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ORIGINAL PAPER

Cumulative exposure to high estradiol levels during the follicular phase of IVF cycles negatively affects implantation

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Abstract *Purpose*: To investigate the effect of the cumulative exposure to estradiol (E_2) during the follicular phase on IVF outcome.

Methods: Patients were stimulated with recombinant FSH after GnRH agonist suppression and had a day 3-embryo transfer. Estrogen exposure was determined as the area under the curve (AUC) for serum E_2 levels measured from the first day of stimulation through the day after hCG administration.

Results: E_2 AUC thresholds for 10th and 90th percentiles were 4704 pg/ml and 16338 pg/ml, respectively. The pregnancy and implantation rates were highest in the 10th–90th percentile group, and were statistically higher in this group than in the >90th percentile group (54.6% vs. 33.3% and 24.8% and 12.9%, respectively, for pregnancy and implantation rates, $P < 0.05$). Recovered mature oocytes, fertilization, and number and mean score of transferred embryos were similar.

Conclusions: High cumulative E_2 exposure during the follicular phase of IVF cycles has detrimental effects on implantation.

Keywords Area under the curve · Controlled ovarian hyperstimulation · Embryo · Estradiol · Implantation

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Introduction

The measurement of serum estradiol (E_2) levels as a steroid product of the granulosa cells has been an accepted criterion for the adequacy of the ovarian response, and serum E_2 levels on the day of hCG administration have been traditionally used to define poor and good responders in controlled ovarian hyperstimulation (COH) cycles [1–3].

High serum E_2 levels achieved through COH in IVF cycles has been claimed to cause lower pregnancy rates either by adverse effects on endometrial receptivity or on oocyte/embryo quality [4–14]. However, other reports concluded that supraphysiologic E_2 levels were not detrimental [15, 16]. Hence, the impact of serum E_2 concentrations on the outcome of IVF/embryo transfer is still under debate.

Most of the above mentioned studies have differed in methodology and cut-off values used for definition of high, normal and low serum E_2 levels. Some studies used peak serum E_2 levels [8, 10, 12, 16] or serum E_2 levels on the day of hCG administration [6, 11, 13]. Other studies, on the other hand, considered the E_2 response curves of the patients [4, 5, 7, 14].

The significance of the E_2 response curve for IVF outcome has been demonstrated for most ovarian stimulation protocols. Jones et al reported that in gonadotropin stimulated cycles the pattern of serum E_2 response during the late follicular phase was a primary determinant of ovarian stimulation results and IVF outcome [4, 17]. Padilla et al suggested that the early E_2 response pattern to leuprolide acetate in a flare protocol was the best early prognostic indicator of IVF outcome [18]. Kolibianakis et al showed that in GnRH antagonist cycles the exposure to high levels of E_2 in the early follicular phase might be a cause of lower pregnancy rates [19].

There are differences in the patterns of E_2 response from the early through the late follicular phase in gonadotropin-stimulated cycles (reviewed in Arslan et al., 2005 -reference # 20). Hence, individual patients' variations of the E_2 response curves might play a role in the final outcome of ovarian stimulation and such variability may be the explanation for contradictory results reported so far. However, to the best of our knowledge, only one study in the literature directly investigated the total effect of E_2 exposure to which the ovum/endometrium were exposed to during the follicular phase of COH cycles [15].

The aim of the present study was to investigate the effect of serum E_2 exposure on oocyte/embryo quality and implantation results in IVF cycles by measuring the cumulative exposure to the steroid throughout the total duration of the follicular phase. In order to avoid variability in the ovarian response observed in different groups of responders, as well as the effects of hormonal -gonadotropin- preparations containing variable FSH:LH ratios, we studied a group of patients with an adequate ovarian reserve under 40 years of age and that were subjected to a single ovarian stimulation protocol using recombinant FSH in GnRH agonist-treated cycles.

