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Follicular fluid steroid hormone levels are associated with fertilization outcome after intracytoplasmic sperm injection

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Abstract

Objective—To investigate the association between hormone levels from individual follicles and fertilization outcome among patients undergoing intracytoplasmic sperm injection (ICSI). Differences in concentrations of selected sex steroids and pituitary hormones in individual follicular aspirates between oocytes that fertilize successfully, those that fail to fertilize, and those that degenerate with ICSI were examined.

Design—Prospective cohort study.

Setting—Academic medical center.

Patient(s)—Women undergoing ovarian stimulation and ICSI.

Intervention(s)—Follicular fluid was sampled by transvaginal ultrasound-guided aspiration of the hyperstimulated ovary. Each follicle was individually aspirated and collected. Intracytoplasmic sperm injection and subsequent embryo culture were performed using standard laboratory technique. Follicular fluid gonadotropin and steroid hormone levels were measured by immunoassay.

Main Outcome Measure(s)—Oocyte fertilization outcome with ICSI.

Result(s)—Oocytes that fertilized normally came from follicles with higher estradiol (adjusted odds ratio [AOR] = 1.28) and testosterone (AOR = 1.35) concentrations compared with those that degenerated with ICSI. Oocytes that fertilized normally also came from follicles with higher estradiol (AOR = 1.14) and progesterone (AOR = 1.09) concentrations compared with those that failed to fertilize.

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Conclusion(s)—The hormonal profile of the follicular fluid yielding a degenerative egg or an egg that fails to fertilize is different from that resulting in normal fertilization. Higher follicular fluid estradiol may be a marker for oocytes that will fertilize normally with ICSI.

Keywords

Follicular fluid; hormone levels; fertilization outcomes; steroid hormones; FSH; estradiol; testosterone; follicular aspirate

To maximize IVF success, ovarian stimulation is performed to generate multiple oocytes in a given cycle of assisted reproductive technology. The regulation of selection, growth, and ovulation of the dominant follicle is a complex process that involves follicle stimulating hormone (FSH), lutenizing hormone (1), and modulation by an intraovarian network of hormonally regulated factors (2). Following ovarian hyperstimulation, follicular aspiration does not always yield a “healthy” egg. Despite nuclear maturation, some oocytes degenerate with ICSI and others fail to fertilize. When faced with these situations, possible etiologies for oocyte degeneration include technical skill of the ICSI operator and intrinsic oocyte “health”; although failed fertilization could be, again, because of the operator skill, but also quality of either the oocyte or sperm. We designed this study to gain a better understanding of the importance of the follicular hormonal biology that supports oocyte competence following ovarian stimulation in determination of fertilization capacity.

Recent work from our center suggests that oocyte recovery has a biologic basis dependent upon the follicular fluid hormonal milieu, with FSH levels being greater in follicles that yield an egg (3). This is not a new concept; in fact, several previous studies have attempted to establish correlations between the concentrations of follicular fluid hormone levels and oocyte “quality.” Andersen (4) found that eggs and embryos from follicles with increased concentrations of androgens and reduced estradiol (E_2)–androgen ratios were less likely to result in pregnancy with IVF. Prior studies evaluating fertilization by conventional insemination were limited in their ability to determine the impact of the oocyte’s nuclear maturity on fertilization outcome (5). Mendoza et al. (6) overcame this limitation by analyzing hormones from follicles yielding oocytes destined for ICSI and found follicular fluid concentrations of progesterone (P), growth hormone (GH), prolactin (PRL), interleukin-1, and tumor necrosis factor to be directly correlated with normal fertilization, embryo morphology, and cleavage. However, they did not control for size of the follicle, which has significant impact on oocyte health and follicular hormonal concentrations (1, 7, 8). Conversely, Asimakopoulos and colleagues (9) recently found no significant difference in steroid and cytokine concentrations comparing oocytes that fertilized normally to those with three pronuclei and those that failed to fertilize following ICSI. They did not, however, evaluate gonadotropin concentrations or evaluate the degenerated egg as a separate fertilization outcome. In addition, although Asimakopoulos et al. measured individual follicular hormone levels, they also did not control for follicular size.

