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ORIGINAL ARTICLE

Are extremely high progesterone levels still an issue in IVF?

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Abstract
Background Premature luteinization of one or more developing follicles complicates 1–2 % of controlled ovarian stimulation cycles for assisted reproduction. The management of this complication is controversial, with cycle cancellation likely representing the most commonly used strategy. The aim of this study was to evaluate the efficacy of the "freeze-all" policy—where the entire cohort of blastocysts is cryopreserved for subsequent frozen-thawed embryo transfer—in treating cases of premature luteinization.
Methods Patients experiencing premature luteinization during controlled ovarian stimulation—identified by extremely high progesterone levels at induction (P levels ≥3.0 ng/ml and/or P/estradiol ratio ≥1, $n = 42$)—were included in a "freeze-all" program and compared to controls undergoing a "freeze-all" program with normal progesterone levels at induction (P < 1.5 ng/ml, $n = 67$).
Results Blastulation rate was comparable between patients with premature luteinization and controls (48.1 ± 20.5 % in Cases vs. 52.3 ± 24.9 % in Controls, $p = 0.36$). Ongoing pregnancy rates after the first frozen-thawed embryo transfer (38.1 % in Cases and 41.0 % in Controls, $p = 0.83$) and cumulative ongoing pregnancy rates after three frozen-thawed embryo transfer cycles (40.5 % in Cases vs. 47.8 % in Controls, $p = 0.55$) were also similar.
Conclusions These results show that extremely marked progesterone elevation throughout controlled ovarian stimulation does not impair blastocyst development and implantation potential in the context of a "freeze-all" strategy. Based on this, adoption of the "freeze-all" strategy represents a valuable tool in treating premature luteinization. In contrast, cycle cancellation—likely the most frequently used method for management of this complication—currently represents a misconduct.

Keywords Premature luteinization · Ovulation · Progesterone · Blastulation · Pregnancy · "Freeze-all" strategy

Abbreviations

AMH	Anti-mullerian hormone
BMI	Body mass index
CI	Confidence interval
COS	Controlled ovarian stimulation
E_2	17β estradiol
FET	Frozen-thawed embryo transfer
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HAS	Human serum albumin
hCG	Human chorionic gonadotropin
HP-hMG	Highly purified human menopausal gonadotropin
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
MII	Metaphase II
OHSS	Ovarian hyperstimulation syndrome
OR	Odds ratio
P	Progesterone

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rFSH	Recombinant FSH
rLH	Recombinant luteinizing hormone
SD	Standard deviation
SSS	Serum substitute supplement

