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Follicular Fluid Transferrin Levels in Preovulatory Human Follicles

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There is transferrin-like protein present in the follicular fluid of stimulated ovarian cycles. The transferrin concentration correlates with the follicular morphologic maturity and steroidogenesis, varies among follicles, and often exceeds serum concentrations. An intermediate range of transferrin concentration is associated with the highest likelihood of oocyte fertilization in vitro. The biological significance of these observations may relate to an optimum degree of follicle maturation.

KEY WORDS: follicular fluid; protein; transferrin.

INTRODUCTION

Transferrin is a glycoprotein of the β-1 globulin series which functions primarily in plasma as a carrier protein for iron. Although transferrin is synthesized in the liver, extrahepatic production of transferrin has recently been demonstrated. In men, cultured Sertoli cells produce a transferrin-like protein (1). Holmes and others have demonstrated a correlation between seminal fluid transferrin concentrations and serum follicle-stimulating hormone (FSH) levels in men with abnormal spermatogenesis (2). More recently, Sueldo *et al.* reported that low seminal fluid transferrin concentrations correlate with a low sperm density and a decreased ability to fertilize human oocytes in vitro (3).

In women, transferrin has been identified as one of the many protein constituents of follicular fluid (4) but has not been related to folliculogenesis, ovarian steroidogenesis, or oocyte development. Transferrin is essential to proliferation of cells in culture (5). The preovulatory follicle is avascular, and thus, since transferrin is important to cell growth, its local concentration might correlate with follicular development. We have previously reported transferrin levels in individual human follicles (6). In this report, follicular fluid transferrin concentrations are related to serum levels of transferrin, follicular fluid steroid concentrations, morphologic characteristics of the oocyte, and the ability of the oocyte to be fertilized and then to cleave in vitro.

MATERIALS AND METHODS

Follicular fluid was obtained from 104 follicles among 48 women undergoing laparoscopic oocyte recovery in a program of in vitro fertilization and embryo transfer (IVF-ET).

Folliculogenesis was stimulated with human menopausal gonadotropin (hMG) in 21 patients yielding 51 analyzed follicles, with clomiphene citrate in 16 patients yielding 33 analyzed follicles, and with a combined protocol of clomiphene and hMG in 11 patients yielding 20 analyzed follicles. All patients received human chorionic gonadotropin (hCG, 10,000 U intramuscularly, after adequate follicular maturity had been estimated by serum estradiol concentrations and ultrasonographic measurements of follicle size. These cri-

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teria varied according to the particular stimulation protocol. Follicle aspiration was performed 34–36 hr after hCG administration.

The initial evaluation utilized randomly available fluid samples from 54 follicles among 23 patients. Subsequent to this, in order to compare follicular fluid transferrin concentrations with the outcome of the in vitro fertilization of the oocytes, we analyzed two fluid samples from each of 25 patients from whom at least two oocytes were recovered. We selected one of those samples to be associated with an oocyte that fertilized and cleaved and the second, with an oocyte which fertilized but failed to cleave. Concurrent sera for transferrin concentrations were available from 16 of the 25 women who provided paired follicular fluid samples.

Only follicular fluid samples free of visible blood contamination were included in both phases of the study. Fluid samples from 104 follicles were analyzed for transferrin; 100 samples were analyzed for estradiol and androstenedione, and 99 for progesterone. Follicular fluid and serum samples were centrifuged at 1500*g* and the supernatants were frozen at $-20°C$ for later analysis.

Oocytes were graded for morphologic maturity using the criteria of Veeck *et al.* (7). Following incubation and insemination in vitro, all oocytes were observed for fertilization and cleavage.

Transferrin was measured by a modification of the immunonephelometric method of Spencer (8). Briefly, buffered anti-human transferrin (Atlantic Antibodies, Scarborough, ME) and a 20-μl aliquot of follicular fluid or serum previously diluted in phosphate-buffered saline (1:20) were added to separate compartments of a multi-sample rotor in an IL Multistat III Micro Centrifugal Analyzer. After incubation and mixing, serial right-angle light-scatter measurements were made and computer-assisted calculations of the change in light intensity were applied to the standard curve. The transferrin concentration of the sample was then calculated. With this assay, all analytical measurements can be completed within 1 hr. The standard curve was linear from 0 through 700 mg/dl, with an interassay coefficient of variation of less than 4%.

