Kinetic Immunonephelometric Determination of Protein Concentrations in Follicular Fluid

Fizza Gulammal-Majid, Steven Ackerman, Lucinda Veeck, Anibal Acosta, and Patricia Pieban

Nonsteroidal biochemical markers of oocyte maturity could presumably aid in oocyte selection and in the timing of insemination for in vitro fertilization. We assessed the usefulness of six potential markers found in follicular fluid. We used kinetic immunonephelometry to measure concentrations of α1-antitrypsin, α2-macroglobulin, antithrombin III, ceruloplasmin, fibrinogen, and plasminogen in 53 fluids and the corresponding plasma from 20 women undergoing in vitro fertilization. Specimens were obtained from both mature and immature follicles from each woman. The respective protein concentrations in follicular fluid could be assayed with a between-assay CV of 1.5% to 3.2%. Analytical-recovery studies indicated that only fibrinogen and antithrombin III concentrations were altered (by 120% and 75%, respectively) during aspiration of the follicle into saline. All protein concentrations were significantly increased in mature follicles, as were the follicular fluid/plasma concentration ratios for α1-antitrypsin, antithrombin III, and ceruloplasmin.

Additional Keyphrases: fertility • markers of oocyte maturity • follicular fluid protein/plasma protein ratio • α1-antitrypsin • antithrombin III • ceruloplasmin • plasminogen • fibrinogen • α2-macroglobulin • in vitro fertilization

During in vitro fertilization, one must correctly estimate the oocyte's developmental stage in order to determine its suitability for fertilization. In most cases, this is assessed solely by microscopic techniques (1). However, the maturational stage of oocytes aspirated before ovulation may be difficult to assess because the ooplasm may be obscured by dense layers of surrounding follicular epithelium; furthermore, the surrounding cells of the cumulus–oocyte complex may not be at the same maturational stage as the oocyte (1). Thus, biochemical markers of oocyte maturity would provide useful information to aid in oocyte selection and assist in timing insemination.

Nayudu et al. (2) suggested that concentrations of specific proteins in follicular fluid may reflect the physiological condition of the follicle. The follicular epithelium acts as a barrier, permitting the passage of plasma proteins at a rate inversely related to their relative molecular mass. As the follicle matures, it becomes more permeable to plasma proteins. Furthermore, the granulosa cell layer evidently secretes various proteins into the follicle (3, 4). In particular, plasminogen and tissue plasminogen activator have been associated with mature (or preovulatory) oocytes and ovulation (5, 6). These investigators (3–6) have suggested that follicular fluid protease-inhibitor concentrations should increase, to prevent unrestrained proteolysis within the follicle at ovulation. Espey (7), comparing ovulation to an inflammatory response, predicted an increase in acute-phase proteins.

We have investigated the use of the follicular fluid concentrations of six proteins as potential indicators of oocyte maturity. Three are protease inhibitors: α1-antitrypsin, α2-macroglobulin, and antithrombin III. We also measured fibrinogen, plasminogen, and the estrogen-responsive acute-phase protein, ceruloplasmin. Alterations in concentrations of α1-antitrypsin and fibrinogen in follicular fluid at the time of ovulation have previously been reported (2). In addition, concentrations of antithrombin III and fibrinogen (8), α1-antitrypsin (9), and plasminogen (10) in peritoneal fluid reportedly increase after ovulation.

Preovulatory oocytes require a 5- to 8-h preincubation before insemination (1). Immature oocytes, which may mature in vitro, typically require 24 h of preincubation. Thus, oocyte status must be determined within 5 to 8 h after aspiration if premature insemination of an immature oocyte is to be prevented. We report here our use of kinetic immunonephelometry, which allows rapid analysis time and good sensitivity, to measure concentrations of several proteins in human follicular fluid aspirated during laparoscopic harvest of eggs for in vitro fertilization.

