Analysis of various factors associated with High-quality embryo formation in GnRH-antagonist protocol

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Introduction. To explore the various factors influencing the formation of high-quality embryos in GnRH-antagonist protocol.

Methods. The clinical data of 1092 patients who underwent in vitro fertilization/ intracytoplasmic monosperm injection-embryo transfer (IVF/ICSI-ET) with GnRH-antagonist protocol in Reproductive Medicine Center of the First Affiliated Hospital of Soochow University were retrospectively analyzed from January 2020 to May 2023. They were divided into two groups according to whether they had obtained high-quality embryos or not, high-quality embryo group and no high-quality embryo group. The differences of the factors were compared between the groups. One-way and logistic regression were used to analyze the correlation between obtaining high-quality embryos with GnRH-antagonist protocol.

Results. Logistic regression analysis showed that patients with anti-Mullerian hormone (AMH)≥4.07ng/ml obtained high-quality embryos as a result of 1.43 times higher in patients with AMH < 4.07ng/ mL [*Odds Ratio (OR)* 1.434, $P < 0.05$], and patients with antral follicle count(AFC)≥16 were 1.46 times more likely to obtain high-quality embryos than patients with AFC < 16 (*OR*1.457, $P < 0.05$).Patients with ≥6 2PNs had 4.97 times the probability of obtaining high-quality embryos as compared with patients with < 6 2PNs (*OR*4.969, *95%CI* 2.095-11.787, $P < 0.001$).

Conclusion. Whether the GnRH-antagonist protocol could obtain high-quality embryos was positively correlated not only with the patients' ovarian reserve function AMH and AFC, but also with the number of 2PN and 2PN rate.

Keywords. GnRH-antagonist protocol; high-quality embryos; infertility

INTRODUCTION

According to the World Health Organization (WHO), 186 million people in the world currently suffer from infertility [1], which has become an important public health problem. The results of a national reproductive health epidemiological survey and analysis showed that the prevalence of infertility in China increased from 12% in 2007 to 18% in 2020 [2].The goal of in vitro fertilization/intra cytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) is to help patients achieve clinical pregnancy in the shortest possible time with safety, efficiency and less interference.Controlled Ovarian stimulation (COS) is an important part of improving the success rate of IVF-ET.With the development of assisted reproductive drugs and the accelerated pace of people's life, the mainstream ovulation stimulation protocol has changed accordingly.Compared with Gonadotropin releasing hormone(GnRH) agonist protocol, GnRH antagonist had shorter stimulation time, lower total cost, more flexible use and easier acceptance by patients.At present, it has become the mainstream ovulation stimulation protocol in many international reproductive centers.GnRH-antagonist protocol has been reported to be comparable to GnRH agonist protocol in terms of clinical, obstetric and perinatal outcomes, and has a lower risk of OHSS, making GnRH-antagonist protocols an appropriate option for patients seeking effective, safe and shorter treatment cycles[3-5].How to select the best ovulation stimulation protocol for patients to improve the rate of high quality embryo formation is a hot and difficult issue in reproductive medicine.At present, the factors related to good embryo genesis of GnRH-antagonist protocol are not very clear.This study retrospectively analyzed the factors related to the formation of high-quality embryos in the GnRH-antagonist protocol, in order to provide clinical reference and promote the optimal application of the GnRH-antagonist protocol.

1.MATERIALS AND METHODS

Patients

Retrospective analysis of clinical data of patients who underwent GnRH-antagonist protocol of IVF/ICSI-ET for assisted conception at the Reproductive Medicine Centre of the First Affiliated Hospital of Soochow University during the period of January 2020-May 2023. Exclusion criteria: chromosomal abnormalities that are not polymorphic in either the man or the woman. A total of 1092 cases were included, and the patients were divided into the group of high-quality embryo group (≥1high-quality embryos, 732 cases) and the group of no high-quality embryo group (0 high-quality embryos, 360 cases) according to whether or not the patients obtained high-quality embryos.