Follicular fluid concentrations of estradiol-17β, androstenedione, and progesterone were determined by radioimmunoassay (RIA) after selective extraction as previously described (9–11). Estradiol and androstenedione were extracted with diethyl ether, while progesterone was extracted with petroleum ether before direct RIA. Antisera to estradiol (anti-estradiol-17β-6-BSA) and progesterone (anti-progesterone-11-BSA) were supplied by Dr. Gordon Niswender (Colorado State University, Fort Collins). Antiserum for androstenedione was supplied by Dr. Fortune Kohen (The Weitzmann Institute of Science, Rehovot, Israel). Steroid values were corrected for recovery and blanks were below the sensitivity of each RIA.

Statistical significance was determined by paired Student's T test, analysis of variance (ANOVA), Kolomogorov–Smirnov test, and chi-square test. Pearson product–moment correlation coefficients were calculated for transferrin with each of the follicular fluid steroids.

RESULTS

Transferrin was present in all follicular fluid samples at concentrations ranging from 3 to 588 mg/dl [mean, 202.2 $\pm$ 12.2 mg/dl (SE)].

The mean transferrin concentration from follicles stimulated by hMG was 197.9 $\pm$ 18.2 mg/dl; that from follicles stimulated by clomiphene citrate, 208.3 $\pm$ 21.6 mg/dl; and that from follicles stimulated by the combined drug protocol, 203 $\pm$ 23.1 mg/dl. These differences are not statistically significant.

Morphologically, seven oocytes were judged to be immature. Follicular fluid from these follicles had a mean transferrin concentration of 24.0 $\pm$ 8.3 mg/dl. This value is significantly less than the mean concentration of 215 $\pm$ 12.1 mg/dl from 97 follicles with oocytes judged to be morphologically mature ($P < 0.001$). Corresponding steroid concentrations measured in these two groups (Table I) demonstrate a similar significant difference among each of the steroids measured between follicles bearing mature and those bearing immature oocytes.

Among the seven oocytes judged to be morphologically immature, two fertilized and cleaved in vitro (FC) and five failed to fertilize (NF). Among the 47 morphologically mature oocytes in the first phase, 30 became fertilized (64%) and 17 were NF. Cleavage occurred in 17 (57%) of the oocytes that fertilized. Combining the total study population, we analyzed fluids from follicles yielding oocytes, with the following outcome: 42 FC, 17 NF, and 38 fertilized but either failed to cleave or underwent degen-

Table I. Concentration of Follicular Fluid Constituents Compared by Morphologic Grade of the Oocyte (Mean ± SE)

	Transferrin (mg/dl)	Estradiol (ng/ml)	Androstenedione (ng/ml)	Progesterone (ng/ml)
Immature	24.0 ± 8.3	15.4 ± 4.3	21.1 ± 6.4	1644.3 ± 1459.5
(*N*)	(7)	(7)	(7)	(7)
Mature	215.0 ± 12.1*	595.6 ± 80.4*	119.2 ± 16.1*	9258.0 ± 1100.01*
(*N*)	(97)	(93)	(93)	(92)

* $P < 0.001$.

eration (FNC). Of those 38 FNC, 25 (66%) appeared to have more than two pronuclei.

Figure 1 demonstrates the relationship of the mean transferrin concentration in serum to that of follicular fluid from each of the classifications of oocyte outcome. Statistically significant differences are noted.

The serum transferrin concentration exceed the follicular fluid transferrin concentration in the FC group in 81% of 16 cases studied but exceeded the follicular fluid transferrin concentration in the FNC group in only 44% ($P < 0.04$). Considering the individual patient with paired follicles, transferrin concentrations in FNC exceeded FC in 81%. There was often a striking transferrin elevation in the fluid from follicles yielding unsuccessful oocytes, more than doubling the associated serum concentrations. Table II shows these data and the statistically significantly higher concentration in FNC compared to FC.

