Long-term effects of repeated superovulation on ovarian structure and function in rhesus monkeys

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Objective: To assess the long-term effects of repeated controlled ovarian hyperstimulation (COH) on ovarian structure and function.
Design: Experimental study.
Setting: Laboratory.
Animal(s): Adult female rhesus macaques.
Intervention(s): A repeated COH rhesus macaque model (superovulation group) with spontaneously ovulating macaques used as controls (normal group) and samples of serum and ovarian tissue collected over a 5-year period.
Main Outcome Measure(s): Steroid hormone levels, and structural, functional, and protein changes in ovaries.
Result(s): The follicular histology, proportion of follicles at each developmental stage, and expression levels of oocyte-specific genes showed no statistically significant differences between the two groups. However, the superovulation group exhibited mitochondrial abnormalities in the granulosa cells and a low expression of genes involved in steroid hormone synthesis compared with the normal group. A comparison of protein expression in the ovaries of both groups using tandem mass tag labeling with mass spectrometry revealed that most of the differentially-expressed proteins were down-regulated in the superovulation group. These proteins were mainly localized in the mitochondria and cytosol, and changes in protein levels in the superovulation group mainly inhibited cell proliferation and differentiation but promoted apoptosis.
Conclusion(s): Our study indicates that repeated COH could change the expression of many proteins in the ovaries even after several years, potentially affecting the development and function of ovarian cells. (Fertil Steril® 2014;102:1452–7. ©2014 by American Society for Reproductive Medicine.)
Key Words: Controlled ovarian stimulation, ovarian follicular structure, proteomics, rhesus macaques, superovulation

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Assisted reproductive technology (ART) is widely used to treat human infertility. Controlled ovarian hyperstimulation (COH) and in vitro maturation (IVM) are two clinical protocols that are routinely used to obtain fully mature fertilization-competent oocytes (1). Studies have estimated that the number of babies born as a result of ART reached a total of 5 million by 2012. Despite improvements in the success of ART, the majority of patients require more than one treatment cycle to achieve pregnancy (2).

Many researchers have explored the effect of repeated ART on the human body. A series of studies have suggested that repeated superovulation may influence the structure and function of the ovary. In mice, repeated ovarian stimulation can induce oxidative damage and mitochondrial DNA mutations in ovaries, increase the incidence of oocyte spindle defects, and decrease the quality of oocytes (3–5). Clinical studies have found that consecutive ovulation induction cycles can impair the ovarian response and/or alter the quality of oocytes (6). However, there is no consensus regarding the adverse effects of repeated ovulation stimulation. Some large-scale retrospective studies have shown that the number of oocytes retrieved over five repeated in vitro fertilization (IVF) cycles remained the same and that there

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was no difference in oocyte quality (7). Age may be a determining factor associated with the decline of both pregnancy and live-birth rates with repetitive ART cycles (2).

Therefore, although an increasing number of studies have focused on the effects of repeated ovulation on ovaries or oocytes, studies conducted to date have failed to reach a definitive conclusion as a result of numerous shortcomings, such as inconsistent drug exposure and use, and relatively short follow-up periods (8). More importantly, many of these studies were retrospective cohort and case-control studies based on human populations, which are not suitable for the assessment of the long-term influence of repeated ovulation stimulation on the ovary. Furthermore, such studies cannot adequately explore the mechanisms by which repeated ovulation influences the reproductive system.

Our study used nonhuman primate rhesus monkeys as the study model to evaluate the structure and function of ovaries that have previously received repeated COH treatments. We believe that this model can remove the influence of other factors, determine whether repeated ovulation stimulations have a long-term effect on the ovaries, and investigate the potential associated underlying mechanisms.

MATERIALS AND METHODS

Animals

We used six adult female rhesus macaques (average age: 13 years) provided by the Kunming Primate Research Center (KPRC), People's Republic of China. Three rhesus macaques received four cycles of ovarian stimulation 5 years previously (superovulation group). The remaining three monkeys received no treatment (normal control group). Ovulation stimulation was performed according to previously published procedures (9). All experiments requiring the use of animals were approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University (permit no. 1403027).

Hormone Measurement

On days 3 and 10 of the menstrual cycle, blood samples were drawn from the animals to quantify serum hormone concentrations. The serum estradiol (E_2) and progesterone (P_4) concentrations were determined by radioimmunoassay (RIA) (10). Serum follicle-stimulating hormone (FSH) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit for monkey FSH (Cusabio).

Tissue Collection

Ovarian tissue samples were collected at approximately day 10 of the menstrual cycle when the follicles were in the proliferative phase. One ovary of each monkey was frozen in liquid nitrogen and used for the extraction of protein and RNA samples. The other ovary was divided into two parts, and fixed in 10% formaldehyde and glutaraldehyde solution, respectively.

Histologic and Ultrastructural Examination

The formaldehyde-fixed ovarian tissues were embedded in paraffin blocks, which were sectioned into 5-μm-thick slices, deparaffinized, and stained with hematoxylin and eosin for histologic examination. The follicles at each different stage were identified according to previously described criteria (11). Each tissue block was serially sectioned, and the numbers of primordial, primary, secondary, antral, and atretic follicles were counted in every 10th section per sample.

For ultrastructural examinations, the glutaraldehyde-fixed tissue blocks were postfixed with 2% OsO_4 and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

Immunohistochemical Analysis

Paraffin-embedded sections were immunostained as previously described elswhere (12) by incubation overnight at 4°C with primary antibodies against Ki67 (1:200; Abcam). An Axioskop 2 microscope (Carl Zeiss) was then used to examine the slides treated with diaminobenzidine, and the ratio of Ki67-positive follicles was calculated.

TUNEL Assay

Analysis of apoptosis was performed using the terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore). The numbers of TUNEL-positive granulosa cells were counted in all follicles in three individual sections per animal.

Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted from the ovaries. SYBR Premix Ex *Taq* II kits (TaKaRa) were used for real-time polymerase chain reaction (PCR). Reactions were performed according to the manufacturer's protocol. Primer sequences and target fragment sizes are listed in Supplemental Table 1 (available online).

Western Blot Analysis

Western blotting of ovarian tissue lysates was performed as previously described elsewhere (12) using anti-RDH11 (1:4,000; Novus Biologicals), anti-RRAS2 (1:1,000; Proteintech), anti-TXNDC17 (1:250; Abcam), and anti-β-actin (1:10,000; Merck Millipore).

Tandem Mass Tag Labeling, SCX Fractionation, and MS Analysis

Proteins were extracted from the ovaries of all the monkeys. Each 100-μg protein sample was reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (13, 14). Tandem mass tag (TMT) labeling (Pierce) was performed according to the manufacturer's protocol with minor modifications. The aliquots were then combined, and the pooled samples were evaporated in a vacuum. Strong cation-exchange chromatography (SCX) fractionation was performed using an UltiMate 3000 high-pressure liquid chromatography (HPLC) system as previously described elsewhere (15–17). A total of 21 fractions were collected at