Materials and Methods

Equipment

For the immunonephelometric analysis we used an automated Immunochemistry System (ICS) with manual mode program gain card M-33 for the antithrombin III and plasminogen assays, and the M-44 card for the fibrinogen assays (all from Beckman Instruments, Inc., Fullerton, CA 92621). We used an antibiotic-zone reader (Fisher Scientific Co., Richmond, VA 23235) for measuring results of the radial immunodiffusion assays.

Reagents

For the nephelometric assays of α1-antitrypsin, α2-macroglobulin, and ceruloplasmin, we adapted commercially available kits (Beckman Instruments) for use with follicular fluid. For the antithrombin III, fibrinogen, and plasminogen assays, we purchased anti-human antithrombin III (Atlantic Antibodies, Scarborough, ME 04074), anti-human fibrinogen and anti-human plasminogen (both from Dako Inc., Santa Barbara, CA 93102), and ICS buffer and ICS diluent (both from Beckman Instruments). Standard human serum, standard human plasma, and lyophilized control plasma (all from Calbiochem-Behring Corp., La Jolla, CA 92037) were also used. For the matrix effects and recovery studies, porcine follicular fluid was used as a base (kindly provided by Dr. Gabriel Bialy, NICHD, NIH, Bethesda, MD 20205). For the radial immunodiffusion assays we used "Nor-Partigen" plates for antithrombin III and fibrinogen and "M-Partigen" plates for plasminogen (all from Calbiochem-Behring Corp.).

All follicular fluids were diluted in Dulbecco’s phosphate-
buffered saline, pH 7.4, containing 40 mg of magnesium chloride and 100 mg of calcium chloride per liter (DSB; Gibco Labs., Grand Island, NY 14072) upon aspiration of the follicle.

Specimen Collection and Handling

Follicular fluid from 53 follicles was obtained from 20 women participating in the In Vitro Fertilization Program (Jones Institute for Reproductive Medicine) during oocyte retrieval for extracorporeal fertilization (11). Ovulation induction protocols included administration of follicitropin (follicle-stimulating hormone) or human menopausal gonadotropin, or both. Follicular fluids were obtained from both mature and immature follicles aspirated from each woman.

Follicles were aspirated into a known volume of DSB. Immediately after collection, we centrifuged the fluids to remove follicular cells. Specimens were kept at 4 °C until assay (within 24 h of collection). The remaining follicular fluid was then stored at −20 °C and assayed later, to determine the stability to freezing.

In addition, citrated whole blood was obtained from each patient before laparoscopy. Plasma was separated by centrifugation (2000 x g, 10 min), then assayed for proteins for comparison with concentrations in follicular fluid.

Nephelometric Assays

Antithrombin III. Control and patients' specimens were diluted 12-fold with ICS buffer. Reconstituted standard human plasma was diluted with ICS buffer to give concentrations of 10, 50, 100, 200, 250, 300, 400, and 500 mg of antithrombin III per liter. The diluted standards, controls, and patients' specimens were centrifuged (2000 x g, 5 min) to remove any precipitate.

The ICS was programmed in the manual mode. We found that a threefold dilution of antiserum and use of the M-33 gain card gave the best overall linearity and the least scatter (10). Using the M-33 gain card, special dilution "X", and a threefold dilution of antiserum (with ICS diluent), we obtained the rate units for standards, samples, and controls. We prepared a standard curve and read the concentrations in the control and patients' samples from it.

Fibrinogen. We diluted control and patients' samples 48-fold with ICS buffer. Reconstituted standard human plasma was diluted with ICS buffer to give 286, 477, 715, 1430, 2860, 3432, and 4230 mg of fibrinogen per liter. We then centrifuged the diluted standards, controls, and patients' samples (2000 x g, 5 min). The ICS was programmed in the manual mode. Using the M-44 gain card, special dilution "X", and a sevenfold dilution of antiserum, we obtained the rate units for the standards, control, and patients' specimens. Concentrations in follicular fluid and plasma were determined in a manner analogous to the antithrombin III assays.

