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粒線體功能對胚胎發育影響之研究(1/3)

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Abstract

Objective: Both nuclear and cytoplasmic factors contribute to oocyte activation, fertilization, and subsequent development; ATP production from mitochondria may play a crucial role. In order to test the hypothesis, we collected several groups of unfertilized oocytes, arrested embryos, and tripronucleate zygotes to evaluate the relationship between mitochondrial gene expression of oocytes/embryos and their fertilizability.

Design: Prospective laboratory research.

Settings: IVF laboratory in a university hospital.

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

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

Main outcome measure(s): Comparison of the expression levels of mitochondrial genes including ND2, COI, COII, ATPase 6, COIII, ND3, ND6 and Cyt b in unfertilized oocytes and arrested embryos.

Result(s): Significantly decreased transcription levels were expressed in unfertilized oocytes and arrested embryos compared with tripronucleate zygotes. The average

expression levels of the 8 determined genes were 4.4±0.7, 6.4±1.1, and 13.2±1.1 arbitrary units in unfertilized oocytes, arrested and tripronucleate embryos, respectively. Significantly decreased expressions of the ATPase 6, CO III, and ND3 genes were determined from samples with the mtDNA 4977-bp common deletion compared with non-deletion group.

Conclusion(s): The present study is the first report to present mitochondrial gene expression levels in human oocytes and embryos. These data support the idea that the down-regulation of mitochondrial RNA by defective oxidative phosphorylation genes possibly affects oocyte quality including fertilization and embryo development.

Key Words: Embryo, Mitochondrion, Oocyte, RT-PCR

Introduction

In eukaryotic cells, mitochondria are specialized organelles that are responsible for the synthesis of ATP. Two distinct genomes exist in all eukaryotic cells. One is located in the nucleus and is transmitted in the Mendelian fashion, while the other is located within the mitochondria and is transmitted through maternal lineage. The respiratory chain comprises 5 enzyme complexes located on the inner mitochondrial membrane. Complex I is the largest of these proteins with at least 26 polypeptides. NAD-linked substrates feed reducing equivalents into the chain via complex I which passes electrons down the chain to ubiquinone. Complex II accepts reducing equivalents from succinate and subsequently passes electrons to ubiquinone. From ubiquinone, electrons pass to complex III, to cytochrome *c*, and then via cytochrome oxidase to oxygen. The electrochemical proton gradient which supplies the energy for complex V to generate ATP is produced by complexes I, III, and IV (1-3).

mtDNA comprises a circular, histone-free molecule composed of 16.6 kb of DNA, present in 1 or more copies in every mitochondrion. There are 13 protein subunits: NADH:ubiquinone oxidoreductase subunit 1 (ND1), ND2, ND3, ND4, ND4L, ND5, and ND6 of complex I; cytochrome b of complex III; cytochrome *c* oxidase subunit 1 (COI), COII, and COIII of complex IV; and subunits 6 and 8 of ATPase of complex V. They are required for oxidative phosphorylation of a total of about 80 subunits, with

the remainder being encoded by nuclear genes and imported into the mitochondrion. mtDNA also contains 2 ribosome subunits and 22 transfer RNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes; mtDNA encodes 13 proteins that are all components of the respiratory chain, whereas nuclear DNA encodes the majority of the respiratory chain proteins, all proteins that regulate replication and transcription of mtDNA, as well as proteins necessary for the biogenesis of mitochondria (2).

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

mtDNA deletions may contribute to mitochondrial dysfunction and the failure of embryonic development (8,11,12). Mitochondrial replication for maintenance of a proper amount of functional mitochondria, transcription for synthesis of mtRNA, and translation for mitochondrial biogenesis during oogenesis is crucial for successful fertilization and embryo development. We examined the expression levels of 8 mitochondrial genes, including ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, and Cyt b genes, in human unfertilized oocytes and abnormal embryos to determine whether decreased expression of mitochondrial genes possibly interferes with developmental capacity.