Introduction

Elevation of progesterone (P) throughout controlled ovarian stimulation (COS) is a well-recognized cause of decreased pregnancy rates in in vitro fertilization (IVF), due to impaired endometrial receptivity negatively affecting fresh embryo transfer at both cleavage and blastocyst stage [1–4]. For this reason, the "freeze-all" strategy—where the entire cohort of embryos/blastocysts is cryopreserved and the transfer is delayed to a later natural or primed cycle—is considered the preferred method for management of P elevation at the end of COS [3, 5, 6]. However, published studies supporting the "freeze-all" strategy in this context and demonstrating that P elevation alters endometrial receptivity while sparing gamete quality have reported several arbitrary thresholds for P elevation ranging from 0.4 to 2.5 ng/ml, without focusing on extremely high P levels [4, 7]. Subtle P elevation during COS is primarily related to a supraphysiologic secretion from multiple healthy mature follicles in response to follicle-stimulating hormone (FSH) and does not reflect a "premature luteinization" [8]. On the contrary, extremely high P levels at the end of COS seem to reveal a premature ovulation of one or more follicles despite pituitary suppression, and no consensus exists on management of patients with this complication [9]. Several controversial strategies have been proposed, ranging from cycle cancellation to ovarian priming with gonadotropins [10], earlier antagonist administration [11], anticipation of human chorionic gonadotropin (hCG) triggering [12], co-treatment with mifepristone [13] or medroxyprogesterone acetate [14] or even aspiration of a single leading follicle [15]. However, data in the literature are very scarce, suggesting that cycle cancellation is likely the most commonly used method for management of extremely high P levels. Interestingly, a report of successful cryopreservation of 6 viable embryos in a patient showing extremely high P levels at induction (P = 5.5 ng/ml) has been published, but no data on subsequent pregnancy outcomes are currently available [16]. In contrast, a recent retrospective, cohort analysis of 4236 routine fresh IVF cycles found a detrimental effect of elevated progesterone on embryo quality, especially if an extremely high threshold (P levels ≥2.5 ng/ml) is used. However, this study as well does not provide pregnancy outcomes [17]. As a matter of fact, the subject deserves further consideration. In fact, with recent advances in cryopreservation and thawing techniques, the quality and implantation potential of frozen embryos are currently similar to those of fresh embryos [6, 16, 18]. Our hypothesis is that also extremely high P levels—reflecting cases of premature luteinization throughout COS—could indeed be managed with a "freeze-all" strategy, rather than with cycle cancellation or any other interventions. However, to the best of our knowledge, no study has so far evaluated whether extremely marked P elevation related to premature ovulation of one or more follicles during COS might have consequences at oocyte/blastocyst level in the context of a "freeze-all" strategy.

The aim of our case–control analysis was therefore to evaluate the impact of a premature luteinization during COS on blastulation rates and subsequent pregnancy outcomes in a "freeze-all" strategy. Because no consensus exists on the definition of premature luteinization [9], it was defined by both the arbitrary threshold for extremely high P elevation based on previous literature (P levels ≥3.0 ng/ml at induction) [4] and/or the non-arbitrary criterion of a progesterone/17β estradiol (P/E_2) ratio ≥1 at induction, as previously described [9, 19–21].

The fate of blastocysts derived from oocytes retrieved after exposure to premature luteinization was compared to the fate of blastocysts cryopreserved in the presence of normal P levels (P levels at induction <1.5 ng/ml) for other contraindications to fresh transfer.

Materials and methods

Study design and populations

This is a non-interventional, retrospective, case–control, observational, single-centre cohort study conducted between January 2012 and September 2014 on patients undergoing blastocyst culture and a "freeze-all" strategy due to premature ovulation throughout COS. Patients were included in the study if at day of ovulation induction they had a P/E_2 ratio ≥1 (with both P and *E* levels expressed as pg/ml) and/or P levels ≥3.0 ng/ml (Cases). The outcomes were then compared to women undergoing blastocyst culture and a "freeze-all" program for other clinical reasons [i.e. risk of ovarian hyperstimulation syndrome (OHSS) or patients' pain] in the presence of normal P levels (P at induction <1.5 ng/ml) (Controls). Controls were matched to cases for age (±1 year), body mass index (BMI) (±3.0 kg/m^2) and duration of infertility (±1 year). Specifically, controls corresponded to the subsequent women with the same baseline characteristics of the included cases and normal P levels (P at induction <1.5 ng/ml). Full ethics committee approval was not required because of the retrospective nature of the study and the anonymized handling of the data. All women provided informed consent for

their clinical data and anonymized records to be used for research purposes.

Controlled ovarian stimulation

COS was performed according to the clinical practice. Either gonadotropin-releasing hormone (GnRH) agonist long protocol or GnRH antagonist daily protocol was used for pituitary down-regulation, and ovarian stimulation was carried out with: (a) recombinant FSH (rFSH) alone; (b) rFSH combined with recombinant luteinizing hormone (rLH); (c) highly purified human menopausal gonadotropin (HP-hMG); or (d) rFSH combined with HP-hMG as previously described [20–22]. Both initial dose and dose adjustments during treatment were chosen on a case-by-case basis according to patients' characteristics. Triggering of ovulation was performed with 5000–10,000 IU of HP-hCG when one or more follicles had reached a diameter ≥16 mm or with GnRH agonist (triptorelin 0.2 ml) in case of risk of OHSS in patients undergoing antagonist protocol.