GnRH-antagonist Protocol

Gonadotropin was activated on day 2 or 3 of the menstrual cycle [Gn,including recombinant human follicle-stimulating hormone (rFSH preparation, Merck Serono, Switzerland),urinary gonadotropin (hMG,Lizhu Pharmaceutical Factory, China)].Different doses of Gn(112.5-300IU/d) were used according to the specific conditions of patients. After 4-5 days of ovulation stimulation, ultrasound adjusted the dose of Gn according to follicular growth and development and sex hormone level until the trigger day. When at least one follicular diameter ≥14mm or serum estrogen level of > 600pg/ mL or serum LH level of> 3 times of basal LH level or serum LH level of > 10mIU/ mL[6], GnRH antagonist was added daily, including cetrorelix (Merck-Serono,Switzerland),ganirelix (Organon, The Netherlands), 0.125-0.25mg /d until trigger day. When the diameter of 3 dominant follicles was ≥17mm or the diameter of 2 dominant follicles was ≥18mm,short-acting triptorelin(GnRHa,Pfizer Pharmaceuticals Ltd.,Switzerland) was injected in the evening of the same day, and ovulation was induced by using a combination of urinary chorionic gonadotropin (hCG, 2000U/strike, Lizhu Pharmaceuticals Ltd., China)2000U triggering. If patients with high risk of ovarian hyperstimulation syndrome (OHSS) were triggered with

triptorelin 0.2mg trigger alone and and not transferred in a fresh cycle, frozen-thawed embryos were transferred after the day of whole embryo freezing.

Laboratory Measurements

Evaluation of Egg Retrieval, Fertilization and Embryo Quality

36-37 hours after trigger drug injection, all follicles ≥10mm in diameter were extracted by transvaginal ultrasound. The method of insemination was selected according to the male sperm quality and medical history of assisted fertilization. Routine IVF/ICSI was performed after the ovum was cultured in vitro for 3-6 hours.On the third day after fertilization, the quality embryos were determined by morphological evaluation. The criteria were as follows: 2PN, 7-9 cells, uniform size, fragmentation <10%.At 5-6 days after fertilization, the blastocyst score method proposed by Gardner et al., 1999[7], was used to determine the high-quality embryos. The criteria were as follows: stage 4 (blastocyst cavity is completely filled with embryos, the total volume of embryos becomes larger, and the zona pellucida becomes thinner) or above, the number of inner cell masses and trophoblast cells is moderate, and grade B or above.

Statistical Analyses

SPSS26.0 software was used for statistical analysis. Measurement data in accordance with normal distribution were expressed as mean ± standard deviation ($\bar{x}\pm s$), and two independent sample T test was used. Median [M(P25,P75)] was used for measurement data that did not conform to normal distribution, and nonparametric Mann-Whitney U test was used.The counting data were expressed as rate (%), and Pearson Chi-square test was used when the conditions were met.Logistic regression analysis was used for univariate and multivariate analysis.When $P<0.05$, the difference was statistically significant.

RESULTS

1.Comparison of General Conditions Between Optimal Embryo Group and Non-optimal Embryo Group

AMH, number of sinus follicles, proportion of basal LH and PCOS in optimal embryo group were significantly higher than those in non-optimal embryo group, and the differences were statistically significant(*Table 1*);Female age, basal E_2, proportion of endometriosis (EMS), proportion of decreased ovarian reserve function (DOR), male age and male factors were significantly lower than those in non-optimal embryo group ($P < 0.05$).There were no significant differences in primary infertility ratio, infertility years, body weight, body mass index (BMI), CA125, triglyceride, fasting blood glucose, basal FSH, basal progesterone, fallopian tube factor infertility ratio, male semen DFI and normal morphology rate between optimal embryo group and non-optimal embryo group($P > 0.05$).

Table 1 General situation of patients in the two groups

grouping	high quality embryo group	Non-high quality embryo group	$t/Z/x^2$	***P***
Number of cycles	732	360		
Female age	31.44±3.98	32.13±4.52	-2.58	0.010
Infertility type % (n)				
Primary infertility	50.82（372/732）	52.50(189/360)	0.27	0.60
Secondary infertility	49.18(360/732)	47.50(171/360)		
Duration of infertility （year）	3（2,5）	3（2,5）	-0.30	0.767
Weight(kg)	58（52,65）	58（52,65）	-0.09	0.925

