報告內容摘要：

Paper 1: Calcium-dependent up-regulation of mitochondrial electron transfer chain gene expressions in human luteinized granulosa cells

Abstract: To evaluate the transcription and translation ability of mitochondria in terminally differentiated granulosa cells, these cells were incubated with ionic calcium. There were dose-dependent increases in RNA expressions of the four genes analyzed from granulose cells cultured in a serial concentration of calcium ions. This effect was abolished when cells were preincubated with the extracellular calcium-chelating agent, Ethylene glycol-bis (2-aminoethylether)-N,N,N=,N=-tetraacetic acid (EGTA). The effect of ionic calcium on both the nuclear- and mitochondrial-encoded subunits also was determined. Expression levels of mitochondrial transcription factor A in RNA significantly increased in granulose cells that were exposed for 24 and 48 hours to 0.5 and 1 _M A23187. The present study is the first report to present calcium-dependent increases in the transcription and translation levels of both nuclear-encoded and mitochondrial-encoded mitochondrial respiratory enzyme subunits and also indicates that mitochondrial transcription factor A is involved in mitochondrial biogenesis.

Key Words: granulosa cells; mitochondria; RT-PCR

Paper 2: Deleted mitochondrial DNA in human luteinized granulosa cells

Abstract: The rearrangement of mitochondrial DNA in luteinized granulose 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 granulose 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.

Key Words: deletion; mtDNA; granulosa cell

Paper 3: Effects of gonadotrophin-releasing hormone agonists on apoptosis of granulosa cells

Abstract: Granulosa cells are known to contribute to maturation of oocytes, and most of the growth factors exert their action via granulosa cells. It has been established that granulosa cell death during follicular atresia and luteolysis results from apoptosis. However, the precise mechanistic pathways of granulose cell apoptosis have not yet been defined. In this study, we determined the proportions of apoptosis in granulosa cells treated with two kinds of gonadotrophinreleasing hormone agonists (GnRHa): buserelin and leuprorelin depot. The incidences of DNA fragmentation of human granulosa cells treated with buserelin and leuprorelin were 54.33% and 39.02%, respectively. The proportions of apoptotic bodies were 6.04% and 4.29%, respectively. There was a significant difference in the proportions of DNA fragmentation between the two kinds of GnRHa-treated granulosa cells. The apoptosis pathway and associated protein expression in granulosa cells treated with GnRHa were also determined. The Bax molecule, a pro-apoptosis protein, was expressed in granulosa cells undergoing apoptosis. In contrast, Bcl-2, an anti-apoptosis protein, could not be detected in the same group of granulosa cells. The distribution of cytochrome *c* determined via immunostaining showed a diffuse pattern, which most likely indicated that cytochrome *c* was translocated from mitochondria into the cytoplasm. Western blotting showed the expressions of caspase-9 and caspase-3 in patients' granulosa cells. The GnRHa effects on granulosa cells indicated a higher incidence of DNA fragmentation and apoptotic bodies in the buserelin-treated than in the leuprorelin depot–treated group. The granulosa cells go through the mitochondria-dependent apoptosis pathway; the indicated pro-apoptosis protein Bax was expressed and induced cytochrome *c* release from mitochondria, which then activated caspase-9 and caspase-3 until cell death occurred.

Key Words: apoptosis; gonadotrophin-releasing hormone; granulosa cell

Paper 4: Abnormal mitochondrial structure in human unfertilized oocytes and arrested embryos

Abstract: To clarify the relationship between mitochondria and embryo development, we collected human unfertilized oocytes, early embryos, and arrested embryos. Unfertilized oocytes and poor-quality embryos were collected, and the ultrastructure of mitochondria was determined by transmission electron micrography. Four criteria for determining the mitochondrial state were mitochondrial morphology, cristae shape, location, and number of mitochondria. In mature oocytes, mitochondria were rounded with arched cristae and a dense matrix and were distributed evenly in the ooplasm. In pronuclear zygotes, the size and shape of mitochondria were similar to those in mature oocytes; however, mitochondria appeared to migrate and concentrate around pronuclei. In this study, 67% of examined unfertilized oocytes had fewer mitochondria in the cytoplasm. A decreased number of mitochondria located near the nucleus was also demonstrated in 60% of arrested embryos. Fewer differentiated cristae were determined in all three arrested

blastocyst stages of embryos. The relative expressions of oxidative phosphorylation genes in oocytes and embryos were also determined. These data imply that inadequate redistribution of mitochondria, unsuccessful mitochondrial differentiation, or decreased mitochondrial transcription may result in poor oocyte fertilization and compromised embryo development.

Key Words: embryo; mitochondria; oocyte

Calcium-dependent up-regulation of mitochondrial electron transfer chain gene expressions in human luteinized granulosa cells

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Objective: To evaluate the transcription and translation ability of mitochondria in terminally differentiated granulosa cells, these cells were incubated with ionic calcium.
Design: Prospective laboratory research.
Setting: In vitro fertilization laboratory in a university hospital.
Patient(s): Granulosa cells were harvested from 50 female patients undergoing IVF.
Intervention(s): Analysis of mitochondrial gene expression by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and of mitochondrial-encoded proteins by Western blot.
Main Outcome Measure(s): Comparison of the RNA expression levels of genes including cytochrome *c* oxidase subunit I (COX I), adenosine triphosphate synthase 6 (ATPase 6), flavoprotein, and succinate-ubiquinone oxidoreductase, and protein levels of COX I and flavoprotein in different calcium ion treatment groups.
Result(s): There were dose-dependent increases in RNA expressions of the four genes analyzed from granulosa cells cultured in a serial concentration of calcium ions. This effect was abolished when cells were preincubated with the extracellular calcium-chelating agent, Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). The effect of ionic calcium on both the nuclear- and mitochondrial-encoded subunits also was determined. Expression levels of mitochondrial transcription factor A in RNA significantly increased in granulosa cells that were exposed for 24 and 48 hours to 0.5 and 1 μM A23187.
Conclusion(s): The present study is the first report to present calcium-dependent increases in the transcription and translation levels of both nuclear-encoded and mitochondrial-encoded mitochondrial respiratory enzyme subunits and also indicates that mitochondrial transcription factor A is involved in mitochondrial biogenesis. (Fertil Steril® 2005;84(Suppl 2):1104–8. ©2005 by American Society for Reproductive Medicine.)

Key Words: Granulosa cells, mitochondria, RT-PCR

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 a mendelian fashion, whereas the other is located within the mitochondria and is transmitted through maternal lineage. Human mitochondrial (mt) DNA 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 of a total of about 80 constituting the oxidative phosphorylation system, with the remainder being encoded by nuclear genes that are imported into the mitochondrion.

Human mtDNA also encodes 2 ribosomal 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 respiratory chain proteins and all of the proteins and enzymes that regulate the replication and transcription of mtDNA (1).

In mammals, an essential component of the mitochondrial transcription initiation complex is mitochondrial transcription factor A (mtTFA), a nuclear-encoded 25-kDa protein that bends and unwinds mtDNA upon binding and that belongs to the high-mobility-group (HMG)-box family of proteins (2, 3). Mitochondrial transcripton factor A regulates both mitochondrial transcription and replication by permitting transcription of the RNA primer to commence from the unwinding L-strand DNA (4). The gene of mtTFA has been isolated in humans and is structured with 7 exons and 6 introns (5).

Granulosa cells play a major role in regulating ovarian physiology, including ovulation and luteal regression (6). Granulosa cells secrete a wide variety of growth factors that

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may attenuate gonadotropin's action in the ovary in paracrine–autocrine processes (7, 8). Most of these factors do not directly affect oocytes but exert their actions via granulosa cells. The presence of granulosa cells appears to be beneficial for oocyte maturation and early development (9). Nevertheless, granulosa cells also might have a negative effect upon oocytes. It has been demonstrated that the increased apoptotic potential in oocytes of aged mice is because of the presence of cumulus cells (10).

