

Deleted Mitochondrial DNA in Human Luteinized Granulosa Cells

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ABSTRACT: The rearrangement of mitochondrial DNA in luteinized granulosa cells was determined in order to evaluate the fertilization capacity of oocytes and the development of embryos. Multiple deletions of mtDNA were found in luteinized granulosa cells from *in vitro* fertilization (IVF) patients. The 4977-base pair (bp) deletion was the most frequent deletion found in human granulosa cells. No significant difference was noted between mtDNA deletions of granulosa cells based on the fertilization capacity of oocytes and the development of embryos. To determine the relationship of proportions of mtDNA rearrangements with the aging process, granulosa cells were grouped into three different cohorts according to maternal age: younger than 32 years, between 32 and 37 years, and older than 37 years. No statistical correlation was noted between patient age and the frequency of occurrence of multiple mtDNA deletions. However, an increase in granulosa cell apoptosis was associated with an increase in mtDNA deletions. Accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and impaired ATP production. We concluded that the accumulation of rearranged mtDNA in granulosa cells might not interfere with fertilization of human oocytes and further embryonic development; it was, however, associated with apoptosis processes.

KEYWORDS: deletion; mtDNA; granulosa cell

INTRODUCTION

Mammalian ovaries include several hundreds of thousands of follicles in the primordial and primary stages during ovarian follicular development, among which

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only limited numbers in each cycle will fully develop and are selected for ovulation, whereas the remaining majority of follicles undergo atresia.^{1,2} Granulosa cells play a major role in regulating ovarian physiology, including ovulation and luteal regression.³ Granulosa cells secrete a wide variety of growth factors that may attenuate gonadotrophin's action in ovaries in paracrine-autocrine processes.^{4,5} Most of these factors do not directly affect oocytes but exert their action via granulosa cells. The presence of granulosa cells appears to be beneficial for oocyte maturation and early development.⁶ Recent studies have suggested that follicular atresia is associated with apoptosis of granulosa cells.^{1,2} Researchers also reported that the incidence of apoptotic bodies in granulosa cells can be used to predict the developmental capacity of oocytes in an IVF program.⁷

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 1,000 mitochondria, and each mitochondrion consists of 2 to 10 copies of mtDNA.⁸ mtDNA comprises a circular, histone-free molecule composed of 16.6 kb of DNA, present in one or more copies in every mitochondrion. Thirteen protein subunits are required for oxidative phosphorylation of a total of about 80 subunits, the remainder of which are encoded by nuclear genes and imported into the mitochondrion. The mtDNA also contains 2 ribosome subunits and 22 transfer RNA. 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 respiratory chain proteins, which are all proteins that regulate replication and transcription of mtDNA.⁹ In recent years, an increasing number of reports have shown that mtDNA deletions are associated with human aging and mitochondrial diseases.^{10,11} In this study, the rearrangement of mitochondrial DNA in luteinized granulosa cells was determined in order to evaluate the fertilization capacity of oocytes and the development of embryos.

MATERIAL AND METHODS

Polymerase Chain Reaction

Oocytes and embryos were stored in 20 μ L of 1X polymerase chain reaction (PCR) buffer containing 0.05 mg/mL of proteinase K, 20 mM of dithiothreitol (DTT), and 1.7 μ M of SDS. After digestion for 1 h at 56°C and 10 min 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 (nucleotide position [np] 8285~8304, CTCTAGAGCCCACTGTAAAG), H2 (np 8781~8800, CGGACTCCTGCCTCACTCAT), H3 (np 9207~9226, ATGACCCACCAATCACATGC), L1 (np 13650~13631, GGGGAAGCGAGGTTGACCTG), L2 (np 14145~14126, TGTGATTAGGAGTAGGGT-TA), L3 (np 15896~15877, TACAAGGACAGGCCCATTTG), and L4 (np 16410~16391, GAGGATGGTGGTCAAGGGAC).

