







Original Article
Biotechnology



Generation of cynomolgus monkey fetuses with intracytoplasmic sperm injection based on the MII-stage oocytes acquired by personalized superovulation protocol

Zhangqiong Huang ^{1,2,†}, Yun Li ^{1,2,†}, Qinfang Jiang ^{1,2}, Yixuan Wang ^{1,2},
Kaili Ma ^{1,2,3,*}, Qihan Li ^{1,2,3,*}

¹Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Kunming 650118, China

²Medical Primate Research Center & Neuroscience Center, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China

³Yunnan Key Laboratory of Vaccine Research Development on Severe Infectious Diseases, Kunming 650118, China

 OPEN ACCESS

Received: Sep 27, 2019

Revised: Feb 29, 2020

Accepted: Mar 2, 2020

*Corresponding authors:

Kaili Ma

Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, 935 Jiaoling Rd, Wuhua District, Kunming 650118, China.

E-mail: mklpumc@gmail.com

Qihan Li

Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, 935 Jiaoling Rd, Wuhua District, Kunming 650118, China.

E-mail: liqihan@imbcams.com.cn

[†]Zhang-Qiong Huang and Yun Li contributed equally to this work.

© 2020 The Korean Society of Veterinary Science

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABSTRACT

Background: Mature oocytes at the metaphase II status (MII-stage oocytes) played an important role in assisted reproductive technology in non-human primates.

Objectives: In order to improve the proportion of MII-stage oocytes retrieval, three different superovulation protocols were performed on 24 female cynomolgus monkeys.







Methods: All the monkeys received once-daily injection of follicle-stimulating hormone (25 international unit [IU]) on day 3 of the menstruation, 3-day intervals, twice daily for 8–12 days until the time of human chorionic gonadotropin (1,500 IU) injection, on the 14–17th day of menstruation collecting oocytes. The difference between protocol I and protocol II was that 0.1 mg the gonadotropin-releasing hormone agonist was injected on day 1 of the menstruation, while the difference between personalized superovulation protocol and protocol II was that oocytes could be collected on the 14–17th day of menstrual cycle according to the length of each monkey.

Results: The total number of oocytes harvested using the personalized superovulation protocol was much higher than that using protocol I ($p < 0.05$), and the proportion of MII-stage oocytes was significantly greater than that from either superovulation protocol I or II ($p < 0.001$ and $p < 0.01$ respectively), while the proportion of immature oocytes at the germinal vesicle was less than that from superovulation protocol I ($p < 0.05$).

Conclusions: The personalized superovulation protocol could increase the rate of MII-stage oocytes acquired, and successfully develop into embryos after intracytoplasmic sperm injection, and eventually generated fetus.

Keywords: Cynomolgus monkeys; assisted reproductive technologies; MII-stage oocytes; personalized superovulation; menstrual cycles; sexual swelling; ovulation point

ORCID iDs

Zhangqiong Huang 
<https://orcid.org/0000-0003-2893-772X>
Yun Li 
<https://orcid.org/0000-0002-4777-5482>
Qinfang Jiang 
<https://orcid.org/0000-0003-1651-1959>
Yixuan Wang 
<https://orcid.org/0000-0002-6285-8235>
Kaili Ma 
<https://orcid.org/0000-0002-5242-3000>
Qihan Li 
<https://orcid.org/0000-0002-8932-4516>

Funding

This work was supported by the National Natural Science Foundation of China (81571254); the Fundamental Research Funds for the Central Universities (3332016116,2016ZX310179-1); CAMS Innovation Fund for Medical Sciences (2016-I2M-2-001,2016-I2M-1-004); the Foundation for the Institute of Pathogen Biology (2015IPB201); National Science and Technology Major Projects for The Drug Innovation Major Project (2018ZX09711001-003-008).

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Ma K, Li Q; Data curation: Huang Z, Li Y; Formal analysis: Huang Z; Funding acquisition: Ma K, Li Q; Investigation: Huang Z, Li Y; Methodology: Huang Z, Li Y; Project administration: Ma K, Li Q; Resources: Jiang Q; Software: Huang Z; Supervision: Ma K, Li Q; Validation: Wang Y; Visualization: Jiang Q; Writing - original draft: Huang Z; Writing - review & editing: Ma K, Li Q.

INTRODUCTION

Non-human primate offspring, including rhesus monkeys (*Macaca mulatta*) [1], cynomolgus monkey (*Macaca fascicularis*) [2], pigtailed macaque (*Macaca nemestrina*) [3], common marmoset (*Callithrix jacchus*) [4], baboon (*Papio spp.*) [5], and African green monkey (*Cercopithecus aethiops*) [6], have been produced by assisted reproductive technologies (ARTs). However, due to the lack of resources, high cost, complex procedures, and the long reproductive cycle of these species, the development speed and success rate of ARTs in monkeys are still far from those of rodents and humans. In particular, the use of ARTs in cynomolgus monkeys remains modest. Compared with rhesus monkeys, the breeding of cynomolgus monkeys is not subject to seasonal restrictions, enabling their increased use in transgenic animal models. Regardless of the animal model used, the number and quality of mature oocytes (at the metaphase II status [MII-stage oocytes]) has long been considered as a primary limiting factor for *in vitro* fertilization (IVF) and embryo transfer (ET). Numerous studies have shown that the quality of mature oocytes *in vivo* exceeds the quality of matured oocytes via *in vitro* maturation [7-10]. Most importantly, the quality of the oocytes directly impacts the quality of the embryos and thus affects the rates of oocytes fertilization, embryonic implantation, and pregnancy [7-11]. Therefore, the high-quality embryos are critically dependent on the superovulation method based on obtaining more mature oocytes, and pregnancy and fetus production depend on not only on high-quality embryos, but also on the synchrony of uterus-embryos.

In the past 20 years, despite a few previous reports on promoting ovulation in non-human primates, the average of all menstrual cycles was used to calculate the regimen and frequency of hormonal injection [5,12,13]. That is, as each monkey's ovulation time is considered to be the mean for all monkeys, the time interval between the injection of the first hormone and the collection of oocytes was identical for all monkeys. There were also some studies that determine the time of oocyte retrieval based on measuring the size of the follicle [14-16]. The total number of oocyte retrieval and proportion of MII-stage oocytes obtained were quite different, and most of the proportion of MII-stage oocytes in the study was less than 50% of the total number of oocytes obtained. In general, it is difficult to determine a technology that can provide consistent and reliable results.

In this study, the different superovulation protocol was used according to the length of the menstrual cycle of each cynomolgus monkey and the total number of oocytes obtained by the typical superovulation protocols based on the average of the menstrual cycle of all cynomolgus monkeys and the proportion of immature oocytes at the germinal vesicle (GV-stage oocytes), immature oocytes at the metaphase I status (MI-stage oocytes), and MII-stage oocytes were compared. After intracytoplasmic sperm injection (ICSI) on MII-stage oocytes from a monkey (#21) in the personalized superovulation group, the obtained embryos were transferred into the oviduct of recipient female selected according to three key factors of "menstrual cycle," "sexual swelling," and "ovulation point," and verified whether these oocytes can generate fetuses.

MATERIALS AND METHODS**Ethics**

The design and procedures of all animal studies were in accordance with the ethical standards detailed in the 1964 Declaration of Helsinki and its later amendments. All the

animal studies were approved by the Experimental Animal Ethics Committee of the Institute of Medical Biology Chinese Academy of Medical Sciences (approval No. 20160801) which are based on the 3R principle (reduction, replacement, and refinement).

Animals

Twenty-four adult female cynomolgus monkeys (*M. fascicularis*; 6–13 years of age; mean, 9.5 years) were divided equally between the three different protocols (eight animals per group) for ovarian stimulation and oocyte collection, and then four (#1, 2, 3, 6 and #9, 13, 14, 16) out of protocol I and protocol II were randomly selected and a repetitive stimulation was performed with personalized superovulation protocol (**Table 1**); the other four females (#25, 26, 27, 28; 4.5–9 years of age) were used as recipients, with menstrual cycles between 28 and 33 days in

Table 1. The total number of oocytes retrieved by three superovulation protocols and the number of oocytes obtained after repeated stimulation by personalized superovulation in the same monkey

Protocol	Code of monkey (#)	Age (yr)	x ± S	Total number of retrieval*	x ± S		
Superovulation protocol I	1	8	9.5 ± 3.1	19	16.4 ± 7.3 [†]		
	2	7		24			
	3	6		18			
	4	12		7			
	5	11		9			
	6	15		8			
	7	10		21			
	8	7		25			
Superovulation protocol II	9	8	9.6 ± 1.9	28	21.6 ± 6.8		
	10	8		32			
	11	10		22			
	12	8		25			
	13	12		20			
	14	13		19			
	15	9		17			
	16	9		10			
Personalized superovulation protocol	17	12	9.6 ± 2.6	15	26.6 ± 9.0 [†]		
	18	13		22			
	19	8		31			
	20	13		17			
	21	7		39			
	22	8		23			
	23	8		38			
	24	8		28			
The first stimulation	Superovulation protocol I	1	9.0 ± 4.1	19	17.3 ± 6.7		
		2		24			
		3		18			
		6		15			
	Superovulation protocol II	9		10.5 ± 2.4		28	19.3 ± 7.4
		13				20	
		14				19	
		16				10	
The second stimulation	Personalized superovulation protocol	1	9.0 ± 4.1	15	22.5 ± 16.1		
		2		45			
		3		22			
		6		15			
	Personalized superovulation protocol	9		10.5 ± 2.4		25	17.0 ± 7.0
		13				20	
		14				14	
		16				9	

The average number of oocytes obtained from the personalized superovulation protocol was 26.6 ± 9.0, less than from superovulation protocol I (16.4 ± 7.3, $p < 0.05$), and no differences between other groups.

