Follicular fluid and serum levels of Inhibin A and pregnancy-associated plasma protein A in patients undergoing IVF

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Objective: To elucidate transport of intrafollicular proteins Inhibin A and pregnancy-associated plasma protein A (PAPP-A) across the follicular fluid (FF)/blood barrier.

Design: A retrospective study.

Setting: IVF lab at a university hospital, academic, and industrial research labs.

Patient(s): Fifty-five women undertook the IVF program.

Main Outcome Measure(s): Concentrations of Inhibin A, PAPP-A, and major serum proteins in FF and serum, total amount of PAPP-A, and Inhibin A in FF.

Result(s): The FF/blood barrier permeability was calibrated using major serum proteins. The FF/serum ratio decreased with the molecular mass of proteins, and their FF and serum concentrations were well correlated. In contrast, concentrations of Inhibin A in paired serum and FF samples showed a weak correlation (r = 0.563), whereas serum and FF concentrations of PAPP-A were independent of each other. The total amount of Inhibin A in FF correlated well with concentrations of Inhibin A in paired serum samples (r = 0.858), whereas the correlation between the total amount of FF PAPP-A and PAPP-A serum concentrations remains poor (r = 0.215).

Conclusion(s): These observations suggest that at the day of oocyte retrieval, FF is a major source of serum Inhibin A but not of serum PAPP-A. (Fertil Steril 2009;91:1739–44. ©2009 by American Society for Reproductive Medicine.)

Key Words: Follicular fluid/blood barrier, protein transport, PAPP-A, Inhibin A, IVF

Ovaries are tissues in which endocrine organs, ovarian follicles, and corpora lutea periodically grow and regress. The developing ovarian follicles are composed of a follicular fluid (FF)-filled antrum containing granulosa cells and oocytes, and a highly vascularized thecal compartment. At ovulation the granulosa cells undergo a transition into luteal parenchymal cells. To control that process, ovarian follicles have to communicate with the environment via FF and blood plasma. In this communication, properties of the FF/blood barrier play an important role, as ovarian proteins facilitate both intraovarian paracrine effects, as well as endocrine feedback effects (1).

Semipermeable barriers between the blood and various body fluids, separating the environments of both fluids, are well-described phenomena. The best-known examples are blood/brain barrier and a selective ultrafiltration of blood plasma proteins into urine. A similar barrier has been reported for the transport of blood proteins into the ovarian FF with permeability inversely proportional to the protein molecular mass (2) or into a combination of protein pI and molecular mass (3). Only proteins with molecular mass <500 kDa were found to be ultrafiltrated from the blood into FF.

To test the FF–blood barrier permeability for the transport of ovarian proteins into the blood, we have selected two proteins secreted by granulosa cells into the FF at the time of oocyte retrieval: Inhibin A (32 kDa) and pregnancy-associated plasma protein A (PAPP-A) (500 kDa).

Inhibin secretion by granulosa cells and the evidence that ovarian Inhibin A and Inhibin B suppress FSH production has been reported by Ericsson and Hsueh (4). Inhibins are heterodimeric glycoprotein hormones composed of one α (18 kDa) and one β (14 kDa) chain linked by disulphide bonds. Inhibin A consists of α–βα subunits and Inhibin B consists of α–ββ subunits (5). Meunier et al. (6) reported the expression of inhibin subunits in various tissues; the inhibin α subunit, however, is predominantly expressed in the gonads.
Inhibin A serum concentrations were measured in samples taken at various time points from the start of ovarian stimulation (7, 8). The presence of a measurable concentration of Inhibin A and Inhibin B in serum of stimulated women suggests that the blood/follicle barrier is permeable for them, although the FF concentration of both Inhibin A and Inhibin B is considerably higher (9).

Pregnancy-associated plasma protein A (PAPP-A) was first isolated from human pregnancy serum 30 years ago (10). Only recently it has been shown that proteolytic activity against Insulin-like growth factor binding protein 4 (IGFBP-4) in human FF is identical to PAPP-A (11).

