization, and development of the embryo (4–6). Deficiencies in mitochondrial ATP production may be associated with impairment of oocyte fertilization or retarded embryonic development at later stages (7, 8). When the mutant mtDNA accumulates to a significant level, a reduction in oxidative phosphorylation efficiency may occur (9, 10). In humans, germ-line cells are derived from primordial germ cells, which are conspicuous in the developing zygote by the third week after conception. Quiescent primordial follicles might not enter meiotic division for a period of up to 40 years, and therefore they are expected to accumulate abundant mutant mtDNA in oocytes which may be unable to produce enough energy because of a dysfunction in the oxidative phosphorylation system. Recent studies have shown that the accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and the failure of embryonic development (8, 11, 12).

Furthermore, mitochondrial replication for maintenance of a proper amount of functional mitochondria, transcription for synthesis of mtRNA, and translation for mitochondrial biogenesis during oogenesis is crucial for successful fertilization and embryo development. We examined the expression levels of eight mitochondrial genes, the ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, and Cyt b genes, in human unfertilized oocytes and abnormal embryos to determine whether decreased expression of mitochondrial genes possibly interferes with developmental capacity.

MATERIALS AND METHODS

Preparation of Oocytes

The Institutional Review Board of Taipei Medical University Hospital approved the study we performed on oocytes and embryos discarded during an in vitro fertilization (IVF) program. Oocytes were obtained from patients who were recruited into an IVF-ET (embryo transfer) program. Ovarian stimulation was performed by desensitization by using a gonadotropin-releasing hormone agonist (GnRH-a) followed by treatment with gonadotropins (FSH and hMG). Ovulation was induced using hCG. Oocytes were then retrieved by transvaginal ultrasonography-guided aspiration 34 to 36 hours after hCG administration. Oocytes were inseminated in vitro with spermatozoa for 16 to 18 hours and subsequently cultured in human tubal fluid (HTF; Irvine, Santa Ana, CA) supplemented with 10% human plasmanate (8). Twenty hours after insemination, cumulus cells were mechanically removed, and oocytes were examined for the presence of pronuclei.

Unfertilized oocytes collected 48 hours after retrieval were prepared for evaluation of mitochondrial gene expression. Abnormal embryos were harvested when embryos were arrested or severely fragmented at two to four blastomeres from unknown causative factors. Tripronucleate embryos obtained 18 to 24 hours after IVF were cultured until they were four to eight blastomeres and then were prepared for analysis. From 48 patients enrolled in the IVF procedures, 75 unfertilized oocytes were donated to our research. In addition, 45 embryos that were abnormally arrested and 24 three-pronuclei (3PN) zygotes unsuitable for embryonic replacement or cryopreservation were also donated and used for the following experiments.

Semiquantitative RT-PCR

Total RNA extracted from oocytes and embryos was used as templates and cDNA was prepared using the RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) kit from Ambion (Austin, TX). The RT-PCR amplifications were performed with 3 μL of cDNA in a total volume of 50 μL of amplification buffer, 40 pmol of specific primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). The sequences of the oligonucleotide primers used in this study are listed as follows: ND2 (Forward, np5101-5120, TAACTACTACCGCATTCCTA; Reverse, np5400-5381, CGTTGTTAGATATGGGGAGT), CO I (Forward, np7041-7060, GTCCTATCAATAGGAGCTGT; Reverse, np7340-7321, CTTCGAAGCG AAGGCTTCTC), CO II (Forward, np7845-7864, CAGACGAGGTCAACGATCCC; Reverse, np8130-8111, GTTTGGTTTAGACGTCCGGG), ATPase 6 (Forward, np8781-8800, CGGACTCCTGCCTCACTCAT; Reverse, np9090-9071, AGAGGGAAGGTTAATGGTTG), CO III (Forward, np9611-9630, CGTATTACTCGCATCAGGAG; Reverse, np9908-9889, GCCAAAGTGATGTTTGGATG), ND3 (Forward, np9981-10000, TGAGGGTCTTACTCTTTTAG; Reverse, np10300-10281, GTTTGTAGGGCTCATGG TAG), ND6 (Forward, np14291-14310, TCATAAATTATTCAGCTTCC; Reverse, np14579-14560, TGATTGTTAGCGGTGTGGTC), Cyt b (Forward, np15506-15525, GACAATTATACCCTAGCCAA; Reverse, np15800-15781, GTCCAATGATGGTAAAAGGG), and GAPDH (Forward, CCTTCATTGACCTCAAC; Reverse, AGTTGTCATGGATGACC).