Each oocyte develops within a distinct microenvironment of granulosa cells and follicular fluid. The goal of this study was to gain a better understanding of the association of the follicular hormonal milieu, on oocyte fate, by evaluating follicular fluid obtained from

follicles that yielded either an egg that normally fertilized, degenerated, or failed to fertilize with ICSI. We hypothesize that differences exist between hormone concentrations in follicular aspirates of those oocytes that fertilize successfully, those that fail to fertilize, or those that undergo degeneration after ICSI.

METHODS

Study Population

Since April of 2005, all IVF patients at the University of California, San Francisco (UCSF) have been offered participation in research to understand and improve IVF outcomes by collection and banking of biologic materials retrieved at the time oocyte harvest. In general, 68.5% of patients who present to our clinic agree to participate in some type of research including having follicular fluid banked during the time period of this study. Patients who consented had follicular fluid aspirated from the first aspirate from each ovary. Following identification of the oocyte, the retrieved fluid was individually collected and banked in a consecutive fashion and linked to the individually cultured oocyte. All follicular fluid from follicles yielding a degenerative egg or an egg that failed to fertilize, after ICSI, was analyzed. Because of the large number of samples and the expense of completion of assays, a random selection of fifty fluids (out of 285), that yielded a two-pronuclei (2PN) zygote, underwent hormonal analysis. For all analyses, only a single follicle per subject was included. This study was approved by the institutional review board at UCSF.

Follicular Size Measurement and Egg Retrieval

Follicular growth was monitored by transvaginal ultrasound (Shimadzu SDU-450XL, Kyoto, Japan), and follicles were measured to obtain a two-dimensional mean diameter. The timing of administration of hCG was based on two or more follicles measuring 18 mm or greater, in mean diameter. Transvaginal ultrasound-guided follicular aspiration was conducted 36 hours post-hCG administration.

Before obtaining follicular fluid, the collection system (needle and tubing) was flushed and cleared so that follicular size could be extrapolated by follicular fluid volume measurement. The first follicle from one ovary was aspirated and collected. Each follicle was pierced with a single lumen needle, emptied completely, and then the needle withdrawn. The tubing was then flushed and the contents collected in a separate tube to assure capture of the corresponding oocyte.

For each follicle, the presence or absence of an egg was recorded immediately and the follicular fluid was placed into a 15-mL sterile Falcon conical tube. The volume of fluid aspirated per individual follicle was recorded and correlated to corresponding follicle size as previously described by Wittmaack et al. (10). If the follicular fluid contained blood the sample was excluded from the study. The follicular fluid was cleared by centrifugation, aliquoted into 5-mL Falcon tubes, and placed at −80°C for later analysis.

Oocyte and Fertilization Assessment

The fertilization method was determined by male infertility diagnosis. Intracytoplasmic sperm injection is routinely performed on patients with a history of failed fertilization, sperm concentration <15 mil/mL, motility <20%, or Kruger morphology <4%. Oocytes were stripped of surrounding cumulus cells and inspected for meiotic maturation. Mature (metaphase II) oocytes were defined by presence of first polar body; immature (germinal vesicle or metaphase I) and degenerative oocytes were identified and excluded from study. Intracytoplasmic sperm injection was performed on metaphase II oocytes using the direct penetration technique. Fertilization results were assessed 16 to 19 hours after ICSI. Fertilization was considered normal by the presence of two pronuclei. Oocyte degeneration was identified by collapse of cytoplasmic contents and separation from the zona (11). Failed fertilization was defined by the absence of pronuclei.

Follicular Fluid Hormones

Before running samples, follicular fluid assays were calibrated to known standards and validated by serial dilution. The following hormone concentrations were quantified in batch and duplicate, and measured with commercially available automated chemiluminescent immunoassays on the DPC-Immulite 1000 (Diagnostic Products, Los Angeles, CA): estradiol (E_2), P, total testosterone (T), PRL, FSH, hCG, and growth hormone (GH). These hormones were selected, guided by published literature, because of either their possible or established association with the outcome of interest or to each other. Each test was run with three controls of low, medium, and high concentrations. Dilutions were performed before measurement of E_2 (1:1,000) and P (1:500), depending on the calibration range. The intraassay coefficient of variations were: E_2 11.9%, P 3.6%, T 8.7%, PRL 6.8%, hCG 5.1%, FSH 3.2%, and GH 11.9%. High or low results were repeated with appropriate dilution. The published serum data for Immulite intraassay coefficient of variations for our range of concentrations are: E_2 7.1%, P 6.3%, T 6.8%, PRL 5.7%, hCG 4.7%, FSH 2.6%, and GH 5.3% (Immulite 1000, Siemens Medical Solutions Diagnostics).

