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Comparison of modified agonist, mild-stimulation and antagonist protocols for *in vitro* fertilization in patients with diminished ovarian reserve

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Abstract

Objective: To compare the efficacy of three protocols for ovarian stimulation in patients with diminished ovarian reserve during *in vitro* fertilization (IVF) treatment.

Methods: This prospective randomized study enrolled patients with diminished ovarian reserve who underwent cycles of IVF or intracytoplasmic sperm injection. The patients were randomly divided into three groups: a modified gonadotrophin releasing hormone (GnRH) agonist protocol (group A); (ii) a mild stimulation protocol (group B); or (iii) an antagonist protocol (group C). Demographic characteristics, clinical variables and pregnancy outcomes were compared between the groups.

Results: A total of 116 patients were enrolled in the study: 54 in group A, 52 in group B and 60 in group C. Group B (32.69%) had a significantly higher cycle cancellation rate compared with groups A (11.11%) and C (16.67%). The early abortion rate of group C (44.44%) was significantly higher than group A (12.50%), but not significantly different from group B (16.67%). There were no significant differences in the clinical pregnancy rates and live birth rates among the three groups.

Conclusion: A modified GnRH agonist protocol achieved a comparable pregnancy rate to those of the mild stimulation protocol and antagonist protocol, whilst having lower cycle cancellation and early abortion rates.

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Introduction

Diminished ovarian reserve (DOR) has always been a difficult problem to address during *in vitro* fertilization (IVF) treatment. When these patients are treated with controlled ovarian hyperstimulation, the incidence of a poor ovarian response is high, which results in a significant reduction in the number of retrieved oocytes and a low IVF success rate.[1] In addition to the classic agonist protocol, a variety of protocols and drugs have been used in patients with a DOR to investigate whether the outcome of IVF can be improved.[2] A randomized controlled trial that compared agonist down regulation and a short flare-up protocol conducted in 200 infertile women $\geq$40 years old showed that the pregnancy rates were 22.7% and 10.9%, respectively.[3] It was also observed that the transferable cycle rate was only 57% and the clinical pregnancy rate per transfer was 17.1% in 500 consecutive natural cycles, which meant that nearly half of all cycles were cancelled.[4] The availability of antagonist has made the mild stimulation cycle and the antagonist cycle possible good alternatives.[5] However, a large prospective randomized trial and a meta-analysis showed that the two regimens did not achieve higher clinical pregnancy rates when compared with agonist protocols.[6,7] To date, there is no consensus on which strategy is the best choice for women with DOR.[8]

A modified agonist protocol was used for a small sample of patients with DOR in a preliminary experiment in our centre. In this specific protocol, patients were given an injection of a large dose of gonadotrophin releasing hormone (GnRH) agonist during the menstrual period; and ovarian stimulation with human menopausal gonadotrophin (hMG) was started 4 weeks later. The pregnancy results of this preliminary experiment were satisfactory, but the data obtained were insufficient to be used for statistical analyses. These preliminary findings suggested that this modified agonist protocol could be used as a suitable alternative for patients with DOR.

In this present study, three protocols (modified GnRH agonist, mild stimulation, and antagonist) were used for patients with DOR. The comparative effectiveness of these three protocols was determined by measuring a range of clinical and laboratory parameters.

Patients and methods

Patient population and study design

This prospective randomized study enrolled patients with DOR in the Reproductive Centre, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China between March 2015 and September 2015. Eligible patients were required to meet all of the following eight inclusion criteria: (i) age $\leq$ 42 years; (ii) serum level of basal follicle stimulating hormone (FSH) $\geq$ 15.0 IU/l, or the ratio of basal FSH to luteinizing hormone (LH) $\geq$3; (iii) total number of antral follicles $\leq$8;

(iv) serum level of anti-Müllerian hormone (AMH) < 1.5 ng/ml; (v) no prior IVF treatment; (vi) body mass index < 23 kg/m^2; (vii) infertility caused only by tubal factors or male factors; (viii) no definite endometriosis, thyroid, adrenal or other endocrine diseases, and no history of ovarian surgery. Patients who met these criteria were numbered consecutively after providing written informed consent.

The study was registered with the Department of Research of the First Affiliated Hospital of Wenzhou Medical University in December 2014 (registration no. 201412016R). The study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University in July 2014 (registration no. GO-2014-09). All patients provided written informed consent.