BMI(kg/m^2)	22.5（20.20,25.00）	22.2（20.40,25.00）	-0.25	0.800
AMH(ng/ml）	4.53（2.78,6.98）	3.55（2.06,5.20）	-6.16	＜0.001
CA125(mIU/L)	14.5（10.8,19.9）	13.80（10.20,19.9）	-1.27	0.206
triglyceride	0.99（0.74,1.37）	0.96（0.73,1.41）	-0.30	0.767
Fasting blood glucose	5.22（4.98,5.51）	5.23（4.98,5.46）	-0.66	0.510
(AFC)	17（12,25）	15（11,20）	-5.67	＜0.001
Basal E_2 level(pg				
/ml)	27.2（20.5,34.8）	29（21.35,36.96）	-2.36	0.018
Basal LH level(IU/L)	4.23（3.08,6.00）	4.05（2.81,5.44）	-2.44	0.015
Basal FSH				
level(IU/L)	7.29（6.15,8.43）	7.42（6.22,8.63）	-1.36	0.174
Basal P level(ng/ml)	0.72（0.50,1.00）	0.72（0.48,1.06）	-0.69	0.490
EMS factor	12.16(89/732)	17.22(62/360)	5.19	0.02
PCOS factor	20.77(152/732)	13.61（49/360）	8.22	＜0.001
Tubal factor	57.92(424/732)	54.44(196/360)	1.19	0.28
DOR factor	6.69(49/732)	16.67(60/360)	26.71	＜0.001
Male factor	18.03(132/732)	24.17(87/360)	5.66	0.02
Male age	32（29,35）	32（30,36）	-2.11	0.035
Sperm DFI(%)	10.95（6.71,17.51）	11.27（6.33,17.54）	-0.17	0.864
Sperm normal form				
rate (%	1.80（1.0,2.5）	1.7（1.0,2.5）	-0.06	0.950

2.Comparison of Ovulation Induction Between Optimal Embryo Group and Non-optimal Embryo Group

The number of cycles in optimal embryo group, the proportion of rFSH combined with HMG, Gn initiation amount and the maximum value of FSH during COS were significantly lower than those in non-optimal embryo group ($P < 0.05$); Total dose of antagonist, total number of days of antagonist, maximum value of LH in COS process, trigger day E_2And the proportion of double trigger drugs was

significantly higher than that of embryo group (P<0.05).Total amount of Gn, total number of Gn days, proportion of antagonist drugs, initial dose of antagonist, minimum LH and maximum P value in COS process, LH, P and E_2 on trigger day in both groups.There was no significant difference in the number of follicles ≥14mm (P > 0.05).

Tab 2 Comparison of ovulation induction outcomes between the two groups

Index	high quality embryo group	Non-high quality embryo group	X^2/Z	P
Number of cycles	1（1,1）	1(1,1)	-2.79	0.005
Gn Start drug % (n)				
rFSH +HMG	19.81(145/732)	26.39(95/360)	6.09	0.010
rFSH	80.19(587/732)	73.61(265/360)		
Dosage of Gn activation	200（187.5,225）	225(187.5,262.5)	-2.63	0.009
Total dosage of Gn used	2025（1687.5,2550.0）	2100(1687.5,2700)	-1.51	0.131
Duration of Gn used (d)	9（9,10）	9(8,10)	-1.82	0.068
Antagonist drug % (n)				
Cetrorelix	86.48(633/732)	89.72(323/360)	2.33	0.13
Ganirelix	13.52(99/732)	10.28(37/360)		
Antagonist initiation dose	0.125（0.125,0.25）	0.125（0.125,0.25）	-1.97	0.049
Total dosage of GnRHant used (mg)	0.625（0.5，1.0）	0.625（0.5，0.875）	-4.84	＜0.001
Total days of GnRHant used(d)	4（4,5）	4(3,5)	-4.67	＜0.001
Maximum FSH value at COS	16.975 （14.478,20.218）	17.97(15.30,21.90)	-3.91	＜0.001
The maximum value of	5.12（3.81,7.32）	4.72(3.45,6.46)	-2.60	0.009