For semiquantitative amplification, each cycle was carried out at 92°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds. The reactions were analyzed after 15,

TABLE 1

Expression ratios of oxidative phosphorylation genes in mtDNA in unfertilized oocytes, arrested embryos, and 3PN embryos compared with GAPDH.

Group	ND2	COI	COII	ATPase6	COIII	ND3	ND6	Cyt b	Average
Unfertilized	4.5 ± 0.6[a]	4.6 ± 0.7[a]	4.7 ± 0.8[a]	4.0 ± 1.0[a]	3.9 ± 0.8[a]	3.8 ± 0.7[a]	4.6 ± 0.5[a]	4.8 ± 0.7[a]	4.4 ± 0.7
Arrested	6.9 ± 1.2[a]	6.9 ± 0.6[a]	6.3 ± 1.1[a]	5.8 ± 1.4[a]	5.5 ± 1.7[a]	5.9 ± 1.4[a]	7.1 ± 1.4[a]	6.9 ± 0.7[a]	6.4 ± 1.1
3PN	13.1 ± 1.1[b]	12.5 ± 0.8[b]	13.1 ± 0.9[b]	13.7 ± 1.3[b]	13.1 ± 1.1[b]	12.9 ± 1.8[b]	13.7 ± 0.6[b]	13.2 ± 0.8[b]	13.2 ± 1.1

Note: Data are presented as mean ± SEM.

[a,b] Values with different superscripts denotes statistically significant difference ($P<.05$; ANOVA).

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20, 25, 30, 35, and 40 cycles to optimize the linear range of amplification. The PCR reactions were optimized with respect to annealing temperature and numbers of PCR cycles. Each PCR product was run through a 2% agarose gel and was visualized with ethidium bromide staining. Cycle-dependent amplification of the housekeeping GAPDH mRNA was almost identical in oocytes, arrested embryos, and 3PN embryos, which allowed semiquantitative comparison of mtDNA PCR products obtained with each sample by densitometric analysis. The relative expression levels of the PCR products were determined using an imaging desitometer, and results were expressed as a ratio of above eight genes divided by GAPDH.

Determination of the 4977-bp Deleted mtDNA

Oocytes and embryos were stored in 20 μL of 1× PCR buffer containing 0.05 mg/mL of proteinase K, 20 mM DTT, and 1.7 μM SDS. After digestion for 1 hour at 56°C and 10 minutes of heat-inactivation of proteinase K at 95°C, this template was then used in the PCR assays. The sequences of the oligonucleotide primers used in this study are listed as follows: H1 (np 8285-8304 CTCTAGAGCCCACTGTAAAG) and L1 (np 13650-13631 GGGGAAGCGAGGTTGACCTG). The mtDNA was amplified in a 100-μL reaction volume containing a final concentration of 1.5 mM $MgCl_2$, 1 mM dNTPs, 20 pmol of each primer, and 1.5 IU Taq polymerase (Life Technologies). Then the following amplification profile was used: 1 cycle of 95°C for 5 minutes; 35 cycles of 95°C for 40 seconds, 58°C for 40 seconds, 72°C for 6 minutes, and 1 cycle of 72°C for 7 minutes, then the mixture was kept at 4°C (8).

The PCR products were examined by agarose gel electrophoresis in which 8 μL of PCR products was separated on a 1.5% agarose gel and stained with ethidium bromide. The PCR products were cloned into a pGEM-T vector (Promega, Madison, WI). We performed DNA sequencing using the Dye Terminator cycle sequencing kit (Applied Biosystems, Foster, CA). Sequencing reactions were read on an ABI Prism Model 377 cycle sequencer (Applied Biosystems).

