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Effect of estradiol levels after hCG trigger on embryo quality, implantation, and IVF outcome, using donor ovarian stimulation protocols

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Abstract

In this study, we aimed at delineating the possible effect of estradiol (E2) levels on the day of β-hCG administration on the ovary by analysingthe Embryo Score (ES) of early day 2, 44hrs post insemination. Oocyte donor ovarian stimulation protocols were thus used in order to avoid any possible counteractions of E2 with the endometrium. All oocytes were microinjected and all embryo transfers were fresh.E2 and E2/oocyte correlated positively with the ES on day 2 of embryo development. In contrast, the E2 and E2/oocyte do not appear to significantly affect the implantation and clinical pregnancy rates. Furthermore, E2 levels on the day of β-hCG administration were positively associated with fast and slowly dividing embryos compared to "ideally" dividing embryos on day 2. E2 levels corresponding to the "ideal" ES ($E2^{16}$) on day 2 sonographically translated into 12 mature oocytes. Embryos with "ideal" ES on day 2 were positively associated with implantation and gestational sac rates, as well as lower spontaneous miscarriage rates, in comparison to the fast and slowly dividing embryos. We propose that a) ES on day 2 of embryo development, 44hrs post insemination, is a valuable prognostic marker of embryo quality in ovarian stimulation protocols and b) an ovarian stimulation protocol may most likely be successful if not more than 12 oocytes are collected, i.e. E2 levels do not exceed 3.309pg/ml or on the day of hCG administration.

Key words: Estradiol, hCG, embryo score on day 2 - 44 hrs post-insemination, embryo quality, donor ovarian stimulation protocols, implantation, miscarriage, clinical pregnancy

Introduction

Estradiol (E2) plays a crucial role in the development and maturation of oocyte as well as in the receptivity of endometrium(Drakakis P et al, 2007). However, there are conflicting studies concerning the correlation of E2 on the day of β-hCGadministration, withimplantation and clinical pregnancy rates. This discrepancy refers not only to the effect of E2 on the outcome of artificial reproductive technology (ART), but also on the type of tissue it affects (ovary, endometrium). Several studies support the idea that high E2 levels on the day of β-hCG administration are associated with higher pregnancy rates, increased number of oocytes and embryos, as well as with better quality embryos (Loutradis D et al, 1999). Additionally, increased E2 levels have been associated with increased embryo quality, albeit with no subsequent increase in pregnancy rate (Kyrou D et al, 2009). On the other hand, some studies support that higher E2 levels are associated with lower fertilization and implantation rates, due to impaired oocyte quality (Pellicer A et al, 1996). Another reason of lower pregnancy rates, in cases of high E2 levels, might be the decrease in endometrium receptivity(Yu Ng EHY et al, 2000). Therefore, the plausible simultaneous effect of E2 on endometrium receptivity, as well as on oocyte development,is reflected on our difficulty to study and understand the effect of high E2 levels on these two different tissues (endometrium, ovary) and the ensuing consequences in assisted reproduction outcomes.

In order to shed more light onto the possible effect of E2 levels on the ovary vs. the endometrium, we decided to include in our study fresh donated oocytes only.We then tried to evaluate the relationship between E2 levels on the day ofβ-hCGadministration and the embryo quality using the Embryo Score (ES) on day 2 of embryo development, 44hrs post insemination, with implantation, clinical pregnancy and spontaneous miscarriage rates.

Material and methods

41 women, aged between 19-29 years old (median age 24± 1.9), donated their oocytes from April 2008 to August 2011, after signing the appropriate informed consent forms (Oocyte donor consent forms). 102 women aged 37-49 years (mean ± SD: 43 ± 2,7) received the donated oocytes, after signing the appropriate informed consent forms (Oocyte recipient consent forms).All consent forms were prepared according to Convention for the protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine, Oviedo (1999) and abiding to the Greek national framework, as it applied by 2011.

Semen observation and evaluation was done using the Olympus CX 41 microscope, 20x magnification. The evaluation of transferring embryos was done with Olympus SZx7.For the ICSI process an Olympus microscope Ix71 was used in magnification 40x with Hoffmann lens ICSI and Holding pipettes. The semen's evaluation was done in association with the WHO, 2010 criteria (WHO 2010).

That is an observational retrospective study – statistical analysis and there was no need to have an ethical committee number. Written informed consent (recipients and donors) had been already signed by all women participated in the already done protocol.