The decline in reproductive ability in women as they age is associated with a loss of follicles and a decrease in oocyte quality. Aging-associated apoptosis increases in follicular granulosa cells and consequently decreases ovarian fecundity (11, 12). In the present study, to determine the mitochondrial biogenesis ability of human granulosa cells, the expressions of the RNA of five genes including cytochrome *c* oxidase subunit I (COX I), ATP synthase 6 (ATPase 6), flavoprotein, succinate-ubiquinone oxidoreductase (SDHA), and mtTFA were determined. Nuclear-encoded flavoprotein and mitochondrial-encoded COX I protein expression levels also were detected.

MATERIALS AND METHODS

Granulosa Cells in Culture

The institutional review board of Taipei Medical University Hospital approved the study, which we intended to perform on granulosa cells discarded during an IVF program. Human granulosa cells were collected from patients who were recruited into an IVF-embryo transfer (IVF-ET) program. Ovarian stimulation was performed by desensitization using a gonadotropin-releasing hormone (GnRH) agonist followed by treatment with gonadotropins (follicle-stimulating hormone (FSH) and human menopausal gonadotropin (hMG)). Ovulation was induced by using human chorionic gonadotropin (hCG).

Oocytes then were retrieved by transvaginal ultrasonography-guided aspiration 34–36 hours after hCG administration. Granulosa cells were harvested from follicular aspirates by centrifugation through Ficoll-Paque, washed, and suspended in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 50 μg/mL of uridine supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. Human granulosa cells were grown at 37°C in a humidified atmosphere with 5% CO_2 for 3 days before the experimental treatment. A23187 was purchased from Sigma (St. Louis, MO). One millimole of A23187 dissolved in dimethyl sulfoxide was prepared freshly before study and diluted into Dulbecco's modified Eagle's medium just before use.

Extraction of RNA and Semiquantitative Reverse-Transcription-Polymerase Chain Reaction Analysis

Total RNA was extracted from cells with Trizol reagent according to the manufacturer's instructions. Total RNA (1–5 μg) was reverse-transcribed into cDNA using oligo(dT)18 as the primer and into MMLV reverse transcriptase (Ambion, Austin, TX), and 1 μL of the cDNA template was used separately to amplify different mRNAs. Amplifications by reverse-transcription polymerase chain reaction (RT-PCR) were performed with 1 mL of cDNA in a total volume of 25 μL of amplification buffer, 10 pmol of specific primers, and 2.5 U of Taq DNA polymerase (Life Technologies, Grand Island, NY).

Sequences of the oligonucleotide primers used in this study are listed as follows: mtTFA (forward, CCGGAGGGTCGCACGCGGGT; reverse, CAGATGAAAACCACCTCAAT), COX I (forward, GTCCTATCAATAGGAGCTGT; reverse, TTCGAAGCGAAGGCTTCTC), flavo-protein (forward, GCTCAGTATCCAGTAGTGGA; reverse, CCCTTCACGGTGTCGTAGAA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward, ACCACAGTCCATGCCATCA; reverse, TCCACCACCCTGTTGCTGTA). The predicted sizes of the mtTFA, COX I, flavoprotein, and GAPDH RT-PCR products were 235, 299, 230, and 453 bp, respectively. For semiquantitative amplification, each cycle was performed at 92°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds. The reactions were analyzed after 15, 20, 25, 30, 35, and 40 cycles, respectively, to optimize the linear range of amplification. The PCR reactions were optimized with respect to the annealing temperature and number of PCR cycles (13).

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 different clones, 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 with an imaging densitometer, and results are expressed as a ratio of analyzed genes divided by GAPDH.

Western Blot Analysis

Total protein was extracted from harvested cells by using lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreitol; 10% glycerol; and 1% Triton X-100) with a protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysate was cleared of cell debris by low-speed centrifugation at 10,000 × *g* for 5 minutes. The protein concentration was measured by using a BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved on polyacrylamide gels, and then proteins were transferred onto a polyvinyldene diflouride membrane (PVDF; Amersham Pharmacia, Piscataway, NJ). Membranes were blocked for 1 hour at 4°C with 10% skim milk in TBST buffer (1 M Tris-HCl, 100 mM NaCl, and 1% Tween-20).

Blots were probed with the following primary antibodies: a monoclonal antibody against cytochrome *c* oxidase subunit I, a monoclonal antibody against flavoprotein (Molecular

Probes, Eugene, OR), or a polyclonal antibody against GAPDH. Blots then were incubated with the appropriate horseradish peroxidase–conjugated anti-IgG antibody. Antibody-bound protein was detected by using enhanced chemiluminescence and exposure to film.

Statistical Analysis

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

RESULTS

To test whether mitochondria in terminal-differentiated granulosa cells preserve their transcription and translation ability, human granulosa cells were incubated with ionic calcium. Expressions in RNA of four genes, COX I, ATPase 6, flavoprotein, and SDHA, were analyzed by semiquantitative RT-PCR. Three different A23187 dosages of 0.25, 0.5, and 1 μM were used to treat human granulosa cells for 48 hours. There were dose-dependent increases in the RNA expressions of the 4 genes analyzed (Fig. 1). Both mitochondrial-encoded mitochondrial protein transcripts, COX I and ATPase6, and nuclear-encoded mitochondrial protein transcripts, flavoprotein and SDHA, were significantly elevated compared with the control. This effect was abolished when granulosa cells were preincubated with the extracellular calcium (Ca^{2+})-chelating agent, Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Fig. 2).

Western blot analysis was used to determine the effect of ionic calcium on nuclear-encoded flavoprotein and mitochondrial-encoded COX I protein expression levels. The effect of ionic calcium on both the nuclear- and mitochondrial-encoded subunits was also time-dependent (Fig. 3). However, calcium induction of protein expression in granulosa cell also was abolished by EGTA. To determine the possible factors participating in mitochondrial respiratory enzyme subunit transcription and translation, mtTFA was examined. Expression levels of mtTFA were determined by treatment of granulosa cells with A23187 (at 0.5–1 μM) for 24 or 48 hours of incubation time.

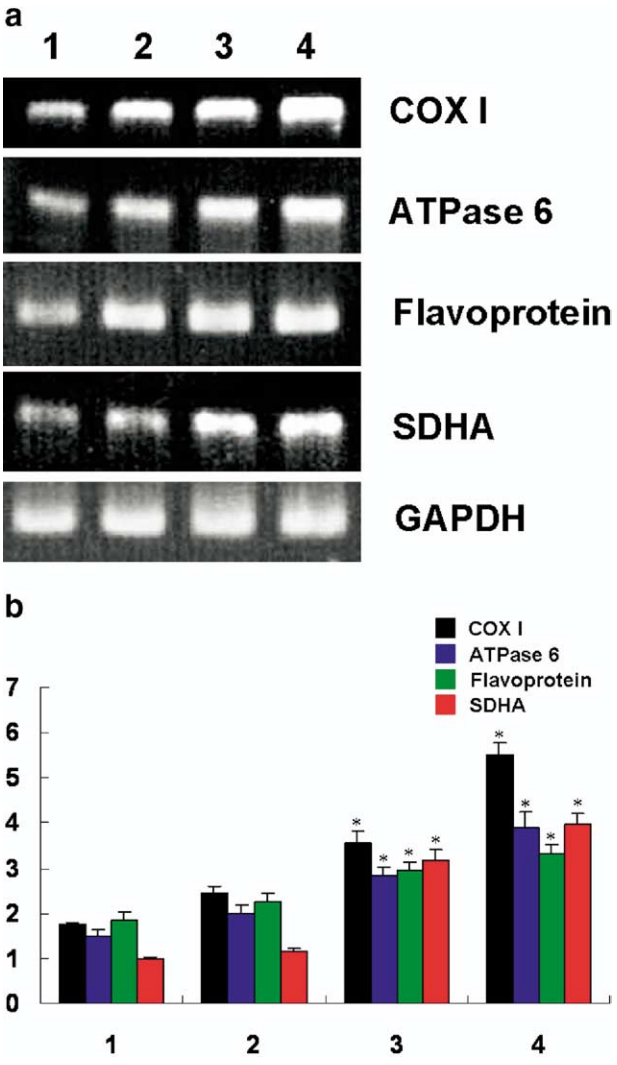
The RNA expression levels of mtTFA significantly increased in granulosa cells exposed for 24 and 48 hours to 0.5–1 μM A23187 (Fig. 4). Taken together, these data show calcium-dependent increases in transcription and translation levels of both nuclear-encoded and mitochondrial-encoded mitochondrial respiratory enzyme subunits and indicate that mtTFA is involved in mitochondrial biogenesis.