Oocyte retrieval, fertilization and embryo culture and evaluation

Oocyte collection was performed 36 h after hCG administration. After 2- to 3-h incubation in human serum albumin (HSA)-supplemented fertilization medium (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA) under oil, selected oocytes were allocated to fresh insemination or intracytoplasmic sperm injection (ICSI). For ICSI, denudation of the cumulus oophorus was performed as previously described [22–24]. Each inseminated or injected oocyte was cultured separately in microdrops of equilibrated HSA-supplemented fertilization medium or serum substitute supplement (SSS, Irvine, CA, USA)-supplemented cleavage medium (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA) under oil. Sixteen–eighteen hours after insemination or ICSI, all oocytes were checked for fertilization as previously described [22–24] and embryos were cultured until blastocyst stage. Blastocyst evaluation was performed according to the Istanbul consensus [25]. Blastocysts were given a rating from 1 to 3 based on the degree of expansion and hatching status. The inner cell mass was scored as follows: 1: prominent, easily discernible, with many cells that are compacted and tightly adhered together; 2: easily discernible, with many cells that are loosely grouped together; 3: difficult to discern, with few cells. The trophectoderm was scored as follows: 1: many cells forming a cohesive epithelium; 2: few cells forming a loose epithelium; 3: very few cells. "Top-quality" blastocyst (Rating 1) was defined as expanded or hatched blastocyst with an inner cell mass scored 1 and multicellular trophectoderm scored 1. Rating 2 was defined as the combination of scores 1 and 2, and Rating 3 was defined as the combination of scores 1, 2 and 3.

Blastocyst vitrification and warming procedures

On days 5–7, all viable blastocysts were cryopreserved by means of the Cryotop device and solutions (Kitazato BioPharma Co., Japan). The first equilibration was carried out in 7.5 % ethylene glycol and 7.5 % dimethylsulphoxide at room temperature for 12 min. Subsequently, blastocysts were transferred into 15 % ethylene glycol, 15 % dimethylsulphoxide and 0.5 M sucrose for 1 min and then placed on the film strip of the Cryotop in a single small drop. The excess solution was removed to leave just a thin layer around each blastocyst, and the Cryotop was submerged into liquid nitrogen. The strip was covered with the cap, and the sample was stored submerged in liquid nitrogen. At warming, the cap was removed under liquid nitrogen and the Cryotop was quickly submerged into 1 ml of 37 °C warming solution containing 1.0 M sucrose for 1 min, then transferred to a room temperature solution containing 0.5 M sucrose and incubated for 3 min. After two subsequent washings in basic medium at room temperature for 5 min each, the blastocyst was placed into culture medium [20 % SSS (Irvine, CA, USA)-supplemented cleavage medium (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA)].

Embryo transfer supplementation and procedures

The number of blastocysts transferred was established according to the American Society for Reproductive Medicine guidelines [26]. Frozen-thawed transfer was performed after endometrial priming with estradiol valerate at 6 mg/day taken orally; both groups underwent luteal phase support with progesterone 600 mg/day administered vaginally and continued through week 12 of pregnancy (treatment was discontinued only in case of miscarriage).

Hormone essays

As a part of routine monitoring procedures of COS, serial determinations of serum E_2 and P levels were performed throughout the treatment and on the day of trigger of ovulation. Samples were tested for progesterone level in a Tosoh AIA fluorimetric system with ST-AIA-PACK immunoessay (Tosoh Corporation) which has a sensitivity of 0.1 ng/ml. The assay was used for the entire duration of the study. Intra-assay and interassay variation coefficients were 10.7 and 12.9 %, respectively. Besides the internal quality control checks performed daily by the institutional laboratory, the assays were calibrated whenever a new reactive batch was used or whenever an outcome outside the normal range