Statistical Analysis

All experiments were repeated at least three times. An ANOVA analysis was used to test statistically significant differences ($P<.05$) between experimental groups.

RESULTS

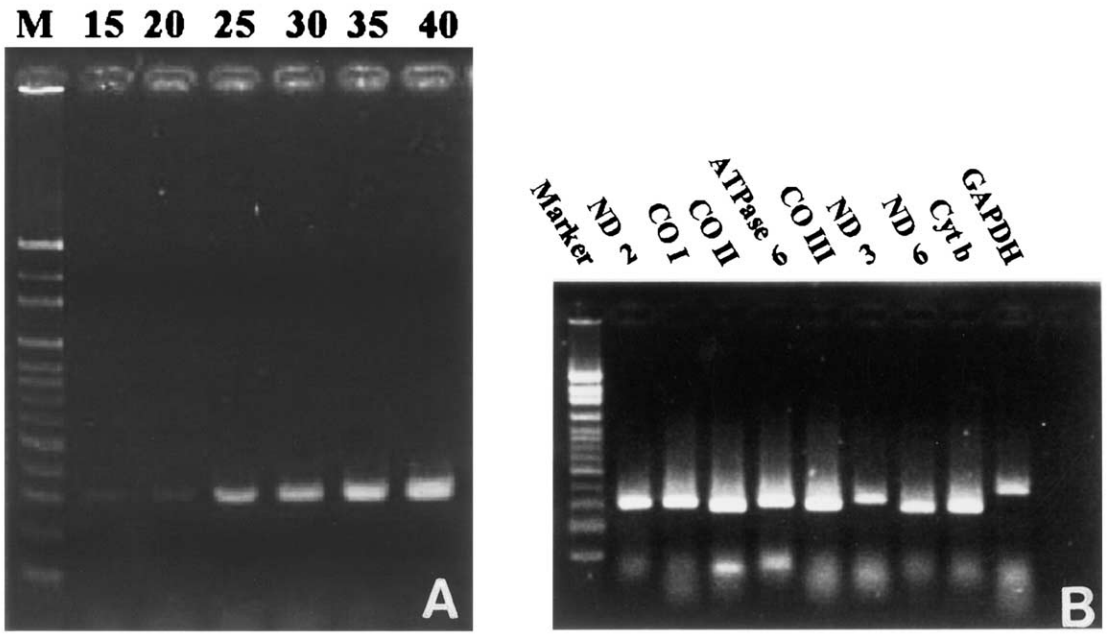
Transcript levels of the ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, and Cyt b genes in oocytes and embryos were determined. To measure the relative expression levels of oxidative phosphorylation genes in each oocyte or embryo, semiquantitative RT-PCR was performed on total RNA extracted from unfertilized oocytes, arrested embryos, and tripronucleate embryos. The PCR products of 15, 20, 25, 30, 35, and 40 cycles were analyzed to determine the optimal amplification conditions for RT-PCR reactions. Twenty cycles were necessary to visualize the ND2 PCR product of the expected size (300 bp). Performing additional cycles enhanced DNA amplification without the appearance of any other band, arguing in favor of the specificity of the reaction (Fig. 1A). As expected, no PCR product was observed after 40 amplification cycles in the control reaction without cDNA by reverse transcription.

The expression levels of all eight examined mitochondrial genes and the GAPDH gene were determined by densitometric analysis of RT-PCR products (see Fig. 1B). To obtain the relative expression levels of the eight genes, each of them was followed by normalization to the GAPDH gene. Samples were collected into three groups according the development stage of oocytes and embryos. We collected 16, 9, and 6 cohorts of unfertilized oocytes, arrested embryos, and tripronucleate zygotes, respectively, with each cohort including three oocytes or embryos from the same donor. The normalized expression ratios of the eight different genes in each sample are presented in Table 1.

