Pituitary Suppression before Frozen Embryo Transfer Is Beneficial for Patients Suffering from Idiopathic Repeated Implantation Failure*

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Summary: Long-term gonadotropin-releasing hormone agonist (GnRHa) administration before *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) in infertile women with endometriosis or adenomyosis significantly enhanced the chances of pregnancy in both fresh and frozen embryo transfer cycles. We hypothesized that long-term GnRHa treatment might also be beneficial for the idiopathic repeated implantation failure (RIF) patients. In the 21 patients receiving GnRHa and hormone replacement therapy (G-HRT) protocols for frozen embryo transfer, their data were compared with those of the 56 of frozen/fresh cycles they had previously undergone (previous protocols). Comparison showed that the finial results were significantly better with G-HRT protocols than with their previous protocols, with pregnancy rate, clinical pregnancy rate, implantation rate and on-going pregnancy rate being 70%, 60%, 40% and 38% respectively with G-HRT protocols, against 17%, 11%, 6.3% and 5% with previous protocols. The results showed that hormonally controlled endometrial preparation with prior GnRHa suppression could be used for patients who had experienced repeated failures of IVF treatment despite having morphologically optimal embryos, and the treatment may help increase the receptivity of the endometrium in these patients.

Key words: idiopathic repeated implantation failure; pituitary suppression; gonadotrophin-releasing hormone agonists; endometrium receptivity

Treatment of infertile couples has progressed immensely during recent years. Pregnancy rate following one cycle of *in vitro* fertilization and embryo transfer (IVF-ET) can be up to 60%. But even in those very successful units, some couples fail to conceive repeatedly. Extensive research has been conducted on the possible causes detrimental to conception, such as ovarian and testicular dysfunctions, poor sperm and oocyte quality[1–3], fallopian transport defects[4], lower endometrial receptivity[5], implantation failures, and endometriosis[6]. Among multiple causes of repeated implantation failure (RIF), various uterine pathologies, such as thin endometrium, altered expression of adhesive molecules and immunological factors, may impair endometrial receptivity whereas genetic abnormalities of the male or female, sperm defects, embryonic aneuploidy or zona hardening are possible embryonic reasons for implantation failure. Endometriosis and hydrosalpinges may adversely influence both uterine microenvironment and embryo quality. While a wide array of strategies have been applied to identify the cause of infertility and promote implantation[7, 8], 8% of RIF patient in our clinic are diagnosed with idiopathic RIF after IVF treatment. Since no evident adverse factors for such failures have been definitely found, the "idiopathic repeated implantation failure" has been posing a tough challenge for both clinicians and researchers.

So far, there is no uniform or consistent definition of RIF in the literature[9, 10]. In this study, we referred to a study which defined RIF as the failure of implantation after two consecutive cycles of IVF, intracytoplasmic sperm injection (ICSI) or frozen embryo replacement cycles when the cumulative number of transferred embryos was no less than four with cleavage-stage embryos and no less than two with blastocysts, with all embryos being of good quality and of appropriate developmental stage[10].

The transfer of cryo-preserved embryos in women with functioning ovaries can be timed with ovulation in natural cycle (NC) or after endometrium preparation with exogenous hormone replacement therapy (HRT)[11–13]. Protocols of controlled endometrial preparation also involve the use of gonadotropin-releasing hormone agonist (GnRHa) prior to steroid administration to suppress ovarian function and generate a synchronization of endometrial and embryo development[14]. Compared to GnRHa programmed cycle, NC or HRT approach is simple and more convenient for both patients and medical workers, and can achieve similar success rates in unclassified infertility couples with a lower financial burden. However, several randomized studies have shown that long-term GnRHa administration before IVF/ICSI in infertile women with endometriosis or adenomyosis significantly enhanced the chances of pregnancy in both fresh[15–17] and frozen cycles[18–20]. On the basis of the data, we were led to hypothesize that long-term GnRHa treatment might

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also be beneficial for the idiopathic RIF patients.

To test our assumption, in this self-control study, we retrospectively examined the effect of long-term G-HRT on endometrial receptivity in women with idiopathic RIF. Parameters in previous failed fresh or frozen transfer cycle were compared with those in G-HRT protocol. The primary end-point was the clinical pregnancy rate per transfer cycle, and the secondary outcome measures were endometrium thickness and implantation rate.