Clinical protocols

Ovarian stimulation

Oocyte donors were stimulated as follows: the long protocol was used in 27 women, the GnRH antagonist protocol in 11 and the short protocol in 3 women. The beginning dose of r-FSH was 150 iu/day for all patients (Gonal-F or Puregon or Merional or Menopur). The mean r- FSH dose during the ovarian stimulation was 225 iu/day. For the ovarian suppression, leuprolide Daronda 2,8 ml was used (20 iu/day). Moreover, Ganirelix, Orgalutran (0,25 mg/day) was

used for the GnRH antagonist protocol. Furthermore, when the follicle's diameter is ≥18 mm or 2 follicles reach a diameter ≥16mm, β-hCGwas given (10000 IU Pregnyl or 250 mcg Ovitrelle).

Oocyte retrieval, embryo transfer, luteal phase support

Follicular aspiration and oocyte retrieval wasperformed by transvaginal ultrasound-guided puncture 35-36 h after the intramuscular administration of β-hCG.Serum E2 levels were either measured on the day of triggering final oocyte maturation with β-hCG or calculated using the algorithm developed by the Geneva Foundation for Medical Education and Research for "Monitoring IVF cycles" (Tawfik E et al, 2012).

Luteal phase support was provided with 200 mg of micronized progesterone administered intravaginally or per osthree times daily from the day after oocyte collection onwards and serum β-hCG was measured 14 days later. Clinical pregnancy was defined as the presence of at least one gestational sac on ultrasound at six gestational weeks.

Ultrasonic examination and laboratory analysis

All ultrasonic measurements were performed using a 7.5 or 6 or 5 MHz vaginal probe (Medison in 24 cases and Phonoace 8000 SE in the remaining 17 donors). E2 levels were measured by the chemiluminescence method, using an Abbott Architect analyzer and commercially available kits (DPC, Los Angeles, California, USA). The sensitivity was 15 pg / ml and the coefficient of variation was 6.3 and 6.4% for E2. In 7 cases E2 was measured on the day of administration of β-hCG. In the remaining 34 cases, where E2 measurement was not performed, it was calculated using the proposed algorithm developed by the Geneva Foundation for Medical Education and Research for "Monitoring IVF cycles" (Tawfik E et al, 2012). This algorithm takes into account the size and number of follicles shown via ultrasonography on the day of administration of β-hCG. The algorithm is as follows:E2 = 291 pg / ml + 180 (x) + 64 (y) + 18.7 (z), where x, y and z represent follicles of size>17 mm, 15 to 16 mm and <14 mm, respectively. The secretion of E2 from follicles of size <14 mm and>17 mm is considered not to be significantly different from follicles of size 14 mm and 17 mm, respectively. It is also considered that there is no difference in E2 production between follicles ≤14 mm, nor between follicles ≥17 mm.

Embryo quality evaluation via Embryo Score (ES) on day 2 of embryo development

The evaluation of embryos was based on their Embryo Score(ES), (Steer CV et al, 1992). ES for each embryo wascalculated by the multiplication of the number of its blastomeres with their grade (from 1 to 4, with 4 being the highest grade) according to morphological and developmental criteria (Table 1).We decided to focus only on day 2 of embryo development, since after this day, the activation of the embryonic genome accents the role of sperm, which may act as a confounding factor in our study. Embryo development on day 2 was assessed at 44hpi (hours post insemination). The ideal blastomere number for a human embryo on day 2, at 42-44hpi, is considered to be 4 (Stylianou C et al, 2012), therefore the ideal ES used in this study for a day 2 embryo was 16 (4x4), i.e. E^{16}. Thus, the ES we used is an indicator of embryo quality, that takes into account the cleavage rate of each embryo.

Statistical Analysis

The parameters evaluated in this study were estradiol levels (E2) and E2/oocyte on the day of β-hCG administration in relation to the Embryo Score (ES), positive or negative β-hCG [β-hCG(+)/β-hCG(-)], presence or absence of gestational sac [GS(+)/GS(-)]. In addition, total sperm count and (a+b) % sperm

Table 1. Quantification of embryo quality using the Embryo Score (ES)

PRESENCE OF FRAGMENTS %	**>50**	**20-50**	**10-20**	**<10**
Blastomere symmetry	significant blastomere asymmetry (>50% difference)	moderate blastomere asymmetry (20-50% difference)	low blastomere asymmetry (<20% difference)	perfect blastomere symmetry
Grade	1	2	3	4
Blastomere number on day 2 of development	N (1.....Z)			
Embryo Score (ES)		Grade x N		

motility in relation to ES, β-hCG(+)/β-hCG(-) and GS(+)/GS(-).

