Follicular fluid contents of hyaluronic acid, follicle-stimulating hormone and steroids relative to the success of in vitro fertilization of human oocytes*†

Ernest Suchanek, Ph.D.‡  
Velimir Simunic, Ph.D.  
Dubravka Juretic, Ph.D.  
Veselko Grizelj, Ph.D.

Department of Obstetrics and Gynecology, University of Zagreb, School of Medicine, Zagreb, Croatia

Objectives: To determine the concentrations of hyaluronic acid, FSH, P, and E2 in the follicular fluid (FF) obtained from IVF-ET patients and to assess the value of these measurements in predicting the outcome of fertilization.

Design: One hundred eleven samples were retrospectively analyzed for the hyaluronic acid and hormone contents.

Setting: University-based tertiary care center.

Patients: Preovulatory FF samples were collected from 67 women undergoing IVF-ET treatment because of tubal absence or obstruction.

Main Outcome Measures: The FF hyaluronic acid and hormone concentrations were compared according to the type of ovulation induction, follicular development, and IVF outcome.

Results: According to the type of ovulation induction, a significantly lower hyaluronic acid concentration was found in FF harvested from the patients treated with GnRH agonist-hMG. No significant correlation was found between FF hyaluronic acid and either morphological maturity of the oocyte-cumulus complex or fertilizability of oocytes. The level of FSH was significantly higher in FF, yielding a mature oocyte-cumulus complex and from which the oocyte obtained successfully fertilized and cleaved. A significant increase in the E2 concentration was found in FF in which mature cumuli oophori were present. The levels of hyaluronic acid significantly correlated with FSH in FF.

Conclusions: Expansion of the human oocyte-cumulus cell complex is an FSH-dependent phenomenon. The data are in agreement with the hypothesis that intrafollicular FSH plays an important role in the secretion of hyaluronic acid by granulosa cells and may act synergistically with E2 to enhance cytoplasmic maturation, resulting in successful fertilization.


Key Words: Follicular fluid, hyaluronic acid, FSH, estradiol, progesterone, ovarian stimulation, in vitro fertilization
proteoglycans and glycosaminoglycans (1). Proteoglycans are large macromolecules consisting of numerous heteropolysaccharide side chains called glycosaminoglycans, covalently linked to a protein core. Their biosynthesis is regulated by FSH (2), prostaglandin E<sub>2</sub>, and transforming growth factor-β1 (TGF-β1) (3). Proteoglycans found in follicular fluid (FF) (4, 5) or secreted by granulosa in vitro (6) contain predominantly chondroitin sulphate. In contrast, cumulus cells secrete hyaluronic acid, indicating a metabolic divergence in glycosaminoglycans between GCs and cumuli.

Interaction between the extracellular matrix and cells has a major effect on the proliferation, differentiation, and organization of cells, but the role of extracellular matrix constituents in the ovarian follicle has not yet been clarified. The purpose of this study was to determine whether the content of human FF is related to the following: [1] the type of ovarian follicle growth induction; [2] the developmental stage of the follicle as judged from the microscopic examination of the follicular cells; and [3] the outcome of IVF treatment. We also investigated the FF contents of hyaluronic acid, FSH, P, and E<sub>2</sub>.

**MATERIALS AND METHODS**

Preovulatory FF samples were obtained from 67 infertile women who, because of tubal absence or obstruction, were undergoing IVF-ET. The selection of patients, oocyte recovery, and IVF procedure have previously been described in detail (7, 8). The median age of the patients (24 to 35 years) was comparable in the three groups. The first group of patients, consisting of 32 women, received clomiphene citrate (CC, Klonifen; Richardson-Merrell-Belupo, Koprivnica, Croatia) in doses of 100 mg/d from days 3 to 7 of the menstrual cycle. The induction of follicular growth from days 8 to 11 was performed by additional 150 to 300 IU/d of hMG (Pergonal; Ares Serono, Aubonne, Switzerland), depending on the growth of dominant follicles and the daily rise in serum E<sub>2</sub>. Follicular aspiration by ultrasound (US)-guided transvaginal technique was performed 34 to 36 hours after a 5,000 IU hCG injection (Profasi; Ares Serono).