DISCUSSION

In this study, cohorts of every surrounding granulosa cell from five IVF oocytes were lysed to harvest total RNA and determine the semiquantative expression level of RNA by RT-PCR. To rule out limitations of RT-PCR, various replication cycles (15, 20, 25, 30, 35, and 40 cycles) were decided upon to identify the optimal cycles with RT-PCR products in a linear range as in a previous study (13). The mtDNA transcripts are polycistronic (14, 15), which means that each gene is separated after precise endonucleolytic excision of the tRNAs from the nascent transcripts. In the present study, we determined that two mtDNA-transcribed genes, COX I

FIGURE 1

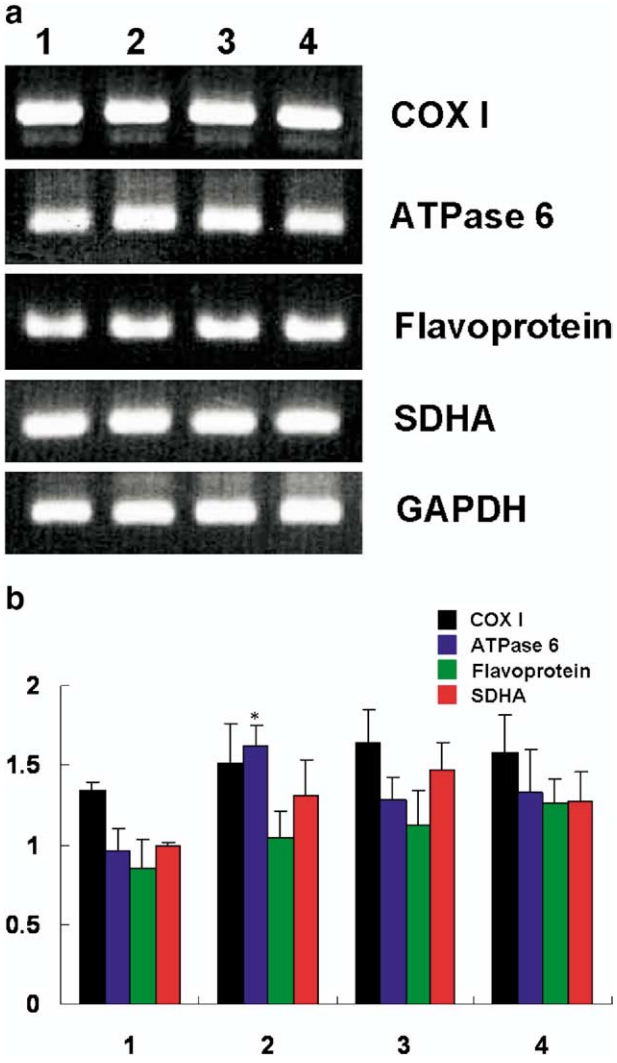
Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of COX I, ATPase 6, flavoprotein, and succinate-ubiquinone oxireductase (SDHA) gene expressions in human granulosa cells treated with three different A23187 dosages for 48 hours. The RNA was extracted and reverse-transcribed into cDNA, and then PCR amplification was respectively performed with primers for the four genes. *Lanes 1* to *4* are 0, 0.25, 0.5, and 1 μM of A23187, respectively.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

FIGURE 2

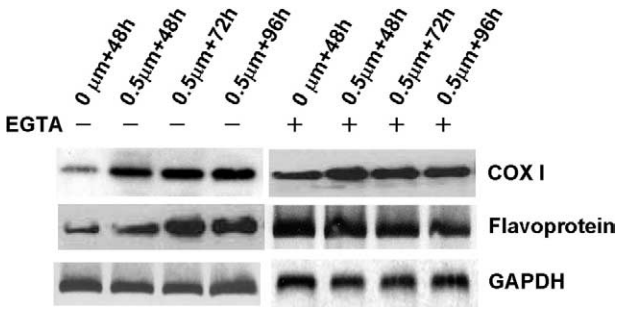
Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of COX I, ATPase 6, flavoprotein, and succinate-ubiquinone oxireductase (SDHA) gene expressions in human granulosa cells pretreated with the Ca^{2+}-chelating agent, EGTA, then treated with three different A23187 dosages for 48 hours. *Lanes 1* to *4* are 0, 0.25, 0.5, and 1 μM of A23187, respectively.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

FIGURE 3

Western blot analysis of nuclear- and mtDNA-encoded proteins in human granulosa cells. COX I = mtDNA-encoded cytochrome *c* oxidase subunit I; flavoprotein = nuclear-encoded flavoprotein; EGTA+ = cells pretreated with the Ca^{2+}-chelating agent, EGTA, then respectively treated with 0.5 μM of A23187 for 48, 72, and 96 hours.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

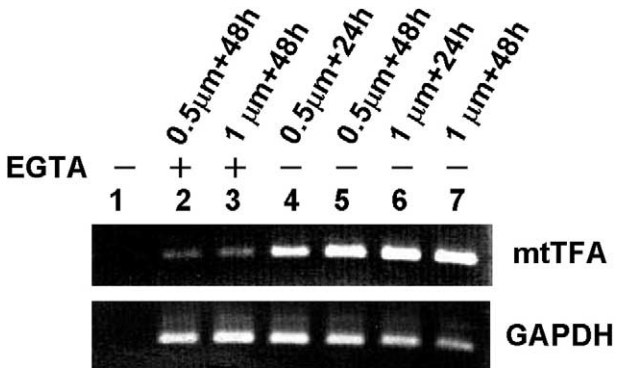
and ATPase6, may be representative of mtDNA gene expression.

Although different mtDNA transcripts encode different protein assemblies to one of five mitochondrial complexes, in our previous report, the expression levels of the eight genes, including ND2, COI, COII, ATPase 6, COIII, ND3, ND6, and Cyt b, 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 cell (13, 16, 17).

Calcium acts as a second messenger in a variety of biological processes, including fertilization, embryo develop-

FIGURE 4

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mtTFA gene expression in human granulosa cells treated with EGTA and/or A23187. *Lane 1,* RNA was extracted and without reverse-transcribed into cDNA, and then PCR amplification was performed with primers for the mtTFA gene. EGTA+ = cells pretreated with the Ca^{2+}-chelating agent, EGTA, then treated with A23187.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

ment, and cell proliferation and death (18, 19). The mechanisms underlying the induction of calcium signals by pituitary FSH or the release of ATP by autonomic nerves in granulosa cells are controversial (20, 21). It is uncertain whether the mobilized cytosolic calcium is caused by mobilization from intracellular sources or to Ca^{2+} influx. Previous studies have provided evidence that cytosolic Ca^{2+} concentration is involved in regulating mitochondrial biogenesis in skeletal muscle (22, 23). In the present study, the induction of RNA and the protein expression levels of extracellular Ca^{2+} on both nuclear- and mitochondrial-encoded respiratory chain subunits were dose and time dependent. Calcium-induced mitochondrial biogenesis was abolished in the presence of EGTA. These novel data demonstrate that extracellular Ca^{2+} can stimulate mitochondrial biogenesis in human granulosa cells.