Proteolysis of IGFBP-4 by PAPP-A is an important mechanism regulating FSH-induced ovarian follicle maturation and steroidogenesis in human granulosa cells (12). Pregnancy-associated plasma protein A is a metalloproteinase composed of two 200-kDa disulfide-bound subunits. The most common form of PAPP-A in circulation is the 500-kDa disulfide-bound 2:2 heterotetrameric complex with the proform of eosinophil major basic protein (proMBP), PAPP-A/proMBP(13). Ovarian follicles and generally reproductive tissues are important but not an exclusive [for review, see (14)] source of PAPP-A in nonpregnant women. The reported blood serum levels of PAPP-A are much lower than the levels found in FF, so despite the various sources of the PAPP-A, we believe that PAPP-A is still a good candidate to our study. Hourvitz et al. (15) reported expression of PAPP-A gene in the ovarian follicles. Sjöberg et al. (16) reported PAPP-A presence in human preovulatory FF, luteinized cells of unruptured follicles, and corpus luteum. Siniosich et al. (17) published a concentration range of PAPP-A in FF between 0.317 and 1.595 IU/L. Conover et al. (11) demonstrated measurable concentrations of PAPP-A (1.604 ± 0.315 IU/L) in FF obtained from dominant ovarian follicles in a standard IVF procedure. There are, however, only a few reports showing a relation between blood and FF PAPP-A concentration and transport of PAPP-A from ovarian follicles across the FF/blood barrier (18, 19).

In the present study, FF and serum concentrations of one large (PAPP-A, 500 kDa) and one small (Inhibin A, 32 kDa) protein of ovarian follicle origin was analyzed in patients undergoing regular IVF treatment. Our hypothesis was that although Inhibin A passes the FF/blood barrier rather freely and its serum and FF concentrations are highly correlated, PAPP-A is, because of its high molecular mass, retained within the ovarian follicles and only acts locally. To evaluate the hypothesis, concentrations of Inhibin A and PAPP-A were analyzed in FF and in paired serum samples collected at the time of the oocyte retrieval.

**MATERIALS AND METHODS**

**Female Patients**

Patients undergoing regular stimulation for IVF were recruited for the study at the Center of Assisted Reproduction, Department of Obstetric and Gynecology, General Teaching Hospital in Prague. A total of 55 women were recruited for that study. Because of serum volume requirements, patients were separated into two groups. Samples of 32 patients were used for the serum protein study and samples of 23 patients were used for the ovarian protein experiment. Two patients of the latter group suffered with ovarian hyperstimulation syndrome and their samples were excluded from the study. The number of growing follicles ranged from 6 to 27 follicles. Follicular fluid and serum samples were obtained with the patient’s permission. All the patients participating in this study signed an institutional review board-approved informed consent form.

All subjects underwent standard treatment protocol: FSH ovarian hyperstimulation using GnRH long agonists or GnRH short antagonist’s protocol with hCG induction of the follicular/egg maturation 36 hours before egg collection.

**Follicular Fluid Aspiration and Blood Processing**

Follicular fluid was obtained from the puncture of dominant ovarian follicles (14 to 22 mm in diameter) or from all follicles as indicated. After oocytes were removed, FF was cleared by centrifugation, and the resulting supernatant was transferred into sterile tubes, frozen at −20°C and stored at −70°C for further analysis. Blood-contaminated FF was excluded from the study. In parallel, samples of blood (5 mL) were taken on the day of oocyte retrieval, allowed to clot, cleared by centrifugation, and the resulting sera were frozen at −20°C and kept at −70°C until assayed.

In the serum protein experiment, only the FF from large dominant follicles was collected and analyzed. In the PAPP-A and Inhibin A experiments, FF from large dominant follicles, as well as FF from all follicles, was collected and analyzed. Fluid from large dominant follicles was collected and analyzed separately. After that, all FF was pooled and analyzed for volume and total concentration of PAPP-A and Inhibin A. The volume and total FF concentration were used to calculate the total amount of FF PAPP-A and FF Inhibin A.

Serum and FF concentrations of Inhibin A were analyzed using enzyme-linked immunosorbent assay kits (Diagnostic System Laboratories, Webster, TX). Concentrations of PAPP-A in serum (ultrasensitive PAPP-A) were analyzed using enzyme-linked immunosorbent assay kits supplied by DRG instruments GmbH (Marburg, Germany). Follicular fluid PAPP-A was assayed by a PAPP-A IRMA kit (Immunotech, Prague, Czech Republic). All the immunodiagnostic kits were processed according to the manufacturer’s instructions. For the analysis of FF Inhibin A, the FF samples were diluted 200-fold with calf plasma (Scantibodies Laboratory, Inc., Santee, CA). The linearity of the dilution was verified (recovery percentages obtained in range 84.6% to 109%).