Materials and methods

Preparation of oocytes

The institutional review board of Taipei Medical University Hospital approved the study which we intended to perform on oocytes and embryos discarded during an IVF program. Oocytes were obtained from patients who were recruited into an IVF-ET program. Ovarian stimulation was performed by desensitization in the previous luteal phase using a gonadotropin-releasing hormone agonist followed by treatment with gonadotropins (FSH and hMG). Ovulation was induced using hCG. Oocytes were then retrieved by transvaginal ultrasonography-guided aspiration 34-36 h after hCG administration. Oocytes were inseminated in vitro with spermatozoa for 16-18 h and subsequently cultured in tissue culture medium supplemented with 10% human plasmanate (8). Twenty hours after insemination, cumulus cells were mechanically removed, and oocytes were examined for the presence of pronuclei. Unfertilized oocytes collected 48 h after retrieval were prepared for evaluation of mitochondrial gene expression. Abnormal embryos were harvested when embryos were arrested at 4 to 8 blastomeres from unknown causative factors. Tripronucleate embryos obtained 18-24 h after IVF were cultured until they were 4 to 8 blastomeres and then were prepared for analysis. From 48 patients enrolled in the IVF procedures, 75 unfertilized oocytes were donated to our research. In addition, 45 embryos that were

abnormally arrested and 24 3PN zygotes unsuitable for embryonic replacement or cryopreservation were also donated and used for the following experiments.

Semiquantitative RT-PCR

Total RNA extracted from oocytes and embryos was used as templates and cDNA was prepared using the RNA extraction and reverse transcription-PCR kit from Ambion, (Headquarters, USA). RT-PCR amplifications were performed with 3 ul of cDNA in a total volume of 50 ul of amplification buffer, 40 pmol of specific primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). The sequences of the oligonucleotide primers used in this study are listed as follows: ND2 (Forward, np5101-5120, TAACTACTACC GCATTCCTA; Reverse, np5400-5381, CGTTGTTAGATATGGGGAGT), COI (Forward, np7041-7060, GTCCTATCAATAGGAGCTGT; Reverse, np7340-7321, CTTCGAAGCG AAGGC TTCTC), COII (Forward, np7845-7864, CAGACGAGGTCAACGATCCC; Reverse, np8130-8111, GTTTGGTTTAGACGTCCGGG), ATPase6 (Forward, np8781-8800, CGGACTCCTGCCTCACTCAT; Reverse, np9090-9071, AGAGGGAAGGTTAATGGTTG), COIII (Forward, np9611-9630, CGTATTACTCGCATCAGGAG; Reverse, np9908-9889, GCCAAAGTGATGTTTGGATG), ND3 (Forward, np9981-10000,

TGAGGGTCTTACTCTTTTAG; Reverse, np10300-10281, GTTTGTAGGGCTCATGG TAG), ND6 (Forward, np14291-14310, TCATAAATTATTCAGCTTCC; Reverse, np14579-14560, TGATTGTTAGCGGTGTGGTC), Cytb (Forward, np15506-15525, GACAATTATACCCTAGCCAA; Reverse, np15800-15781, GTCCAATGATGGTAAAA GGG), and GAPDH (Forward, CCTTCATTGACCTCAAC; Reverse, AGTTGTCATGGATG ACC).

For semiquantitative amplification, each cycle was carried out at 92 ^{o}C for 30 s, 58 ^{o}C for 30 s, and 72 ^{o}C for 60 s. 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 annealing temperature and numbers of PCR cycles. Each PCR product was run through a 2% agarose gel and was visualized with ethidium bromide staining. Cycle-dependent amplification of the housekeeping GAPDH mRNA was almost identical in oocytes, arrested embryos, and 3PN embryos, which allowed semiquantitative comparison of mtDNA PCR products obtained with each sample by densitometric analysis. The relative expression levels of the PCR products were determined using an imaging desitometer, and results were expressed as a ratio of 8 genes divided by GAPDH.

Determination of the 4977-bp deleted mtDNA

Oocytes and embryos were stored in 20 μl of 1x PCR buffer containing 0.05 mg/ml of proteinase K, 20 mM DTT, and 1.7 μM SDS. After digestion for 1 h at 56 ^{o}C and 10 min of heat-inactivation of proteinase K at 95 ^{o}C, this template was then used in the PCR assays. The sequences of the oligonucleotide primers used in this study are listed as follows: H1 (np 8285-8304 CTCTAGAGCCCACTGTAAAG) and L1 (np 13650-13631 GGGGAAGCGAGGTTGACCTG). The mtDNA was amplified in a 100-ul reaction volume containing a final concentration of 1.5 mM $MgCl_2$, 1 mM dNTPs, 20 pmole of each primer, and 1.5 IU Taq polymerase (Life Technologies). Then the following amplification profile was used: 1 cycle of 95 ^{o}C for 5 min; 35 cycles of 95 ^{o}C for 40 s, 58 ^{o}C for 40 s, 72 ^{o}C for 6 min, and 1 cycle of 72 ^{o}C for 7 min, then the mixture was kept at 4 ^{o}C (8). PCR products were examined by agarose gel electrophoresis in which 8 ul of PCR products was separated on a 1.5% agarose gel and stained with ethidium bromide. 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, CA). Sequencing reactions were read on an ABI Prism Model 377 cycle sequencer (Applied Biosystems).

