Impact of highly purified urinary FSH and recombinant FSH on haemostasis: an open-label, randomized, controlled trial

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BACKGROUND: It has recently been suggested that recombinant FSH administration may result in an increased risk of venous thrombosis. An open-label, randomized, controlled trial was carried out to compare the impact of urinary and recombinant FSH on haemostasis. METHODS: Fifty infertile women were randomized, using a random number generator on a personal computer, to receive either highly purified urinary FSH (u-hFSH) or recombinant human FSH (r-hFSH); a starting dose of 150 IU. Human chorionic gonadotrophin 10000 IU was administered once there was at least one follicle >18 mm. The luteal phase was supported with progesterone 50 mg/day for at least 15 days. Fifty normally menstruating women were recruited as controls. Repeated measurements of estradiol, progesterone, prothrombin time (PT) expressed as INR, activated partial thromboplastin time (APTT) ratio, fibrinogen (FBG), factor VIII (FVIII), normalized activated protein C ratio (nAPC ratio), antithrombin III activity (AT), protein C activity (PC), protein S activity (PS), tissue-type plasminogen activator antigen (t-PA), type 1 plasminogen activator inhibitor (PAI), prothrombin fragments 1+2 (F1+2), were performed during both hyperstimulated and natural cycles, and at onset of the following menstruation or at 8 weeks of pregnancy.

RESULTS: At the end of gonadotrophin administration PT INR increased in the u-hFSH group, while AT and t-PA significantly decreased. In the patients treated with r-hFSH, only F1+2 significantly decreased. No significant changes were observed in the control group. In the luteal phase FBG increased significantly in all groups. In the u-hFSH group no other significant changes were noted compared to pre-ovulatory values, while compared to baseline values AT, PS and t-PA significantly decreased. In the r-hFSH group during the luteal phase PT INR significantly decreased, but did not differ from baseline levels. Other parameters such as FBG, FVIII, t-PA, rose significantly, but only FVIII and FBG values were significantly higher than baseline levels. In the women who became pregnant a significant increase in t-PA and a significant decrease in PS at the midluteal phase were observed. After one month all the haemostatic parameters returned to baseline value if pregnancy failed to occur, while in the pregnant women a significant increase in FVIII and a significant decrease in PS were observed. CONCLUSIONS: Ovarian stimulation with recombinant FSH does not influence coagulation and fibrinolysis significantly, as already reported for urinary gonadotrophins. The moderate changes induced by both treatments are no longer detectable after 4 weeks.

Key words: haemostasis/highly purified urinary FSH/menstrual cycle/pregnancy/recombinant FSH

Introduction

In recent years there has been great debate as to the use of urinary and recombinant gonadotrophins. A large cohort of studies has compared the clinical efficacy of different gonadotrophin preparations in women undergoing ovarian stimulation. However, little attention has been paid to the safety of these treatments and only very limited data are available on the safety of urinary versus recombinant gonadotrophins (for a review, see Gleicher et al., 2003).

The first report about the risk of thromboembolic events associated with the use of urinary gonadotrophins was published in 1965 (Mozes et al., 1965). Many studies have
demonstrated that ovarian stimulation with urinary gonadotrophins may induce significant changes in the coagulation and fibrinolytic system (Phillips et al., 1975; Kim et al., 1981; Aune et al., 1991, 1993; Hanss et al., 1993; Bremme et al., 1994; Lox et al., 1995; Falkon et al., 1995; Biron et al., 1997; Lox et al., 1998; Magnani et al., 1999). Yet although assisted reproduction technologies have spread widely throughout the world over the past two decades and millions of women have been treated with urinary gonadotrophins, the incidence of clinical thrombosis so far reported is low (Ludwig et al., 2000).

Recombinant FSH preparations became available a few years ago. Reports of complications are scanty. However, recently, two cases of subclavian deep venous thrombosis (DVT) associated with the use of recombinant FSH have been reported (Loret de Mola et al., 2000). These findings have suggested that r-hFSH may induce a higher rate of DVT either by increasing estradiol levels, changing haemostasis factors directly, or by another, as yet unknown, mechanism. To date, there are few data on the impact of recombinant FSH both on coagulation and fibrinolytic systems in patients undergoing controlled ovarian hyperstimulation.

The aim of this study was to compare the effects of highly purified urinary FSH and recombinant human FSH treatment on haemostasis parameters.