Randomization and treatment protocols

Patients enrolled in the study were randomized to receive one of three treatment protocols using random number generation software from the SPSS® statistical package, version 17.0 (SPSS Inc., Chicago, IL, USA) for Windows®: (i) a modified GnRH agonist protocol; (ii) a mild stimulation protocol; or (iii) an antagonist protocol. If the patient did not receive fresh embryo transfer only due to endometrial abnormalities, then data from the patient were not be included in the statistical analysis. Those patients who did not undergo embryo transfer because of ovulation induction and embryo formation were still included in the study.

Patients in group A received a modified GnRH agonist protocol: 0.04 mg/kg long-acting GnRH agonist triptorelin (IPSEN PHARMA SAS, Boulogne-Billancourt, France), up to a maximum of 3.75 mg, intramuscular injection was given once only on the third day of the menstrual cycle. The patient then had a withdrawal vaginal bleed accompanied by intimal detachment. Then, hMG (Shanghai Livzon Pharmaceutical Company, Zhuhai, China) was intramuscularly injected 28 days after the injection of GnRH agonist. The initial dose of hMG was determined according to the bodyweight of the patient: 225 IU/day for a bodyweight $\leq$ 60 kg and 300 IU/day for a bodyweight > 60 kg. Follicular development was monitored as described below and the dose of hMG was adjusted by 75 IU more or less per day.

Patients in group B received the mild stimulation protocol. On the third day of the menstrual cycle, 5 mg letrozole (Jiangsu Hengrui Medicine Company, Jiangsu, China) was taken orally every day for 5 days. Then, 75 IU hMG was intramuscularly injected daily from the next day after the oral drug administration. Follicular development was monitored as described below and the dose of hMG was adjusted by 75 IU more or less per day. When the maximum follicular diameter reached 14 mm, 0.25 mg GnRH antagonist cetrorelix (Merck Serono Europe, Solna, Sweden) was subcutaneously administered daily until the end of ovarian stimulation period.

Patients in group C received the antagonist protocol. On the third day of the menstrual cycle, 150 IU hMG was intramuscularly injected daily. Follicular development was monitored as described below and the dose of hMG was adjusted by 75 IU more or less per day. When the maximum follicular diameter reached 14 mm, 0.25 mg GnRH antagonist cetrorelix was subcutaneously administered daily until the end of ovarian stimulation period.

Monitoring of follicular development

All three groups of patients were subject to ultrasonographic monitoring by the same doctor (R.Y.). When the maximum follicle diameter reached 14 mm, the monitoring

was performed every 2 days. When at least one follicle had a diameter ≥18 mm, 10 000 IU human chorionic gonadotrophin (hCG; Shanghai Livzon Pharmaceutical Company) was intramuscularly injected once and oocytes were collected after 36 h. Then for luteal phase support, 60 mg progesterone (Xianju Pharmaceutical Company, Hangzhou, China) was intramuscularly injected daily and 20 mg dydrogesterone (Solvay Pharmaceuticals, Veenendaal, The Netherlands) was taken orally daily from the day when the oocytes were retrieved. For patients with an endometrial thickness of <7 mm as determined by ultrasonography on the day of the hCG injection, 4 mg oestradiol valerate (Progynova®; Schering, Berlin, Germany) was taken orally daily until embryo transfer.

Oocyte retrieval and in vitro *fertilization*

The laboratory procedures were performed according to the routine operating protocols of the Reproductive Centre, First Affiliated Hospital of Wenzhou Medical University and described previously.[9] The oocytes were retrieved, washed and then placed in Vitrilife G-IVF™ solution (Vitrilife Sweden, Göteborg, Sweden). Oocytes were incubated for 4 h in a humidified incubator with 5% O_2 and 6% CO_2 at 37°C, followed by either IVF or intracytoplasmic sperm injection (ICSI) procedures depending upon the sperm quality of the patient's male partner. Embryos were transferred on the third day. Based on the provisions of the Chinese Ministry of Health, one or two embryos are allowed for embryo transfer. The number can be increased to three, but no more than three, if no high-quality embryos are obtained. Embryo quality grading was based on the standards described previously.[10] A high-quality embryo was defined as having at least six cells generated from cleavage in 3 days, with generally uniform size and morphology and < 20% cell debris.