Statistical Analysis

All experiments were repeated at least 3 times. ANOVA analysis was used to

determine statistically significant differences ($p < 0.05$) between experimental groups.

Results

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

The expression levels of all 8 examined mitochondrial genes and the GAPDH gene were determined by densitometric analysis of RT-PCR products. In order to obtain the relative expression levels of the 8 genes, each of them was followed by normalization to the GAPDH gene. Samples were collected into 3 groups according the development stage of oocytes and embryos, which were unfertilized oocytes, arrested embryos, and tripronucleate zygotes. We collected 16, 9, and 6 cohorts of

unfertilized oocytes, arrested embryos, and tripronucleate zygotes, respectively, with each cohort including 3 oocytes or embryos from the same donor. The normalized expression ratios of the 8 different genes in each sample are presented in Fig. 2 and Table 1. These 8 gene transcription levels remained at similar levels in the same groups, with no significant differences between analyzed genes in each cohort. However, there were statistically significant decreases in transcript levels expressed in unfertilized oocytes and arrested embryos compared to 3PN embryos. The average ratios of the expression of the 8 different genes were 4.4±0.7, 6.4±1.1, and 13.2±1.1 in unfertilized, arrested, and 3PN embryos, respectively.

In our previous study, the 4977-bp rearranged mtDNA was observed to exist in oocytes and embryos, and resulted in compromised developmental capacity (8). In order to determine whether rearranged mtDNA in oocytes and embryos affects expression levels of mtDNA genes, mitochondrial RNA expression was also classified by gathering 2 groups according to samples with or without the 4977-bp rearranged mtDNA (Tables 2). In a comparison of cohorts with and these without the 4977-bp deletion, there were significantly decreased RNA expressions of the ATPase 6, CO III, and ND 3 genes in unfertilized oocytes and arrested embryos harboring the 4977-bp deletion. However, there were no differences in 3PN embryos with or without the 4977-bp deletion.

Discussion

Unknown factors causing unfertilized oocytes and arrested embryo development may be due to cytoplasmic defects of the oocytes. In particular, the organization and continued metabolic activity of mitochondria are necessary features of cytoplasmic maturation and resumption of meiosis (7,13). Accumulation of mtDNA rearrangements, decreased mtDNA copy number, and mtRNA expression reflecting mitochondrial defects may compromise the maturation of oocytes. The 4977-bp deletion is the most common mtDNA deletion associated with human aging processes as well as in oocytes and embryos (8,14,15). In our previous study, the 4977-bp mtDNA rearrangement was found to have frequencies of 66.1% in unfertilized oocytes, 34.8% in arrested embryos, and 21.1% in tripronucleate (3PN) embryos (8). There was a significant increase in the proportion of deleted mtDNA in unfertilized oocytes. Accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and impaired ATP production, and may also interfere with fertilization of human oocytes and subsequent embryonic development (8,11). Frequencies of the 4977-bp deleted mtDNA in oocytes have also been determined and reported by other studies (11,16,17). The 4977-bp deletion causes the removal of major structural genes containing ATPase 6 and 8, cytochrome oxidase III, and NADH-CoQ oxidoreductase (ND3, ND4, ND4L, and ND5). The deleted genes in this rearranged

mtDNA may result in impaired gene expression by decreasing the expression of the deleted genes. This deletion also creates a chimeric gene, which fuses the 5'-portion of ATPase 8 and the 3'-portion of ND5 genes of mtDNA. Therefore, the deleted or truncated genes in this rearranged mtDNA may result in impaired gene expression by decreasing the expression of the deleted genes by producing transcripts of fused genes. Consistently, lower expression levels of ATPase6, CO III, and ND3 genes in unfertilized oocytes and arrested embryos with the 4977-bp deleted mtDNA than other genes outside of the deleted region were determined in this study. The decreased transcription reflected rearranged mtDNA in unfertilized oocytes and arrested embryos. However, compensatory transcription may take place in 3PN zygotes with rearranged mtDNA. There were no significantly decreased expression levels determined in 3PN zygotes with or without the 4977-bp deleted mtDNA (Table 2).

