行政院國家科學委員會補助專題研究計畫成果報告

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※ 人類粒線體基因突變與卵細胞老化相關性之研究 ※

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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC89－2314－B－038－082－

執行期間：　89 年 8 月 1 日至　90 年 7 月 31 日

計畫主持人：高淑慧

共同主持人：趙湘台

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：台北醫學大學生物醫學技術研究所

中 華 民 國　89　年　10　月　31 日

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計畫參與人員：

一、計畫中文摘要：

卵細胞的功能缺陷，常成為受孕力降低及女性不孕症的重要影響因素。因此，對於造成卵細胞老化或缺陷的形成因素的探討更顯為急迫。在卵子生成(Oogenesis)及成熟過程(maturation)，以及胚胎發育及分化過程，都須藉由粒線體所提供的能量(ATP)，才能夠順利完成各個成長過程。粒線體的功能會受基因異常、缺氧及氧化性壓迫而造成呼吸功能缺陷，並且直接減少 ATP 的合成。高反應性氧分子及自由基這會經由缺陷性的呼吸作用而產生，並且對細胞內的 DNA, RNA, 蛋白質等產生廣泛性的氧化性破壞。目前，我們已經初步由榮民總醫院及國泰醫院收集了 24 例由接受試管嬰兒治療而未授精之卵細胞（unfertilized oocytes 或 degenerative oocytes）、各階段分裂停止之授精卵（如 1 cell, 2-cell 或 4-cell），以及 12 例顆粒細胞的標本。並針對這些卵細胞的粒線體DNA的斷損突變及解毒酵素的基因突變（包括 *glutathione-S- transferase GSTM1,GSTT1,GSTP1*）基因型的鑑定及表現量加以定量。實驗結果於無法授精的卵細胞及其顆粒細胞中出現 mtDNA 斷損片段約 3400bp, 3940bp, 4590bp，且同病患的 *GSTM1*gene 為 null mutation。由結果顯示病患帶有 null type mutation 的 *GSTM1* gene，常伴隨有粒線體 DNA 的斷損突變。以 primer-shift PCR 的方法分析斷損突變，其片段長度隨引子的設計作平移，目前我們正進行 DNA 定序分析。

此外，本研究更進一步針對不孕症治療中進行超排卵步驟對卵巢及卵細胞及受精卵所產生的影響進行研究。以 C57BL/6J mice 注射 5 IU/mice 的 PMSG 及 HCG 分別注射同劑量，不同的注射週次(從 1 週次至 6 週次)，將取出之卵巢、排出之受精卵、輸卵管及子宮，分析因超排卵所引發之傷害，分析其粒線體DNA的斷損突變包括點突變及片段突變，並且以 RT-PCR 定量熱休克蛋白(heat sock protein HSP70 及 HSP90) mRNA的表現量。由結果顯示 C57BL/6J mice 隨著刺激週次的增加，其排卵率、受精率、胚胎分裂率皆呈現降低，胚胎

死亡率增加。六週次的刺激超排卵遠較於一週次的刺激超排卵產生大量的HSP70 mRNA大量表現及mice mtDNA片段突變(length mutation)的增加大量表現。

我們期望本計畫之研究成果能使我們對於粒線體基因突變在卵細胞及卵丘細胞的的老化及受孕力降低上所扮演的角色有進一步的瞭解。此外，藉此超排卵的研究能更進一步了解以賀爾蒙治療所引發的傷害，並且能尋找出更適當的治療方式，使不孕症的療程能有更進一步的療效。

關鍵詞：粒線體、斷損突變、卵細胞、老化、超排卵

二、計畫英文摘要：

Females of most mammalian species, including humans, experience reproductive declined with age. The fertilization ability and developmental competence of human embryos appear to be directly related to the metabolic capacity of mitochondria. Mitochondrial dysfunctions resulting from a variety of intrinsic and extrinsic influences, including genetic abnormalities, hypoxia and oxidative stress, can profoundly deplete the level of ATP generation in oocytes, which in turn may result in aberrant chromosome segregation or development arrest. Deletions and point mutations in oocyte mitochondrial DNA may subtend metabolic deficiencies or replication disorders in some infertile women and in women with advanced age. We hypothesized that some unfertilized oocytes and cleavage-arrest embryos from women undergoing IVF would contain the mtDNA mutations (deletions, point mutation, and depletion) and that oocytes and ovaries from older women, whose oocytes tend to have low development potential, would be more likely contain mtDNA mutations than oocytes from younger women.

