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ASSISTED REPRODUCTION TECHNOLOGIES

Delayed blastocyst development is influenced by the level of progesterone on the day of trigger

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Abstract

Purpose To evaluate the association between progesterone (P) level on the day of trigger and time to blastulation in IVF cycles.
Methods This was a retrospective cohort study with autologous IVF cycles performed at our Institution from January 2019 to December 2021. A total of 1109 IVF cycles were included. The primary outcome was to compare time to blastulation in terms of percentage of expanded (grade 3) blastocysts on day 5 according to progesterone level at trigger.
Results A total of 3517 blastocysts were analyzed. After dividing progesterone level in quartiles (Q1, $P < 0.50$ ng/ml; Q2 0.50 ng/ml $\leq P \leq 0.78$ ng/ml; Q3, 0.79 ng/ml $\leq P \leq 1.15$ ng/ml; Q4, $P > 1.15$ ng/ml), we observed a delay in blastocyst development according to the increasing level of progesterone at trigger (analysis by rank, P-value = 0.01). After adjusting for confounding factors at the multivariate analysis, the percentage of day 5 blastocysts was reduced for Q3 (− 13.8%, 95% CI from − 20.5 to − 7.0%, $p < 0.001$) and Q4 (− 7.7%, 95% CI from − 15.5 to 0.0%, $p = 0.05$) compared to Q1 (reference).
Conclusions Progesterone levels on day of trigger correlate to the percentage of expanded (grade 3) blastocysts on day 5 and a delayed blastocyst development day 5 is expected for high progesterone levels.

Keywords Elevated progesterone · Progesterone at trigger · Time to blastulation · day5-blastocyst

Introduction

During the last decades, Premature Progesterone Elevation (PPE), defined as the rise of progesterone (P) serum levels on the day of ovulation trigger during a controlled ovarian stimulation (COS), has been extensively studied in a progressively narrowing interest for its role on IVF outcomes.

Firstly, the role of PPE on endometrial maturation has been elucidated, with consistent evidence of embryo implantation impairment and reduced clinical pregnancy rate in fresh embryo transfers [1, 2]. However, the key clue deriving from these important insights and demonstrations relies on the clarification that a premature P surge determines a prematurely advanced endometrium, shifting the expected and desired window of implantation [3, 4]. In this regard, the optimum clinical management has been extensively investigated, with a clear consensus about the freeze-all strategy [5, 6].

After finding the best clinical management protocol to avoid an impact on endometrial receptivity, questions arise about the possible effect that P elevation could have on oocyte quality, embryo development, and morphology.

In 2016, the study by Huang et al. [7] demonstrated a detrimental effect of elevated progesterone on the quality of day 3 embryos. Our group also confirmed this result for embryos at blastocyst stage, with progesterone levels showing an inverse relation with top-quality blastocyst formation [8]. This detrimental effect of progesterone on the quality of embryos was also reported by other studies [9, 10], although there is still a lack of consensus [11]. The presence of a deleterious impact of PPE on embryo competence was also investigated by Racca et al. [12], reporting the association of PPE with a decrease in embryo utilization and cumulative live births rate. However, a subsequent multicenter retrospective study, which evaluated

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only freeze-all cycles, reported no differences in cumulative live birth rate [13].

Therefore, considering the increasing evidence of an effect of progesterone on embryo quality, we can postulate that an indirect effect of P in embryo–endometrial asynchrony may also be exerted through a delayed blastocyst development. Following this insight, we have hypothesized that P levels during a COS could hamper the blastocyst development. For this reason, our study aims to investigate the impact that P levels, detected on the day of trigger, may have on time to blastulation, performing a large retrospective analysis. Our analysis could lead to an intriguing conclusion regarding embryological outcome summarized as follows: is delayed blastocyst development associated with elevated progesterone levels in COS? Should the embryologist expect a different day5 blastocyst rate in COS cycles with elevated progesterone levels?

Materials and methods

Study design

This was a non-interventional, observational, retrospective, single-center cohort study of autologous IVF/ICSI cycles with blastocyst culture performed from January 2019 to December 2021 at our infertility unit Centro Scienze Natalità, San Raffaele Scientific Institute, Milan, Italy. The aim was to compare the effect of progesterone level at trigger on time to blastulation. Data were obtained from our fertility center database.

Patients

Patients aged 18–42, undergoing controlled ovarian stimulation for homologous IVF\ICSI cycles were screened for inclusion. A detailed flow chart of the study process is shown in Fig. 1. We excluded all cycles (i) with indications to preimplantation genetic testing (PGT), (ii) with missed serum progesterone level on the day of induction, (iii) with ovulation already occurred based on P level at trigger of ≥ 3 ng/ml. This threshold for extremely high P elevation was selected as an arbitrary threshold derived from previous literature [14]. Moreover, we excluded all cycles where at least one expanded blastocyst was not transferred or cryopreserved (grade 3, see "Embryo culture and grading" section for more details).