Luteal phase support using progesterone, which is necessary for the preparation of embryo implantation and development, as described above, was used for 2 weeks until the determination of pregnancy. The serum hCG level was measured by immunofluorescence assay (Roche Diagnostics, Mannheim, Germany) 2 weeks after the embryo transfer. Clinical pregnancy referred to an intrauterine gestational sac and embryo heartbeat observed during an ultrasound scan using an ALOKA SSD-5500 ultrasound machine (ALOKA, Tokyo, Japan) 28–30 days after the embryo transfer. Early pregnancy abortion referred to the disappearance of the fetal heartbeat during the first 12 weeks after pregnancy.

Demographic and clinical parameters

The demographic and clinical parameters that were included in the analyses were as follows: age, duration of infertility, antral follicle count, basal serum FSH value, basal LH value, basal oestradiol (E2) value, AMH value, amount of hMG administered, the number of days of hMG use, levels of LH, E2 and progesterone on the hCG injection day, endometrial thickness, number of retrieved oocytes, proportion of metaphase II (MII) oocytes, fertilization rate, the number of embryos transferred, the cycle cancellation rate, clinical pregnancy rate and live birth rate.

Statistical analyses

The sample size was estimated according to the clinical pregnancy rate. Briefly, in order to meet a 20% significant difference and 80% test effectiveness in pregnancy rate among patients with DOR with the test protocols and general clinical treatment, each group had to contain at least 52

patients. Given the possibility of treatment cancellation, the study aimed to enrol 60 patients for each group. The sample size was calculated using an online computing program.[11]

All statistical analyses were performed using the SPSS® statistical package, version 17.0 (SPSS Inc.) for Windows®. Data are presented as mean ± SD or *n* (%). Analysis of variance was used to compare continuous data and two groups of data were compared using *post-hoc* Tukey Honestly Significant Difference (HSD) test. Fisher's exact test or χ^2-test was used to compare categorical data. A P-value < 0.05 was considered statistically significant.

Results

This study enrolled 166 patients: 54 in group A, 52 in group B and 60 in group C. Group A received 20 IVF cycles, 32 ICSI cycles and two IVF/ICSI cycles. Group B received 16 IVF cycles and 36 ICSI cycles. Group C received 32 IVF cycles, 26 ICSI cycles and two IVF/ICSI cycles. The mean ± SD age, infertility duration, antral follicle count, basal serum levels of FSH, LH, E2, and serum level of AMH were as shown in Table 1. There were no significant differences between the three groups of patients.

The mean ± SD dose of GnRH agonist (triptorelin) used in group A was 2.73 ± 0.85 mg. At 28 days after injection, the mean ± SD serum LH level was 0.85 ± 0.24 IU/l and the mean ± SD E2 level was 80.61 ± 18.97 pmol/l in Group A. The dose of hMG, the number of days of hMG stimulation, and serum LH, E2 and progesterone levels on the hCG injection day for the three groups are shown in Table 2.

Table 3 shows the outcomes of the *post-hoc* Tukey HSD test comparison between the three groups for the dose of hMG, the number of days of hMG stimulation, and serum LH, E2 and progesterone levels on the hCG injection day. The hMG dose and number of days of hMG stimulation in group B were significantly lower than those of group A ($P < 0.05$ for both comparisons), but there were no significant differences when groups A and B were compared with group C. On the day of hCG injection, the level of serum LH was significantly lower in group A compared with groups B and C ($P < 0.05$ for both comparisons). The level of serum E2 in group C was significantly higher compared with group B ($P = 0.024$), but there was no significant difference compared with group A.

Table 1. Demographic and clinical characteristics of female patients with diminished ovarian reserve enrolled in this randomized study of the comparative effectiveness of three protocols for *in vitro* fertilization.

Characteristic	Group A $n = 54$	Group B $n = 52$	Group C $n = 60$
Age, years	35.43 ± 3.84	37.32 ± 4.63	36.70 ± 3.59
Duration of infertility, years	7.64 ± 6.13	7.07 ± 5.11	5.77 ± 5.56
Antral follicle count	5.64 ± 1.39	5.44 ± 1.90	6.23 ± 2.64
Basal FSH, IU/l	17.27 ± 7.22	16.24 ± 5.77	15.92 ± 5.75
Basal LH, IU/l	6.30 ± 4.01	5.47 ± 5.60	5.89 ± 5.36
Basal E2, pmol/l	131.54 ± 59.52	173.09 ± 86.72	188.20 ± 61.90
AMH, ng/ml	0.86 ± 0.31	0.79 ± 0.28	0.85 ± 0.42

Data are presented as mean ± SD.

