Do increased levels of progesterone and progesterone/estradiol ratio on the day of human chorionic gonadotropin affects pregnancy outcome in long agonist protocol in fresh *in vitro* fertilization/ intracytoplasmic sperm injection cycles?

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ABSTRACT

BACKGROUND: The effect of elevated levels of serum progesterone (P_4) and estradiol (E_2) on the day of human chorionic gonadotropin and their cut-off value on *in vitro* fertilization (IVF) outcomes is still not clear. **AIMS:** The aim was to evaluate the association between serum P_4, E_2 and progesterone/estradiol ratio (P_4/E_2) on pregnancy outcome in IVF/intracytoplasmic sperm injection (ICSI) cycles with long agonist protocol. SETTING AND DESIGN: Retrospective, single center, cohort study. **MATERIALS AND METHODS:** A review of complete data of 544 women undergoing fresh IVF/ICSI cycles (539 cycles) with long agonist protocol from January 2012 to February 2014 was done. Data were stratified into Three groups according to the number of oocytes retrieved: low (≤4 oocytes obtained), intermediate (5–19 oocytes obtained), and high ovarian response (≥20 oocytes obtained). **STATISTICAL ANALYSIS:** Fishers exact test/Chi-square was carried for comparing categorical data. Receiver operating characteristics analysis was performed to determine the cut-off value for P_4 and P_4/E_2 detrimental for pregnancy. **RESULTS:** A negative association was observed between pregnancy rate (PR) and serum P_4 and P_4/E_2 levels with no effect on fertilization and cleavage rate. The overall cut-off value of serum P_4 and P_4/E_2 ratio detrimental for pregnancy was found to be 1.075 and ≥0.35, respectively. Different P_4 threshold according to the ovarian responders were calculated, 1.075 for intermediate and 1.275 for high responders. Serum E_2 levels were not found to be significantly associated with PR. **CONCLUSION:** Serum P_4 levels and P_4/E_2 ratio are a significant predictor for pregnancy outcome without affecting cleavage and fertilization rate while serum estradiol levels do not seem to affect PR.

KEY WORDS: Estradiol, *in vitro* fertilization/intracytoplasmic sperm injection, progesterone, progesterone/estradiol ratio

INTRODUCTION

Pregnancy outcomes during *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles may be influenced by supra-physiological concentrations of estradiol (E_2) and progesterone (P_4).[1] The current use of both Gonadotropin-releasing hormone (GnRH) agonist and antagonist, highly effective to prevent luteinizing hormone (LH) surge, has limited the need for determination of serum P levels. Still, studies have shown a rise in P_4 levels in 35% (5–35%) of GnRH agonists cycles and 38% (20–38%) with GnRH antagonists[1-3] but the effect on IVF outcome is still unclear. The mechanisms that account for this P_4 rise are not clearly understood. Recently, this elevated P_4 has been linked with the number of mature follicles and secretion of P_4 in late follicular phase.[4-6] Several questions have been raised regarding this P_4 elevation at the time of human chorionic gonadotropin (hCG) administration. The underlying mechanism of its effect on IVF success rate is not yet clear. Several

studies-deny any association between P_4 and pregnancy rate (PR)[7-9] while others show a negative association.[10-12] In several studies, the increasing P_4 levels have shown an adverse effect on oocyte maturation, fertilization, or early cleavage[13] while other studies have denied the concept of poor embryo quality[14-16] and found it to be associated with impaired endometrial receptivity as a consequence of disturbed endometrial development and maturity[6,17] and gene expression.[18] Different detrimental cut-off values of P_4 and P_4/E_2 has also been determined, but a consensus could not be reached.

This study aims to investigate the relationship between P_4 and E_2 levels on the day of hCG administration with PR, oocyte number, fertilization and cleavage rate in GnRH long agonist protocol in nondonor fresh IVF/ICSI cycles.

MATERIALS AND METHODS

This is a retrospective, single-center cohort study of patients undergoing IVF/ICSI treatment. Complete data of 544 women who underwent nondonor fresh IVF/ICSI cycles between January 2012 and February 2014 with long agonist protocol was reviewed. The primary or combined indications for fertility treatment were tubal pathology (45.1%), male subfertility (22.7%), endometriosis (10.7%), polycystic ovarian syndrome (5.9%), unexplained infertility (14.4%). To eliminate the confounding factors that might affect the outcome, following inclusion criteria was kept: Age < 40 years, follicle stimulating hormone (FSH) <10, antimullerian hormone (AMH) >1, fresh cycles (frozen cycles excluded). The study involves no violation of animal or human rights.

