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Effects of rhFSH regimen and time interval on ovarian responses to repeated stimulation cycles in rhesus monkeys during a physiologic breeding season

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Abstract

We studied the effects of repeated stimulation by recombinant human FSH (rhFSH) at various time intervals during a physiologic breeding season in rhesus monkeys. Ovarian recovery and responses were assessed by ultrasonography, serum steroid concentrations, number of oocytes retrieved, and in vitro blastocyst development following IVF. One group underwent a single stimulation regimen with 18 IU rhFSH i.m., followed by 1000 IU hCG, and serum steroid concentrations and ovarian status were determined in the following three menses. Another group was stimulated as before and then allocated into three subgroups; each subgroup was re-stimulated once at the beginning of the ensuing first, second, or third menses. In the final experiment, one group was stimulated with 37.5 IU rhFSH, whereas another group received 18 IU rhFSH. In subsequent cycles, all were re-stimulated twice with 18 IU rhFSH at time intervals of two menstrual cycles (MCs). At the first menses after stimulation, serum progesterone concentrations were significantly higher and the ovaries larger than before stimulation. Monkeys that were re-stimulated at the first menses responded poorly; at the second menses, progesterone concentrations and ovarian size recovered, but the number of oocytes retrieved from re-stimulated monkeys was still significantly reduced. However, animals that were re-stimulated in two MCs later responded well (i.e., percentage of the animals responding, oocytes recovered, and potential for fertilization and blastocyst formation). In conclusion, rhesus monkeys were likely to have similar ovarian responses to repeated stimulation with the same regimen spaced at least two MCs apart.

Keywords: Rhesus monkey; Ovarian recovery; Repeated ovarian stimulation; rhFSH; Ultrasonography

1. Introduction

Substantial progress in the application of assisted reproductive technologies in non-human primates over the last two decades has resulted in the routine

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production of in vitro-derived embryos and the use of embryo transfer to establish pregnancy [1–3]. These successes were followed by the development of somatic cell nuclear transfer research [4–6], which depends upon a large number of oocytes being retrieved by ovarian stimulation. However, the availability rhesus monkey oocytes is impeded by the high cost of ovarian stimulation, limited numbers of animals, and the availability of rhesus monkeys for only approximately half of the year, due to their physiologic breeding season [2]. Protocols for ovarian stimulation of the rhesus monkey have been developed that use gonadotropins extracted from animal or human urine and/or pituitaries [7–16]. Recently, recombinant human FSH (rhFSH) used for ovarian stimulation improved the reliability and results [17–19]. In previous studies, we reported that lower dosages of rhFSH not only reduced the cost of ovarian stimulation, but also improved results, as the number of oocytes retrieved increased and the developmental potential with improved IVF [20,21]. However, the effects of repetitive treatments with low doses of rhFSH and the optimal time interval for subsequent treatments in the same breeding season are unknown. Therefore, the objective of this study was to assess rhesus ovarian responses to repeated treatment cycles using various time intervals and protocols.

2. Materials and methods

2.1. Animals and chemicals

All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of Kunming Primate Research Center. Adult female rhesus monkeys were housed in individual cages in a controlled environment (20–24 °C, humidity 40–60%) and exposed to a 08:00–20:00 h light cycle. Vaginal bleeding was monitored daily to detect the menstrual cycle (MC) and the onset of menses. Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Ovarian stimulation and oocyte recovery

During the September to March physiologic breeding season, 73 adult females (mean age: 6.4 years; mean weight: 5.6 kg) were subjected to ovarian stimulation. Treatment with rhFSH (Gonal F, Laboratories Serono SA, Aubonne, Switzerland) was initiated 1–3 days after the onset of menses. Two regimens of rhFSH were used. Regimen I consisted of 37.5 IU of rhFSH given i.m. twice daily, 10–12 h apart, for 8 days [19,22]. Regimen II consisted of 18 IU of rhFSH, also given twice daily for 8 days [20]. Animals that exhibited increases of serum estrogen and more than five follicles (at least 3 mm diameter) combined for both ovaries were defined as ''responders'' and were given 1000 IU hCG (i.m.) at 21:00 h of Day 9 [20]; otherwise, they were characterized as ''poor responders'' and removed from the study. Ovaries were imaged with a Diasus^{TM} ultrasound system (Dynamic Imaging Ltd., Livingston, Scotland, UK) with 10–22 MHz linear-array transducers, and mean ovarian diameter was calculated by averaging the maximum length and width. Oocyte collection was performed through laparoscopic follicular aspiration [20].