Semiquantitative RT-PCR

Total RNA extracted from human granulosa cells 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). RT-PCR amplifications were performed with 3 μ L of cDNA in a total volume of 50 μ L of amplification buffer, 40 pmol of specific primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). Sequences of the oligonucleotide primers used in this study are listed as follows: ND2 (forward, np 5101~5120, TAACTACTACCGCATTCCTA; reverse, np 5400-5381, CGTTGTTAGATATGGGGAGT), and GAPDH (forward, CCTTCATTGACCTCAAC; reverse, AGTTGTCATGGATGACC). For semiquantitative amplification, each cycle was carried out at 92°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The reactions were analyzed after 15, 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.

RESULTS

We attempted to determine whether the existence of mtDNA deletions in luteinized granulosa cells affects the fertilization capacity of oocytes and the development of embryos. DNA was extracted from granulosa cells to determine the extent of mtDNA rearrangement by PCR using multiple pairs of primers. Multiple deletions of mtDNA were found in luteinized granulosa cells from IVF patients. The 4977-bp deletion was the most frequent deletion in human granulosa cells. There was no significant difference between mtDNA deletions of granulosa cells with the fertilization capacity of oocytes and the development of embryos. To determine the relationship of proportions of mtDNA rearrangements with the aging process, granulosa cells were grouped into three different cohorts according to maternal age: younger than 32 years, between 32 and 37 years, and older than 37 years. Frequencies of occurrence of mtDNA deletions in human granulosa cells of different age cohorts are listed in TABLE 1. Percentages of 4977-bp rearranged mtDNA were 43.8%, 39.1%, and 47.4% in the three age-stratified cohorts, respectively. There was no statistical correlation between patient age and the frequency of occurrence of the 4977-bp mtDNA deletion or with multiple mtDNA deletions. Whether rearranged mtDNA coexisted

TABLE 1. Frequency of occurrence of mtDNA deletions in human granulosa cells of different age cohorts

Type of rearranged mtDNA	Percentage of rearranged mtDNA (%)		
	<32 ^a	32–37 ^a	>37 ^a
4977 bp	43.8 (7/16)	39.1 (9/23)	47.4 (9/19)
Multiple	56.3 (9/16)	52.2 (12/23)	57.8 (11/19)

^aMaternal age (years).

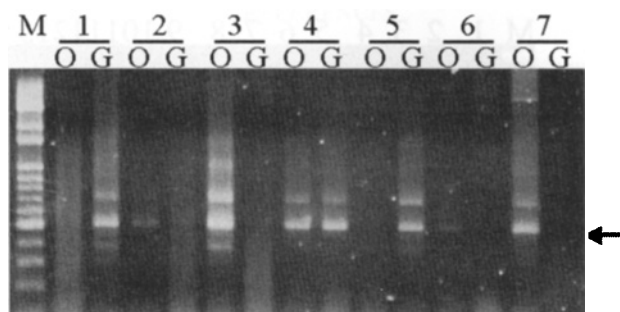


FIGURE 1. Multiple deletions of mtDNA in granulosa cells and oocytes. *Lane 1*, 26 years old; *lane 2*, 30 years old; *lane 3*, 32 years old; *lane 4*, 35 years old; *lane 5*, 36 years old; *lane 6*, 38 years old; and *lane 7*, 40 years old. O, oocyte; G, granulosa cells. Arrow indicates a 4977-bp mtDNA deletion.

in oocytes and surrounding granulosa cells was also determined. The data showed that mtDNA deletions were randomly distributed between oocytes and granulosa cells; higher proportions of rearranged mtDNA were found in oocytes (FIG. 1, lanes 2, 3, 6, and 7), and higher frequencies were determined in granulosa cells (FIG. 1, lanes 1 and 5). The independent existence of rearranged mtDNA in oocytes and granulosa cells is shown in FIGURE 1. Expression levels of mitochondrial RNA in granulosa cells of different age groups were determined using a semiquantitative RT-PCR (FIG. 2). Transcripts of mitochondrial NADH dehydrogenase subunit 2 (ND2) were determined and normalized to the GAPDH gene, with no statistically significant differences among different age cohorts.

