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Double ovarian stimulation during the follicular and luteal phase in women ≥38 years: a retrospective case-control study

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KEY MESSAGE
Double ovarian stimulation could increase the chances of achieving pregnancy for older women, by accumulating more oocytes/embryos in a short time. Luteal-phase ovarian stimulation could be carried out following almost any follicular-phase stimulation such as GnRH-a long and short protocol, GnRH-A protocol, mild stimulation protocol or MPA pituitary down-regulation protocol.

A B S T R A C T

Previous studies have shown that double ovarian stimulation could obtain more oocytes in women with poor ovarian response. This retrospective case–control study aimed to investigate the efficacy of double ovarian stimulation in older women. One hundred and sixteen women aged ≥38 years who received double ovarian stimulation were assigned to the study, with 103 divided into four groups according to follicular-phase ovarian stimulation protocols, including gonadotrophin-releasing hormone agonist short protocol (n = 27), gonadotrophin-releasing hormone antagonist protocol (n = 32), mild stimulation protocol (n = 21) and medrocyprogesterone acetate (MPA) pituitary down-regulation protocol (n = 23). Numbers of oocytes retrieved and available embryos after double ovarian stimulation were more than double those obtained after follicular-phase ovarian stimulation alone. In total 81.90% of patients had available embryos, and the cancellation rate decreased from 37.07% to 18.10%. Forty-eight cases underwent 50 cryopreserved embryo transfer cycles, with a 22.00% clinical pregnancy rate. The implantation rate (10.53% versus 10.67%) was similar between the embryos derived from first and second stimulations. The results suggest that double ovarian stimulation could increase the chances of achieving pregnancy by accumulating more oocytes/embryos in a short time, which might serve as a useful strategy for older women.

Introduction

It is widely acknowledged that with increasing age, women suffer from a diminished ovarian reserve, poor response to gonadotrophin and a significant increase in aneuploidy oocytes, leading to a decline in fertility, which accelerates after the age of 35 (Spandorfer et al., 2007). Also, age is now considered to be a crucial and independent influencing factor on the outcomes of IVF/intracytoplasmic sperm injection (ICSI) (Gomaa et al., 2012; Ronel et al., 2000). It has been reported

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that even when conception is achieved through IVF–embryo transfer, advanced maternal age (AMA) patients are at a higher risk of miscarriage and lower live birth rate (LBR), and there might be no deliveries for women over 44 years attempting IVF. Some authors have even suggested that women attempting IVF should be limited to 45 years old (Ronel et al., 2000; Spandorfer et al., 2007).

Based on an analysis of 400,135 treatment cycles, it was found that there was a strong association between the number of oocytes and LBR. LBR rose with an increasing number of oocytes, especially for women over 38 years, and the number of oocytes might be a reasonable surrogate outcome to use in IVF practice and research (Sunkara et al., 2011). It was reported that the clinical pregnancy rate (CPR) was only 14% and the cycle cancellation rate of no available embryos was as high as 40% for women with fewer than five oocytes (Saldeen et al., 2007), and that the outcomes of IVF/ICSI would be improved with increasing number of oocytes retrieved, especially for those with poor ovarian response or AMA (Briggs et al., 2015). Therefore, various ovarian stimulation protocols have been explored to obtain more mature oocytes and available embryos for AMA patients (Pacchiarotti et al., 2016). A potential alternative strategy to accumulate more available oocytes and embryos in sequential stimulation cycles has been proposed for poor responders, with fewer transfer cancellations and higher cumulative pregnancy rate (Cobo et al., 2012).

