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The relationship between embryo quality assessed using routine embryology or time-lapse videography and serum progesterone concentration on the day of ovulatory trigger in *in vitro* fertilization cycles

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ABSTRACT

Objective: To investigate the relationship between elevated serum progesterone levels (EP) on the day of ovulatory trigger, live birth rates, and the growth of resulting embryos. **Methods:** A total of 836 *in vitro* fertilization (IVF) cycles with 4 478 embryos in conventional culture were retrospectively analyzed, together with an additional 90 IVF cycles producing 618 embryos from culture and assessment using the Embryoscope™ time-lapse system. **Results:** In cycles using conventional culture, serum progesterone per follicle ≥ 14 nmol/L (median 0.42 nmol/L/follicle, range 0.05–3.50 nmol/L/follicle) was a significant negative predictor of live-birth (ROC AUC = 0.395, 95% CI 0.345–0.445; $P=0.000$) as were progesterone/estradiol ratio (0.442, 0.391–0.494; $P=0.027$) and progesterone per oocyte (0.374, 0.326–0.421; $P=0.000$) but not progesterone alone (0.470, 0.419–0.521; $P>0.05$). Women with an EP/follicle (>0.42 nmol/L/follicle) had reduced live birth rates if they were ≥ 35 yrs (14.4% vs. 24.2%, $P<0.05$) but not <35 years (35.3% vs. 37.4%, ns). Despite reduced pregnancy rates, cycles with EP/follicle in women ≥ 35 years produced similar proportions of “good” and “top” quality embryos in conventional culture compared to women with low progesterone/follicle, and no difference in abnormalities of cleavage (direct cleavage or reverse cleavage), multinucleation or timings of division (pronuclear fading to 2-cell, 3-cell, 4-cell and 5-cell; cc2 and S2) observed with time-lapse videography. **Conclusions:** EP/follicle ≥ 14 nmol/L (>0.42 nmol/L/follicle) adversely affects embryo implantation in women aged ≥ 35 years, but not <35 yrs. However, no adverse features were seen in the embryos from these affected cycles in terms of morphological appearance, abnormal patterns of cleavage, or morphokinetic timings.

1. Introduction

An elevated serum progesterone (EP) concentration on the day of ovulatory trigger was first reported in the early 1990's[1], showing

an adverse impact on the pregnancy outcome following in vitro fertilization (IVF) treatment. However, no conclusive consensus has been achieved in the last two decades due to inconsistent conclusions drawn by a large number of publications as summarized in a few recent meta-analysis review articles[2–4]. Furthermore, the controversy is also illustrated in these reviews as they came up with different conclusions depending on the period reviewed, reflecting continued debate between the emerging papers[2–4].

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Although the exact mechanism behind EP is still not clear[5, 6], the adverse impact of EP on the endometrial environment was supported by both direct[7–9] and indirect[3, 10] evidence. Whilst the majority of previous studies focussed on this apparent effect of endometrial asynchronicity, several have investigated the effect of EP on the quality of subsequent embryos via either direct static observations[11], indirect evidence with the use of subsequent frozen embryo transfer cycles[3, 10–12] or separation of the embryo and endometrium by studying donor oocyte cycles[3, 13]. Again, the conclusions were inconsistent, suggesting possible variability between different populations of patients, a limitation or insensitivity in the methods used to detect differences between embryos, or a combination of the two.

The developmental performance of embryos during *in vitro* culture is associated with both maternal and paternal factors. The use of implantation rates as an end-point is only informative about the best embryos that were selected for transfer, whereas the examination of the whole population of embryos obtained following controlled ovarian stimulation may well help to assess the full impact upon oocyte quality of a potentially disturbed hormonal milieu. In addition, paternal confounding factors are more likely to be present in intracytoplasmic sperm injection (ICSI) cycles if the treatment is indicated due to abnormal semen quality. Indeed, time-lapse study has shown the abnormal growth pattern of reverse cleavage (RC) following ICSI to be associated with reduced progressive sperm motility[14]. The vast majority of recently published large-scale retrospective studies regarding EP, each with more than 1 000 cycles analyzed, included a mixture of conventional IVF and ICSI cycles [10–12, 15–18] thereby failing to acknowledge the confounding variable of semen quality. The effect of male factors could be largely eliminated if conventional IVF cycles alone were considered when using embryo development to reflect oocyte quality. Furthermore, time-lapse imaging has recently been introduced into human IVF practice providing more information about embryo growth through continuous monitoring[19]. Timing the parameters of early embryonic developmental events have been successfully linked with blastulation[20], euploidy status[20, 21], and implantation results[19], with abnormal cleavage division patterns of observed embryos also being associated with subsequent implantation potential[14, 22, 23]. Time-lapse videography would be ideally suited to assess embryos derived from oocytes obtained from different hormonal milieu.

