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# 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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中文摘要：

以促性腺刺激素(gonadotropin)誘導超排卵(superovulation)為人工生殖技術中重要的治療技術，以荷爾蒙刺激卵巢，可使大量的卵泡(follicles)生長與發育，排出進行至metaphase II 的成熟卵。臨床上的不孕症治療便是使用超排卵的刺激方式來大量取得已發育到 metaphase II 的卵以進行人工受精(IVF)。於哺乳類中，發現以標準劑量單次刺激超排卵降低受孕力及胚胎植入前和植入後存活率皆產生影響。若以高劑量刺激，則發生非整倍數卵(oocyte anueploidy)、胚胎死亡率(embryo mortality)、胎兒發育遲緩障礙(fetal growth retardation)、和先天性異常(congenital abnormalities)的頻率將昇高。本計劃將針對重複性刺激卵巢超排卵造成的卵細胞缺陷或對卵巢有傷害性的成因及機轉加以探討。我們以 gonadotropin 從一週次至六週次重複刺激老鼠卵巢，並且追蹤細胞與生化的影響，以瞭解卵細胞的受孕力下降與何種機制相關。我們發現對老鼠重複性施打刺激卵巢超排卵後，分別收集卵巢、肝及子宮等組織。觀察發現於多週次刺激組的老鼠卵巢產生嚴重充血並水腫。我們分析排卵數量及卵細胞品質隨刺激週次的增加而降低。於多週次刺激組的胚胎中 apoptosis 的數目增加，且胚胎的發育能力(即胚胎於體外發育至囊胚期)降低，細胞質內粒線體凝集現象，此為老化的卵細胞的特性。此外，我們針對卵巢、肝及子宮等檢體分析 lipid peroxidation, 8-OH-dG 的測定。實驗發現 8-OH-dG 及 lipoperoxide 的含量隨刺激週次的增加而顯著性增加。此外，我們更進一步分析粒線體 DNA 突變的產生，發現共有3種不同的粒線體DNA 突變產生，分別 4700 bp 及 4900 bp 的斷損突變以及點突變。這顯示了多週次刺激對老鼠產生對組織的破壞，並且影響胚胎與卵細胞的品質。胚胎與卵細胞的功能缺陷,常成為受孕力降低及女性不孕症的重要影響因素。因此,對於多週次的超排卵治療造成卵細胞老化或缺陷的形成因素的探討及引發傷害的機轉的研究更顯為急迫。對於多週次的超排卵治療，必需找到更適合的間隔時間並減緩傷害的方式。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。

**Abstract**

Superovulation by injection of

exogenous gonadotropin is still the fundamental method to produce in vivo derived embryos for embryo transfer in women. In mammals, 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. At higher doses, increased frequencies of oocyte aneuploidy, embryo mortality, fetal growth retardation and congenital abnormalities have been reported. Ovarian and oocyte biological ageing may possibly adverse implications for human oocyte competence with repeated hyperstimulation. The extent to which oocyte competence declines after repeated cycles of ovarian stimulation in some women undergoing infertility treatment, or is associated with adverse changes in ovarian function that are stimulation-related, is unknown. What an important factor in determining the normality of pre-ovulatory maturation during subsequent natural or stimulated cycles is currently under investigation. In this study, reduced competence for the human oocyte has been associated with degenerative embryos upsurge during embryo culture, and failed to develop into blastocyst stage in the three, four, five, and six stimulation cycles. On the other hand, the numbers of ovulated oocytes were decreased in the groups with ovarian stimulation. More aggregated mitochondria were found in the cytoplasm of the repetitively stimulated embryos. Furthermore, higher amount oxidative damages including 8-OH-dG and lipoperoxide contents (malondialdehyde) were also revealed in the ovaries and livers with more cycle numbers of ovarian stimulation. The higher proportion of mtDNA large-scale deletions were also shown in the more cycle numbers of ovarian stimulation. There were three mutated bands were detected, the molecular sizes were approximately about 700 bp, 420 bp, and 200 bp. However, an understanding of the relationship between oocyte competence and ovarian responses to stimulation in the mouse may provide insights into the origin of oocyte defects and the biology of ooplasmic ageing that could be of clinical relevance in the diagnosis and treatment of human infertility