Controlled Ovarian Stimulation protocols and hormone measurements

Controlled ovarian stimulation was performed by administration of recombinant FSH (r-FSH) or highly purified human menopausal gonadotrophin (hMG), starting from day 2 or 3 of the menstrual cycle under GnRH antagonist pituitary suppression initiated on day 6. When three or more leading follicles reached a diameter ≥ 17 mm, final oocyte maturation was triggered with 10,000 IU of high purified (HP)-hCG or GnRH agonist 0.2 ml in case of risk of ovarian hyperstimulation syndrome (OHSS) (presence of 25 follicles with a diameter ≥ 12 mm on the day of triggering).

Oocyte retrieval occurred 36 h later, and insemination was achieved with conventional IVF or ICSI. Fresh embryo transfer was performed after three or 5 days after insemination. In the case of freeze-all cycles or presence of supernumerary embryos after a fresh ET, blastocysts were cryopreserved on day 5, 6 or 7. Cycles monitoring, oocyte retrieval, IVF/ICSI, fresh ET and blastocyst cryopreservation were all performed in accordance with our standard procedures.

Serum P on the day of hCG administration was measured with Tosoh AIA fluorimetric system with ST-AIA-PACK immunoassay (Tosoh Corporation) which has a sensitivity of 0.1 ng/ml. The assay was used for the entire duration of the study.

Embryo culture and grading

After collection, follicular fluids were screened for cumulus-oocyte complexes (COCs). Once identified, COCs were incubated into pre-equilibrated IVF medium (Quinn's Advantage Fertilization, Cooper Surgical®) supplemented with human serum albumin (HSA) (SAGE In-vitro Fertilization, Cooper Surgical®) at 37 °C in controlled atmosphere (6% CO_2 and 5% O_2) for 3 h and then, allocated to conventional IVF or ICSI. For conventional IVF, COCs were placed into a 4-well dish with pre-equilibrated IVF medium (Quinn's Advantage Fertilization, Cooper Surgical®) supplemented with HSA overlaid with mineral oil (Oil for Tissue Culture, Cooper Surgical®) and incubated overnight with 25,000 motile spermatozoa per well at 37 °C in controlled atmosphere. For ICSI, denudation of COCs was performed by a brief exposure to HEPES-buffered medium (Quinn's Advantage Medium with HEPES, Cooper Surgical®) supplemented with HSA and containing 20 IU/ml of hyaluronidase solution (FUJIFILM Irvine Scientific®). Once initial cumulus-cell dissociation was observed, further denudation was carried out removing the corona cells through denuding pipettes of decreasing diameter. After denudation, the oocytes were thoroughly washed and examined under an inverted microscope to assess integrity and nuclear status, and only metaphase II (MII) oocytes were considered for injection. ICSI was performed in a Petri culture dish containing pre-warmed