The aims of the present study were therefore to 1) restrict analysis to IVF cycles to minimize paternal confounding variables, 2) determine the impact of EP upon live birth rates, 3) assess embryo quality in IVF cycles using both conventional and time-lapse culture systems, and 4) examine the relationship between embryo growth and the level of progesterone in maternal serum seen on the day of ovulatory trigger.

2. Materials and methods

2.1. Patient management and gamete collection

Conventional embryology data were collected retrospectively from 617 patients with a total of 836 conventional IVF treatment cycles performed at 3 participating clinics, between January 2007 and June 2013. Retrospective morphokinetic embryology data were obtained using the Embryoscope™ at one of these 3 clinics between July 2013 and October 2014, with a total of 90 IVF cycles included. Retrospective data analysis has been approved by the institutional research ethics committees, and informed consent was provided by both partners of couples using Embryoscope™ as the culture option for the embryos.

Pituitary suppression was administered in the female patients with the use of either GnRH agonist or antagonist as previously described[14]. Serum progesterone level was measured on the day of ovulatory trigger in all 3 laboratories using Siemens Centaur CP automated analyzers, and the number of ovarian follicles with a diameter ≥ 14 mm determined by transvaginal ultrasonography was noted. The oocyte-cumulus complexes (OCC) were collected under ultrasound guidance thirty-six hours after the ovulatory trigger injection with hCG, either 10 000 IU Pregnyl (Organon) or 500 IU Ovidrel (Merck Serono). Sperm samples were processed within 2 hours of oocyte collection from samples either collected by the male partners via masturbation with an abstinence period of 2–5 days, or thawed from cryopreserved donor samples. Sperm with good motility were separated from fresh ejaculates or the post-thaw frozen samples with the use of 95%:50% density gradients (Puresperm, Nidacon) centrifugation. Four to six hours post oocyte collection, the OCCs were mixed with the prepared sperm sample at a ratio of 50 000 sperm per oocyte, with a maximum 5 OCCs per dish, being co-incubated overnight within a tri-gas regulated MINC incubator (Cook Medical, Queensland, Australia) at 37 °C. Fertilization was confirmed by the visualization of two pronuclei (2PN) 16–18 hours post insemination. Fertilized oocytes were then transferred to different types of incubators for further culture before replacement or storage.

One or two embryos were transferred on day 2, 3 or 5 after insemination depending on availability of embryo selection and treatment plan. All cycles with a positive pregnancy test (serum β hCG > 25 iu/L) 14 days after oocyte collection were followed up and the outcome of the pregnancy confirmed. Live birth rates were used to indicate a successful treatment outcome.

2.2. Embryology in MINC incubators

Fertilized oocytes were incubated in MINC incubators using premixed gas (6% CO₂, 5% O₂, balance N₂) for 2 to 5 days of culture before replacement or storage. Embryos were observed daily for grading. A simple coding system has been employed which allows for more consistency between operators and between clinics. The

embryologist records the date and time of the observation and the number of intact blastomeres seen. The embryologist estimates the embryos as symmetrical or asymmetrical and also estimates the degree of fragmentation into 3 possible categories (<10%, 10%-40% and >40% of the total volume of the embryo). A computer algorithm then grades each embryo into 4 possible grades with grade 1 being the best and grade 4 the worst quality embryos. This grading is based primarily on the principle of on-time cleavage and most emphasis is placed on this factor. Each embryology code is assigned a grade in priority order of cell numbers, fragmentation and symmetry. This system is a rudimentary morphokinetic system of grading.

2.3. Embryology in Embryoscope™ time-lapse incubator

Fertilized oocytes were loaded into Embryoscope™ time-lapse incubator perfused with 6% CO₂, 5% O₂, balance N₂ as described previously[14] with images taken every 10 mins at 7 focal planes. Morphokinetic analysis of embryos was performed with the Embryoviewer® software, with time from insemination to pronuclear fading (tPNF), time from PNF to 2-(t2_pnf), 3-(t3_pnf), 4-(t4_pnf), and 5-cell (t5_pnf) stages, and time intervals between cell stages such as cc2 (t3-t2) and s2 (t4-t3) recorded. Abnormal biological events were also noted, including multinucleation (MN) in at least one blastomere at the 2- or 4-cell stage, RC as described in a previous publication[14], and direct cleavage 2-3 cells (DC) which is defined as cc2<5 hours[23].