Statistical Analysis

Analysis of variance was performed to compare patient and cycle characteristics by the fertilization outcome: normal fertilization (2PN), failed fertilization, or degeneration after ICSI. Next, the concentrations of follicular fluid E_2, P, T, FSH, hCG, PRL, and GH where compared among the eggs by fertilization outcome. The association between the follicular fluid hormone concentrations and the fertilization result were checked for linearity. Multivariate logistic regression was performed to determine the magnitude of the association for each hormone on the fertilization outcome, adjusting for follicular fluid volume. Next, each patient characteristic as well as follicular fluid FSH and hCG were added to the model to determine if any of the added variables were confounders. A sensitivity analysis was done including only fluid from those follicles from lupron suppression protocols to exclude the impact of stimulation selection. STATA version 9.0 (Stata Corporation, College Station, TX) was used for statistical analysis. Tests were declared statistically significant for a value of $P<.05$.

RESULTS

The follicular fluid bank contains data from fluid that yielded 285 2PN zygotes, 55 oocytes that failed to fertilize with ICSI, and 38 oocytes that degenerated with ICSI. Follicular fluid hormone levels were measured in all follicles that yielded volumes between 2 cc and 9 cc (indicating follicular size 16–25 mm) of nonbloody fluid, and either an egg that degenerated or one that failed to fertilize with ICSI. We measured follicular fluid hormone levels from 48 follicles that yielded an egg that failed to fertilize and 29 follicles that yielded an egg that degenerated with ICSI attempt. Follicular fluid hormone levels were also measured from 50 randomly chosen follicles that yielded an MII oocyte that became a 2PN zygote. The MII recovery rate in this study population was 80% of oocytes retrieved.

Baseline patient characteristics were similar between the three groups for age, day 3 FSH, number of oocytes retrieved, and number of embryos transferred (Table 1). The peak serum E_2 (2,448 vs. 1981 pg/mL, $P<.05$) was significantly higher in the normal fertilization group versus those that degenerated.

Among the follicular fluid hormone concentrations, E_2, P, and T levels were significantly different between the three fertilization outcomes ($P=.003$, .018, and .004, respectively) (Table 2). No differences were detected in the other hormones measured.

Relationship between Follicular Fluid Hormone Concentrations from Follicles That Yielded an Oocyte That Fertilized Normally versus One That Degenerated with ICSI

The mean E_2 and T concentrations from a follicle that resulted in an oocyte that fertilized normally was significantly higher compared with that of a follicle yielding an oocyte that degenerated with ICSI (adjusted odds ratio [AOR] = 1.28 $P=.01$, 95% confidence interval [CI] = 1.05,1.56; AOR = 1.35 $P=.02$, 95% CI 1.05,1.73, respectively) (Table 3). Thus, for every 100 ng/mL increase in follicular fluid E_2 there is 28% greater odds of the follicle yielding an ooctye that fertilizes normally; and for every 100 ng/dL increase in T there is a 35% higher odds of the follicle yielding an oocyte that fertilizes normally. Other steroid and gonadotropin concentrations were similar between the two groups.

Relationship between Follicular Fluid Hormone Concentrations from Follicles That Yielded an Oocyte That Fertilized Normally versus One That Failed to Fertilize with ICSI

Oocytes that fertilized normally with ICSI were retrieved from follicles with significantly higher concentrations of E_2 and P than those that yielded an MII oocyte that failed to fertilize (AOR = 1.14, $P=.05$, 95% CI = 1.00, 1.20, AOR = 1.09, $P=.01$, 95% CI = 1.02, 1.17, respectively) (Table 4). Thus, for every 100 ng/mL increase in follicular fluid E_2 or P there is 14% or 9%, respectively, greater odds of yielding an oocyte that fertilizes normally than one that fails to fertilize. Other steroid and gonadotropin concentrations were similar between the groups.