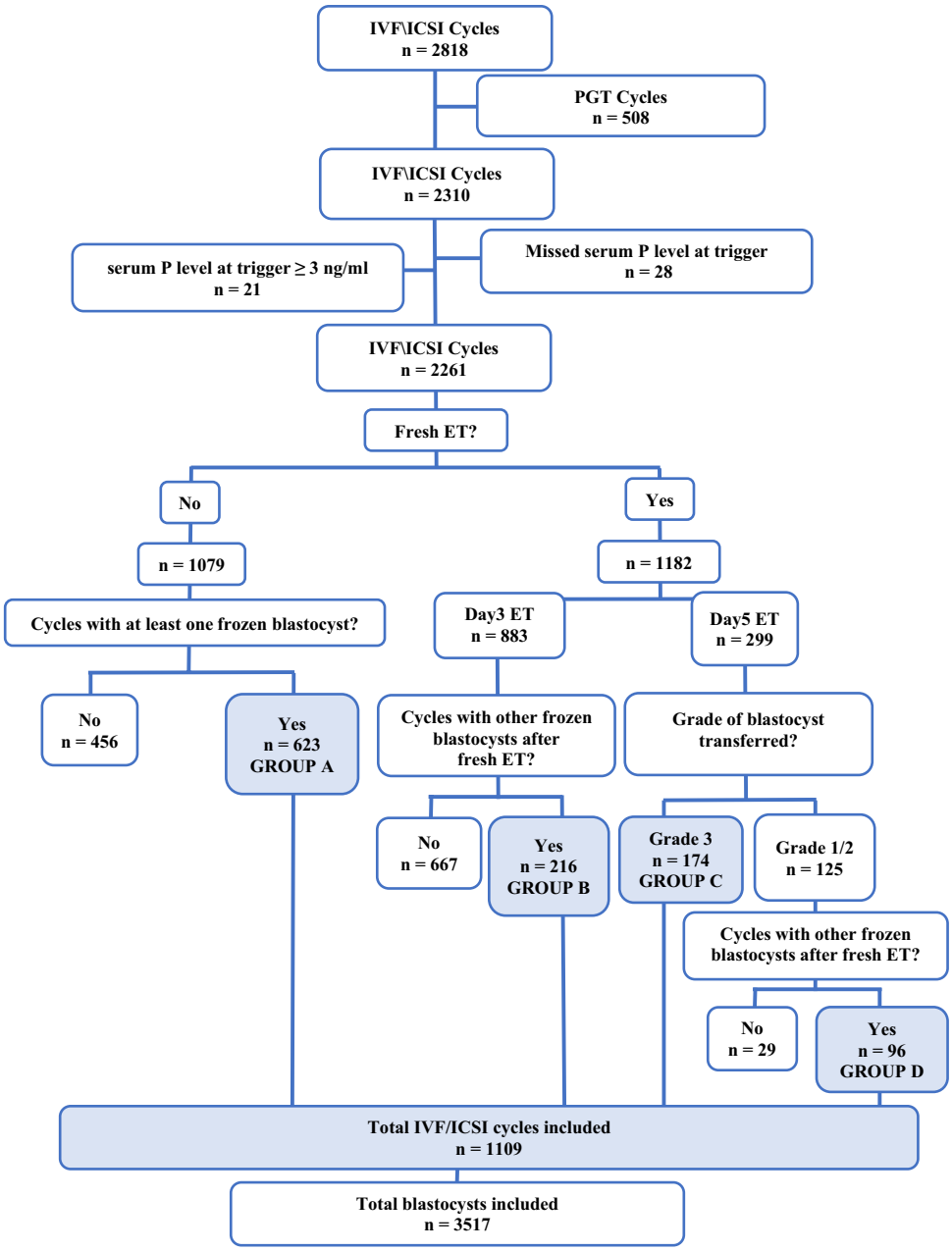

Fig. 1 Flow diagram showing selection of IVF\ICSI cycles. Included cycles are colored in light blue and named as GROUP A, B, C or D

HEPES-buffered medium microdroplets supplemented with HSA and one drop of PVP (polyvinylpyrrolidone solution with HSA-7%, FUJIFILM Irvine Scientific®) covered by mineral oil. Injected oocytes were then rinsed and placed into pre-equilibrated culture medium (CSC-NX, Irvine Scientific, or Quinn's Advantage Cleavage-Blastocyst, Cooper Surgical®) supplemented with Serum Substitute Supplement (SSS) (FUJIFILM Irvine Scientific®) in a controlled atmosphere incubator.

Fertilization was assessed 17 ± 1 h after insemination and a media change-over was performed (CSC-NX, Irvine Scientific, or Quinn's Advantage Cleavage, Cooper Surgical®) supplemented with SSS. After a media change-over in day 3 post-insemination (CSC-NX, Irvine Scientific, or Quinn's Advantage Blastocyst, Cooper Surgical®) supplemented with SSS, embryos were cultured until blastocyst stage, in a single drop of culture media under a controlled humidified atmosphere. Blastocyst evaluation was performed according to the Istanbul Consensus [15] on day 5, day 6 and day 7 at 116 ± 2 h, 140 ± 2 h and 164 ± 2 h post insemination, respectively. In case the patient was eligible for embryo transfer on day 5, this was carried out regardless of the grade of expansion of the blastocysts. However, blastocysts transferred with an expansion grade lower than 3 were removed from the study and treated as missing data since the timing for reaching grade 3 expansion was not known. On the other

hand, only expanded blastocysts (grade 3), with the inner cell mass (ICM) and the trophectoderm (TE) graded as 1–3, were considered suitable for cryopreservation on day 5, 6 and 7. Expanded grade 3 blastocysts were defined as blastocysts in which the cavity is greater than the original volume of the embryo and the zona pellucida is thin.

Primary outcome

The primary outcome was to compare time to blastulation in terms of percentage of expanded (grade 3) blastocysts on day5 [(N of grade 3 blastocysts transferred or frozen on day 5) / (total N of grade 3 blastocysts obtained) * 100] according to progesterone level at trigger. Only grade 3 blastocysts were considered for the study in order to have a reliable and objective outcome to determine the time to blastocyst.

Data collection

Variables of interest included the patient's baseline characteristics, COS parameters and laboratory outcomes. Data regarding age, height, weight, Body Mass Index (BMI), main cause of infertility, controlled ovarian stimulation data (total gonadotropin dose, duration of stimulation, estradiol and progesterone level at trigger), number of oocytes retrieved, fertilization technique and number of blastocysts on day5, day6, and day7 were recorded.

Ethical approval

All patients signed a written informed consent agreeing to deliver their own anonymous information for research purposes (ID: BC-GINEOS, date of approval: 09/02/2012, San Raffaele Hospital Ethics Committee).