2.4. Statistical analysis

Proportion data were analyzed via *Chi* square or Fisher exact test. The predictive value of continuous data on live birth outcome was assessed with the use of the receiver operating characteristic (ROC) and area under the ROC curve (AUC) with 95% Confidence Interval (CI). Continuous data were expressed as mean±SD, and compared via student t-test. All statistical analysis was carried out with the use of the Statistical Package for the Social Sciences 20.0 (SPSS). *P*<0.05 was considered statistically significant.

3. Results

3.1. Live birth rates and choice of progesterone threshold

AUC [95% CI] showed the overall occurrence of live birth could

not be predicted at the time of ovulatory trigger by the serum progesterone concentration alone (AUC=0.470 [0.419-0.521], *P*>0.05). However, negative predictors of live birth were the progesterone/estradiol ratio (AUC=0.442 [0.391-0.494], *P*=0.027), progesterone per follicle ≥14 mm (AUC=0.395 [0.345-0.445], *P*=0.000) and progesterone per oocyte (AUC=0.374 [0.326-0.421], *P*=0.000). Of the two strongest predictors, progesterone per follicle had the advantage that the information was available before the woman underwent surgery for the collection of oocytes, and was calculated using follicle number and size which is widely used to time the ovulatory trigger. Progesterone per follicle was therefore used in subsequent analyses with the median concentration of 0.42 nmol/L/follicle being chosen as the arbitrary threshold to separate high and low values. Further examination of the live birth rates per transfer, taking into account the women's age, is shown in Table 1. Women ≥35 years had significantly reduced live birth rates compared to those <35 years whether the progesterone per follicle was low or high. Whilst rates were similar for the younger women between the two progesterone groups, the live birth rate per transfer for the older women was significantly reduced when the progesterone per follicle was high compared to their low progesterone counterparts.

Table 1

Live birth rates for women with low (≤0.42 nmol/L/follicle) or high (>0.42 nmol/L/follicle) progesterone per follicle ≥14 mm on the day of ovulatory trigger according to their age at egg collection.

Age	Progesterone (nmol/L/follicle)		Total
	≤0.42	>0.42	
<35 years	40/107 (37.4%) ^c	18/51 (35.3%) ^d	58/158 (36.6%) ^c
≥35 years	58/240 (24.2%) ^{ac}	42/291 (14.4%) ^{ad}	100/531 (18.8%) ^c
Total	98/347 (28.2%) ^b	60/342 (17.5%) ^b	158/689 (22.9%)

^{a-c}Groups with same superscript are significantly different, *P*<0.05.

3.2. Prevalence of high progesterone per follicle

Table 2 shows the prevalence of EP/follicle on the day of ovulatory trigger in 617 patients according to the number of repeat treatment cycles. Approximately half (314/617, 50.9%) of the women experienced an elevated progesterone per follicle in at least one cycle. Of the 137 women having two or more cycles, 48 (35.0%) were affected by EP in all repeat cycles.

Table 2

Prevalence of elevated serum progesterone concentration per follicle ≥14 mm (>0.42 nmol/L/follicle) on the day of ovulatory trigger in 836 IVF cycles from 617 patients and its incidence in repeat treatment cycles.

Number of patients	Number of repeat cycles (%)				Total
	1 cycle	2 cycles	3 cycles	≥4 cycles	
Nil cycles affected	265	29	7	2	303 (49.1%)
1 cycle affected by high progesterone	215	29	7	2	253 (41.0%)
2 cycles affected by high progesterone	-	27	10	1	38 (6.2%)
3 cycles affected by high progesterone	-	-	10	2	12 (1.9%)
4 cycles affected by high progesterone	-	-	-	11	11 (1.8%)
Total	480 (77.8%)	85 (13.8%)	34 (5.5%)	18 (2.9%)	617 (100.0%)

3.3. Patients and cycles

Table 3 demonstrates the clinical features and outcomes of all 836 IVF cycles using conventional embryo culture. In both age groups (<35 and \geq 35 years), the EP/follicle group was significantly older, had a significantly lower serum oestradiol concentration, higher serum progesterone concentration, and lower number of oocytes collected, although there was no difference in the proportion of oocytes fertilized.

3.4. Embryo quality and growth

As shown in Table 3, the younger women with EP/follicle had a significantly higher proportion of embryos with better morphology but no difference in the proportion that gave rise to live babies. Conversely, the older women with EP/follicle had similar embryo quality to their low progesterone counterparts but a reduced proportion that went on to become a baby. Table 4 shows the embryo development and incidences of abnormal biological events which

Table 3

Clinical features and outcomes of 836 IVF cycles between January 2007 and June 2013 according to whether or not serum progesterone (P4) concentration per follicle \geq 14 mm was high (>0.42 nmol/L/follicle) or not on the day of ovulatory trigger.