In this study, we collected 24 oocytes and embryos from women undergoing IVF program in the department of OB/GYN in Taipei-Veterans General Hospital and Cathy Hospital. The samples were collected due to unfertilization or cleavage-arrest in 1 cell, 2-cell, and 4-cell stage. By using the PCR and RT-PCR methods, we examined the genetic integrity of human degenerative oocytes and embryos. Large-scale deletions and point mutation of mtDNA and the mutations of detoxification enzymes including *glutathione-S-transferase (GST M1, GSTP1)*. There are several types of deleted mtDNA were generated from the aged oocyes and cumulus cells. Three types of length mutation were generated from 3400bp, 3940bp, and 4590bp. The primer shift PCR was also proceeded on for excluding miss-annealing amplification. The one who carried the deleted mtDNA was examined with a null type of *GSTM1*.

The present study aims to shed light of abnormal oocytes ovulated by aged females. In order to reach this goal, cellular and biochemical traits of ovulated from C57B/6J female mice retrieved after exogenous ovarian hyperstimulation for

different cycles from one to six cycles. Decreased numbers of ovulated oocytes were collected from the treated mice with repeated ovarian stimulation for six cycles. Furthermore, the fertilization rate, cleavage rate, and development capacity were also decreased. The expression of heat shock protein HSP70 was dramatically increased in the overstimulated ovaries. Large scale deletions of mice mtDNA were also examined in the overstimulated ovaries.

Taken together, the results will underlie the mutation of mtDNA and gene expression in mitochondria may play some roles in pathophysiology of oocyte senescence.

Keywords : mitochondrial DNA, deletion, oocyte, ageing, superovulation

三、研究計畫之背景及目的：

Introduction

The post-ovulatory ageing of mammalian oocytes is elucidated to be associated with a reduction in oocyte viability and potential for development (1). The developmental competence of mouse and human early embryos appears to be directly related to the metabolic capacity of a finite complement of maternally inherited mitochondria (2). Mitochondrial dysfunctions resulting from a variety of intrinsic and extrinsic influences, including genetic abnormalities, hypoxia and oxidative stress, can profoundly influence the level of ATP generation in oocytes and early embryos, which in turn may result in aberrant chromosome segregation or developmental arrest. Recently, Tarin (3) proposed a mechanism based on 'the oxygen radical-mitochondrial injury hypothesis of ageing (4) to explain the effects of post-ovulatory ageing on mammalian oocytes. This mechanism ascribes a key role in senescent process to oxygen radical damage to mitochondria may both decrease the functionally intact mitochondria and ATP levels, and increase the proportion of reactive oxygen species (ROS) by the electron transport chain.

In mammals, mtDNA are the primary genetic elements that can profoundly effect development, metabolism, and reproductive performance (6-8). In the human, significant differences in net ATP content occur among mature oocytes from the same and different patients, and these differences are not only oocyte-specific but appear to be associated with embryo developmental competence (9,10). In this respect, several investigators have suggested that the maternal age-associated reduction in embryo developmental competence may be related to an inadequate capacity to generate ATP at levels sufficient to support normal chromosomal segregation (11) or normal biosynthetic mitotic, and physiological activities of blastomeres (9,10).

Mitochondrial dysfunction leading to oxidative damage and apoptosis, hypoxia, and deletions or point mutations in the oocyte mitochondrial genome (mtDNA) (12-15), especially in oocytes of older woman (12), are the types of adverse influences that may contribute to reduce mitochondrial function in the oocyte. There are more than 150

deletions and insertions of human mtDNA, which were accumulated with age and are responsible for certain neuromuscular diseases. In previous studies of Barrit's group, mtDNA rearrangements were detected in 50.5% of the oocytes (n = 295) and 32.5% of the embryos (n = 197). This represents a significant difference in the percentage of mtDNA rearrangements between oocytes and embryos. Twenty-three novel mtDNA rearrangements with deletions, insertions and duplications were found. There was no significant age-related increase in the percentage of human oocytes or embryos that contained mtDNA rearrangements.