There were three experiments in this study. In the first experiment, we assessed ovarian recovery after stimulation by Regimen II. Serum steroid concentrations and ovarian size were monitored in monkeys ($n = 26$) during the following three menses after stimulation, with the aim of understanding the potential for repeated ovarian stimulation.

The second experiment evaluated the effect of time interval between cycles on the ovarian responses to repeated stimulation. Monkeys ($n = 22$) were first stimulated by Regimen II and then divided into three subgroups for repeated treatments. The first subgroup ($n = 6$) was re-stimulated with Regimen II at the beginning of the first menstrual cycle after initial stimulation. The second ($n = 6$) and third ($n = 10$) subgroups were re-stimulated with the same regimen at the beginning of the second and the third menses, respectively, during the same physiologic breeding season.

The third experiment investigated the consistency of ovarian responses to three consecutive ovarian stimulation cycles and the effect of high dose and reduced dose rhFSH regimen treatments on subsequent repeated treatments. Monkeys were stimulated with Regimen I ($n = 15$) or with Regimen II ($n = 10$) for the first stimulation cycle. Each group then received another two stimulation cycles with Regimen II, with time intervals of two MCs (Fig. 1).

2.3. IVF and embryo culture

To assess developmental competence, freshly collected mature oocytes were inseminated as in our previous reports [20,23]. Briefly, hyperactivated sperm and mature oocytes (MII) from the collection were co-incubated for 12–16 h at 37 °C in a humidified atmosphere of 5% CO_2. Fertilized oocytes were cultured for embryonic development under mineral

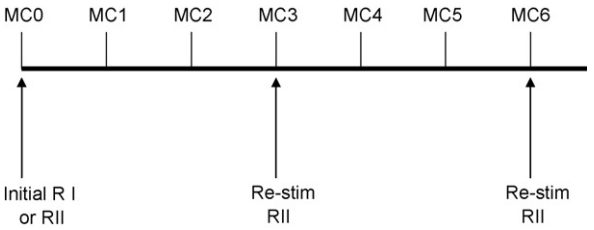

Fig. 1. Timeline for Experiment 3. After the initial stimulation with either RI or RII, the rhesus monkeys were re-stimulated twice at intervals of two cycles during the same physiologic breeding season. MC, Menstrual cycle; RI, Regimen I; RII, Regimen II; Re-stim, re-stimulated.

oil in 50 μL drops of HECM-10 [24] containing 10% fetal bovine serum for up to 7 days at 37 °C in humidified air containing 5% CO_2. The culture medium was changed every other day. Progress of embryo growth was monitored daily.

2.4. Hormone assays

To quantify serum concentrations of estradiol (E2) and progesterone (P4), blood samples were drawn from conscious animals by saphenous venipuncture in Experiment 1. Collections were made on the first and last day of ovarian stimulation and again on the first day of subsequent menses after the conclusion of ovarian stimulation. Serum concentrations of E2 and P4 were determined by RIA [13]. The intra- and inter-assay coefficients of variation were all <10%.

2.5. Statistical analysis

Results obtained are presented as the mean ± S.D. unless stated otherwise. The proportion of responders was analyzed by Fisher's exact test between the original cycle and repeated cycles or within the first, second, and third stimulation cycles. For statistical purposes, the number of oocytes recovered was transformed by square root, and the embryo development rates were transformed by arcsine of the square root prior to ANOVA. Paired Student's *t*-test comparisons were made between consecutive stimulation cycles in the same monkeys [20]. The measurements of ovarian sizes and serum concentrations of steroids at various time points relative to ovarian stimulation were log-transformed prior to statistical analysis. They were compared with the values at various time points by repeated-measures ANOVA, followed by a Student's *t*-test with the Bonferroni method. Values with $P < 0.05$ were considered different.