Biogenesis of the mitochondrial oxidative phosphorylation enzyme complex requires the symphonious expression of mtDNA and nuclear genes which both encode mitochondrial proteins and their control factors. One of these control factors is mtTFA, which plays a major role in regulating mtDNA transcription and replication (4, 24, 25). Our data indicate increased expression levels of mtTFA mRNA in human granulosa cells after Ca^{2+} stimulation (Fig. 4). The data presented in this study also showed that both mitochondrial biogenesis and mtTFA gene expression were induced by Ca^{2+}.

Cell responses to environmental changes including energy demands should be reflected in the physiological state of the mitochondria. This correlates well with Ca^{2+}-mediated increases in mitochondrial biogenesis in muscle (23) and mitogen-stimulated effects in human lymphocytes (26) and murine splenocytes (27). However, more data are needed to clarify the correlation between mtTFA and mitochondrial biogenesis in this study. This experimental evidence indicated that Ca^{2+} is one of the signals that mediate mitochondrial biogenesis in differentiated luteinized granulosa cells.

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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 evaluae the fertilization capacity of oocytes and the development of embryos.

MATERIAL AND METHODS

Polymerase Chain Reaction

Oocytes and embryos were stored in 20 μL of 1X polymerase chain reaction (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 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, TGTGATTAGGAGTAGGGTTA), 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 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). 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)

[a]Maternal age (years).

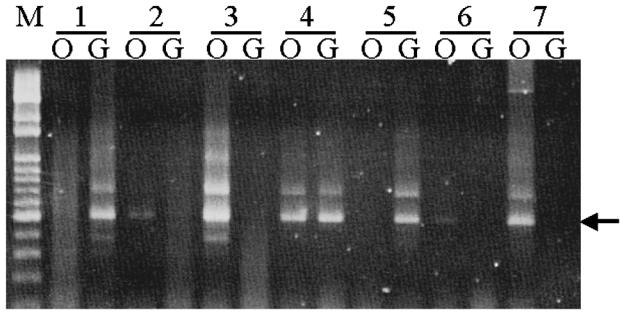

FIGURE 1. Multiple deletions of mtDNA in granulosa cells and oocytes. *Lane 1*, 26 years old; *lane 2*, 30 years old; *lane 3*, 32 years old; *lane 4*, 35 years old; *lane 5*, 36 years old; *lane 6*,38 years old; and *lane 7*, 40 years old. O, oocyte; G, granulosa cells. *Arrow* indicates a 4977-bp mtDNA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

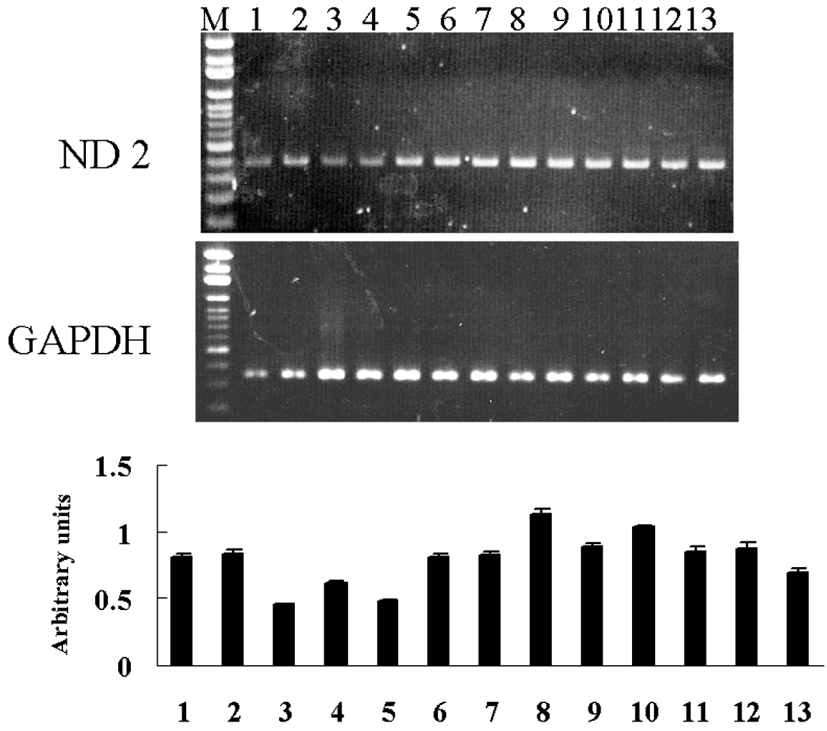

FIGURE 2. Expression levels of mtRNA in granulosa cells of different age groups. *Lanes 1–3*, 25–30 years old; *lanes 4–6*, 30–35 years old; *lanes 7–10*, 35–38 years old; and *lanes 11–13*, 38–42 years old.

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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Effects of Gonadotrophin-Releasing Hormone Agonists on Apoptosis of Granulosa Cells

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ABSTRACT: Granulosa cells are known to contribute to maturation of oocytes, and most of the growth factors exert their action via granulosa cells. It has been established that granulosa cell death during follicular atresia and luteolysis results from apoptosis. However, the precise mechanistic pathways of granulosa cell apoptosis have not yet been defined. In this study, we determined the proportions of apoptosis in granulosa cells treated with two kinds of gonadotrophin-releasing hormone agonists (GnRHa): buserelin and leuprorelin depot. The incidences of DNA fragmentation of human granulosa cells treated with buserelin and leuprorelin were 54.33% and 39.02%, respectively. The proportions of apoptotic bodies were 6.04% and 4.29%, respectively. There was a significant difference in the proportions of DNA fragmentation between the two kinds of GnRHa-treated granulosa cells. The apoptosis pathway and associated protein expression in granulosa cells treated with GnRHa were also determined. The Bax molecule, a pro-apoptosis protein, was expressed in granulosa cells undergoing apoptosis. In contrast, Bcl-2, an anti-apoptosis protein, could not be detected in the same group of granulosa cells. The distribution of cytochrome *c* determined via immunostaining showed a diffuse pattern, which most likely indicated that cytochrome *c* was translocated from mitochondria into the cytoplasm. Western blotting showed the expressions of caspase-9 and caspase-3 in patients' granulosa cells. The GnRHa effects on granulosa cells indicated a higher incidence of DNA fragmentation and apoptotic bodies in the buserelin-treated than in the leuprorelin depot–treated group. The granulosa cells go through the mitochondria-dependent apoptosis pathway; the indicated pro-apoptosis protein Bax was expressed and induced cytochrome *c* release from mitochondria, which then activated caspase-9 and caspase-3 until cell death occurred.

KEYWORDS: apoptosis; gonadotrophin-releasing hormone; granulosa cell

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INTRODUCTION

During follicular development, only a few follicles are selected for ovulation, whereas the remaining follicles undergo atresia. Recent studies have suggested that follicular atresia is associated with the apoptosis of granulosa cells.[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 the ovary in paracrine–autocrine processes.[4,5] Most of these factors do not directly affect oocytes but exert their actions via granulosa cells. The presence of granulosa cells appears to be beneficial for oocyte maturation and early development.[6] The downregulating effects of a gonadotrophin-releasing hormone agonist (GnRHa) are related to the frequency of administration and the prolonged occupation of GnRH receptors by the agonist.[7]

It has been established that granulosa cell death during follicular atresia and luteolysis results from apoptosis. However, the precise mechanistic pathways of granulosa cell apoptosis have not yet been defined. Mitochondria may be intimately involved in apoptosis, and some apoptosis-associated proteins have been determined to be present in granulosa cells.[8,9] Granulosa cells are known to contribute to the maturation of oocytes, and most growth factors exert their action via granulosa cells. Researchers have also reported that the incidence of apoptotic bodies in granulosa cells can predict the developmental capacity of oocytes in an *in vitro* fertilization (IVF) program.[10] In this study, we wanted to determine whether there is a different proportion of apoptosis in granulosa cells of patients treated with two kinds of GnRHa: buserelin and leuprorelin depot. The apoptotic pathway and associated protein in GnRHa-treated granulosa cells were determined.