1 MATERIALS AND METHODS

1.1 Patients

This retrospective self-control study enrolled 21 idiopathic RIF patients who had been on G-HRT protocol in the Reproductive Medical Center of the Sixth Affiliated Hospital of Sun Yat-sen University between May 2010 and September 2014 and had previously experienced 56 repeated failed cycles. All patients included in the study satisfied the following inclusion criteria: (1) aged under 42 years old; (2) having been diagnosed as having RIF against the Coughlan C criteria[10]; (3) having normal uterine cavity as assessed by ultrasonography, hysterosalpingography and hysteroscopy. The exclusion criteria included: (1) presence of hydrosalpinges; (2) having been diagnosed as having endometriosis or adenomyosis; (3) having undergone myomectomy; (4) endometrium thickness less than 7 mm; (5) having auto-immune diseases; (6) possessing abnormal karyotypes.

1.2 G-HRT Protocol

The first intramuscular injection of leuproreline acetate (Diphereline@; Ipsen, France) at 2.5 mg was administered during the early follicular phase of the menstrual cycle to G-HRT patients. Then, a second injection of leuproreline acetate was given 28 days later. HRT protocol commenced 15 days later. The patients were asked to orally take estradiol (E2) valerate (Progynova; Schering, Germany) for at least 10 days, and then progesterone was intramuscularly injected at 40 mg twice a day when the endometrial thickness was greater than 7 mm. Embryo transfer (ET) was performed on the fourth day of progesterone administration for cleavage stage embryo transfer or on the sixth day for blastocyst embryo transfer. Estrogen and progesterone were continued until a serum beta human chorionic gonadotropin (hCG) assay was conducted 14 days after ET. If the hCG assay yielded a positive result, the patient was put on ultrasonographic monitoring to determine fetal viability until approximately the 7th week of gestation. Pregnancy rate was defined by the number of positives of hCG assay 14 days after ET. Clinical pregnancy was defined by the presence of a gestational sac and a live fetus on transvaginal ultrasound at the 7th week of gestation. Implantation rate was defined as number of gestational sacs observed divided by the number of embryos transferred. On-going pregnancy was defined as live fetus beyond the 12th week of gestation.

1.3 Blood Sample Assays

Serum levels of estradiol (pg/mL) and progesterone (ng/mL) were determined in all patients on the day of ET by employing fluorescence polarization immunoassay and the Abbott AXSYM assay. The inter- and intra-assay coefficients of variation were both at 10%.

1.4 Embryo Grading System

Cleavage-stage embryo was graded on a 1.0–2.5 scale, in terms of the number of cells, percentage of fragmentation and cell symmetry: 1.0, 6–8 cells with less than 5% fragmentation, equal blastomeres; 1.5, 6–8 cells with less than 10% fragmentation, equal blastomeres; 2.0, 4–6 cells with less than 25% fragmentation almost equal blastomeres; 2.5, cell number did not double in 24 h with less than 30% fragmentation, unequal blastomeres; 3.0, cells did not divide with over 50% fragmentation and unequal blastomeres.

Blastocyst was rated on a 3-point scale in accordance with a simplified grading system formulated by the Society for Assisted Reproductive Technology (SART), grading system. Briefly, 1.0 (Good): inner cell mass (ICM) graded A or blastocysts graded A or B; 2.0 (fair): ICM graded B or TE graded A, B or C; 3.0 (poor): any ICM graded C.

1.5 Statistical Analysis

Regression analysis was used to assess 7 parameters (age, duration of infertility, number of embryo transferred, score of embryo transferred, endometrium thickness, serum progesterone and estradiol level on embryo transfer day) potentially related to clinical pregnancy rate, with G-HRT taken as a grouping variable. Data were expressed as $\bar{x}\pm s_x$ and analyzed by using SPSS version 13.0 (SPSS Inc., USA). Mean values were analyzed using the two-tailed *t*-test for parametric data, and ratios were compared by using the *Chi*-square test. A *P*-value less than 0.05 was considered to be statistically significant.

2 RESULTS

In the 21 patients receiving G-HRT protocols for frozen embryo transfer, their data were compared with those of the 56 of frozen/fresh cycles they had previously undergone (previous protocols). Table 1 shows the demographic data of two groups. Nine patients had primary infertility and 12 had secondary infertility, with the major causes of their infertility including: tube factor (n=10), male-caused factor (n=5) and unexplained reasons (n=6). Compared with their previous protocols, patients undergoing the G-HRT protocol were at an elder age (33.75 years *vs.* 30.88 years), had lower ovarian reserve, as revealed by their higher basal FSH (6.24 U/L *vs* 5.15 U/L), lower basal antral follicle count (4.84 *vs* 7.35) and longer duration of infertility (6.13 years *vs* 4.00 years). Mean serum CA-125 was at 42.57 IU/L in the 21 patients on G-HRT protocol.