*Total number include GV-stage, MI and MII-stage; [†] indicates a significantly difference ($p < 0.05$).

length, and weights between 3 kg and 6 kg. In addition, 12 monkeys with similar conditions to the former were used for hormone testing. Two sexually mature male monkeys (#41, 42; 6 years of age) were selected to collect sperm; all the monkeys were provided by the Institute of Medical Biology, Chinese Academy of Medical Science, and Peking Union Medical College.

Superovulation protocols

We investigated three protocols for superovulation in cynomolgus monkeys. Superovulation protocol I: female cynomolgus monkeys that had not previously received ovarian stimulation were administered single intramuscular injection of 25 international unit (IU) follicle-stimulating hormone (FSH; Livzon Pharmaceuticals, China) on day 3 of the menstrual cycle. On the fourth day of the menstrual cycle, they were injected twice daily with 25 IU FSH for eight days, followed by an intramuscular injection of human chorionic gonadotropin (hCG, 1,500 IU, Livzon Pharmaceuticals) on the 12th day of the menstrual cycle, 35–36 h after collecting oocytes (i.e., oocyte harvest was on the 13th day of the menstrual cycle; **Fig. 1A**). Superovulation protocol II: female cynomolgus monkeys that had not previously received ovarian stimulation, the difference from superovulation protocol I being a subcutaneous injection of 0.1 mg of gonadotropin-releasing hormone agonist (GnRH-a, Ferring Pharmaceuticals, Germany) given on the first day of the menstrual cycle (**Fig. 1B**). Personalized superovulation protocol: regardless of whether female cynomolgus monkeys had previous ovulation induction experience, they were given a subcutaneous injection of 0.1 mg GnRH-a on the first day of their menstrual cycle. After a day's interval, on the third

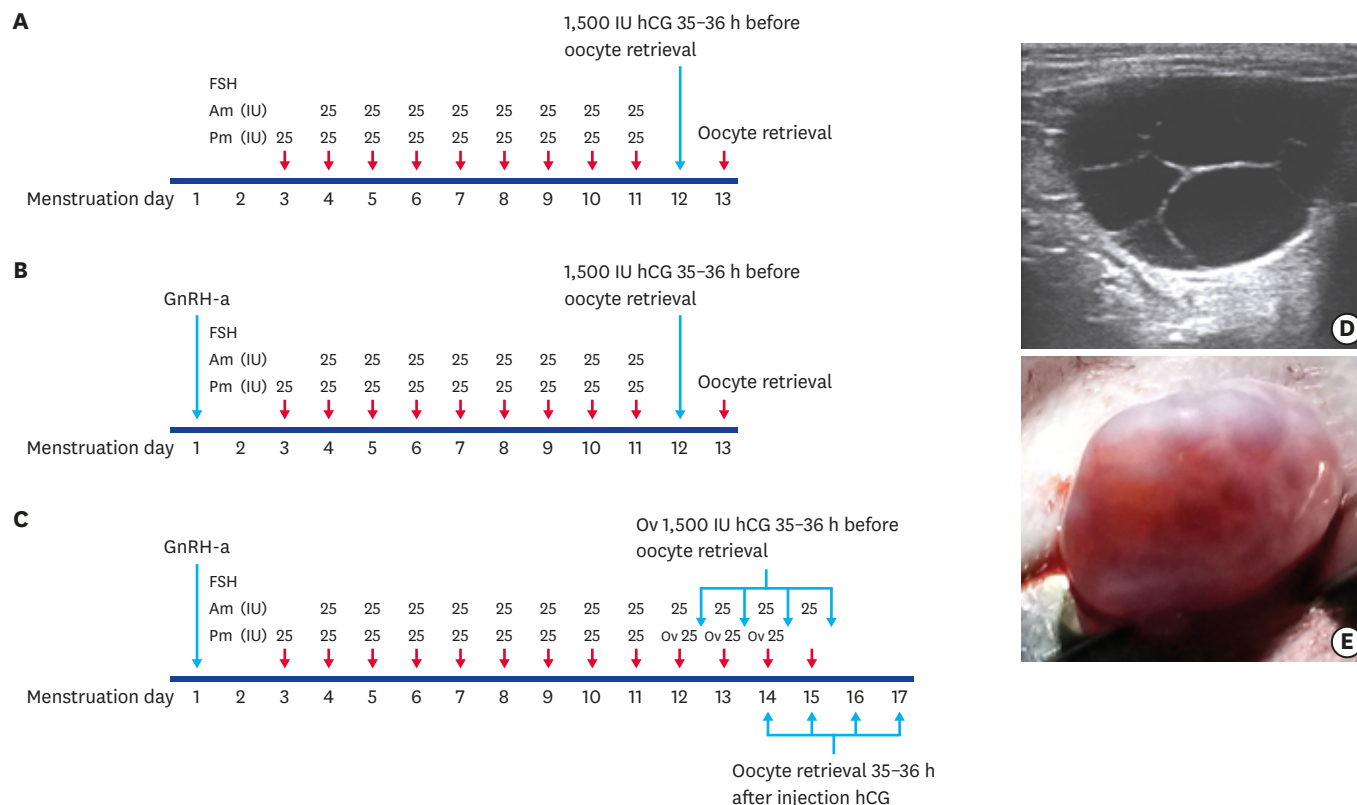


Fig. 1. Superovulation protocols and the proportion of oocytes retrieval. (A) Superovulation protocol I. (B) Superovulation protocol II. (C) Personalized superovulation protocol. (D) B-type ultrasound images of the ovary and follicles after ovulation induction with personalized superovulation protocol. (E) Surgical extraction of ovary after ovulation induction with personalized superovulation protocol. FSH, follicle-stimulating hormone; IU, international unit; hCG, human chorionic gonadotropin; GnRH-a, gonadotropin-releasing hormone agonist.

day of the menstrual cycle, an intramuscular injection of FSH (25 IU) was administered once a day. On the fourth day of the menstrual cycle, they were injected twice daily with FSH for 8–12 days. The final FSH (25 IU) was administered once daily on the 12–15th days based upon the length of each monkey's menstrual cycle. If the menstrual cycle was 28 days, hCG (1,500 IU) was injected on the 12th or 13th day, with oocyte harvest 35–36 h later (i.e., on the 14th or 15th day of the menstrual cycle). If the menstrual cycle was 30 days, hCG (1,500 IU) was injected on day 13 or 14, followed by oocyte collection 35–36 h later (on the 15th or 16th day of menstrual cycle). The time of oocyte retrieval was based on the principle of menstrual cycle length/2 ± 1, and the latest date for harvesting oocytes was on day 17 of the menstrual cycle (**Fig. 1C**). After ovulation induction by personalized superovulation, the size of monkey follicles was confirmed by B-ultrasound (**Fig. 1D**).

Oocyte retrieval and *in vitro* culture

For oocyte retrieval (usually 35–36 h after hCG injection), all female monkeys were anesthetized with 3% pentobarbital sodium at a dose of 25 mg per kg of body weight. Ovaries were exposed through a small incision in the middle of the lower abdomen (**Fig. 1E**), and the contents of large follicles (1 cm ± 5 mm in diameter) were aspirated through a 25-gauge needle connected to a 5.0 mL syringe. Cumulus oocyte complexes (COCs) were harvested by aspiration with the medium (TALP-HEPES [Tyrode's Albumin Lactate Pyruvate, Caisson Labs, USA], supplemented with 5 mg/mL bovine serum albumin [Sigma-Aldrich, USA] and 5 IU/mL of heparin [Sigma-Aldrich]) under a stereomicroscope (Nikon SMZ745; Nikon, Japan). COCs were rinsed with TALP-HEPES supplemented with 0.1% hyaluronidase (Sigma-Aldrich) for removing cumulus cells. The maturation status of the oocytes was evaluated under an inverted microscope (Leica DMI6000B; Leica Microsystems, Germany) at ×100 or ×200 magnification, and GV, MI, and MII oocytes were counted for statistical analysis after washing. Oocytes (both immature and mature) were cultured in 50 µL drops of defined medium (HECM9 medium [Gibco; Invitrogen, USA], containing 10% fetal bovine serum [Sigma-Aldrich], and 10% GLU [Sigma-Aldrich]) under a layer of embryo-tested mineral oil (Sigma-Aldrich) at 37°C in 5% CO₂ and 95% compressed air at high humidity (Thermo Forma 3131; Thermo Fisher Scientific, USA).

Sperm collection

Sperm was collected by electric stimulation of an adult male cynomolgus monkey penis. The sperm was washed twice through TALP-HEPES supplemented with 5 mg/mL bovine serum albumin and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded. Then we added 1.0 mL TALP-HEPES supplemented with 5 mg/mL bovine serum albumin, and rested for 5 min, took upstream sperm for ICSI.