No significant between-group differences ($P \geq 0.05$); groups were compared using analysis of variance.

FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, oestradiol; AMH, anti-Müllerian hormone.

Table 2. Comparison of the dose of human menopausal gonadotrophin (hMG), the number of days of hMG stimulation, and serum hormone levels on the human chorionic gonadotrophin (hCG) injection day for the female patients with diminished ovarian reserve enrolled in this randomized study.

Characteristic	Group A $n = 54$	Group B $n = 52$	Group C $n = 60$	Statistical significance[a]
hMG dose, IU	3045.92 ± 785.13	925.56 ± 463.82	1413.33 ± 557.15	$P = 0.017$
Duration of hMG stimulation, days	14.2 ± 3.8	6.4 ± 2.7	9.3 ± 3.1	$P = 0.038$
LH on hCG injection day, IU/l	0.91 ± 0.53	5.94 ± 4.25	7.25 ± 3.81	$P = 0.012$
E2 on hCG injection day, pmol/l	3407.92 ± 3890.53	2182.25 ± 1876.99	7510.21 ± 5530.72	$P = 0.034$
Progesterone on hCG injection day, nmol/l	3.82 ± 2.70	2.53 ± 1.47	3.15 ± 2.39	NS

Data are presented as mean ± SD.

[a]The three groups were compared using analysis of variance.

LH, luteinizing hormone; E2, oestradiol; NS, no significant between-group difference ($P \geq 0.05$).

Table 3. Comparison using the *post-hoc* Tukey Honestly Significant Difference test of the dose of human menopausal gonadotrophin (hMG), the number of days of hMG stimulation, and serum hormone levels on the human chorionic gonadotrophin (hCG) injection day for the female patients with diminished ovarian reserve enrolled in this randomized study.

Group comparisons	hMG dose, IU	Duration of hMG stimulation, days	LH on hCG injection day, IU/l	E2 on hCG injection day, pmol/l
A versus B	$P = 0.012$	$P = 0.023$	$P = 0.018$	NS
A versus C	NS	NS	$P = 0.009$	NS
B versus C	NS	NS	NS	$P = 0.024$

LH, luteinizing hormone; E2, oestradiol; NS, no significant between-group difference ($P \geq 0.05$).

Patient disposition and the reasons for cancelled treatment cycles are outlined in Figure 1. A number of patients had their treatment cycles cancelled in the follicular development, ovulation, fertilization and cleavage stages. The treatment cycle cancellation rates for groups A, B and C were 11.11% (6/54), 32.69% (17/52) and 16.67% (10/60), respectively. Group B had the significantly highest cycle cancellation rate compared with groups A and C ($P < 0.05$ for both comparisons).

There were not significant differences between the three groups in terms of endometrial thickness on the hCG injection day, number of oocytes obtained, proportion of MII oocytes, fertilization rate, number of embryos obtained, proportion of high quality embryos and number of transferred embryos (Table 4).

Group A had 16 cases of clinical pregnancy, giving a clinical pregnancy rate per embryo transfer of 33.33% (16/48), a clinical pregnancy rate for the overall starting group population of 29.63% (16/54) and an early abortion rate of 12.50% (2/16). Group B had 12 cases of clinical pregnancy, giving a clinical pregnancy rate per embryo

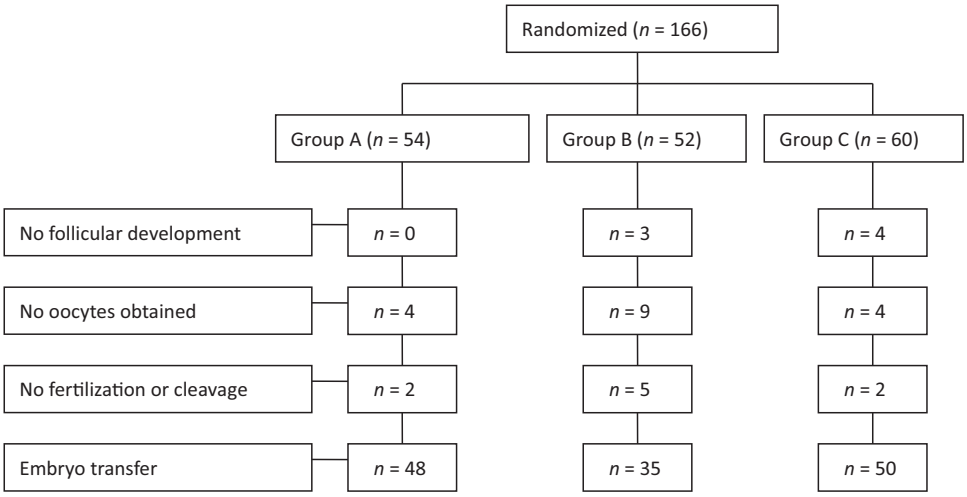

Figure 1. Patient flow through this randomized study of the comparative effectiveness of three protocols for *in vitro* fertilization showing the reasons for treatment cycle cancellation.