Material and methods

Patients and study design

We retrospectively evaluated IVF cycles performed during a 4-year period in our institution between January 2001 and January 2005. Inclusion criteria were: women $<$40 years of age, who underwent COH with recombinant FSH (rFSH) under pituitary desensitization with a GnRH agonist (Lupron, Leuprolide Acetate, TAP Pharmaceuticals, Deerfield, IL) in a long protocol [20], and who had a day-3 embryo transfer. Patients $\geq$40 years old, with a single ovary, having had a coasting period longer than 2 days during COH [21], using another COH protocol and/or who had a blastocyst transfer, were excluded from the analysis. Patients who had a cycle cancellation before oocyte retrieval were also not included in the study. Only the first stimulation cycle of each woman was taken into consideration and a total of 272 women were eligible to undergone evaluation according to the determined inclusion and exclusion criteria. There were 129 cycles of conventional IVF and 143 cycles of ICSI. This was an exempt study approved by the Institutional Review Board.

Stimulation protocol and cycle monitoring

The GnRH agonist leuprolide acetate was initiated on day 21 of the preceding luteal phase (0.5 mg/d sc), continued until menses, and dropped to 0.25 mg/d until triggering ovulation. Ovarian stimulation was started on cycle day 3 at a dose of 225–300 IU/d sc of rFSH and continued for the first 3 to 4 days until cycle day 6 or 7. Thereafter the gonadotropin dose was adjusted according to the individual's ovarian response using a step down regimen. Blood samples were collected in the morning and serum E_2 levels were measured on day 3 (t_1, first day of stimulation), day 6 or 7 (t_2), day 8 or 9 (t_3), day 10 (t_4) and every day until the day after hCG administration (t_{n+1}).

Serum E_2 was assayed with a microparticle enzyme immunoassay (IMX, Abbott Laboratories, IL, USA). The intra-assay and inter-assay coefficients of variation were 8.04 and 8.09%, respectively. The serum E_2 area under the curve (AUC) was calculated as the sum of partial areas obtained according to the following formula [22]:

$$\text{Area} = (t_2 - t_1) \times (\text{EC}_1 + \text{EC}_2)/2 + (t_3 - t_2) \times (\text{EC}_2 + \text{EC}_3)/2 + \ldots + (t_n - t_{n-1}) \times (\text{EC}_{n+1} - \text{EC}_n)/2$$

where EC3, EC4, EC5 and EC_{n+1} where serum E_2 concentrations on the days of stimulation cycle matched by the corresponding "t" values.

Human chorionic gonadotropin (hCG) (10 000 IU) was administered intramuscularly to trigger ovulation when at least two follicles were $\geq$17 mm in diameter. Transvaginal follicular aspiration was performed under ultrasound guidance 34–36 after hCG injection. Gamete processing, embryo culture procedures and transfer techniques were previously described [23]. On the day of embryo transfer, embryo quality (cleavage and morphology) was assessed using the criteria of Veeck [24] with a modification to assign the best quality embryos by 5 and poorest quality embryos by 1. An individual embryo quality score was calculated by multiplying the number of blastomeres times the morphology grade. A cumulative embryo score per transfer was calculated by adding the scores of all individual embryos and was then divided by the number of embryos to obtain a "mean score of transferred embryos" (MSTE) [25].

Uterine transfer of embryos was performed on day 3. The luteal phase was supported with 600 mg/day of micronized progesterone vaginally. A clinical pregnancy was defined following identification of a gestational sac with a heartbeat by transvaginal ultrasound in patients with normally rising serum β-hCG levels. An ongoing pregnancy was defined as a continuing pregnancy after 20 weeks gestation. The implantation rate was defined as the number of gestational sacs divided by the number of embryos transferred.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. Patients were subdivided into three groups according to the 10th and

90th percentiles of E_2 AUC (<10th percentile, 10th to 90th percentile and >90th percentile). These percentile levels were chosen based on previous reports [6, 14]. Comparisons between groups of continuous outcomes were performed by Student's t-test, Mann-Whitney U Test, analysis of variance with LSD post-hoc test or the Kruskal-Wallis rank sum test, as appropriate, after testing for normal distribution by the Kolmogorov-Simirnov test. Nominal data were analyzed by χ^2 test. The presence of significant associations between parameters was assessed with either Pearson's or Spearman's correlation.

Analyses were performed with SPSS (SPSS Inc. Chicago, USA) for Windows (version 9.05). Results are presented as mean ± standard error. *P* values <0.05 were considered significant.