Most traditional ovarian stimulation protocols target antral follicles of the follicular phase, and usually start from the early follicular phase. For several years, a luteal-phase ovarian stimulation protocol has been used in women for emergency fertility conservation in medical indications such as ovarian surgery or malignant disease, through cryopreservation of mature oocytes/embryos (Maman et al., 2011; Von et al., 2009). It was found that the oocytes and embryos originating from luteal-phase ovarian stimulation (LPS) had a similar developmental potency as those from follicular-phase ovarian stimulation (FPS), and a satisfactory pregnancy outcome could be obtained after subsequent cryopreserved embryo transfers (Kuang et al., 2014b). A recent study from Kuang's (2014a) team demonstrated that double ovarian stimulations in the follicular and luteal phases of the same menstrual cycle could increase pregnancy opportunities for poor responders by accumulating more available embryos.

Aiming to improve the IVF–embryo transfer outcomes of AMA patients, the efficacy of double ovarian stimulation during the follicular and luteal phase for women aged 38 years or older and its cryopreserved embryo transfer outcomes were evaluated in this study.

Materials and methods

Subjects

This is a retrospective case-control study, and all enrolled subjects received IVF/ICSI treatment with double ovarian stimulation in the follicular and luteal phase at the Reproductive Centre of the 105th Hospital of PLA (The People's Liberation Army) from March 2014 to March 2017. The inclusion criteria were: all enrolled subjects were 38 years or older with a normal menstruation and at least one follicle 6–11 mm in diameter observed under ultrasonography when oocyte retrieval was finished in FPS. Exclusion criteria included: uterine malformation, intrauterine adhesion, ≥ grade 3 endometriosis, history of tuberculosis or pelvic operation. All enrolled subjects were counselled about the efficacy and procedure of double ovarian stimulation, and provided written informed consent. The study was approved by the Reproductive Medical Ethics Committee of the 105th Hospital of PLA on 1 January 2014.

There were 116 subjects assigned in this study, and the levels of basic FSH, LH, oestradiol and anti-Müllerian hormone (AMH) of all subjects were detected on day 3 of the menstrual cycle. The parameters including duration and total dose of gonadotrophin (Gn) administered, the levels of serum LH, oestradiol and progesterone on the day of human chorionic gonadotrophic (HCG) administration, the number of oocytes retrieved and metaphase II (MII) oocytes, fertilization rate, cancellation rate due to no embryos suitable for transfer, top-quality embryos and cryopreserved embryos in all groups, the outcomes of cryopreserved embryo transfers in the first and second stimulations were recorded and compared. The data were further analysed in 103 out of 116 subjects divided into four groups according to their ovarian stimulation protocols of the follicular phase, including GnRH-a short protocol (group 1, n = 27), GnRH-A protocol (group 2, n = 32), mild stimulation protocol (group 3, n = 21) and MPA pituitary down-regulation protocol (group 4, n = 23); the remaining 13 subjects received GnRH-a long protocol and were excluded from further classification due to there being too few samples.

Ovarian stimulation

Follicular-phase stimulation

The protocols of FPS included a GnRH-a long and short protocol, a GnRH-A protocol, a mild stimulation protocol and a MPA pituitary down-regulation protocol (Wang et al., 2016). When the oestradiol level was >80 pg/ml, the patients would receive pretreatment with low-dose oestrogen (4–6 mg, daily) in the mid-luteal phase of the previous cycle. The starting dose of Gn in FPS was 150–300 IU FSH based on the patient's age, hormone profile, AFC and body mass index (BMI). Serial ultrasonographic monitoring of follicle development and evaluations of serum FSH, LH, oestradiol and progesterone were carried out during the ovarian stimulation. When one or two leading follicles had reached 18 mm in diameter, 250 μg recombinant human chorionic gonadotrophin (rHCG; Merck, Germany) was administered, and oocytes retrieved from the follicles ≥12 mm in diameter were transvaginally retrieved under ultrasound guidance 36 h later, while the follicles ≤11 mm in diameter were left for LPS.

Luteal-phase stimulation

Transvaginal ultrasound examination was carried out when oocyte retrieval was finished in FPS. When there were at least one or more follicles 6–11 mm in diameter observed, LPS was performed with 225 IU human menopausal gonadotrophin (HMG) (Menotropins for Injection; Livzon Pharmaceutical Group Co., Ltd, China) daily within 1–3 days of oocyte retrieval. When one or two follicles had reached 18 mm in diameter, 250 μg rHCG was administered. Oocytes were retrieved transvaginally under ultrasound guidance 36 h after rHCG administration.