Materials and methods

Study groups

FSH treatment groups. Fifty infertile Caucasian women scheduled to undergo ovarian hyperstimulation for assisted reproduction at the Department of Reproductive and Developmental Sciences of the University of Trieste, Istituto per l’Infanzia, ‘Burlo Garofolo’, I.R.C.C.S. of Trieste, were included in the study. Inclusion criteria were: aged between 28 and 38 years; normal ovulatory cycles; FSH, LH, prolactin, testosterone free and deidroepiandrosterone sulphate within normal range on cycle day 3; no treatment with clomiphene citrate, testosterone or drugs known to influence haemostasis for at least 6 months preceding the study. Exclusion criteria were: history of thromboembolic events; chronic diseases; body mass index (BMI) <18 or >27 kg/m²; past or current abuse of alcohol; >5 cigarettes per day; regular intake of drugs.

Control group. Fifty comparable volunteer Caucasian women were concurrently enrolled as controls. Inclusion criteria were: aged between 28 and 38 years; regular menstrual cycles lasting from 26 to 30 days; previous three cycles of regular length; no administration of sex steroids or drugs known to interfere with haemostasis within the previous 6 months. The exclusion criteria were the same as for infertile subjects.

Study design

The study was designed as an open-label, randomized, controlled trial comparing the effects of u-hFSH and r-hFSH on haemostasis parameters. After confirming eligibility for the study, informed consent was obtained from each subject. The infertile women were randomly allocated into two equally sized treatment groups using a random number generator on a personal computer. The patients were randomized by one of the study nurses. After randomization, patients received treatment protocol and appropriate instructions from one of the study gynecologists. Fifty, nonrandomized, comparable women were included in the untreated control group. The study was conducted in accordance with the guidelines proposed in The Declaration of Helsinki. The study was approved by the ‘Comitato Tecnico-Scientifico’ of ‘Istituto per l’Infanzia, Burlo Garofolo, I.R.C.C.S.’ of Trieste.

Study protocol

Starting on cycle day 2, infertile patients received u-hFSH (Metrodin HP; Serono, Rome, Italy) or r-hFSH (Gonal F; Serono, Rome, Italy) 150 IU/day s.c. Ovarian response was monitored with vaginal ultrasound and plasma estradiol. The first control was performed on day 6 of the cycle. The FSH dose was then adjusted according to the response. Human chorionic gonadotropin 10000 IU i.m. was given to induce ovulation when at least one follicle ≥18 mm in diameter was present. The luteal phase was supplemented with 50 mg progesterone in oil (Prontogest, Amsa, Italy) daily i.m. beginning 3 days after hCG administration and continuing for at least 15 days. On the 18th day after hCG administration, a pregnancy test was performed. If positive, progesterone was continued for 14 days.

Blood samples for measurement of haemostasis parameters, estradiol and progesterone, were taken from all subjects on cycle day 2 before starting gonadotropin administration, on cycle day 6, on the day of hCG administration, and 10 days after hCG administration. In patients who failed to achieve pregnancy, measurements were repeated on day 2 of the following menstruation, providing one month of wash-out. In patients who became pregnant, measurements of haemostasis parameters, estradiol and progesterone serum levels were repeated after a comparable period at 8 weeks of pregnancy. In the control group, the first blood sample was collected on cycle day 2. To standardize the different cycle lengths, the timing of the successive blood samples was calculated personally. The presumed first day of the next menstruation, estimated on the basis of the length of the previous three cycles, was taken as day 0. The second, third and fourth blood samples were taken on days −21, −14, −7. The fifth sample was collected on day 2 of the following menstrual cycle.

Laboratory analyses

Blood samples were obtained between 8:30 and 9:30 a.m. after a 12-h fast. Subjects remained at rest for a period of 15 min before venipuncture. Blood was taken from an antecubital vein with minimal stasis using standard venipuncture techniques into serum tubes.