## Introduction

Despite the recent development of the technology of in vitro production of embryos, superovulation by injection of exogenous gonadotropin is still the fundamental method to produce in vivo derived embryos for embryo transfer in women. Ovulation stimulation permits the growth and development of supernumerary dominant follicles and the ability to time the initiation of pre-ovulatory oocyte maturation.

However, the yield and quality of embryo raised after ovarian hyperstimulation for *in vitro fertilization* (IVF) are variable and unpredictable owing to variations in ovarian response, fertilization rate and embryo development.[1] 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 gonadotrophins.[2,3] At higher doses, increased frequencies of oocyte aneuploidy,[4] embryo mortality, fetal growth retardation and congenital abnormalities have been reported.[5] Reduced viability with ovarian stimulation is often attributed to adverse 'maternal' factors such as inadequate uterine synchrony or receptivity.[5]

In clinical IVF therapy, it is common for women to have undergone several cycles of ovarian stimulation before pregnancy is achieved. Outcome data from some studies indicate no significant change in pregnancy rates after as many as eight cycles of ovarian stimulation for IVF.[6] However, maternal age-related depletion of ovarian reserve that normally begins in women after about age 30-33 years is a significant factor in the quality and competence of oocytes and preimplantation embryos in both normal and stimulated cycles.[7] For example, several recent studies have demonstrated that a diminished ovarian reserve that is age-related, or occurs prematurely in women with early age menopause,[8] is associated with increased frequencies of trisomic pregnancies. In this respect, physiological changes in the ovary have been suggested to adversely influence oocyte competence.[9] In contrast, a rapid decline in fecundity after the second IVF cycle in women of different ages[10] has been observed and these authors suggested that regardless of age, this number of cycles may represent an important threshold related to the probability of pregnancy occurring on subsequent attempts. The extent to which oocyte competence declines after repeated cycles of ovarian stimulation in some women undergoing infertility treatment, or is associated with adverse changes in ovarian function that are stimulation-related, is unknown.

Recently, Davis's group[11] had discussed with respect to ovarian and oocyte biological ageing and possibly adverse implications for human oocyte competence with repeated hyperstimulation. Reduced competence for the human oocyte has been associated with spindle malformations and chromosomal misalignments. What an important factor in determining the normality of pre-ovulatory maturation during subsequent natural or stimulated cycles is currently under investigation. In this regard, it was found that multiple rounds of ovarian stimulation in

the rat altered hormonal homeostasis, [12] and these changes may have a direct effect on oocyte competence. Therefore, in a situation that repeated cycles of exogenous gonadotrophins used for induction of multiple folliculogenesis were suggested to affect oocyte quality and maturation.

For the human, successful outcomes after multiple cycles of ovarian stimulation and ovulation induction for intrauterine insemination or IVF must be improved. Whether or how disorders in ovarian structure and physiology associated with repeated stimulation may influence oocyte competence remains to be determined. However, an understanding of the relationship between oocyte competence and ovarian responses to stimulation in the mouse may provide insights into the origin of oocyte defects and the biology of ooplasmic ageing that could be of clinical relevance in the diagnosis and treatment of human infertility