The protocol of double ovarian stimulation is presented in **Figure 1**.

IVF and embryo cryopreservation

According to our standard protocol, all retrieved oocytes were fertilized with either conventional IVF or ICSI 2–4 h after oocyte retrieval,

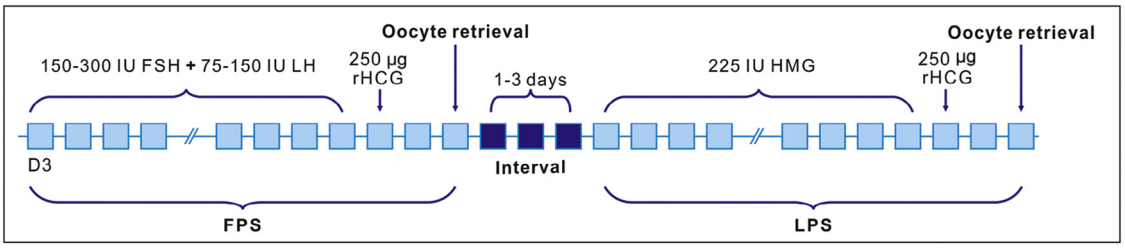

Figure 1 – The protocol of double stimulation during the follicular and luteal phases. Each square represents a day of the cycle. FPS = follicular-phase ovarian stimulation; HMG = human menopausal gonadotrophin; LPS = luteal-phase ovarian stimulation; rHCG = recombinant human chorionic gonadotrophin.

and embryos were cultured individually until day 3 to day 6. The embryos were graded according to Cummins et al. (1986). All top-quality cleavage embryos were cryopreserved on the third day (D3) after oocyte retrieval and the other available embryos were cultured for blastocyst until the fifth/sixth day (D5/D6). All available embryos including D3 and D5/D6 from both follicular and luteal-phase stimulations were cryopreserved with a commercial vitrification/warming kit (Kitazato Biopharma Co., Shizuoka, Japan), and all vitrification/warming steps were carried out according to the manufacturer's instructions. The available embryos were defined as normal fertilized embryos if they had five or more blastomeres and fragmentation <50% on day 3, and those with six or more blastomeres with fragmentation <20% were defined as top-quality embryos. Blastocysts equal or superior to grade 4BC or 4CB on day 5/6 were defined as available (Yin et al., 2016).

Vitrified-warmed embryo transfer

All patients who underwent cryopreserved embryo transfer received hormone replacement treatment for endometrial preparation with 4–8 mg oestradiol valerate tablets (Progynova; Bayer Schering, Guangzhou, China) on day 3 to 5 of menstruation. When endometrial thickness reached >8 mm, 40 mg of progestin (Xianju, Taizhou, China) was injected intramuscularly once a day for 3 or 5 days according to the stage of transferred embryos, then two or three warmed embryos were transferred. All patients received 40 mg progestin daily for corpus luteum support after cryopreserved embryo transfer, in addition to the original dose of oestradiol valerate. Clinical pregnancy was defined as the presence of a gestational sac under ultrasound examination 30–35 days after cryopreserved embryo transfer. Once the clinical pregnancy was confirmed, corpus luteum support was continued until 10 weeks of gestation. Embryonic developmental arrest or spontaneous abortion at less than 12 weeks of pregnancy was defined as early abortion.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical comparisons were carried out using Student's t-test, one-way analysis of variance, chi-squared test and Fisher's exact test where appropriate. All statistical analyses were carried out with SPSS 17.0 (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

The basic characteristics and hormonal profile of all 116 subjects including age, BMI, cause and duration of infertility, attempts at previous IVF cycles, the levels of basal FSH, LH, oestradiol and AMH are summarized in **Table 1**. The mean age of the patients in this study was 42.12 ± 2.68 (range 38–48) years. Ovarian reserve markers were mean basal FSH, 10.83 ± 5.88 IU/l (range 0.11–31.35); mean AMH, 0.77 ± 0.98 ng/ml (range 0.04–5.96); mean oestradiol, 40.55 ± 51.41 pg/ml (1.09–262.40); and mean antral follicle count (AFC), 6.72 ± 5.23 (range 1–30) follicles, and 49.14% (57/116) of the patients had at least one failed IVF attempt, suggesting that most enrolled cases were low ovarian reserve.

