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Elevated follicular cortisone level is a negative predictor of clinical pregnancy in women undergoing fresh embryo transfer

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ABSTRACT

Background: Although numerous studies have investigated the potential correlation between follicular fluid (FF) steroid concentrations and *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes, few have accounted for the effect of controlled ovarian hyperstimulation regimes on FF steroid concentrations.

Objective: To comprehensively compare follicular steroid concentrations between women stimulated with gonadotropin-releasing hormone agonist (GnRHa) and antagonist (GnRHant) protocols and to explore the associations between FF steroid concentrations and IVF/ICSI outcomes.

Methods: A total of 295 infertile women undergoing IVF/ICSI from January 2018 to May 2020 were enrolled. Eighty-four and 211 women received GnRHa and GnRHant protocols, respectively. Seventeen steroids in FF were quantified by liquid chromatography tandem mass spectrometry (LC–MS/MS), and the correlation of follicular steroids with clinical pregnancy was explored.

Results: Follicular steroid concentrations were similar between the GnRHa and GnRHant groups. Follicular cortisone levels were adversely associated with clinical pregnancy in fresh embryo transfers. Receiver operating characteristic (ROC) analysis revealed an area under the ROC curve (AUC) of 0.639 (95% confidence interval = 0.527–0.751, $p = 0.025$) for predicting non-pregnancy, with an optimal cutoff value of 15.81 ng/mL (sensitivity = 33.3%, specificity = 94.1%). Women with FF cortisone concentrations ≥15.81 ng/mL were fifty times less likely to achieve clinical pregnancy in fresh embryo transfers than those with FF cortisone levels below this threshold (adjusted OR = 0.019, 95% confidence interval = 0.002–0.207, $p = 0.001$) after adjusting for age, body mass index, baseline serum progesterone levels, serum levels of luteinizing hormone, estradiol and progesterone on human chorionic gonadotropin day, ovarian stimulation protocols, and the number of transferred embryos.

Conclusions: There was no significant difference in intrafollicular steroid levels between GnRHa and GnRHant protocols, and intrafollicular cortisone level ≥15.81 ng/mL was found to be a strong negative predictor of clinical pregnancy in fresh embryo transfers with high specificity.

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1. Introduction

The follicular fluid (FF) provides a microenvironment critical for oocyte development, maturation and quality [1,2]. It is composed of plasma exudates and secretions by granulosa cells and theca cells under the regulation of gonadotropins. Steroid hormones, integral to FF, play pivotal roles in folliculogenesis and oocyte competence [3]. With the introduction of assisted reproduction technology (ART), FF is routinely available at oocyte retrieval and provides a valuable resource for analyzing intrafollicular hormonal milieu [4,5]. A number of studies [6–10] have analyzed FF samples from women undergoing controlled ovarian hyperstimulation (COH) in an attempt to identify a relationship between steroid concentrations and *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes. However, the results of these studies are inconsistent due to the diversity of COH protocols and the possible inaccuracy of immunoassays for steroids quantification.

Gonadotropin-releasing hormone (GnRH) agonists (GnRHa) and antagonists (GnRHant) have been widely used to prevent premature luteinization during COH for IVF/ICSI [11]. GnRHa suppress gonadotrophin secretion through pituitary desensitization and GnRH receptor depletion by long-term administration, whereas GnRHant rapidly inhibit gonadotrophin secretion by competitively blocking the GnRH receptors [12]. GnRH receptors are also expressed in extrapituitary tissues such as ovary [13,14]. In addition, GnRH may act as an autocrine factor by stimulating the mitogen-activated protein kinases in human granulosa cells [15]. Given the different mechanisms of action of GnRHa and GnRHant, it is unclear whether these agents could disrupt autocrine and paracrine signaling of GnRH in human ovarian cells and therefore result in divergent follicular steroid hormonal milieus that could affect IVF/ICSI outcomes. To date, only one study [16] has compared the effects of these two COH strategies on follicular steroids, but the study had a relatively small sample size (20 GnRHa vs. 16 GnRHant) and measured only three steroid hormones using radioimmunoassays. Immunoassays can suffer from cross-reactivity with similar analytes and standardization issues between labs [17], while liquid chromatography tandem mass spectrometry (LC–MS/MS) is increasingly becoming the state-of-the-art method for steroid hormone quantifications due to small sample volumes, fast analysis times, greater sensitivity and specificity, and simultaneous multi-analyte quantitation capability [18]. Therefore, the aims of our study were to comprehensively compare follicular steroid concentrations between women stimulated with GnRHa and GnRHant protocols by quantifying 17 endogenous steroids using LC–MS/MS and to explore the associations between FF steroid concentrations and IVF/ICSI outcomes.