**Aim of this study**

In this study, we will focus on clarifying which factor(s) are contributed to adverse oocyte competence after repeated ovarian hyperstimulation. If extrinsic factors differentially influence the normality of meiotic maturation *in vivo*, the mouse may offer a clinically relevant system in which to ask why oocytes are affected. The detrimental effects in ovaries and affect oocyte quality will be discussed in this study. Multiple approaches will be addressed as follows. In the first, we plan to examine the oocyte quality and embryo developmental competences that are collected from repeated stimulaton. In order to check membrane potential, viability, spindle configuration, and chromosome alignment, all of the oocytes and embryos are stained with fluorescent dye such as JC-1, TUNEL, antitubulin-FITC, and PI. We will examine the alteration in pre-ovulatory events, especially in ovaries whether with accumulation of damages and altered hormonal homeostasis. In this regard, we will analyze whether the accumulation of mouse mitochondrial DNA rearrangements and oxidative stress markers such as contents of lipid peroxides and oxidized 8-deoxyguanosine (8-OHdG). Recently, much attention has been focus on reactive oxygen species (ROS) in the regulation of luteal function and ovulation. ROS are that excess ROS will elicit oxidative stress and apoptosis. ROS plays a paradoxical role on the luteaotropic effects. For this reason, we will measure whether oxidative damages existed in the repeated mouse ovary. In the preliminary results, the differential accumulation of mtDNA mutations, malondialdehyde and 8-OH-dG were existed in the ovaries with repeated stimulation. The

oxidative damages were proportional to repeated stimulation cycles. On the other way, the damages were also identified in the uterus and livers with multiple rounds of stimulation.

**Results**

*1. Declined development competence of embryos*

In this study, we detected the development competence of embryos from differentially repeated stimulation (Fig.3 and 4). Large populations of embryos were degenerative and failed to develop into blastocyst stage in the three, four, five, and six stimulation cycles (Table 2). The fertilization rates were seen to be similar in all tested groups. On the other hand, the ovulated oocytes were decreased in the groups with ovarian stimulation. After staining with JC-1(Fig. 4), we observed the embryos by using con-focal microscope. More aggregated mitochondria which were found in aged embryos were located in the cytoplasm of embryos with repeated stimulation cycles.[18]

*2. Oxidative damages in the ovaries and livers*

We detected the oxidative damages including 8-OH-dG and lipoperoxide contents (malondialdehyde) (Tab 3 and 4). It was strong depicted that higher amounts of oxidative damages were existed in the ovaries and livers with more cycle numbers of ovarian stimulation. There were positive correlation between oxidative damages and cycles of repeated ovarian stimulation. The oxidative damages were existed in the ovaries, livers, and uterus. The higher amount was examined in the ovaries than in livers in the same stimulated cycle groups.

*3. Accumulation of large-scale deletion of mouse mtDNA with repeated stimulation*

The higher proportion of mtDNA large-scale deletions were also shown in the more cycle numbers of ovarian stimulation. Using the primer sets L8265-H13375, we detected large-scale deletions of mtDNA in repeated stimulated ovaries. The 5100 bp band was amplified from the wild type mtDNA. There were three mutated bands were generated, the molecular sizes were approximately about 700 bp, 420 bp, and 200 bp (Fig. 5).

4. Differential expression of HSP70 and IL-6

The higher relative amount of HSP70 mRNAs were detected in the livers with stimulation. No significant increase in HSP70 and mRNAs were found in the stimulated ovaries. The higher relative amount of IL6 mRNAs were detected in the livers with stimulation. No significant increase in IL6 mRNAs were found in the stimulated ovaries. (Fig.6 and 7).

Table 1: 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 2. Declined oocyte qualities were revealed with repeated ovarian stimulation

| Cycle No | Mice No. | Collection No. | Degeneration Oocytes No. | GV | MII |
|---|---|---|---|---|---|
| 1 | 3 | 28 | 6 | 4 | 18 |
| 3 | 4 | 29 | 15 | 2 | 12 |
| 6 | 5 | 21 | 15 | 5 | 1 |

Abbreviation, GV, geminal vesicle; MII, metaphase of meiosis II

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

| Cycle | Day | | | Day | | | Day | | | Day | | | Day | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | |
| | A | 1C | 2C | A | 2C | 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 |

Abbreviations, A, apoptosis; 1C, fertilized one-cell stage; 2C, two-cell stage; 4C, four-cell stage; 8C, eight-cell stage; M, morula stage of embryo; B, blastocyst.