The IVF outcomes of one and double ovarian stimulations

As shown in **Table 2**, the total dose of Gn in FPS and LPS was 1882.02 ± 958.89 IU and 1728.66 ± 937.11 IU, respectively, and there was no significant difference in total dose of Gn between FPS and LPS. The levels of FSH and LH on the day of HCG administration

Table 1 – Basic characteristics of the subjects (*n* = 116).

Parameter	Values
Age (years)	42.12 ± 2.68 (38–48)
BMI (kg/m^2)	23.58 ± 2.88 (18.07–31.63)
Infertility duration (years)	5.21 ± 4.83 (1–22)
Primary infertility	13/116 (11.21%)
Secondary infertility	103/116 (88.79%)
Basal FSH (IU/l)	10.83 ± 5.88 (0.11–31.35)
Basal LH (IU/l)	4.78 ± 3.17 (0.47–22.14)
Basal oestradiol (pg/ml)	40.55 ± 51.41 (1.09–262.40)
AMH (ng/ml)	0.77 ± 0.98 (0.04–5.96)
AFC (n)	6.72 ± 5.23 (1–30)
Previous IVF cycles (n)	
0	59
1–2	42
≥3	15

Values presented as mean ± SD (range) or number (%).
AFC = antral follicle count; AMH = anti-Müllerian hormone; BMI = body mass index.

Table 2 – The cycle characteristics of double stimulation (*n* = 116).

Characteristic	First oocyte retrieval	Second oocyte retrieval	Double oocyte retrieval	*P*-value 1[a]	*P*-value 2[b]
Gn duration (days)	8.26 ± 3.52	7.53 ± 3.39	15.26 ± 4.90	NS	<0.001
Total dose of Gn (IU)	1882.02 ± 958.89	1728.66 ± 937.11	3578.24 ± 1385.20	NS	<0.001
No. of follicles on trigger day					
≤11 mm	1.69 ± 2.48	0.56 ± 0.91	-	<0.001	-
≥14 mm	2.66 ± 2.29	3.34 ± 2.70	-	0.007	-
FSH (IU/l) on trigger day	23.26 ± 6.47	20.82 ± 4.34	-	<0.001	-
LH (IU/l) on trigger day	5.23 ± 4.01	1.35 ± 2.50	-	<0.001	-
Oestradiol (pg/ml) on trigger day	1027.24 ± 917.95	1343.15 ± 1058.33	-	0.004	-
Progesterone (ng/l) on trigger day	1.42 ± 3.63	14.76 ± 12.10	-	<0.001	-
No. of oocytes retrieved	2.33 ± 1.99	3.50 ± 3.55	5.83 ± 4.60	0.002	<0.001
No. of MII oocytes	1.93 ± 1.70	2.80 ± 3.15	4.73 ± 4.01	0.003	<0.001
No. of fertilized oocytes	1.72 ± 1.54	2.56 ± 2.96	4.28 ± 3.67	0.003	<0.001
No. of cleaved embryos	1.60 ± 1.47	2.40 ± 2.72	4.00 ± 3.42	0.002	<0.001
No. of top-quality embryos	1.04 ± 1.33	1.35 ± 1.40	2.42 ± 2.11	0.031	<0.001
No. of cryopreserved embryos	1.17 ± 1.34	1.38 ± 1.36	2.57 ± 1.99	NS	<0.001
Fertilization rate (%)	73.70 (199/270)	73.15 (297/406)	73.37 (496/676)	NS	NS
Top-quality embryos rate (%)	65.05 (121/186)	57.55 (160/278)	60.56 (281/464)	NS	NS
Cancellation rate (%)	37.07 (43/116)	28.45 (33/116)	18.10 (21/116)	NS	0.001