3. Results

3.1. Analysis of ovarian recovery after a single rhFSH stimulation cycle

Ovaries in monkeys stimulated by Regimen II significantly increased in size during ovarian stimulation, and gradually shrank in the following menses in Experiment 1 (Fig. 2). Mean ovarian diameter was 6.8 mm at the beginning of stimulation and increased to 13.6 mm at oocyte collection ($P < 0.05$; Table 1). The diameter decreased to 8.5 mm at the onset of the first menses after the conclusion of ovarian stimulation. At that time, it was still larger than the original 6.8 mm size ($P < 0.05$). Most of this residual increase was due to the presence of a CL (Fig. 2). The ovarian diameter in the following second and the third menses returned to the base size (Table 1). Concomitantly, the plasma concentration of E2 was 70.9 pg/mL at the beginning of ovarian stimulation and increased to 230.2 pg/mL at the time of oocyte collection (Table 1). After that, the E2 level returned to baseline for the following three menses. The P4 concentration was 7.3 ng/mL at oocyte collection and 3.2 ng/mL at the onset of the first menses, both of which were higher than 1.8 ng/mL at the beginning of ovarian stimulation ($P < 0.05$).

3.2. The effects of various intervals between stimulation cycles on subsequent ovarian responses

There were a sharp decline in the proportion of responders and recovered oocytes in monkeys that were re-stimulated with the same regimen starting at the onset of the first menses after the conclusion of ovarian stimulation by Regimen II in Experiment 2 (Table 2). The number of oocytes recovered, 12.0, was significantly reduced in the animals re-stimulated immediately following the completion of the first ovarian stimulation. However, the difference in proportion of responder animals was not significant ($P = 0.06$), probably due to the small sample size. When the second stimulation cycle was started at the beginning of the second menses after the first cycle, the proportion of responders did not change significantly (Table 2), but the number of oocytes retrieved from the second cycle was reduced compared to the first ($P < 0.05$). When the second stimulation cycle was started at the onset of the third menses after the first cycle, both the proportion of responders and the number of oocytes retrieved in repeatedly stimulated monkeys were similar in the second cycle compared to the first (Table 2).