2. Subjects and methods

2.1. Subjects

A total of 295 infertile women who underwent IVF/ICSI using either GnRHa or GnRHant stimulation protocols were recruited from January 2018 to May 2020 at the Reproduction Medicine Center, International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University. This study was approved by the Ethics Committee (No. GKLW2017-81), and informed consent was obtained from all participants. The study included women who met the following inclusion criteria: 1) female, aged between 20 and 43 years; 2) regular menstrual cycles; 3) infertility with male factor, tubal factor, or unexplained etiology; and 4) both ovaries present, with no morphological abnormalities. Participants were excluded if they had any of the following: 1) chromosomal abnormalities; 2) premature ovarian failure, endometriosis, uterine abnormalities, gynecological tumors, or a history of ovarian surgery; 3) endocrine abnormalities, such as polycystic ovary syndrome, hyperprolactinemia, thyroid dysfunction, and hypopituitarism; 4) serious and unstable diseases, such as cardiovascular, liver, or kidney disease; and 5) a history of failed IVF/ICSI cycles.

2.2. Ovarian stimulation protocols and IVF/ICSI outcomes assessment

The COH protocols for the patients were determined by a physician to either GnRHa long protocol or GnRHant protocol. For the GnRHa long protocol, the GnRHa triptorelin (Decapeptyl, 0.1 mg/d; Ferring, Malmo, Sweden) was started at the mid-luteal phase of the preceding cycle. After pituitary down-regulation, gonadotropin (Gn) was added until the day of human chorionic gonadotropin (hCG) administration. For the GnRHant protocol, Gn was started on the 2nd or 3rd day of the menstrual cycle without previous oral contraceptive pretreatment. The GnRH antagonist cetrolelix (Cetrotide, Merck-Serono, Switzerland) was added at a dose of 0.25 mg per day starting when the leading follicle reached a diameter of 14 mm and continued until the day of hCG administration. For both protocols, hCG was subcutaneous administered to trigger ovulation when two or more follicles reached a diameter of $\geq$18 mm, or when three or more follicles reached a diameter greater than 17 mm. Oocyte retrieval was performed under transvaginal ultrasound guidance 34–36 h after the hCG administration. Retrieved oocytes were fertilized *in vitro* by either conventional insemination or ICSI depending on semen quality [19]. Fertilization results were assessed 18–20 h after insemination, and successful fertilization was considered when two pronuclei were present. The resulting embryos were monitored for cell number and morphological quality as previously described [20] every day. Embryo grade was determined by a team of experienced embryologists according to the Istanbul consensus workshop on embryo assessment [21]. Useable embryos were day 3 embryos with $\geq$6 blastomeres, <35% fragments, and without multinucleation. High-quality embryos were those embryos with an even cleavage, 7–9 blastomeres, and <10% fragments on day 3. Only high-quality and useable embryos were transferred or cryopreserved. Embryos not eligible for transfer or cryopreservation on the cleavage stage were cultured up to day 6. Grading of blastocyst was performed according to Gardner blastocyst grading system [22,23], and only blastocysts categorized as type A or B were chosen for transfer or cryopreservation. The oocyte utilization rate was calculated by the number of useable embryos divided by the number of oocytes retrieved, and the high-quality embryo rate was

calculated by the number of high-quality embryos divided by the number of oocytes retrieved.

Fresh or frozen embryo transfers were performed on the patients as clinically indicated [24]. The number of embryos transferred was determined according to the American society for Reproductive Medicine's embryo transfer guidelines [25]. The pregnancy outcomes of the first embryo-transfer were evaluated in this study. We defined biochemical pregnancy as serum β-hCG >100 IU/L fourteen days after transfer and clinical pregnancy as the presence of fetal cardiac activity on an ultrasound examination 4–5 weeks after transfer. Implantation rate was defined as the number of gestational sacs per the number of embryos transferred.

2.3. Collection and processing of follicular fluid samples

The follicular fluid (FF) samples used in this study were collected from the first aspirated ovarian follicle measuring 16–18 mm in diameter during oocyte retrieval. Only FF samples from follicles without visible blood contamination were used. The collected FF was transferred into sterile polypropylene tubes and centrifuged at 1200 rpm for 10 min to isolate FF from cellular components and debris. The supernatant was then stored at −80 °C until analysis.

Table 1
Demographic and clinical characteristics of women undergoing IVF/ICSI according to COH protocols.