Plasminogen. Control and patients' specimens were diluted sixfold with ICS buffer. Reconstituted standard human serum was diluted with ICS buffer to give 10, 25, 50, 100, 150, 200, and 300 mg of plasminogen per liter. The diluted standards, controls, and patients' specimens were then centrifuged (2000 x g, 5 min).

A fourfold dilution of the antisera and use of the M-33 gain card gave the best overall linearity and the least scatter. Concentrations in follicular fluid and plasma were determined as in the antithrombin III assays.

α1-Antitrypsin, α2-Macroglobulin, and Ceruloplasmin. We determined concentrations of these proteins in follicular fluid and plasma by adapting commercially-available kits and using the automated ICS mode.

Radial Immunodiffusion Assays

For comparison with results by the nephelometric assays, we also determined antithrombin III, fibrinogen, and plasminogen concentrations in follicular fluid by radial immunodiffusion, according to the manufacturer's instructions. The resulting precipitin rings were measured after 48 h at room temperature (equilibrium conditions).

Aspirated Oocyte Characterization

The histological staging of oocyte maturity was done by the In Vitro Fertilization Laboratory according to a generally accepted procedure (1). Both the oocyte and cumulus cells were classified as "mature" or "immature.”

Matrix Effects and Recovery Studies

Because no pure human follicular fluid was available, we used porcine follicular fluid containing low concentrations of the immunologically reactive proteins. We fortified this fluid with all six proteins to give final concentrations of 267, 190, 120, 115, 715, and 50 mg of α1-antitrypsin, α2-macroglobulin, antithrombin III, ceruloplasmin, fibrinogen, and plasminogen, respectively, per liter. Protein concentrations were then determined directly, and in two- and fourfold dilutions of the porcine follicular fluid in DSB, because the human follicular fluids were similarly diluted during aspiration.

Statistical Procedures

Patients' results were tested for gaussian distribution by use of the Kolmogorov–Smirnov test for “goodness of fit.” Comparison of central tendencies of the distribution were assessed by using the Mann–Whitney U test, with use of an IBM 4381 computer and the Statistical Package for the Social Sciences (13).

Results

Because all the follicular fluids obtained by aspiration were diluted one to four volumes of DSB, we investigated the effects of dilution (of the fortified porcine fluid) on the analytical recovery for each protein. We then determined the respective protein concentrations in the undiluted and diluted (two- and fourfold, with DSB) samples. For α1-antitrypsin, α2-macroglobulin, ceruloplasmin, and plasminogen, recoveries (n = 3) ranged between 89% and 111%, with no apparent effect of dilution per se. However, for fibrinogen, a greater apparent recovery was observed (undiluted: 105%, both dilutions: 120%), and for antithrombin III an apparent lower recovery (undiluted: 98%, diluted: 75% and 74% for the two- and fourfold dilutions, respectively).

Between- and within-assay precision data are shown in Table 1. Results are for pooled follicular fluid. The within-assay CVs ranged between 1% and 6% and between-assay CVs from 1.5% to 3.2%. Detection limits for these assays were, for the diluted samples, 180, 20, 80, 10, 280, and 10 mg/L for α1-antitrypsin, α2-macroglobulin, antithrombin III, ceruloplasmin, fibrinogen, and plasminogen, respectively. We chose to base these detection limits on the value of the lowest standard for each assay, because we observed marked nonlinearity at protein concentrations below these values, making quantification difficult.