Table 2 compares the clinical variables and treatment outcomes of the two protocols. Equal number (1.78 and 2.04) of embryos was transferred and their thickness was similar (10.84 and 11.39). Serum progesterone and estradiol levels on the embryo transfer day were significantly lower in patients on the G-HRT protocol. A total of 36 embryos were transferred in the G-HRT group, including 9 (25%) cleavage stage embryos and 27 (75%) blastocyst embryos, while 114 embryos were transferred in their previous protocols, including 60 (52.63%) cleavage stage embryos and 54 (47.67%) blastocyst embryos. The quality of embryos transferred was not significantly different between the two protocols. Comparison showed that the finial results were significantly better with

G-HRT protocol than with their previous protocols, with pregnancy rate, clinical pregnancy rate, implantation rate and on-going pregnancy rate being 70%, 60%, 40% and 38% respectively with G-HRT protocol, against 17%, 11%, 6.3% and 5% with previous protocols.

Table 1 Demographic data of the two protocols

Parameters	G-HRT protocol	Previous protocols	*P* value
Number of patients with primary infertility	9		
Number of patients with secondary infertility	12		
Major cause of infertility (*n*)			
Tube	10		
Male-caused	5		
Repeated AIH failure (unexplained)	6		
Serum CA-125 (IU/L)		42.57±32.08	
Age (years)	33.76±1.39	30.88±0.67	0.047
Body mass index (kg/m^2)	21.45±0.61	20.57±0.58	0.3
Duration of infertility (years)	6.13±0.83	4.00±0.32	0.0054
Average cycle number	4.17±0.53	2.75±0.27	0.014
Basal FSH (U/L)	6.24±0.37	5.15±0.31	0.03
Basal LH (U/L)	4.51±0.55	4.17±0.60	0.68
Basal E2 (pg/mL)	45.06±3.99	43.37±4.12	0.76
Antral follicle count (*n*)	4.84±0.43	7.35±0.80	0.01

Table 2 Clinical variables and treatment outcomes of the two protocols

	G-HRT protocol	Previous protocols	*P* value
Endometrium thickness on embryo transfer day (mm)	10.84±0.52	11.39±0.35	0.42
Estradiol level at embryo transfer day (pg/mL)	148.2±14.87	759.8±142.7	0.0037
Progesterone level at embryo transfer day (ng/mL)	19.31±2.58	40.55±4.93	0.0042
Mean number of embryo transferred	1.78±0.13	2.04±0.10	0.15
Total number of D3 embryo transferred	9 (25%)	60 (52.63%)	
Score of D3 embryo transferred	1.43±0.20	1.44± 0.072	0.94
Total number of D5 blastocyst transferred	27 (75%)	54 (47.67%)	
Score of blastocyst transferred	1.67±0.098	1.91±0.13	0.22
Pregnancy rate	0.70±0.11	0.17±0.058	0.0012
Clinical pregnancy rate	0.60±0.11	0.11±0.042	<0.0001
Implantation rate	0.40±0.086	0.063±0.026	<0.0001
On-going pregnancy rate	0.38±0.08	0.05±0.03	<0.0001

Table 3 Logistic regression analysis of factors associated with clinical pregnancy rate

	Beta	S.E.	Wald	Sig.	OR
GnRHa	2.74	0.64	18.46	0.000	15.48
Constant	–2.12	0.43	24.08	0.000	0.12

To identify the potential factors associated with the significantly improved pregnancy rate in G-HRT group, we subjected all potential factors (age, infertility duration, number of embryo transferred, score of embryo transferred, endometrium thickness, serum progesterone and estradiol level) to logistic regression. After all the other potential confounding factors were eliminated, the analysis revealed that that G-HRT strategy was the only factor that was significantly associated with enhanced clinical pregnancy rate (table 3).

3 DISCUSSION

In unclassified IVF cycles, similar success rate was achieved after transfer of cryopreserved-thawed embryos by using either NC, HRT or hormonally controlled endometrial preparation with prior GnRHa suppression[21]. In addition, the use of GnRHa may also present some disadvantages: The treatment cycle is prolonged with increased dosage of hormone administration, the protocol might pose more financial burden and the patients may suffer from menopausal symptoms resulting from agonist-induced hypo-estrogenic state. Due to aforementioned reasons, pituitary suppression has never been used as the first choice for frozen embryo transfer. In fact, 21 patients with idiopathic RIF in our series had undergone RIF with fresh embryos transfer, hormone replacement or natural frozen embryo transfer cycle and GnRHa suppression regime was used as a last resort for just protocol adjustment. However, several researches revealed that for patients with endometriosis, GnRHa treatment substantially improved pregnancy outcomes with both fresh and frozen ET cycle[19]. Therefore, in this study, we tried to test whether GnRHa suppression could benefit certain patients who had experienced idiopathic RIF.