Statistical analysis

Data collection ended in December 2021 and analysis was performed in January 2022. To avoid potential biases related to the assumption of linear relationship between serum progesterone levels and day 5 blastulation rate, cycles were divided into distinct groups according to the serum progesterone levels on the day of trigger: the 1st quartile (Q1; $P < 0.50$ ng/ml); the 2nd quartile (Q2; 0.50 ng/ml $\leq P \leq 0.78$ ng/ml); the 3rd quartile (Q3; 0.79 ng/ml $\leq P \leq 1.15$ ng/ml); the 4th quartile (Q4; $P > 1.15$ ng/ml). In order to maximize the sample size of the study, all cycles where at least one expanded blastocyst was transferred or cryopreserved were included in the analysis and classified accordingly to the presence of (A) freeze-all cycles (B) day 3 embryo transfer; (C) day 5 embryo transfer with grade 3 blastocysts; (D) day 5 embryo transfer without grade 3 blastocysts (Fig. 1). This classification allowed us to analyze also cycles where some embryos were not included because of transfer on day 3 or because they were transferred in day 5 with an expansion grade lower than 3 (due to unknown time for complete blastulation), while maintaining the possibility of correcting the analysis for potential differences related to these clinical options.

The distribution of the blastulation rate on day5 was investigated using the Shapiro–Wilk test. Since the variable did not follow a normal distribution, we used the Kruskal–Wallis test to assess differences between groups (progesterone quartiles). Subsequently, we evaluated separately each possible confounding factor associated with blastulation rate on day 5 using a univariate analysis. Specifically, we analyzed with a Univariate Generalized Linear Model (GLM) woman's age, weight, height, BMI, cause of infertility, AMH levels, total dose of FSH administered, estrogen levels, duration of COS, number of oocytes retrieved, day of transfer and type of treatment IVF\ICSI. Factors that were significantly associated with blastulation rate on day 5 in the univariate analysis and factors intrinsically associated with progesterone levels (BMI and estrogen at induction) were considered confounders and thus included as covariates in the Multivariate Generalized Linear Model to assess the association between day 5 blastulation rate and progesterone levels, defined by the four quartiles. Notably, we considered only confounders that precede ovarian pick-up, so that they could not themselves be an effect of progesterone levels. The first quartile (Q1) was used as the reference group. GLM results are reported as beta values (representing, for the primary outcome, the difference in terms of percentage of day 5 blastocysts between each progesterone quartile and the reference quartile) and their confidence intervals.

We conducted a power analysis using G*Power 3 [16] that resulted in a sample size of 621 blastocysts per progesterone group in order to detect a 10% difference in day 5 blastocyst rates, with 95% power and alpha of 0.05.

The results are presented as n (%) for categorical variables, mean ± Standard Deviation for continuous variables. For all statistical tests, we considered statistically significant a p-value < 0.05. Data analysis and graphical plots were performed using software RStudio version 2022.07.2 + 576 (RStudio, Boston, MA, USA) and package ggplot2 [17].

Results

A total of 1109 COS cycles were included in this study (Table 1). The mean age of women at the time of egg retrieval was 36.1 (±3.8), with no significant differences between quartiles. Overall, among the various main infertility causes, we found the following percentages: male factor 239 (21.6%),

Table 1 Demographics, baseline serum characteristics and IVF\ICSI data of study population according to progesterone quartiles