Results

Two hundred and seventy two patients constituted the study group, and 270 reached embryo transfer. Two patients had no fertilization. Of them 36% (99/270) had male factors, 25% (67/270) had tubal factors, 10% (27/270) had ovulatory dysfunction, 14% (37/270) had endometriosis and 7% (18/270) of patients had more than one infertility factor. Twenty-two patients (8%) had no determined etiologic cause for infertility (unexplained). The mean duration of infertility was 5.3 ± 2.2 years (range 1.5–13).

The overall clinical pregnancy and ongoing pregnancy rates per cycle, and the multiple pregnancy rates were 50.7%, 43.0% and 17.3%, respectively. Demographic and cycle specific data by clinical pregnancy outcome are presented in Table 1 (138 pregnant and 134 non-pregnant cases). The mean age of the pregnant women was slightly albeit significantly lower than the non-pregnant ones (33.09 vs. 33.94, $p < 0.05$). The number of embryos transferred was comparable in both groups. The mean score of transferred embryos was significantly higher in the pregnant group (27.97 vs. 23.03, $p < 0.001$). However, the mean serum E_2 levels on the day of hCG administration and the mean follicular phase E_2 AUC were not statistically different among groups. There were 6 patients (all in the in the high E_2 group) that developed mild-moderate ovarian hyperstimulation syndrome without the need for hospitalization or intravenous fluid replacement.

The E_2 AUC thresholds for the 10th and 90th percentiles in all patients were 4704 pg/ml and 16338 pg/ml, respectively. Cycle outcome according to the three groups of patients established based on the 10th and 90th percentiles of E_2 AUC is presented in Table 2 (<10th percentile, 10th to 90th percentile and >90th). The distributions of patients in these groups based upon treatment during the four-year study period were similar (not shown). The percentage of patients with PCOS was higher in the >90th group (12/27 patients, 44.4%) compared to the <10th percentile group (4/26 patients, 15.4%) and the 10th to 90th percentile group (51/217 patients, 23.5%) ($p < 0.05$). The diagnosis of PCOS was reassessed for each patient retrospectively based on the Rotterdam criteria [26].

There was a significant difference between groups regarding the total number of oocytes recovered and the number of frozen embryos with the highest values observed in the >90th percentile group. Importantly, groups were comparable in the percentage of MII oocytes recovered, percentage of fertilization (defined as the presence of two nuclei, for inseminated oocyte-cumulus complexes and microinjected oocytes), the number of embryos transferred and the mean

Table 1 Demographic and cycle specific data by clinical pregnancy outcome

	Pregnant ($n = 138$)	Not pregnant ($n = 134$)	*P*
Age (years)	33.09 ± 3.50	33.94 ± 3.64	<0.05
Gravidity	0.83 ± 1.39	0.70 ± 1.03	NS
BMI (kg/m2)	25.72 ± 5.67	25.43 ± 5.38	NS
Day 3 FSH (IU/L)	6.56 ± 1.79	6.77 ± 2.01	NS
Day 3 E_2 (pg/mL)	42.78 ± 17.86	42.96 ± 16.26	NS
Day of hCG administration	12.22 ± 1.01	12.25 ± 0.94	NS
E_2 value on hCG day (pg/mL)	2676 ± 1302	2552 ± 1402	NS
Peak E_2 value (pg/mL)	2767 ± 1491	2610 ± 1547	NS
Follicular phase E_2 AUC (pg/mL)	10300 ± 5764	10266 ± 6555	NS
Endometrial thickness on hCG day (mm)	10.45 ± 2.22	10.28 ± 2.34	NS
Total dose of rFSH used (IU)	2316 ± 949	2626 ± 961	<0.05
Number of oocytes aspirated	12.94 ± 6.35	11.81 ± 5.15	NS
Percentage of MII oocytes	77.74 ± 16.46	79.83 ± 19.53	NS
Percentage of fertilization	50.59 ± 17.64	60.59 ± 19.97	NS
Number of embryos frozen	2.80 ± 3.69	2.36 ± 3.30	NS
Number of embryos transferred	2.99 ± 0.71	2.98 ± 0.87	NS
Mean score of transferred embryos	27.97 ± 9.26	23.03 ± 8.73	<0.001

Note. NS: not significant.