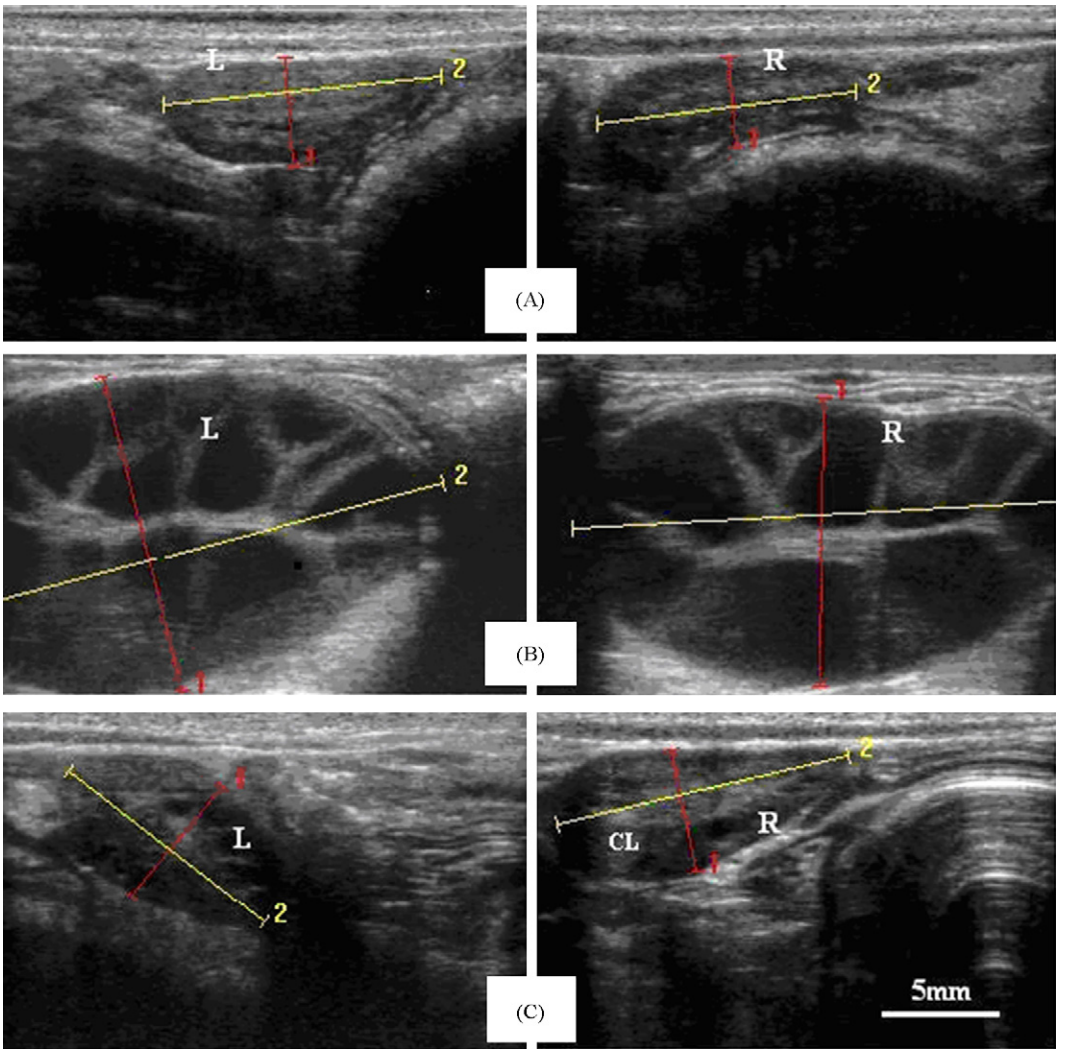

Fig. 2. Ovarian ultrasound imaging in a rhesus monkey subjected to ovarian stimulation with Regimen II. (A) At the beginning of hormonal stimulation, the mean diameter of the ovaries was 7.2 mm. (B) Just before oocyte collection, there were many intact follicles and the mean ovarian diameter was 16.0 mm. (C) At the onset of the first menses after the conclusion of ovarian stimulation, the mean ovarian diameter was 8.1 mm, which included a corpus luteum (CL). L, Left ovary; R, right ovary; bar, 5 mm.

Table 1
Mean (±S.D.) ovarian diameter and serum steroid hormone concentrations in rhesus monkeys ($n = 26$) at various time points before and after ovarian stimulation

Time points	Ovarian diameter (mm)	E2 (pg/mL)	P4 (ng/mL)
Between Days 1 and 3 of initial menses, prior to ovarian stimulation	6.8 ± 0.9^{a}	70.9 ± 16.2^{a}	1.8 ± 0.2^{a}
At oocyte collection	13.6 ± 4.3^{b}	230.2 ± 86.3^{b}	7.3 ± 0.3^{c}
At the onset of first menses after stimulation	8.5 ± 1.5^{c}	56.9 ± 20.2^{a}	3.2 ± 0.3^{b}
At the onset of second menses	7.1 ± 0.9^{a}	87.4 ± 14.3^{a}	2.0 ± 0.2^{a}
At the onset of third menses	6.4 ± 0.8^{a}	75.8 ± 16.9^{a}	2.2 ± 0.3^{a}

All monkeys were stimulated once by Regimen II. Within a column, means without common superscript letters (a–c) differ (a, b and b, c, $P < 0.01$; a, c, $P < 0.05$).

Table 2
Responding animals and mean (±S.D.) number of oocytes recovered from rhesus monkeys following repeated stimulation (with various intervals between stimulations)

Time of second stimulation	Cycle	Responding animals (%)	Number of oocytes recovered
Beginning of first MC after first stimulation ($n = 6$)	First	6 (100)[a]	28.5 ± 17.1[a]
	Second	2 (33)[a]	12.0 ± .0.7[b]
Beginning of second MC ($n = 6$)	First	6 (100)[a]	33.6 ± 19.2[a]
	Second	5 (83)[a]	17.0 ± 8.5[b]
Beginning of third MC ($n = 10$)	First	10 (100)[a]	28.2 ± 10.1[a]
	Second	10 (100)[a]	31.4 ± 5.9[a]

All monkeys were stimulated by Regimen II. Within a treatment category (interval for second stimulation), means without common superscript letters (a and b) differ ($P < 0.05$). Recovered oocytes included both MII and MI stages.

3.3. Ovarian responses to three consecutive stimulation cycles

In Experiment 3, unexpectedly, in monkeys first stimulated with the high dose of rhFSH (Regimen I) followed by two cycles with Regimen II, the proportion of responders significantly decreased to 87 and 53% in the second and third cycles, respectively, compared to the first cycle (Table 3). The number of oocytes retrieved also diminished from 39.5 to 24.2 in the second cycle and 22.5 in the third cycle ($P < 0.05$). The rates of fertilization and development to the blastocyst stage were not different among the three groups (Table 3). In contrast, the proportion of responders among monkeys stimulated with only half the dose of FSH stimulation (Regimen II) for three consecutive cycles did not change over the three-cycle study period. Furthermore, there were also no differences in the number of oocytes recovered or rates of fertilization and development to the blastocyst stage.