Specimens were collected using 3.8% sodium citrate (9/1 v/v blood/anticoagulant) for blood clotting studies, and centrifuged at 1500 g for 15 min at 4°C. Plasma was collected and tested for prothrombin time (PT) expressed as INR (STA Neoplastin Plus, Roche, Milan, Italy), activated partial thromboplastin time (APTT) ratio (STA APTT LT, Roche, Milan, Italy), fibrinogen (FBG) (Thrombin 90 U, Roche, Milan, Italy), antithrombin III activity (AT) (AT III Chromogenic Assay, Roche, Milan, Italy). The remainder of the assays were performed on plasma stored at −80°C until used. Factor VIII (FVIII) activity measurement was carried out using FVIII-deficient plasma and expressed as a percentage of normal activity (STA Factor VIII, Roche, Milan, Italy). Protein C activity (PC) and protein S activity (PS) were determined in a functional assay (STA Protein C and STA Protein S clotting, Roche, Milan, Italy). Tissue-type plasminogen activator antigen (t-PA) was measured using a sensitive microELISA method (Asserachrom t-PA, Boehringer, Milan, Italy) and type 1 plasminogen activator inhibitor (PAI) by the determination of the residual amidolytic activity following the addition of a known amount of single-chain t-PA in the plasma (Coatest PAI, Ortho, Milan, Italy). The prothrombin fragments 1+2 (F1+2) were determined using an
ELISA method (Enzignost F1+2, Behring, L’Aquila, Italy). Resistance to activated Protein C (APC) was determined by the ratio of APTT in the presence of an excess of Factor V Reagent Plasma, measured in the absence and presence of APC (APC Resistance V, Instrumentation Laboratory, Milan, Italy). The assay gives a 2.5-fold prolongation of the coagulation surface induced clotting time when APC has been added. The APC ratio of a sample was then compared to the APC ratio of pooled normal plasma. The results were expressed as the normalized APC ratio (nAPC ratio); nAPC ratio <0.8 was considered the cut-off which distinguishes abnormal from normal samples to represent APC resistance.

Serum 17β-estradiol and progesterone were determined by means of a solid-phase, ligand-labeled, competitive chemiluminescent immunoassay with an Immulite Analyzer (Immulite Estradiol and Immulite Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) and results were expressed in pg/mL and ng/mL respectively.

Objectives and outcome measurements

The objective of the study was to evaluate the impact of u-hFSH and r-hFSH on haemostasis by comparing changes in the diverse parameters during ovarian stimulation and the luteal phase.

The primary outcome measurements were the differences between the changes in haemostatic parameters induced by the use of u-hFSH and r-hFSH.

Secondary outcome measurements included the changes in haemostatic parameters in the women who failed to achieve pregnancy and in the women who became pregnant.

Sample size

It was assumed that differences in changes in haemostatic parameters between u-hFSH and r-hFSH use which are 80% or more over standard deviation were to be considered clinically important. To have 80% power and a 2-tailed α error of 0.05 to detect these differences, 25 subjects in each study group were required.
Table I. Main characteristics of the women who were enrolled in the study

<table>
<thead>
<tr>
<th></th>
<th>u-hFSH HP (n = 25)</th>
<th>r-hFSH (n = 25)</th>
<th>Controls (n = 50)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (years)</td>
<td>34.2 ± 3.1</td>
<td>33.5 ± 3.9</td>
<td>33.9 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (mean ± SD) (kg/m²)</td>
<td>21.8 ± 2.5</td>
<td>21.5 ± 2.3</td>
<td>21.6 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Infertility duration (mean ± SD)</td>
<td>3.2 ± 1.7</td>
<td>3.5 ± 1.8</td>
<td>3.9 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Unexplained infertility (%)</td>
<td>15/25 (60)</td>
<td>13/25 (52)</td>
<td>13/25 (52)</td>
<td>NS</td>
</tr>
<tr>
<td>Male infertility (%)</td>
<td>10/25 (40)</td>
<td>12/25 (48)</td>
<td>12/25 (48)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values in parentheses are numbers of cases.
*Statistics for between-group differences were determined by *Kruskal–Wallis ANOVA test, *Mann–Whitney U-test and *χ²-test.

Table II. Stimulation characteristics of patients receiving HCG

<table>
<thead>
<tr>
<th></th>
<th>u-hFSH HP (n = 23)</th>
<th>r-hFSH (n = 24)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days of FSH stimulation required</td>
<td>7.8 ± 1.8</td>
<td>7.1 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total dose of FSH required (no. of FSH 75 IU ampoules)</td>
<td>13.8 ± 4.1</td>
<td>13.1 ± 3.7</td>
<td>NS</td>
</tr>
<tr>
<td>No. of follicles ≥ 16 mm diameter on day of HCG</td>
<td>3.5 ± 2.7</td>
<td>3.5 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol on day HCG (pg/mL)</td>
<td>815 ± 63</td>
<td>808 ± 62</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol at midluteal phase (pg/mL)</td>
<td>423 ± 222</td>
<td>409 ± 215</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone at midluteal phase (ng/mL)</td>
<td>37.8 ± 17.5</td>
<td>39.7 ± 18.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD.
*Statistics for between-group differences were determined by Mann–Whitney U-test.