	GnRH agonist (n = 84)	GnRH antagonist (n = 211)	*P* value
Age (years)	31 (29, 33)	31 (28, 34)	0.736
BMI (kg/m^2)	21.6 (20, 24.3)	21.5 (19.6, 23.7)	0.470
Cycle length (days)	30 (28, 30)	30 (28, 32)	0.402
Duration of infertility (years)	2.75 (1.5, 4)	3 (2, 4)	0.876
Etiology of infertility			0.101
Male factor (n, %)	29 (34.5%)	65 (30.8%)	
Tubal (n, %)	54 (64.3%)	130 (61.6%)	
Unexplained (n, %)	0 (0%)	16 (7.6%)	
Types of infertility			0.576
Primary infertility (n, %)	48 (57.1%)	113 (53.6%)	
Secondary infertility (n, %)	36 (42.9%)	98 (46.4%)	
Baseline serum FSH (IU/L)	6.9 (5.7, 8.2)	7.8 (6.4, 9.0)	**0.001**
Baseline serum LH (IU/L)	3.75 (2.7, 5)	4.1 (2.9, 5.4)	0.190
Baseline serum FSH/LH	0.54 (0.38, 0.76)	0.52 (0.37, 0.71)	0.508
Baseline serum PRL (μg/L)	12.61 (9.45, 17.53)	13.45 (10.6, 17.13)	0.423
Baseline serum E2 (pmol/L)	145.5 (94, 193.5)	144 (98, 193)	0.992
Baseline serum T (nmol/L)	1.3 (0.8, 1.8)	1.5 (1.025, 1.8)	0.133
Baseline serum P (nmol/L)	1.2 (0.7, 2.0)	1.5 (0.9, 1.95)	0.105
Serum AMH (ng/mL)	4.37 (2.71, 6.26)	4.11 (2.43, 5.89)	0.590
AFC (n)	11 (9, 14)	10 (7, 13)	**0.004**
Dose of gonadotropins used (IU)	2381 (2025, 2925)	2100 (1763, 2625)	**0.002**
Duration of COH (days)	10 (9, 12)	9 (8, 10)	**0.000**
Serum LH on HCG day (IU/L)	1.5 (0.7, 2.5)	2 (1.1, 3.3)	**0.001**
Serum E2 on HCG day (pmol/L)	10198 (6429, 15007)	9741 (5982, 15237)	0.738
Serum P on HCG day (nmol/L)	2.95 (2.00, 4.68)	3.1 (2.1, 4.4)	0.660
EMT on HCG day (mm)	10 (9, 12)	10 (9, 11.5)	0.116
Treatment type			0.490
IVF (n, %)	60 (71.4%)	139 (65.9%)	
ICSI (n, %)	22 (26.2%)	69 (32.7%)	
Combined IVF and ICSI (n, %)	2 (2.4%)	3 (1.4%)	
Follicles ≥14 mm (n)	10 (8, 14)	10 (6, 12)	0.061
Retrieved oocytes (n)	13 (8, 16)	10 (7, 15)	0.055
Fertilization rate (%)	75.6 (58.4, 89.3)	70.0 (55.6, 85.7)	0.406
Cleavage rate (%)	100 (100, 100)	100 (100, 100)	0.737
Oocyte utilization rate (%)	50 (31.3, 68.0)	44.4 (33.3, 62.5)	0.336
High-quality embryo rate (%)	33.3 (14.3, 50.0)	29.2 (14.3, 50.0)	0.595
Transfer type			0.657
Fresh (n/total, %)	29/78 (37.2%)	68/198 (34.3%)	
Frozen (n/total, %)	49/78 (62.8%)	130/198 (65.7%)	
Number of embryos transferred			0.986
Single-embryo transfer (n/total, %)	46/78 (59.0%)	117/198 (59.1%)	
Double-embryo transfer (n/total, %)	32/78 (41.0%)	81/198 (40.9%)	
Biochemical pregnancy (n/total, %)	44/78 (56.4%)	88/198 (44.4%)	0.073
Clinical pregnancy (n/total, %)	33/78 (42.3%)	70/198 (35.4%)	0.282
Implantation rate (n/total, %)	35/110 (31.8%)	87/279 (31.2%)	0.903

Data are presented as median (25th, 75th percentile) or number (percentage). Bold values indicate statistically significant differences.
Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; E2, estradiol; T, testosterone; P, progesterone; AMH, anti-Müllerian hormone; AFC, antral follicle count; hCG, human chorionic gonadotropin; EMT, endometrial thickness; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

2.4. Hormone measurements

Serum hormones and follicular steroids analysis were performed as previously described [26]. Briefly, serum hormones were analyzed by chemiluminescence immunoassays or enzyme-linked immunosorbent assays (ELISA), while the 17 steroids in FF were quantified by LC-MS/MS.