Pearson product–moment coefficients demonstrated a statistically significant positive correlation between the transferrin concentration and the concentration of each of the steroids ($P < 0.0001$). This pattern held when analyzed for each of the three drug protocols. Furthermore, with the exception of androstenedione in the FNC group, there was a significant positive correlation between transferrin and the steroids in all three classifications of oocyte outcome.

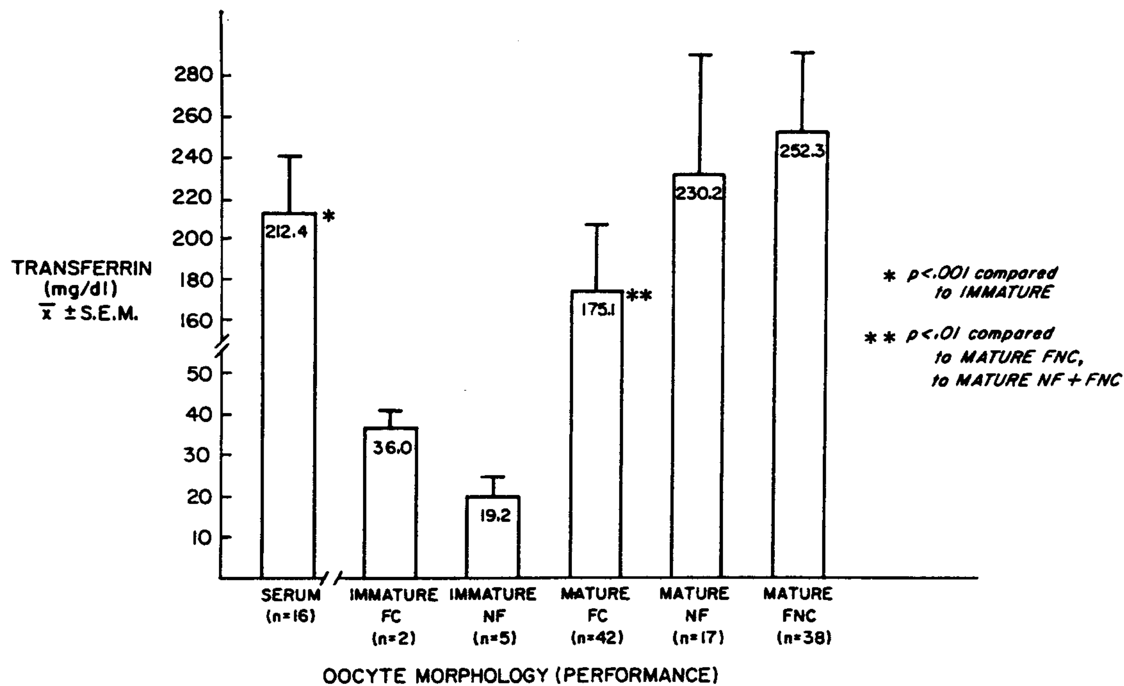

Fig. 1. Comparison of mean transferrin concentration in serum versus follicular fluid yielding mature or immature oocytes that either fertilized and cleaved (FC), failed to fertilize (NF), or fertilized but failed to cleave (FNC).

Table II. Comparison of Transferrin Concentration (mg/dl) in Serum and Follicular Fluid in 16 Patients with Paired Follicles of Different Outcomes

	Serum	Fertilized, cleaved	Fertilized, noncleaved
	281	102	140
	240	588	564
	144	126	152
	221	99	253
	202	168	222
	241	162	158
	153	136	160
	95	85	149
	278	181	256
	264	208	224
	208	182	132
	157	160	220
	283	35	96
	200	210	438
	198	144	156
	234	144	371
Mean	212.4	170.6	230.7*
SE	13.6	30.1	31.6
N	16	16	16

* $P < 0.01$ compared to fertilized, cleaved.