Table 4. Comparison of the treatment outcomes for the female patients with diminished ovarian reserve enrolled in this randomized study.

Outcome	Group A $n = 54$	Group B $n = 52$	Group C $n = 60$
Endometrial thickness, mm	9.64 ± 2.72	8.99 ± 3.05	10.30 ± 3.64
Number of oocytes obtained	5.25 ± 2.64	2.65 ± 2.37	4.90 ± 3.55
Proportion of MII oocytes, %	0.83 ± 0.18	0.87 ± 0.23	0.93 ± 0.11
Fertilization rate, %	0.71 ± 0.20	0.71 ± 0.34	0.74 ± 0.25
Number of embryos obtained	3.24 ± 1.07	1.73 ± 0.65	3.31 ± 0.82
Proportion of high quality embryos, %	0.34 ± 0.06	0.31 ± 0.04	0.33 ± 0.05
Number of embryos transferred	2.24 ± 0.57	1.86 ± 0.96	2.36 ± 0.81

Data are presented as mean ± SD.
No significant between-group differences ($P \geq 0.05$); groups were compared using analysis of variance.
MII, metaphase II.

transfer of 34.29% (12/35), a clinical pregnancy rate for the overall starting group population of 23.08% (12/52) and an early abortion rate of 16.67% (2/12). Group C had 18 cases of clinical pregnancy, giving a clinical pregnancy rate per embryo transfer of 36.00% (18/50), a clinical pregnancy rate for the overall starting group population of 30.00% (18/60) and an early abortion rate of 44.44% (8/18). There was no significant difference among the three groups in the clinical pregnancy rate per embryo transfer or the clinical pregnancy rate for the overall starting group population. The early abortion rate for group C was significantly higher than that of group A ($P = 0.04$), but the difference was not significant compared with group B.

By September 2016, all pregnancies had been delivered. The live birth rates in group A, group B and group C were 22.92% (11/48), 22.86% (8/35), and 16.00% (8/50)

per embryo transfer, respectively. Meanwhile, the live birth rates in group A, group B and group C were 20.37% (11/54), 15.38% (8/52) and 13.33% (8/60) for the overall starting group populations, respectively. There were no significant differences between the three groups.

Discussion

This present study compared the efficacy of three different ovarian stimulation protocols for IVF treatment in patients with DOR. At present, there is no unified definition of DOR.[12] Serum FSH and AMH levels and antral follicle count are generally considered to be the most valuable and most commonly used indicators of DOR,[13] but the cut-off values of these indicators remain controversial. In this present study, patients were required to meet both criteria of basal FSH levels and antral follicle count according to standards reported in the literature.[13] Age is also considered to be an independent factor affecting the ovarian response in IVF treatment,[14] so patients included in this study were $\leq$42 years old. Previous unpublished data from our group has shown that the success rates of IVF for patients with DOR above a certain age, for example $>$ 42 years, using various ovulation induction protocols were $<$15%, which was comparable to that of a natural cycle. In our opinion, if patients with DOR are $>$ 42 years old, then a natural cycle without any follicle stimulation should be recommended.

The mild stimulation protocol was once thought to be more appropriate for younger women and for those with a higher ovarian response.[15] When compared with the conventional agonist protocol in patients with DOR, the mild stimulation protocol resulted in fewer oocytes, a thinner endometrium and a higher rate of rejection, but a similar clinical pregnancy rate.[6,16] In the present study, the cycle cancellation rate was highest in the mild stimulation group (group B) compared with the other two groups, which meant that a larger proportion of patients in group B required further IVF treatment, thus reducing the economic advantage provided by the mild stimulation protocol. The number of oocytes retrieved was nonsignificantly lower in the mild stimulation group when compared with the other two groups, which reduced the number of embryos available for transfer. Theoretically, this would affect the cumulative pregnancy rate. Like clomiphene, letrozole is also commonly used in the mild stimulation protocol.[17] It reduces oestrogen production by inhibiting aromatase and has less effect on the endometrium than clomiphene.[17] In the present study, there was no significant difference in endometrial thickness between the mild stimulation group and the other two groups, suggesting that letrozole might provide better pregnancy outcomes than clomiphene.

