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Multiple rearrangements of mitochondrial DNA in unfertilized human oocytes

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Objective: To determine the rearrangement of mitochondrial DNA (mtDNA) in unfertilized human oocytes and compromised embryos to evaluate the fertilization capacity of oocytes.

Design: Prospective laboratory research.

Setting: IVF laboratory in a university hospital.

Patient(s): One hundred twenty-four unfertilized oocytes, 98 arrested embryos, and 45 tripronucleate (3PN) embryos from 65 female patients undergoing in vitro fertilization (IVF).

Intervention(s): Unfertilized oocytes and poor quality embryos were collected 48 hours after IVF.

Main Outcome Measure(s): Comparison of the frequency of mtDNA deletions and fertilization rates of oocytes.

Result(s): Multiple deletions of mtDNA were found in unfertilized oocytes and arrested embryos obtained from IVF patients. A 4977-bp deletion was the most frequent deletion in human oocytes and embryos. About 66.1% of the unfertilized oocytes, 34.8% of the arrested or fragmented embryos, and 21.1% of the 3PN embryos harbored the 4977-bp deletion of mtDNA. There was a significant increase in the proportion of deleted mtDNA in unfertilized oocytes.

Conclusion(s): Accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and impaired ATP production. We conclude that the accumulation of rearranged mtDNA may interfere with fertilization of human oocytes and further embryonic development. (Fertil Steril® 2002;77:1012–7. ©2002 by American Society for Reproductive Medicine.)

Key Words: Embryo, infertility, mtDNA rearrangement, oocyte, tripronucleate

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In eukaryotic cells, mitochondria are specialized organelles that catalyze the formation of ATP. Two distinct genomes exist in all eukaryotic cells. One is located in the nucleus and is transmitted in a Mendelian fashion, whereas the other is located in mitochondria and is transmitted by maternal inheritance. Most human somatic cells contain about 1000 mitochondria, and each mitochondrion consists of 2 to 10 copies of mitochondrial DNA (mtDNA) (1). There is a higher copy number of mtDNA in mature oocytes but with just one mtDNA molecule per organelle (2). The mtDNA copy number of a bovine oocyte was estimated to be 260,000, which is about 100 times higher than that of somatic cells in animals (3). The number of mtDNA in a mouse oocyte was approximately 92,000 (2), and that of the human oocyte was estimated to be about 100,000 (4).

Human mtDNA is a circular, histone-free molecule composed of 16.6 kb of DNA, present in one or more copies in every mitochondrion. It encodes 13 protein subunits out of a total of about 80 constituting the oxidative phosphorylation system, the remainder being encoded by nuclear genes and imported into the mitochondrion. Human mtDNA also encodes 2 ribosome RNAs and 22 transfer RNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes. Whereas mtDNA encodes 13 polypeptides that are the components of the respiratory system, nuclear DNA encodes the majority of the respiratory chain proteins and all of the proteins and enzymes that regulate replication and transcription of mtDNA (5).

First proposed by Harman (6), the free radical theory is one of the most comprehensive

theories of aging. It suggests that living organisms age because of accumulation of oxygen radical–induced cellular damage and that mtDNA is a possible target of free radical attack during the aging process. In humans, germ-line cells are derived from primordial cells, which are conspicuous in the developing zygote by the third week after conception. Following sexual differentiation, the number of germ cells increases through mitotic division from a few hundred to several million, reaching its peak in humans at 20 weeks of gestational age (7). Quiescent primordial follicles might not enter meiotic division for a period of up to 40 years, and therefore they have been postulated to accumulate considerable mtDNA mutations.