	All cycles	Q1	Q2	Q3	Q4	*P*-value*
		< 0.50	0.50–0.78	0.79–1.15	> 1.15	
Number of cycles	1109	268	279	290	272	
Age at oocytes retrieval (years)	36.1 ± 3.8	36.1 ± 4.1	36.0 ± 3.9	36.0 ± 3.7	36.1 ± 3.8	ns
BMI (kg/m^2)	22.1 ± 3.8	22.0 ± 4.5	22.6 ± 3.8	22.0 ± 3.7	21.4 ± 3.2	0.008
Basal AMH (ng/ml)	2.9 ± 2.5	2.7 ± 2.8	2.7 ± 2.2	3.0 ± 2.5	3.1 ± 2.6	0.02
Main cause of infertility						ns
- Male factor	239 (21.6)	63 (23.5)	56 (20.1)	67 (23.1)	53 (19.5)	
- Maternal age	211 (19.0)	57 (21.3)	56 (20.1)	50 (17.2)	48 (17.6)	
- Idiopathic	199 (17.9)	38 (14.2)	53 (19.0)	57 (19.7)	51 (18.8)	
- Diminished ovarian reserve	152 (13.7)	46 (17.2)	31 (11.1)	38 (13.1)	37 (13.6)	
- Endometriosis	120 (10.8)	23 (8.5)	29 (10.4)	34 (11.7)	34 (12.5)	
- Endocrine factor	116 (10.5)	31 (11.6)	28 (10.0)	31 (10.7)	26 (9.6)	
- Tubal factor	72 (6.5)	10 (3.7)	26 (9.3)	13 (4.5)	23 (8.4)	
COS characteristics						
- Total dose of r-FSH\hMG (IU)	1746.3 ± 1092.7	1434.8 ± 1072.6	1715.3 ± 1025.7	1865.8 ± 1057.3	1957.7 ± 1148.9	< 0.001
- Length of stimulation (days)	10.3 ± 7.8	9.6 ± 2.0	9.7 ± 2.0	11.0 ± 1.8	10.6 ± 2.3	< 0.001
- E2 levels on the day of ovulation trigger (pg/ml)	2453.6 ± 1479.6	1613.6 ± 1042.1	2165.9 ± 1064.0	2681.9 ± 1335.4	3331.7 ± 1789.3	< 0.001
- P level on the day of ovulation trigger (ng/ml)	0.90 ± 0.50	0.34 ± 0.10	0.63 ± 0.08	0.95 ± 0.10	1.57 ± 0.40	< 0.001
- Number COC retrieved	10.3 ± 5.8	8.7 ± 5.9	9.7 ± 5.1	10.8 ± 5.5	12.01 ± 6.2	< 0.001
Fertilization method						0.01
- ICSI	1028 (92.7)	261 (97.4)	261 (93.5)	262 (90.3)	244 (89.7)	
- FIVET-ICSI	64 (5.8)	4 (1.5)	14 (5.1)	23 (8.0)	23 (8.5)	
- FIVET	17 (1.5)	3 (1.1)	4 (1.4)	5 (1.7)	5 (1.8)	
Freeze-all cycles (GROUP A)	623 (56.2)	101 (37.7)	127 (45.5)	155 (53.4)	240 (88.2)	< 0.001
Day 3 ET (GROUP B)	216 (19.5)	68 (25.4)	66 (23.7)	64 (22.1)	18 (6.6)	
Day 5 ET	270 (24.3)	99 (36.9)	86 (30.8)	71 (24.5)	14 (5.1)	
- Grade 3 (GROUP C)	174 (15.7)	68 (25.4)	55 (19.7)	44 (15.2)	7 (2.6)	
- Grade 1\2 (GROUP D)	96 (8.6)	31 (11.5)	31 (11.1)	27 (9.3)	7 (2.6)	
Total number of blastocysts	3517	762	889	940	926	< 0.001
- Day 5 blastocyst	1280 (36.4)	317 (41.6)	343 (38.6)	296 (31.5)	324 (35.0)	
- Day 6 blastocyst	1979 (56.3)	403 (52.9)	474 (53.3)	565 (60.1)	537 (58.0)	
- Day 7 blastocyst	258 (7.3)	42 (5.5)	72 (8.1)	79 (8.4)	65 (7.0)	

Data are expressed as mean ± standard deviation (SD) or number (%)

* Kruskal–Wallis test was used for continuous variables, while categorical data were analyzed with Chi-Square test

BMI, Body Mass Index; *AMH*, antimullerian hormone; *COS*, controlled ovarian stimulation; *FSH*, Follicle-Stimulating hormone; *E2*, estrogens; *P*, progesterone; *COC*, cumulus-oocyte complex; *ET*, embryo transfer

advanced maternal age 211 (19%), unexplained infertility 199 (17.9%), diminished ovarian reserve 152 (13.7%), endometriosis 120 (10.8%), endocrine factors 116 (10.5%), and tubal dysfunction 72 (6.5%). Regarding the COS, we noticed an average FSH\hMG total dose of 1746.3 IU and 10.3 days of stimulation with a mean oocyte retrieval of 10.3 (±5.8). In particular, total dose of r-FSH\hMG administered, length of stimulation, E2 levels on the day of ovulation trigger and number of COC retrieved increased according to progesterone level (from Q1 to Q4) with a significant *p*-value.

ICSI was the elective insemination approach in 1028 cases (92.7%), while IVF was performed in 17 (1.5%) cycles and a split ICSI/IVF method was adopted in 64 (5.8%). We obtained a total of 3517 blastocysts divided as follows: day 5 blastocysts were 1280 (36.4%), day-6 blastocysts were 1979 (56.3%), and day-7 blastocysts were 258 (7.3%). Considering all the COS cycles included, for 623 (56.2%) we adopted the freeze-all strategy and cryopreserved all the blastocysts obtained. Of the remaining, 270 (24.3%) cycles included a fresh blastocyst embryo transfer and 216 (19.5%) a fresh

day3 cleavage embryo transfer (see Table 1 for detailed COS characteristics and laboratory and embryological outcomes).