Table 2 Cycle outcome according to the three groups of patients established based on the 10th and 90th percentiles of E_2 AUC

E_2 (AUC) (number of cycles)	<10 percentile 26	10–90 percentile 217	>90 percentile 27	*P* value
Number of ICSI cycles (%)	17 (65.3)	112 (51.6)	15 (55.5)	NS
Age (years)	34.48 ± 4.04[a]	33.59 ± 3.39	31.89 ± 4.26[a]	<0.05
Day 3 FSH (IU/L)	7.61 ± 2.41	6.70 ± 1.84	5.46 ± 1.04	NS
E_2 value on hCG day (pg/mL) (min. – max.)	1009 ± 269[b,c] (273–1533)	2501 ± 935[b,d] (850–5975)	5140 ± 1504[c,d] (2046–8165)	<0.001
Peak E_2 (pg/mL) (min. – max.)	1009 ± 269[b,c] (273–1533)	2501 ± 935[b,d] (850–5975)	5893 ± 1666[c,d] (3530–9550)	<0.001
Endometrial thickness on hCG day (mm)	10.26 ± 2.47	10.41 ± 2.31	10.07 ± 1.84	NS
Number of oocytes recovered	7.63 ± 3.77[e,f]	12.17 ± 4.91[e,g]	18.99 ± 8.25[f,g]	<0.001
Percentage of MII oocytes	75.57 ± 22.75	78.63 ± 17.98	83.16 ± 12.03	NS
Percentage of fertilization	53.25 ± 22.86	61.05 ± 18.84	64.33 ± 11.10	NS
Number of embryos frozen	0.56 ± 1.89[h,j]	2.42 ± 3.21[h,k]	5.93 ± 4.73[j,k]	<0.001
Number of embryos transferred	2.74 ± 1.23	3.04 ± 0.73	2.81 ± 0.62	NS
Mean score of transferred embryos	22.35 ± 8.43	25.80 ± 9.45	26.59 ± 8.83	NS
Clinical pregnancy rate	37.0%	54.6%[m]	33.3%[m]	<0.05
Implantation rate	16.9%	24.8%[n]	12.9%[n]	<0.05

Note. NS: not significant.

[a,b,c,d,e,f,g,h,j,k,m,n] statistically significant letters differences between groups.

score of transferred embryos. Endometrial thickness was comparable among groups. On the other hand, the clinical pregnancy rate was highest in the 10th–90th percentile group and it was significantly higher in this group than in the >90th percentile group (54.6% vs. 33.3%, respectively, $p < 0.05$). Similar results were observed for the implantation rate (24.8% versus 12.9% in the 10th–90th percentile group versus >90th percentile group, respectively, $P < 0.05$).

Pearson correlation analysis showed that the patients who had higher E_2 AUC values (>90th percentile group) were younger ($r = -0.176$, $p < 0.005$), required less gonadotropin stimulation ($r = -0.301$, $p < 0.001$) despite having higher number of oocytes retrieved ($r = 0.517$, $r < 0.001$), and had more frozen embryos ($r = 0.374$, $p < 0.001$) compared to the other two groups. There was no correlation between E_2 AUC and the mean score of transferred embryos, the percentage of MII oocytes or the percentage of fertilization.

There was no significant difference in the clinical pregnancy rate when groups were established according to the 10th and 90th percentiles of serum peak E2 levels (44.4%, 52.3% and 44.4% in the <10th, 10th–90th, and >90th percentile groups, respectively, $p > 0.05$).

The impact of variables such as females' age, gravidity, BMI, day 3 serum FSH and E_2 levels, type of fertilization (IVF or ICSI), endometrial thickness, number and mean score of transferred embryos and E_2 AUC were analyzed by logistic regression analysis. A binary logistic regression model was built with -2 log likelihood of 345.098 and χ^2 of 26.294 ($p = 0.0018$). The mean score of transferred embryos was the most significant factor in the determination of clinical pregnancy ($p < 0.001$) (Table 3). Age had a negative impact on pregnancy outcome ($p < 0.05$).