Most human somatic cells contain about 1000 mitochondria, and each mitochondrion consists of 2 to 10 copies of mtDNA (18). There is a higher copy number of mtDNA in mature oocytes but with just 1 mtDNA molecule per organelle (4). 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 (19). The number of mtDNA molecules in a mouse oocyte was approximately 92,000 (4), and

that of the human oocyte was estimated to be about 100,000 (16). Recently, 2 reports quantified the mtDNA copy number in unfertilized human oocytes obtained after IVF failure using real-time PCR (20,21). The former study found an average of 314,000 mtDNA copies in 18 oocytes, and the latter found an average of 193,000 mtDNA copies in 113 unfertilized oocytes. Steuerwald *et al.* reported that no correlation was identified between the mtDNA concentration in either polar bodies or cytoplasmic samples and their corresponding oocytes (20). In contrast, the average mtDNA copy number was significantly lower in cohorts suffering from fertilization failure compared to cohorts with a normal rate of fertilization (21). The important role of mitochondria in embryonic development has been determined by experiments with mice whose mitochondrial transcription factor A gene was disrupted by the gene knockout technique (22). Heterozygous knockout mice exhibited a reduced mtDNA copy number and respiratory chain deficiency in the heart. Homozygous knockout embryos exhibited severe mtDNA depletion with defects in oxidative phosphorylation and had abundant enlarged mitochondria with abnormal cristae. That study suggested that the maternal contribution of functional mitochondria was sufficient to maintain oxidative phosphorylation during early embryonic development.

In a previous study, Piko and Taylor (4) reported that mouse mitochondrial DNA does not replicate during preimplantation development but is transcribed actively

from the 2-cell stage. There is about a 30-fold rise during cleavage through the blastocyst stage (23). There are no experimental data concerned with mitochondrial expression levels in different stages of human embryos. Increased expression of mtDNA will accompany embryo development, and lower transcription ability may interfere with oocyte fertilization or embryo development. Since amounts of RNA in single oocytes or embryos were too low to determine the expression level of specific genes by Northern blotting, in this study, cohorts of every 3 oocytes or embryos were lysed to harvest total RNA and determine the semiquantative expression level of RNA by RT-PCR. In order to rule out limitations of RT-PCR, various replication cycles were determined to identify the optimal cycles with RT-PCR products in a linear range. The average expression proportions of the 8 studied genes were 4.4, 6.4, and 13.2 arbitrary units in unfertilized oocytes, arrested embryos, and 3PN embryos, respectively. The ND2, CO I, CO II, ATPase 6, CO III, ND3, ND6, and Cyt b gene transcription levels remained at similar levels in the same groups. mtDNA transcripts are polycistronic, which means that each gene is separated following precise endonucleolytic excision of the tRNAs from the nascent transcripts. Although different mtDNA transcripts encode different protein assemblies to 1 of 5 mitochondrial complexes, in our study, the expression levels of the 8 genes were similar in unfertilized oocytes, arrested embryos, and tripronucleate

zygotes. Consistently, mtDNA transcripts are polycistronic (24,25), with different mtRNA expression levels showing the same pattern in the same oocyte. The same expression level representing the overall defective transcription was observed in unfertilized oocytes. There was a higher expression level in 3PN embryos compared to unfertilized oocytes and arrested embryos. In this study, arrested embryos were collected at the 2- to 4-cell stage, and 3PN zygotes were collected at the 8-cell stage; all had normal growth rates. In normal development, the stage with 2 to 4 blastomeres may have more than twice the expression level of mtRNA compared to unfertilized oocytes. Mitochondrial transcription appeared to still be arrested from unfertilized oocytes to the 4-cell stage of the examined arrested embryos. However, there was no significant difference in expression levels between unfertilized oocytes and arrested embryos in this study. Oocytes resuming transcription may correlate with sufficient production of ATP. Differences in the amount of ATP generated by mature human oocytes may be related to the fertilization potential and developmental competence of an embryo (7).

There was almost a 3-fold higher expression level in tripronucleate zygotes compared to unfertilized oocytes. Mitochondrial RNA expression did not seem to be modified in embryos developed with abnormal 3 pronuclei. A probable hypothesis to explain the existence of 3 pronuclei would be normal fertilization by a single

injected spermatozoon along with the non-extrusion of a second polar body (26,27). There are no reports implying that cytoplasmic or mitochondrial dysfunction can cause the formation of 3PN zygotes. Injection with the cytoplasm of 3PN zygotes may enhance the clinical pregnancy rate in patients with repeated implantation failure (28). This implies that 3PN zygotes may have sufficient amounts of mtDNA content and mtDNA transcripts for embryo development. Decreased expression levels of the ATPase 6 gene in unfertilized oocytes compared with early cleavage-stage embryos have also been reported (29). In conclusion, the current study is the first report to present mitochondrial gene expression levels in human oocytes and embryos. These data support the idea that the down-regulation of mitochondrial RNA possibly affects oocyte fertilization and embryo development.

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