In recent decades, clinical experience with the use of antagonists for ovarian stimulation protocols for IVF has rapidly accumulated. Unlike agonist, which is likely to cause the pituitary over-inhibition and decreased ovarian response,[18] the inhibitory effect of the antagonist on the pituitary gland rapidly declines following its withdrawal, and it was therefore thought to be useful for patients with DOR.[19] Another advantage of the antagonist protocol is the reduction of ovarian hyperstimulation (OHSS),[20] although in patients with DOR who are more likely to present with low ovarian response states, OHSS is correspondingly rare.[21] Meta-analyses suggest that there is no difference in the clinical pregnancy rate between the antagonist and agonist protocols for patients with DOR, and that the antagonist protocol has a shorter gonadotrophin treatment duration and faster follicular growth.[7,22] These advantages are similar to those of the mild stimulation protocol.

When antagonists were used in patients with normal ovarian function, some studies suggested that the clinical pregnancy rate was lower than the agonist protocol.[23,24] This might have been due to the antagonist exerting a negative effect directly or indirectly on endometrial receptivity, such as by reducing the expression of the important regulatory protein HOXA10 in endometrial stroma[25] or by promoting premature maturation of the endometrium.[26] In this present study, the antagonist protocol (group C) achieved a similar clinical pregnancy rate as the agonist protocol (group A), but the early miscarriage rate was as high as 44.44%. In our opinion, this might be due to intimal damage during the antagonist protocol. Decreased endometrial receptivity and abnormal protein expression might cause the development of embryos to stagnate.[25,26]

The ultra-long GnRH agonist IVF protocol is suitable for patients with endometriosis.[27] In response to large doses and long durations of GnRH agonist treatment, cytotoxicity and oxidative stress in the pelvic cavity and ovary may be significantly reduced, leading to an improvement in the embryo implantation rate and pregnancy rate of patients with endometriosis.[28] Previous research also confirmed that GnRH agonist could increase the concentration of melatonin in the follicular fluid, which was suggested to be a powerful free radical scavenger and to have a broad spectrum antioxidant effect.[29] Therefore, it was hypothesized that increased GnRH agonist dose and treatment duration would also be beneficial to patients with DOR because diminished ovarian function in these patients has been correlated with insufficient free radical scavenging and excessive oxidative stress.[30]

Compared with the conventional agonist protocol, the dose of GnRH agonist used in the modified agonist regimen in the present study was increased to 0.04 mg/kg and the interval before hMG injection was increased to 4 weeks. This increased the inhibitory effects on the cytotoxic factors and oxidative stress in the pelvic cavity, and even on the endometrium.[28] In contrast, a longer interval before hMG injection could lead to a recovery of the pituitary and ovaries from deep inhibition, rather than there being a low response to gonadotrophin. In this present study, the number of retrieved oocytes in the agonist group (group A) was not less than that in the antagonist group (group C), suggesting that the ovaries were not over suppressed as long as the stimulation time was appropriate, which was similar to findings in a previous report.[31] In this present study, hMG was used as the ovarian stimulation drug, since the right amount of LH would be beneficial for patients with poor ovarian response,[32,33] especially after pituitary suppression. hMG is frequently used for ovulation induction in patients with diminished ovarian function.[34]

In conclusion, there was no significant difference in the pregnancy rate among patients with DOR treated with the three different protocols in this present study. The cycle cancellation rate was higher in the mild stimulation group compared with the other two groups due to the milder follicular stimulation. The antagonist group had the highest rate of abortion compared with the other two groups, possibly due to intimal damage. The modified agonist protocol resulted in the greatest amount of hMG administered and the longest duration of hMG administration because of pituitary suppression. However, higher doses of hMG and longer durations of ovarian stimulation are acceptable if the pregnancy outcomes can be improved. Compared with the other two protocols, the modified agonist protocol showed a nonsignificant higher live birth rate per overall starting group population, suggesting that more research in larger sample sizes

is needed to optimize the ovarian stimulation protocol for patients with DOR in the future.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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