2.5. Statistical analysis

Normal distribution of continuous data was tested by Kolmogorov–Smirnov test. Non-normally distributed variables were presented as median (interquartile range), while categorical variables were expressed as number (percentage). Comparisons between groups were appropriately made using Mann-Whitney *U* test or Kruskal-Wallis test for continuous variables, and chi-square test or Fisher's exact test for categorical variables. Bonferroni correction was performed to adjust for multiple comparison testing. Receiver operating characteristic (ROC) curves were generated to evaluate the predictive value of follicular cortisone concentrations on non-pregnancy after fresh embryo transfer. The cutoff value was obtained using Youden Index, which is defined as the maximum sum of sensitivity and specificity minus one. Binary logistic regression with a forward stepwise (likelihood ratio) method were performed to assess the odds ratio (OR) of FF cortisone levels on the likelihood of clinical pregnancy.

Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS software version 22.0.

3. Results

3.1. FF steroid concentrations were comparable between GnRHa and GnRHant protocols

Demographic, baseline, and treatment-related characteristics of women undergoing GnRHa and GnRHant protocols are shown in Table 1. A total of 295 women were enrolled in this study, with 84 women receiving GnRHa protocol and 211 receiving GnRHant protocol. Of those, 97 patients received fresh embryo transfer, 179 received frozen embryo transfer, and 19 patients did not undergo embryo transfer. Among those who did not undergo this procedure, 3 were due to lack of fertilization, 5 were due to no useable embryos, and 11 were due to personal affairs. No significant difference was observed between the GnRHa and GnRHant groups in terms of demographic features such as age and body mass index (BMI), nor in the distribution of infertility characteristics, such as infertility duration, etiology, and types. Baseline serum follicle-stimulating hormone (FSH) concentrations were lower in the GnRHa group compared to the GnRHant group, while baseline luteinizing hormone (LH) levels, serum LH/FSH concentrations ratio, and other hormone levels were comparable between the two groups. Compared with GnRHant group, women in GnRHa group presented significantly higher antral follicle count, received a higher gonadotropins dosage and had a longer duration of COH and significantly lower serum LH levels on hCG day. The distributions in treatment type, transfer type and the number of embryos transferred were similar in both groups. No significant difference was observed in IVF/ICSI outcomes, such as the number of oocytes retrieved, fertilization rate, oocyte utilization rate, high-quality embryo rate, pregnancy rate, and implantation rate between the two groups.

Table 2
Follicular fluid steroid concentrations of women undergoing GnRH agonist and antagonist protocols.

Steroids	GnRH agonist (n = 84)	GnRH antagonist (n = 211)	*P* value
Pregnenolones			
Pregnenolone	117.6 (61.359, 222.811)	128.346 (74.394, 263.421)	0.207
17OH-Pregnenolone	7.470 (5.722, 11.667)	8.180 (6.007, 12.935)	0.433
Progestins			
Progesterone	119.724 (94.808, 190.091)	131.631 (92.889, 189.068)	0.939
17OH-Progesterone	673.2 (569.3, 802.7)	689.0 (548.9, 815.0)	0.780
Androgens			
DHEAS	1049.4 (807.0, 1342.6)	1049.0 (800.0, 1406. 2)	0.908
Androstenedione	0.702 (0.225, 4.474)	1.093 (0.339, 5.283)	0.105
Testosterone	0.054 (0.016, 0.239)	0.07 (0.021, 0.315)	0.279
DHT	0.089 (0.044, 0.225)	0.120 (0.066, 0.203)	0.150
Estrogens			
Estrone	28.85 (18.74, 43.63)	31.64 (19.79, 44.49)	0.639
Estradiol	602.2 (380.5, 824.3)	613.4 (451.7, 813.6)	0.504
Estriol	3.524 (2.559, 5.727)	3.890 (2.720, 5.547)	0.696
Glucocorticoids			
11-Deoxycortisol	2.108 (1.238, 3.163)	2.353 (1.568, 3.196)	0.283
Cortisol	52.38 (42.23, 63.34)	53.39 (45.13, 64.09)	0.654
Cortisone	12.83 (10.47, 16.33)	13.06 (10.93, 16.03)	0.564
Mineralocorticoids			
11-Deoxycorticosterone	32.44 (27.37, 38.60)	32.47 (26.60, 40.60)	0.692
Corticosterone	2.146 (1.695, 2.899)	2.430 (1.813, 3.079)	0.204
Aldosterone	0.023 (0.012, 0.031)	0.023 (0.015, 0.033)	0.224

Data are presented as median (25th, 75th percentile) in ng/mL.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone.

