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Recombinant Human Gonadotropins for Rhesus Monkey Superovulation: Age and the Stimulation Interval are Determinant to the Fecundity

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Abstract: The objective of this study was to analysis the key factors which may be determinative to Superovulation (SO) in rhesus monkey. Total 361 rhesus monkeys were stimulated with recombinant human gonadotropins and Ova Pick Up (OPU) was conducted by laparoscopic follicular aspiration. Number of total ova and oocytes at Metaphase I (MI) and Metaphase II (MII) (MI+MII) from each retrieval was calculated, MI+MII≥10 was defined as effective SO-OPU while MI+MII<10 was defined as non-effective SO-OPU. Parameters including age, month in breeding season, stimulated times and stimulation interval were compared between effective and non-effective SO-OPU. The results showed that 5-10 years old rhesus monkeys were ideal oocytes donors while 3-4 years old and >10 years old monkeys included high rate of poor responders. SO conducted from late October to next February harvested similar fecundity while the rate of poor responders increased in March which imply proper SO breeding season should be from late October to next February. In addition, SO-OPU outcomes were gradually decreased along with the increased times of repeated stimulation. Further analysis showed that stimulation interval affected monkeys' response to recombinant human gonadotropins. When the interval was <2 menstrual cycles, the fecundity compromised significantly. In conclusion, the key determinative factors to SO-OPU should be animal's age and stimulation interval in rhesus monkey stimulated with recombinant human gonadotropins.

Key words: Superovulation, recombinant human gonadotropins, age, stimulation interval, fecundity, rhesus monkey

INTRODUCTION

The rhesus monkey has been one of the most widely used laboratory animal in medical and biological research due to its close relationship to human being in anatomy, genetics and physiology. Rhesus monkey has similar characteristic of menstrual cycle to human, it is an ideal model to study the biology of human reproduction. Embryos are basic materials to explore series of scientific problem in reproductive and developmental biology. However, it is difficult to obtain plenty of human embryos because of ethical and jurally limitation. To obtain embryos from rhesus monkey is still difficult but much feasible compare to from human.

Collection of oocytes from rhesus monkey is the basic procedure for *in vitro* embryology research. In order to obtain ample quantitative and fine qualitative oocytes, animals are often administrated exogenous gonadotropins to cause Superovulation (SO) (Bavister *et al*., 1986; Zelinski-Wooten *et al*., 1995).

Researchers have paid great effort on SO in rhesus monkey for improving the yield of oocytes retrieval. These studies found many factors affect SO outcome which including age (Hull *et al*., 1996; Nichols *et al*., 2010; Yang *et al*., 2009), dose of gonadotropins (Yang *et al*., 2007), physiological condition and repeated ovarian stimulation (Bavister *et al*., 1986; Ottobre and Stouffer, 1985; Yang *et al*., 2009), Initiation timing (Chen *et al*., 2011), etc.

However, little attention has been paid to workout which factor(s) would be determinant to SO outcome which caused instability, variability and high inefficiency

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in some treatments. Therefore, researchers have retrospectively analyzed and summarized the data from 361 SO treatments in rhesus monkeys.

MATERIALS AND METHODS

Animals: All animal procedures were approved in advance by the Institutional Animal Care and Use Committee at the Kunming Biomed International (KBI) and Kunming Institute of Zoology, Chinese Academy of Sciences. Rhesus monkeys aged 3-15 years old (Body weight was about 4-8 kg) were selected as oocyte donors and caged individually in a controlled environment (Temperature 20-24°C, humidity 40-60% and lighting cycle from 08:00-20:00 h). Vaginal bleeding was checked at least twice a day to detect the onset of menses.

Superovulation (SO) and Ovum Pick Up (OPU): SO was conducted in breeding season of rhesus monkey which lasted from late October to next March. According to the ages of animals, female rhesus monkeys were divided into three groups: group 1, puberty, 3-4 years old; group 2, adult, 5-10 years old and group 3, pre-menopause, 11-15 years old. All selected donors were administrated rhFSH (Gonal F, Laboratories Serono SA, Aubonne, Switzerland) to ovarian stimulation by intramuscular injection during the physiologic breeding season as described before (Yang *et al*., 2008). Treatment was random initiated on 1-5 days after the onset of menses. The 37.5 IU of rhFSH was given for the first injection and 18 IU of rhFSH was given twice daily for 8 days. 1000 IU of rhCG (Recombinant human chorionic gonadotropin alpha for injection, Merck Serono) was injected on day 9th. For oocyte retrieval, animals were anesthetized with anesthetic agents ketamine (10-12 mg kg^{-1}) and atropine (0.3-0.4 mg) and analgesic agent morphine (0.05 mg kg^{-1}) given intramuscularly.