Statistics Package for Social Sciences was employed to analyze the data of the study. Chi-square tests and Student's t-test was used. Mann-Whitney test and Kruskal-Wallis were also applied. For the evaluation of correlation between two quantitative variables, the Pearson correlation coefficient was used if the distribution was normal. Statistical significance was set at $p<0.05$ and analyses were conducted using STATA statistical software (STATA Corp., College Station, Texas, USA).

Results

Demographic and clinical characteristics

41 women, aged between 19-29 years old (median age 24± 1.9), donated their oocytes to 102 recipient women aged 37-49 years (mean ± SD: 43 ± 2.7). The infertility of recipient oocyte donors ranged from 2 to 7.8 years (mean ± SD: 5.2±1.7). 27 oocyte donors were stimulated with the long protocol, 11 with the GNRH antagonist protocol and 3 with the short protocol (Table 2a).

553 oocytes were donated and ICSI was performed in all cases resulting in 404 zygotes (Table 2b; 73.1% fertilization rate) and 102 embryo transfers. Depending on their quality, a maximum of three embryos were transferred on day 2in corresponding recipient women. From 102 recipient women 58 had a positive β-hCG test (57%) and 33 women revealed the presence of at least one gestational sac (32%) using transvaginal ultrasound. There was 1 case of ectopic pregnancy (1%). All demographic and clinical characteristics of oocyte donor and recipient women can be seen in Table 2a and 2b. Cases with inadequate information about ovarian stimulation, number and quality of embryos or the embryo transfer's outcome were excluded from our study.

Effect of estradiol levels (E2) on Embryo Score(ES) onday 2 of embryo development

The correlation between E2 levels andE2/oocyte reached in oocyte donors on the day of β-hCG administration with ES on day 2 of all embryos in recipient women,was conducted using Pearson correlation coefficient (Table 3). The Pearson correlation coefficient for E2 vs. ES was calculated to be 0.78 with $p=0.038$, whereas for E2/oocyte vs ES was found 0.53 $p<0.001$, both correlations using diagnostic graphs PP-plot and QQ-plot. This means that we found a positive correlation between E2 and E2/oocyte with the ES on day 2.

ESs on day 2 of all embryos were then divided into 3 categories for ($ES^{<16}$, ES^{16}, $ES^{>16}$). Slowly developing were considered embryos with $ES^{<16}$ and fast developing were considered the embryos with $ES^{>16}$ for day 2 of embryo development. 122 embryos had an $ES^{<16}$, 114 were ES^{16}, and 168 were $ES^{>16}$ on day 2 of their development. The difference of mean E2 levels of donors on the day of β-hCG injection

Table 2a. Demographic characteristics of oocyte donors/recipients

VARIABLE	MEAN VALUE ± SD
Donors	41
Recipients	102
Donors age (years)	24± 1.9(19-29)
Recipients age (years)	43±2.7(37-49)
Infertility years	5.2±1.7
Cycles with long protocol	27
Cycles with short protocol	3
Cycles with GnRH antagonists	11

Table 2b. Clinical characteristics of oocyte donor/recipients

VARIABLE	MEAN VALUE ± SD
Estradiol (E2) *(pg/ml)	3214± 287
E2/oocyte(pg/ml)	258.4± 43.1
No retrieved oocyte/cycle	13.4± 5.3
No oocytes	553
% fertilization (ICSI)	404/553(73.1%)
No embryo transfers (ETs)1	102
% implantation rate2	58/102 (57%)
% clinical pregnancy rate3	33/102 (32%)
% ectopic pregnancy	1/102(1%)
N sperm (x 106)	68.7±7.9
% (a+b) sperm mobility	33.7±4.6

*On day of β-hCG administration, 1: maximum 3 embryos transferred on day 2, 2: No of women with β-hCG(+)/ETs, 3: No of women carrying gestational sac(s) (GS)/ETs.

Table 3. Correlation of estradiol levels (E2) and estradiol levels per oocyte (E2/oocyte) with Embryo Score (ES) on day 2 of embryo development.

	PEARSON CORRELATION COEFFICIENT	P- VALUE
E2* vs. ES	0.78	0.038
E2/oocyte vs. ES	0.53	0.001

*E2 levels on day of β-hCG administration, ES: Embryo Score.

between the three above categories was shown to be statistically significant (Table 4, p=0.04). Therefore, on day 2 of embryo development, the increased E2 levels (3618±216) of donors seem to be positively associated with rapidly dividing embryos ($ES^{>16}$), the lower E2 levels (2978±142) with slowly dividing embryos ($ES^{>16}$), while the ideal ES^{16} was associated with E2= 3309±189, in recipient women.