In recent years, an increasing number of reports have shown that mtDNA mutations are associated with human aging and mitochondrial diseases (8–10). Large-scale deletions of mtDNA are responsible for specific neuromuscular diseases, the most common being Kearns-Sayre syndrome (KSS) or chronic progressive external ophthalmoplegia (CPEO) syndrome. A 4977-bp deletion occurs in a "hot spot" region, which is flanked by two 13-bp direct repeats beginning at positions 8470 and 13,447, respectively (11, 12). So far, more than 150 types of mtDNA rearrangements have been reported (13). Deleterious mtDNA rearrangements cause cellular energy deficiencies and result in clinical disorders. In human oocytes, the mitochondria must produce and store all the energy required for the resumption of meiosis II, fertilization, and development (14, 15). Deficiencies in mitochondrial ATP production may be associated with the impairment of oocyte fertilization. In human in vitro fertilization (IVF), differences in the amount of ATP generated by mature human oocytes may be related to fertilization potential and developmental competence of an embryo (16).

To detect and characterize rearrangements of mtDNA in human oocytes and embryos, the unfertilized oocytes and arrested and tripronucleate (3PN) embryos were collected with consent from infertile women of different age groups undergoing IVF. Total cellular DNA from all of these samples were subjected to molecular analysis by polymerase chain reaction (PCR) and DNA sequencing.

MATERIALS AND METHODS

Human Oocytes and Embryo Collection

This study was approved by the institutional review board of Taipei Medical University Hospital. From 65 patients enrolled in an IVF program, 124 unfertilized oocytes were donated to our laboratory for research. In addition, 98 embryos that were abnormally arrested, and 45 3PN embryos unsuitable for embryonic replacement or cryopreservation were also donated and used for the following experiments.

Polymerase Chain Reaction

Oocytes and embryos were stored in 20 μL of 1× PCR buffer containing 0.05 mg/mL of proteinase K, 20 mM of dithiothreitol (DTT), and 1.7 μM of SDS. After digestion for 1 hour at 56°C and 10 minutes of heat-inactivation of proteinase K at 95°C, the total DNA in the solution was then used as template for the PCR assay. The sequences of the oligonucleotide primers used in this study are listed as follows: H1 (np 8285–8304, CTCTAGAGCCCACTGTAAAG), H2 (np 8781–8800, CGGACTCCTGCCTCACTCAT), H3 (np 9207–9226, ATGACCCACCAATCACATGC), L1 (np 13650–13631, GGGGAAGCGAGGTTGACCTG), and L2 (np 14145–14126, TGTGATTAGGAGTAGGGTTA), L3 (np 15896–15877, TACAAGGACAGGCCCATTTG) and L4 (np 16410–16391, GAGGATGGTGGTCAAGGGAC).

The desired mtDNA fragment was amplified in a 100-μL reaction mixture containing a final concentration of 1.5 mM of $MgCl_2$, 1 mM of dNTPs, 20 pmol of each primer, and 1.5 IU of Taq polymerase (Life Technologies, Grand Island, NY). 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 it was kept at 4°C. For nested PCR, 5 μL of the first PCR product was used as a template. The strategy of second-round PCR was developed to amplify products only when mtDNA rearrangements were present. The procedure was carried out using the same PCR conditions as mentioned above. 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.

DNA Sequencing

The PCR products were cloned into a pGEM-T vector (Promega, Madison, WI). DNA sequencing was performed using the Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were read on an ABI Prism Model 377 cycle sequencer (Applied Biosystems).

Statistical Analysis

Chi-square analysis was used to determine statistically significant differences ($P<.05$) between experimental groups.

RESULTS

To clarify the relationship between mitochondrial defect and the fertilization potential of oocytes, we collected 124 unfertilized oocytes, 98 compromised embryos, and 45 3PN embryos. Total cellular DNA was extracted from these donated samples and examined for mtDNA mutations with PCR using nested primers. Multiple PCR fragments with lengths shorter than expected were observed after PCR amplification of deleted mtDNA with specific primer sets (Table 1). Thirteen mtDNA rearrangements were identified, and the deletion junction was determined by direct sequencing of the PCR products. Table 1 shows the length of deletions, deletion junctions, and primers used to characterize the 13 established mtDNA deletions.

TABLE 1

Types of mitochondrial DNA rearrangements in human unfertilized oocytes, arrested embryos, and 3PN embryos.