OPU was conducted from animals by laparoscopic follicular aspiration (STORZ, Germany) 32-35 h after rhCG administration (Yang *et al*., 2007). Follicular contents were placed into HEPES-buffered TALP (Modified Tyrode solution with albumin, lactate and pyruvate) medium (Bavister *et al*., 1983) containing 0.3% Bovine Serum Albumin (BSA) at 37°C.

Oocytes were stripped of cumulus cells by mechanical pipetting after brief exposure (<1 min) to hyaluronidase (0.5 mg mL^{-1}) to allow classification of nuclear maturity as Prophase I (PI; intact germinal vesicle), Metaphase I (MI; no germinal vesicle, no polar body) and Metaphase II (MII; one polar body). Immature oocytes in either PI or MI stages were cultured in a 50 µL drop of medium CMRL-1066 containing 10% FBS, 10 IU mL^{-1} of porcine FSH and 10 IU mL^{-1} of ovine LH at 37°C in humidified 5% CO_2 in air for up to 24 h.

Follicular development monitor by ultrasonography: Follicular development was monitored by ultrasonography on day 6th of SO by Diasus ultrasound system (Dynamic Imaging Ltd., Livingston, Scotland, UK) with 10-22 MHz linear-array transducers. If there were no obvious or only 2-3 predominant follicles on each ovary, the SO was considered as non-effective and succeeding treatment would be canceled. Before ultrasonography detection, animals were anesthetized with ketamine (10-12 mg kg^{-1}).

Statistical analysis: Duration of menstrual cycle and quantity of SO and OPU were recorded. Number of ova, MI and MII oocytes from donor animals were collected. MI+MII≥10 was defined as effective SO-OPU while MI+MII<10 was defined as non-effective SO-OPU. Data were expressed as the Mean±SEM (Standard Error of the Mean) and analyzed on SPSS software using the least significant difference test. Significant difference was defined as $p<0.05$ and extreme difference was defined as $p<0.01$.

RESULTS

The effect of age on SO-OPU in rhesus monkeys: The menses of rhesus monkeys at different ages was observed and those who had onset of menses in 3 successive cycles were shown in Table 1. The duration of menstrual cycle and OPU outcomes were compared according to animal's ages.

The mean duration of menstrual cycle in puberty monkeys (23.72±0.75 days) was significantly shorter than those who were >5 years old ones (about 28 days) ($p<0.05$). These data showed that ages of rhesus monkey determine the duration of menstrual cycle which had important effect on animal's response to recombinant human gonadotropins.

To further analyze the effect of age on SO-OPU outcomes, 361 female oocytes donor rhesus monkeys were divided into 3 groups including puberty (3-4 years old, n = 54), adult (5-10 years old, n = 231) and pre-menopause (>10 years old, n = 31). On the day 6th of SO, monkeys were monitored by ultrasonography, good responders were injected subsequent rhFSH and rhCG.

Table 1: Duration of menstrual cycle of monkeys at different ages

Groups	No. of animal	Duration of menstrual cycle (days)
Puberty (3-4 years old)	22	$23.72±0.75^{a}$
Adult (5-10 years old)	31	$27.85±0.51^{b}$
Pre-menopause (11-15 years old)	18	$28.15±0.50^{b}$

a, b Denotes significant difference, $p<0.05$

Table 2: SO-OPU outcomes of oocytes donor rhesus monkeys in different ages

Groups	No. of SO	No. of OPU (%)	(MI+MII)≥10 (%)	No. of ova/n	No. of (MI+MII)/n
Puberty (3-4 years old)	54	46 (85.19)	34 (73.91)	44.85±3.70	25.03±2.41
Adult (5-10 years old)	231	212 (91.77)	156 (73.58)	48.40±2.05	26.82±1.34
Pre-menopause (11-15 years old)	76	66 (85.71)	47 (69.32)	43.02±2.96	24.53±1.99

Table 3: SO-OPU results of re-stimulated rhesus monkeys

Re-stimulation	No. of SO	No. of OPU	(MI+MII)≥10 (%)	No. of ova/n*	No. of (MI+MII)/n*
0	150	140	105 (70.00)	46.04±2.46[a]	27.26±1.76[a]
Once	87	80	56 (64.37)	46.55±3.09[a]	25.89±2.15[a]
Twice	66	59	40 (60.61)	55.13±4.46[b]	27.55±2.35[a]
3 times	35	33	25 (71.43)	37.88±2.73[c]	20.12±1.29[b]
≥4 times	23	19	13 (56.52)	43.00±3.50[ac]	22.08±1.76[ab]

[a-d]Different letter in same row showed that the mean number of ova or oocytes at MI plus MII was significantly different among groups (p<0.05). [ab, ac, bc]meant the marked value had no significant difference between ab and a or b, ac and a or c, bc and b or c. *No. of ova and treatment animal was calculated according to effective SO-OPU [(MI+MII) ≥10]