Table 1 Baseline characteristics of Cases and Controls

Baseline characteristics (mean ± SD)	Cases (premature luteinization) ($n = 42$)	Controls ($n = 67$)	p
Age (years)	34.3 ± 4.7	33.9 ± 4.4	NS
BMI (kg/m^2)	22.0 ± 2.7	21.3 ± 2.7	NS
Duration of infertility (years)	2.1 ± 0.8	2.0 ± 0.8	NS
AMH (ng/ml)	2.0 ± 1.4	3.8 ± 3.1	<0.05
FSH (IU/L)	6.1 ± 2.4	6.9 ± 4.0	NS
LH (IU/L)	7.2 ± 2.0	6.2 ± 4.4	NS

NS Not significant

was observed. Furthermore, external quality control assessment was performed bimonthly at the National Research Committee; no actions of correction had to be performed during the study period.

Outcome variables

The main outcome of our study was to compare blastulation rates in patients who experienced premature ovulation during COS and controls. Clinical and ongoing pregnancy rates after the first frozen embryo transfer (FET) and cumulative clinical and ongoing pregnancy rates after three FET cycles were also compared. Clinical pregnancy was defined as the presence of at least one intrauterine gestational sac on transvaginal ultrasound performed on week 5–6 after FET. Ongoing pregnancy was defined as the presence of at least one viable foetus on transabdominal ultrasound performed on week 10–11 after FET in all clinical pregnancies. Cumulative outcomes were calculated based on the first three FET cycles by dividing the total number of clinical or ongoing pregnancies observed by number of patients (%).

Statistical analysis

Data analysis was performed using Statistics Package for Social Sciences version 18.0 (PASW Statistics 18.0, Chicago, Illinois) and G*Power 3.1 (Erdfelder, Faul and Buchner, 1996). Continuous variables were tested for normal distribution using the Kolmogorov–Smirnov test and subsequently analysed using either the Student's t test (normal data distribution) or the Mann–Whitney U test (skewed data) to compare two means (normal data distribution) or medians (skewed data distribution) where appropriate. Categorical variables were analysed using the Chi-square test or Fisher's exact test. Results are expressed as mean ± standard deviation (SD) or median (range) and odds ratio (OR) with 95 % confidence interval (95 %CI). A p value <0.05 was considered statistically significant. Power analysis was used to assess that with an expected blastulation rate of 50 % per used MII oocyte, 816 oocytes were required to exclude a difference of 10 % in blastocyst formation rate with a power of 80 % and two-tailed p values <0.05. Considering a mean number of eight MII-phase oocytes used for each patient, $n = 34$ Cases and $n = 68$ Controls were thus needed to complete this study.

Results

A total of 109 women undergoing COS and a "freeze-all" program were included. Patients with premature luteinization (Cases, $n = 42$) were identified based on a P/E_2 ratio ≥1 ($n = 11$), or P levels ≥3.0 ng/ml at induction ($n = 7$), or both a P/E_2 ratio ≥1 and P levels ≥ 3.0 ng/ml at induction ($n = 24$). Women with normal P levels at induction (P < 1.5 ng/ml) and undergoing a "freeze-all" program for other contraindication to fresh blastocyst transfer (i.e. risk of OHSS, patient's abdominal pain) were included as controls (Controls, $n = 67$). Main baseline characteristics of included patients are listed in Table 1. As shown, patients with premature ovulation and controls are comparable in terms of all major baseline characteristics except for basal anti-mullerian hormone (AMH) (2.0 ± 1.4 ng/ml in Cases and 3.8 ± 3.1 ng/ml in controls, mean ± SD, $p < 0.05$), reflecting the fact that patients who served as controls were undergoing a "freeze-all" strategy mainly due to risk of ovarian hyperstimulation syndrome (OHSS). Controlled ovarian stimulation and embryologic data are presented in Table 2. Number of oocytes retrieved was similar between Cases and Controls (12.6 ± 6.5 vs. 15.0 ± 6.8, respectively, mean ± SD, $p = 0.06$) and so was percentage of mature oocytes (metaphase two or MII oocytes, 71.6 ± 21.6 % vs. 69.7 ± 20.6 %, respectively, mean ± SD, $p = 0.20$). Also blastulation rate was not decreased in patients with premature luteinization compared to controls (48.1 ± 20.5 % in Cases vs. 52.3 ± 24.9 % in the Control group, $p = 0.36$), and the proportion of top-quality blastocysts was as well comparable (Table 2). Importantly, also blastocyst survival rate following warming was similar between Cases (98.1 ± 10.1 %, mean ± SD) and controls (98.3 ± 10.7 %, mean ± SD, $p = 1.0$). Table 3 shows the pregnancy