Stimulation protocol

Patients underwent controlled ovarian hyperstimulation (COH) with use of a GnRH agonist long protocol. The pituitary down-regulation was achieved by subcutaneous injection of 1 mg of leuprolide acetate daily from the midluteal phase of the preceding cycle. Ovarian stimulation was done with 150–300 IU recombinant FSH follitropin alpha (Gonal-F, Merck Serono) SC (subcutaneously) and dose adjusted according to the response. Recombinant Chorionic Gonadotropin alpha (250 mg; Ovitrelle) was given to trigger ovulation when at least two-three leading follicles reached a mean diameter of 18 mm. Serum P_4 and E_2 levels were measured on the day of hCG administration by the chemiluminescent immunoassay using Access 2 Immunoassay system (Beckman Coulter) in the same laboratory. Oocyte pickup was done 36 h after hCG administration. Oocytes were cultured in G-IVF plus media (VITROLIFE) containing 10% of human serum albumin with gentamicin as an antibacterial agent and inseminated with motile sperm prepared by the two-layer percoll gradient method. Fertilization was defined as oocytes with two pronuclei 16–20 h after insemination. Embryos were transferred to G-IVF plus media and were classified by blastomere equalization and cytoplasmic fragment. Day 3 or 5 embryo transfer was done depending upon the number of embryos, and excess good-quality embryos were cryopreserved for subsequent frozen embryo transfer cycles with different grades of embryos (1–3). An ongoing pregnancy was defined as the pregnancy test done after 14 days of embryo transfer with a positive heartbeat by ultrasound at 6 weeks of gestation.

Grouping of high, intermediate and poor ovarian responders

Patients were categorized into 3 groups according to the number of oocytes retrieved: Low (≤4 oocytes), intermediate (5–19 oocytes) or high ovarian response (≥20 oocytes).

Six groups according to P_4 levels

Group A <1.0, group B: 1.0–1.25, group C 1.26–1.50, group D 1.51–1.75, group E 1.76–2.0 and group F > 2.0.

Five groups according to E_2 levels

Group A (<1000 pg/ml), group B (1000–2000 pg/ml), group C (2000–3000 pg/ml), group D (3000–4000 pg/ml), and group E (>4000 pg/ml).

Statistical analysis

All the statistical analyses were carried out using Statistical package for the social sciences (SPSS) IBM version 19.0 (Chicago, Illinois). Data were expressed as mean, standard deviation (SD) or frequencies and percentages. For comparing categorical data, Chi-square/ Fishers exact test was carried out as appropriate. Receiver operating characteristics (ROC) analysis was performed to determine the cut-off value for P_4 and P_4/E_2 at an approximately equivalent sensitivity and specificity, which may discriminate between pregnancy and nonpregnancy. A $P < 0.05$ was considered to be statistically significant.

RESULTS

A total of 544 ovarian stimulation cycles were included in the study. Of these, 5 cycles need to be cancelled as no oocyte could be retrieved. Of the total 539 cycles with long agonist protocol, 319 (59.1%) were conventional IVF cycles and 220 (40.8%) were ICSI cycles. Of all, 146 (27.08%) clinical pregnancies were noted. Baseline characteristics such as age, body mass index, serum AMH, FSH, LH (follicular phase), total gonadotropin dose, endometrial thickness on day of hCG, E_2 and P_4 on the day of hCG, number of oocytes retrieved, fertilization rate, cleavage rate and PR for all the subjects among the different ovarian responders are shown in Table 1.

Overall, the mean age of the patients in our study was 31.6 ± 3.6 years (21–40). Average (SD) values of P_4 among different ovarian responders (≤4 oocytes, 5–19 oocytes and ≥20 oocytes) were 1.06 (0.7), 1.3 (0.8) and 1.4 (0.8), respectively, with an increasing value among high responders. Serum E_2 levels and numbers of mature oocytes retrieved also showed an increasing trend in high responders with a statistically significant difference between the three groups ($P < 0.01$).

We analyzed the correlation between the serum P_4 levels on fertilization rate, cleavage rate and PR among the different groups [Table 2]. It was found that PR was affected significantly with the change in serum P_4 levels. The total number of oocytes retrieved also varied significantly between the groups showing increasing trend with P_4 values.

The trend of PR according to P_4 levels is as shown in Figure 1. PR showed a significantly decreasing trend (Chi-square trend in proportion = 8.25; $P = 0.004$) with increasing level of P_4 with maximum PR in the range of 1.01–1.25, but after a serum P level of 2 ng/ml, relatively stable effect was seen.