The magnitude of the association was not different when accounting for follicular fluid hCG (AOR = 1.06, $P=.033$, 95% CI = 1.00, 1.2), or FSH (AOR = 1.05, $P=.062$, 95% CI = 1.00, 1.12). In addition, no differences were observed in the outcomes when limiting analysis to fluid collected from follicles aspirated from patients undergoing lupron suppression

protocols. Further, no patient specific characteristics diminished the predictor's (E_2, P, or T) significant effect on the fertilization outcomes.

DISCUSSION

The hormonal profile of the follicular fluid that yields an egg that degenerates or fails to fertilize with ICSI is distinctly different from that of normal fertilization. This suggests oocyte "health" is a significant factor in ICSI fertilization success.

Our findings differ from the recent study by Asimakopoulos et al. (9), where no differences between the steroid products in follicular fluid from follicles that yield a healthy egg versus one that fails to fertilize were identified. Our current study controls for follicular fluid volume, and therefore follicular size, and also looks at the follicular fluid hormonal milieu from a follicle that yields an egg that degenerates with ICSI. Limiting this study to ICSI fertilization allowed us to specifically investigate the relationship between the intrafollicular homone levels and the specific fertilization outcome by controlling for nuclear maturation.

A lower E_2 level was found and persisted when controlled for follicular fluid volume in follicles yielding an egg that degenerated with ICSI or one that failed to fertilize. No difference in gonadotropin levels was seen between the groups. The decrease in the granulosa cell steroid products suggests that these supporting cells are failing despite supraphysiologic gonadotropin stimulation. This is consistent with type B atresia, whereby granulosa cell demise precedes that of the oocyte (5).

Does this difference suggest something inherently different in the follicle, within the oocyte itself, or between subjects? Follicles from the same patient can yield oocytes with inherent differences in fertilization and developmental capacity. For example, Van Blerkom et al. (12) found that similar-sized follicles from the same patient had significant differences in dissolved oxygen content associated with resultant cleavage differences. Our data suggest the local follicular environment may play a key role in the observed differences in the developmental capacities. Further studies, with a larger patient population, and multiple follicles per patient, are required to address patient-specific issues.

One limitation of this study is the potential increased variability of the coefficients of variations for the assays of some of the follicular fluid hormones measured. Likely some of the variability is because of the high levels of the hormones in the follicular fluid that requires serial dilutions, introducing larger variability, thus yielding higher coefficients of variance than would be expected for serum. This increased variability should theoretically attenuate the results making it less likely to detect a difference. Interestingly, we still find significant differences in these follicular fluid hormone levels.

The design of this study did not allow us to answer if follicular fluid hormone concentrations could predict pregnancy rates or embryo quality. Others have attempted to look at this (13). Yet, the ideal study should involve aspirating each follicle, measuring the follicular fluid hormones, and culturing the embryos individually allowing assessment of additional outcomes while still controlling for follicular fluid volume. Additionally, the analysis would have to confirm the fate and be limited to 100% implantation or failure to allow for the most

accurate information. However, the feasibility of such a study has proven difficult, as it would require multiple vaginal punctures. In the future, it is possible that we will be able to assess all transferred embryos where we had follicular fluid obtained to answer this valuable question.

FSH and hCG are known to increase follicular E_2 levels (14). Adjusting for FSH and hCG in the analysis did not account for the observed differences in E_2 levels between the specific fertilization outcomes.

Follicular fluid hCG, used to promote nuclear maturation, had no predictive value for normal fertilization and presumed cytoplasmic maturation. Follicular fluid E_2 levels, however, correlated with oocyte competence as defined by the ability to undergo normal fertilization. Not surprisingly, serum E_2 levels were also greater in patients whose follicle yielded an MII oocyte that became a 2PN. Serum E_2 levels were also greater per follicle and per egg retrieved suggesting there is a global increase in granulosa cell capacity. We hypothesize E_2 likely serves as a surrogate marker rather than having a causal role in cytoplasmic maturation, and perhaps will some day help us counsel patients regarding fertilization expectations.