Table 2 IVF-ICSI cycle characteristics and outcomes in Cases and Controls

COS cycle characteristics	Cases (premature luteinization) ($n = 42$)	Controls ($n = 67$)	p
E_2 at induction (pg/ml)	2294 ± 1538	2877 ± 1508	NS
P at induction (ng/ml)	3.0 ± 1.4	0.7 ± 0.5	<0.0001
Oocytes retrieved (*n*)	12.6 ± 6.5	15.0 ± 6.8	NS
MII oocytes (%)	71.6 ± 21.6	69.7 ± 20.6	NS
Oocytes used (*n*)	8.5 ± 3.3	8.9 ± 3.2	NS
Fertilization rate (%)	71.3 ± 18.3	74.4 ± 16.6	NS
Blastulation rate (%)	48.1 ± 20.5	52.3 ± 25.0	NS
Cryopreserved blastocysts (*n*)	2.8 ± 1.5	3.3 ± 1.8	NS
Rating 1 (%)	14.3 ± 25.2	12.7 ± 21.4	NS
Rating 2 (%)	34.7 ± 34.4	31.9 ± 31.8	NS
Rating 3 (%)	48.4 ± 35.9	41.4 ± 36.0	NS

NS Not significant

Table 3 FET cycles pregnancy outcomes in Cases and Controls

Pregnancy outcomes	Cases (premature luteinization) ($n = 42$)	Controls ($n = 67$)	p
After first FET cycle			
Clinical pregnancy rate (%)	46.5	42.4	NS
Ongoing pregnancy rate (%)	38.1	31.0	NS
After three FET cycles			
Cumulative clinical pregnancy rate (%)	53.5	53.0	NS
Cumulative ongoing pregnancy rate (%)	40.5	47.8	NS

NS Not significant

outcomes of the "freeze-all" program in the study participants. Pregnancy rates were comparable between the study groups. In particular, observed ongoing pregnancy rates after the first FET were 38.1 % in Cases and 41.0 % in Controls, $p = 0.83$. Also after three FET cycles, comparable cumulative clinical (53.5 % in Cases and 53.0 % in Controls, $p = 1.0$) and ongoing pregnancy rates (40.5 % in Cases vs. 47.8 % in Controls, $p = 0.55$) were observed in patients with premature luteinization and controls. Subgroup analyses were done, separately grouping patients based on only one of the two definition criteria for premature luteinization (patients with P/E_2 ratio ≥ 1, $n = 35$ and patients with P $\geq$ 3.0 ng/ml at induction, $n = 31$), and no significant differences between each group and Controls were revealed in terms of baseline characteristics and outcomes (data not shown).