Gn = gonadotrophin; MII = metaphase II; NS = not significant.
[a] *P*-value 1 for the comparison between the first and second retrieval.
[b] *P*-value 2 for the comparison between the first and double retrieval. Values are presented as mean ± SD unless otherwise stated.

were significantly lower (both $P < 0.001$) and the levels of oestradiol and progesterone were significantly higher ($P = 0.004$ and $P < 0.001$, respectively) in LPS compared with FPS. The number of oocytes retrieved and MII oocytes ($P = 0.002$ and $P = 0.003$, respectively), fertilized oocytes ($P = 0.003$), cleaved embryos ($P = 0.002$), top-quality embryos ($P = 0.031$) in LPS were higher than those in FPS, while no significant differences in number of cryopreserved embryos were found between FPS and LPS (1.17 ± 1.34 versus 1.38 ± 1.36). The cancellation rate (37.07%) owing to no available oocytes, immature oocytes, non-fertilization or poor-quality embryos in FPS was higher than LPS (28.45%), but did not reach statistical significance. However, the number of oocytes retrieved, MII oocytes, fertilized oocytes, cleaved embryos, top-quality embryos and cryopreserved embryos in double stimulation increased and the cancellation rate of no available embryos reduced (37.07% versus 18.10%) significantly compared with FPS.

Out of 116 subjects receiving double stimulation, a total of 270 and 406 oocytes were collected in the first and second attempts, respectively. There were 81.90% (95/116) of patients who had viable embryos cryopreserved, and no available embryos were identified in the remaining 21 cases owing to no oocytes retrieved, immature oocytes, non-fertilization or poor-quality embryos. No ovarian hyperstimulation syndrome (OHSS) occurred in any patient receiving double ovarian stimulation.

IVF outcomes of double ovarian stimulation according to follicular-phase ovarian stimulation protocol

As shown in **Table 3**, whichever protocol of FPS was carried out, the number of oocytes retrieved, MII oocytes, fertilized oocytes, cleaved embryos, top-quality embryos and cryopreserved embryos increased significantly after double stimulation.

Table 3 – Cycle characteristics of double stimulation under different follicular-phase stimulation protocols.