Table III. Haemostatic parameters changes during ovarian stimulation with u-hFSH (n = 23)

<table>
<thead>
<tr>
<th></th>
<th>Cycle day 2</th>
<th>Cycle day 6</th>
<th>HCG day</th>
<th>Midluteal</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (INR)</td>
<td>1.02* (0.99–1.05)</td>
<td>1.03 (1.00–1.06)</td>
<td>1.08* (1.04–1.11)</td>
<td>1.03 (0.99–1.07)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PTT (Ratio)</td>
<td>0.98 (0.94–1.02)</td>
<td>0.97 (0.92–1.01)</td>
<td>0.95 (0.91–1.00)</td>
<td>0.94 (0.91–0.98)</td>
<td>NS</td>
</tr>
<tr>
<td>FBG (mg%)</td>
<td>293* (272–319)</td>
<td>293* (243–344)</td>
<td>273* (252–294)</td>
<td>352* (321–384)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FVIII (%)</td>
<td>95.9 (81.9–109.9)</td>
<td>96.5 (83.7–109.4)</td>
<td>95.8 (80.9–110.6)</td>
<td>103.2 (86.6–119.8)</td>
<td>NS</td>
</tr>
<tr>
<td>ATIII (%)</td>
<td>105.4* (99.8–110.9)</td>
<td>100.1 (94.0–106.3)</td>
<td>97.5* (91.8–103.2)</td>
<td>98.9 (94.2–103.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PC %</td>
<td>113.7 (104.1–123.4)</td>
<td>107.5 (98.8–116.2)</td>
<td>104.6 (95.2–114.0)</td>
<td>104.2 (95.8–112.6)</td>
<td>NS</td>
</tr>
<tr>
<td>PS (%)</td>
<td>98.2* (90.1–106.4)</td>
<td>100.1* (91.5–110.0)</td>
<td>96.6 (89.4–103.7)</td>
<td>85.5* (75.0–96.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>t-PA (ng/mL)</td>
<td>3.9* (3.2–4.7)</td>
<td>3.5 (2.8–4.2)</td>
<td>3.0* (2.1–3.9)</td>
<td>3.3* (2.5–4.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PAI (AU/mL)</td>
<td>15.8 (13.0–18.5)</td>
<td>14.8 (12.3–17.3)</td>
<td>14.4 (11.9–16.8)</td>
<td>14.4 (12.4–16.5)</td>
<td>NS</td>
</tr>
<tr>
<td>F1+2 (nmol/L)</td>
<td>0.71 (0.54–0.88)</td>
<td>0.69 (0.53–0.84)</td>
<td>0.65 (0.50–0.80)</td>
<td>0.69 (0.55–0.83)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means and 95% CI.
*Friedman non-parametric repeated measures test.
Dunn’s multiple comparisons post-hoc test: *versus b; ^versus c; ~versus d; $versus e; †versus f; P < 0.05; ²versus l; P < 0.01; ³versus g; P < 0.001; ⁴versus h; P < 0.001; ⁵versus i; P < 0.001; ⁶versus j; P < 0.01; ⁷versus k; P < 0.05; ⁸versus m; P < 0.05.

Statistical analyses

Statistical analyses of all baseline parameters for inter-group differences were performed by means of Kruskal–Wallis analysis of variance (ANOVA) test. Statistically significant differences between the u-hFSH and r-hFSH group were examined by the Mann–Whitney U-test. The *χ² test was used to compare categorized measures when appropriate. Multiple comparisons of paired data during natural and hyperstimulated cycles were performed with Friedman’s repeated measures test followed by Dunn’s multiple comparisons post-hoc test. The differences between the changes in the haemostatic parameters at end of FSH administration and in the luteal phase in the u-hFSH and in the r-hFSH group were also assessed using the Mann–Whitney U-test. Statistical significance was assigned when P < 0.05.

Results

A total of 100 subjects (50 infertile patients and 50 controls) who were eligible were recruited in the study, which was carried out between January 2001 and December 2002 (Figure 1). The main characteristics of the three groups were not statistically different (Table I). Gonadotrophin administration was ceased before completing the study with regard to two patients in the u-hFSH group and one patient in the r-hFSH group as they were at risk of developing ovarian hyperstimulation syndrome. Accordingly, three cycles were cancelled and were excluded from the analysis. There were no significant differences between the two groups in the stimulation characteristics of the patients receiving hCG (Table II). The pregnancy rate per started cycle was 28% for r-hFSH and 24% for u-hFSH (not significantly different). Neither important adverse events nor side effects were observed in each intervention group. In the control group 9 of the 50 eligible volunteers were excluded from the analysis: five women had an anovulatory cycle, as assessed by low serum progesterone concentrations in the midluteal phase (<5 ng/ml) and four
women did not complete the measurements as required by the protocol for personal reasons.

