Evaluation of endometrial activin A secretion for prediction of pregnancy after intrauterine insemination

Pasquale Florio, Ph.D., M.D., Luca Bruni, M.D., Letizia Galleri, Ph.D., Fernando M. Reis, Ph.D., M.D., Lavinia E. Borges, M.D., Caterina Bocchi, M.D., Pietro Litta, M.D., Vincenzo De Leo, M.D., and Felice Petraglia, M.D.

Department of Pediatrics, Obstetrics and Reproductive Medicine, Section of Obstetrics and Gynecology, University of Siena, Siena; and Department of Gynecological Science and Human Reproduction, University of Padua, School of Medicine, Padua, Italy

Objective: To measure activin A concentrations in uterine washing fluid collected from women with couple infertility undergoing intrauterine insemination (IUI).

Design: Retrospective case-control study.

Setting: Tertiary university center for women’s care.

Patient(s): Fifty women, of whom 25 became pregnant after up to three IUI attempts and 25 did not.

Intervention(s): Endocrine and clinical evaluation, semen analyses and hypo-osmotic swelling test, ovarian stimulation, endometrial thickness measurement by ultrasound, uterine washing fluid collection by sonohysterography, and IUI.

Main Outcome Measure(s): Activin A measurement by enzyme-linked immunosorbent assay, receiver operating characteristics curve analysis, and pregnancy rates after IUI; sensitivity, specificity, positive and negative likelihood ratios of activin A, and endometrial thickness for pregnancy prediction.

Result(s): Activin A was measurable in all samples evaluated, and the levels (mean ± SEM) were statistically significantly higher in the pregnant (0.08 ± 0.01 ng/mL) than in the nonpregnant (0.02 ± 0.001 ng/mL) group. Activin A at the cut off of 0.04 ng/mL achieved a sensitivity of 76.0% (95% CI, 54.9%–90.6%) and a specificity of 100% (95% CI, 86.2%–100%) as a single marker for prediction of pregnancy.

Conclusion(s): Activin A is secreted into uterine lumen; the levels in endometrial washing fluids were higher in women who subsequently became pregnant after IUI, so its measurement may be useful in predicting successful implantation. (Fertil Steril 2010;93:2316–20. ©2010 by American Society for Reproductive Medicine.)

Key Words: Endometrium, endometrial thickness, implantation, pregnancy, unexplained infertility, sonohysterography, sensitivity, specificity

Successful implantation results from a coordinated series of events that allows the establishment of a timely dialogue between an invasive blastocyst and receptive endometrium. On this regard, the endometrium controls the implantation of blastocyst, and the process of decidualization is an essential preparative event both to enable and to regulate embryo implantation through the involvement of locally expressed endometrial factors (1). On the other hand, the evidence that a 100% pregnancy rate cannot be achieved by increasing the number of embryos (2) has reinforced the hypothesis that factors that are presumably of endometrial origin may drive implantation, accounting for endometrial receptivity. We evaluated whether activin A may have a role in the presence of a receptive endometrium.

Activin A, a dimeric growth factor belonging to the transforming growth factor beta (TGF-β) superfamily (3), plays an important role in endometrial differentiation, trophoblast invasion, and embryo implantation (4). It is now well established that activin A is expressed throughout the endometrial cycle in increasing amounts, with the highest levels during the secretory phase (5–10), and that the human endometrium also expresses activin A receptors (11, 12) and both binding proteins, namely follistatin (FS) (7, 12) and follistatin-related gene (FLRG) (13, 14). Moreover, activin A is secreted by the human endometrium as decidualization progresses, as supported both by in vitro (15, 16) and in vivo (7) evidence. Indeed, in vitro cultured human endometrial stromal cells produce activin A with the onset of decidualization (15), and in...
vivo levels in uterine washing fluid correlate with endometrial thickness and with the day of menstrual cycle (7).

Because it has been postulated that the local endometrial secretion of activin A may be involved in embryo implantation (17, 18), we investigated whether uterine washing fluid activin A levels differed according to the achievement of pregnancy in women undergoing intrauterine insemination (IUI), and if so, whether activin A measurement has any potential clinical usefulness in predicting implantation.