In the second group of 27 patients, follicular growth was induced with 225 IU of hMG on days 2 and 3 of cycle, then daily administration of 150 IU hMG, followed by increasing doses of hMG in a stepwise manner. Human chorionic gonadotropin in a dose of 10,000 IU was administered 34 to 36 hours before oocyte retrieval.

Eight patients of the third group received 200 μg buserelin acetate (GnRH-agonist (GnRH-a, Suprefact; Behringwerke, Marburg, Germany) intranasally three times a day (at 7.00 A.M., 3.00 P.M., and 11.00 P.M.) commencing on day 21 of the previous cycle. This was continued until the day of hCG administration. Ovarian US and serum E<sub>2</sub> assay were performed 14 to 21 days after the beginning of GnRH-a therapy. When the serum E<sub>2</sub> concentration reached <20 pg/mL (0.073 nmol/L), the following treatment for superovulation was initiated: 225 IU of hMG for 3 days, followed by 150 IU/d thereafter. The dose of hMG was adjusted according to the response to stimulation. As previously stated, 10,000 IU of hCG were administered when three follicles were >16 mm diameter corresponding to serum E<sub>2</sub> > 500 pg/mL (1.8 nmol/L).

The aspirates were examined microscopically, oocyte-corona-cumulus complexes identified, removed from the FF, and processed for fertilization. Dilution of FF with aspiration medium during egg retrieval was omitted because it may have an obvious effect on follicular concentration measurements. The degree of morphological maturity of the complexes was estimated according to the criteria described by Laufer et al. (9). Sperm were prepared by the swim-up technique, and the motile sperm were used for insemination.

The FFs were centrifuged at 1,000 × g, the volume of cell-free liquid was measured, and fluids were stored (−20°C) until assayed. A total of 111 samples, excluding blood-stained ones, were subjected to analysis.

The FF concentration of hyaluronic acid was measured by RIA (Pharmacia Diagnostics AB, Uppsala, Sweden). The test is based on the use of specific hyaluronic acid binding proteins isolated from bovine cartilage (10). The detection limit of the assay is <5 ng/mL (5 μg/L). There is no measurable interference of chondroitin sulphate, keratan sulphate, or fibronectin from FF. Precision studies for this assay performed at our laboratory showed the intra-assay and interassay coefficients of variation to be 4.5% and 8.9%, respectively. The levels of FSH, P, and E<sub>2</sub> in FF were measured by RIA (Biodata S.p.A., Rome Italy), and the details have been reported previously (7).

Statistical analysis was made to test the significance of differing levels of hyaluronic acid and hormones among the groups classified according to either the type of follicular growth induction, the
morphology of the oocyte-corona-cumulus complex, or the development of fertilized oocytes. Values are expressed as means ± SEM. Differences among treatment groups were analyzed using either one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA, depending on whether the data were normally distributed; otherwise, the data were compared using the χ² test. A possibility of <0.05 indicated statistical significance. Correlations were estimated using linear regression analyses and Pearson’s coefficients of contingency.

RESULTS

Characteristics of the follicles and oocytes obtained by aspiration are shown in Table 1. No significant differences were noted in any parameter of oocytes, except that the significantly (P < 0.001) lower total dose of hMG was administered to patients receiving CC. The maturity scores of oocytes, fertilization, and cleavage rates were comparable among the patients.

Concentrations of hyaluronic acid, FSH, P, and E₂ in preovulatory FF measured in this study are shown in Table 2. A significantly lower content of hyaluronic acid (P = 0.019) was found in FF harvested from the patients treated with GnRH-a-hMG than in those administered CC-hMG or hMG only. No significant relationship existed between FF hyaluronic acid and the morphological maturity of the oocyte-corona-cumulus complex and fertilizability of oocytes.