Table 3. The content of 8-OH-dG and lipoperoxides of mouse ovaries were characterized. The mouse ovaries where collected from which with repeated ovarian stimulation by gonadotropin administration. The higher contents of 8-OH-dG were detected in the ovaries with six-repeated stimulation cycles.

| No. of Cycle | Lipoperoxide (pmol/mg protein) | 8-deoxyguanosine (8-OH-dG/dG, $x10^{-3}$%) |
|---|---|---|
| 1 | 2.156 ± 0.024 | 0.086 ± 0.014 |
| 2 | 2.756 ± 0.376 | 3.487± 0.658 |
| 3 | 2.987 ± 0.746 | 5.438 ± 1.633 |
| 4 | 3.255 ± 0.926 | 6.225 ± 1.677 |
| 5 | 3.651 ± 1.004 | 8.462 ± 2.889 |
| 6 | 4.579 ± 1.278 | 10.941 ± 2.546 |

Table 4. The content of 8-OH-dG and lipoperoxides of mouse livers were characterized. The mouse livers where collected from which with repeated ovarian stimulation by gonadotropin administration. The higher contents of 8-OH-dG were detected in the livers with six-repeated stimulation cycles.

| No. of Cycle | Lipoperoxide (pmol/mg protein) | 8-deoxyguanosine (8-OH-dG/dG, $x10^{-3}$%) |
|---|---|---|
| 1 | 1.536 ± 0.058 | 0.042 ± 0.004 |
| 2 | 1.832 ± 0.405 | 2.354 ± 0.586 |
| 3 | 1.934 ± 0.357 | 4.859 ± 1.110 |
| 4 | 2.255 ± 0.780 | 5.837 ± 1.577 |
| 5 | 2.291 ± 0.037 | 6.447 ± 2.331 |
| 6 | 2.469 ± 0.787 | 9.198 ± 1.785 |

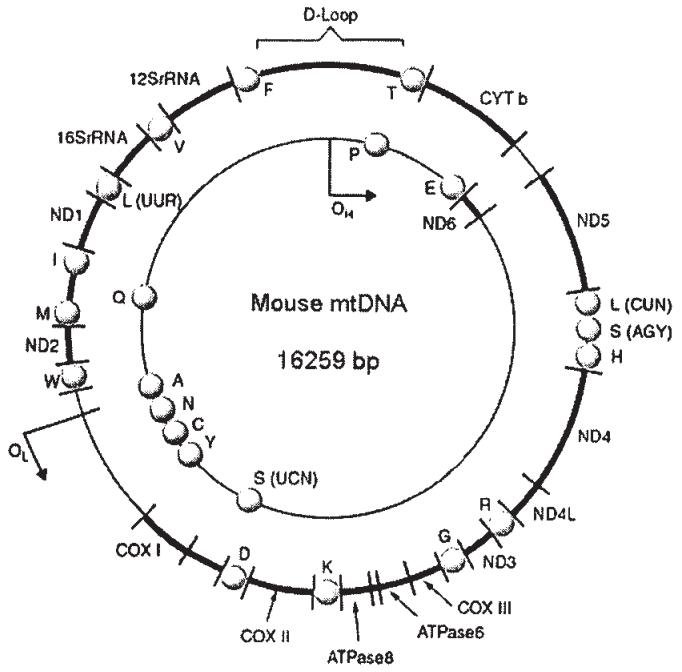


Fig. 1 Histogram of mouse mitochondrial DNA.