FF steroid concentrations in the GnRHa and GnRHant groups are presented in Table 2. No significant difference was found between the values. Steroid enzyme activities in the steroidogenic pathway assessed by product-to-precursor concentration ratios as previously reported [26] did not differ between the two groups either (Supplementary Table 1). To control for the possible effect of age on endocrine hormones, we additionally analyzed the FF steroid hormone levels separately for women aged ≥38 years and <38 years in both the GnRHa and GnRHant groups. Clinical and treatment-related characteristics are presented in Supplementary Table 2. Though some minor differences were observed among the four groups, no significant difference in FF steroid hormone levels were found between the GnRHa and GnRHant groups within the same age range, as shown in Supplementary Table 3. Therefore, the following analysis of the relationship between FF steroid hormones levels and IVF/ICSI outcomes were not stratified by COH regimens.

3.2. *Follicular cortisone level is a negative predictor of clinical pregnancy in women undergoing fresh embryo transfer*

Thirty-four out of 97 (35.1%) women undergoing fresh embryo transfer have achieved clinical pregnancy, with lower basal serum progesterone levels and serum LH levels on hCG day, and a higher proportion of GnRHa protocol and double-embryo transfer compared to those not pregnant (Supplementary Table 4). Follicular cortisone levels were significantly lower in pregnant women than that in non-pregnant women (Table 3), and this difference remained significant (adjusted OR = 0.781, 95% confidence interval (CI) = 0.667–0.915, $p = 0.002$) after adjusting for age, BMI, baseline serum progesterone levels, serum LH levels on hCG day, serum E2 and progesterone levels on hCG day, COH protocols, and the number of embryos transferred. In women undergoing frozen embryo transfer, sixty-nine out of 179 (38.5%) have achieved clinical pregnancy, with a slightly lower serum prolactin (PRL) levels, a lower percentage of singleton embryo transfer, and lower concentrations of follicular dehydroepiandrosterone sulfate (DHEAS) compared to non-pregnant women (Supplementary Tables 5 and 6). However, after adjusting for age, BMI, baseline serum PRL levels, the number of embryos transferred, and serum LH, E2, and progesterone levels on hCG day, the concentrations of follicular DHEAS were not found to be significantly different between pregnant and non-pregnant women (adjusted OR = 1.000, 95% CI = 1.000–1.000, $p = 0.946$). This suggests that follicular DHEAS may not be a significant factor in clinical pregnancy outcomes in frozen embryo transfer.

To further investigate the effect of follicular cortisone concentrations on clinical pregnancy rates, patients were subdivided into four groups according to their FF cortisone concentration quartiles (Q1-Q4): Q1 group, cortisone ≤10.78 ng/mL; Q2 group, 10.78 < cortisone ≤12.96 ng/mL; Q3 group, 12.96 < cortisone ≤16.17 ng/mL; and Q4 group, cortisone >16.17 ng/mL. Demographic data, baseline hormone levels, COH parameters and embryo transfer characteristics were similar across the quartiles for FF cortisone concentrations, except for lower basal serum PRL levels and stimulated serum progesterone levels in the Q1 group (Supplementary Table 7). The clinical pregnancy rates for women undergoing fresh embryo transfer were 46.7%, 36.8%, 40.7% and 9.5% in the Q1, Q2, Q3 and Q4 groups, respectively (Fig. 1), and the difference was statistically significant ($p = 0.042$). Clinical pregnancy rates for women undergoing frozen embryo transfer were 36.6%, 35.6%, 40.0% and 41.7% in the Q1-Q4 groups, respectively (Fig. 1), with no significant difference ($p = 0.925$) among the four groups. ROC analysis (Fig. 2) showed the area under the ROC curve (AUC) of FF cortisone concentrations for predicting non-pregnancy in women undergoing fresh embryo transfer was 0.639 (95% CI = 0.527–0.751,

Table 3
Follicular fluid steroid concentrations of women with and without clinical pregnancy after fresh embryo transfer.