Discussion

This study showed that the implantation and pregnancy rates in cycles with highest levels of E_2 AUC (>90th percentile) were significantly lower than in patients with a normal E_2 response (patients with a E_2 AUC between 10th–90th percentile). Since the number and maturity of recovered oocytes, the mean embryo scores and the number of embryos transferred were similar among the groups, we speculate that this difference may be due to a cumulative effect of supraphysiological serum E_2 levels on endometrial receptivity.

In a similar study, Levi et al compared outcome of IVF patients and of recipients of oocyte donation prepared by exogenous hormone replacement [15]. There was no difference in implantation rates between the groups despite a significantly higher mean E_2 AUC in the IVF patients. Contrary to the results of our study, these authors concluded that exposure of the developing endometrium to COH did not inhibit implantation or affected pregnancy. While in our study the serum E_2 AUC was calculated as the sum of partial areas obtained according to the formula described earlier including the total period of stimulation from the start of gonadotropin administration throughout the day after hCG, it is not evident how these authors calculated E_2 AUC and its duration. Nonetheless, the overall mean E_2 AUC for both of the groups in the study of Levi et al. (IVF and recipients) was relatively low (in the range of 2445 to 3059 pg/ml) compared to the

Table 3 Logistic regression model: impact of significant parameters on clinical pregnancy

Parameters	β	exp (β)	95% confidence intervals for exp (β)		p
			Lower	Upper	
Mean score of transferred embryos	0.069	1.072	1.038	1.107	0.000
Age	− 0.0967	0.908	0.837	0.985	0.020

Note. β, coefficient; exp (β): odss ratio.

value that we found in our study (where the overall mean value was 10283 pg/ml). Besides, the mean peak E_2 level (5893 pg/ml) that we observed in the group with high E_2 AUC (<90th percentile) was high compared to the mean peak serum E_2 level (3004 pg/ml) determined by Levi et al. in the IVF patients. This difference suggests that the group with very high levels of E_2 AUC (>90th percentile) analyzed in our study is composed of true high responder patients [20] that may have not been included in the study by Levi et al.

In a prospective study performed for evaluation of differences in ovarian responses to rFSH and hMG, Balasch et al found mean higher E_2 AUC levels for the patients in the hMG stimulated group (7096 pg/ml vs. 4511 pg/ml), but both oocyte and embryo qualities were lower in this group [27]. It is worth mentioning that both values were higher than the mean levels presented in the study by Levi et al. However, the study design did not allow excluding the possibility that the type of gonadotropin preparation influenced oocyte/embryo quality. In addition, the E_2 AUC was calculated by using serum hormone concentrations during the first 16 days of stimulation although there was a significant difference between groups regarding the length of stimulation.

In vitro and *in vivo* animal and human studies have presented evidence for the possible detrimental effects of high E_2 levels on oocyte and embryo quality [7, 28–31]. Contrarily, Ng et al reported no differences in the number of blastomeres per embryo comparing groups of patients with different levels of E_2 levels on the day of hCG administration [32]. However the implantation rates were lower in patients with lower E_2 levels. It has also been shown that high serum E_2 concentrations (>5450 pg/ml on the day of hCG administration) in fresh IVF cycles did not impair implantation and pregnancy rates in subsequent cryopreserved-thawed embryo transfer cycles [13]. In fresh oocyte donation cycles, Pena et al showed a significant positive correlation between peak E_2 levels and the average embryo quality scores [16]. Consequently, implantation rates were higher in the group with higher peak E_2 levels (>3000 pg/ml). Papageorgiou et al also showed higher number of good quality embryos and better pregnancy rates in patients with E_2 above the 90th percentile on the day of hCG [14]. Our results are similar to the results of Ng's study in that the percentage of MII oocytes recovered, the fertilization rate and the mean scores of transferred embryos were not different between the groups, supporting the concept that high E_2 levels have no detrimental effect on oocyte and embryo quality.