Table III demonstrates the mean concentration of transferrin, estradiol, androstenedione, and progesterone in follicles yielding morphologically mature oocytes with three different outcomes. Steroid concentrations were not significantly different. However, among these groups, the transferrin concentration in the group that fertilized and cleaved (FC) was significantly lower than the mean of the two unsuccessful groups (NF + FNC).

When fertilization success was stratified by transferrin concentration, the likelihood of successful fertilization and cleavage appeared to be related to the transferrin concentration. Only two of seven (29%) morphologically immature oocytes, each with low transferrin levels (<60 mg/dl), progressed successfully. Of the 97 morphologically mature oocytes, 40 were associated with follicular fluid transferrin concentrations that exceeded 220 mg/dl (an approximation of expected serum concentrations) and only 11 of these (28%) developed appropriately. On the other hand, 31 of 57 (54%) of those morphologically mature oocytes derived from follicles with transferrin concentrations less than 220 mg/dl successfully fertilized and cleaved (P = 0.015).

DISCUSSION

The transferrin concentration in follicular fluid from human ovaries has been reported previously as one among many protein components (4). Our observations suggest that, in stimulated cycles, the follicular fluid transferrin concentration reflects the gross morphologic grade of oocyte maturity and correlates with the steroidogenic capacity of the follicle. The concentration of transferrin varies between different follicles in the same patient and may be two to three times that of serum. Since none of the samples was bloody, the contribution by proteins derived from traumatic contamination with serum would be neglible and would then be further diminished by dilution with follicular fluid. Thus, contamination could not account for the transferrin concentrations observed.

It is not yet clear whether this transferrin-like protein is synthesized locally, as in the testis, or is derived by a transudative concentrating mechanism during maturation of the preovulatory follicle. In work in progress, however, our group has demonstrated the presence of transferrin receptors on human and rat granulosa cells in increasing

Table III. Relationship of Follicular Fluid Constituents to Results of IVF with Morphologically Mature Eggs (Mean ± SE)

	Transferrin (mg/dl)*	Estradiol (ng/ml)	Androstenedione (ng/ml)	Progesterone (ng/ml)
FC	175.1 ± 16.6	633.9 ± 154.7	99.3 ± 30.0	8106.7 ± 1597.8
(N)	(42)	(38)	(38)	(38)
NF	230.2 ± 28.9	740.6 ± 229.6	114.8 ± 27.3	11949.3 ± 3458.4
(N)	(17)	(17)	(17)	(16)
FNC	252.3 ± 19.3	492.3 ± 63.4	141.1 ± 30.5	9276.2 ± 1567.8
(N)	(38)	(38)	(38)	(38)
ANOVA	0.01	0.5	0.5	0.5

* Transferrin: successful IVF (FC) vs unsuccessful IVF (NF + FNC), P = 0.009.

numbers with progressive follicular maturation (12). In light of these findings, the apparent relevance of seminal fluid transferrin to male reproductive capacity and the importance of transferrin to satisfactory cell culture growth, we hypothesize that follicular fluid transferrin may be relevant to the stage of proliferation and maturation of the granulosa-cell component of the ovarian follicle. This level of maturity may also relate to the condition of the oocyte.

Our data indicate a range of follicular fluid transferrin concentration that is associated with optimum oocyte fertilization. Morphologically immature oocytes, associated with a low transferrin concentration, have a low likelihood of successful progression unless the incubation time is extended. Among morphologically mature oocytes, those with a high transferrin concentration often fail to fertilize or, if they do, frequently either exhibit polyspermia and/or fail to progress. However, our observations suggest that mature oocytes associated with a transferrin concentration less than 220 mg/dl have the highest rate of satisfactory in vitro fertilization. Whether this cutoff is in fact biologically related to the serum concentration of transferrin, or only coincidentally so, is not yet known.

These preliminary observations raise important questions about the relationship of transferrin, an iron-binding protein, to folliculogenesis and oogenesis. Iron utilization via transferrin and the transferrin receptor has been shown to be essential for populations of rapidly dividing mammalian cells. Further work to elucidate these mechanisms in the follicle is under way.

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