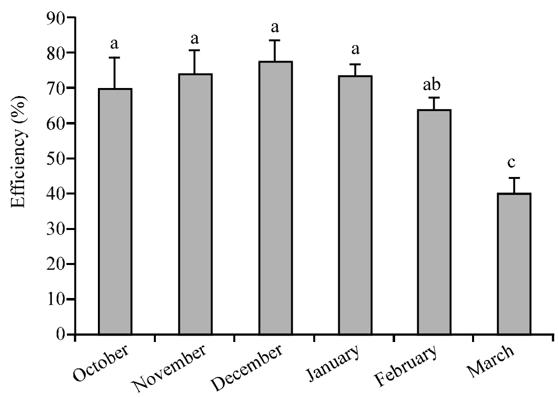

Fig. 1: Compare of SO-OPU efficiency of different month in breeding season in rhesus monkey. Efficiency = [(No. of (MI+MII)≥10 OPU/No. of SO)×100%]. [a-c]Denotes significant difference, p<0.05

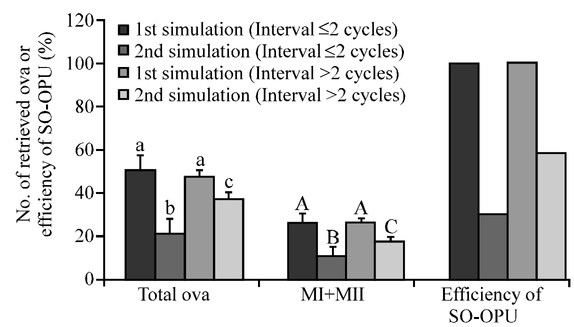

Fig. 2: Compare of influence of re-stimulation interval on SO-OPU efficiency and fecundity in rhesus monkeys re-stimulated in one breeding season when interval is ≤2 cycles, the mean number of ova and efficiency of SO-OPU was significantly decreased compared with those interval >2 cycles (p<0.05)

After OPU, number of MI+MII oocytes and rate of OPU [(No. of OPU/No. of SO)×100%] and effective SO-OPU [(No. of (MI+MII)≥10 OPU/No. of SO)×100%] was shown in Table 2.

Monthly difference of SO-OPU outcomes in breeding season: Little attention was paid to the difference of SO-OPU result in each month during the breeding season of rhesus monkeys. In this study, 217 SO treatments of adult female rhesus monkeys (5-10 years old) in three breeding seasons were chosen to be oocytes donors and SO were started on day 1-4 of menses. As defined before, number of MI+MII oocytes ≥10 was considered effective SO-OPU. So, only the efficiency of SO was referred here. SO and OPU result in each month was shown in Fig. 1. After analysis the data of 3 breeding seasons, the efficiency of OPU [(MI+MII)≥10] in each month was 70% (14/20), 74.47 (35/47), 77.55 (38/49), 73.33 (33/45), 63.41 (26/41) and 40% (6/15), respectively. These data imply that the optimal months for SO in rhesus monkey are October to next February.

The effect of re-stimulation on SO-OPU fecundity in rhesus monkey: Some rhesus monkeys in KPRC were used as oocytes donor up to 8 times. Without regard to other parameter such as animal's age, SO initiated time point and month in breeding season, 361 donors were divided into 5 groups according to times of re-stimulation. In general, efficiency of SO-OPU and mean ova and mature oocytes (MI+MII) was gradually decreased along with the increase of re-stimulation (Table 3).

However, some individual had potential to be continuously sensitive to recombinant gonadotropins which caused a non-linear relationship between re-stimulation and SO-OPU fecundity. Importantly, we found that if the animals were continuously stimulated in one breeding season, the interval of re-stimulation had crucial effect on efficiency and fecundity of SO-OPU (Table 4). When the re-stimulation interval >2 cycles, the efficiency and fecundity was significantly lower than the 1st stimulation but much higher than that of interval ≤2 cycles (Fig. 2).

Table 4: SO-OPU results of re-stimulated in one season at different interval in rhesus monkeys

Re-stimulation	No. of SO	NO. of OPU	(MI+MII)≥10 (%)	No. of ova/n*	No. of (MI+MII)/n*
Interval >2 cycles (In one season)	43	37	25 (58.14)	36.63±3.67[a]	16.93±2.25[A]
Interval ≤2 cycles (In one season)	17	11	5 (29.41)	20.50±5.84[b]	10.50±3.56[B]

