Oocyte Morphology and Early Zygote Cleavage Does Not Appear to Be Related to Intrafollicular Concentrations of Inhibin-A or -B¹

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Purpose: The aim was to investigate the relationship of intrafollicular inhibin dimers A and B with human oocyte morphology and subsequent embryo potential.

Methods: Sixty-eight oocytes were isolated from 31 women undertaking intracytoplasmic sperm injection (ICSI). Estradiol, progesterone, testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, sex hormone-binding globulin, inhibin-A and inhibin-B was assayed in corresponding follicular fluid.

Results: The mean ($\pm$ SD) concentration for inhibin-A was 9.7 ± 9.8 ng/ml (range, 1.1–60.0 ng/ml) and for inhibin-B was 269.4 ± 185.2 ng/ml (range, 33.1–811.0 ng/ml). In a correlation matrix there were no marked relationships ($r < 0.556$) between inhibin and steroid or gonadotropin concentrations. Similarly, when inhibin concentrations were divided according to whether the oocytes had mature or immature cumulus complexes, were viable or necrotic, were meiotically immature or mature, became fertilized or not, or had different embryo gradings after cleavage, no statistically significant difference could be seen between groupings.

Conclusions: Because the range of values was large and the data often skewed, neither inhibin dimer has discriminatory power to reflect the potential of the oocyte.

KEY WORDS: Oocyte maturation; follicular fluid; inhibin-B; inhibin-A; assisted reproductive technology.

INTRODUCTION

Oocyte maturation preovulation is a complex process mediated through a number of mechanisms including

production by granulosa cells of growth factors, cytokines, and inhibins. Gonadotropin modulation of follicular concentrations of these paracrine factors may ultimately be responsible for the precise regulation of oocyte function (1). Several studies have examined the relationship of intrafollicular compounds to pregnancy rates including the effects of steroids (2). None have proved particularly useful in determining the pregnancy potential of oocytes recovered for assisted reproductive technology (ART) so that the gross morphology of the surrounding cumulus cells (3) and phenotypic appearance of the oocyte remain the method of choice for embryologists whether or not data support (4) or refute (5) the correlation of visible morphology with pregnancy.

Inhibin similarly has been measured in human periovulatory peritoneal fluid (6) and in follicular fluid (7), but while correlation with pregnancy outcome has been attempted (8–10), the status of the oocyte has usually been ignored. Since Alak et al. (11) have shown inhibin to significantly increase the percentage of rhesus monkey metaphase II (MPII) oocytes that fertilize compared to controls, and Stock et al. (12) have suggested that inhibin-A plays an important role during the final stages of bovine oogenesis, concentrations of inhibin in follicular fluid may be a useful marker of oocyte performance in human ART. This premise has been further indicated because histochemical studies of bovine cumulus–oocyte complexes (COC) have suggested that inhibin may modulate the action of activin on oocyte maturation (13). The objective of this study therefore was to investigate any correlation of intrafollicular inhibin on human cumulus–oocyte cell morphology, oocyte meiotic maturity, and subsequent embryo development in women being treated by ART.