Improved understanding of oocyte–granulosa cell biology may lead to advancements in our understanding of ovarian response to stimulation and subsequently to improvements in ovarian stimulation protocols. This would allow optimization of the intrafollicular hormonal environment, and potentially, improve oocyte competence.

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TABLE 1

Patient and cycle characteristics.

	Degenerated egg (mean ± SD)	Failed fertilization (mean ± SD)	2PN (mean ± SD)	*P* value
Oocyte age (y)	36.1 ± 5.3	33.8 ± 6.3	33.8 ± 6.6	.41
Day 3 serum FSH (IU/L)	7.5 ± 2.2	8.2 ± 3.6	6.8 ± 2.6	.27
Cycle day of hCG	9.9 ± 1.4	9.9 ± 1.5	9.9 ± 1.4	.58
Peak serum estradiol (pg/mL)	1,981.1 ± 1,061	2,154.4 ± 1,341	2,448.2 ± 1,309	<.05
Peak serum estradiol per egg recovered (pg/mL)	164.5 ± 51.6	170.8 ± 62.7	181.5 ± 81.3	<.02
Number of oocytes retrieved	12.9 ± 6.0	14.4 ± 7.9	15.7 ± 8.5	.22
Follicular volume from single follicle (mL)	3.5 ± 2.2	4.2 ± 2.4	3.9 ± 2.1	.26
Number of embryos transferred	2.4 ± 0.9	2.5 ± 1.0	2.8 ± 1/3	.99

TABLE 2

ANOVA comparison of follicular fluid hormone levels between different fertilization outcomes.

	Degenerated egg mean ± SD (n = 29)	Failed fertilization mean ± SD (n = 48)	2PN mean ± SD (n = 50)	*P* value
Estradiol (ng/mL)	359.1 ± 211.6	438.7 ± 317.8	596.4 ± 354.7	.003
FSH (IU/L)	8.36 ± 6.3	8.0 ± 5.6	9.43 ± 6.3	.491
Progesterone (μg/mL)	10.5 ± 6.8	7.5 ± 5.1	11.7 ± 8.5	.018
hCG (IU/L)	94.6 ± 53.7	103.5 ± 60.7	99.6 ± 61.0	.817
Prolactin (ng/mL)	291.0 ± 224.1	316.4 ± 117.8	275.4 ± 139.0	.671
Testosterone (ng/dL)	362.4 ± 344.9	617.5 ± 390.6	563.8 ± 257.8	.004
Growth hormone (μg/mL)	1.7 ± 1.1	2.4 ± 2.6	1.8 ± 1.2	.163

TABLE 3

Multivariate analysis: effect of follicular fluid hormone levels on the odds of normal fertilization versus degeneration with ICSI.

	AOR[a]	*P* value	95% CI
Estradiol (per 100 ng/mL)	1.28	.01	1.05; 1.56
FSH (IU/L)	1.01	.62	0.94; 1.10
Progesterone (μg/mL)	1.02	.63	0.95; 1.08
hCG (IU/L)	1.00	.58	0.99; 1.01
Prolactin (ng/mL)	0.99	.98	0.99; 1.00
Testosterone (per 100 ng/dL)	1.35	.02	1.05; 1.73
Growth hormone (μg/mL)	1.19	.44	0.77; 1.83

Note: AOR = adjusted odds ratio; CI = conficence interval.

[a]Adjusted for follicular fluid volume.

TABLE 4

Multivariate analysis: effect of follicular fluid hormone levels on odds of normal versus failed fertilization.

	AOR[a]	*P* value	95% CI
Estradiol (per 100 ng/mL)	1.14	.05	1.00; 1.20
FSH (IU/L)	1.03	.25	0.97; 1.11
Progesterone (per 100 μg/mL)	1.09	.01	1.02; 1.17
hCG (IU/L)	0.99	.87	0.99; 1.01
Prolactin (ng/mL)	1.00	.36	0.99; 1.00
Testosterone (ng/dL)	0.93	.28	0.82; 1.06
Growth hormone (μg/mL)	0.85	.25	0.65; 1.12

Note: AOR = adjusted odds ratio; CI = confidence interval.

[a] Adjusted for follicular fluid volume.