These eight gene transcription levels remained at similar levels in the same groups, with no statistically significant differences among analyzed genes in each cohort. However, there were statistically significant decreases in transcript levels expressed in unfertilized oocytes and arrested embryos compared with 3PN embryos. The mean ratios of the

FIGURE 1

(**A**) Optimal amplification cycles of RT-PCR. After reverse transcription of total RNA from oocytes, the ND2 transcript was amplified with 15, 20, 25, 30, 35, and 40 cycles. (**B**) Semiquantitation of mitochondrial RNAs by RT-PCR. *Lanes 1 to 9* represent the PCR products of the ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, Cyt b, and GAPDH genes.



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expression of the eight different genes was 4.4 ± 0.7, 6.4 ± 1.1, and 13.2 ± 1.1 in unfertilized, arrested, and 3PN embryos, respectively (see Table 1).

In our previous study, the 4977-bp rearranged mtDNA was commonly observed to exist in oocytes and embryos, and resulted in compromised developmental capacity (8). To examine whether rearranged mtDNA in oocytes and embryos affects expression levels of mtDNA genes, mitochondrial RNA expression was also classified according to samples with or without the 4977-bp deletion (Table 2). The frequencies of 4977-bp mtDNA were 62.7%, 31.1%, and 20.8% in unfertilized oocytes, arrested embryos, and 3PN embryos, respectively. In a comparison of cohorts with and these without the 4977-bp deletion, there were statistically

TABLE 2

Expression ratios of oxidative phosphorylation genes in mtDNA with or without 4977-bp deletion in unfertilized oocytes, arrested embryos, and 3PN embryos compared with GAPDH.

Group	ND2	CO I	CO II	ATPase 6	CO III	ND3	ND6	Cyt b
Unfertilized oocyte								
With 4977 bp deleted mtDNA	4.5 ± 0.7[a]	5.0 ± 0.7[a]	4.6 ± 1.0[a]	2.6 ± 1.0[c]	2.6 ± 0.9[c]	2.5 ± 0.6[c]	4.7 ± 0.4[a]	5.0 ± 0.7[a]
Without 4977 bp deleted mtDNA	4.4 ± 0.6[a]	4.2 ± 0.7[a]	4.8 ± 0.5[a]	4.5 ± 0.9[a]	4.3 ± 0.7[a]	4.2 ± 0.6[a]	4.5 ± 0.6[a]	4.6 ± 0.7[a]
Arrested embryo								
With 4977 bp deleted mtDNA	7.7 ± 0.4[a]	6.9 ± 0.7[a]	6.3 ± 0.5[a]	4.1 ± 0.6[c]	3.6 ± 0.6[c]	4.4 ± 0.6[c]	6.4 ± 0.3[a]	7.1 ± 1.1[a]
Without 4977 bp deleted mtDNA	6.5 ± 1.3[a]	6.9 ± 0.5[a]	6.3 ± 1.4[a]	6.6 ± 0.6[a]	6.5 ± 1.1[a]	6.7 ± 0.9[a]	7.4 ± 1.6[a]	6.8 ± 0.6[a]
Tripronucleate embryo								
With 4977 bp deleted mtDNA	13.9 ± 0.9[b]	13.4 ± 0.1[b]	13.0 ± 1.8[b]	14.8 ± 0.6[b]	13.5 ± 2.3[b]	14.3 ± 0.6[b]	13.9 ± 0.9[b]	12.9 ± 0.4[b]
Without 4977 bp deleted mtDNA	12.7 ± 1.0[b]	12.0 ± 0.6[b]	13.1 ± 0.5[b]	13.1 ± 1.3[b]	13.0 ± 0.5[b]	12.2 ± 1.8[b]	13.6 ± 0.6[b]	13.4 ± 1.0[b]

Note: Data are presented as mean ± SEM.

[a,b,c] Values with different superscripts denotes statistically significant difference ($P < .05$; ANOVA).