The median values for the percentage of day 5 blastocyst were compared in the four progesterone quartiles groups using a Kruskal–Wallis test (Table 2). For the 1st quartile (Q1; P < 0.50 ng/ml) the median day 5 blastocysts rate accounted for 33.3% [Inter Quartile Range (IQR): 0–67.8%]; in the 2nd quartile (Q2; 0.50 ng/ml ≤ P ≤ 0.78 ng/ml) the median day 5 blastocysts rate accounted for 25.0% (IQR: 0–60.0%); in the 3rd quartile (Q3; 0.79 ng/ml ≤ P ≤ 1.15 ng/ml) the median day 5 blastocysts rate accounted for 18.3% (IQR: 0–50.0%); lastly, in the 4th quartile (Q4), which included *P* values > 1.15 ng/ml, the median day 5 blastocysts rate was 25.0% (IQR: 0–50.0%). The same results are presented in Fig. 2, together with the complete distribution of the data in the different progesterone quartiles.

In order to estimate the effect of progesterone on the percentage of blastocysts observed on day 5 and at the same time correct for confounding factors, we performed a univariate/multivariate GLM analysis (Table 3). In the univariate GLM, the model showed a progressively marked decrease in day5 blastocyst development percentage for progesterone Q3 ($p < 0.001$) and Q4 ($p = 0.01$). This result is intriguing and suggests an inverse correlation between progesterone levels at the day of trigger and day5 blastocyst development. More specifically, the decrease was more marked for progesterone Q3 (− 10.5% day 5 blastocyst vs progesterone Q1, 95% CI from − 16.4 to − 4.6%), compared to Q4 (− 7.8% day 5 blastocyst vs progesterone Q1, 95% CI from − 13.8 to − 1.8%). In the univariate GLM, age, AMH and FSH total dose, and the cycle groups defined according to presence/timing of

Table 2 Percentage of day 5 blastocyst (median) according to progesterone quartiles

	Q1 < 0.50 *n* = 268	Q2 0.50–0.78 *n* = 279	Q3 0.79–1.15 *n* = 290	Q4 > 1.15 *n* = 272	*p*-value*
% Day 5 blastocyst, median (IQR)	33.3 (0–67.8)	25.0 (0–60)	18.3 (0–50)	25.0 (0–50)	0.013

Fig. 2 Violin plot. The graph shows, for each quartile of progesterone levels, the density profile of cycles at different percentage values of day5 blastocyst. The boxplot inside the violin plot indicates the interquartile range and the median of each group

Table 3 Univariate and Multivariate Generalized Linear Model (GLM) estimates for the association between day 5 blastocyst formation and progesterone levels

	Univariate GLM			Multivariate GLM		
	B*	95% CI	*p*-value	B*	95% CI	*p*-value
Progesterone quartiles						
Q1	ref			ref		
Q2	−4.27	(−10.22; 1.67)	0.159	−5.37	(−11.91; 1.16)	0.107
Q3	−10.48	(−16.38; -4.59)	**<0.001**	−13.78	(−20.51; -7.03)	**<0.001**
Q4	−7.79	(−13.77; -1.80)	**0.010**	−7.72	(−15.46; 0.00)	**0.050**
Age at pick-up (years)	−0.76	(−1.30; -0.21)	**0.006**	−0.19	(−0.99;0.59)	0.625
BMI (kg/m^2)	0.23	(−0.31; 0.78)	0.401	−0.07	(−0.66;0.51)	0.796
AMH (ng/ml)	1.26	(0.40; 2.13)	**0.004**	0.76	(−0.32; 1.86)	0.170
Cause of infertility						
- Male factor	ref			ref		
- Maternal age	−8.07	(−14.64; −1.49)	**0.016**	−3.29	(−12.03; 5.43)	0.459
- Idiopathic	2.22	(−4.45;8.89)	0.514	4.61	(−2.45; 11.69)	0.201
- Diminished ovarian reserve	1.49	(−5.72; 8.71)	0.684	5.44	(−2.39; 13.27)	0.173
- Endometriosis	1.06	(−6.71; 8.85)	0.788	6.95	(−1.40; 15.31)	0.103
- Endocrine factor	5.97	(−1.90; 13.84)	0.137	1.35	(−7.73; 10.45)	0.769
- Tubal factor	3.10	(−6.25; 12.45)	0.516	6.60	(−3.49; 16.69)	0.200
COS characteristics						
- Total dose of r-FSH\hMG (IU)	−0.005	(−0.007; −0.002)	**<0.001**	−0.002	(−0.004; 0.0005)	0.122
- Length of stimulation (days)	0.14	(−0.11; 0.41)	0.265			
- E2 levels at ovulation trigger (pg/ml)	0.0001	(−0.001; 0.001)	0.851	0.002	(0.0002; 0.0042)	**0.028**
- Number COC retrieved	0.08	(−0.27;0.44)	0.637			
Fertilization method						
- ICSI	ref					
- FIVET-ICSI	7.08	(−1.92; 16.08)	0.123			
- FIVET	2.30	(−14.78; 19.39)	0.791			
Freeze-all cycles (GROUP A)	ref			ref		
Day 3 ET (GROUP B)	−3.89	(−9.11;1.32)	0.144	−1.48	(−8.06; 5.09)	0.657
Day 5 ET						
- Grade 3 (GROUP C)	20.49	(14.83; 26.15)	**<0.001**	21.80	(14.96; 28.62)	**<0.001**
- Grade 1\2 (GROUP D)	−28.38	(−35.62; −21.13)	**<0.001**	−24.18	(−32.98; −15.38)	**<0.001**