To assess an optimum P_4 level for an equivalent sensitivity and specificity for pregnancy status, ROC analysis was carried out. The area under curve (AUC) 0.58 (95% confidence interval [CI]: 0.53–0.63) was significant ($P = 0.006$). Overall, P_4 level was found to be 1.075 for an equivalent sensitivity and specificity value of 55% [Figure 2] with positive and negative predictive value of 31.3% and 77%, respectively. For intermediate and high responders, the cut-off value was found to be 1.075 for an equivalent sensitivity and specificity value of 56% and 1.275 for the sensitivity and specificity value of 62%, respectively, while it could not be predicted for low responders as AUC was not statistically significant.

Table 3 shows the correlation of E_2 on the day of hCG with fertilization, cleavage and PR. Bivariate logistic regression analysis revealed that the variable E_2 on day of hCG alone is not a significant predictor of pregnancy status, fertilization rate and cleavage rate although the number of oocytes retrieved increased significantly with an increase in E_2 levels.

Impact of P_4/E_2 ratio on the day of human chorionic gonadotropin

To assess threshold level of P_4 to E_2 ratio (P_4 [ng/ml] $\times 1000/E_{2[pg/ml]}$) for PR, ROC analysis was carried out. AUC 0.58 (95% CI: 0.53–0.64) was found to be statistically significant ($P = 0.003$). For an equivalent sensitivity and specificity value (56%) the corresponding value of P_4/E_2

Table 1: Baseline characteristics of subjects in three groups according to ovarian response

Characteristics	Low responders (≤4 oocytes)	Intermediate responders (5-19 oocytes)	High responders (≥20 oocytes)	Overall	*P*
Number of cycles (*n*)	76	423	40	539	
Age (years)	33.0±3.3	31.4±3.5	29.9±4.2	31.6±3.6	<0.001
BMI (kg/m^2)	25.2±3.9	25.4±4.0	24.8±4.0	25.4±4.0	0.6
FSH (follicular phase) (mIU/mL)	6.4±1.9	5.9±2.0	6.2±2.1	6.0±2.0	0.1
LH (mIU/mL)	4.5±2.3	4.8±2.7	5.1±4.0	4.7±2.7	0.6
AMH (ng/mL)	2.6±1.3	3.3±1.7	4.1±1.7	3.2±1.7	<0.001
Total gonadotropin dose (IU)	4049.5±1214.4	3468.3±1143.5	2947.6±1002.4	3511.8±1170.5	<0.001
Endometrial thickness (on hCG day) (mm)	9.2±1.7	9.4±1.7	10.0±1.9	9.4±1.7	0.1
E_2 on hCG day (pg/mL)	2061.8±1714.9	3735.1±2246.1	5452.0±3298.6	3622.8±2397.0	0.00
P_4 on hCG day (ng/mL)	1.06±0.7	1.3±0.9	1.4±0.8	1.2±0.8	0.04
No of oocytes retrieved	3.0±0.9	9.9±5.5	23.0±3.7	9.9±5.5	<0.001
Cleavage rate (%)	92.8	92.3	88.4	92.1	0.3
Fertilization rate (%)	72.6	69.5	64.5	69.6	0.09
Pregnancy rate (%)	10.5	27.6	52.5	27.1	<0.001

BMI=Body mass index, FSH=Follicle stimulating hormone, LH=Luteinizing hormone, AMH=Antimullerian hormone, hCG=Human chorionic gonadotropin, E_2=Estradiol, P_4=Progesterone

Table 2: Comparison of fertilization rate, cleavage rate, pregnancy rate and number of oocytes retrieved in different groups according to serum P_4 levels

	Group A (*P*<1.0) (*n*=234)	Group B (*P*=1.0-1.25) (*n*=96)	Group C (*P*=1.26-1.50) (*n*=69)	Group D (*P*=1.51-1.75) (*n*=33)	Group E (*P*=1.75-2.0) (*n*=29)	Group F (*P*>2.0) (*n*=78)	Overall (*n*=539)	*P*
Fertilization rate (%)	69.0	68.8	69.1	68.8	69.4	66.4	69.6	0.8
Cleavage rate (%)	91.6	92.9	93.6	95.0	89.7	92.5	92.1	0.70
Pregnancy rate (%)	30.2	35.8	23.5	25.0	14.3	16.9	27.3	0.004
Oocytes retrieved (mean±SD)	8.7±4.9	11.0±5.4	9.8±6.0	11.0±7.6	11.4±5.5	11.0±5.0	9.9±5.4	0.01

SD=Standard deviation, P_4=Progesterone

ratio was found to be 0.35 [Figure 3]. PR (31.4%) among the patients having ≤ 0.35 P_4/E_2 ratio was significantly ($P = 0.047$) higher compared with 23.4% observed among the patients having value >0.35. However, fertilization and cleavage rates were not significantly ($P > 0.05$) different between the two categories. Similar ROC analysis was carried out according to the ovarian responders. As AUC was not significant, therefore P_4/E_2 cut-off value could not be predicted.