Steroids	Clinical pregnancy (n = 34)	No clinical pregnancy (n = 63)	*P* value
Pregnenolones			
Pregnenolone	74.057 (34.063, 222.613)	122.77 (71.986, 223.449)	0.067
17OH-Pregnenolone	6.804 (5.898, 10.135)	8.892 (6.316, 13.878)	0.107
Progestins			
Progesterone	110.8 (88.2, 171.0)	132.4 (92.4, 187.6)	0.634
17OH-Progesterone	762.0 (589. 6, 859.7)	740.3 (560.4, 797.5)	0.345
Androgens			
DHEAS	907.4 (777.0, 1072.4)	1050.1 (753.8, 1383.4)	0.341
Androstenedione	0.358 (0.162, 3.423)	0.716 (0.281, 1.913)	0.107
Testosterone	0.026 (0.009, 0.223)	0.044 (0.02, 0.101)	0.082
DHT	0.128 (0.048, 0.217)	0.089 (0.053, 0.192)	0.892
Estrogens			
Estrone	28.976 (19.189, 45.765)	24.89 (18.49, 43.598)	0.661
Estradiol	610.5 (364.5, 777.2)	560.5 (426.0, 806.0)	0.874
Estriol	3.706 (2.565, 5.243)	3.779 (2.586, 5.183)	0.982
Glucocorticoids			
11-Deoxycortisol	1.852 (1.233, 2.796)	2.372 (1.504, 3.24)	0.121
Cortisol	45.044 (40.055, 50.893)	49.095 (40.325, 58.654)	0.072
Cortisone	11.78 (8.853, 13.614)	13.633 (10.749, 16.432)	**0.025**
Mineralocorticoids			
11-Deoxycorticosterone	32.33 (27.156, 38.135)	33.725 (29.654, 41.416)	0.183
Corticosterone	2.009 (1.483, 2.544)	2.399 (1.67, 3.158)	0.171
Aldosterone	0.021 (0.011, 0.028)	0.026 (0.015, 0.034)	0.153

Data are presented as median (25th, 75th percentile) in ng/ml. Bold values indicate statistically significant differences.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone.

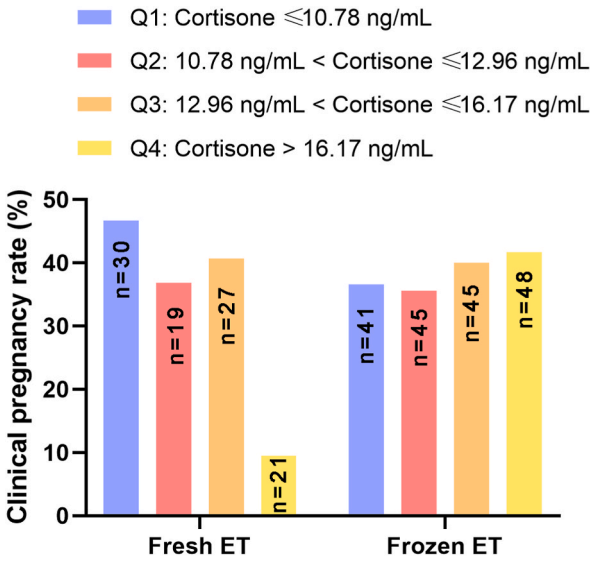

Fig. 1. Clinical pregnancy rates in fresh/frozen embryo transfer across FF cortisone quartiles.
Clinical pregnancy rates across FF cortisone quartiles were statistically different in fresh embryos transfers ($p = 0.042$), but comparable in frozen embryo transfers ($p = 0.925$).
Abbreviations: ET, embryo transfer.

$p = 0.025$), with an optimal cutoff value of 15.81 ng/mL (sensitivity = 33.3%, specificity = 94.1%). Among the women with follicular cortisone concentrations >15.81 ng/mL, only two out of 23 achieved clinical pregnancy, whereas 32 out of 74 women with FF cortisone concentrations <15.81 ng/mL achieved clinical pregnancy. Logistic analysis (Table 4) revealed that women with FF cortisone concentrations ≥15.81 ng/mL were eight times less likely to achieve clinical pregnancy after fresh embryo transfer compared to those with FF cortisone concentrations <15.81 ng/mL (unadjusted OR = 0.125, 95% CI = 0.027–0.572, $p = 0.007$). After adjusting for age, BMI, baseline serum progesterone levels, serum LH levels on hCG day, serum E2 and progesterone levels on hCG day, COH protocols, and the number of embryos transferred, the odds of clinical pregnancy in women with FF cortisone levels ≥15.81 ng/mL were 50 times less than those with levels below this threshold (adjusted OR = 0.019, 95% CI = 0.002–0.207, $p = 0.001$).