**MATERIALS AND METHODS**

**Patients and Study Design**

We did a retrospective case-control study on a group of healthy women who underwent IUI as the first-line therapy for infertility related to cervical factor, mild male factor, or unexplained infertility. Informed consent was obtained from all participants before inclusion in the study, which was approved by the local human investigation committee.

A total of 50 women with age ranging from 28 to 42 years (mean age ± SD, 35 ± 4.1 years) whose infertility duration ranged from 3 to 8 years (mean ± SD, 4.6 ± 2.8 years) were enrolled. Pregnancy was assessed 4 weeks after the IUI by human chorionic gonadotropin (hCG) measurement and ultrasonographic detection of fetal heart activity, and accordingly patients were subdivided in pregnant (n = 25) and nonpregnant (n = 25) groups. The latter group was selected from a consecutive series of 43 patients treated contemporarily with the former group. Cases (pregnant) and controls (nonpregnant) were matched for woman’s age (maximum 2 years of difference) and for the presence of unilateral tubal factor or male factor infertility or uterine fibroids.

A complete medical history was obtained and a physical examination performed for each woman. All patients had tubal patency proven by hysterosalpingography, and none had evidence of ovarian cyst at transvaginal ultrasound (Real Time Ultrasound Scan Equipment, Siemens Sonoline Elegra Millennium Edition, with a transvaginal probe at 4.5–7.0 MHz). The menstrual cycle day was calculated on the basis of the last menstrual period and confirmed by transvaginal ultrasound scans. All women underwent endocrine evaluation.

All men underwent andrologic evaluation, with at least two semen analyses and hypo-osmotic swelling tests. Semen samples were collected by masturbation after 48 to 72 hours of sexual abstinence. Semen volume, sperm concentration, motility, and morphology were measured according to standard World Health Organization criteria. The hypo-osmotic swelling test was performed after examination of standard semen parameters. Men affected by severe infertility (sperm concentration less than 10 × 10⁶/mL, progressive motility less than 15%, total motility less than 30%, and normal morphology less than 30%) were considered ineligible for the study. Sperm for IUI were prepared using a conventional layering technique. For this, approximately 1.0 mL of medium (Sperm Preparation Medium; Medi-Cult, Jyllinge, Denmark) was layered onto 1.0 mL of semen, and the specimen was incubated at 37°C for 45 minutes. The supernatant was removed and was used for treatment. All women were treated for up to three consecutive cycles of controlled gonadotropin-induced monoovulation followed by IUI. Only three consecutive cycles of the same treatment were evaluated to prevent carryover effects of ovarian stimulation treatment from affecting results.

Ovarian stimulation was conducted with recombinant follicle-stimulating hormone (FSH, follitropin beta; Puregon, NV Organon, Oss, the Netherlands), starting at a daily dose of 50–100 IUI on the third day of the cycle. Before starting controlled ovarian stimulation treatment, transvaginal ultrasound examinations were performed every other day from the 5th day of treatment until the mean diameter of the dominant follicles reached 14 mm; examinations were then performed daily.

Human chorionic gonadotropin (10,000 IU; Profasi, Serono, Rome, Italy) was administered when the follicle reached a mean diameter of at least 18 mm. Intrauterine inseminations were performed 30 to 36 hours after hCG administration. Intrauterine insemination was performed using a Frydman catheter. The cervix was exposed, and the catheter was passed into the uterus to about 0.5 cm from the top of the uterine cavity, as predicted by a previous ultrasound measurement. The sperm were then expelled.

**Collection of Endometrial Washing Fluid**

Endometrial wash fluid was collected during the periovulatory phase of the first IUI cycle, on the same day of hCG prescription. The women were submitted to sonohysterography by using a Siemens Sonoline SL-2 (Siemens, Erlangen, Germany) with a transabdominal and transvaginal probe of 3.5 MHz and 5.0–7.5 MHz, respectively. After a first evaluation of uterine dimensions and measurement of the endometrial thickness, under transvaginal sonography guidance, a pediatric Foley catheter was inserted into the uterine cavity, and the balloon of the catheter was inflated with 2 mL of sterile saline solution (9 g/L NaCl). The same volume solution (2 mL) was then gradually flushed into the uterine cavity via the opening connected to the inner lumen, and endometrial measurements were taken. Afterward, gentle suction via the same opening was applied to recover the fluid. The washing fluid was centrifuged (3000 rpm for 10 minutes) to discharge the cellular component, then stored at −20°C until assay.