Tables III and IV summarize the effects of gonadotrophin administration on the haemostatic parameters. An increase in PT values expressed as INR occurred both in patients receiving u-hFSH and in patients receiving r-hFSH at the end of gonadotrophin administration, and a decrease occurred during the luteal phase. Hence, PT INR luteal values were not significantly different in comparison with baseline in both groups. No significant changes in APTT, FBG, and FVIII were
Ovarian stimulation and haemostasis

Table VII. Mean haemostatic parameters changes during luteal phase from baseline

<table>
<thead>
<tr>
<th></th>
<th>u-hFSH HP (n = 23)</th>
<th>r-hFSH (n = 24)</th>
<th>Controls (n = 41)</th>
<th>u-hFSH HP versus r-hFSH</th>
<th>u-hFSH HP versus controls</th>
<th>r-hFSH versus controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (INR)</td>
<td>0.01 ± 0.06</td>
<td>−0.01 ± 0.06</td>
<td>0.02 ± 0.06</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PTT</td>
<td>−0.04 ± 0.07</td>
<td>−0.02 ± 0.05</td>
<td>−0.01 ± 0.10</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FVIII (%)</td>
<td>5.22 ± 23.10</td>
<td>20.30 ± 24.81</td>
<td>4.12 ± 17.31</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ATIII (%)</td>
<td>−7.17 ± 9.48</td>
<td>−1.70 ± 8.99</td>
<td>−1.39 ± 10.48</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>t-PA (ng/mL)</td>
<td>−13.28 ± 27.13</td>
<td>−4.35 ± 26.14</td>
<td>−1.08 ± 21.12</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>PAI (AU/mL)</td>
<td>−0.93 ± 5.49</td>
<td>−2.30 ± 7.72</td>
<td>−0.20 ± 5.28</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AT (%)</td>
<td>90.1 ± 13.02</td>
<td>93.0 ± 15.93</td>
<td>92.7 ± 13.47</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD.
*Statistics for between-group differences were determined by Mann–Whitney U-test.

Table VIII. Haemostatic parameters changes in stimulated women who failed to achieve pregnancy (n = 34)

<table>
<thead>
<tr>
<th></th>
<th>Cycle day 2</th>
<th>Cycle day 6</th>
<th>HCG day</th>
<th>Midluteal</th>
<th>Cycle day 2 (following cycle)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (INR)</td>
<td>1.01 (0.98–1.04)</td>
<td>1.02 (0.98–1.05)</td>
<td>1.04 (1.00–1.08)</td>
<td>1.01 (0.98–1.04)</td>
<td>1.02 (0.98–1.06)</td>
<td>NS</td>
</tr>
<tr>
<td>PTT</td>
<td>0.99 (0.94–1.04)</td>
<td>0.98 (0.94–1.03)</td>
<td>0.99 (0.93–1.04)</td>
<td>0.95 (0.90–1.00)</td>
<td>0.98 (0.92–1.04)</td>
<td>NS</td>
</tr>
<tr>
<td>FVIII (%)</td>
<td>88.5 (75.2–101.8)</td>
<td>88.6 (76.4–100.8)</td>
<td>89.8 (76.8–102.8)</td>
<td>100.9 (84.7–117.0)</td>
<td>94.1 (83.6–110.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ATIII (%)</td>
<td>112.6 (107.4–114.7)</td>
<td>110.2 (106.0–114.4)</td>
<td>106.6 (101.5–111.2)</td>
<td>109.8 (103.6–115.9)</td>
<td>111.6 (105.4–112.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PS (%)</td>
<td>622.5 (109.0–135.9)</td>
<td>122.2 (109.7–134.7)</td>
<td>121.2 (108.6–133.8)</td>
<td>109.1 (93.8–124.5)</td>
<td>127.4 (116.3–138.5)</td>
<td>NS</td>
</tr>
<tr>
<td>nAPC ratio</td>
<td>0.98 (0.94–1.03)</td>
<td>0.99 (0.94–1.04)</td>
<td>0.99 (0.95–1.03)</td>
<td>0.99 (0.95–1.04)</td>
<td>0.96 (0.89–1.03)</td>
<td>NS</td>
</tr>
<tr>
<td>PS (%)</td>
<td>70.0 (78.8–101.5)</td>
<td>93 (81.3–104.7)</td>
<td>92 (80.6–103.4)</td>
<td>90.1 (73.8–106.3)</td>
<td>90.4 (78.3–102.6)</td>
<td>NS</td>
</tr>
<tr>
<td>t-PA (ng/mL)</td>
<td>4.3 (3.4–5.3)</td>
<td>4.5 (3.3–5.7)</td>
<td>3.9 (2.7–5.1)</td>
<td>4.3 (3.2–5.5)</td>
<td>4.4 (3.4–5.4)</td>
<td>NS</td>
</tr>
<tr>
<td>PAI (AU/mL)</td>
<td>18.0 (15.2–21.9)</td>
<td>18.4 (15.7–21.1)</td>
<td>18.2 (16.1–20.3)</td>
<td>18.1 (15.0–21.2)</td>
<td>16.7 (14.6–18.9)</td>
<td>NS</td>
</tr>
<tr>
<td>F1+2 (nmol/L)</td>
<td>0.66 (0.44–0.87)</td>
<td>0.72 (0.47–0.98)</td>
<td>0.62 (0.48–0.76)</td>
<td>0.58 (0.42–0.74)</td>
<td>0.64 (0.46–0.82)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means and 95% CI.
*Friedman non-parametric repeated measures test.
**Dunn’s multiple comparisons post-hoc test: a versus b: P < 0.05; b versus c: P < 0.001; c versus d: P < 0.001; d versus e: P < 0.001.
observed in either treatment group during the follicular phase. In the luteal phase a significant increase in FBG and no significant change in APTT were observed in both groups, while only the patients treated with r-hFSH showed a significant increase in FVIII.