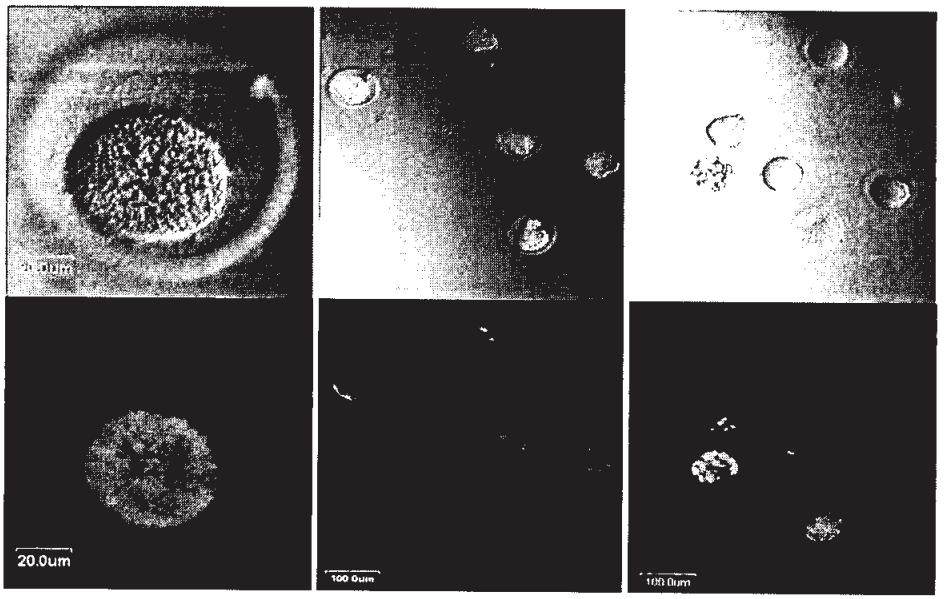


Fig 2 Morphological change in the oocytes with repeated stimulated cycles. A, C, and E were visualized under phase contrast microscopy. B, D, and F were stained with JC-1and visualized under confocal microscopy. A and B, oocytes were with one cycle stimulation; C and D, with 3 repeated cycles; E and F, with six repeated stimulation cycles.

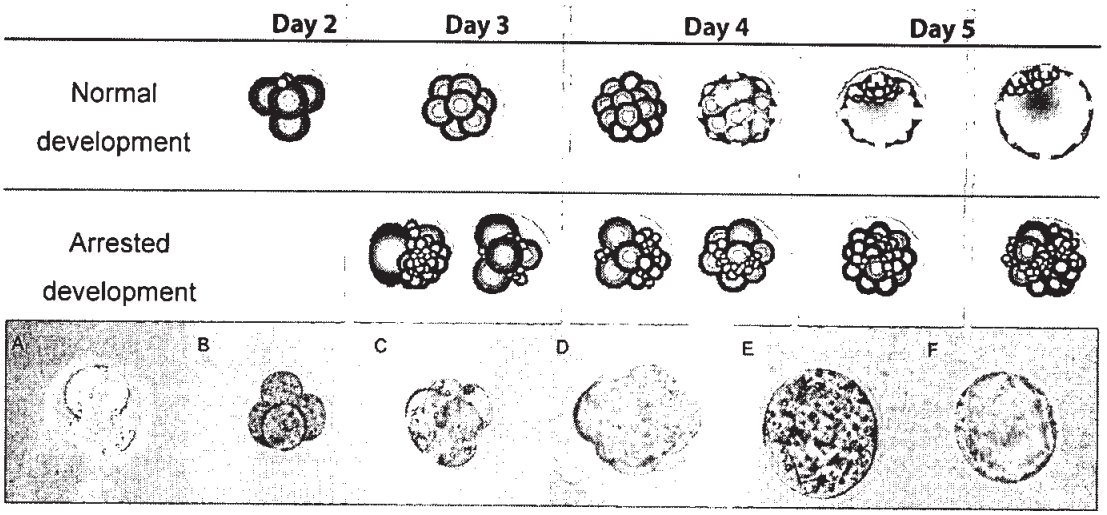


Fig 2. Morphology of embryos from normal control and repeated stimulation mouse were observed under phase-contrast microscope. A, 2-cells; B, 4-cells cell; C, 8-cells; D, morula; E, blastocyst I; F, blastocyst II.