Ten major serum proteins were analyzed using the protein analysis system Immage (Beckman Coulter Inc., Fullerton,
CA) and IVD kits supplied by the manufacturer. Total protein concentration was analyzed on the biochemistry Synchron LX 20 system (Beckman Coulter Inc.) using an IVD kit supplied by the manufacturer.

**Statistical Analysis**

The concentration data and FF/serum ratios in the paired samples were subjected to linear regression analysis and the resulting correlation coefficients were tested by using Fisher Z transformation at a 95% confidence interval.

**RESULTS**

Permeability of the FF/blood barrier was analyzed using 10 major proteins of human serum: serum albumin, immunoglobulin G, immunoglobulin M, Haptoglobin, Transferrin, \( \alpha \)-1-acid glycoprotein, \( \alpha \)-1-antitrypsin, \( \alpha \)-2-macroglobulin, and Apoprotein A. Concentrations were analyzed in paired samples of FF and serum. Individual concentrations of each protein in the paired samples were then used to calculate the FF/serum ratio, and the resulting ratios were plotted against the molecular mass of individual protein (the logarithmic scale was used to display the molecular mass). The resulting correlation between molecular mass of individual proteins and their FF/serum ratios is shown in Figure 1. It was clearly demonstrated that the FF/serum ratio was dependent on protein molecular mass \((r = 0.883)\). The FF concentration of immunoglobulin M was very low (mean concentration = 0.079 g/L) and the FF of \( \alpha \)-2-macroglobulin was below the detection limit of the diagnostic kit used (0.04 g/L). Haptoglobin was not used for the calculation because of its molecular mass variability (80–160 kDa).

We applied the same procedure to proteins of ovarian origin: Inhibin A and PAPP-A. We expected the FF/serum ratio of PAPP-A to be much higher than the FF/serum ratio of Inhibin A, because of the high molecular mass of PAPP-A. However, the data obtained did not fully support that expectation, because the mean FF/serum ratio of PAPP-A ranged from 85 to 851, whereas the FF/serum ratio of Inhibin A was between 99 and 595.

A logical explanation of this observation is that serum PAPP-A originates predominantly from different sources than the ovary. To test this hypothesis, we correlated individual FF concentrations of PAPP-A and Inhibin A with their respective concentrations in paired serum samples. Inhibin A showed weak but clear correlation between its FF and serum concentrations, whereas no correlation was found for PAPP-A. Serum proteins, however, displayed much better correlation, especially the small ones. \( \alpha \)-1-Acid glycoprotein is shown as an example in Figure 2.

There is no simple comparison between blood and ovarian proteins in terms of FF/blood distribution. Blood is a large pool of proteins, and thus the number and volume of FF in growing ovarian follicles do not affect the concentrations of blood proteins in FF. The same is not true in the case of ovarian proteins. The amount of an ovarian protein passing across the FF/blood barrier may be dependent on the total amount of this protein in FF rather than that of the FF protein concentration. So, if serum PAPP-A and Inhibin A predominantly originate from FF, their serum concentrations should be in good correlation with the total amount of PAPP-A or Inhibin A in FF.

To test the origin of serum Inhibin A and serum PAPP-A, we used the above assumption and set up the following experiment. FF was collected not only from large predominant follicles, but from all follicles. The total amount of Inhibin A and PAPP-A in FF was calculated as follows:

\[
\text{Volume (FF total)} \times \text{Concentration in FF}
\]

FF total volumes ranged from 17.5 to 85.5 mL, with a mean and median volume of 31 and 25.5 mL/patient, respectively. The calculated amounts of total FF PAPP-A and Inhibin A in individual FF samples were plotted against their respective concentrations in paired serum samples (Fig. 3). Results of Inhibin A showed a good correlation between the total amount of FF Inhibin A and its serum concentration in paired samples \((r = 0.858)\), whereas no satisfactory correlation was found in the case of PAPP-A \((r = 0.215)\).
The number of follicles also correlates with serum Inhibin A, but a weaker correlation than the total amount was found ($r = 0.647$, results not shown).