The level of FSH was significantly higher (P = 0.0027) in FF, yielding a morphologically mature oocyte-corona-cumulus complex than in those of intermediate maturity. A significantly higher amount of FSH (P = 0.016) was found in FF from which the oocytes obtained were successfully fertilized and cleaved to the stage of two to eight blastomeres, as compared with the samples in which fertilization failed or triploidy occurred.

No significant difference was observed between the FF levels of P in relation to various types of ovulation induction or to the morphological maturity of cumuli oophori. Success rate of IVF was not related to the FF content of P.

The lowest concentrations of FF E₂ (P = 0.007) were determined in patients treated with hMG only for the induction of ovulation. Follicular fluid in which morphologically mature cumuli oophori were present contained a significantly higher (P = 0.007) concentration of E₂ than the FF samples with less mature cumuli. However, no significant differences were observed between the FF levels of E₂ in relation to the fertilization of oocytes in vitro. No correlation was found between FF levels of hyaluronic acid, FSH, P, and E₂, and success rate of oocyte fertilization when Pearson’s coefficient of contingency was performed (P between 0.055 and 0.514, respectively).

The levels of hyaluronic acid in FF were found to correlate positively with FSH (r = 0.205, P = 0.034) and inversely with E₂ concentration (r = −0.341, P = 0.0029). Inverse correlation was found between the P and E₂ contents of FF (r = −0.259, P = 0.023), whereas no significant correlation was found between hyaluronic acid and steroid hormone levels and neither the total dose of hMG administered per cycle nor the volume of FF.

DISCUSSION

The GCs play an integral role in establishing a feasible environment conducive to oocyte development. In parallel with oocyte maturation, the oocyte-cumulus complex starts to accumulate extracellular matrix substances that are secreted locally by cumular GCs.

The findings of our study demonstrated the presence of hyaluronic acid in human preovulatory FF. Its origin was not determined; however, it was unlikely to be derived from the blood because the mean hyaluronic acid concentrations in human FF were found to be twofold higher than that in peripheral plasma (9–27 ng/mL [9–27 μg/L]) (11). In women, an increase in the viscosity of antral fluid has been observed in mature preovulatory follicles

Table 1 Characteristics of Follicles and Oocytes Used for IVF in Relation to Ovulation Induction

<table>
<thead>
<tr>
<th>Total hMG dose (IU)*</th>
<th>CC-hMG</th>
<th>HMG</th>
<th>GnRH-a-hMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of follicles</td>
<td>51</td>
<td>41</td>
<td>19</td>
</tr>
<tr>
<td>FF volume (mL)*</td>
<td>5.2 ± 0.3</td>
<td>4.6 ± 0.3</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Oocyte-cumulus complex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature</td>
<td>37 (72.5)‡</td>
<td>30 (73.2)</td>
<td>14 (73.5)</td>
</tr>
<tr>
<td>Intermediate mature</td>
<td>14 (27.5)</td>
<td>11 (26.8)</td>
<td>5 (26.3)</td>
</tr>
<tr>
<td>Oocytes not fertilized</td>
<td>14 (27.4)</td>
<td>10 (24.4)</td>
<td>5 (26.3)</td>
</tr>
<tr>
<td>Oocytes fertilized</td>
<td>34 (66.7)</td>
<td>25 (61.0)</td>
<td>12 (63.2)</td>
</tr>
<tr>
<td>Triploidy</td>
<td>3 (5.9)</td>
<td>6 (14.6)</td>
<td>2 (10.5)</td>
</tr>
</tbody>
</table>

* Values are means ± SEM.
† P < 0.001.
‡ Values in parentheses are percents.
Table 2  Hyaluronic Acid, FSH, P, and E2 Levels in Preovulatory FF in Relation to the Type of Follicular Growth Induction, Morphological Maturity of Oocyte-Corona-Cumulus Complex, and Fertilizability of Oocytes*