MATERIALS AND METHODS

Granulosa Cells in Culture

The institutional review board of Taipei Medical University Hospital approved the study, which we intended to perform on granulosa cells discarded during an IVF program. Human granulosa cells were collected from patients who were recruited into an IVF–embryo transfer (ET) program. Ovarian stimulation was performed by desensitization by using a GnRHa followed by treatment with gonadotropins (follicle-stimulating hormone [FSH] and human menopausal gonadotrophin [hMG]). Ovulation was induced using human chorionic gonadotrophin (hCG). Oocytes were then retrieved by transvaginal ultrasonography–guided aspiration 34–36 h after hCG administration. Granulosa cells were harvested from follicular aspirates by centrifugation through Ficoll–Paque, washed, and suspended in Dulbecco's modified Eagle's medium containing 50 μg/mL uridine supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Human granulosa cells were grown at 37°C in a humidified atmosphere with 5% CO_2 for 3 days before experimental treatment.

Determination of Apoptosis

Apoptosis was assayed using terminal deoxynucleotidyl transferase–mediated dUDP nick-end labeling (TUNEL) and propidium iodide. DNA fragmentation and

apoptotic bodies were counted under a fluorescence microscope. Immunocytochemical staining of granulosa cells was carried out with antibodies specific for Bcl-2, Bax, cytochrome *c*, and caspase-3, as well as an LSAB-2 kit (Santa Cruz Biotechnology, Santa Cruz, CA). The first antibodies were mouse anti–human Bcl-2, goat anti–human Bax, rabbit anti–human cytochrome *c*, and mouse anti–human caspase-3 (1/200 dilution), respectively. The secondary antibodies and substrate were provided by the LSAB-2 kit, and then crystal mount was added before histomounting. The staining phenomenon of granulosa cells was examined under a light microscope. Double staining of the mitochondria and cytochrome *c* of granulosa cells with MitoTracker Red and rabbit anti–human cytochrome *c* antibody (1/200 dilution), and the secondary antibody, fluorescein isothiocyanate–conjugated mouse immunoglobulin G (IgG; at a 1/200 dilution), was then carried out, respectively. The results of double staining were classified into punctate and diffuse patterns by fluorescence microscopy to assess the translocation of cytochrome *c* from mitochondria. Western blot analysis of granulosa cells was determined specifically for caspase-3 and caspase-9. Granulosa cells were extracted with cell lysis buffer on ice for 30 min, and the total protein was detected with bicinchoninic acid assay. Aliquots containing 10 μg of cell lysates were separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with specific antibodies: mouse anti–human caspase-3 (at a 1/200 dilution, stained with the precursor form), goat anti–human caspase-3 p20 (at a 1/200 dilution, stained with the p20 subunit and precursor form), and rabbit anti–human caspase-9 p35 (at a 1/200 dilution, stained with the p35 subunit and precursor form). The secondary antibodies were horseradish peroxidase (HRP)–conjugated mouse, goat, and rabbit IgGs (at a 1/1000 dilution). Blots were then developed using an enhanced chemiluminescence (ECL) system.

RESULTS

The incidences of DNA fragmentation of human granulosa cells treated with buserelin and leuprorelin were 54.33% and 39.02%, respectively. The proportions of apoptotic bodies were 6.04% and 4.29%, respectively. There was a significant difference in the DNA fragmentation proportions between granulosa cells treated with the two kinds of GnRHa. After buserelin depot treatment, patients had shorter oocyte recovery times than those treated with leuprorelin depot (TABLE 1). The expressions of apoptosis-associated proteins in granulosa cells were investigated. The Bax molecule, a pro-apoptosis protein, was expressed in granulosa cells undergoing apoptosis. In contrast, Bcl-2, an anti-apoptosis protein, was not detected in the same group of granulosa cells. Bax, cytochrome *c*, and caspase-3 were all expressed in the cytosol of patients' granulosa cells (FIG. 1). We also explored the subcellular distribution of cytochrome *c*, a well-known apoptogenic factor that acts upstream of caspase-3 activation, to determine whether cytochrome *c* participates in granulosa cell apoptosis. The distribution of cytochrome *c* immunostaining showed a punctate pattern that coincided with localization of mitochondria in granulosa cells without apoptosis. However, many cells showed a diffuse pattern, which mostly likely indicates that cytochrome *c* is translocated from the mitochondria. Western blotting was used to ex-

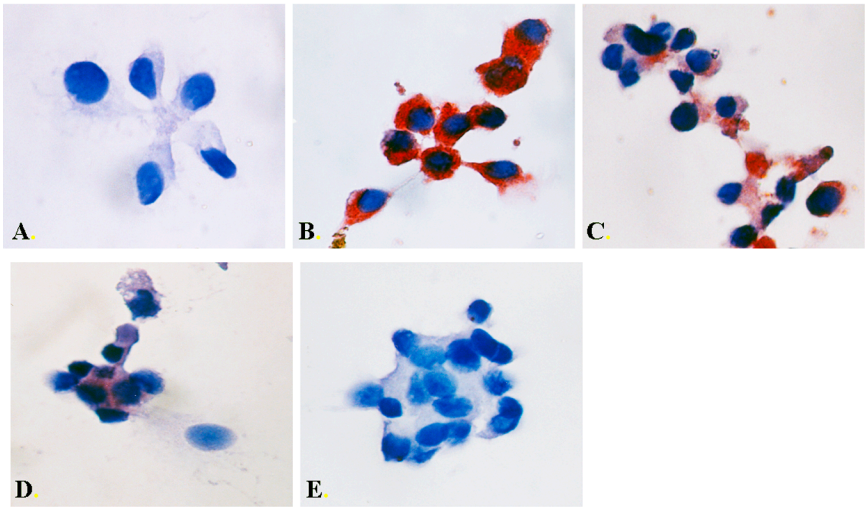

FIGURE 1. Immunocytochemical staining of granulosa cells. Granulosa cells were stained with specific antibodies and the LSAB-2 kit (red) and counterstained with hematoxylin (blue). (**A**–**D**) Granulosa cells were stained with specific antibodies against human Bcl-2, Bax, cytochrome *c*, and caspase-3, respectively. (**E**) Granulosa cells were stained without the first antibody as a negative control.

TABLE 1. Correlation of gonadotrophin-relating hormone agonists on granulosa cells and clinical factors

Pattern	Buserelin	Leuprorelin
DNA fragmentation (%)	55.55 ± 0.03*	39.02 ± 0.07
Apoptotic bodies (%)	6.04 ± 0.88	4.29 ± 0.74
Development index[a]	3.74 ± 0.15	4.06 ± 0.15
Fertilization (%)	63.85 ± 4.1	58.76 ± 6.1
Day of oocytes recovery	13.42 ± 0.3*	14.57 ± 4.2*
Age	34.04 ± 1.03	33.43 ± 1.04
Number of oocytes	7.13 ± 0.84	8.71 ± 2.16
E2 (before HCG)	1158.7 ± 204.9	1850.33 ± 63.81
P4 (before HCG)	1.14 ± 0.14	1.49 ± 0.29

*t-Test, $P < 0.05$.

[a]Development index: unfertilized oocytes were scored 0; dead embryos, 1; fully fragmentated embryos, 2; arrested embryos, 3; embryos with a slow cleavage rate, 4; and embryos with a normal cleavage rate, 5. The total score of each patient was determined, and the development index (total score/total oocyte number) was calculated for each patient.