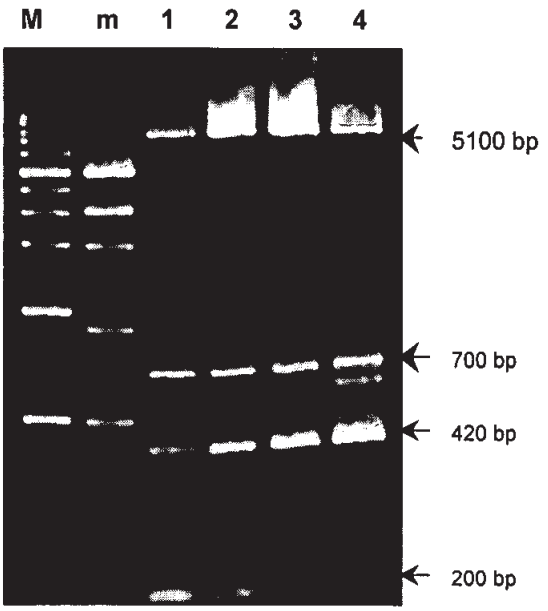


Fig 3. Large-scale deletions of mtDNA were examined in mouse ovaries with differentially repeated stimulation bylong-range PCR. Using the primer set L8265-H13375, we detected deletions of mtDNA in the repeated stimulated ovaries. The 5100 bp band was amplified from the wild type mtDNA. There were 3 mutated bands were generated, the sizes were approximately about 700 bp, 420 bp, and 200 bp. Lane M, 100 bp DNA ladder; lane m: 1Kb DNA ladder, lane1, 1 stimulation cycle; lane 2, three repeated cycles, lane 3, five cycles; and lane 4, 6 cycles stimulation.

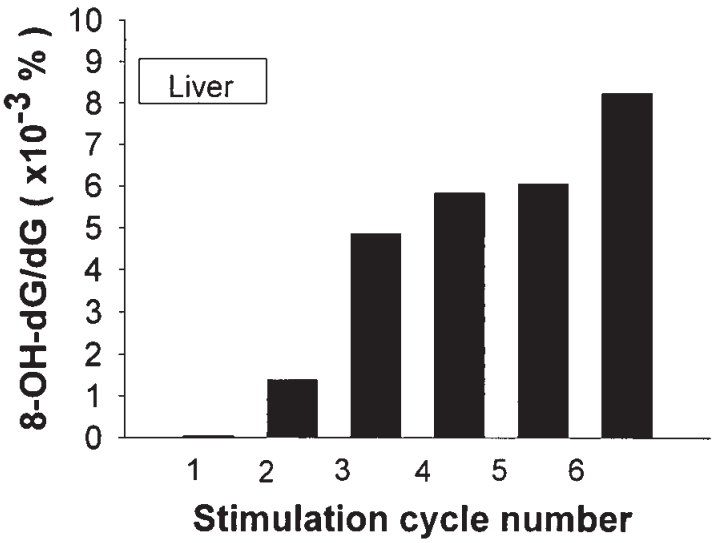


Fig 4 Biomarkers of oxidative damage were examined in the stimulated ovaries and livers with different cycles. Higher contents of 8-OH-dG were detected in the repeated cycles from two to six cycles.