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MATERIALS AND METHODS

Patients

Because each oocyte was to be observed individually, only couples undertaking intracytoplasmic sperm injection (ICSI) were considered for this study, since oocytes are denuded of cumulus cells soon after collection from their follicle. Thus, in each of the 31 women (mean $\pm$ SD age, 33.7 ± 5.6 years; range 23.0–46.3; mean $\pm$ SD body mass index, 22.5 ± 5.4) from whom oocytes were collected, controlled ovarian hyperstimulation was achieved by pituitary desensitization and down-regulation using gonadotropin-releasing hormone (GnRH) agonists leuporelin acetate (Lucrin, Abbott Australasia, Sydney, Australia) or nafarelin acetate (Synarel, Searle Pharmaceutical Products, Sydney, Australia) and subsequent stimulation with follicle-stimulating hormone (FSH) [Metrodin HP or Gonal F (29 women), Serono Australia Pty Ltd, Sydney, Australia, or Puregon (2 women), Organon Pty Ltd, 31-35 Sirius Rd., Lane Cove, NSW 2066, Australia]. The protocols were flexible depending on patient requirement and response with exogenous gonadotropin doses ranging from 750–11,250 IU (mean $\pm$ SD 2400 ± 1950 IU). Final maturation of oocytes was induced with 10,000 IU hCG (Profasi, Serono) 38 hr before oocyte collection performed by transvaginal ultrasound-directed needle aspiration when two or more follicles were >16 mm in diameter and estradiol (E_2) concentrations were >2000 pmole/liter (mean $\pm$ SD 6525 ± 3198 pmole/liter). ICSI was performed between 40 and 42 hr post-human chorionic gonadotropin (hCG) injection. Luteal supplementation was by either progesterone pessaries (200 mg daily: 15 women) or hCG injection (4×2000 IU: 16 women). Sperm for microinjection were isolated from fresh ejaculates (23 men), percutaneous epididymal aspirations (5 men), nonfrozen surgically collected testicular tissue (2 men), or frozen donated semen (1 couple).

Follicular Fluid Collection

The operating surgeon aspirated individual antral follicles using a 16-gauge single-lumen needle (Cook Australia: K-OPS-2125-RWH-BF-ET) continuing suction until the follicle collapsed or the flow stopped. The dead space within the needle and tubing was then suctioned by removal of the needle from the vagina. If there was any doubt about the individuality of follicular fluid collection or whether more than one oocyte was found in the aspirate, these tissues were not included

in the study. Because of the difficulty of isolating aspirates from individual follicles free of extraneous blood contamination, only a few oocytes for study were ever collected from individual patients and generally from large Graafian follicles.

After isolation of the oocyte cumulus mass the aspirate volume was measured before any remaining follicular cells were separated by centrifugation at 500 g for 10 min. The supernatants were stored at -20°C until assayed for E_2 , progesterone (P), testosterone (T), sex hormone-binding globulin (SHBG), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (Prl), and inhibin-A and -B.

Oocyte Preparation and Classification

Cumulus–oocyte complexes (COC) were described as either immature or mature. In the former there was no expansion of the cells and they were tightly packed around the oocyte to a maximum of approximately 5 oocyte diameters. Mature COCs showed the classic expansion of cells into a stringy mass but still apposed to the oocyte. All oocytes had their cumulus cells removed by 15- to 30-sec exposure to 80 IU/ml hyaluronidase (Sigma Type VIII Bovine Testis Enzyme, St. Louis, MO), followed by repeated washing through HEPES-HTFM (Irvine Scientific, Santa Ana, CA) + 10% deactivated autologous maternal serum. Coronal cells were mechanically removed by aspirating the oocytes through finely drawn glass Pasteur pipettes. The denuded oocytes were graded prior to microinjection and then cultured in four-well Nunc dishes (Nunc, Medos, Australia) in bicarbonate buffered-HTFM (Irvine Scientific, Santa Ana, CA) + 10% deactivated maternal serum in an atmosphere of 6% CO_2 in air at 37°C .

Oocytes were considered suitable for microinjection if they had an intact zona pellucida and oolemma and had reached MP II with one clearly visible polar body in the perivitelline space. The characteristics and stages of oocytes unsuitable for injection were as follows: (a) prophase I stage oocytes that had a clear germinal vesicle (GV) in the cytoplasm; (b) metaphase I (MPI) stage oocytes where GV breakdown had occurred but the first polar body had not been extruded into the perivitelline space; and (c) oocytes with structural faults leading to necrotic or grossly misshapen oocytes.