ICSI

The MII-stage oocytes derived from a monkey that was induced by personalized superovulation protocol were cultured at least 2 h before ICSI. ICSI was conducted using an inverted microscope (Leica DMI6000B; Leica Microsystems) with attached micromanipulators, briefly, separately placed mature oocytes and sperms into two 10 µL droplets of TALP-HEPES containing 5 mg/mL bovine serum albumin, and the motile spermatozoa were immobilized in 7% polyvinylpyrrolidone. The oocyte was fixed in holding pipette, and the first polar body (PB) was placed at 12 o'clock, the injection pipette penetrated oocytes at 3 o'clock (**Fig. 2A and B**) passed through the zona pellucida and oocyte membrane; negative pressure was used to attract lightly broken membranes after the sperm was slowly injected into the oocyte, and then the injection pipette was gently withdrawn. After the

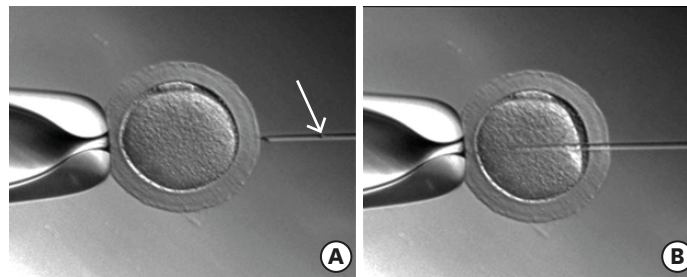


Fig. 2. Intracytoplasmic sperm injection. (A) MII-stage oocytes are held so that the first polar body is positioned at 12 o'clock and a single sperm (aspirated tail first) is pushed to the tip of the injection pipet (arrow). (B) The injection pipet is inserted into oocyte cytoplasm through the zona pellucida at 3 o'clock. MII-stage oocytes, mature oocytes at the metaphase II status.

sperm injection of all MII-stage oocytes was finished, the oocytes were transferred to HECM9 medium and incubated at 37°C in 5% CO₂ and 95% compressed air.

Recipients selection

Recipients selection: healthy, sexually mature, and normal menstrual cycle female cynomolgus monkeys were selected as recipients. It is normal to observe at least three or more menstrual cycle before these monkeys were used as surrogate recipients. We then accurately observed and judged the time when “sexual swelling” appeared and disappeared. The characteristics of young female monkeys with sexual swelling in the early stage of sexual maturity are as follows: the vaginal area is swollen and reddened subcutaneous groin subcutaneously forms two saclike protrusions of testicular size as main features, and sexual swelling has a smooth, shiny appearance and is deep, intense red color, periodic changes (**Fig. 3A**); non-sexual period: there is no swelling in the vaginal area, there are no bulges in the subcutaneous groin, and the entire area is very light in color (**Fig. 3B**). Sexual swelling in adult female monkeys includes the following observations: turgescence area extended to anus and thighs, the perineal skin is fully distended with no wrinkles and smooth and most intense bright red color (**Fig. 3C**); Non-sexual period: there is no expansion in the perineal area, it is full of fine wrinkles, and the color is muted (**Fig. 3D**). Normal sexual swelling occurs during ovulation, which was on day 13–19 after menstruation. After observing the change in the color of sexual skin in surrogate candidates, it was confirmed by laparoscopy whether there was an ovulation point on the ovary of the surrogate candidates when preparing for transplantation (**Fig. 3E**), and if not, the surrogate candidate was abandoned. From the observation of sexual swelling, the window period for ET could last 6–9 days.

ET and pregnancy detection

During the 6- to 9-day window of ET, when embryos developed to the 2–4-cell stage, they were transferred into the oviduct of the surrogate female (**Fig. 3F**). The specific operation of the transplant is to insert the trocar into the abdominal region while avoiding blood vessels near the ovary. The trocar was removed and the incision was expanded to 1–2 cm by cutting the skin, clamping the ovary adipose with a clip, and pulling out the ovary and oviduct. The embryos were aspirated into a thinned Pasteur pipet (PP-9-90PL; ORIGIO, USA) under a stereomicroscope (Nikon SMZ745; Nikon). The Pasteur pipet was then inserted into the oviduct fimbria of the recipient and transfer the embryos to oviduct by blowing the pipet. Pregnancy was confirmed by ultrasonography on day 30 after the transfer.

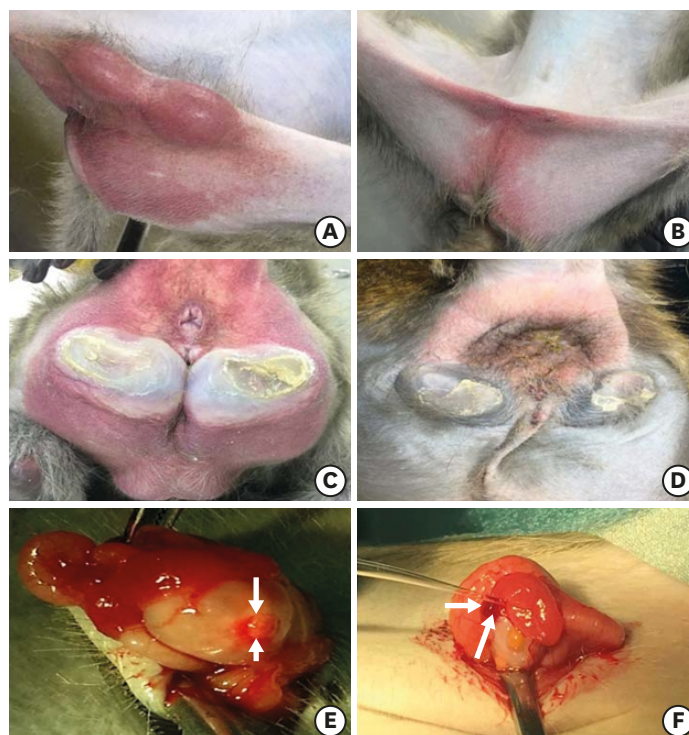


Fig. 3. The characteristics of sexual skin of female cynomolgus monkeys and ovarian features of synchronous surrogate mothers. (A) Ovulation characteristics of young female in the early stage of sexual maturity: the vaginal area is swollen, reddened and subcutaneous groin subcutaneously forms two saclike protrusions of testicular size as the main features, and it has a smooth, shiny appearance and is deep, intense red color, periodic changes. (B) Non-ovulation characteristics of young female: the turgescent area obviously narrowing and color decrease. (C) Ovulation characteristics of adult female: the turgescent area extended to anus and thighs, the perineal skin is fully distended with no wrinkles and smooth and most intense bright red color. (D) Non-ovulation characteristics of adult female: the turgescent area obviously narrowing, limit to reproductive anal area and tail root, and the labia and clitoris have many wrinkles with an overall pinkish-red color. (E) A new ovulation point (arrow) on the ovary of surrogate mother. (F) Ovary with a new ovulation point (arrow), the Pasteur pipet (bar = 15–20 mm) containing the embryos is inserted into the oviduct of the surrogate mother from the fimbria of fallopian tubes and the embryos is gently blown into the oviduct.

Hormone detection

On the third day after menstruation, we took 1 ml of blood from the hindlimb vein of female monkeys and centrifuged it at 3,000 rpm for 20 min. The serum was refrigerated and sent to the Kunming Kingmed Institute for Clinical Laboratory to detect estradiol (E2) concentration by chemiluminescence.

Parentage verification of aborted fetus

Parentage verification of the aborted fetus were done by DNA typing of 20 microsatellite markers: extracting DNA from the hind limbs of three fetuses and oocytes donor. Blood was taken from sperm donor and surrogate mothers to extract DNA. Twenty locus-specific primers were designed according to references [17,18] and each primers contained a fluorescent dye (FAM/HEX) (Table 2), PCR amplification of short tandem repeats (STRs) was performed as follows: firstly, pre-denaturation at 95°C for 1 min, then was amplified with 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, followed by a 30-min extension at 60°C. The multiplexed reaction containing 10 × Buffer I (Beijing Microread, China), 2.5mM dNTP, --F+R (5μm FAM or HEX –labeled) (Takara, Japan), HSTaq (Takara) and DNA template, then PCR were carried out in an ABI GeneAmp9600. After PCR, the PCR product, internal size standard ORG 500 (Beijing Microread), and deionized formamide (0.5:8.5) were mixed and added to the 96-plate. This was followed by capillary electrophoresis on an ABI 3730XL DNA analyzer to obtain the raw data. The raw data file detected by the 3730XL was