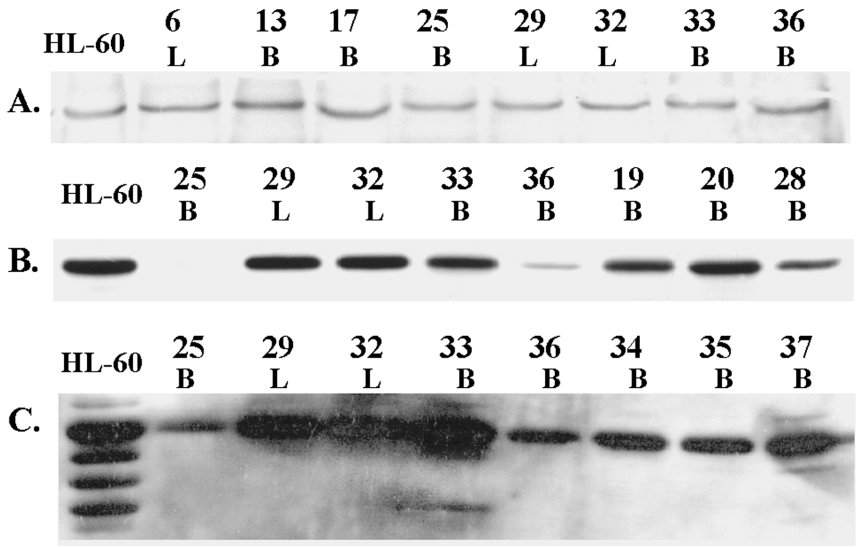

FIGURE 2. Western blot analysis of granulosa cells for caspase-3 and caspase-9. Aliquots containing 10 μg of cell lysates were separated by electrophoresis on12% SDS-PAGE gels and transferred to PVDF membranes with HL-60 serving as a positive control. Membranes were incubated with specific antibodies and horseradish peroxidase–conjugated secondary antibodies, and blots were developed using an enhanced chemiluminescence (ECL) system. Immunoblot analysis: a 34-kDa precursor form of caspase-3 was stained (**A**), caspase-3 p20, a 34-kDa p20 subunit, and the precursor form were stained (**B**), and caspase-9 p35, a 48-kDa p35 subunit, and the precursor form were stained (**C**).

amine the expression of caspase in granulosa cells, and the results indicated that caspase-9 and caspase-3 were activated in patients' granulosa cells (FIG. 2).

DISCUSSION

In this study, granulosa cells treated with buserelin showed a higher incidence of DNA fragmentation than the group treated with leuprorelin depot. After buserelin treatment, patients also showed a shorter oocyte recovery time than those treated with leuprorelin. Apoptosis-positive nuclei were detected in some granulosa cells cultured for 48 h in the presence of FSH, and apoptosis-positive nuclei were less abundant than in granulosa cells in untreated control cultures.[11] However, in the presence of GnRHa, apoptosis-positive nuclei were obviously more abundant than in untreated granulosa cells.[11] GnRHa has attracted attention as a therapeutic agent for synchronizing follicle growth. However, the use of GnRHa agonist neither increases the number of oocytes obtained nor improves the quality of oocytes.[11,12] More atretic oocytes were acquired when GnRHa was used simultaneously with FSH than when FSH was used alone.[11,13] Those reports are consistent with this study concerning the stimulatory effect of GnRHa on granulosa cell apoptosis.

The expressions of apoptosis-associated proteins in granulosa cells were determined in this study. The data indicate that the pro-apoptosis protein Bax was expressed and that cytochrome *c* release was induced from mitochondria; then caspase-9 and caspase-3 were activated until cell death occurred. Incubation of granulosa cells with ATP or staurosporine was also found to possibly trigger apoptosis.[9,14] Activation of caspases works concurrently with mitochondrial dynamics during apoptosis. The mechanism of apoptosis is conserved across species and is known to occur with a cascade of sequential activation of initiator and executioner caspases.[15,16] These studies suggest that GnRHa triggers apoptosis in human granulosa cells and that the downstream apoptotic cascade may act at least in part through mitochondria.

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Abnormal Mitochondrial Structure in Human Unfertilized Oocytes and Arrested Embryos

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ABSTRACT: To clarify the relationship between mitochondria and embryo development, we collected human unfertilized oocytes, early embryos, and arrested embryos. Unfertilized oocytes and poor-quality embryos were collected, and the ultrastructure of mitochondria was determined by transmission electron micrography. Four criteria for determining the mitochondrial state were mitochondrial morphology, cristae shape, location, and number of mitochondria. In mature oocytes, mitochondria were rounded with arched cristae and a dense matrix and were distributed evenly in the ooplasm. In pronuclear zygotes, the size and shape of mitochondria were similar to those in mature oocytes; however, mitochondria appeared to migrate and concentrate around pronuclei. In this study, 67% of examined unfertilized oocytes had fewer mitochondria in the cytoplasm. A decreased number of mitochondria located near the nucleus was also demonstrated in 60% of arrested embryos. Fewer differentiated cristae were determined in all three arrested blastocyst stages of embryos. The relative expressions of oxidative phosphorylation genes in oocytes and embryos were also determined. These data imply that inadequate redistribution of mitochondria, unsuccessful mitochondrial differentiation, or decreased mitochondrial transcription may result in poor oocyte fertilization and compromised embryo development.

KEYWORDS: embryo; mitochondria; oocyte

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INTRODUCTION

Mature oocytes contain approximately 10^5 mitochondria, but these are structurally undifferentiated compared with those of later embryo stages.[1,2] Throughout oogenesis and early embryogenesis, mitochondria in germ cells differ in appearance from those of somatic cells. Mitochondria in female germ cells assume a unique spherical profile, and an elongated mitochondrial morphology can be observed after implantation.[3,4] There are significant differences in net ATP content between oocytes, and low concentrations of ATP are generated in oocytes and early embryos.[5] Mitochondrial function can affect the physiology of embryos in many ways. This organelle has been recognized as the "powerhouse" of the cell because of its role in oxidative metabolism. The electron transfer chain consists of four respiratory enzyme complexes arranged on the mitochondrial inner membrane.

In recent years, an increasing number of reports have shown that mtDNA mutations are associated with human aging and mitochondrial diseases.[6–8] Declining mitochondrial function in older women may contribute to declining fertility.[9,10] Male subfertility and sperm dysfunction are also associated with defective mitochondrial function.[11,12] The loss of mitochondrial activity in oocytes obtained from aging couples therefore may contribute to lower embryo development and pregnancy rates.[13] To determine the relationships of mitochondrial structure and location with the ability for embryo development, we compared the ultrastructure of mitochondria through oocytes to early embryos by electron microscopy.

MATERIALS AND METHODS

Human Oocytes and Embryo Collection

This study was approved by the institutional review board of Taipei Medical University Hospital. Unfertilized oocytes were donated to our laboratory for research from patients enrolled in an *in vitro* fertilization program. In addition, embryos that were abnormally arrested and tripronucleus zygotes unsuitable for embryonic replacement or cryopreservation were also donated and used for the following experiments. Fresh human oocytes were obtained after informed consent in cases in which the donation of these oocytes to the research program would have little effect on the outcome of an *in vitro* fertilization cycle.

Electron Microscopy

Human oocytes and early embryos were fixed for 2 h in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M cacodylate buffer, washed in 0.1 M cacodylate buffer containing 0.2 M sucrose three times, and postfixed for 2 h in 1% osmium tetroxide. Dehydration was achieved by a graded series of 35, 50, 75, 95, and 100% ethanol, respectively. Samples then were infiltrated in a mixture of ethanol and spurr (Electron Microscopy Sciences, Fort Washington, PA) and were embedded in spurr. Ultrathin sections were cut on a Leica AG ultramicrotome, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and photographed on a Hitachi T-600 electron microscope.