Understandably, just because we were unable to identify the causes of infertility in our series that does not mean that there is no cause for the disorder. In fact, it was reported that women who had undergone multiple unsuccessful IVF cycles were finally found to have concomitant uterine adenomyosis and a prominent aggregation of macrophages within the superficial endometrial glands, which potentially interfered with embryo implantation[22]. Long-term pituitary suppression with

GnRHa for at least 6–8 weeks plus prednisolone (15 mg) reportedly could greatly elevate embryo implantation rate[22]. Adenomyosis is present in a large proportion of women with sub-fertility, particularly those with endometriosis and/or symptoms suggestive of menorrhagia and dysmenorrhoea[15].

It has also been demonstrated that even mild endometriosis, which may not be found during IVF cycles, may cause subfertility via a number of mechanisms [23, 24], such as ovulation disturbance, inhibition of ovum uptake, dysfunction of the oviducts, recurrent abortions[22], decrease in implantation[6], modified response to the immune system[25] and endoperitoneal inflammation[26]. Kuivasaari *et al* reported that despite significantly younger age of their subjects, implantation rates in IVF treatment cycle were lower in patients with stage Ⅲ/Ⅳ endometriosis as opposed to either those with stage Ⅰ/Ⅱ disease or a control group with tubal infertility[24]. A database analysis examined three prospective randomized trials including 163 endometriosis patients undergoing 3 to 6 months of pre-cycle GnRHa treatment. The study found that this intervention resulted in significantly improved rates of both live birth (OR: 9.1%; 95% CI: 1.08 to 78.22) and clinical pregnancy (OR: 4.28; 95% CI: 2.0 to 9.15)[27]. The underlying mechanism of this effect has not been clearly established. It has been suggested that, apart from the primary mechanisms of pituitary suppression and decreased circulation estrogen, GnRHa may lower the concentrations of peritoneal fluid metalloproteinase tissue inhibitors, down-regulate peritoneal fluid inflammatory proteins, and promote apoptosis and expression of pro-apoptotic proteins[28–30]. Other researchers exhibited that GnRHa may significantly decrease the expression of endometrial nitric oxide synthase[31]. A great deal of research effort has been directed at understanding the role of endometrial β3 integrin. GnRHa administration for 3 months to women with stage Ⅰ/Ⅱ endometriosis could lower aberrant endometrial β3 integrin expression by 64%[32]. In a murine model with decreased endometrial β3 integrin, leukemia-inhibitory factor expression and uterine receptivity resulting from ovarian stimulation were partially restored after GnRHa administration[33].

On the other hand, a meta-analysis revealed that inclusion of GnRHa to the luteal support scheme could greatly promote live birth rate. The possible mechanism might be that GnRHa exerted a direct effect on embryo and/or on endometrium[34]. The long-term effect of GnRHa, integrated into G-HRT protocol, might also be favorable, since a second GnRHa injection could still be effective during the embryo implantation phase.

Circulating cytokine levels are influenced by ovarian stimulation or higher estrogen level. Lower estradiol level in G-HRT cycle could decrease circulating cytokine level in RIF patients[35], as TNF-α levels were inversely correlated to estradiol levels and RIF was associated with elevated TNF-α level[36]. Some cases of RIF might result from local dys-regulation of the normal expression or action of various cytokines. Elevated endometrial NK cells, dys-regulation of interleukins 12, 15 and 18[37], lower estradiol level during the G-HRT protocol might also be close to natural hormone status that provides a stable implantation microenvironment and help inhibit wave-like activity of hyperactive endometrium[38].

It should be mentioned that the inherent nature of retrospective study made it difficult to exclude the interference of various confounding factors. After these patients had experienced repeated failures in our centre, a number of measures could have been applied, such as hysteroscopic examination, blastocyst transfer, antibody detection, intravenous immuglobin, and heparin treatment for patients with thrombophilia[39]. We conducted a logistic regression analysis covering 7 confounding factors, and the results confirmed that G-HRT was the only factor that had a positive effect on clinical pregnancy rate. Further prospective cohort studies are warranted to investigate the exact molecular mechanism underlying the improved endometrial receptivity in patients with idiopathic RIF treated with G-HRT protocol.

In conclusion, our study showed that hormonally controlled endometrial preparation with prior GnRHa suppression could be used for patients who had experienced repeated failures of IVF-ET treatment despite having morphologically optimal embryos and the treatment may help increase the receptivity of the endometrium in these patients.

Conflict of Interest Statement

The authors declare no conflict of interest.

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