Effect of estradiol levels (E2) on implantation, clinical pregnancy and spontaneous miscarriage rates

From 102 recipient women, 58 had a positive pregnancy test. There was no statistically significant differencesin the mean levels of E2 and E2/oocyte reached in oocyte donors on the day of β-hCG trigger with regard to implantation and clinical pregnancy rates (Table 5, p=0.25, p=0.34 and p=0.54, p=0.58, respectively). Moreover, of the 58 recipient women who got pregnant, 33 were found to carry at least one gestational sac [β-hCG(+) /GS(+)] and 25 suffered spontaneous miscarriage [β-hCG (+) /GS(-)]. No statistically significant difference was found in the mean E2 levels reached in donors in relation to the spontaneous miscarriage rates (Table 5, p=0.67).

Since we were not able to identify which embryo implanted,we decided to calculate the mean Embryo Score (mES) of the transferred embryos, so that we could correlate it with the implantation, clinical pregnancy and spontaneous miscarriage rate for each embryo transfer.Therefore, a new stratification of the mean ES on day 2 was done ($mES^{<16}$, $ES^{16}$and $mES^{>16}$).The highestimplantation ratewas found in the mES^{16}(53.4 %) group with statistically

Table 4. Correlation of estradiol (E2) levels with the 3 categories of Embryo Score (ES) on day 2 of embryo development

ES	$ES^{<16}$	ES^{16}	$ES^{>16}$	P-VALUE
E2	2978±142	3309±189	3618±216	0.04

Table 5. Estradiol levels (E2) in association with implantation, clinical pregnancy and spontaneous miscarriage rates.

	E2	P-VALUE	E2/OOCYTE	P-VALUE
β-hCG (+)[1]	2698±482		263±38	
β-hCG (-)	3352±514	0.25	229±29	0.34
GS(+)[2]	2878±462		271±34	
GS(-)	3458±571	0.54	248±22	0.58
β-hCG (+) /GS(+)	3248±273			
β-hCG (+)/ GS(-)	3093±206	0.67		

1: implantation, 2: clinical pregnancy, GS: gestational sac

significant difference, in comparison to the other two categories (Table 6; $mES^{<16}$ =15.6%, $mES^{>16}$ =31%, p=0.003). Among women who got pregnant [β-hCG(+)], the highest clinical pregnancy rate was observed in the ideal mES^{16} category (71%), with statistically significant difference, in comparison to the other two groups [Table 6; $mES^{<16}$ (45%), $mES^{>16}$ (39%), p=0.042]. It is also noteworthy, that statistically significant less spontaneous miscarriages occurred in the mES^{16} category(29%), compared to the other two categories [Table 6; $mES^{<16}$ (55%), $mES^{>16}$ (61%), p=0.042].

Discussion

It is known that highE2 levels on the day of β-hCG triggering result in a higher number of collected oocytes, thus leading to an increased number of embryos. However, studies have shown that when more than 11 oocytes are collected, fertilization rate decreases (Pellicer A et al, 1989). In fact the oocytes that were not fertilized were found to exhibit an increased incidence of cytoplasmic immaturity (Tarín J J et al, 1992). Moreover, when 20 oocytes are collected, both fertilisation rate and maturity status of oocytes significantly deteriorate (Gelety T J et al, 1995).

Our study showed that in oocyte donor stimulation cycles E2 levels and E2/oocyte on the day of β-hCG trigger, do not affect implantation and clinical pregnancy rates. E2 and E2/oocyte levels, however, were positively associated with the ES on day 2. We also found that ES was positively associated with implantation and clinical pregnancy rate. Conversely, the highest spontaneous miscarriage rates were observed in the slow ($ES^{<16}$, 55%) and fast ($ES^{>16}$, 61%) compared to the ideal embryos on day 2 (ES^{16}, 29%), in agreement with Roberts SA (Roberts SA et al, 2010) who suggested that both "fast" and "slow"

Table 6. Correlation of mean Embryo Score (mES) on day 2 of development with implantation, clinical and spontaneous miscarriage rate

MES	MES<16	MES16	MES>16	P- VALUE
β-hCG(+)	9(15.6%)	31(53.4%)	18(31.0%)	
β-hCG(-)	16(36.3%)	19(43.2%)	9(20,5%)	0.003
GS(+)	4(45%)	22(71%)	7(39%)	
GS(-)	5(55%)	9(29%)	11(61%)	0.042
Total	9(15,6%)	31(53,4%)	18(31%)	

dividing embryos are less likely to result in pregnancy. We also found that the E2 levels associated with the ideal ES on day 2 were $E2^{16}$=3.309±189pg/ml. The levels of E2/oocyte which were found to be associated with a positive pregnancy test [β-hCG(+)] as well as a clinical pregnancy [GS(+)] , were 263pg / ml and 271 pg / ml, respectively, thus permitting us to argue that our ideal $E2^{16}$ could be related back to an ideal number of oocytes, which in our case is 12.