4. Discussion

To our knowledge, this is the first report to assess ovarian recovery, ovarian responses, retrieved oocytes, and in vitro developmental potential of IVF-produced embryos in rhesus monkeys after repetitive ovarian stimulation during the same physiologic breeding season. Our study was based upon results from a previous study [20] in which a cycling monkey was stimulated three times with a reduced-dose gonadotropin regimen during the same physiologic breeding season. That study yielded superior results; each of the cycling monkeys produced approximately 80 mature oocytes in a breeding season.

Repetitive treatments, based on considerations of donor ages [25,26], gonadotropins used [16,26,27], stimulation protocols [16,28], and gonadotropin dosages [20,21], could provide alternative strategies for increasing the efficacy of ovarian stimulation in the rhesus monkey [29]. However, there are discrepancies in the results reported in this field. In some studies, repeated ovarian stimulation induced formation of oocytes with low competency, perhaps due to oxidative damage and mitochondrial DNA mutations [30]. For these oocytes, there may be in vivo compensatory mechanisms that optimize the developmental competence of ovulated oocytes, as in mice [31]. Other reports demonstrated that ovarian responses in women [32–35], wildlife [36], and cattle [37] were the same in

Table 3
Mean (±S.D.) effects of initial rhFSH regimen and interval of re-stimulation in rhesus monkeys in the same breeding season

Number	Cycle	FSH regimen	Responding animals (%)	Number of oocytes recovered	Developmental stage (%)	
					Fertilized	Blastocysts
15	First	I	15 (100)[a]	39.5 ± 17.8[a]	91.1 ± 12.3[a]	41.5 ± 17.7[a]
	Second	II	13 (87)[a,b]	24.2 ± 10.9[b]	82.7 ± 13.8[a]	51.6 ± 20.3[a]
	Third	II	8 (53)[b]	22.5 ± 14.8[b]	90.6 ± 10.2[a]	41.4 ± 19.2[a]
10	First	II	10 (100)[a]	34.0 ± 15.3[a]	87.8 ± 9.2[a]	58.3 ± 22.3[a]
	Second	II	10 (100)[a]	31.4 ± 14.5[a]	90.6 ± 11.4[a]	62.1 ± 15.2[a]
	Third	II	10 (100)[a]	34.4 ± 11.2[a]	83.9 ± 14.5[a]	54.8 ± 23.8[a]

Within a group, means without common superscript letters (a and b) differ ($P < 0.05$). Recovered oocytes included both MII and MI stages. %Fertilized = (ova exhibiting two pronuclei/MII oocytes) × 100. %Blastocysts = (blastocysts/fertilized oocytes) × 100. Five to seven freshly collected MII oocytes from each responding monkey were used in IVF.

consecutive cycles if the same ovarian stimulation protocol was used in each cycle.

In the present study, the response to repeated ovarian stimulations with a low dose rhFSH spaced two MCs apart in rhesus monkeys was assessed in terms of oocyte recovery and developmental potential. However, there were inconsistencies in monkeys first treated with the high dose rhFSH and then in subsequent cycles with a reduced dose of rhFSH. For instance, the number of animals responding in the second and third cycles was reduced compared to the first cycle, as were the number of oocytes recovered; however the rate of fertilization of recovered oocytes and the rate of blastocyst formation was not reduced. This could have been the result of the initial high dose of rhFSH inducing a desensitization of the ovary to the subsequent lower doses. The poor responses to repeated stimulation also might have been due to repeated hCG administration. This issue should be addressed with serial endocrine investigations in future studies.

Ovarian recovery and status at the beginning of each cycle play pivotal roles in the success of ovarian stimulation. Although hCG has a longer half-life in vivo than rhFSH [38], it does not prolong the duration of the MC [2]. In the present study, the MC was 18–23 days. Corpus luteum formation or luteinized follicles were induced after ovarian stimulation and remained present at the onset of new menses. Secretions from luteinized tissue could impair the potential for oocyte development [39]; this may account for repeated treatments resulting in reduced responses when they were spaced less than two MCs apart. In the present study, the animals were stimulated three times in the same breeding season. By the third MC after ovarian stimulation, hormone concentrations, ovarian integrity, and activity were fully recovered, resulting in an improved response to repeated ovarian stimulation. Furthermore, the repetitive treatments did not affect subsequent reproductive ability [29].

In conclusion, repeated ovarian stimulations with a low dose of rhFSH spaced at least two MCs apart could be a successful strategy for addressing the high cost and limited availability of rhesus monkeys for research in reproductive biology.

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