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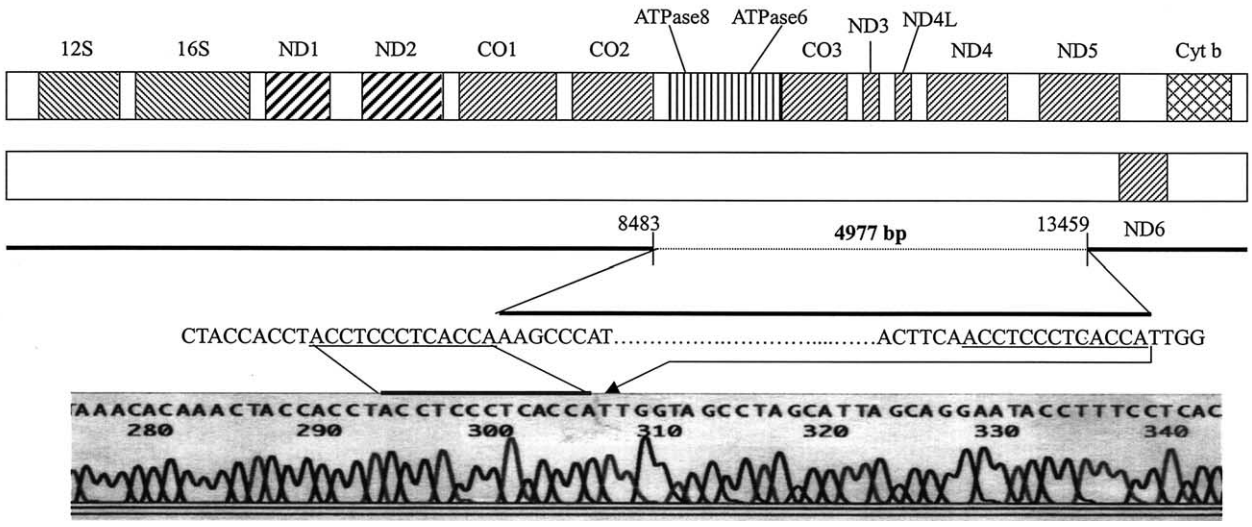
FIGURE 2

The relationship between defective mtDNA gene expression in oocytes and embryos with common 4977-bp deletion.



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significant decreases in RNA expressions of the ATPase 6, CO III, and ND 3 genes in unfertilized oocytes and arrested embryos harboring the 4977-bp deletion. However, there were no differences in 3PN embryos with or without the 4977-bp deletion (Fig. 2).

DISCUSSION

Unknown factors causing oocytes to remain unfertilized and abnormal embryo development may be related to cytoplasmic defects of the oocytes. In particular, the organization and continued metabolic activity of mitochondria are necessity for cytoplasmic maturation and resumption of meiosis (7, 13). Accumulation of mtDNA mutation, decreased mtDNA copy number, and decreased mtRNA expression reflecting mitochondrial defects may compromise the maturation of oocytes. The 4977-bp deletion, the most common mtDNA deletion associated with human aging processes, is found in oocytes and embryos (8, 14, 15). In this study, the frequencies of 4977-bp mtDNA were 62.7%, 31.1%, and 20.8% in unfertilized oocytes, arrested embryos, and 3PN embryos, respectively. The results similar with our previous report, the frequencies of 66.1% in unfertilized oocytes, 34.8% in arrested embryos, and 21.1% in tripronucleate (3PN) embryos (8).

There was a statistically significant increase in the proportion of deleted mtDNA in unfertilized oocytes. Accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and impaired ATP production, and may also interfere with fertilization of human oocytes and subsequent embryonic development (8, 11). Frequencies of the 4977-bp deleted mtDNA in oocytes have also been determined and reported by other studies (11, 16, 17). The 4977-bp deletion causes the removal of major structural genes containing ATPase 6 and 8, cytochrome oxidase III, and NADH-CoQ oxidoreductase (ND3, ND4, ND4L, and ND5) (see Fig. 2). The deleted genes in this rearranged mtDNA may result in impaired gene expression by decreasing the expression of corresponding genes in H strand of circular mtDNA.