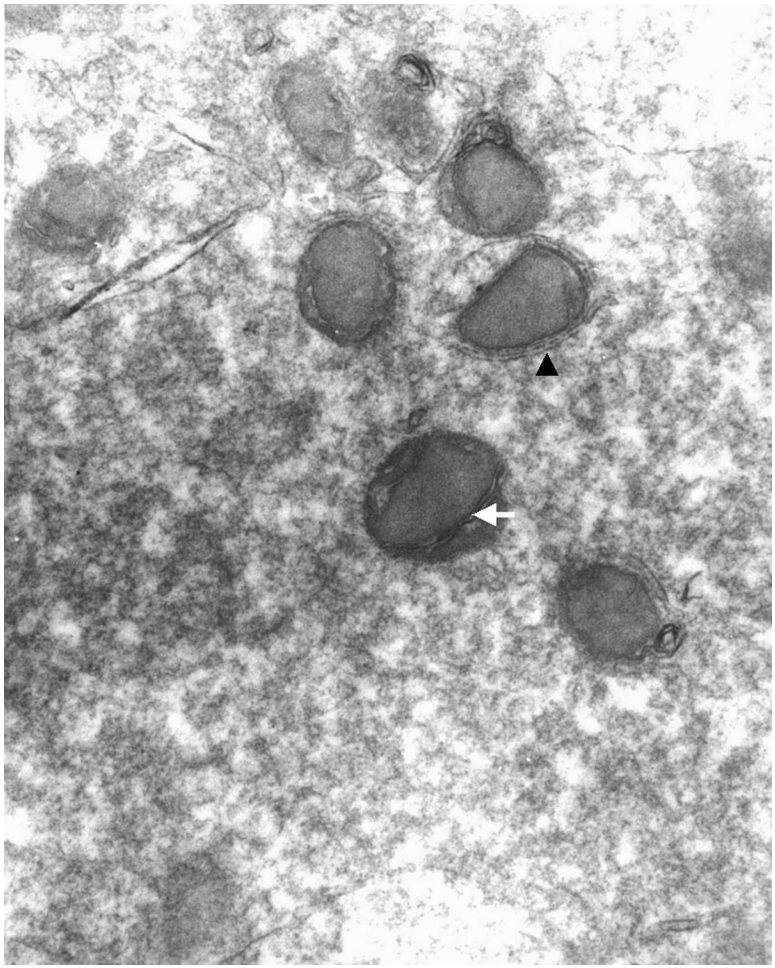

FIGURE 1. Ultrastructure of mitochondria in mature oocytes. The arched cristae were determined by electron microscopy. Mitochondrial cristae had an arched shape and were located in the mitochondria periphery (*arrow*). The smooth endoplasmic reticulum was also present together with mitochondria (*arrowhead*). Original magnification ×25,000.

RESULTS

To study the ultrastructure of mitochondria in human oocytes and early embryos, we examined normal mitochondrial structure and location by electron microphotography. In mature human oocytes, mitochondria are the prominent organelle. Mitochondria were rounded and possessed a dense matrix. Mitochondrial cristae had an arched shape and were located in the mitochondria periphery (FIG. 1). The smooth endoplasmic reticulum was also present together with mitochondria (FIG. 1). Some complexes existed in mature oocytes, which consisted of mitochondria aligned

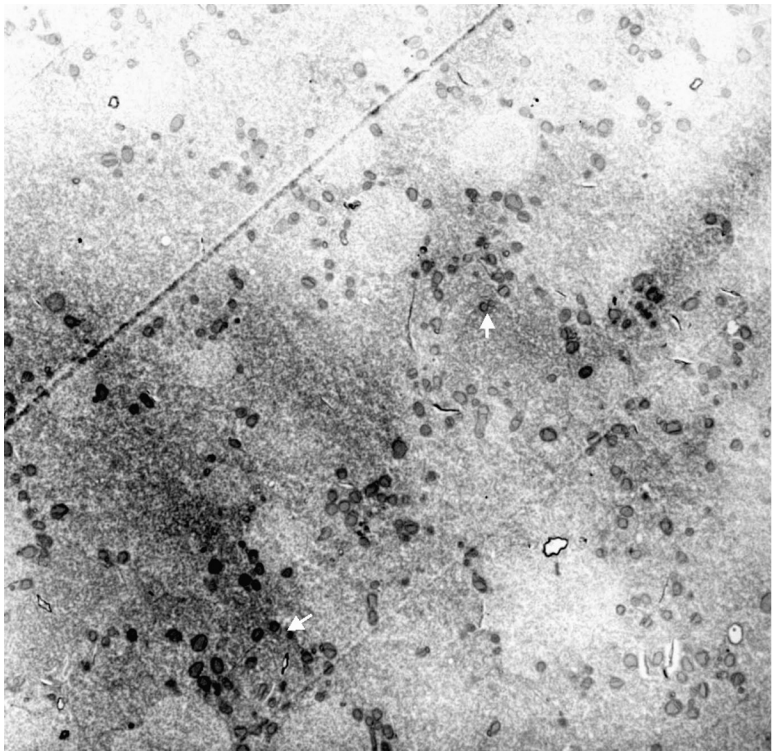

FIGURE 2. Multivesicular complexes distributed in mature oocytes. Mitochondria are arranged around vesicules and form multiple complexes scattered evenly throughout the ooplasm. Multivesicular complexes are indicated (*arrows*). Original magnification ×3,000.

around a vesicule. Multivesicular complexes were randomly distributed in mature oocytes (FIG. 2). Pronuclear zygotes had a similar size and shape compared with mature oocytes. The multivesicular complexes were also still observed at this stage. The mitochondria migrated and were concentrated around the pronuclei. In the eight-cell stage of embryos, mitochondria that were more elongated were seen together with rounded elements, and the cristae were more differentiated (FIG. 3). Some of the mitochondria began to form transverse cristae in the blastocysts (FIG. 4). A decrease in the number of mitochondria was also observed.

To determine whether the number and differentiation patterns of mitochondria affect the ability of oocyte fertilization and embryo development, we also collected 12 unfertilized oocytes and 15 arrested embryos to study their mitochondrial structure and location. There were multiple vacuoles and fewer mitochondria in unfertilized oocytes compared with functional mature oocytes. Eight unfertilized oocytes with less than 100 mitochondria were examined. Significantly decreased numbers of mitochondria located near the nucleus were observed in arrested embryos. Nine of 15 arrested embryos were characterized by this phenomenon. Peripheral arched cristae that were insufficiently differentiated to transverse cristae also were determined in

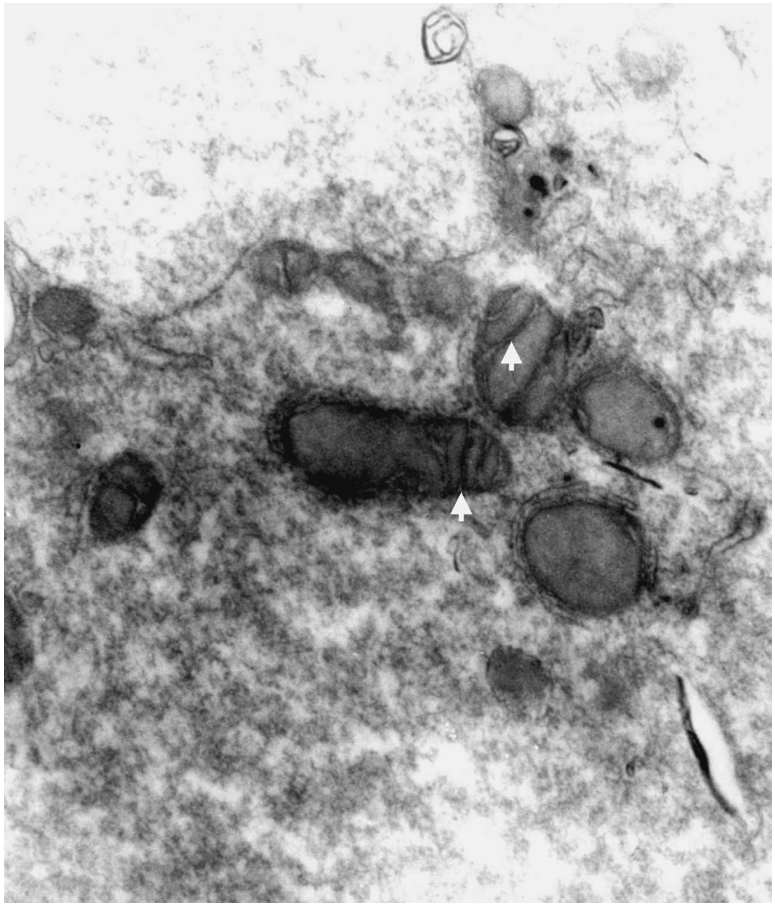

FIGURE 3. Electron microscopic examination of the eight-cell stage of an embryo. There are both round and elongated mitochondria in the embryo. Mitochondria are differentiated with few transverse cristae (*arrows*). Original magnification ×25,000.

all three arrested blastocysts examined. These data indicate that a reduced number of mitochondria may affect the fertilization potential of oocytes. Arrested embryos may occur because of a lack of redistribution of mitochondria or successful mitochondrial differentiation.