Regarding the coagulation inhibitors, no significant changes were observed during gonadotrophin administration in the r-hFSH group, while a significant decrease in AT was noted in the u-hFSH group. In the luteal phase, PS values decreased significantly in comparison with baseline in women treated with u-hFSH.

No significant changes were observed in nAPC ratio in either treatment group.

Among fibrinolysis parameters, no change in PAI was observed, while t-PA decreased significantly in the follicular phase in the u-hFSH group and increased significantly in the luteal phase in the r-hFSH group. Considering the changes compared to baseline values, t-PA luteal values in the u-hFSH group fell significantly, while in the r-hFSH group the differences were not significant.

In women receiving r-hFSH, F1+2 were significantly lower at the end of the follicular phase compared to baseline, but this difference was no longer detectable at the midluteal phase. No change was observed in women receiving u-hFSH.

In the control group, haemostatic parameters showed minimal changes during the menstrual cycle (Table V). These changes were always within the normal range and were not statistically significant, except for PT INR, FBG, AT, and t-PA. However, only FBG levels showed a significant increase in the luteal phase, and returned to baseline at the beginning of the following cycle.

At the end of ovarian stimulation, the changes in haemostatic parameters caused by r-hFSH and u-hFSH administration were not significantly different, with the exception of F1+2 (Table VI). Similar results were found comparing the changes observed in the r-hFSH group and the control group. Comparing the u-hFSH group with the control group, significant differences were noted in PT INR, AT, and PS changes.

In the luteal phase, differences between the two FSH preparations only reached significance for the changes in FBG and tPA levels (Table VII). Both u-hFSH and r-hFSH treatment resulted in a statistically significant increase in FBG and in a significant decrease in PC levels in comparison with the control group. In the u-hFSH group a significant decrease in PS was observed compared with the control group. In the r-hFSH group significant differences were noted in PT INR, AT, and PS levels.

Comparing the women who failed to achieve pregnancy with the women who became pregnant, we noted no differences during ovarian stimulation, other than a significant increase in t-PA levels and a significant decrease in PS levels at the midluteal phase in the pregnant women (Tables VIII and IX).
All the haemostatic parameters returned to baseline after 1 month if pregnancy failed to occur, while, after the same time period, in the pregnant women a significant increase in FVIII levels and a significant decrease in PS compared to pre-treatment values were observed. At 8 weeks of gestation the nAPC ratio values were significantly decreased in comparison with luteal values, but did not differ from baseline levels.

### Discussion

Several studies have investigated the effects of ovarian stimulation on blood coagulation and fibrinolysis and conflicting results have been reported. This might be explained by different treatment regimens, by different timing of blood sample collection, or by the size of the studies. Most of these studies evaluated small series of women, treated with clomiphene citrate (CC) and/or hMG. Few studies investigated haemostasis during the luteal phase, none had a control group, and none evaluated the effects of ovarian stimulation over a period of time. Furthermore, in most of the studies, ovarian stimulation protocols included preliminary down-regulation with GnRH analogue or oral contraceptives (OC) administration and in many cases baseline haemostasis evaluation was performed only after pituitary desensitization, hence the results might have been influenced by estrogen suppression and/or by GnRH analogue administration or by OC administration.