DISCUSSION

This study has analyzed the correlation between serum P_4, E_2 and P_4/E_2 ratio on hCG day with PR. As a secondary outcome, the effect on cleavage and fertilization rate was also studied. The results have shown that serum P_4 levels and P_4/E_2 ratio is a significant predictor for PR while the E_2 levels had no significant association. PR showed a significantly decreasing trend with an increasing level of P_4 and P_4/E_2 levels. Different cut-off levels for P_4 and P_4/E_2 were determined among different ovarian responders. Fertilization and cleavage rate were not affected by either P_4 or E_2 levels.

The potential effect of type of responders on the association between P_4 elevation and probability of pregnancy was also explored. Earlier there have been three studies in which data were analyzed according to the type of ovarian response.[19,20] Recent study by Xu *et al*. has concluded that there is a significant decrease in ongoing PR and implantation rate with *P* elevation in all ovarian responses to COH.[6] Our study has also shown results in agreement with that study and different cut-off values for P_4 among intermediate and high responders were predicted.

A positive correlation between number of mature follicles and secretion of P_4 in late follicular phase have been reported in earlier studies.[5] In the present study also, an increasing pattern of serum *P* level was observed with a significant ($P < 0.05$) difference between the three types of responders thus supporting the concept that increasing P_4 levels is a reflection of the number of follicles and not due to premature luteinization.

Till now, there have been numerous studies which have evaluated the association of P_4 elevation with PR with conflicting results. Our results were in agreement with a recent meta-analysis published in 2013 which have reported a detrimental effect of P_4 elevation on PR in range of 0.8–1.1 ng/ml (odds ratio: 0.79).[21] The present study has used ROC analysis which is a preferred method to identify optimal thresholds to define these detrimental cut-offs and the value was found to be 1.075 for an equivalent (53%) level of sensitivity and specificity.

Serum E_2 concentration on the day of hCG alone was not found to be a significant predictor of pregnancy status

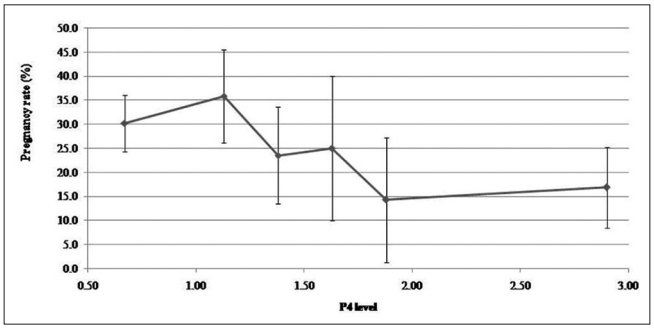

Figure 1: Trend of pregnancy rate with 95% confidence limits according to P_4 levels

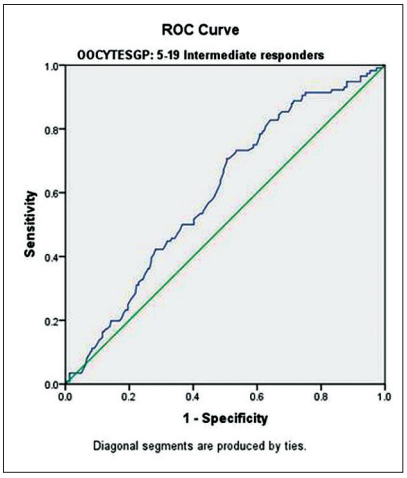

Figure 2: Receiver operating characteristic curve for defining optimal detrimental cut-off value for P_4 on human chorionic gonadotropin day

Table 3: Comparison of fertilization rate, cleavage rate, pregnancy rate and number of oocytes retrieved in different groups according to serum E_2 levels

	Group A (<1000 pg/mL) (*n*=35)	Group B (1000-2000 pg/mL) (*n*=111)	Group C (2000-3000 pg/mL) (*n*=120)	Group D (3000-4000 pg/mL) (*n*=86)	Group (>4000 pg/mL) (*n*=187)	Overall (*n*=539)	*P*
Fertilization rate (%)	75.2	67.3	71.6	67.1	69.9	69.6	0.13
Cleavage rate (%)	94.5	91.7	92.4	90.6	92.4	92.1	0.77
Pregnancy rate (%)	14.3	26.4	28.6	31.4	26.9	27.3	0.42
Oocyte retrieved (mean±SD)	5.1±2.7	7.1±4.2	8.5±3.5	13±6.0	13.6±5.4	10.4±5.7	0.001