Furthermore, we also explored the detrimental effects on the ovaries with repeated ovarian stimulation by using gonadotropin. Ovarian hyperstimulation and ovarian induction permits the growth and development of supernumberary follicles and the ability to the time of the initiation of the preovulatory oocyte maturation. The success of clinical IVF has been largely attribute to the generation of such follicles and the ability to retrieve metaphase II oocytes at the high frequency. In mammals such as mice, rats and hamster, reduced fertility and pre- and post-implantation mortality have been indicated as consequences of a single round of ovarian stimulation using standard doses of gonadotropin (16). At higher doses, increased frequencies of oocyte aneuploidy, embryo mortality, fetal growth retardation and congenital abnormalities have been reported. Reduced viability with ovarian stimulation is often attribute to adverse maternal factors such as inadequate uterine synchrony or receptivity, and chromosome defects in the oocyte whose lethality is expressed during preimplantation stages. Here, we examined whether repeated ovarian stimulation affected the quality of mouse oocytes at the cellular and biochemical levels, and whether types of defects associated with incompetent or compromised human oocytes occurred in gonadotropin-treated mice.

In order to investigate the possible pre-existence of mtDNA mutation in unfertilized oocytes and degenerative embryos and which were treated with repeated ovarian stimulation, we perform following approaches may provide new insights into mtDNA maintenance, segregation, and transmission. We collected 24 oocytes and 12 cases of granulose cells from the women who unfertilized metaphase II oocytes or cleavage-arrest embryos from the IVF program at the Veterans General Hospital-Taipei and Cathy Hospital. We analyzed the existence of mtDNA deletion, and *GSTM1* genotyping by the PCR analysis. Furthermore, we also examined the alternation of gene expression of stress-induced gene in this study.

四、研究方法及成果：

1. Collection of samples

We collected unfertilized metaphase II oocytes from the IVF program at the Veterans General Hospital-Taipei, Cathay Hospital, and Taipei Medical University Hospital. The study was conducted prospectively and including unfertilized oocyte from successive patients. Woman received a GnRH analogue on day 21 of

the preceding cycle and then hMG daily until at least two follicles exceeded 1.8 cm in diameter, when they received 10,000 IU hCG. Thirty-four hours latter, oocytes are retrieved and cultured in warmed modified human tubal fluid (HTF) medium. Oocytes are incubated with washed sperm in HTF medium at 37°C in 5% CO_2 under oil for 48 hours. Unfertilized metaphase II oocytes are defined as which fail to exhibit a second polar body and two pronuclei after 48 hours. Unfertilized MII oocytes and cumulus cells are collected and store at -20 °C until use.

2. Ovarian stimulation schedule

Cycles of ovarian stimulation were initiated in 5-6 week old C57B/6J mice according to the standard protocol of 5 IU of pregnant mare serum gonadotropin (PMSG) administered s.c. followed in 48 hr by 5 IU of human chorionic gonadotropin (HCG) and then mating with male mice. One to six cycles were performed continuously. Embryos were collected from the oviduct ampulla at 38 hr post HCG on each round. Embryos were cultured at 37 °C, 5% CO_2 in M16 medium.

3. Oocyte DNA extraction

At the end of the incubation period, the unfertilized oocyte-granulosa cell complex will be stripped of adherent granulosa cells by gentle pipeting. All the oocytes are then checked by microscopy for morphological changes and no sperm binding. The unfertilized oocyte is incubated at 56 °C for 2 hr in 50 µl lysis buffer containing 2% SDS and 50 mM Tris-HCl (pH 8.3), and boiled for 10 min. All the DNA samples are finally conserved in 200 µl of 10 mM Tris-HCl, pH 8.3.