DISCUSSION

In the present study, FF and serum concentrations of 10 major serum proteins and two proteins of ovarian follicle origin, PAPP-A and Inhibin A, were analyzed in patients undergoing regular IVF. Based on our own experiences with the passage of serum proteins across the FF/blood barrier and on the results of previous studies (2, 3), we expected a clear difference in the FF/serum distribution between the large PAPP-A and the small Inhibin A. There is, however, a recent study suggesting a selective transport processes rather than mere filtration across the blood/follicle barrier (20). Our hypothesis was that although Inhibin A passes the FF/blood barrier rather freely, and its serum and FF concentrations are highly correlated, PAPP-A is, because of its high molecular mass, retained within the ovarian follicles and acts only locally. To evaluate the hypothesis, concentrations of Inhibin A and PAPP-A were analyzed in FF and in paired serum samples collected at the time of oocyte retrieval.

The distribution of PAPP-A between FF and blood serum, however, did not fit to the proposed model. One of the logical explanations was that PAPP-A in ovarian follicles did not contribute substantially to the PAPP-A serum concentration, at least not at the time of oocyte retrieval.

Pregnancy-associated plasma protein A (PAPP-A) was first isolated from human pregnancy serum in 1974 (10), as one of the proteins of placental origin found in high concentrations in the blood of pregnant women. More than 20 years later, several laboratories reported the presence of PAPP-A as a novel protease activity against IGFBP-4 in culture media of several cell types such as human fibroblasts (21), human osteoblasts (22), or human coronary artery smooth muscle cells (23). Because PAPP-A was shown to be expressed by a variety of cell types, it should be no surprise that tissues other than reproductive ones may be the dominant source of PAPP-A in the blood circulation of nonpregnant women.

Sinosich et al. (17) reported that PAPP-A FF concentrations between 0.317 and 1.595 IU/liter, but failed to detect PAPP-A in the circulation of normal nonpregnant adults because of the lack of sensitive PAPP-A assay. Recently, Wittfooth et al. (24) reported features of a new type of PAPP-A assay showing 2.20 mIU/L as a median concentration of PAPP-A in acute coronary syndrome patients. Similarly, at a cutoff level of 2.9 mIU/L, PAPP-A was shown to be an independent predictor of an adverse outcome in patients with acute coronary syndrome (25). The cutoff concentration is well above the median of the PAPP-A serum concentration found in this study (1.48 mIU/L), so the PAPP-A serum concentration at the time of

The results support the hypothesis that at the day of oocyte retrieval, most of serum PAPP-A does not originate from FF, and that there is no significant amount of PAPP-A transported across the FF/blood barrier. That model fits well with the proposed physiologic role of studied ovarian proteins, where Inhibin A has to reach remote receptors, while PAPP-A cleaves IGFBP4 within the ovarian follicle.
Correlation between the total amount of ovarian proteins in FF and their serum concentration in paired samples. Results of Inhibin A and PAPP-A measurement are shown. Data were analyzed using linear regression analysis. The FF and serum samples were collected on the day of oocyte retrieval. The FF from all follicles was collected, pooled, and analyzed in this experiment.

The observed FF concentrations of Inhibin A are in agreement with previous reports (27, 28). However, we found a poor correlation between serum and FF Inhibin A concentrations in paired samples. Better results have been obtained when the number of follicles was correlated with Inhibin A concentrations in serum (results not shown). Clearly, the best correlation was found between the total amount of FF Inhibin A and its serum concentrations. Our explanation is that blood has a much larger pool of proteins than FF, so although concentrations of blood proteins in FF are independent of the total volume of FF in growing ovarian follicles, the concentration of an ovarian protein in circulation is dependent on its amount in FF. Other parameters used to calculate a correlation with the Inhibin A serum level—protein concentration in FF and the number of growing oocytes—only provide an incomplete picture of the process, and the resulting correlations are worse than in the case of FF’s total amount.

Total FF protein appears to be a useful parameter, and will certainly be used in further studies.


References