SD=Standard deviation, E_2=Estradiol

Table 4: Recent studies showing the cut-off levels of P_4 or P_4/E_2 levels in women undergoing fresh IVF cycles

Study	Cycles/pt	Type of study	P_4 (ng/mL) or P_4/E_2 threshold	Method	Protocol	Day of ET
Azem *et al*.[25]	280/201	Retrospective	0.9	Arbitrary	GnRH agonist	Day 2/3
Bosch *et al*.[2] 2010	4032/not reported	Retrospective study	>1.5	Arbitrary	Different COS protocols	Not reported
Seow *et al*.[26] 2010	233/233	Prospective	1.2	ROC	Antagonist	Day 3
Yu *et al*.[27] 2010	200/200	Retrospective	0.9 and 3	Arbitrary	Agonist (short or long)	Not reported
Xu *et al*.[6]	11,055/11,055	Retrospective study	>1.5 for poor responders >1.75-intermediate >2.25-high responders	Arbitrary	Long agonist	Day 2 or 3
Rezaee *et al*.[28]	38/38	Prospective	1.2	Arbitrary	Long agonist	Day 2
Cetinkaya *et al*.[29] 2013	526/129	Observational	P/E_2 0.48	ROC	GnRH antagonist	Day 2 or 3
Bu *et al*.[30] 2014	4651/not reported	Retrospective	>1.60 for poor >2.24 for intermediate >2.50-high responders	-	Different COS protocols	Day 2-6
Wu *et al*.[1] 2012	2510/2510	Retrospective	1.05	ROC	Short protocol in 1970 pt and long luteal protocol in 951 pt	Day 3
Present study	539/544	Retrospective	>1.075-overall >1.075-intermediate responders >1.275-high responders	ROC	Long agonist protocol	Day 2/3 or 5

E_2=Estradiol, P_4=Progesterone, IVF=*In vitro* fertilization, ET=Embryo transfer, GnRH=Gonadotropin releasing hormone, COS= Controlled ovarian stimulation

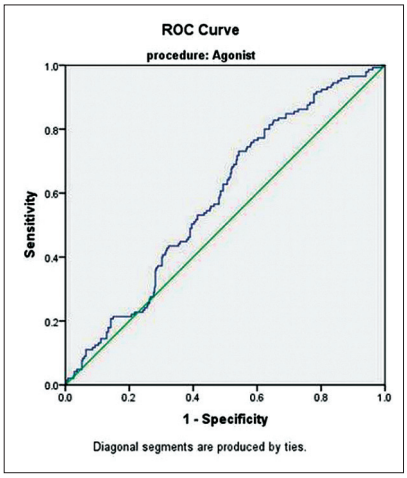

Figure 3: Receiver operating characteristic curve for defining optimal detrimental cut-off value for P_4/E_2 ratio on human chorionic gonadotropin day

which is in consensus with previous studies by Kyrou *et al*. (2012) and Yu Ng *et al*.[22,23]

Not only the P_4 levels alone but also P_4/E_2 has nowadays been considered to be a reasonable option for prediction of pregnancy outcome. A prospective cohort study by Elgindy published in 2012 have found a cut-off levels of P_4 >1.5 ng/ml and P_4/E_2 >0.55 as detrimental to pregnancy.[24] In our study, P_4/E_2 ratio ≤0.35 was found to be associated with a significantly higher PR compared to those having value >0.35 although no significant association with fertilization and cleavage rates was found. Table 4 are summarizes the most recent studies with different threshold cut-offs of serum P_4 and P_4/E_2 levels.[1,2,6,25-30]

Either cryopreservation of pronuclear or cleavage stage embryos or blastocyst transfer has been suggested as a strategy to overcome this problem. A recent study by Corti *et al*. in 2013 has concluded that fresh blastocyst transfer does not completely overcome the detrimental effects of progesterone rise at hCG on pregnancy outcome.[31]

CONCLUSION

The present study has shown a negative association of increasing P_4 levels and P_4/E_2 with PR. It still remains uncertain whether freezing the embryos and transferring them later in frozen-thawed cycles will be a solution to improve the pregnancy outcome when P_4 levels are high. More randomized studies are required to evaluate the effectiveness of frozen cycles in comparison to fresh cycles to overcome the detrimental effect of elevated P_4 or P_4/E_2 ratio.

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