Table 2. The 20 STRs locus and primers used in this study

Locus	Rhesus chromosome location	Forward primer (5' to 3')	Reverse primer (5' to 3')	Fluorescent dye
1-D1S548*	1	GAAGTCATTGGCAAAGGAA	GCCTCTTTGTTGCAGTGATT	5'FAM
2-D2S1333*	12	CTTTGTCTCCCCAGTTGCTA	TCTGTCATAAACCGTCTGCA	5'FAM
3-D2S149*	13	GCTTAGTGGGAAATGTAACCT	GCCTGGCTCCTCTAATACCT	5'FAM
4-D3S1768†	2	GGTTGCTGCCAAAGATTAGA	CACTGTGATTTGCTGTTGGA	5'FAM
5-D4S2365*	5	AGTAATCTTCAACTGCATCACC	ATGCCAAGGATGGTGAGTTA	5'FAM
6-D5S1457*	6	TAGGTTCTGGGCATGTCTGT	TGCTTGGCACACTTCAGG	5'FAM
7-D6S1691†	4	AGGCAGAAATTTGCCTC	GCTGCTCCTGTATAAGTAATAAAC	5'FAM
8-D6S276†	4	TTCCAGTGATACATCAATCAAATCA	GGGTGCAACTTGTTCCTCCT	5'FAM
9-D6S501*	4	GCTGGAACTGATAAGGGCT	GCCACCCTGGCTAAGTTACT	5'FAM
10-D7S513†	3	AGTGTTTTGAAAGTTGTAGTTAAT	ATATCTTTCAGGGGAGCAGG	5'FAM
11-D7S794†	3	GCCAAATCTCTAACAATCC	TATGCCCATGTGTTAGGGTT	5'HEX
12-D8S1106†	8	GCGGCATGTTTTCTACTTT	TTCTCAGAATTGCTCATAGTGC	5'HEX
13-D10S1412†	9	GCTCCCTGCGTACTGAGATA	GCACAGTACTTCCCTCCA	5'HEX
14-D11S925†	14	AGAACCAAGGTCGTAAGTCCTG	TTAGACCATTATGGGGCAA	5'HEX
15-D12S364*	11	CCAGTCTGAACAGGC	CCCTGGAAGTCCCATC	5'HEX
16-D16S409*	20	TGAATCTTACATCCCATCCC	AGTCAGTCTGTCAGAGGTG	5'HEX
17-D17S1300*	16	TAGTGTGTATATGTATGCATGCA	TGCAGATATCTGCTTTTGGC	5'HEX
18-D18S72†	2	GCTAGATGACCCAGTTCCC	CAAGAGAGCCCTTTGGTTT	5'HEX
19-D19S228*	19	AGCTAATATGGCAGAATCTTGAC	GCAGGATCGCAGTGCTT	5'HEX
20-MFGT21†	8	AACTTCAGTAAGATAAGGACC	CCTGAGGCTGGACTTTAT	5'HEX

STR, short tandem repeat.

*STR primers were designed according reference (Siddiqui et al., 2009 [18]); †STR primers were designed according reference (Kanthaswamy et al., 2010 [17]).

imported into the analysis software Genemapper ID3.2 for analysis. According to genetic principles and genetic laws, half of the alleles in the progeny genotypes are from the father and the other half are from the mother.

Statistical analysis

All the data were expressed as the means ± SD of the means and were analysed statistically by using GraphPad Prism software version 7.0 (GraphPad Prism, USA). The one-way analysis of variance was used to analyze the number and proportion of oocytes obtained by protocol I, protocol II, and personalized protocol. For comparison of data between the same monkeys that had undergone the second ovulation, a paired *t*-test (when the pairing was effective, $p < 0.05$) or an unpaired *t*-test (when the pairing was not effective, $p > 0.05$) were used. The results were considered different if $p < 0.05$, obvious different if $p < 0.01$, and highly significantly different (if $p < 0.001$).

RESULTS

Comparison of oocytes retrieval by three different superovulation protocol

Twenty-four adult female cynomolgus monkeys (mean, 9.5 years) were used in evaluating the effect of ovarian stimulation and oocyte collection. The retrieved oocytes were classified into maturational stages: 1) GV (immature oocytes, intact germinal vesicle); 2) MI (immature oocytes, no germinal vesicle, no PB); and 3) MII (mature oocytes, with the first PB). A total of 131, 173, and 213 oocytes were collected from animals of superovulation protocol I, II and personalized superovulation protocol respectively (**Table 1**). Compared with superovulation protocol I, although protocol II used 0.1 mg GnRH-a, the oocytes harvest time was the 13th day of menstruation, and it did not affect the average number of oocytes obtained (16.4 ± 7.3 vs. 21.6 ± 6.8). The personalized superovulation protocol determined the time of harvesting oocytes according to the length of the menstrual cycle of each monkey, compared to protocol I, it increased the average number of oocytes obtained (26.6 ± 9.0 vs. 16.4 ± 7.3 $p < 0.05$) (**Table 1**)

and reduced the proportion of GV-stage oocytes ($15.0\% \pm 5.6\%$ vs. $41.3\% \pm 25.5\%$, $p < 0.05$) (**Fig. 4A**). Although the proportions of MI-stage oocytes obtained by the three protocols were not different (**Fig. 4B**), the personalized superovulation protocol has significantly increased the proportion of MII oocytes ($58.5\% \pm 9.9\%$), which was highly significantly greater than that from either superovulation protocol I ($21.5\% \pm 19.6\%$, $p < 0.001$) or obvious greater than that from the superovulation protocol II ($33.2\% \pm 5.1\%$, $p < 0.01$) (**Fig. 4C**). With respect to the same monkey that has been stimulated with protocol I (#1, 2, 3, and 6) and protocol II (#9, 13, 14, and 16) were randomly selected using the personalized superovulation protocol for repeated ovulation induction, and there had no difference in the total number of oocytes (**Table 1**). The percentage of GV-stage oocytes between the first ovulation induction using superovulation protocol I and the second induction using the personalized superovulation protocol was different ($51.0\% \pm 25.9\%$ vs. $14.6\% \pm 11.4\%$, $p < 0.05$) (**Fig. 4D**), and there was no difference in the percentage of MI-stage oocytes (**Fig. 4E**), however, the difference in the percentage of MII-stage oocytes was obvious ($12.8\% \pm 9.9\%$ vs. $51.5\% \pm 24.9\%$, $p < 0.05$) (**Fig. 4F**). The percentages of GV, MI, and MII oocytes obtained from the first ovulation induction using the superovulation protocol II and the second ovulation induction using the personalized superovulation protocol were not different (**Fig. 4G-I**), but oocyte progression within the ovary from the GV-stage to the MI and MII-stages from the personalized superovulation protocol showed a tendency to increase (**Fig. 4J-L**).

Embryonic development after ICSI

ICSI was performed on 22 MII-stage oocytes within 4 h post-isolation from one monkey that was based on personalized superovulation method. Out of the 22 MII-stage oocytes, 21 of them (95.4%) had an extruded second PB after the injection and culture for 7–12 h, at least 20 of 22 (90.9%) displayed distinct pronuclei (**Fig. 5A**) within 12–18 h post-ICSI. Additionally, 20–24 h after ICSI, 15 of 20 zygotes developed to the 2-cell stage (**Fig. 5B**) and 3-cell stage (**Fig. 5C**). Four embryos cleaved to the 4-cell stage within 26–28 h post-ICSI (**Fig. 5D**).

Comparison of the difference in peak time of E2

A total of 12 female cynomolgus monkeys with normal menstrual cycle were tested for E2 concentration; the data is shown in **Fig. 6**. The earliest peak time was the 7th day after menstruation, and the latest peak occurred on the 20st day after menstruation, there was a huge difference between individuals.

ET outcomes

To verify whether MII-stage oocytes from the personalized ovulation protocol could develop into fetus, 2–4-cell cleaved stage embryos were transferred into the oviducts of four female recipients for pregnancy establishment under laparoscopic guidance. There was ovulation point on the ovary of female recipients by observing the sexual swelling and confirmed by laparoscopy (**Fig. 3E and F**, arrow). Of these seven embryos at 2-cell stage were transplanted to three female surrogates, and the other four embryos at 3- to 4-cell stage were transplanted to one female surrogate. Thirty days after transplantation, pregnancy was confirmed in two female recipients by ultrasound examination, one of them carried only gestational sacs (figure not shown), and one of the females with three fetuses (triplet) had preterm births on the 103rd day after ET (**Fig. 7A**). The rest of the surrogates were not pregnant.

Parentage verification of aborted fetus

STRs DNA analyses was used by Liu et al. [19] and Tachibana et al. [20] to detect DNA from cloned cynomolgus monkey by somatic cell nuclear transfer. We performed STRs analysis on