DISCUSSION

An effective SO should be conducted at proper breeding season according to menstrual cycle. Similar to other animals, selection of oocytes donor rhesus monkey is a necessary process. This study demonstrated that animal's age and stimulation interval might be the determinant factors to fecundity of superovulation in rhesus monkey. Puberty of female rhesus monkey begins about at 30 months (2.5 years old) and menarche emerges at 32 months (2.6 years old) (Wilson and Kinkead, 2008). According to monitor of onset of menses and body weight, monkeys aged 3-4 years old and body weight >4.0 kg were designated puberty group. While 5-10 years old ones belonged to adult group and 11-15 years old, pre-menopause group. Interestingly, the data show that the duration of menstrual cycle in puberty was significantly shorter than adult ones. In the experiment, protocol of SO was same to each animal, without regard to age and body weight. Optimal dose of rhFSH and rhCG and treatment period for adult animal may be suboptimal to puberty or pre-menopause one. This may be the reason that caused high differential SO outcomes in puberty and pre-menopause. Whether, it necessary to set up individual procedure according to animal's age, body weight and menstrual cycle is still need future proof but undoubtedly, animal's age is a key fact which affect fecundity of SO in rhesus monkeys. Another effect of age to fecundity is aging of ovaries (Nichols *et al*., 2010; Schramm *et al*., 2002). Undergrown physiological function in pubertal and gradually decrepit reproductive system in aged rhesus monkey caused poor response to gonadotropins.

Bavister *et al*. (1986) found that rhesus monkey became refractory to repeated ovarian stimulation by Pregnant Mare Serum Gonadotropin (PMSG). Long time administration of human Chorionic Gonadotropin (hCG) produced antibody in rhesus monkeys (Ottobre and Stouffer, 1985). These antibodies did not alter the regulation of the menstrual cycle but attenuated the response to exogenous human gonadotropins (Ottobre and Stouffer, 1985).

PMSG was replaced by short half-life product FSH which was extracted from pituitary glands and urine and now recombinant (De Placido *et al*., 2000; Fisch *et al*., 1995; Ludwig *et al*., 2003). The rhFSH is effective to stimulation of superovulation in human and in rhesus monkeys and to injection of rhFSH will not result in the formation of antibodies in human, even in rhesus monkeys. However, rhCG is still widely used in monkeys for oocytes maturation which maybe the mainly cause to produce antibodies in repeat stimulated rhesus monkeys (Ottobre and Stouffer, 1985).

Recombinant Luteinizing Hormone (LH) is ideal substitution to hCG and recombinant rhesus monkey gonadotropins will be the optimal future choice (Ludwig *et al*., 2003; VandeVoort and Tarantal, 2001). Another important concern of SO in rhesus monkey is the arrangement of repeated stimulation which including repeated times and interval.

Therefore, in order to analyze the influence of these factors to SO-OPU outcomes, 361 rhesus monkeys at different ages from 3 breeding seasons were divided into 5 groups according to the times of repeated stimulation. We found that the efficiency and oocytes yield decreased along with the increase of SO frequency. This showed the repeated stimulation affect SO outcomes in rhesus monkey but this influence is not decisive. The previous investigation found that the repeated ovarian stimulations would be successful when the interval of re-stimulation was >2 menstrual cycles (Yang *et al*., 2008).

The present data has verified and supported that conclusion. Compared to those whose duration of re-stimulation was >2 menstrual cycles when the duration was <2 cycles, the efficiency and fecundity decreased significantly. One possible reason might be the surgical operations affect the physiological status of animal but similar result was obtained even after minimally invasive surgery laparoscopic follicular aspiration was introduced. Poor responders increased in animals re-stimulated <2 cycles imply the rhCG might still be the mainly cause to this consequence.

The data also showed the period of breeding season and stimulation initiated time point had effect on fecundity, these results remind us that the proper season of SO should be conducted from late October to next February. In this study, researchers did not show the results of developmental potential of oocytes. This is because of two reasons. Firstly, the oocytes obtained were used as materials in different subsequent experiments such as *in vitro* fertilization, transgenic, nuclear transfer or chimeric research.

It is difficult to compare the development of oocytes and embryos among those trials. Secondly, the previous studies had compared developmental capacity of oocytes obtained from different SO regimen (Yang *et al*., 2008), from different ages of animals (Yang *et al*., 2009) and seasonal influence (Zheng *et al*., 2001). These data found oocytes developmental capacity varied in non-breeding seasonversus breeding season and prepuberty versus adult (Yang *et al*., 2009; Zheng *et al*., 2001). In the present study, the oocytes donor animals were aged from 3-16 years which should be normal reproductive stage in rhesus monkeys.

Besides, all SO treatment was performed in breeding season. Therefore, the efficiency and mean ova and number of MI+MII oocytes could be useful to judge the fecundity of SO in rhesus monkey.

CONCLUSION

In this study although, many factors could affect the outcomes of superovulation in rhesus monkeys stimulated with recombinant gonadotropins, animal's age and the stimulation interval might be the determinant of the fecundity.

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