Large population studies have revealed that when ovarian stimulation results in more than 15 oocytes to be collected, live birth rates do not increase (Ji J et al, 2013). Others suggest that women undergoing IVF can only produce a maximum of two euploid and viable embryos, therefore suggesting that ovarian stimulation strategies should be as less invasive as they can, in order to reduce interference with ovarian physiology (Baart EB et al, 2007).Polar body analyses have shown that ovarian response to stimulation is positively related to oocyte aneuploidy and that the proportion of euploid oocytes is negatively related to the number of units of FSH per oocyte and per MII oocyte obtained (Gianaroli L et al, 2010). It has been recently suggested that above a certain threshold of gonadotropin doses, no more competent oocytes can be obtained, since fertilization rate and blastocyst/ oocyte ratio decreased significantly (Arce JC et al, 2014). In a study performed in oocyte donors by Rubio, where gonadotropin doses were lowered in the same woman, but still the same number of oocytes were collected, the embryo aneuploidy rate decreased (Rubio C et al, 2010). Others have shown that E2 levels have a concentration-dependent effect on the pregnancy outcome, suggesting an optimal range of E2 level for achieving a successful pregnancy depending on age: 3000-4000 pg/mL for women <38 years and 2000-3000 pg/mL for women ≥38 years (Joo BS et al, 2010).

We found that the E2 levels associated with the ideal ES on day 2 were $E2^{16}$=3.309±189pg/ml. It has been proposed that E2>5.000 pg/ml can be considered an acceptable upper limit (Wu CH et al, 2007), while others suggest that the limit of 2.000pg/ml is neither favorable nor harmful to the outcome of IVF (Bianco K et al, 2009). An optimal range of the E2 levels during a fresh IVF-ET cycle was recently proposed to be 1000 to 3148 pg/mL, with regard to the quality of oocytes retrieved, implantation rates, as well as the incidence of LBW outcomes (Li X et al, 2019). Moreover, oocyte exposure to high E2 levels during ovarian stimulation protocols was recently found to decrease pregnancy rates and increase the risk of preterm birth and small for gestational age outcomes (SGA) following frozen embryo transfers (Cai J et al, 2019), a finding not confirmed by others (Huang J et al, 2020).

Probably, the inability to delimit a commonly accepted maximum E2 value is due to the different ages of the studied donors/women (Kong GW et al, 2009), different protocols, or due to the so called different "ovarian sensitivity index" (Labarta E et al, 2017).It is also possible that different E2 levels may affect the endometrium and ovary differently. The high limit of>5,000 pg/ml is likely to affect the endometrium (Wu CH et al, 2007), while lower levels of ≤2,978 and ≥3,600 pg/ml, as our study shows, may be enough to affect the oocyte, rendering the ensuing embryos "slow" and "fast", respectively.The fact that E2 was found to significantly relate to ES in our study, as recorded between 44 hpi on day 2, combined to the finding that ES significantly affected the implantation, clinical pregnancy and spontaneous miscarriage rates, enables us to propose ES as a new potential predictor of embryo viability, similar to early cleavage introduced by researchers on the day of fertilization (day 1: 24-27hpi) (Fancsovits P et al, 2005).We therefore propose that a) ES on day 2, 44hrs post insemination, of embryo development is a valuable prognostic marker of embryo quality in ovarian stimulation protocols and b) an ovarian

stimulation protocol may most likely be successful if E2 levels,on the day of hCG administration, do not exceed 3.330 pg/ml, i.e. not more than of 12 oocytes are collected.

In the present study we found that a) estradiol (E2) levels and E2/oocyte are not associated with the implantation and pregnancy rates significantly, b) E2 levels significantly correlate with the ES on day 2 of embryo development in a positive manner, c) E2 levels corresponding to the ideal ES on day 2 was found to be e $E2^{16}$=3.309 pg/ml, which sonographically translated into 12 mature oocytes, d) the highest implantation and lowest spontaneous miscarriage rate was found in the mES^{16} (53.4 % and 29%, respectively) group of embryos with statistically significant difference, in comparison to the other two categories. We therefore propose that a) ES on day 2, 44hrs post insemination, of embryo development is a valuable prognostic marker of embryo quality in ovarian stimulation protocols and b) an ovarian stimulation protocol may most likely be successful if E2 levels do not exceed 3.309pg/ml on the day of hCG administration.

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Declarations

Not applicable

Disclosure of interest

The authors declare that they have no competing interest.

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