Measurement of Analytes

As part of the routine monitoring of patients for ICSI, peripheral venous serum concentrations of E_2

were performed. The follicular fluid concentrations of E_2 , P, T, LH, FSH, and Prl were measured using the same commercially available system (ACS180, Chiron Corp, PO Box 158, Ferntree Gully, VIC 3156, Australia) after suitable dilution to allow reading from the most sensitive part of the assay curve. The inter- and intraassay variation for all analytes was <8%. SHBG was determined using DELFIA (Australian laboratory Services, PO Box 193, Rockdale, NSW 2216, Australia). Similarly inhibin-A and Inhibin-B dimers were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) technique (Sero-tec Ltd, 22 Bankside, Station Approach, Kidlington, Oxford OX5 1JE, England). The inter- and intra-assay variation for each dimer at a concentration of 15 pg/ml was 7%.

RESULTS

Two hundred and seventy-three oocytes were collected from the 31 women (median 9, range 1–26). Generally (17/31) two embryos were transferred (range 0–3) 2 days later, and 10 of the 31 couples had supernumerary embryos suitable for cryopreservation. All but one woman progressed to transfer of embryos and 6/30 (20%) had a positive pregnancy and detectable fetal heart beat 6 weeks later. For the woman who did not progress to transfer, failure of fertilization was related to the quality of the single oocyte rather than the sperm. Sixty-eight oocytes (25% of those collected) were able to be isolated from individual follicles without obvious contamination and aspirates were available for analysis.

The aspirated follicles ranged in volume between 1.0–20.0 ml (mean $\pm$ SD, 6.4 ± 3.8 ml) (equivalent to follicular diameters of 12–34 mm if a sphere was assumed) and intrafollicular concentrations of inhibin-A ranged between 1.1 and 60.0 ng/ml with a normal distribution about a mean ($\pm$ SD) of 9.7 ± 9.8 ng/ml. The equivalent value for inhibin-B were 33.1–811.0 ng/ml range and 269.4 ± 185.2 ng/ml. Table I details the relationship between all analytes in follicular fluid [including the free androgen index (FAI), calculated as testosterone/SHBG $\times$ 100], demonstrating that there were no marked correlations ($r < 0.556$).

Tables II and III present summary statistics for inhibin-A and inhibin-B concentrations split according to whether the oocytes had mature or immature COC, were MI, MII, or GV oocytes, contained structural faults within the oocyte, became fertilized or not, or had different embryo gradings after cleavage. Again,

no significant difference could be seen between groupings, the ranges were large, and the data often skewed, suggesting that neither inhibin dimer has discriminatory power to reflect the status of the oocyte whether expressed as a concentration (Tables II and III) or total inhibin per follicle (Figs. 1 and 2). Each “box” in Figs. 1 and 2 represents the interquartile range of the data (25th to 75th percentile) and is notched at the median value (50th percentile). The 5th and 95th percentiles are represented by the “whiskers” either side of the box and outliers as individual points. If notches overlap, the distribution of values are not significantly different.

Similar groupings of the other analytes showed the same scatter of data with large variation. The individual concentrations [mean $\pm$ SD, median, minimum, interquartile range (IQR) and maximum concentrations, respectively] in follicular fluid were: E_2 (1249.3 ± 342.6 , 1254.5, 478.4, 512.7, 2267.0 nmole/liter). P (31.6 ± 12.7 , 30.7, 8.0, 17.5, 59.9 nmole/liter); T (46.5 ± 19.3 , 43.5, 21.0, 13.5, 150.0 nmole/liter); SHBG (110.8 ± 48.3 , 104.9, 42.3, 58.1, 238.1 nmole/liter); FAI (50.6 ± 30.1 , 43.1, 15.9, 27.7, 127.3); LH (0.3 ± 0.8 , 0.2, <0.01, 0.2, 7.0 IU/liter); FSH (36.7 ± 4.3 , 36.0, 28.0, 5.0, 61.0 IU/liter); and Prl (36.7 ± 22.1 , 36.5, 2.1, 30.8, 88.1 IU/liter).