We assessed stability of the diluted specimens to storage
Table 1. Precision of the Assays of Proteins in Follicular Fluid

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Within-assay</th>
<th></th>
<th>Between-assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD),</td>
<td>CV, %</td>
<td>Mean (SD),</td>
<td>CV, %</td>
</tr>
<tr>
<td>mg/L</td>
<td></td>
<td></td>
<td>mg/L</td>
<td></td>
</tr>
<tr>
<td>α₂-Antitrypsin</td>
<td>347.8 (5.9)</td>
<td>1.6</td>
<td>1058 (18)</td>
<td>1.7</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>63.9 (6)</td>
<td>0.9</td>
<td>931 (24)</td>
<td>2.6</td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td>23.5 (1.0)</td>
<td>4.0</td>
<td>239 (6)</td>
<td>2.6</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>70.8 (0.3)</td>
<td>0.4</td>
<td>219 (7)</td>
<td>3.2</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>28.8 (9)</td>
<td>3.0</td>
<td>2900 (4.3)</td>
<td>1.5</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>17.0 (1.0)</td>
<td>6.1</td>
<td>103 (2)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

n = 10 throughout.

at −20 °C for two weeks and six months by comparing values for fresh specimens with those found after such storage. We observed no storage effects for α₂-antitrypsin, antithrombin III, ceruloplasmin, or plasminogen. α₂-Macroglobulin concentrations increased significantly after six months of storage; fibrinogen concentrations decreased after two weeks of storage (Table 2).

We compared the results by kinetic immunonephelometry for the assays developed in our laboratory (antithrombin III, fibrinogen, and plasminogen) with those obtained for the same specimens assayed with commercially available radial immunodiffusion kits. Figure 1 shows results for antithrombin III, fibrinogen, and plasminogen. For kinetic immunonephelometry, results tended to be 8% and 5% lower for antithrombin III and plasminogen, respectively; they tended to be 5% higher for fibrinogen.

Table 3 shows mean (and SD) protein concentrations in follicular fluid. These concentrations had a gaussian distribution (Kolmogorov-Smirnoff test). Follicular fluids from follicles containing mature oocytes (as assessed histologically) had significantly higher mean concentrations of proteins than did those from follicles containing immature oocytes.

Follicular fluid/plasma protein ratios for follicles containing mature oocytes approached (α₂-antitrypsin and ceruloplasmin) or exceeded (antithrombin III and plasminogen) unity for proteins with relative molecular masses ≤151 000. Ratios were proportionately lower for higher-molecular-mass proteins. For fluids from follicles containing immature oocytes, ratios approached unity for antithrombin III and plasminogen, while ratios were less than unity for the other proteins. Follicular fluid/plasma protein ratios for fluid from mature follicles were significantly higher for α₂-antitrypsin, antithrombin III, and ceruloplasmin. Details are given in Table 4.

Discussion

Analytical recoveries of fibrinogen and antithrombin III appeared to differ between undiluted specimens and those diluted with DBS, but recoveries for the two- and fourfold dilutions were similar, suggesting that results for these proteins in individual fluids may validly be compared. Results by kinetic immunonephelometry compared well with those obtained by radial immunodiffusion, and the former method is simpler and faster, providing results within 45 min of receipt of the specimen. Thus, this technique provides data well within the required time interval in which decisions must be made as to whether an oocyte is suitable for insemination.

Concentrations of α₂-antitrypsin, antithrombin III, α₂-

Fig. 1. Concentrations of antithrombin III (A), fibrinogen (B), and plasminogen (C) in follicular fluid, as determined by kinetic immunonephelometry and radial immunodiffusion.

Units on both axes: mg of protein per liter.
Table 3. Protein Concentrations Compared in Follicular Fluid from Mature and Immature Oocytes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mature (n = 33)</th>
<th>Immature (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD), mg/L [μmol/L]</td>
<td></td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>1121 (.259)</td>
<td>598 (380)*</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>227 (85)</td>
<td>139 (97)*</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>145 (66)</td>
<td>84 (71)*</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1221 (416)</td>
<td>600 (419)*</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>203 (51)</td>
<td>133 (70)*</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>87 (24)</td>
<td>56 (29)*</td>
</tr>
</tbody>
</table>

*Significantly different from values for mature oocytes (Mann-Whitney U Test): *P < .001, *P < .005.

macroglobulin, ceruloplasmin, fibrinogen, and plasminogen were all significantly higher in fluid from follicles containing mature oocytes—not unexpectedly, because the follicle becomes more permeable to proteins on maturation (3, 14).