Only minimal changes in PT values have been reported in women treated with hMG (Kim et al., 1981; Lox et al., 1995). Using FSH, we found similar results although we did observe, in both the u-hFSH and the r-hFSH group, an increase in PT values expressed as INR at end of gonadotrophin administration, and a decrease during the luteal phase. However, these changes were slight and within the normal range. Furthermore, in both groups no significant differences were appreciable during the luteal phase compared to baseline values. Hence urinary and recombinant FSH do not appear to influence the extrinsic pathway of coagulation significantly. The PT INR increase at the end of ovarian stimulation could be an estrogenic effect. Indeed, we observed the same trend in PT INR values in the control group. This effect might be explained by factor VII (FVII) decrease. We did not evaluate FVII levels, however, some studies have shown that FVII levels decrease during ovarian stimulation (Bremme et al., 1994) and during the menstrual cycle (Kapiotis et al., 1998). A reduction in FVII is normally due to binding of FVII/FVIIa to tissue factor (TF) or FVIIa to AT (McVey, 1999). Thus, an increase in INR may have been caused by a rise in procoagulant activities.

The studies that investigated hMG effects on haemostasis found follicular increase and luteal decrease in APTT values, but all these changes were still well within the normal ranges (Kim et al., 1981; Lox et al., 1995). In our study, no significant changes were observed regarding APTT ratio values both in FSH-treated patients and in the control group.

Several earlier studies had shown a significant increase in FBG levels during ovarian stimulation (Kim et al., 1981; Aune et al., 1991; Bremme et al., 1994; Biron et al. 1997). This event was generally explained as an estrogen-related effect. Our findings do not support this hypothesis, since in our study FBG increased significantly only in the luteal phase both in the treatment groups and in the control group, while FBG levels were not modified at the end of ovarian stimulation or before spontaneous ovulation. The only study which evaluated FBG before and after pituitary desensitization by GnRH-analogue administration showed a significant FBG decrease after ovarian suppression and a return to baseline level at estradiol peak before hCG administration (Hanss et al., 1993), in accordance with our results. We found a luteal FBG increase both in the stimulated and in the natural cycle, according to previous studies (Cederblad et al., 1977; Lebecch et al., 1990). Therefore, FBG increase does not appear to be related to FSH administration and might be a progesterone-related effect, or a consequence of an inflammatory reaction associated with ovulation (Espey, 1980).

Previous studies evaluating hMG or CC and hMG have shown an increase in FVIII during estradiol increase (Kim et al., 1981; Bremme et al., 1994; Lox et al., 1995), but no significant changes associated with progesterone increase were observed (Bremme et al., 1994). No data are available on the effects of u-hFSH or r-hFSH. In our study, u-hFSH adminis-

| Table IX. Haemostatic parameters changes in stimulated women who became pregnant (n = 13) |
|----------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                 | Cycle day 2 INR | Cycle day 6 INR | HCG day INR | Midluteal INR | 8th week of pregnancy |
| PT (INR)                        | 1.01 (0.97–1.05) | 1.00 (0.96–1.04) | 1.03 (0.98–1.08) | 0.97 (0.93–1.02) | 0.98 (0.95–1.01) |
| PTT (Ratio)                     | 0.94 (0.90–0.98) | 0.95 (0.89–1.01) | 0.94 (0.90–0.99) | 0.94 (0.90–0.98) | 0.94 (0.89–0.99) |
| FBG (mg%)                       | 306 (286–326)    | 313 (237–390)    | 290 (265–315)    | 375 (336–413)    | 323 (284–362)    |
| FVIII (%)                       | 91.5 (73.6–109.5)| 89.4 (75.7–103.2)| 88.9 (68.3–109.5)| 107.3 (83.8–103.8)| 118 (83.8–152.2) |
| ATIII (%)                       | 107.8 (103.2–112.2)| 110.4 (103.6–117.1)| 103.6 (98.4–108.8)| 103.1 (96.2–109.95)| 101.8 (96.0–107.5) |
| PC %                            | 121.7 (108.6–134.8)| 115.8 (98.4–133.1)| 105.4 (88.1–122.6)| 112.8 (98.5–127.2)| 116.4 (107.6–125.1) |
| nAPC ratio                      | 1.00 (0.97–1.04) | 0.99 (0.97–1.03) | 1.02 (0.99–1.04) | 1.00 (0.97–1.04) | 0.96 (0.95–0.98) |
| FS (%)                          | 105% (94.2–115.8)| 92.7 (80.5–104.9)| 97.9 (86.4–109.4)| 100.8 (80.1–110.1) | 104.9 (80.4–88.2) |
| t-PA (ng/mL)                    | 4.1 (1.5–4.1)    | 2.8 (1.3–3.9)    | 3.9 (2.9–4.9)    | 4.2 (3.2–5.2)    | 5.3 (3.2–7.7)    |
| PAI (AU/mL)                     | 16.5 (11.4–21.6) | 17.1 (13.7–20.5) | 15.2 (11.3–19.2) | 14.1 (10.6–17.7) | 15.6 (14.4–18.9) |
| F1+2 (nmol/L)                   | 0.75 (0.58–0.92) | 0.60 (0.50–0.69) | 0.59 (0.48–0.69) | 0.65 (0.42–0.89) | 0.66 (0.56–0.76) |