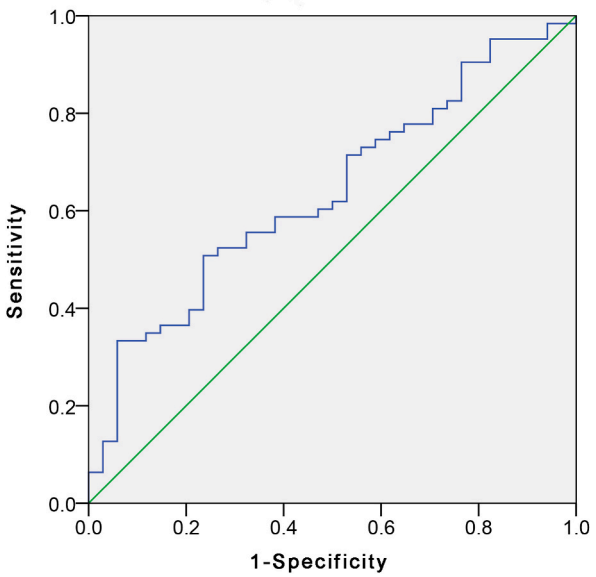

Fig. 2. ROC curve of FF cortisone levels on the probability of non-pregnancy in fresh embryo transfer.
The area under curve (AUC) was 0.639 (95% confidence interval = 0.527–0.751, $p = 0.025$). The optimal cutoff value of FF cortisone levels was 15.81 ng/mL (sensitivity = 33.3%, specificity = 94.1%).

Table 4
Logistic models of follicular cortisone concentrations predicting pregnancy in women undergoing fresh embryo transfer.

	Unadjusted model		Adjusted model[a]	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Cortisone <15.81 ng/mL	1	N/A	1	N/A
Cortisone ≥15.81 ng/mL	0.125 (0.027–0.502)	0.007	0.019 (0.002–0.207)	0.001

Abbreviations: OR, Odds ratio; CI, confidence interval; N/A, not applicable.
[a] Adjusted for age, BMI, baseline serum progesterone levels, serum LH, E2 and P levels on hCG day, COH protocol, and the number of embryo transferred.

4. Discussion

Numerous studies [6,9,27] have attempted to identify an association between intrafollicular steroid levels and IVF/ICSI outcomes. However, few studies have investigated the FF steroid concentrations under different ovarian stimulation protocols. Given the conflicting findings regarding the relationship of FF steroid levels and IVF/ICSI outcomes, we simultaneously measured 17 steroid hormones in the steroidogenic pathway using LC-MS/MS to evaluate whether ovarian steroidogenesis is affected by COH protocols. To the best of our knowledge, this is the first study to comprehensively compare FF steroid concentrations by LC-MS/MS between GnRH agonist and antagonist regimes, the most used COH protocols for IVF/ICSI.

In the present study, the baseline characteristics, ART treatment type and embryo transfer type were comparable between GnRHa and GnRHant protocols except for a lower baseline serum FSH levels in the GnRHa group. However, the ratios of LH/FSH concentrations were similar between the two groups. IVF/ICSI outcomes, specifically the number of oocytes retrieved, fertilization rate, and pregnancy rate, were similar in both protocols in our study, supporting the comparable IVF/ICSI outcomes between the two protocols as previously reported [28–30]. As for FF steroid concentrations, no significant difference was observed between the two protocols, indicating the intrafollicular steroid environment is not affected by the type of GnRH analogues (agonist or antagonist) during COH, which could explain the comparable IVF/ICSI outcomes between the two COH protocols to some extent. These results are in line with previous studies [31,32], demonstrating that treatment with either GnRHa or GnRHant does not significantly affect ovarian steroidogenesis *in vivo* or *in vitro*. However, Garcia-Velasco et al. [16] reported that patients treated with GnRHant showed lower intrafollicular estradiol levels than those treated with GnRHa. This inconsistency may be due to the small sample size and the use of radioimmunoassays for steroids quantification in their study.