*Expressed as variation in the percentage of blastocysts on day5. *P*-values in bold indicate statistically significant associations

the embryo transfer were also significantly correlated with the observed percentage of day5 blastocyst. In addition, we performed a multivariate analysis including all the variables that were statistically significant at the univariate, adding also the BMI and estrogen levels at the day of trigger, as specified in the methods section. The multivariate GLM (Table 3) confirmed previous results, with progesterone Q3 still having a more marked decrease in percentage of day 5 blastocysts (−13.8% day 5 blastocyst vs progesterone Q1, 95% CI from −20.5 to −7.0%, $p<0.001$), compared to Q4 (−7.7% day 5 blastocyst vs progesterone Q1, 95% CI from −15.5 to 0.0%, $p=0.05$). Also, the estrogen levels at the day of trigger were significantly ($p=0.03$) correlated with day5 blastocyst development, although the percentage variation is minimal and inconsistent to draw possible insights.

Finally, a multivariate GLM analysis with the same characteristics as the previous one was conducted exclusively on group A (freeze-all cycles, $n=623$, Supplementary Table 1). This analysis was conducted to exclude a bias related to the heterogeneity of cycles included. Group A was selected since this is the only group with (i) complete data for time to blastulation for the whole embryo cohort and (ii) no inclusion criteria based on the presence of a grade 3 day 5 blastocyst. The results confirmed the validity of the previous estimates, albeit with reduced statistical significance due to the reduction in the sample size.

Discussion

To the best of our knowledge, this is the first study assessing the possible association between serum progesterone level on the day of trigger during COS and time to blastulation. Our results revealed a decreased blastulation rate on day 5 according to the increase in progesterone level. In other words, it is more likely to observe a fully expanded blastocyst on day 5 when the progesterone level at trigger is low (Q1 vs. Q3 and Q4). The effect estimates were similar for groups Q3 (0.79 ng/ml ≤ P ≤ 1.15 ng/ml) and Q4 (P > 1.15 ng/ml). Still, the design of the study and the sample size do not allow to make inferences on the possibility of a threshold effect vs. the case of a continuous effect. Furthermore, this result was confirmed even after adjusting for confounding factors. Therefore, our analysis may show an intriguing result regarding laboratory and embryological outcomes during COS cycle, giving new insights into the progesterone role in assisted reproductive technology (ART).

Thanks to all the efforts made during the last decades in understanding the complexity of reproductive biology, pregnancy is known to be the result of the multiple interactions between a competent embryo and a well-developed endometrium at the optimal place and optimal time. The effect of progesterone on the endometrial implantation window shift has been widely addressed [1–3]. In this contest, the freeze-all strategy has been extensively studied as the best approach to avoid the endometrial impairment of P increase during COS [5, 6], and Racca et al. [13] showed that progesterone elevation during COS did not impair clinical live birth rate (CLBR) if a freeze-all strategy was adopted. However, when approaching the PPE question in ART, some issues still need to be clarified from the embryo's side. As a matter of fact, questions remain on the possibility that P levels effects could impair oocyte quality and the subsequent embryo development [10]. Regarding this specific topic of interest, few studies evaluated differences in blastocyst quality.

Interest in the correlation between P levels and oocyte and embryo quality was driven forward by Huang et al. in 2016 [7]. The results of his study, which analyzed 4236 fresh IVF cycles, demonstrated a negative effect of elevated progesterone levels on the day of ovulation trigger, on top quality embryo rate, regardless of the basal FSH, the total gonadotropin, the age of the woman and the time of ovarian stimulation. In particular, considering three different P cut-offs, the quality embryo rate was significantly different between serum progesterone levels < 2.0 ng/ml and > 2.0 ng/ml. In 2017, our group showed that this effect is also present at the blastocyst level, reporting progesterone as one of the predictors of the top-quality blastocyst formation rate, with an inversely proportional effect [8]. The analysis was conducted using progesterone as a continuous variable, identifying a P level > 1.49 ng/ml as the best cut-off to detect the effect on the top-quality blastocyst formation rate.