Discussion

Our study is the first to investigate the effectiveness of the "freeze-all" strategy for management of accidental premature luteinization in IVF, and it shows that premature luteinization throughout COS is not associated with impaired outcomes if all blastocysts are cryopreserved for subsequent FET. Premature ovulation of one or more follicles during COS complicates a small proportion of IVF cycles, and no consensus exists on definition and management of this condition. Based on previous literature, premature luteinization can indeed be defined either by the non-arbitrary criterion of a P/E_2 ratio ≥ 1 at induction [9, 19–21] or by extremely high P levels $\geq$3.0 ng/ml at induction [4]. Also, clinical management ranges from cycle cancellation to anticipation of hCG triggering [12] or co-treatment with medroxyprogesterone acetate [14], but the very few data presented in the literature likely indicate that cycle cancellation represents the most frequently used method for management of extremely high P levels. However, with recent advances in cryopreservation and thawing techniques, our results seem to prove our hypothesis that cases of premature luteinization throughout COS could be managed with a "freeze-all" strategy. In fact, oocytes retrieved, fertilization rate, blastulation rate, quality of cryopreserved blastocysts and survival rate after thawing were not impaired in patients experiencing premature luteinization compared to good-prognosis patients undergoing a "freeze-all" strategy for other contraindications to fresh transfer (i.e. risk of OHSS or patients' pain) in the presence of normal P levels (P levels at induction <1.5 ng/ml). Also

clinical pregnancy and ongoing pregnancy rates after FET were comparable. Importantly, cumulative pregnancy outcomes after three FET cycles were not affected as well, suggesting that the competence of the whole cohort of oocytes retrieved is not impaired by desynchronization of developing follicles and occurrence of premature ovulation. To overcome potential biases, in our study premature luteinization was defined by either P levels ≥3.0 ng/ml at induction or a P/E_2 ratio ≥1 at induction. Moreover, subgroup analyses were done grouping patients based on each of the two definition criteria separately, and no significant differences were revealed between each group and controls (data not shown). Interestingly, in contrast with our results, a recent study including patients with extremely high progesterone levels (i.e. P levels at induction ≥2.5 ng/ml) found a significant detrimental effect on cumulative live birth rates after IVF [27]. However, in this study, patients initially underwent a fresh embryo transfer regardless of extremely high P levels, thus evidently affecting the outcomes. Our data also reassure about COS protocols performed in the luteal phase, where the cohort of oocytes is exposed to extremely high P levels throughout maturation, and support the "random start" in cancer patients undergoing fertility preservation [28, 29]. Moreover, if confirmed, our results would suggest that progesterone assessment itself might be considered useless in the context of COS for segmented cycles (i.e. fertility cryopreservation, oocyte-donation cycles or any indication to a "freeze-all" strategy). Conversely, P level assessment might be useful on the day of frozen-thawed embryo transfer. In fact, it has been recently found to be a predictor of pregnancy outcomes, even if cut-off values are still to be established [30, 31]. The fact that we have confirmed our results by using both an arbitrary threshold based on previous literature (P ≥ 3.0 ng/ml at induction) [4] and a non-arbitrary biological criterion (P/E_2 ratio ≥1 at induction) [9, 19–21] for definition of premature luteinization represents a strength of our findings. A limitation of our study is the small number of patients included. However, it should be considered that this limitation is related to the uncommon incidence of premature luteinization among patients subjected to COS: the occurrence of this complication among patients at our institution in the last 3 years has indeed been calculated at 1.7 %. In any case, further validation of our results in larger trials with pregnancy rates as primary outcome will be of interest. Also the case–control design of our study could represent a limitation. However, because cycle cancellation is likely the most commonly used method for management of extremely high P levels in IVF, a prospective randomized study comparing the "freeze-all" strategy to the standard care (i.e. cycle cancellation) would be not feasible.

This study supports the adoption of the "freeze-all" strategy in cases of extremely marked P elevation related to premature ovulation of one or more follicles during COS, without important consequences at oocyte/embryo level. Based on these observations, cycle cancellation in case of very high P levels before hCG triggering represents a misconduct.

Author's contribution VSV, VP, PL, QL, CM and PE participated in designing, analysis of results and preparation of manuscript. GP, MM and MM participated in the collection of data. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent All women provided informed consent for their clinical data and anonymized records to be used for research purposes.

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