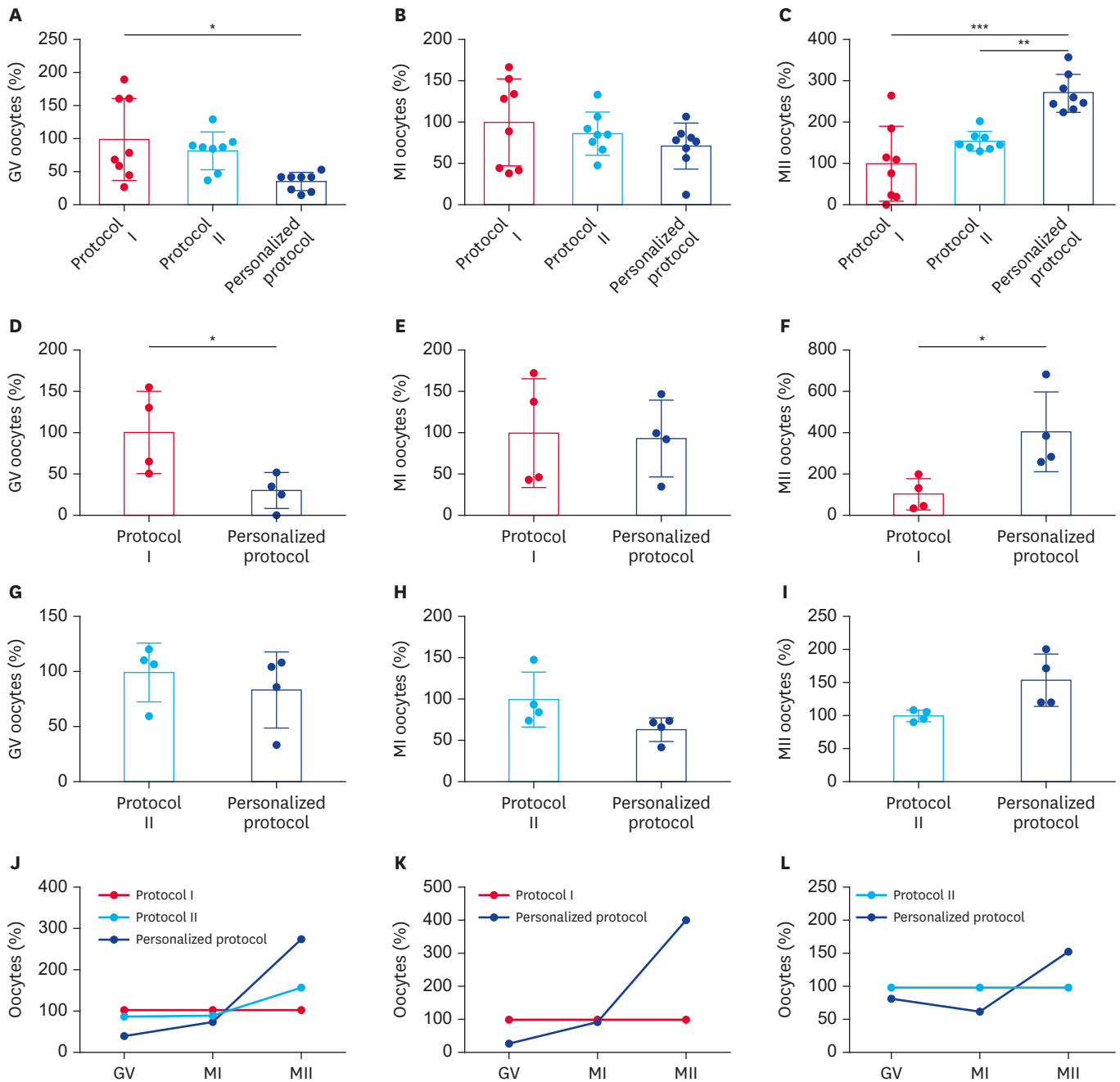


Fig. 4. (A-C) The proportions of GV-stage, MI and MII-stage oocytes after ovulation induction by three protocols. (A) The proportion of GV-stage oocytes from the personalized superovulation protocol were $15.0\% \pm 5.6\%$, less than from superovulation protocol I ($41.3\% \pm 25.5\%$). (B) The proportions of MI-stage oocytes after ovulation induction by three protocols were not different. (C) The proportion of MII-stage oocytes retrieved from the personalized superovulation protocol was $58.5\% \pm 9.9\%$, highly significantly greater than the $21.5\% \pm 19.6\%$ from superovulation protocol I and obvious greater than the $33.2\% \pm 5.1\%$ from superovulation protocol II. (D-F) Results of the same monkey (#1, 2, 3, and 6) stimulating with superovulation protocol I and the personalized superovulation protocol. (D) The proportion of GV-stage oocytes between the first ovulation induction using superovulation protocol I and the second induction using the personalized superovulation protocol was different ($51.0\% \pm 25.9\%$ vs. $14.6\% \pm 11.4\%$). (E) There was no difference in the proportion of MI-stage oocytes. (F) There was difference in the proportion of MII-stage oocytes ($12.8\% \pm 9.9\%$ vs. $51.5\% \pm 24.9\%$). (G-I) Results of the same monkey (#9, 13, 14, and 16) stimulating with superovulation protocol II and the personalized superovulation protocol. The proportions of GV, MI and MII-stage oocytes obtained from the first ovulation induction using the superovulation protocol II and the second ovulation induction using the personalized superovulation protocol were not different. (J-L) Oocyte progression within the ovary from the GV-stage to the MI and MII-stages by three protocols. The proportion of MII-stage oocytes from the personalized superovulation protocol relative to the superovulation protocols I and the superovulation protocol II showed a tendency to increase. GV-stage oocytes, immature oocytes at the germinal vesicle; MI-stage oocytes, immature oocytes at the metaphase I status; MII-stage oocytes, mature oocytes at the metaphase II status. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

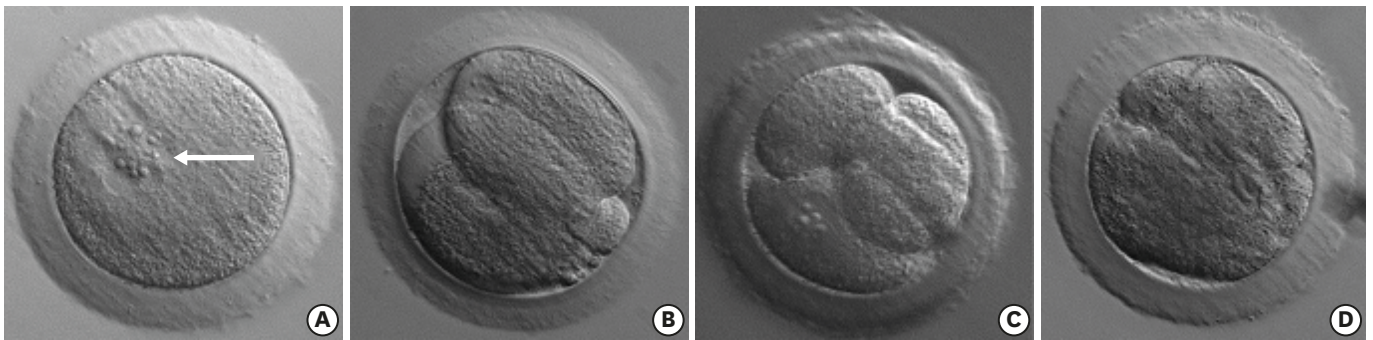


Fig. 5. Embryonic development. (A) Zygotes occurred distinct pronuclei within 12–18 h post-ICSI, after several hours, the pronuclei were fused (arrow). (B) Zygotes develop to 2-cell stage 20–24 h after ICSI. (C) Zygotes develop to 3-cell stage 24–26 h after ICSI. (D) Zygotes develop to 4-cell stage 26–28 h after ICSI. ICSI, intracytoplasmic sperm injection.

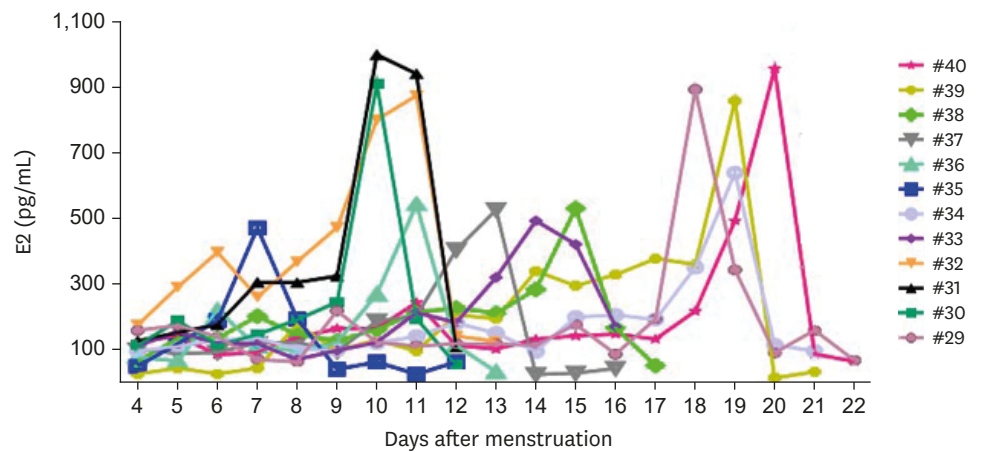


Fig. 6. The results of E2 detection. E2 detection: serum E2 concentrations during days 7–20 after menstruation of female cynomolgus monkeys, one monkey (#35) first appeared E2 peak on day 7 after menstruation, another (#40) peaked on day 20 after menstruation. E2, estradiol.

oocyte donor, sperm donor, surrogate mothers, and aborted fetuses using 20 microsatellite DNA loci, DNA from the hind limbs of aborted fetuses “b” and “c.” Half of the 20 STRs were from the oocyte donor and half from the sperm donor. Half of the D12S364 locus of fetus “a” was from the sperm donor, and the other half may not show a genetic relationship with the oocyte donor due to mutation or deletion, but the other 19 STRs loci were half from the sperm donor and half from the oocyte donor. These genetic analyses confirmed the fetuses “a,” “b,” and “c” have a parent-child relationship with oocyte donor and sperm donor. Six animals STRs were shown in **Fig. 7B and C** and complete information on 20 STRs loci were shown in **Supplementary Table 1**.