Characteristic	Group 1 (n = 27)		Group 2 (n = 32)		Group 3 (n = 21)		Group 4 (n = 23)	
	First retrieval	Double retrieval	First retrieval	Double retrieval	First retrieval	Double retrieval	First retrieval	Double retrieval
No. of follicles on trigger day								
≤11 mm	0.96 ± 1.29	0.67 ± 1.00	2.34 ± 3.75[a]	0.78 ± 1.04[a]	1.14 ± 1.53[b]	0.38 ± 0.80[b]	2.22 ± 2.07[c]	0.39 ± 0.66[c]
≥14 mm	2.81 ± 1.62	3.07 ± 2.06	2.31 ± 1.93	3.28 ± 2.91	2.00 ± 0.95[a]	3.38 ± 3.09[a]	1.83 ± 1.11[b]	2.83 ± 1.50[b]
No. of oocytes retrieved	2.33 ± 1.96[a]	5.30 ± 3.04[a]	2.19 ± 1.67[b]	5.47 ± 4.20[b]	1.86 ± 0.96[c]	5.76 ± 5.60[c]	1.83 ± 1.27[d]	4.57 ± 2.68[d]
No. of MII oocytes	2.00 ± 1.62[a]	4.52 ± 2.82[a]	1.84 ± 1.78[b]	4.22 ± 4.05[b]	1.52 ± 0.98[c]	5.00 ± 5.43[c]	1.52 ± 1.08[d]	3.65 ± 2.25[d]
No. of fertilized oocytes	1.63 ± 1.39[a]	3.96 ± 2.70[a]	1.66 ± 1.49[b]	3.81 ± 3.38[b]	1.48 ± 0.93[c]	4.57 ± 5.44[c]	1.39 ± 1.12[d]	3.48 ± 2.35[d]
No. of cleaved embryos	1.41 ± 1.31[a]	3.48 ± 2.41[a]	1.63 ± 1.50[b]	3.66 ± 3.29[b]	1.43 ± 0.93[c]	4.19 ± 4.82[c]	1.26 ± 1.14[d]	3.35 ± 2.31[d]
No. of top-quality embryos	0.93 ± 1.33[a]	2.07 ± 2.02[a]	1.10 ± 1.67[b]	2.33 ± 2.39[b]	1.06 ± 0.94[c]	2.22 ± 1.73[c]	0.87 ± 1.06[d]	2.43 ± 2.11[d]
No. of cryopreserved embryos	1.00 ± 1.33[a]	2.26 ± 2.01[a]	1.00 ± 1.39[b]	2.19 ± 2.10[b]	1.00 ± 0.89[c]	2.14 ± 1.74[c]	1.09 ± 1.12[d]	2.70 ± 2.20[d]
Fertilization rate (%)	69.84 (44/63)	74.83 (107/143)	75.71 (53/70)	69.71 (122/175)	79.49 (31/39)	79.34 (96/121)	76.19 (32/42)	76.19 (80/105)
Top-quality embryos rate (%)	65.79 (25/38)	59.57 (56/94)	61.54 (32/52)	59.83 (70/117)	70.00 (21/30)	51.14 (45/88)	68.97 (20/29)	72.73 (56/77)

Note: Same superscript letters in a row indicate statistically significant differences ($P < 0.05$). Group 1 = GnRH-a short protocol in FPS; Group 2 = GnRH-A protocol in FPS; Group 3 = mild stimulation protocol in FPS; Group 4 = MPA pituitary down-regulation protocol in FPS.
FPS = follicular-phase ovarian stimulation; MII = metaphase II.

Table 4 – Outcomes of cryopreserved embryo transfer cycles using embryos originating from double ovarian stimulation.

	Total	Embryos from first oocyte retrieval	Embryos from second oocyte retrieval	*P*-value
No. of patients (*n*)	48	16	32	-
Age (years)[a]	40.91 ± 2.95 (38–47)	40.20 ± 2.77 (38–45)	41.50 ± 3.20 (38–47)	NS
Transfer cycles (*n*)	50	16	34	-
Embryos transferred (*n*)	113	38	75	-
Embryo survival rate (%)	96.58 (113/117)	100 (38/38)	94.94 (75/79)	NS
Clinical pregnancy rate (%)	22.00 (11/50)	25.00 (4/16)	20.59 (7/34)	NS
Implantation rate (%)	10.62 (12/113)	10.53 (4/38)	10.67 (8/75)	NS
Early abortion rate (%)	9.09 (1/11)	0 (0/4)	14.29 (1/7)	NS

[a] Mean ± SD (range).

Cryopreserved embryo transfer outcomes of double ovarian stimulation

As shown in **Table 4**, 48 cases underwent 50 cryopreserved embryo transfer cycles by January 2017, whose mean age was 40.91 ± 2.95 (range 38–47) years. Out of 117 warmed embryos, 113 survived, giving an embryo survival rate of 96.58%. The CPR per transfer was 22.00% (11/50); four pregnancy cases originated from FPS, and seven from LPS. The implantation rate (10.53% versus 10.67%), CPR (25.00% versus 20.59%) per transfer and early abortion rate (0 versus 14.29%) were all comparable between the embryos originating from FPS and LPS.