Parameters	<35 years		\geq 35 years	
	P4/follicle \leq 0.42	P4/follicle $>$ 0.42	P4/follicle \leq 0.42	P4/follicle $>$ 0.42
No. cycles	129	68	291	348
No. Agonist cycles (%)	63(48.8)	42(61.8)	143(49.1)	192(55.2)
No. Antagonist cycles (%)	66(51.2)	26(38.2)	148(50.9)	156(44.8)
Age of women (years, mean \pm SD)	31.3 \pm 2.8 ^a	32.5 \pm 2.0 ^a	39.1 \pm 2.6 ^e	40.1 \pm 2.8 ^e
Peak E2 (pmol/L, mean \pm SD)	7 266 \pm 4 546	6 279 \pm 4 293	6 763 \pm 3 992 ^h	4 946 \pm 3 412 ^h
Days of FSH (mean \pm SD)	11.0 \pm 2.1 ^b	12.0 \pm 3.9 ^b	11.0 \pm 2.4	11.1 \pm 2.0
P4 (nmol/L, mean \pm SD)	2.3 \pm 1.1 ^c	3.8 \pm 2.1 ^c	2.3 \pm 1.1 ⁱ	3.4 \pm 1.8 ⁱ
No. oocytes collected (mean \pm SD)	12.2 \pm 6.4 ^d	8.7 \pm 6.1 ^d	10.4 \pm 5.9 ^j	6.1 \pm 4.4 ^j
Total No. oocytes collected	1568	592	3022	2122
No. oocytes fertilized (%)	1 027(65.5)	376(63.5)	1 792(59.3)	1 283(60.5)
No. Top quality embryos (%)	424(41.3) ^e	184(48.9) ^e	766(42.7)	579(45.1)
No. Good quality embryos (%)	576(56.1) ^f	245(65.2) ^f	1 026(57.3)	773(60.2)
No. embryo transfers	107	51	240	291
No. embryos transferred	130	60	335	421
No. babies born (%)	45(34.6)	21(35.0)	61(18.2) ^k	46(10.9) ^k

^{a-k}Values with same superscript are significantly different, $P<0.05$.

Table 4

Embryo development and incidences of abnormal biological events of embryos cultured in an Embryoscope™ time-lapse incubator from an additional 90 IVF cycles between July 2013 and October 2014 according to whether or not serum progesterone (P4) concentration per follicle \geq 14 mm was high (>0.42 nmol/L/follicle) or not on the day of ovulatory trigger.

Parameters	<35 years		\geq 35 years	
	P4/follicle \leq 0.42	P4/follicle $>$ 0.42	P4/follicle \leq 0.42	P4/follicle $>$ 0.42
No. embryos	265	94	144	115
No. embryos \geq 6 cells by day 3(%)	192(72.5)	63(67.0)	94(65.3)	69(60.0)
Reverse cleavage (RC) (%)	58(21.9)	20(21.3)	36(25.0)	30(26.1)
Direct cleavage (DC) (%)	8/237(3.4)	3/85(3.5)	11/131(8.4)	6/96(6.3)
Multinucleation@2-cell (%)	60/254(23.6)	23/90(25.6)	35/143(24.5)	34/109(31.2)
Multinucleation@4-cell (%)	17/226(7.5)	7/83(8.4)	13/123(10.6)	8/87(9.2)
tPNF (n)	24.2 \pm 2.9(256) ^a	27.0 \pm 5.6(88) ^a	25.9 \pm 3.7(133) ^f	24.7 \pm 3.5(106) ^f
t2_pnf (n)	3.9 \pm 4.8(246)	3.2 \pm 2.8(87)	3.8 \pm 4.0(132)	3.8 \pm 3.9(103)
t3_pnf (n)	14.0 \pm 3.0(229) ^b	15.2 \pm 3.7(82) ^b	15.4 \pm 4.1(120)	15.0 \pm 3.6(90)
t4_pnf (n)	15.3 \pm 4.4(218) ^c	16.6 \pm 4.2(80) ^c	16.5 \pm 5.0(114)	16.0 \pm 4.2(81)
t5_pnf (n)	25.3 \pm 3.7(196) ^d	27.9 \pm 4.5(71) ^d	27.4 \pm 3.9(95)	27.6 \pm 3.1(71)
cc2 (t3-t2) (n)	10.8 \pm 1.7(229) ^c	12.0 \pm 2.0(82) ^c	12.1 \pm 3.0(120)	11.8 \pm 2.7(90)
s2 (t4-t3) (n)	1.5 \pm 3.3(218)	1.8 \pm 3.5(80)	1.6 \pm 3.2(114)	1.7 \pm 3.3(81)