LH at cos				
The lowest LH value at cos	1.33（0.94,1.99）	1.34(0.93,1.92)	-0.71	0.478
The maximum value of P at cos	1.34（1.04,1.74）	1.31(1.03,1.68)	-0.62	0.534
E_2 level on HCG day	3289.1（2039.05,4908.0）	2359.15(1570.60,3979.15)	-6.50	＜0.001
LH level on HCG day	2.14（1.43,3.38）	2.32(1.43,3.40)	-0.78	0.437
P level on HCG day	1.2（0.90,1.61）	1.15(0.85,1.56)	-1.85	0.065
E2/ number of follicles ≥14mm on HCG day	277.94（215.14,351.83）	275.23(203.75,360.26)	-0.40	0.691
Trigger mode % (n)				
Single trigger	25.41(186/732)	35.83(129/360)	12.78	＜0.001
Double trigger	74.59(546/732)	64.17(231/360)		

3.Comparison of Ovulation Induction Laboratory Outcomes Between the Optimal Embryo Group and the Non-optimal Embryo Group

The number of punctured follicles, the number of harvested oocytes, the rate of harvested oocytes, the number of mature oocytes (MII), the rate of mature oocytes, the number of normal fertilization, the rate of normal fertilization and the number of cleavage in the optimal embryo group were significantly higher than those in the non-optimal embryo group, and the difference was statistically significant ($P < 0.001$).There were no significant differences in forward motile sperm count (PR) and fertilization method between the two groups on the fertilization day ($P > 0.05$).

Tab3 Analysis of ovulation induction laboratory outcomes between the two groups

	high quality embryo group	Non-high quality embryo group	X^2/Z	P
Number of follicles punctured	13.5（9,19.75）	11(7,14)	-7.70	＜0.001

Number of oocytes retrieved	10（7,14）	7(4,11)	-8.98	＜0.001
Rate of oocytes retrieved (%)	74.46(8197/11008)	69.85(2864/4100)	32.38	＜0.001
Mature oocytes (MII)	7（5,10）	4(2,7)	-12.06	＜0.001
Rate of MII % (n)	71.04(5823/8197)	62.01(1776/2864)	80.43	＜0.001
Total number of PR sperm after treatment	7.30（5.22，9.94）	6.79(4.70,9.95)	-1.20	0.23 1
Fertilization method % (n)				
IVF	79.37(581/732)	74.44(268/360)	3.39	0.07
ICSI	20.63(151/732)	25.56(92/360)		
Nnormal fertilized oocyte rate(2PN ,%)	7（5,10）	4(2,6)	-12.50	＜0.001
	72.24(5628/7791)	64.69(1676/2591)	53.16	＜0.001
Cleavage number	8（6,11）	5(3,7)	-11.88	＜0.001

4. Logistic Regression Analysis of Factors Related to the Formation of High-quality Embryos

Took the acquisition of high-quality embryos as the dependent variable, There would bE_2 statistical differences ($P < 0.05$) variables: female age, male age, AMH, AFC, bE_2, proportion of bLH, EMS, proportion of PCOS, proportion of DOR, proportion of male factors, whether rFSH was combined with HMG at the initiation, amount of Gn initiation, total amount of antagonist, total number of days of antagonist, maximum value of FSH during COS, maximum value of LH during COS, and HCG day E_2, trigger method, number of punctured follicles, number of harvested oocytes, number of mature oocytes, number of normal fertilization, number of cleavage, etc.

Table 4 Logistic univariate regression analysis of factors related to high quality embryo genesis

Index	*OR*	*95%CI*	***P***
Female age	0.806	0.599~1.085	0.155
Male age	0.740	0.474~1.155	0.184

AMH	1.864	1.443-2.410	＜0.001
AFC	1.831	1.419-2.362	＜0.001
bE_2	0.754	0.585-0.971	0.029
bLH	1.0	0.777-1.287	1.0
Number of cycles	0.954	0.692-1.314	0.773
Dosage of Gn activation	1.034	0.803-1.330	0.798
Total dosage of GnRHant used (mg)	1.089	0.841-1.409	0.519
Total days of GnRHant used(d)	1.086	0.808-1.460	0.584
Maximum FSH value at COS	1.057	0.821-1.360	0.668
The maximum value of LH at cos	1.161	0.902-1.495	0.247
E_2 level on HCG day	1.123	0.872-1.446	0.368
EMS or not	1.103	0.762-1.597	0.604
PCOS or not	0.953	0.690-1.318	0.773
DOR or Not	1.148	0.747-1.764	0.529
Male factor	0.891	0.660-1.202	0.449
Whether to add HMG when starting	1.250	0.916-1.706	0.159
Single trigger or Not	0.971	0.734-1.283	0.834
Number of follicles punctured	2.260	1.747-2.923	＜0.001
Number of oocytes retrieved	2.459	1.896-3.190	＜0.001
MII number	3.864	2.963-5.040	＜0.001
2PN number	4.259	3.253-5.576	＜0.001
Cleavage number	3.425	2.625-4.468	＜0.001