Deletion type	Deleted length (bp)	Primer pairs	PCR product (bp)	Deletion junction	Direct repeat sequence
I (13/13)[a]	4977	H1+L1	389	8470–13446	ACCTCCCTCACCA
I (12/12)	7436	H1+L4	690	8637–16072	CATCAACAACCG
I (12/12)	4237	H3+L2	702	9486–13722	TTCGCAGGATTT
I (10/10)	4642	H3+L2	297	9431–14072	CCAAAAAGGC
I (9/9)	4365	H3+L2	574	9579–13943	AATCCCCTA
I (7/7)	7150	H1+L3	462	8581–15730	GCCGCAG
I (7/7)	5228	H1+L2	633	8771–13998	CTAACCT
I (6/7)[b]	6130	H2+L3	986	9189–15318	CCT(A)GCA
I (6/7)	5007	H1+L2	854	8987–13993	TAG(C)CCT
II	4921	H1+L1	445	8503–13423	
II	7276	H1+L4	850	8712–15987	
II	6827	H2+L3	289	8810–15636	
II	4036	H3+L2	903	9697–13732	

Note: Type I deletions bear direct repeats flanking the breakpoints at the 5′ and 3′ ends of the deletion. Type II deletions do not exhibit such a feature.

[a] Nucleotide number of the direct repeat.

[b] Six out of seven nucleotides are direct repeat sequence.

Hsieh. mtDNA rearrangements in unfertilized oocytes. Fertil Steril 2002.

These deletions may be grouped into two classes: type I deletion, which is the dominant one bearing the same nucleotide sequences flanking the breakpoints at the 5′ and 3′ ends of the deletion; and type II deletion which does not harbor such direct repeat sequences. Nine of the 13 deleted mtDNA molecules belong to type I deletion, whose direct repeat sequences are shown in Table 1. The occurrence of multiple mtDNA deletions was 77.9% for unfertilized oocytes, 38.2% for arrested and fragmented embryos, and 26.3% for 3PN embryos (Table 2). The proportions of increased deleted mtDNA are significant in unfertilized oocytes compared to arrested embryos or 3PN embryos. However, there is no statistically significant difference between arrested embryos and 3PN embryos.

The 4977-bp deletion was the most common one observed in this study. We also determined the frequency of occurrence of such rearranged mtDNA in three groups of samples. The incidence was 66.1% in unfertilized oocytes, 34.8% in arrested embryos, and 21.1% in 3PN embryos (Table 3). Therefore, there was a significant increase in the proportion of deleted mtDNA in unfertilized oocytes ($P<.001$). These data suggest that deleted mtDNA may interfere with oocyte fertilization, especially if the 4977-bp mtDNA deletion occurs.

The samples were grouped into three different cohorts according to maternal age: younger than 32 years old, between 32 and 37 years old, and older than 37 years. The frequencies of occurrence of the 4977-bp deletion and multiple deletion of mtDNA in embryos and oocytes by different age cohorts are presented in Table 4. Although there was a slight increase of mtDNA with 4977 bp and multiple deletions of unfertilized oocytes in patients aged over 37 as compared with those aged less than 32, there was no statistical correlation between patient age and the frequency of occurrence of the 4977 bp mtDNA deletion.

TABLE 2

Frequency of occurrence of mtDNA deletions in human unfertilized oocytes, arrested embryos, and 3PN embryos by nested PCR.

	No. analyzed	No. amplified	No. deletions	Frequency (%)
Unfertilized oocytes	124	109	85	77.9[a]
Arrested embryos	98	89	34	38.2[a]
3PN embryos	42	38	10	26.3[a]

Note: The occurrence of any one or more than one of the 13 deletions was considered as positive when counting the frequency of occurrence of mtDNA deletions.

[a] Statistically significant difference ($P<.001$) by χ^2 analysis.

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TABLE 3

Frequency of occurrence of the 4977 bp deleted mtDNA in human unfertilized oocytes, arrested embryos, and 3PN embryos by nested PCR.

	No. analyzed	No. amplified	No. deletions	Frequency (%)
Unfertilized oocytes	124	109	72	66.1[a]
Arrested embryos	98	89	31	34.8[a]
3PN embryos	42	38	8	21.1[a]

[a] Statistically significant difference ($P<.001$) by χ^2 analysis.