DISCUSSION

The 4977-bp deletion is the most common mtDNA deletion in human oocytes and embryos.^{12,13} Our previous study showed that frequencies of 4977-bp deleted mtDNA were 66.1%, 34.8%, and 21.1% in unfertilized oocytes, arrested embryos, and 3PN embryos, respectively.¹³ The 4977-bp mtDNA rearrangement may remove major structural genes containing Fo-F1-ATPase (ATPase 6 and 8), cytochrome oxidase III (CO III), and NADH-CoQ oxidoreductase (ND3, ND4, ND4L, and ND5). This deletion also creates a chimeric gene, which fuses the 5'-portion of ATPase 8 and 3'-portion of the ND5 gene of mtDNA. These mutated genes may cause impaired gene expression by decreasing the expression of the deleted genes or by producing transcripts of fused genes. In this study, the age-independent existence of rearranged mtDNA in granulosa cells indicates that the aging process did not play a major role in the accumulation of mtDNA mutations in granulosa cells.

To evaluate the relation between mitochondrial gene expression of granulosa cells and oocyte fertilization ability, transcripts of mitochondrial NADH dehydrogenase subunit 2 (ND2) were determined. The mtDNA transcripts are polycistronic,^{14,15} which means that each gene is separated following precise

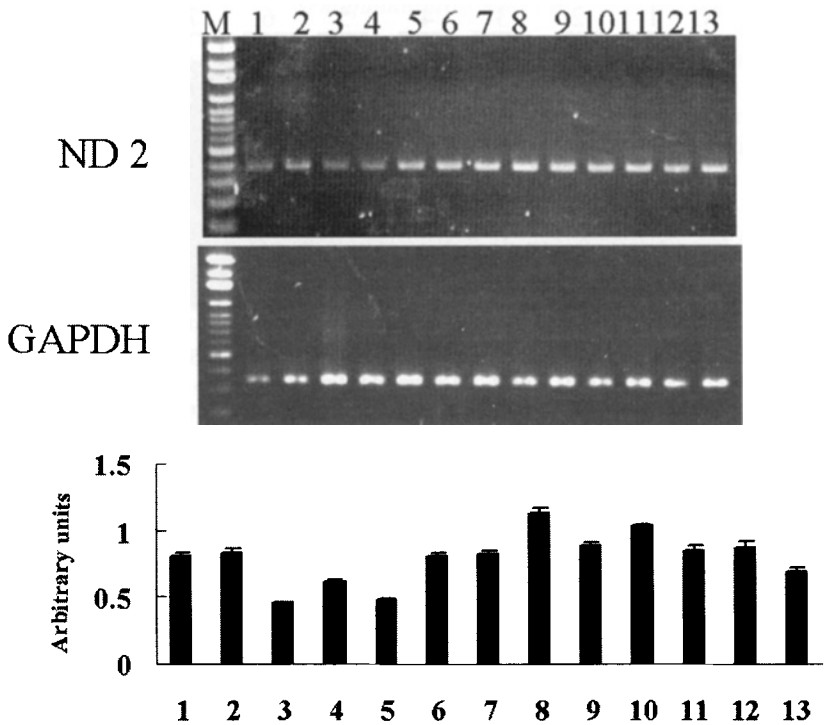


FIGURE 2. Expression levels of mtRNA in granulosa cells of different age groups. Lanes 1–3, 25–30 years old; lanes 4–6, 30–35 years old; lanes 7–10, 35–38 years old; and lanes 11–13, 38–42 years old.

endonucleolytic excision of the tRNAs from nascent transcripts. The polycistronic mtDNA transcripts are consistent, with different mtRNA expression levels showing the same pattern in human oocytes.¹⁶ In this study, we first determined the ND2 transcript in order to estimate mitochondrial gene expression in granulosa cells. No statistically significant differences in mtRNA expression patterns were noted among the different age cohorts. The present evidence of the independent existence of rearranged mtDNA in oocytes and granulosa cells supports the accumulation of rearranged mtDNA in granulosa cells possibly not interfering with fertilization of human oocytes and further embryonic development.

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