DISCUSSION

In the past decade, the role of inhibin in the human ovarian cycle has been extensively studied (14,15). However, while it has been suggested that inhibin may be a better marker of follicular development than E_2 in ART patients whose pituitary's have been desensitized (16) a well-controlled, randomized, assessor-blind study (17) and a case-controlled study (18) have refuted this. Similarly, while Seifer et al. (19) and Lindheim et al. (20) have suggested a poorer response to ovulation induction and reduced pregnancy rates in ART in women with low early follicular-phase inhibin-B concentrations, this predictor has not been widely accepted. A further reflection of the low predictive value of inhibin measurements in ART was exemplified by the study of Norman et al. (21) who showed that pregnancies that ended in miscarriages tended to have lower concentrations of inhibin in the luteal phase, but no reliable discrimination could be found between the clinical conditions of blighted ovum and of spontaneous abortion.

However, the microenvironment of the follicle presents a different scenario to the measurement of inhibin in peripheral venous plasma and Billiar et al. (6),

Table I. A Correlation Matrix of Follicular Fluid Endocrine and Inhibin Dimer Concentrations^a

	Volume	P	E ₂	T	FSH	SHBG	FAI	LH	Prl	Inhibin-B
P	0.32									
E ₂	0.30	0.07								
T	0.16	0.06	0.56							
FSH	-0.03	-0.08	-0.01	-0.15						
SHBG	0.24	-0.15	0.27	0.08	-0.13					
FAI	0.01	0.20	0.21	0.54	0.07	-0.68				
LH	-0.02	-0.15	0.15	0.15	0.02	-0.13	0.31			
Prl	-0.19	0.15	-0.14	-0.24	0.13	-0.29	-0.02	-0.11		
Inhibin-B	-0.29	-0.23	-0.03	-0.16	0.12	-0.17	-0.05	-0.12	0.16	
Inhibin-A	0.41	0.01	0.23	0.13	-0.05	0.06	0.12	-0.09	0.08	0.07

^a Progesterone (P), estradiol (E₂), testosterone (T), sex hormone binding globulin (SHBG), prolactin (Prl).

Table II. Summary Statistics for Inhibin-A Concentration (ng/ml) in Follicular Fluid and the Associated Status of the Oocyte and Subsequent Embryo Development^a

	N	Mean	SD	Median	Min	IQR	Max
Immature COC	14	6.7	6.0	5.5	1.1	6.4	21.1
Mature COC	54	10.5	10.4	8.1	2.0	9.9	60.0
Abnormal oocytes	9	5.0	3.6	4.1	1.1	5.7	11.4
GV oocyte	1			21.1			
MI oocytes	2	8.8		8.8	2.0		15.6
MII oocytes	56	10.3	10.3	8.1	2.0	9.5	60.0
Fertilized (2PN)	39	10.7	11.3	7.9	2.0	10.2	60.0
Unfertilized	12	8.7	9.3	5.8	2.0	10.1	34.0
Abnormally activated (1 or 3PN)	2	12.1		12.1	11.0		13.3
Embryo grade 3	13	8.9	8.2	6.7	2.0	10.9	29.5
Embryo grade 4	16	8.7	5.3	8.1	2.0	3.7	19.6
Embryo grade 5	9	17.6	19.5	8.8	2.0	20.6	60.0

^a Min, IQR, and Max = minimum, interquartile range, and maximum values, respectively.

because they were able to measure inhibin in peritoneal fluid, speculated that inhibin may have potential implications for oocyte maturation and early embryogenesis within the oviduct. Several publications have detailed inhibin concentration in follicular fluid but none have

related these to oocyte morphology or meiotic maturity because classic IVF was used. Unlike previous reports this study has utilized gametes obtained for ICSI, thus allowing observation of oocytes from the time of their collection with no masking of visual status by cumulus

Table III. Summary Statistics for Inhibin-B Concentration (ng/ml) in Follicular Fluid and the Associated Status of the Oocyte and Subsequent Embryo Development^a