The variable with difference was analyzed univariate: AMH, AFC, basal E_2,

number of stimulated follicles, number of harvested oocytes, number of mature oocytes, number of normal fertilization and number of cleavage were independent variables, and the median of the total sample of independent variables was controlled. Multivariate Logistic regression analysis showed that AMH, AFC and number of normal fertilization were independent factors influencing whether the antagonist program could obtain high-quality embryos (*OR* > 1, *P* < 0.05).Patients with AMH≥4.07ng/ mL and AFC≥16 were 1.4 times more likely to obtain high-quality embryos than those with AMH < 4.07ng/ml and AFC < 16.2PN number ≥6 significantly increased the rate of high-quality embryos (*OR* 4.969, *P* <0.001).

Table 5 Logistic multifactor regression analysis of factors related to high quality embryogenesis

Index	Population median	*OR*	*95%CI*	***P***
AMH	4.07	1.434	1.035-1.986	0.030
AFC	16	1.457	1.054-2.013	0.023
bE2	27.8	0.905	0.688-1.189	0.473
Number of follicles	12			
punctured		0.973	0.642-1.474	0.898
Number of oocytes	9			
retrieved		0.849	0.524-1.375	0.506
MII number	6	0.789	0.352-1.766	0.564
	6		2.095-11.78	
2PN number		4.969	7	＜0.001
Cleavage number	7	1.207	0.687-2.121	0.512

DISCUSSION

Obtaining high quality embryos is the key to the success of assisted reproductive technology.At present, antagonist program has become the mainstream ovulation induction program in IVF treatment, and ovulation induction program is the key

factor to obtain high-quality embryos and increase the clinical pregnancy rate.In this study, the proportion of high-quality embryo gainers and non-gainers in patients with GnRH-antagonist protocol was 67.03% (732/1092) and 32.97% (360/1092) respectively, that is, about one-third of patients failed to obtain high-quality embryos. What are the related factors of lack of high-quality embryos?

From the perspective of ovarian function of patients, the commonly used indexes for evaluating ovarian reserve function at present include AMH, AFC, basal FSH and basal E_2, age, ovarian surgery history, etc.In this study, patients were divided into excellent embryo group and no excellent embryo group according to whether they obtained high-quality embryos. Logistic regression analysis results showed that patients with AMH≥4.07ng/ mL obtained high-quality embryos were 1.43 times higher than those with AMH < 4.07ng/ml (*OR*1.434, $P < 0.05$), and patients with AFC≥16 had a 1.46 times higher probability of obtaining high-quality embryos than those with AFC < 16 (*OR*1.457, $P < 0.05$).There was no significant difference in basal FSH between the two groups.In this study, the age of optimal embryo group and basal E_2Although the difference was lower than that of the non-optimal embryo group, the correlation was not significant after regression analysis. Age, base E_2It was not an independent factor affecting the acquisition of high-quality embryos of antagonists, and may be related to the fact that the mean age of patients in both groups was less than 35 years old.Therefore, it can be speculated that whether the antagonist program can obtain high-quality embryos is positively correlated with AMH and AFC in the ovarian function indexes of patients. Patients with high AMH and more AFC may have better ovarian reactivity, and the probability of obtaining high-quality embryos is increased.