<table>
<thead>
<tr>
<th></th>
<th>Hyaluronic acid</th>
<th>FSH</th>
<th>P†</th>
<th>E2†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/mL</td>
<td>mIU/mL</td>
<td>µg/mL</td>
<td>ng/mL</td>
</tr>
<tr>
<td><strong>Induction of ovulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC-hMG (n = 51)</td>
<td>65.7 ± 6.7</td>
<td>12.7 ± 2.0</td>
<td>12.5 ± 1.8</td>
<td>217.9 ± 19.1</td>
</tr>
<tr>
<td>HMG (n = 41)</td>
<td>60.6 ± 3.5</td>
<td>9.8 ± 1.3</td>
<td>10.9 ± 1.2</td>
<td>155.3 ± 13.6</td>
</tr>
<tr>
<td>GnRH-a-hMG (n = 19)</td>
<td>36.0 ± 6.7</td>
<td>10.6 ± 1.3</td>
<td>10.3 ± 1.5</td>
<td>237.0 ± 19.1</td>
</tr>
<tr>
<td>Significance</td>
<td>P &lt; 0.02</td>
<td>NS‡</td>
<td>NS</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td><strong>Morphological maturity of oocyte-corona-cumulus complex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate mature (n = 25)</td>
<td>53.3 ± 8.7</td>
<td>12.5 ± 1.5</td>
<td>10.3 ± 1.7</td>
<td>204.3 ± 10.9</td>
</tr>
<tr>
<td>Mature (n = 86)</td>
<td>61.4 ± 4.3</td>
<td>12.6 ± 1.5</td>
<td>10.9 ± 0.7</td>
<td>141.7 ± 27.2</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>P &lt; 0.03</td>
<td>NS</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td><strong>Fertilization of oocyte</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonfertilized (n = 26)</td>
<td>53.3 ± 4.7</td>
<td>9.4 ± 2.2</td>
<td>12.7 ± 2.4</td>
<td>179.8 ± 16.3</td>
</tr>
<tr>
<td>2 to 8 blastomeres (n = 75)</td>
<td>63.4 ± 5.2</td>
<td>12.7 ± 1.6</td>
<td>11.2 ± 1.1</td>
<td>193.4 ± 16.3</td>
</tr>
<tr>
<td>Triplody (n = 10)</td>
<td>51.0 ± 8.7</td>
<td>9.8 ± 1.3</td>
<td>10.3 ± 1.5</td>
<td>187.9 ± 35.4</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>P &lt; 0.02</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Values are means ± SEM.
† Conversion factors to SI units, 3.18 for P values and 3.67 for E2 values.
‡ NS, not significant.

(12). Bellin et al. (4) have demonstrated the presence of chondroitin and heparan sulfates in human FF, which is in agreement with compositional studies detailed for other species (6). Increased concentrations of heparan sulphate were observed in follicles with mature, expanded cumulus complexes, indicative of the preovulatory stage of each individual follicle.

Our results are consistent with these findings: FF from mature follicles appeared to contain an elevated concentration of hyaluronic acid when compared with the follicles of intermediate maturity, but the difference was not statistically significant. A significant decrease of hyaluronic acid was observed in the follicles from GnRH-a-hMG induction. The use of GnRH-a in patients undergoing IVF may influence the GC function and/or oocyte maturation, as shown by Pellicer et al. (13). The authors have demonstrated that human granulosaluteal cells treated with GnRH-a are less luteinized after hCG administration than the cells treated with CC and gonadotropins. Various types of the oocyte-cumulus cell morphology could be a result either of the lack of hyaluronic acid secretion or of secretion of collagen-degrading enzymes and plasmin, which usually are fated for follicular rupture, but may affect the extracellular matrix of cumulus cells in vitro as well (14). In agreement with the results mentioned, P levels tended to be lower in the FF obtained from the patients treated with GnRH-a-hMG, although not significantly different from those of patients treated with CC-hMG or hMG for follicular growth induction. Decreased preovulatory production of P may have resulted from the GnRH-a treatment because GnRH has been shown to stimulate the P-metabolizing enzyme 20α-hydroxy-steroid dehydrogenase (15), resulting in accumulation of 20α-hydroxyprogesterone.