	N	Mean	SD	Median	Min	IQR	Max
Immature COC	14	174.6	96.0	182.9	35.5	156.6	381.4
Mature COC	54	294.0	195.2	239.2	33.1	260.3	811.0
Abnormal oocytes	9	150.4	79.7	135.7	35.5	130.1	261.6
GV oocyte	1			178.1			
MI oocytes	2	221.4		221.4	61.3		381.4
MII oocytes	56	291.9	191.9	239.2	33.1	253.2	811.0
Fertilized (2PN)	39	308.2	211.6	254.2	33.1	278.8	811.0
Unfertilized	12	261.1	131.8	250.0	62.2	169.2	528.7
Abnormally activated (1 or 3PN)	2	206.2		206.2	169.3		243.1
Embryo grade 3	13	202.0	101.2	210.1	49.3	108.1	400.2
Embryo grade 4	16	369.6	243.2	295.3	33.1	336.0	811.0
Embryo grade 5	9	346.2	225.2	388.9	47.5	329.8	783.7

^a Min, IQR, and Max = minimum, interquartile range, and maximum values, respectively.

Fig. 1. Box plots of the distribution of intrafollicular inhibin-A and -B content per follicle grouped according to oocyte status.

cells. The study therefore has specifically concentrated on the practicality of using inhibin as a marker of oocyte function in routine ART.

Intrafollicular concentrations of inhibin in this study corresponded well to published data and have confirmed the marked increase in concentration of inhibin-B over inhibin-A (22). The lack of any relationship between inhibin and other follicular analytes confirms the work of Sudik et al. (23) and Reichard et al. (10). The only consistent correlation appears to be with follicular size. Thus Reichard et al. (10) found a correlation between inhibin concentration and follicular size ($r = 0.75$), E_2 ($r = 0.57$), and P ($r = 0.82$) concentration, but the follicles they studied ranged in size between 4.8 and 20 mm in diameter. Similarly, Lam-

bert-Messerlian et al. (24) collected aspirates from women with and without polycystic ovarian syndrome and found that inhibin-A increased with follicular volume and inhibin-B decreased in "normal" women but not in patients with polycystic ovaries. Neither study reported on oocyte morphology. In this study of antral follicles from women being treated by ART, little association with follicular volume ($r < 0.4$), inhibin, or steroid concentration could be demonstrated ($r < 0.56$).

Similarly, most of the oocytes were fully mature in this study (56/68; 82.3%) and the large range of data for inhibin suggests that, for antral follicles at least, there is no relationship between follicular inhibin and oocyte maturity. The potential of oocytes to resume meiosis after ICSI and successfully cleave also is unrelated to intrafollicular inhibin concentrations. Also, there were marked differences in inhibin concentrations between follicles from the same woman from whom only MII oocytes were collected. Inhibin values from follicles containing necrotic and immature oocytes overlapped with those from follicles where viable oocytes were obtained (Tables II and III). In contrast to this, Andersen (8) measured inhibin in 23 follicular fluids that each gave an oocyte known to result in a clinical pregnancy after ART and compared it with data from follicles obtained from women who failed to conceive after embryo transfer. While he did find an association with immunoreactive inhibin and pregnancy outcome, he did not comment on oocyte morphology.

Thus the data from this study and those previously published on intrafollicular inhibin concentrations appear to lack consistency. For any method to be a useful predictor of outcome it should satisfy several conditions. It must be technically easy and cost-effective to apply, have a low failure rate, and give a rapid turnaround result. The measurement of inhibin in follicular fluid meets none of the above requirements, and thus helps neither the embryologist nor the patient. The measurement of intrafollicular inhibin A or B appears to have no predictive value for the maturity of the cumulus-oocyte complex, the meiotic status, fertilization capacity, or cleavage potential of the oocyte and resulting embryo.

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Fig. 2. Box plots of the distribution of intrafollicular inhibin-A and -B content per follicle grouped according to embryo status (grade 5 is "best").

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