From the perspective of infertility factors, although infertility factors are not independent factors of whether the antagonist program can obtain high-quality embryos, there is a statistical difference in the infertility factors between the optimal embryo group and the non-optimal embryo group in this study.EMS, DOR and male factor combined patients had a lower rate of optimal embryo in GnRH-antagonist

protocol, while PCOS patients had a higher rate of optimal embryo in GnRH-antagonist protocol.Some scholars have proposed that the GnRH-antagonist protocol is the first-line treatment of COS in PCOS patients. The pregnancy rate of GnRH GnRH-antagonist protocol in PCOS patients is similar to that of GnRH agonist regimen, and the incidence of OHSS complications in the GnRH-antagonist protocol is lower[8].Patients with endometriosis may be better candidates for GnRHa agonist regiments.It has been reported that for young DOR patients, GnRH-a agonist has a lower ET cancellation rate and a higher implantation rate and live birth rate than GnRH-ant[9].

According to COS process analysis, gn-initiated drugs with a high proportion of rFSH alone had a higher rate of optimal embryos, while rFSH+HMG had a lower probability of obtaining optimal embryos.These results suggest that exogenous FSH alone may be sufficient to promote follicular development, while hMG may not improve embryo status at all ages.This is consistent with literature reports[10-11].In this study, Gn activation was smaller in the optimal embryo group, which may be related to better ovarian function in the optimal embryo group.The total amount and days of use of antagonists in the optimal embryo group were higher than those in the non-optimal embryo group.This is inconsistent with the report that "duration of antagonist has no effect on treatment outcome"[12]A.In this study, the maximum VALUE of FSH in the optimal embryo group was 16.97 (14.478,20.218) mIU/ mL lower than that in the non-optimal embryo group, and the maximum value of LH was 5.12 (3.81,7.32) mIU/ mL higher than that in the non-optimal embryo group.The development of oocytes requires the synergistic action of FSH and LH, indicating that excessive FSH may damage egg quality, and appropriately high LH is beneficial to improve egg quality and embryo quality.Optimal embryo group trigger date E_2Higher, so that the optimal embryo group egg capture rate is high. The rate of high quality embryos obtained by GnRHa combined with hCG was higher than that obtained by single trigger.Through the "dual effect" of endogenous LH and exogenous hCG LH,

double trigger may improve the problem of low egg maturation rate in some patients treated with hCG or GnRHa alone[13-16].

This study is the first to compare whether female triglyceride, fasting blood glucose, male sperm normal morphology rate and DFI are related to the formation of high-quality embryos when using GnRH-antagonist protocols, and the results show no significant differences.

The results of ovulation induction showed that the number of punctured follicles, obtained oocytes, MII, 2PN and cleavage in the optimal embryo group were significantly higher than those in the non-optimal embryo group.Logistic regression analysis showed that 2PN number was an independent factor influencing the formation of no high-quality embryos (*OR* 4.969, *95%CI* 2.095-11.787, *P* <0.001). 2PN rate was significantly positively correlated with the rate of high-quality embryos.As we all know, 2PN rate reflects oocyte quality, laboratory culture conditions and operation techniques to a certain extent.Therefore, clinicians and laboratory embryologists should strengthen exchanges and cooperation, do a good job in quality control, and provide guarantee for obtaining more 2PN embryos, so as to lay a good foundation for obtaining high-quality embryos.

In conclusion, whether the GnRH-antagonist protocol can obtain high-quality embryos is not only positively correlated with the ovarian reserve function AMH and AFC, but also positively correlated with the basal E_2, egg number, mature egg number, cleavage number and other factors, especially with 2PN number, 2PN rate.There was no retrospective analysis in this study, the sample size was small, and stratified analysis of patients' age or infertility factors was not conducted, which had certain limitations. Later, the sample size will be expanded to further clarify the key factors of high-quality embryogenesis and select the population more suitable for the use of GnRH-antagonist protocol in clinical application.

ACKNOWLEDGEMENTS

We thank all the embryologists and nurses of the First Affiliated Hospital of Soochow

University, data recording, and other clinical assistance.

AUTHOR CONTRIBUTIONS

The present work was designed by Weiqin Zhou. Data extraction and analysis were performed by Yanping Pan and Ziwei Zhao. Yanping Pan ,Nan Wang and Zhinan Wu participated in the data collection. Caiping MAO, Fei Xia, Qi He, Weiqin Zhou and Jingfang Liu participated in revisions to the article. All authors have read and approved the final manuscript.

FUNDING

There was no funding for this study.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author or first author on reasonable request.

DECLARATIONS

Consent for publication

Competing interests

The authors declare that they have no conflict of interest. There was not any company involved in this project.

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