In our study, a significant correlation was observed between FF concentrations of FSH and the degree of oocyte-cumulus cell complex maturation. Mature oocyte-cumulus cell complexes were obtained from the follicles that contained higher levels of FSH. Although no significant difference was found between the content of hyaluronic acid in FF derived from mature and intermediate mature follicles, a significant correlation between hyaluronic acid and FSH concentration in FF appears to support the idea that expansion of the human oocyte-cumulus cell complex is an FSH-dependent phenomenon and that intrafollicular FSH plays a major role in the process of mucification.

The studies of Eppig (2, 16) have clearly shown that the synthesis and deposition of hyaluronic acid by isolated mouse oocyte-cumulus cell complexes are stimulated by FSH, but not by LH, suggesting a specific biosynthetic process that is stimulated directly by FSH. The action of FSH is probably mediated through cyclic adenosine monophosphate (cAMP) formation because synthetic cAMP analogues, adenylate cyclase activators and phosphodiesterase inhibitors, produce the same effect. Follicle-stimulating hormone and dibutylryl cAMP increase the hyaluronic acid synthesis only by
intact oocyte-cumulus cell complexes (17). Under the same stimulatory conditions, cumulus cells isolated around the oocyte do not synthesize hyaluronic acid unless they are co-cultured with isolated oocytes. Buccione et al. (18) and Salustri et al. (3, 19) have proposed that a soluble factor(s) produced by the oocyte is critically involved in the hyaluronic acid synthesis by cumulus cells when exposed to FSH. This factor(s), which remains to be identified, acts independently or at the step after the increase in cAMP induced by FSH. Differences in the hyaluronic acid synthesis in subregions of the preovulatory follicle depend on the variations in the local concentration of the oocyte factor(s) to which the cells are exposed (19). As confirmed recently by Salustri et al. (3), mural GCs also respond in vitro to the oocyte factor(s) with greatly increased hyaluronic synthesis. Unlike cumulus cells, FSH is not required for maximal stimulation of hyaluronic acid synthesis by mural GCs, in part because these cells produce prostaglandin E_2 that can substitute for FSH in promoting oocyte-cumulus complex expansion. Of several growth factors studied, only TGF-β1 stimulated hyaluronic acid synthesis in both cell types.

The levels of FSH were significantly higher in FF from which successfully fertilized ova were derived. The FF content of E_2 was found to be significantly increased in mature follicles. Follicle-stimulating hormone and E_2 may act synergistically with LH or hCG to enhance cytoplasmic maturation, resulting in fertilization of the oocyte. Samples of FF from which, after IVF, triploid pre-embryos were obtained, contained lower hyaluronic acid, FSH, E_2, and P concentrations, suggesting that this impairment may be consequential to follicular immaturity. Our findings are consistent with the results obtained by Laufer et al. (20).

Steroids are not involved in cumulus expansion and mucification (21). The steroidogenic activity of cumulus cells may have some role in other processes. Numerous investigations have attempted to determine steroidogenic characteristics of FF for predicting pregnancy outcome. Only a few studies (22, 23) have shown a significant relationship between FF E_2 concentrations and the success of IVF. In general, FF steroid levels and their ratios are of limited value because they either fail to vary significantly according to oocyte evolution or with the morphological appearance of the oocyte-cumulus complex (24). In a very recent study, Andersen (25) demonstrated that oocytes in IVF treatment have an increased chance of resulting in pregnancy when the E_2:androge ratio of FF is high. The FF E_2:P ratio was found not useful in pinpointing pregnancy-associated follicles.

Acknowledgments. Danko Dobec, B.C., Visnja Hlavati, B.C., and Setar Halimi, Ph.D., made contributions to this investigation at the Laboratory for Human Reproduction, Department of Obstetrics and Gynecology, Zagreb University School of Medicine, Zagreb, Croatia.

REFERENCES

13. Pellicer A, Tarin JJ, Miró F, Sampaio M, De los Santos MJ,