Given the similar effect of GnRHa and GnRHant on ovarian steroidogenesis, the following analysis of relationship between FF steroid levels and IVF/ICSI outcomes was not stratified by COH protocols. An interesting finding of this analysis was that elevated FF cortisone concentrations were detrimental to pregnancy outcomes in fresh embryo transfers. Grouped by quartiles of FF cortisone concentrations, the clinical pregnancy rates of fresh embryo transfer in the Q1, Q2, and Q3 groups were around 40%, and plummeted to 9.5% in the Q4 group. Given the significant difference of basal serum progesterone levels between pregnant and non-pregnant women undergoing fresh embryo transfer, correlation between FF cortisone levels and basal serum progesterone levels was investigated, and no significant correlation ($r = 0.110$, $p = 0.320$) was observed, suggesting that the adverse effects of FF cortisone on pregnancy were independent of basal progesterone levels. Consistent with our results, two studies [33,34] have reported clinical pregnancy rates were associated with significantly lower follicular cortisone levels, but the cutoff value was not identified. ROC analysis in our study identified that FF cortisone levels ≥15.81 ng/mL were associated with a fifty-fold decrease in the likelihoods of clinical pregnancy in fresh embryo transfers than levels below that value after adjusting for age, BMI, baseline serum progesterone levels, serum LH levels on hCG day, COH protocols, and the number of embryos transferred. While our sample size was not large, we noted that this cut-off value had an excellent specificity (94.1%) for non-pregnancy in fresh embryo transfer, which suggested that with intrafollicular cortisone level above this threshold, clinical pregnancy in fresh embryo transfer is almost predetermined to fail and that clinicians should consider alternatives.

Implantation is a crucial step in achieving a successful pregnancy, but it can fail due to a range of factors, such as impaired embryo development potential, suboptimal endometrial receptivity, or altered embryo-endometrial dialogue [35]. In the present study, we found that FF steroid hormones levels were not associated with endometrial thickness ($r = -0.135$, $p = 0.211$), the most used marker of endometrial receptivity. Furthermore, clinical pregnancy rates were not affected by intrafollicular cortisone levels in frozen embryo transfers, indicating the embryo development potential was not impaired. Therefore, we speculated that elevated FF cortisone levels may lead to implantation failure by impairing embryo–endometrial dialogue. It is reported that high doses of cortisone was capable of leading to implantation failure by delaying ovo-implantation in the rat [36], corroborating our hypothesis. Additionally, glucocorticoids are reported to affect local inflammation and immune response in the endometrium [37], potentially affecting embryo implantation. The use of glucocorticoids in assisted reproduction to improve the embryo implantation rate has been proposed, though controversial [38–40]. A Cochrane meta-analysis [41] of 13 randomized controlled trials (RCTs) found that glucocorticoid therapy led to no significant improvement in pregnancy rates. However, their subgroup analysis that focused only on fresh IVF cycles showed significantly higher pregnancy rates in glucocorticoid treatment groups [41], supporting the potential effect of glucocorticoids on implantation in fresh embryo transfers together with our results. Our research presents important implications for clinical practice. First and foremost, our findings suggest that FF cortisone concentrations could be closely monitored during IVF/ICSI cycles. Once these concentrations exceed a certain threshold level, a "freeze-all" strategy would be recommended, whereby all embryos are cryopreserved for a future cycle of frozen embryo transfer. Additionally, the results of our study indicate that treatment with glucocorticoids might

improve pregnancy outcomes during fresh embryo transfer. Of course, further studies are warranted to verify our observations and elucidate the underlying mechanisms.

Two limitations of the present study should be mentioned. First, this was an observational study rather than a RCT, which means there may be confounding factors that could influence the results. However, the GnRHa and GnRHant groups had comparable clinical characteristics, such as age, BMI, distribution of infertility etiology, and basal serum hormones, which could minimize the selection bias. Additionally, when investigating the effect of intrafollicular cortisone levels on clinical pregnancy rates, we adjusted for confounding factors potentially affecting clinical pregnancy rates, such as age, BMI, ovarian stimulation regimens, and so on. Second, FF steroid measurements from the first aspirated ovarian follicle were not necessarily representative of other follicles in the ovary, and may not match the fate of the corresponding single oocytes. Therefore, the results of our study have to be interpreted with caution. However, it is impractical to measure steroids from all follicles in clinical routine, and a large overlap and strong correlation was observed between metabolomic features across follicles within a woman [42]. Therefore, the results of the first aspirated ovarian follicle may give us a hint.

To summarize, administration of GnRHa and GnRHant during COH have similar effects on ovarian steroidogenesis. Women with FF cortisone concentrations $\geq$15.81 ng/mL were 50 times less likely to achieve clinical pregnancy in fresh embryo transfers compared to those with FF cortisone levels below this threshold, which may contribute to the criteria for cryopreservation of embryos to be used in future embryo transfers.

Author contribution statement

Zuwei Yang and Jiexue Pan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Chengliang Zhou and Jianzhong Sheng: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Li Jin and Hefeng Huang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17492.

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