This deletion also creates a chimeric gene, which fuses the 5′-portion of ATPase 8 and the 3′-portion of ND5 genes of mtDNA. Therefore, the deleted or truncated genes in this rearranged mtDNA may result in decreasing the expression of the deleted genes by producing transcripts of fused genes. In this study we demonstrated that lower expression levels of ATPase6, CO III, and ND3 genes in unfertilized oocytes and arrested embryos with the 4977-bp deleted mtDNA were significant than other genes outside of the deleted region. The decreased transcription reflected rearranged mtDNA in unfertilized oocytes and arrested embryos. However, compensatory transcription may take place in 3PN zygotes with rearranged mtDNA. There was not statistically significant decrease in expression levels in 3PN zygotes with or without the 4977-bp deletion in mtDNA, indicating the sperm factor plays an important role in 3PN formation.

Piko and Taylor (4) reported that mouse mitochondrial DNA does not replicate during preimplantation development but is transcribed actively from the two-cell stage. There is about a 30-fold rise during cleavage through the blastocyst

stage (18). To date, there is no experimental data to show mitochondrial expression levels in different stages of human embryos. Increased expression of mtDNA will accompany with embryo development, and lower transcription ability may interfere with oocyte fertilization or embryo development. Because amounts of RNA in single oocytes or embryos were too low to determine the expression level of specific genes by Northern blotting, in this study cohorts of every three oocytes or embryos were lysed to harvest total RNA and determine the semiquantitative expression level of RNA by RT-PCR. To rule out limitations of RT-PCR, various replication cycles were decided to identify the optimal cycles with RT-PCR products in a linear range. The average expression proportions (mean ± SEM) of the eight studied genes compared with the control was 4.4 ± 0.7, 6.4 ± 1.1, and 13.2 ± 1.1 in unfertilized oocytes, arrested embryos, and 3PN embryos, respectively.

The ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, and Cyt b gene transcription levels remained at similar levels in the same groups. The mtDNA transcripts are polycistronic (19, 20), which means that each gene is separated following precise endonucleolytic excision of the tRNAs from the nascent transcripts. Although different mtDNA transcripts encode different protein assemblies to one of five mitochondrial complexes, in our study the expression levels of the eight genes were similar in unfertilized oocytes, arrested embryos, and tripronucleate zygotes. The polycistronic mtDNA transcripts are consistent, with different mtRNA expression levels showing the same pattern in the same oocyte. The lower expression level representing the overall defective transcription was observed in unfertilized oocytes. There was a higher expression level in 3PN embryos compared with unfertilized oocytes and arrested embryos. In this study, arrested or severely fragmented embryos were collected at the two-cell to four-cell stage, and 3PN zygotes were collected at the four-cell to eight-cell stage with normal growth rates.

In normal embryonic development, the stage with two to four blastomeres may have more than twice the expression level of mtRNA compared with unfertilized oocytes. In fact, mitochondrial transcription appeared to be hampered from unfertilized oocytes to the four-cell stage of the examined arrested embryos, and hence there was no statistically significant difference in expression levels between unfertilized oocytes and arrested embryos in this study. Oocytes with intact mtDNA resumed with transcription may correlate with sufficient production of ATP. Differences in the amount of ATP generated by mature human oocytes may be related to the fertilization potential and developmental competence of an embryo (7).

There was almost a threefold higher expression level of the oxidative phosphorylation subunits in 3PN zygotes compared with unfertilized oocytes. Mitochondrial RNA expression did not seem to be modified in embryos developed with abnormal 3PN. The existence of 3PN would be normal fertilization by a single injected spermatozoon along with the nonextrusion of a second polar body or merely a polyspermy (21, 22). In addition, injection with the cytoplasm of 3PN zygotes may enhance the clinical pregnancy rate in patients with repeated implantation failure (23). This implies that 3PN zygotes may have sufficient amounts of mtDNA content and mtDNA transcripts for embryo development. Our report does not contradict the contention that decreased expression levels of the ATPase 6 gene in unfertilized oocytes compared with early cleavage-stage 3PN embryos (24).

In conclusion, the current study is the first report to present globally decreased mitochondrial gene expression levels in human compromised oocytes and embryos. These data support the notion that the down-regulation of mitochondrial RNA possibly affects oocyte fertilization and embryo development.

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