**Activin A Measurement**

A commercially available assay was used to analyze serum samples for activin A (Serotec, Kidlington, Oxford, United Kingdom), according to the manufacturer’s instructions and as previously described elsewhere (18). Measurements were performed in duplicate, the assay had a limit of detection of 0.010 ng/mL, with intra-assay and interassay coefficients of variation for quality control samples of 2.0% and 4.5%, respectively. Cross-reaction with the various inhibin-related proteins was less than 0.5%.
Endocrinologic Assessment

Hormone (follicle-stimulating hormone, luteinizing hormone, prolactin, and testosterone) measurements were performed with commercially available kits (Diagnostic Systems Laboratories, Webster, TX) that had intrassay and interassay coefficients of variance determined by the manufacturer as routine quality control material <10%.

Statistical Analysis

The Kolmogorov-Smirnov test showed experimental values to have a Gaussian distribution; therefore, data are expressed as the mean ± standard error of mean (SEM). Differences between groups were analyzed with an unpaired Student’s t-test, and the Pearson correlation coefficient was calculated to evaluate the linear correlation between activin A concentration and endometrial thickness. After adjusting for various clinical variables (age, tubal damage, uterine fibroids, and male factor infertility), we performed multiple logistic regression analysis with pregnancy as the dependent variable to analyze whether activin A concentration or endometrial thickness were associated with successful implantation.

We used a receiver operating characteristic (ROC) curve analysis (19) to estimate the sensitivity, specificity, and positive and negative likelihood ratios of activin A concentration and endometrial thickness for pregnancy prediction. \( P < .05 \) was considered statistically significant.

RESULTS

Activin A was measurable in all samples of endometrial washing fluids: the levels were statistically significantly \( (P < .0001) \) higher \((0.80 ± 0.01 \text{ ng/mL})\) in uterine washing fluid collected from women who became pregnant than from those who did not \((0.022 ± 0.001 \text{ ng/mL})\) (Fig. 1). Moreover, in patients who became pregnant activin A statistically significantly correlated \((\text{Pearson } r = 0.9164, P < .0001)\) with endometrial thickness; such a correlation was absent in the nonpregnant women \((\text{Pearson } r = 0.2523, P = .2236)\) (Fig. 2).

A multiple logistic regression analysis with pregnancy as the dependent variable showed a positive correlation with activin A concentration \((\text{adjusted odds ratio } 31.7, P < .001)\), whereas no statistically significant correlations were found with clinical variables (age, presence of tubal damage, and male factor infertility).

We evaluated sensitivity and specificity, positive and negative likelihood ratios, and the area under ROC curve (AUC) for activin A concentration and endometrial thickness as diagnostic tests. Activin A levels at a cutoff value of 0.04 ng/mL had a sensitivity of 68.0% \((95\% \text{ CI, } 48.4\%–82.8\%))\) and a specificity of 48.0% \((95\% \text{ CI, } 30.0\%–66.5\%))\) for predicting successful implantation \((\text{AUC, } 0.079; 95\% \text{ CI, } 0.469\%–0.751\%))\) with positive and negative likelihood ratios of 1.31 and 0.67, respectively. The AUC for activin A concentration

Endometrial thickness at a cutoff value of 6.0 mm had a sensitivity of 68.0% \((95\% \text{ CI, } 48.4\%–82.8\%))\) and a specificity of 48.0% \((95\% \text{ CI, } 30.0\%–66.5\%))\) for predicting successful implantation \((\text{AUC, } 0.079; 95\% \text{ CI, } 0.469\%–0.751\%))\) with positive and negative likelihood ratios of 1.31 and 0.67, respectively.
was statistically significantly \((P<.0001)\) greater than that for endometrial thickness (difference between the AUCs, 0.310; 95% CI, 0.183%–0.437%) (see Fig. 3).