DISCUSSION

Statistical analysis after washing

Research in humans has shown that the physiological cycle of each individual is typically most irregular, and the variability in menstrual cycle length can range from 24 to 35 days due to varying lengths of the follicular phase [21,22]. The E2 peak in cynomolgus monkeys is similar to that in humans because it occurs one day before the peak in luteinizing hormone

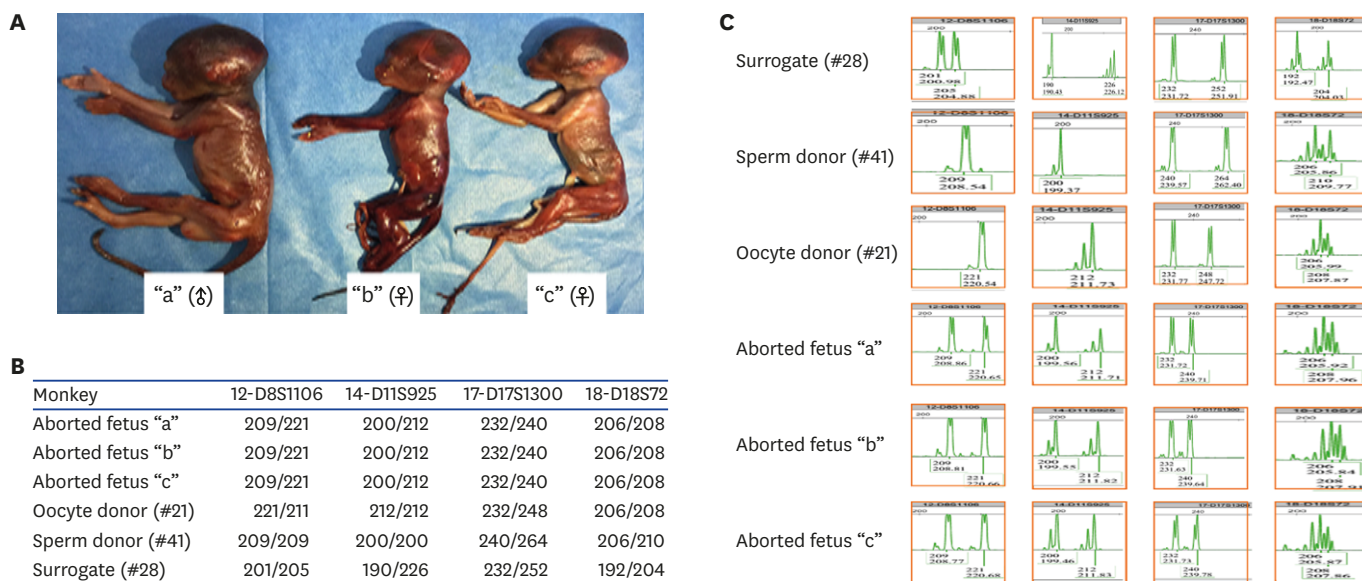


Fig. 7. Parentage verification of aborted fetus. (A) Image of spontaneous abortion of fetuses "a," "b," and "c" on day 103 post-embryos transfer. (B) Four examples of STRs from fetus "a," "b," and "c," oocyte donor, sperm donor and surrogate, half of them were from the oocyte donor and half from the sperm donor. Complete list of STRs are shown in **Supplementary Table 1**. (C) Examples of electrophoresis signal about amplification products of D8S1106, D11S925, D17S1300, D18S72 sites for fetus "a," "b," and "c," oocyte donor, sperm donor and surrogate, showing fetus "a," "b," and "c" have parent-child relationship with oocyte donor and sperm donor. STR, short tandem repeat.

(LH) concentration [23]. The menstrual cycle in the cynomolgus monkey consists of a 12–14 days follicular phase, a 3-day ovulatory phase, and a 14–16 days luteal phase [24]. Although the lengths of the ovulatory and luteal phases are consistent between cynomolgus monkeys and humans, the follicular phase is more variable in duration in cynomolgus monkeys, and as a result, affects the subsequent ovulatory phase [23,25]. Thus, the length of the menstrual cycle is different for each monkey, and the timing of ovulation varies. It is unscientific to calculate an ovulation time based on the average of the menstrual cycle of all monkeys. In this study, a personalized superovulation protocol was developed based on the differences among individual menstrual cycles. It was compared with the typical superovulatory protocols based on the average of all menstrual cycles, the total numbers of oocytes retrieved and proportion of MII-stage oocytes were significantly higher than those with protocol I and II, and it ($58.5\% \pm 10\%$) exceeded those observed previously for other cynomolgus monkeys ($47.6\% \pm 32.1\%$ MII) [26], baboons (range, 20–43% MII) [27], or rhesus monkeys (range, 44–53% MII) [14,15]. Moreover, these studies do not consider the different menstrual cycle of each monkey, and only determine the oocyte retrieval time according to the B-ultrasound monitoring follicle size. In previous research, we also used ultrasound monitoring of follicles, but we needed to monitor them 2–3 times to reach our goal, which was both time-consuming and laborious, and caused an emergency response to the monkey. Through careful observation, we determined that the skin of many monkeys changed throughout its menstrual cycle, in the middle of the menstrual cycle, the period of "menstrual cycle length/2 \pm 1" was the best time to harvest mature oocytes. Additionally, we repeatedly confirmed by ultrasound that the follicle growth was very good at this time, so we did not observe the growth of follicles with ultrasound, avoiding the effects on monkeys and improving work efficiency. The total number of retrieved oocytes and the proportion of MII-stage oocytes clearly demonstrated that more MII-stage oocytes following personalized ovarian stimulation were retrieved during the natural ovulatory cycle of female cynomolgus monkeys. Similar

to the situation in humans, each monkey's reproductive cycle is not identical, and the ovulation time of each individual cannot be calculated solely using the average for all monkey menstrual cycles [28,29].

For the same monkey, the first ovulation induction used superovulation protocol I (#1, 2, 3, and 6), after 5–6 menstrual cycles, the second ovulation induction used personalized superovulation protocol, the proportion of MII-stage oocytes significantly higher than the first ovulation induction. The first ovulation induction using the superovulation protocol II (#9, 13, 14, and 16), the second ovulation induction with personalized protocol also increased the proportion of MII-stage oocytes, and the proportion of oocytes in GV, MI, and MII-stage gradually increased, although the difference was not significant, which may be due to the small sample size, however, this tendency appeared to be quite flat in superovulation protocol I and superovulation protocol II. Despite previous research having shown that repeated ovarian stimulations could reduce the quantity and quality of the oocytes [30–32], the analysis of data in the present study did not show this particular result, but instead showed an increased proportion of MII-stage oocytes by adjusting the superovulation protocol based on the length of each monkey's menstrual cycle, thereby allowing the effective utilization of this precious resource.

A comparison of the results of superovulation protocol II and the personalized superovulation protocol revealed that the use of GnRH-a in combination with the length of the individual menstrual cycle of cynomolgus monkeys also served the role of GnRH-a in ovulation induction. The biological activity of GnRH-a is 50–200 times higher than that of endogenous GnRH for the GnRH receptor [33]. Therefore, its affinity to GnRH receptors substantially increased [34]. It also causes LH and FSH hypersecretion (flare-up) [35]. After GnRH-a has induced a state of hypoestrogenism, exogenous FSH is given to stimulate ovarian follicles, in addition to synchronization with the naturally occurring FSH peak, followed by the use of hCG to trigger oocyte release [36]. Based on this pharmacological feature, GnRH-a has been universally used for ovarian hyperstimulation cycles in human assisted-reproduction technologies to obtain high-quality oocytes to improve clinical pregnancy rate [37–39]. We found our results to be similar with those obtained in human studies. Therefore, we speculate that GnRH-a may inhibit the early LH peak and synchronize follicular development to increase the number of retrieved oocytes, which subsequently increases the related ovulation-induction parameters and pregnancy efficiency.

Ovulation during the natural menstrual cycle requires participation of LH/FSH peaks, in particular, the oocyte maturation process requires an appropriate LH peak, natural ovulation usually occur 35–36 h after the appearance of the endogenous LH/FSH peak [40]. The personalized superovulation protocol incorporated the menstrual cycle length of each cynomolgus monkey, thereby allowing to determine the timing of oocyte retrieval based on the equation: $\text{menstrual cycle length}/2 \pm 1$, and then pushing this time 35–36 h forward for the time of hCG injection. This new time is close to the time of natural ovulation and consistent with the monkey's reproductive physiological laws.

The primary focus of ET is how to ensure the synchronization of the recipients, there are currently two methods that are commonly used. The first method is used to detect the concentration of E2, 2–3 days after the peak of serum E2 is the transplant window, the corresponding embryo can be transferred into the oviduct [41,42]. However, this method has some problems, because in our previous research, all the selected recipients need to be tested

for serum E2 daily for about 8–19 days. This process is cumbersome and time consuming, and the peak of serum E2 occurs at a wide range from 7 to 20 days after menstruation (**Fig. 6**), such a large difference in time will mean that the same batch of embryos can be transferred on the 8th day of menstruation, but some need to be cultured *in vitro* for up to 13 days before they can be transferred. Cynomolgus monkey embryos can develop to blastocysts *in vitro* culture for seven days (about 168 h), as culturing up to 13 days will inevitably greatly reduce the quality of the embryos. The second method is that the female recipients had an ovulation point on the ovary, and the surrogate mother of the first somatic cloned monkey in the world was also selected by observing the ovulation point on the ovary [19]. This is a highly reliable method for ensuring uterine-embryo synchrony. However, in previous studies, we found that monkeys have normal menstrual cycles, but no ovulation point. Therefore, relying on the monkey's menstrual cycle to go directly to the ovulation point, there is uncertainty, which may reduce the efficiency of obtaining a surrogate mother.