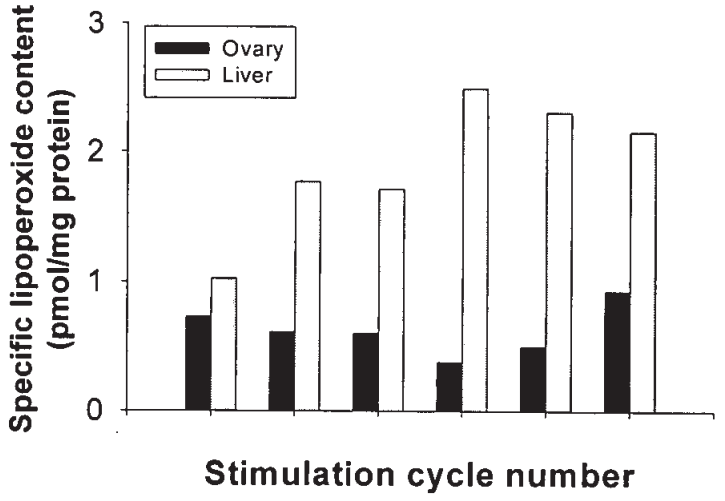


Fig 5 The biomarkers of oxidative damage were examined in the stimulated ovaries and livers with different cycles. The differential responses were found in the livers and ovaries with repeated stimulations. The higher proportions of lipoperoxides were detected in the livers.

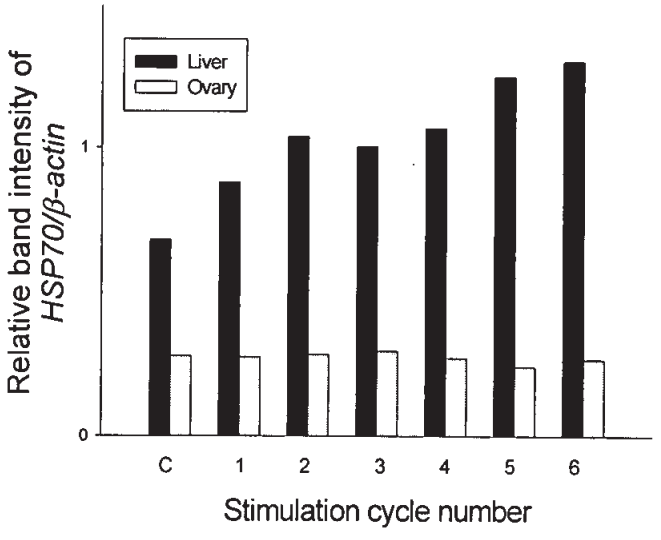


Fig 6 RT-PCR products amplified from heat shock protein (HSP70) mRNAs in mouse ovaries and livers. β-actin was used as internal control. The higher relative amount of HSP70 mRNAs were detected in the livers with stimulation. No significant increase in HSP70 and mRNAs were found in the stimulated ovaries.

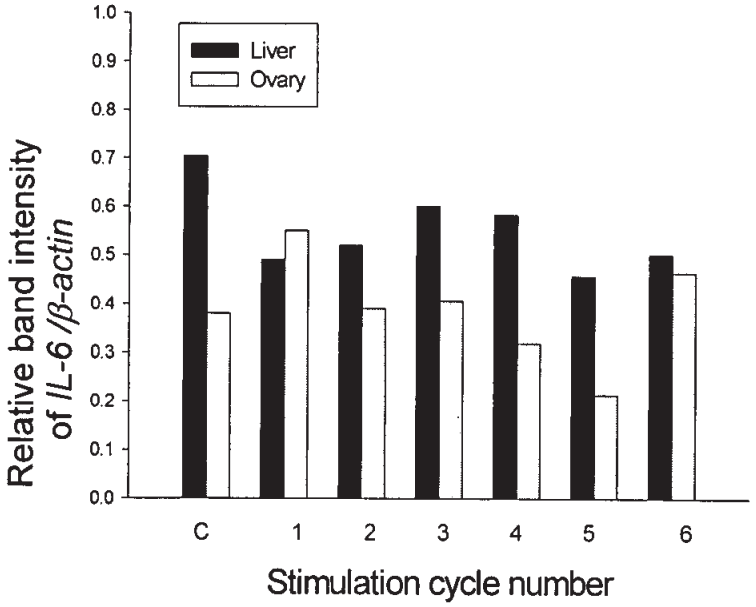


Fig 7 RT-PCR products amplified from interleukin 6 (IL6) mRNAs in mouse ovaries and livers. β-actin was used as internal control. The higher relative amount of IL6 mRNAs were detected in the livers with stimulation. No significant increase in IL6 mRNAs were found in the stimulated ovaries.