On the other hand, Paulson et al. reported on inhibitory effects of COH on embryo implantation through a decrease in endometrial receptivity as evidenced in a comparison of ovum donation and fresh IVF cycles [9]. In another study focused on a selected population of high responders, Simon et al showed that E_2 levels >3000 pg/ml were detrimental to implantation and pregnancy [33]. However, much higher serum E_2 levels (>5000 pg/ml) have been proposed for the critical cut-off value [8, 13, 34]. In agreement with the latter group of studies, we also found a significantly lower implantation rate in a group of patients with a mean peak serum E_2 of 5893 pg/ml (>90th percentile E_2 AUC group). The fact that these patients were significantly younger and that age was one of the most significant determinants of pregnancy by regression analysis (Table 3) strengthens this conclusion. However, the peak serum E_2 level was in the range of 3350 to 9550 pg/mL and 12 out of the 27 patients in the group had a peak E_2 <5000 pg/ml. Hence, it is possible that patients in the study by Simon et al. could have had high levels of E_2 AUC despite the relatively lower peak E_2 levels (>3000 pg/ml).

It also caught our attention that, in the study made by Ng et al. [13], patients with low E_2 levels on the day of hCG administration (<10000 pmol/L) had significantly lower implantation and pregnancy rates than patients with high E_2 levels (>20000 pmol/L). The patients with E_2 levels in between those two groups had the highest implantation and pregnancy rates. The authors attributed the lower number of embryos transferred as a possible cause for the lower pregnancy rate observed in the group with low E_2 levels.

In our study, the group with low E_2 AUC (<10th percentile) also demonstrated a trend toward lower implantation and pregnancy rates compared to the group with intermediate or normal (10th–90th percentile) E_2 AUC levels. The mean number of transferred embryos and the mean quality scores of embryos transferred were not different between these two groups. Posthoc analysis revealed that at least 74 patients were needed in the <10th percentile group to detect a significant level (alpha) of 0.05 with a power of 80%, which means that a population with approximately three times more patients is needed for evaluation. Hence,

although it is speculative, there might be an optimal range of E_2 levels that maximizes the receptivity of endometrium. Exposure to low amounts of E_2 and its effects on endometrial receptivity should be further evaluated in future studies.

In the regression analysis the mean score of transferred embryos and age were the first and second most powerful predictors of clinical pregnancy. Our results are in accordance with those of Terriou et al. [35] in that the embryo score appeared to be a better predictor of pregnancy than the number of transferred embryos. In the regression analysis, however, there was no significant contribution of the E_2 AUC levels in determining pregnancy. This might be due to the possible non-linear correlation between E_2 levels and clinical pregnancy (lower pregnancy rates both in the $<$10th and $>$90th percentile groups) and/or intertwined roles of other parameters (e.g. the effects of the amount of gonadotropins used and patient's age). Other possibilities, at least in theory, are that the lower pregnancy rate in the higher E_2 AUC patients could potentially be due to other direct effects of ovarian hyperstimulation and/or PCOS on the endometrium rather than high E_2 levels *per se*. However, to the best of our knowledge, there are no studies in the literature addressing the direct effect of ovarian hyperstimulation syndrome (in cycles without any preventive intervention e.g. coasting) and/or PCOS on endometrial receptivity performed on matched patients based on similar and high serum E_2 levels.

Because they are assumed to be able to eliminate embryonic factors, oocyte donation cycles have been used to evaluate many aspects of implantation in IVF cycles. Most of the retrospective studies performed in oocyte donation cycles could not find any effect of different E_2 levels in recipients on pregnancy rates [36, 37]. In a case control study performed on 542 oocyte donation cycles, Garcia-Velasco et al investigated the factors in oocyte recipients who shared oocytes from the same donor and showed discordant pregnancy outcome [38]. None of the recipient's factors, including serum E_2 levels, were related to discordant pregnancy outcomes. In fact, all recipients in these studies had serum E_2 levels in the physiological range. On the other hand, Paulson et al. compared ovum donation and fresh IVF cycles matched by cycle characteristics and reported decreased endometrial receptivity suggesting detrimental effects of COH on embryo implantation through supraphysiologic levels of steroid production [9].

In conclusion, the results of this study demonstrated that the cumulative effect of very high levels of E_2 during the total duration of the follicular phase of COH had an adverse effect on implantation and pregnancy. This is in agreement with a previous report from our program that an extraordinarily high response (E_2 greater than 5000 pg/ml) may be detrimental to implantation as it can be associated with severe down regulation of the expression of endometrial progesterone receptors [39]. On the other hand, the present study showed that different levels of E_2 exposure did not likely affect oocyte and embryo quality during this period.

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