In summary, in order to avoid the above shortcomings, we fully considered the possible factors affecting ovulation according to the reproductive physiology characteristics of non-human primates, the synchronization of surrogate females was ensured efficiently and accurately based on three points of menstrual cycle, sexual swelling, and ovulation point. It is worth noting that the sexual skin characteristics of estrus between young female monkeys and adult female monkeys are different. After observing the color change of sexual skin of surrogate candidate with normal menstruation, more than 98% of monkeys can clearly see the ovulation point by laparoscopy. From the observation of sexual swelling, the window period for ET can last 6–9 days, which can improve the utilization of surrogate candidate. Tardif et al. [43] also clearly pointed out that the sexual swelling was a very reliable way to predict the time of ovulation in monkeys.

Based on the previous personalized ovulation induction and comprehensive method of selecting recipients, we successfully obtained two pregnant monkeys as a result. Three fetuses were aborted on day 103. This does not necessarily mean that offspring can be obtained easily by ICSI, because ours was the first case in which a monkey had three fetuses based on the personalized superovulation in a nonhuman primate. This study did not show the results of fertilization rates for the oocytes from each of these protocols. This is because the oocytes obtained were used as materials in different subsequent experiments such as IVF and transgenic (including different genetic modifications) research. It is difficult to compare the development of oocytes and embryos among these protocols.

In conclusion, our study concluded that the personalized superovulation protocol increased the retrieval rate of MII-stage oocytes, and after ICSI, the embryos were successfully obtained and transplanted into the oviducts of the surrogate mothers selected according to the menstrual cycles, sexual swelling, ovulation point on the ovary, and the pregnancy was successful. The cynomolgus monkey fetus was acquired. The highlights of superovulation and selective recipients in this study were shown in **Fig. 8**. This study exhibited that the personalized superovulation combined with the selection of surrogate mother synchronization according to the menstrual cycles, sexual swelling, and ovulation point on the ovary is a simple and reliable method for the generation of the cynomolgus monkey fetus.

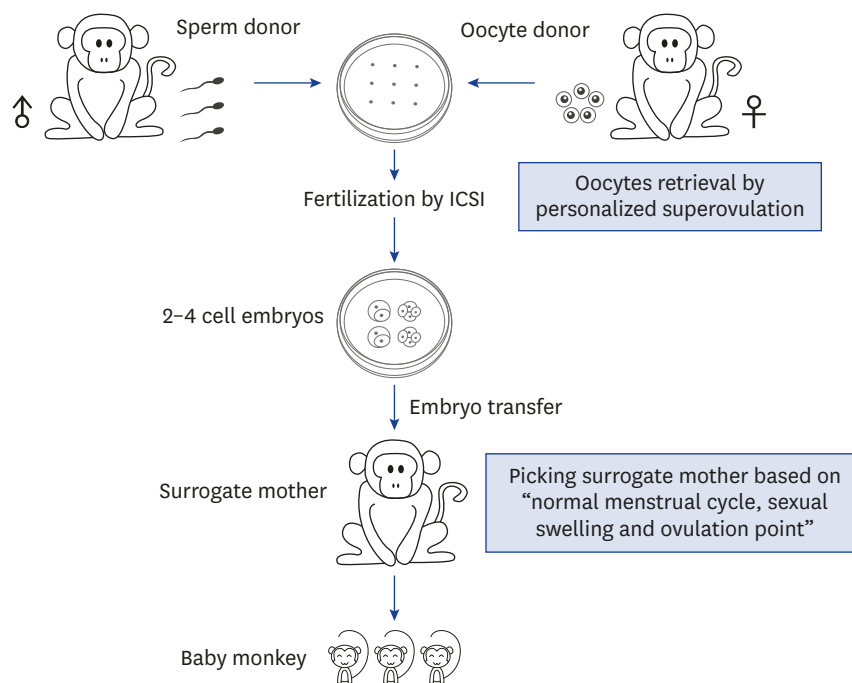


Fig. 8. The highlights of superovulation and picking surrogate mother in female cynomolgus monkeys in this study. Firstly, sperms were collected from adult male cynomolgus monkey and oocytes were acquired by personalized superovulation protocol incorporated the menstrual cycle length of each cynomolgus monkey. After ICSI, the MII-stage oocytes could develop into embryos successfully. Then the 2–4-cell embryos were transplanted into the oviducts of the surrogate mothers selected according to the “menstrual cycles,” “sexual swelling,” “ovulation point,” and the pregnancy was successful. At last, the cynomolgus monkey fetus was acquired. ICSI, intracytoplasmic sperm injection; MII-stage oocytes, mature oocytes at the metaphase II status.

ACKNOWLEDGMENTS

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Table 1

Complete lists of STRs examined for aborted fetus “a,” “b,” and “c”

[Click here to view](#)

REFERENCES

1. Bavister BD, Boatman DE, Collins K, Dierschke DJ, Eisele SG. Birth of rhesus monkey infant after *in vitro* fertilization and nonsurgical embryo transfer. *Proc Natl Acad Sci U S A*. 1984;81(7):2218-2222. [PUBMED](#) | [CROSSREF](#)
2. Balmaceda JP, Gastaldi C, Ord T, Borrero C, Asch RH. Tubal embryo transfer in cynomolgus monkeys: effects of hyperstimulation and synchrony. *Hum Reprod*. 1988;3(4):441-443. [PUBMED](#) | [CROSSREF](#)

3. Kubisch HM, Gagliardi C, Williams VM, Ribka EP, Ratterree MS. *In vitro* fertilization in the pigtailed macaque (*Macaca nemestrina*). *Theriogenology*. 2006;66(4):749-754.
[PUBMED](#) | [CROSSREF](#)
4. Sasaki E, Suemizu H, Shimada A, Hanazawa K, Oiwa R, Kamioka M, Tomioka I, Sotomaru Y, Hirakawa R, Eto T, Shiozawa S, Maeda T, Ito M, Ito R, Kito C, Yagihashi C, Kawai K, Miyoshi H, Tanioka Y, Tamaoki N, Habu S, Okano H, Nomura T. Generation of transgenic non-human primates with germline transmission. *Nature*. 2009;459(7246):523-527.
[PUBMED](#) | [CROSSREF](#)
5. Simerly CR, Castro CA, Jacoby E, Grund K, Turpin J, McFarland D, Champagne J, Jimenez JB Jr, Frost P, Bauer C, Hewitson L, Schatten G. Assisted reproductive technologies (ART) with baboons generate live offspring: a nonhuman primate model for ART and reproductive sciences. *Reprod Sci*. 2010;17(10):917-930.
[PUBMED](#) | [CROSSREF](#)
6. Shimozawa N, Nakamura S, Takahashi I, Hatori M, Sankai T. Characterization of a novel embryonic stem cell line from an ICSI-derived blastocyst in the African green monkey. *Reproduction*. 2010;139(3):565-573.
[PUBMED](#) | [CROSSREF](#)
7. Ko DS, Lee SH, Park DW, Yang KM, Lim CK. Pregnancy and fertilization potential of immature oocytes retrieved in intracytoplasmic sperm injection cycles. *Clin Exp Reprod Med*. 2015;42(3):118-125.
[PUBMED](#) | [CROSSREF](#)
8. El Hajj N, Haaf T. Epigenetic disturbances in *in vitro* cultured gametes and embryos: implications for human assisted reproduction. *Fertil Steril*. 2013;99(3):632-641.
[PUBMED](#) | [CROSSREF](#)
9. Dupont C, Bavister BD, Armant DR, Brenner CA. Rhesus macaque embryos derived from MI oocytes maturing after retrieval display high rates of chromosomal anomalies. *Hum Reprod*. 2009;24(4):929-935.
[PUBMED](#) | [CROSSREF](#)
10. Schramm RD, Paprocki AM, VandeVoort CA. Causes of developmental failure of *in-vitro* matured rhesus monkey oocytes: impairments in embryonic genome activation. *Hum Reprod*. 2003;18(4):826-833.
[PUBMED](#) | [CROSSREF](#)
11. Álvarez C, García-Garrido C, Taronger R, González de Merlo G. *In vitro* maturation, fertilization, embryo development & clinical outcome of human metaphase-I oocytes retrieved from stimulated intracytoplasmic sperm injection cycles. *Indian J Med Res* 2013;137(2):331-338.
[PUBMED](#)
12. Stouffer RL. Pre-ovulatory events in the rhesus monkey follicle during ovulation induction. *Reprod Biomed Online*. 2002;4 Suppl 3:1-4.
[PUBMED](#) | [CROSSREF](#)
13. Chen Y, Ji S, Niu Y, He X, Xie Y, Tang X, Ji W. Recombinant human gonadotropins for rhesus monkey superovulation: age and the stimulation interval are determinant to the fecundity. *J Anim Vet Adv*. 2011;10:3303-3307.
14. Nusser KD, Mitalipov S, Widmann A, Gerami-Naini B, Yeoman RR, Wolf DP. Developmental competence of oocytes after ICSI in the rhesus monkey. *Hum Reprod*. 2001;16(1):130-137.
[PUBMED](#) | [CROSSREF](#)
15. Hewitson L, Takahashi D, Dominko T, Simerly C, Schatten G. Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. *Hum Reprod*. 1998;13(12):3449-3455.
[PUBMED](#) | [CROSSREF](#)
16. Yin H, Duffy DM, Gosden RG. Comparative maturation of cynomolgus monkey oocytes *in vivo* and *in vitro*. *Reprod Biol Endocrinol*. 2006;4(1):14.
[PUBMED](#) | [CROSSREF](#)
17. Kanthaswamy S, Kou A, Satkoski J, Penedo MC, Ward T, Ng J, Gill L, Lerche NW, Erickson BJ, Smith DG. Genetic characterization of specific pathogen-free rhesus macaque (*Macaca mulatta*) populations at the California National Primate Research Center (CNPRC). *Am J Primatol*. 2010;72(7):587-599.
[PUBMED](#) | [CROSSREF](#)
18. Siddiqui RA, Sauermann U, Altmüller J, Fritzer E, Nothnagel M, Dalibor N, Fellay J, Kaup FJ, Stahl-Hennig C, Nürnberg P, Krawczak M, Platzer M. X chromosomal variation is associated with slow progression to AIDS in HIV-1-infected women. *Am J Hum Genet*. 2009;85(2):228-239.
[PUBMED](#) | [CROSSREF](#)
19. Liu Z, Cai Y, Wang Y, Nie Y, Zhang C, Xu Y, Zhang X, Lu Y, Wang Z, Poo M, Sun Q. Cloning of macaque monkeys by somatic cell nuclear transfer. *Cell*. 2018;172(4):881-887.e7.
[PUBMED](#) | [CROSSREF](#)