To evaluate the predictive value (15) of each assay in assessing oocyte maturity (as established by microscopic techniques), we chose the lower limit reference value to be 2 SD from the mean concentration of each protein in follicles containing mature oocytes. With these criteria, the predictive value of a result above the lower limit of the reference range ranged from 75 to 83% for each of these proteins.

Using radial immunodiffusion, Shalgi et al. (3) reported mean (and SD) fibrinogen concentrations in follicular fluid from follicles of undetermined maturational stage to be 1100 (315) mg/L, with a mean (SD) follicular fluid:plasma ratio of .38 (.12). Our data agree well with their values. Nayudu et al. (2) reported that the fibrinogen:IgG ratios for follicular fluid decreased with increasing maturity of the follicle. These investigators used immunoelectrophoresis to quantify follicular fluid protein concentrations, and reported ratios rather than (as here) absolute protein concentrations. The apparent decrease in fibrinogen with increasing maturity of the follicle would appear to differ from the findings of our study. However, it is possible that as the follicle matures, more IgG (with a smaller relative molecular mass of 160 000) than fibrinogen crosses the "blood–follicle" barrier. This would cause an apparent decrease in the ratio of fibrinogen to IgG, even though the absolute concentration of fibrinogen in the fluid might increase.

Both Shalgi et al. (3) and Edwards (14) have reported detecting α2-macroglobulin in human follicular fluid.

e et al. did not quantify the protein, but Edwards reported a follicular fluid:serum mean ratio for this protein of 0.12 in fluids from follicles of undetermined maturity. Our results agree well with those of Edwards (mean ratios for fluid from mature and immature follicles were 0.14 and 0.12, respectively).

Absolute concentrations of α1-antitrypsin, antithrombin III, ceruloplasmin, and plasminogen in follicular fluid have not previously been reported. Nayudu et al. (2) reported the α1-antitrypsin:IgG ratio to be increased with increasing follicular maturity. We observed both increased absolute concentrations of this protein as well as an increased follicular fluid:plasma ratio in mature follicles.

Of particular interest is the apparent increased concentration of antithrombin III in fluid from follicles with mature oocytes relative to the concentrations in plasma. Increased concentrations of antithrombin III have also been reported in peritoneal fluid at the time of ovulation (6). Concentrations of antithrombin III reportedly are decreased at the time of ovulation (16); thus our finding of a mean follicular fluid:plasma ratio of 1.31 from follicles with mature oocytes suggests that some of the protein may originate from cells within the follicle. Possibly the blood–follicle barrier also actively transports antithrombin III into the follicle. Similarly, plasminogen had a mean follicular fluid:plasma ratio of 1.15 in follicles with mature oocytes. Therefore, assay of these two proteins in follicular fluid may provide the most reliable information about oocyte maturity. Further studies with a larger group of women are needed to confirm these findings.

We thank the staff at The Jones Institute for Reproductive Medicine involved in processing the specimens, and the Chemistry Laboratory at Children’s Hospital of the King’s Daughters, Norfolk, VA, for use of the ICS.

References

Table 4. Ratios for Some Protein Concentrations in Follicular Fluid and Plasma for Fluid from Follicles Containing Mature and Immature Oocytes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mature (n = 33)</th>
<th>Immature (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>M.</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>0.98 (.41)</td>
<td>64 (.38)*</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>1.31 (.40)</td>
<td>91 (.40)*</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>1.15 (.41)</td>
<td>98 (.34)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>1.00 (.33)</td>
<td>70 (.35)*</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.44 (.16)</td>
<td>38 (.15)</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>0.14 (.08)</td>
<td>12 (.04)</td>
</tr>
</tbody>
</table>

*Significantly different from values for mature oocytes (Mann-Whitney U test): *P < .01, *P < .001.