4. Synthesis of oligonucleotide primers

Oligonucleotide primers used for the amplification of the target sequences of mtDNA are chemically synthesized by Roche Molecular System, Inc. (Branchburg, NJ). The nucleotide sequences and sizes of the PCR products obtained from these primer pairs are summarized in Tables 2-4.

5. Mice Oocyte RNA extraction

Total RNA was isolated using RNAzol B solution from oocytes, embryos, and ovarian.

6. Polymerase chain reaction (PCR)

The desired target sequence of mtDNA will be amplified from 15-20 ng of each DNA sample in a 50 l reaction mixture containing 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of Ampli-Taq® DNA polymerase (Perkin-Elmer/Cetus, Roche Molecular System, Inc., Branchburg, NJ), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.3 (16). PCR is carried out for 35 cycles in a DNA thermal cycler (Perkin-Elmer/Cetus) using the thermal profile of denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec, and primer extension at 72°C for 40 sec.

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Table 1: Oligonucleotide primers used for the analysis of the deletions in mitochondrial DNA and mutation in the detoxification enzymes of human gametes.

Primer pair	Sequences	Product (bp)
Mitochondrial DNA		
L258-H640		383
L258	5'-CAGCCACTTTCCACACAGAC-3'	
H640	5'-GGGGTGATGTGAGCCCGTCT-3'	
L3304-H3753		450
L3304	5'-AACATACCCATGGCCAACCT-3'	
H3753	5'-AATGATGGCTAGGGTGACTT-3'	
L8150-H13650		524
L8150	5'-CCGGGGGTATACTACGGTCA-3'	
H13650	5'-GGGGAAGCGAGGTTGACCTG-3'	
Genomic DNA		
GSTM1		271
Forward	5'-CTGCCCTACTTGATTGATGGG-3'	
Reverse	5'-CTGGATTGTAGCAGATCATGC-3'	
GSTT1		450
Forward	5'-TTCCTTACTGGTCCTCACATCTC-3'	
Reverse	5'-TCACCGGATCATGGCCAGCA-3'	
GSTP1		176
Forward	5'-ACCCCAGGGCTCTATGGGAA-3'	
Reverse	5'-TGAGGGCACAAGAAGCCCCT-3'	
GSHPx		379
Forward	5'-GCAGAGCCGGGGACAAGAGAA-3'	
Reverse	5'-CTGCTCTTTCTCTCCATTGAC-3'	
CYP1		176
Forward	5'-GAACTGCCACTTCAGCTGTCT-3'	
Reverse	5'-CCAGGAAGAGAAAGACCTCCCAGCGGGCCA-3'	
β-Actin		315
Forward	5'-ATCATGTTTGAGACCTTCAA-3'	
Reverse	5'-CATCTCTTGCTCGAAGTCGA-3'	

Table 2: Oligonucleotide primers used for the analysis of the deletions and point mutation in mtDNA of mouse gametes retrieved from ovarian hyperstimulation.

Primer pair	Sequences	Product(bp)
Mitochondrial DNA		
L8265-H13375		5100
L8265	5'-AATTACAGGCTTCCGACACA-3'	
H13375	5'-TTTAGGCTTAGGTTGAGAGA-3'	
L1953-H2473		15746
L1953	5'-GAGGTGATGTTTTTGGTAAACAGGCGGGGT-3'	
H2473	5'- GGTTCGTTTGTTCAACGATTAAAGTCCTACGTG -3'	
L1794-H2501		15588
L1794	5'-GGTTATCCGAGTTGTTATACGCC-3'	
H2501	5'-ACGTGATCTGAGTTCAGACCG-3'	
Genomic DNA		
β-Actin		315
Forward	5'-GGTTCCTAGCCCTGAGGCTC-3'	
Reverse	5'-ACTTGCGGTGCACGATGGAGG-3'	

Table 3. The developmental competence of mouse embryos were characterized. The mouse embryos where collected from which with repeated ovarian stimulation by gonadotropin administration.