Despite the publication of further confirmations of the effect of progesterone on the quality of blastocysts [9, 10], it should be noted that other papers failed to confirm these results. The relationship between P levels and the number of oocytes retrieved and euploid embryos was investigated by Kofinas et al. [18]. In particular, P values on the day of trigger seemed not to affect the number of eggs retrieved and the number of chromosomally normal embryos available for transfer in a subsequent embryo transfer cycle. Also, Hernandez-Nieto et al., in a study evaluating PGT cycles, found no differences in euploid blastocyst rate and implantation rate [11]. Neves et al. [19] also compared the P levels with the quality of embryos. In this study, blastocyst formation rate, embryo euploidy rate, live birth rate in the first frozen embryo transfer, and the CLBR were not significantly different between the two groups (P < 1.5 ng/ml and P > 1.5 ng/ml). The same results were obtained from Turgut et al. [20], concluding that top-quality embryo development and blastulation rate were not affected by progesterone elevation. It still deserves to be considered that the same study reports a very significant effect of PPE on the quality of blastocysts on day 5 [20], which the authors did not further investigate.

Despite the excellent research value of these previous studies, none have further investigated the relationship between time to blastulation and serum progesterone levels at trigger.

Our study may reveal a missing link between PPE and IVF outcomes. Indeed, we demonstrated that the P levels on the day of ovulation trigger before ovarian pick-up are negatively correlated to day 5 blastulation rate. For this reason, a first practical consideration relies on laboratory expectations: a greater percentage of day 6 or day 7 blastocysts should be expected when dealing with COS cycles with high P levels on the day of trigger. In addition, the correlation between progesterone and time to blastocyst described in the principal analysis and adjusted for confounding factors, this effect is also evident in Table 1 and Table 2, where unadjusted day 5 blastocyst rates are described for the different progesterone groups. However, it should be emphasized that no intervention can be suggested based on present data.

Biochemical reasons for the relationship between P levels at ovulation trigger and blastocyst development still lack, but insights can be drawn from animal studies. For example, in 2010, Carter et al. investigated the effect of elevated P levels on cattle blastocyst development [21]. They found that high P levels did not affect the proportion of embryos developing to the blastocyst stage, but did result in subtle changes to the transcriptome of the embryo.

On this topic, a second practical consideration lies in the laboratory expectations for COS cycles with high levels of P per day of trigger induction: one might expect a forced

shift towards a freeze-all approach due to the absence of blastocyst on day 5. Embryologists should not consider this a failure in laboratory results since no evidence of a different implantation potential for subsequent frozen-thawed embryo transfer is available to date. However, time to blastulation has been reported to be associated with euploidy [22, 23] and this may suggest a correlation between progesterone levels and euploidy rate. Hernandez-Nieto et al. investigated this possibility [11], finding no difference in this parameter between patients with elevated progesterone ($P > 2.0$ ng/ml) and patients with normal values (defined as $P \leq 2.0$ ng/ml). Nonetheless, considering the difference between this cut-off and the progesterone values reported in the present study, it might be worthwhile to conduct a similar investigation on euploidy rates without any threshold for progesterone values.

Finally, although the effect of progesterone on embryo development does not appear to influence the cumulative live birth rate, a better understanding of this effect could still improve PPE management, for example, by reducing the time to pregnancy. In fact, one can speculate on the possibility of improving embryo-endometrial synchrony of frozen-thawed embryo transfer based on progesterone levels at the trigger and the day of blastocyst freezing.

Strengths and limitations

To the best of our knowledge, this was the first study investigating the relationship between progesterone levels on the day of trigger during COS and time to blastulation. Originality, rigorous methodology, and the relatively large sample size could be considered the points of strength. However, the potential variability between our laboratory protocols and blastocyst culture media used compared to those of other reproductive centers may limit the external validity of our findings. The single-center retrospective nature of our analysis could be defined as the major weakness of our study. Additionally, our center presents a fertilization policy strongly oriented to ICSI (92.7%), which may affect the reproducibility of results in centers with a greater IVF/ICSI rate.

Conclusions

In conclusion, progesterone levels on the day of trigger during controlled ovarian stimulation cycles are correlated to the blastocyst development, and a decrease in day 5 blastocyst rate is expected for high although "safe" (≤ 3 ng/ml) progesterone levels. Considering the new insight of progesterone impact on time to blastulation, further studies on freeze-all cycles should consider clinical outcomes in FET stratified per day of blastocyst development and per progesterone level at the day of trigger. New evidence could then arise and provide suggestions on appropriate management of FET deriving from elevated progesterone levels, according to the day of blastocyst development.

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Declarations

Conflict of interest E.P. reports grant and personal fees from MSD, grants from Ferring, from IBSA, grants and personal fees from Merck, grants from TEVA, grants from Gedeon Richter, not related to the present study. All the other authors have no conflicts of interest to declare.

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