Discussion

A large number of studies have demonstrated that oocyte quantity and quality are age-dependent, and associated with the outcomes of IVF–embryo transfer (Navot et al., 1994; van Rooij et al., 2003). It was found that the chance of a live birth was 20–40% for oocyte numbers <6, rising to 50–70% when oocyte numbers were 6–12, suggesting that the LBR would increase with more oocytes obtained (Stanger and Yovich, 2013). Therefore, various ovarian stimulation protocols resulting in more oocytes retrieved have been proposed in order to obtain higher cumulative pregnancy rates.

According to the traditional theory of folliculogenesis, antral follicles are recruited from the late luteal phase of the preceding menstrual cycle under the action of FSH, then a single follicle is selected to develop during the follicular phase, while high levels of progesterone in the luteal phase may inhibit the levels of FSH and LH through negative feedback to the pituitary, resulting in follicular quiescence (Baerwald et al., 2003; McNatty et al., 1983). However, it has been shown that 68% of women with normal menstrual cycles exhibited two waves of follicle development during an inter-ovulatory interval (IOI) and 32% exhibited three waves (Baerwald et al., 2003). The small antral follicles in the luteal phase may not necessarily be in atresia, but may rather be in the early stages of follicular development (Baerwald et al., 2003; Yang et al., 2013), and follicular quiescence under high levels of progesterone could be removed and further developed when an adequate dose of gonadotrophin was administrated (Kuang et al., 2014a, 2014b; Zhu et al., 2015). The increasing evidence of successive waves of antral follicle development during the human menstrual cycle indicated that oocytes could be available continuously under the stimulation of gonadotrophins in one menstrual cycle.

Several researchers have reported that luteal-phase ovarian stimulation or even random-start ovarian stimulation could be performed in emergency fertility preservation and the number of oocytes retrieved and top-quality embryos originating from LPS increased significantly compared with FPS (Maman et al., 2011; Nayak and Wakim, 2011; Xu and Li, 2013). Another retrospective cohort study confirmed that there was no elevated rate of abnormality at birth in LPS (Chen et al., 2015). In addition, 3-year-old twin babies born from transferring embryos originating from LPS have been identified as having similar physical and psychological development to those conceived naturally (Kuang et al., 2013). Based on these findings, LPS following follicular-phase stimulation during the same cycle (double ovarian stimulation) were proposed, and the results showed that double ovarian stimulation could obtain more oocytes and available embryos in a single menstrual cycle (Kuang et al., 2014a; Vaiarelli et al., 2017; Zhang et al., 2015). Moreover, a recent study demonstrated that follicular versus LPS during the same menstrual cycle in a reduced ovarian reserve population could obtain a similar euploid blastocyst formation rate (Ubaldi et al., 2016). The present study also demonstrated that the oocytes retrieved and available embryos in double ovarian stimulation were more than double those originating from first oocyte retrieval, and viable embryos cryopreserved in double ovarian stimulation (2.57 ± 1.99) were significantly higher than one stimulation in the follicular phase (1.17 ± 1.34). Meanwhile, the cancellation rate of no viable embryos in double ovarian stimulation decreased significantly compared with one stimulation in the follicular phase (18.10% versus 37.07%), suggesting that double stimulation during the same menstrual cycle might be a promising alternative or a rescue approach for AMA patients.

In contrast with previous studies which showed that the mean dose of Gn in the second stimulation was significantly increased compared with the first stimulation (Buendgen et al., 2013; Kuang et al., 2014a, 2014b), our results showed that there was no significant difference in dose of Gn between FPS and LPS (1882.02 ± 958.89 IU versus 1728.66 ± 937.11 IU). The possible explanation might be that all the patients in this study were age advanced and the ovarian response to Gn was poor.

In previous studies, double ovarian stimulation has usually been used when the first stimulation was carried out with a mild stimulation protocol or a GnRH-A protocol, and ovulation was triggered with GnRH-a (Kuang et al., 2014a; Ubaldi et al., 2016; Zhang et al., 2015). With the advantages of almost complete prevention of OHSS and inducing an endogenous surge of FSH in addition to the LH surge similar to the natural mid-cycle, the GnRH-a trigger for final maturation of oocytes has been considered a valuable and alternative strategy to classical HCG triggering (Humaidan et al., 2012; Kol and Itskovitzeldor, 2010). However, the GnRH-a trigger had a limitation, in that it could not be used in a GnRH-a pituitary down-regulation

protocol, in which the flare-up effect of the GnRH-a trigger is blocked as GnRH receptors in the pituitary are already occupied.