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DISCUSSION

The results obtained in this study show that both human oocytes and embryos harbor multiple rearrangements of mtDNA. The primordial oocyte is formed during fetal development and remains in a dyctiate state of meiotic prophase until it resumes meiosis later in life (17). Thus, an oocyte can stay in this resting phase for more than 40 years. It has been suggested that the production of reactive oxygen species (ROS) during such a long period can lead to a decline in oocyte quality (18). It is unclear whether damage caused by ROS occurs during the long primordial follicle stage or during follicular growth. Primordial oocytes may have a low oxygen demand, possibly further reflecting the low number of mtDNA copies (19, 20).

The environment is anaerobic in primordial oocytes, and this further reduces the chance of ROS formation. However, oocyte growth is accompanied by a 100-fold increase in the copy numbers of mtDNA, indicating an increase in oxygen-mediated metabolism and ROS formation (19, 20). Thus, mutant mtDNAs may accumulate in oocytes during the long period of oogenesis. The occurrence of rearranged mtDNA is significantly reduced if the oocytes are capable of fertilization.

Two plausible mechanisms elucidate the tendency for a higher proportion of mtDNA deletions in unfertilized oocytes. One, accumulation of mtDNA rearrangements in the process of oogenesis, causes mitochondrial dysfunction. Inadequate amounts of ATP are produced via oxidative phosphorylation of defective mitochondria, which will result in an insufficient energy supply for efficient completion of meiosis I of an oocyte into a mature oocyte and for fertilization. Second, defective mitochondria may lose membrane potential and then release cytochrome *c* or other mitochondria-associated apoptosis-inducing factors, which in turn trigger germ cells into the apoptotic pathway.

In females of many species, over half of the oocyte population dies by apoptosis before birth (21). Krakauer et al. (22) have reported this death of germ cells as a developmental solution to the accumulation of mutations in mitochondria, proposing that the prenatal oocyte apoptosis effectively removes oocytes carrying mutant mitochondria. Moreover, mitochondria influencing the fate of oocytes has also been proven by microinjecting small numbers of mitochondria into mouse oocytes, which prevents these oocytes with inherently high rates of apoptosis from undergoing programmed cell death (23).

The 4977-bp deletion is the most common mtDNA deletion in human oocytes and embryos, with frequencies of

TABLE 4

Frequency of occurrence of mtDNA deletions in human unfertilized oocytes, arrested embryos, and 3PN embryos of different age cohorts.

Sample	Type of rearranged mtDNA	Percentage of rearranged mtDNA (%)		
		<32[a]	32–37[a]	>37[a]
Unfertilized oocytes	4977 bp	56.3 (27/48)	74.3 (26/35)	73.0 (19/26)
	multiple	68.8 (33/48)	82.9 (29/35)	88.5 (23/26)
Arrested embryos	4977 bp	42.9 (9/21)	36.2 (17/47)	23.8 (5/21)
	multiple	47.6 (10/21)	36.2 (17/47)	33.3 (7/21)
3PN embryos	4977 bp	9.1 (1/11)	23.5 (4/17)	30.0 (3/10)
	multiple	27.3 (3/11)	23.5 (4/17)	30.0 (3/10)

[a] Maternal age (year).

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66.1% in unfertilized oocytes, 34.8% in arrested embryos, and 21.1% in 3PN embryos. Additionally, several investigators have already analyzed the 4977-bp deletion commonly found in the affected tissues of patients of mitochondrial myopathy and aging-associated tissues (11, 24–26). The 4977-bp deletion causes the removal of major structural genes containing Fo-F_1-ATPase (ATPase 6 and 8), cytochrome oxidase (CO III), and NADH-CoQ oxidoreductase (ND3, ND4, ND4L, and ND5). This deletion also creates a chimeric gene, which fuses the 5′-proportion of ATPase 8 and 3′-proportion of ND5 genes of mtDNA. Therefore, the deleted or truncated genes in this rearranged mtDNA may result in impaired gene expression by decreasing expression of the deleted genes or by producing transcripts of fused genes.