**DISCUSSION**

Our study first found that activin A is detectable in uterine washing fluid in higher concentration in women who became pregnant after IUI, thus leading us to suggest that endometrial activin A is somehow related to implantation and that its secretion may signal endometrial receptivity. In this regard, it is well known that the endometrium expresses activin A and its receptors (4–9, 12) and that activin A stimulates endometrial cell decidualization in vitro (15, 16), an initial process for implantation and placental development (1). Moreover, even in vitro, the addition of activin A is reported to stimulate the outgrowth of cytotrophoblast cells and their differentiation into extravillous anchoring trophoblast (20).

These findings and our data together would support the hypothesis that the endometrial secretion of activin A may be part of the complex molecular dialogue between the endometrium and the blastocyst, finalized to allow implantation. Some intriguing observations, however, have challenged this concept, such as the increased activin A release by eutopic endometrial cells of women with endometriosis (21). Activin A also increases modestly the apoptosis index in rat blastocysts in vitro (22), although it is unknown whether this influences embryo survival or implantation. What emerges from this apparent controversy is that activin A, like other cytokines (17), is an important paracrine agent in endometrial physiology that can also be overproduced in some pathological circumstances.

Our study also confirms that activin A is secreted by the human endometrium (7, 11) and it is measurable in uterine washing fluid (7). However, the correlation between activin A and the endometrial thickness in women who became pregnant after IUI merits further discussion. Indeed, we had already found that activin A is secreted in higher amounts in the secretory than in the proliferative phase of the menstrual cycle, and that uterine washing fluid concentrations statistically significantly correlate with endometrial thickness and with the day of the menstrual cycle in fertile women (7). Activin A subunits are highly expressed in the decidualizing endometrium: the expression of \(\beta A\) subunit is up-regulated in decidualized stromal cells (6); it is differentially expressed in human endometrial stromal cells decidualized in vitro in response to progesterone and progestins (6, 15, 17) or cyclic adenosine monophosphate (cAMP) (16) compared with nondecidualized cells; after the onset of decidualization, the expression of \(\beta A\) subunit is up-regulated in decidualized stromal cells (17).

Therefore, on the basis of these and our data, we can conclude that the secretion of activin A into uterine lumen may be regarded as a signal of an endometrium that is becoming receptive to the implantation process. Although we did not perform uterine fluid sampling during the implantation window of an IUI cycle for obvious ethical reasons, our observations made in the periovulatory phase are still relevant for understanding endometrial development in preparation to implantation. This hypothesis is further sustained by data reporting reduced expression of activin A in samples of endometrium collected from women with recurrent pregnancy loss (23) as well as its reduced concentration (24) and expression (25) in women with ectopic pregnancy. In other words, activin A in the endometrium may be part of the local molecular repertoire evoked in modulating extracellular matrix modification (26) and molecular cues (27) that are implicated in ending endometrium with this receptivity, as in the case of reproduction. Activin A would operate locally but also on human preimplantation embryos, which express activin receptors in all their cells (28).

We evaluated patients undergoing IUI after ovarian stimulation with exogenous gonadotropins, which leads to the question of what influence ovarian stimulation has on the production of endometrial activin A. We are not aware of any previous study that has evaluated endometrial activin A production in women under the influence of exogenous gonadotropins, and our study design did not include a nonstimulated control group for this kind of comparison. Although the endometrium expresses gonadotropin receptors and responds directly to hCG (29, 30), it is still unknown whether local activin A production is directly regulated by gonadotropins.
Activin A is measurable in the endometrial washing fluid, its concentrations were higher in the women who became pregnant after IUI, and its measurement may be a promising prognostic biochemical marker for implantation. Whatever the role of activin A in human endometrium, the putative clinical usefulness of its measurement in uterine washing fluid warrants discussion. Indeed, we found that activin A concentration above the cutoff level increased the likelihood of subsequent pregnancy with high specificity and positive likelihood ratio combined with acceptable sensitivity. These findings could prompt further study of biochemical marker assessment of endometrial receptivity such that patients with a higher chance of successful implantation could be identified earlier, possibly preventing unnecessary interventions. However, real estimation of pregnancy probability depends on the pretest probability that is given by all known prognostic factors in fertility treatments, mainly the woman’s age.

REFERENCES