Proportion of embryos with DC in each group was based on the number of embryos that had completed 3-cell stage; Proportion of embryos with multinucleation at 2- or 4-cell stage in each group was based on the number of embryos that have reached 2- or 4-cell stage respectively; Timing parameters are expressed as mean \pm SD in hours, and embryos with direct cleavage were excluded; ^{a-f}Values with same superscript are significantly different, $P<0.05$.

were detected by the time-lapse analysis in a further 90 IVF cycles. There was no difference for either age range between the high and low progesterone/follicle groups for the proportion of embryos reaching ≥ 6 cells by Day 3, or showing abnormal patterns of cleavage namely reverse cleavage, direct cleavage or multinucleation. However, EP/follicle in the younger women was associated with a slower time from insemination to PNF, slower times from PNF to 3-cell, 4-cell and 5-cell, and an extended cell cycle time from 2- to 3-cell. For the older age range, EP/follicle was associated with a quicker time from insemination to PNF but all other morphokinetic parameters were similar.

4. Discussion

EP is a potentially abnormal hormonal profile which has received much attention over the years. Originally thought to be a result of premature luteinisation[24], EP can occur in the presence of low LH[25], although reduced implantation can occur with the advancing of the endometrium by elevated circulating progesterone in the absence of a classical LH surge[26]. There are many definitions of EP used by different authors, and the prevalence varies according to the cut-off values used for the progesterone level[3, 10] although one must be cautious about comparing thresholds as different commercial progesterone assays do show different performance characteristics [27, 28], the expression of progesterone concentration on its own or as a ratio with estradiol[29], and the population of women investigated [11, 12, 16, 17, 30–33]. Furthermore, the degree of ovarian response has been identified as a factor in affecting the impact of EP, with a sliding scale of cut-off values being proposed according to the number of oocytes collected[11, 15]. The present study did not see a clear clinical effect of elevated progesterone alone, but the effect of EP expressed per follicle did and this may well reflect the influence of the ovarian response (as gauged by the number of follicles ≥ 14 mm) when used as a denominator. This adjustment allowed a clearer analysis of pregnancy rates according to age, and indeed an EP/follicle was seen to be associated with lower implantation rates in the older women but not the younger women. Apart from examining the prevalence of EP in consecutive treatment cycles, the present study also investigated its occurrence in the repeat cycles of the same patients. Only 48/137 (35.0%) women were affected by EP in all cycles suggesting that the phenomenon is cycle rather than patient specific.

Whilst high progesterone levels have been shown to affect oocyte maturation in-vitro using a mouse model[34], the effect on oocytes and embryos of elevated progesterone in the human is not clear. Using oocyte donors and recipients as a means of separating the potential effects of progesterone upon oocyte and endometrium,

pregnancy rates in the recipients were either not affected[35, 36] or even improved[37]. The examination of subsequent frozen embryo transfer in patients has also been used to try and separate the potential effects in the original cycle, and a simple analysis of pregnancy rates showed no difference when the embryos originated from a high progesterone cycle[38], but a more comprehensive appraisal of cumulative pregnancy rates did show a significant reduction in implantation with embryos exposed to a higher progesterone level in the original cycle[15]. Whilst there has been very little in the literature looking at the impact of elevated progesterone on embryo development other than blastulation rates [39], the present study has shown using continuous monitoring during culture within the Embryoscope™ time-lapse incubator no evidence of embryo morphokinetics usually indicative of reduced implantation potential in the older women despite a reduced pregnancy rate. Indeed, there was a short time to PNF in the older EP/follicle group and this is allegedly associated with either better subsequent embryo quality judged by time-lapse monitoring[40, 41] or higher implantation potential in a conventional embryo culture system[42]. There was also no difference in the occurrence of abnormal patterns of cleavage associated with reduced implantation potential such as multinucleation[43–45] and reverse cleavage[14].

In conclusion, EP/follicle ≥ 14 mm adversely affects embryo implantation in women aged ≥ 35 years, but not < 35 yrs. However, no adverse features were seen in the embryos from these affected cycles in terms of morphological appearance, abnormal patterns of cleavage, or morphokinetic timings.

Conflict of interest statement

We declare that we have no conflict of interest.

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