No evidence has been shown to indicate that such kinds of deletions will interfere with amplification and stability of new truncated mtDNA. Adversely, Shoffner et al. (27) proposed that the faster replication of deleted mtDNA (it is shorter than a normal mtDNA) might give it a selective advantage in propagation. However, no replicative advantage for the smaller deleted molecules has been reported in other studies (28). We suggested that the deleted mtDNA accumulated in oocytes might interfere with the fertilization ability. Therefore, the deletion rate appears higher in eggs than embryos.

Thirteen different mtDNA deletions were identified in human oocytes and embryos in this study. Nine of the 13 harbored direct repeats in the junction of mtDNA deletions. These deletions may be grouped into two types: one bearing direct repeats flanking the breakpoints at the 5′ and 3′ ends of the mtDNA deletion (type I), and the others contain no such feature (type II). It seems that more than one mechanism of deletion is operating in causing these mtDNA deletions. As previously proposed, deletions with direct repeats are probably caused by slippage mispairing at the direct repeats during mtDNA replication (27). Oocytes often contain deleted mtDNA other than the 4977-bp deletion, which demonstrates the presence of heteroplasmy of mtDNA. The frequency of deletions in human embryos is lower than that in oocytes, suggesting that superior oocytes with fewer mtDNA deletions have been selected by nature for fertilization during IVF or intracytoplasmic sperm injection (ICSI) procedures.

Using a nested PCR strategy, our results show a higher frequency of the 4977-bp deleted mtDNA in oocytes (66.1%, n = 109) when compared with results of Chen et al. (4) (49%, n = 104), Keefe et al. (29) (43%, n = 51), and Brenner et al. (30) (32.8%, n = 74). In this study there was no correlation between the 4977-bp deletion in human oocytes and embryos with the age of the patient. However, only one report showed a significant association between patient age and this deletion (29). It suggests that this deletion is not a suitable indicator for reproductive senescence. The plausible explanation may be that the previous three studies including this study were all qualitative rather than quantitative studies. Therefore, further quantitative study is mandatory and is undergoing in our laboratory to clarify the association between maternal age and deleted mtDNA in human oocytes and embryos.

Many factors cause fertilization failure after in vitro insemination (31, 32); one of the reasons is chromosomal abnormalities (33, 34). Numerous chromosomal disorders and structural defects arise during meiotic metaphase, therefore predisposing a fertilized oocyte to developmental failure (35, 36). These chromosomal nondisjunctions may result from defective mitochondrial function at the stage of chromosomal segregation (37, 38). A wide range of ATP contents has been detected in meiotically mature oocytes from the same and different patients. However, a higher potential for continued embryogenesis and further implantation is correlated with oocytes with higher ATP concentrations (16). The efficiency of mitochondrial respiration in oocytes and preimplantation embryos is closely correlated with the programmed rate of embryo development (39). A deficiency in mitochondrial ATP production may be associated with various cellular and chromosomal disorders and the failure of oocyte fertilization and embryo development.

The ICSI procedure assumes that only a single spermatozoon is microinjected in each oocyte, but sometimes some zygotes show three pronuclei (3PN) instead of the expected two by 16 to 18 hours after ICSI (40, 41). The most probable hypothesis to explain the existence of 3PN would be normal fertilization by a single injected spermatozoon along with the nonextrusion of a second polar body (40, 42). There were no reports implying that cytoplasmic or mitochondrial dysfunction would cause formation of 3PN zygotes. Decreased ATPase 6 gene expression was found in unfertilized oocytes compared with 3PN embryos (43). Injection of the cytoplasm of 3PN embryos may enhance the clinical pregnancy rate in patients with repeated implantation failure (44). Our findings about decreased proportions of multiple deleted mtDNA (26.3%) and the 4977 deletion (21.1%) in 3PN embryos may also indicate the normal growth rates of 3PN embryos. In conclusion, our results imply that oocytes with mutant mtDNA exist among mature oocytes, being especially higher in unfertilized oocytes, and that the decrease in energy supply may interfere with oocyte fertilization and embryo development.

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