Cycles	Day1			Day2			Day3			Day4			Day5		
	A	1 c	2c	A	2 c	4c	A	4c	8c	A	8c	M	A	M	B
1	4	16	8	5	4	15	4	4	11	5	3	7	3	3	4
2	6	12	4	8	6	2	5	2	1	3	0	0	0	0	0
3	8	10	2	5	4	3	4	3	0	0	0	0	0	0	0
4	10	6	2	5	2	1	3	0	0	0	0	0	0	0	0
5	9	6	1	6	1	0	1	0	0	0	0	0	0	0	0
6	5	3	0	3	0	0	0	0	0	0	0	0	0	0	0

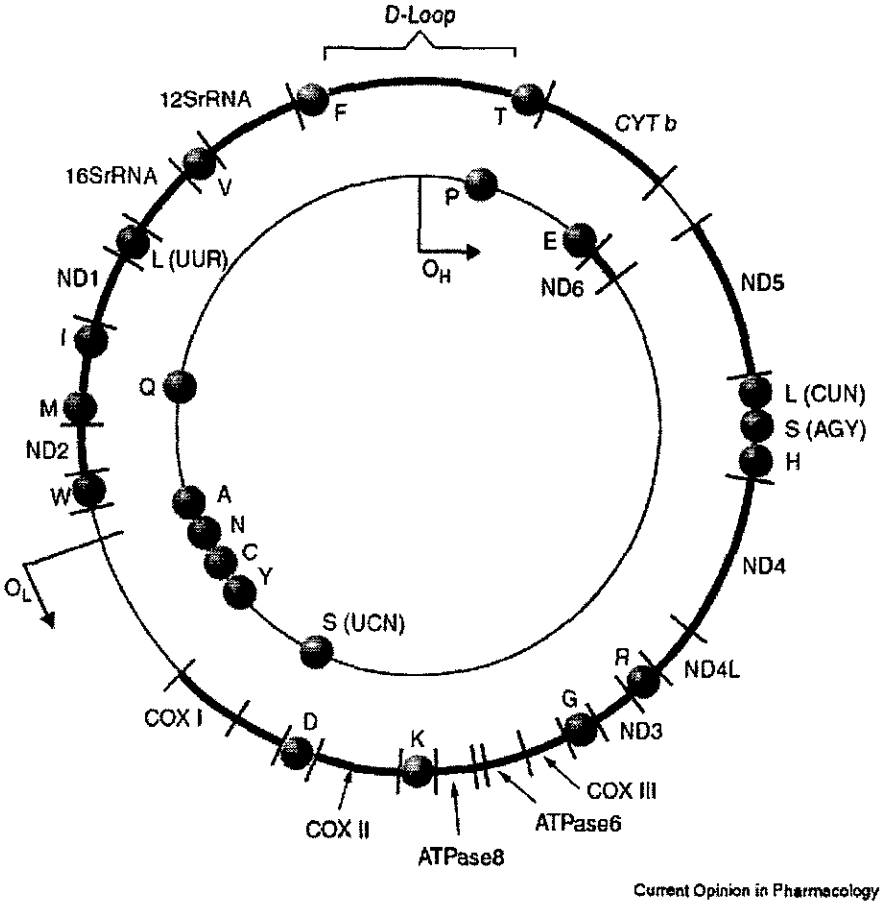

Fig. 1 Histogram of human mitochondrial DNA.

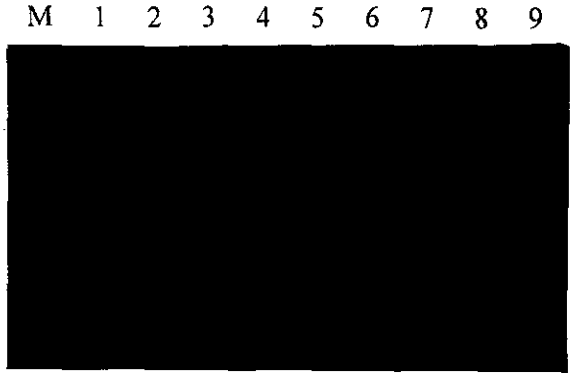

Fig. 2 Electrophorectogram of the PCR products amplified from the granulosa cells. Different length products were amplified by using primer pairs. The primer pairs were used as following: Lane 1, the 450 bp products was generated with primer pair for GSTT1; Lane 2, the 176 bp products were amplified for GSTP1; Lane 3, the 271 bp products were generated for GSTM1; Lane 4, the 220 bp products were amplified for HSP70; Lane 5, the 315 bp products were amplified for β-Actin; Lane 6, the 383 bp products were amplified with L258-H640 primer pair; Lane 7, the 450 bp products were amplified with L3304-H3757 primer pair; Lane 8, no PCR product was generated by using the primer pair L8150-H13650; Lane 9, the 524 bp PCR product was generated by using the primer pair L8150-H13650 from the 4,977 bp deleted mitochondrial DNA.