In this study, the application of double ovarian stimulation was expanded. Instead of GnRH-a, HCG was used as first trigger, and second ovarian stimulation was performed following various FPS protocols including a GnRH-a long and short protocol, a GnRH-A protocol, a mild stimulation protocol and a MPA pituitary down-regulation protocol. The results showed that second ovarian stimulation could achieve comparable outcomes to the first, with the advantage of obtaining more MII oocytes, suggesting that second ovarian stimulation in the luteal phase could be carried out following almost any follicular-phase stimulation protocols when HCG was used as first trigger, except for patients at high risk of OHSS. Moreover, no OHSS, premature LH surge or spontaneous ovulation occurred during LPS. The possible explanation might be that all the patients in this study were at low risk of OHSS due to advanced age and low ovarian reserve, and the number of oocytes obtained from two stimulations were low (2.33 ± 1.99 versus 3.50 ± 3.55), the oestradiol level was not high (1027.24 ± 917.95 versus 1343.15 ± 1058.33 pg/ml). Otherwise, the level of progesterone in the second stimulation was significantly higher than in the first stimulation (14.76 ± 12.10 versus 1.42 ± 3.63 ng/l, $P < 0.001$), which might inhibit the pituitary through negative feedback, playing a role in pituitary down-regulation as GnRH-a, to prevent premature LH surge and allow ovaries to respond to exogenous gonadotrophin efficiently (Pelican et al., 2008).

It is known that dominant follicles (>10 mm in diameter) with abundant FSH/LH receptors may be luteinized, with atresia occurring under high levels of LH/HCG (Baerwald et al., 2003). Therefore, in the present study, the antral follicles that ranged in diameter from 6 to 11 mm were targeted for continuous ovarian stimulation because the antral follicles <5 mm in diameter might not respond to gonadotrophin. It was found that the embryos originating from FPS and LPS had a similar development and implantation potential, suggesting that the first trigger with HCG may not influence the results of the second stimulation when antral follicles were no more than 11 mm in diameter.

Because there was asynchrony between the endometrium and the embryo following LPS, it is usually recommended that embryos are vitrified as cryopreserved embryo transfers in subsequent cycles (Kato et al., 2012). In view of the overwhelming evidence of higher implantation rate and better delivery outcomes as well as low risk of OHSS, cryopreserved embryo transfers have been considered as a viable alternative to fresh embryo transfers (Maheshwari et al., 2012). This study further confirmed that the embryos originating from FPS and LPS had a similar development and implantation potential, and a double ovarian stimulation protocol could obtain a satisfactory and higher CPR (22.00%) in cryopreserved embryo transfer cycles, compared with other reports concerning AMA patients (Roque et al., 2013; Wang et al., 2001).

The main limitation of this study is the retrospective design, with the inherent problems relating to selection bias in patients assigned to treatment, and the outcomes of cryopreserved embryo transfers to cited date only including small sample sizes. A large cohort study and prospective randomized trials with LBR are needed to further elucidate the benefits of double ovarian stimulation for AMA patients.

As is known, chromosomal aneuploidy of the embryo is the crucial cause of infertility and poor outcomes of IVF–embryo transfer, and more chromosomally abnormal embryos are derived from women older than 37 years (Platteau et al., 2005), so AMA patients have a short fertility time left. Double ovarian stimulation could increase the chance of achieving pregnancy for AMA patients through accumulating more oocytes and top-quality embryos in a short time, with the advantages of low risk and less cost compared with conventional ovarian stimulation protocols. This approach might therefore serve as a useful strategy for AMA patients, especially for those who obtain no viable embryos in conventional FPS.

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