DISCUSSION

In our studies, mitochondria present peripherally arched to transverse cristae from mature oocytes to the blastocyst stage. The dynamic nature of cristae may be caused by proteins, which mediate electron transport and oxidative phosphorylation, being bound to the inner mitochondrial membrane. The varied crista structures repre-

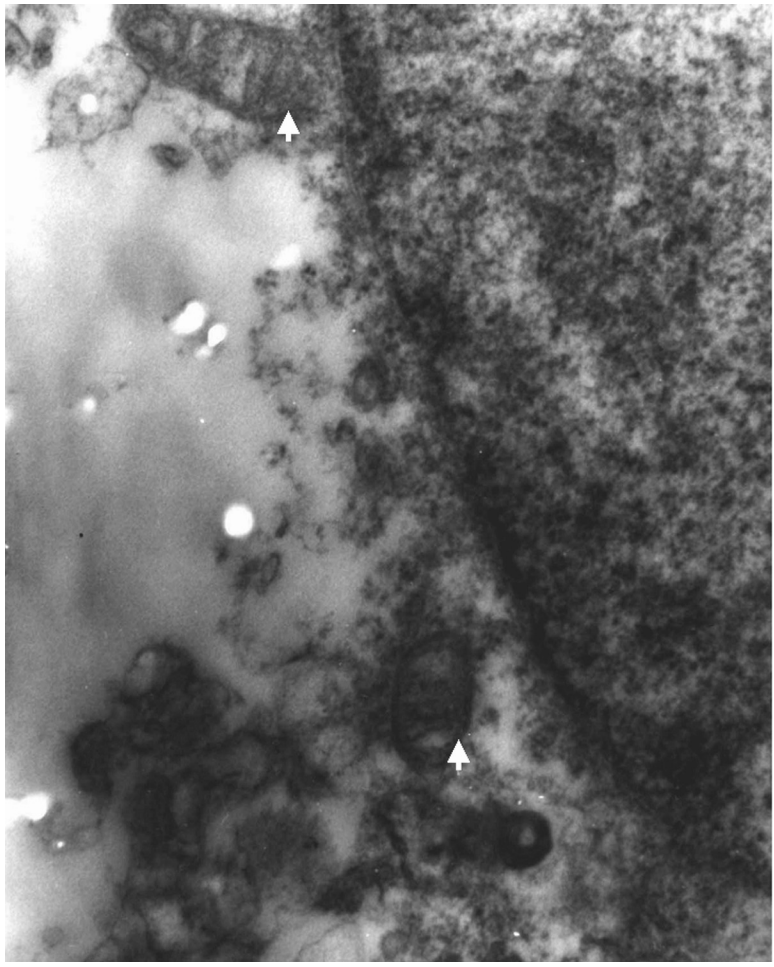

FIGURE 4. Electron microscopic examination of a blastocyst. Some of the mitochondria are differentiated with fully transverse cristae (*arrows*). Original magnification ×25,000.

sent mitochondria that progress from the arrested to the active state with embryo development. The relationship between energy production and cristae area has been shown in other studies. Proportional increases in respiratory chain enzymes and cristae surface areas have been observed.[14] The high energy demand of cells is met by an increase in the surface area of cristae.[15] Cristae differentiation may provide an efficient energy power supply for embryo development. Mitochondrial cristae change from a tubulovesicular pattern to a sparse, lamellar configuration in primordial germ cells during differentiation into oogonia.[16] Throughout oogenesis to early embryogenesis, despite cristae changes, mitochondria are also differentiated into various

shapes to fit the energy requirements of different developmental stages. Mitochondria vary considerably in size and structure depending on their source and metabolic state. Mitochondria in mature oocytes assume a unique spherical profile. The arrested state of round mitochondria in ovulatory oocytes was also reported by other groups.[4] Postfertilization changes in mitochondria are characterized by a gradual transition from round or oval mitochondria with a dense matrix and few arched cristae to forms that are more elongated, possessing a lighter matrix and more numerous cristae oriented transverse to the long axis of the mitochondria.[16] Increased mitochondrial metabolism appears to coincide with a decrease in density of the mitochondrial matrix and an increase in the number of cristae.

In mature oocytes, mitochondria are the prominent organelles and are evenly distributed in the cytoplasm. After fertilization, mitochondria become concentrated in the center of the oocyte, around the developing pronuclei (FIG. 3). The mitochondria are persistently located around the nucleus from fertilization to the early developmental stage. Pronuclear formation and fusion presumably require energy. Mitochondria were reported to move close to the nucleus along microtubules to satisfy this energy requirement.[17,18] The observation that mitochondrial DNA replication in somatic cells is preferentially located close to the nucleus,[19] with human pachytene oocytes giving the appearance of a necklace of mitochondria around the nucleus,[20,21] implies that mitochondria migrate close to the nucleus when replication is required in both germ cells and somatic cells. In immature oocytes, mitochondrial aggregation is granular and clumped. Maturation of oocytes to metaphase I or II leads to the appearance of evenly distributed mitochondria.[13] Mitochondria evenly distributed in the cytoplasm are translocated to the perinucleus area as embryos develop.

There is a decrease in the number of mitochondria in normal blastocysts compared with mature oocytes. This may result from the original mitochondria segregating into the blastomeres without biogenesis of mitochondria from fertilization to the blastocyst stage. With oocyte maturity at ovulation, mitochondrial amplification[22] and mtDNA replication cease.[23] The gap between oogenesis and resumption of new mtDNA synthesis means that mitochondria are diluted and partitioned into multiplying daughter blastomeres. At ovulation, each oocyte contains around 10^5 mitochondria.[5] The mtDNA does not replicate until gastrulation in diverse species.[22–24] In arrested embryos, we also observed that fewer mitochondria existed in the cytoplasm. There were not enough mitochondria to supply energy for embryo development because of less-functional mitochondria or defective mitochondria in aging oocytes.

The average expression proportions of the eight studied genes were 4.4, 5.8, and 12.9 in unfertilized oocytes, arrested embryos, and tripronucleus zygotes, respectively. Higher expression levels in tripronucleus zygotes compared with unfertilized oocytes and arrested embryos were determined.[5] In this study, the arrested embryos collected at the two- to four-cell stage and tripronucleus zygotes collected at around the eight-cell stage had normal growth rates. In previous studies, Piko and Taylor reported that mouse mtDNA does not replicate during preimplantation development but is transcribed actively from the two-cell stage.[26] There is an approximately 30-fold increase during cleavage through the blastocyst stage.[22] Embryos with normal growth rates are assumed to have more than two times the expression level compared with unfertilized oocytes. However, there were no significant differences in expression levels between unfertilized oocytes and arrested embryos. Reduced mitochon-

drial transcription may affect the development of embryos. There was a three-fold greater expression level in 3PN compared with unfertilized oocytes. Mitochondrial RNA expression does not seem to be modified in embryos developing with abnormal tripronucleus. The expression of the ATPase 6 gene in unfertilized oocytes decreases compared with that in early cleavage–stage embryos.[27] We previously determined multiple deletions of mtDNA in unfertilized oocytes and arrested embryos, as well as significant increases in the proportion of deleted mtDNA in unfertilized oocytes.[28] It is probable that there is a minimum requirement for ATP content for normal embryo development including chromosomal segregation, normal mitosis, and physiological events. Fully differentiated mitochondria, successful translocation, an optimal amount of mitochondria, and sufficient transcripts may be the minimum requirements for embryo development. Our study results provide some criteria for selecting adequately developed oocytes.

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