20. Tachibana M, Sparman M, Sritanandomchai H, Ma H, Clepper L, Woodward J, Li Y, Ramsey C, Kolotushkina O, Mitalipov S. Mitochondrial gene replacement in primate offspring and embryonic stem cells. *Nature*. 2009;461(7262):367-372.
[PUBMED](#) | [CROSSREF](#)
21. Lenton EA, Landgren BM, Sexton L, Harper R. Normal variation in the length of the follicular phase of the menstrual cycle: effect of chronological age. *Br J Obstet Gynaecol*. 1984;91(7):681-684.
[PUBMED](#) | [CROSSREF](#)
22. Reed BG, Carr BR. The normal menstrual cycle and the control of ovulation. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, Dungan K, Grossman A, Hershman JM, Kaltsas G, Koch C, Kopp P, Korbonits M, McLachlan R, Morley JE, New M, Perreault L, Purnell J, Rebar R, Singer F, Trencle DL, Vinik A, Wilson DP, editors. *Endotext*. South Dartmouth: MDText.com, Inc.; 2000.
23. Shimizu K. Reproductive hormones and the ovarian cycle in macaques. *J Mamm Ova Res*. 2008;25:122-126.
24. Weinbauer GF, Niehoff M, Niehaus M, Srivastav S, Fuchs A, Van Esch E, Cline JM. Physiology and endocrinology of the ovarian cycle in macaques. *Toxicol Pathol*. 2008;36(7S):7S-23S.
[PUBMED](#) | [CROSSREF](#)
25. Esch EV, Cline JM, Buse E, Wood CE, Rijk EPCT, Weinbauer GF. Summary comparison of female reproductive system in human and the cynomolgus monkey (*Macaca fascicularis*). *Toxicol Pathol*. 2008;36 7_suppl:171S-172S.
[CROSSREF](#)
26. Kim JS, Yoon SB, Jeong KJ, Sim BW, Choi SA, Lee SI, Jin YB, Song BS, Lee SR, Kim SU, Chang KT. Superovulatory responses in cynomolgus monkeys (*Macaca fascicularis*) depend on the interaction between donor status and superovulation method used. *J Reprod Dev*. 2017;63(2):149-155.
[PUBMED](#) | [CROSSREF](#)
27. Nyachio A, Spiessens C, Mwenda JM, Debrock S, D'Hooghe TM. Improving ovarian stimulation protocols for IVF in baboons: lessons from humans and rhesus monkeys. *Anim Reprod Sci*. 2009;110(3-4):187-206.
[PUBMED](#) | [CROSSREF](#)
28. Yoshida T, Nakajima M, Hiyaoka A, Suzuki MT, Cho F, Honjo S. Menstrual cycle lengths and the estimated time of ovulation in the cynomolgus monkey (*Macaca fascicularis*). *Jikken Dobutsu*. 1982;31(3):165-174.
[PUBMED](#) | [CROSSREF](#)
29. Ji W. *Reproduction and Breeding of Rhesus Monkey*. Beijing: Science Press; 2013.
30. Abbott DH, Nicol LE, Levine JE, Xu N, Goodarzi MO, Dumesic DA. Nonhuman primate models of polycystic ovary syndrome. *Mol Cell Endocrinol*. 2013;373(1-2):21-28.
[PUBMED](#) | [CROSSREF](#)
31. Lee JE, Kim SD, Jee BC, Suh CS, Kim SH. Oocyte maturity in repeated ovarian stimulation. *Clin Exp Reprod Med*. 2011;38(4):234-237.
[PUBMED](#) | [CROSSREF](#)
32. Van Blerkom J, Davis P. Differential effects of repeated ovarian stimulation on cytoplasmic and spindle organization in metaphase II mouse oocytes matured *in vivo* and *in vitro*. *Hum Reprod*. 2001;16(4):757-764.
[PUBMED](#) | [CROSSREF](#)
33. Lovejoy DA, Corrigan AZ, Nahorniak CS, Perrin MH, Porter J, Kaiser R, Miller C, Pantoja D, Craig AG, Peter RE, Vale WW, Rivier JE, Sherwood NM. Structural modifications of non-mammalian gonadotropin-releasing hormone (GnRH) isoforms: design of novel GnRH analogues. *Regul Pept*. 1995;60(2-3):99-115.
[PUBMED](#) | [CROSSREF](#)
34. Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, Hardy SL, Millar RP, Cernea M, Coolen LM, Lehman MN. Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology*. 2013;154(11):4259-4269.
[PUBMED](#) | [CROSSREF](#)
35. Mehl NS, Khalid M, Srisuwatanasagul S, Swangchan-Uthai T, Sirivaidyapong S. GnRH-agonist implantation of prepubertal male cats affects their reproductive performance and testicular LH receptor and FSH receptor expression. *Theriogenology*. 2016;85(5):841-848.
[PUBMED](#) | [CROSSREF](#)
36. van Loenen AC, Huirne JA, Schats R, Hompes PG, Lambalk CB. GnRH agonists, antagonists, and assisted conception. *Semin Reprod Med*. 2002;20(4):349-364.
[PUBMED](#) | [CROSSREF](#)
37. Wu L, Ren XL, Chen W, Huang B, Zhou YF, Jin L. Influence of different gonadotropin-releasing hormone agonist administration methods on pregnancy outcomes of patients undergoing *in-vitro* fertilization-embryo transfer. *Curr Med Sci*. 2019;39(3):437-441.
[PUBMED](#) | [CROSSREF](#)

38. Dumont A, Dewailly D, Plouvier P, Catteau-Jonard S, Robin G. Does polycystic ovarian morphology influence the response to treatment with pulsatile GnRH in functional hypothalamic amenorrhea? *Reprod Biol Endocrinol*. 2016;14(1):24.
[PUBMED](#) | [CROSSREF](#)
39. De Jong D, Van Hooren EG, Macklon NS, Mannaerts BM, Fauser BC. Pregnancy and birth after GnRH agonist treatment for induction of final oocyte maturation in a woman undergoing ovarian stimulation for ICSI, using a GnRH antagonist (Orgalutran/Antagon) to prevent a premature LH surge: a case report. *J Assist Reprod Genet*. 2001;18(1):30-33.
[PUBMED](#) | [CROSSREF](#)
40. Hoff JD, Quigley ME, Yen SS. Hormonal dynamics at midcycle: a reevaluation. *J Clin Endocrinol Metab*. 1983;57(4):792-796.
[PUBMED](#) | [CROSSREF](#)
41. Niu Y, Yu Y, Bernat A, Yang S, He X, Guo X, Chen D, Chen Y, Ji S, Si W, Lv Y, Tan T, Wei Q, Wang H, Shi L, Guan J, Zhu X, Afanassieff M, Savatier P, Zhang K, Zhou Q, Ji W. Transgenic rhesus monkeys produced by gene transfer into early-cleavage-stage embryos using a simian immunodeficiency virus-based vector. *Proc Natl Acad Sci U S A*. 2010;107(41):17663-17667.
[PUBMED](#) | [CROSSREF](#)
42. Sun Q, Dong J, Yang W, Jin Y, Yang M, Wang Y, Wang PL, Hu Y, Tsien JZ. Efficient reproduction of cynomolgus monkey using pronuclear embryo transfer technique. *Proc Natl Acad Sci U S A*. 2008;105(35):12956-12960.
[PUBMED](#) | [CROSSREF](#)
43. Tardif S, Carville A, Elmore D, Williams LE, Rice K. Reproduction and breeding of nonhuman primates (chapter 8). In: Abee CR, Mansfield K, Tardif S, Morris T, editors. *Nonhuman Primates in Biomedical Research*. San Diego: Academic Press; 2012, 197-249.