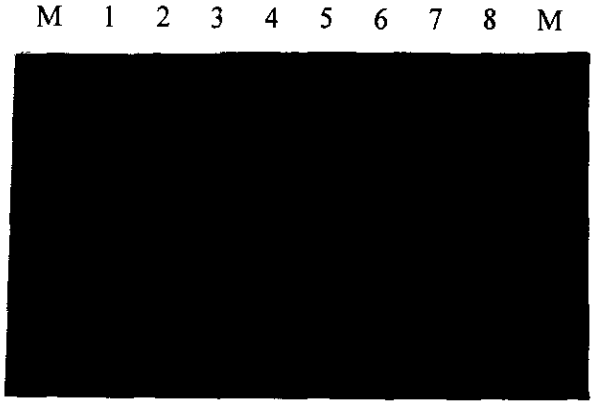

Fig. 3 Electrophorectogram of the PCR products amplified from the unfertilized human oocytes. Different length products were amplified by using primer pairs. The primer pairs were used as following: Lane 1, the 450 bp products was generated with primer pair for GSTT1; Lane 2, the 176 bp products were amplified for GSTP1; Lane 3, the 271 bp products were generated for GSTM1; Lane 4, the 220 bp products were amplified for HSP70; Lane 5, the 315 bp products were amplified for β-Actin; Lane 6, the 383 bp products were amplified with L258-H640 primer pair; Lane 7, the 450 bp products were amplified with L3304-H3757 primer pair; Lane 8, the 524 bp PCR product was generated by using the primer pair L8150-H13650 from the 4,977 bp deleted mitochondrial DNA; Lane M, 100bp DNA ladder.

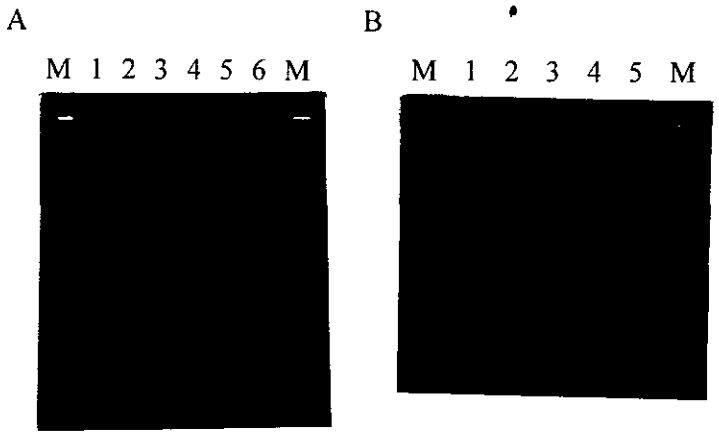

Fig. 4 Electrophorectogram of the PCR products amplified from human oocytes (A) Different length products were amplified by using different primer pairs L8251-H14020 (Lane 1 and 2), 8811-14020 (Lane 3 and 4), L8811- H13650 (Lane 1 and 2). The samples were used as following: Lane 1; 35 yr old woman 1; Lane 2, 28 yr old woman 2; Lane 3; 35 yr old woman 1; Lane 4, 28 yr old woman 2; Lane 5, 35 yr old woman 1; Lane 6, 28 yr old woman 2 ; Lane M, 100bp DNA ladder. (B) PCR products were amplified by using different primer pairs for GSTM1. Lane 1, lane 2, and lane 5 were shown as null type of GSTM1. Lane 3 and Lane 4 were normal GSTM1 individuals.