Values are means and 95% CI.

*Friedman non-parametric repeated measures test.

Dunn’s multiple comparisons post-hoc test: a versus b: *P < 0.05; c versus e: *P < 0.01; d versus i: *P < 0.05; e versus f: *P < 0.05; g versus h: *P < 0.05; h versus j: *P < 0.05; i versus j: *P < 0.05; k versus l: *P < 0.05; l versus m: *P < 0.05; m versus n: *P < 0.05; n versus o: *P < 0.05; o versus p: *P < 0.05; p versus q: *P < 0.05; q versus r: *P < 0.05; r versus s: *P < 0.05.

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tation led to no changes in FVIII levels, nor were any significant changes observed in the control group. On the contrary, in the r-hFSH group we found a significant luteal increase in FVIII, compared to both baseline and hCG-day values. The cause and the significance of different trends in FVIII levels observed during u- and r-hFSH treatment are unclear. It is known that FVIII acts as an acute phase reactant in several clinical conditions associated with acute physical stress (Grant, 1990) and it has been demonstrated that factor FVIII is an independent cardiovascular risk factor (Cortellaro et al., 1992; Gorog et al., 1998).

The decrease in AT during ovarian stimulation has been consistently described in women treated with different hMG-based regimens (Kim et al., 1981; Aune et al., 1991; Bremme et al., 1994; Lox et al., 1995; Biron et al., 1997). We observed the same trend in the u-hFSH group, while no significant changes were observed in women treated with r-hFSH. It has been suggested that an AT decrease during ovarian stimulation could be an estrogenic effect or a result of plasma volume expansion induced by the estrogens increase (Kim et al., 1981). However, these hypotheses are not consistent with our findings, as AT was not significantly influenced by r-hFSH treatment, nor did it change significantly during the normal menstrual cycle.

Earlier studies investigating the effects of ovarian stimulation on PC levels reached discordant conclusions. A significant decrease in PC associated both with estradiol and progesterone increase was observed with a combination of CC and hMG (Bremme et al., 1994), while using hMG alone a not significant follicular decrease was noted (Lox et al., 1995). Other authors (Biron et al., 1997) using GnRH analogue and hMG reported a significant decrease before hCG administration and no changes during the luteal phase, although pre-treatment assessment was not performed. The results of a more recent study (Curvers et al., 2001) seem to suggest a different effect of r-hFSH compared to u-hFSH. Indeed, in women stimulated with r-hFSH, a slight increase in Protein C activity at down-regulation and during the other phases of treatment was reported. Our results do not confirm this finding. We observed no differences between the urinary and recombinant group. Protein C levels showed a not significant decrease both in women receiving u-hFSH and in women receiving r-hFSH. We would like to note that in the study by Curvers et al. (2001) the patients received OC for 3–6 weeks before ovarian stimulation. Therefore, the results of this study might have been significantly influenced by OC pre-treatment. Indeed, it has been demonstrated that both second and third generation OC increase PC levels (Tans et al., 2000).

In addition, with regard to the PS trend during ovarian stimulation, conflicting results were reported in earlier studies. Using CC and hMG a slight, but still significant, increase at estradiol peak, but no luteal changes, was found (Bremme et al., 1994). The effects of urinary gonadotrophin administration with preliminary GnRH analogue down-regulation were investigated in two studies (Falkon et al., 1995; Biron et al., 1997). In the first, PS levels showed no changes, but luteal levels were not evaluated (Falkon et al., 1995). In the second study, a decrease at the end of stimulation, but no change during the luteal phase, was noted (Biron et al., 1997). However, in this study assessment before ovarian suppression was not performed. In our study, in women treated with u-hFSH PS levels were unchanged during estradiol increase, whereas luteal levels of PS were significant lower in comparison with baseline. On the contrary, recombinant FSH treatment does not seem to significantly affect PS: PS levels at hyperstimulation and during the luteal phase were not different compared to baseline.

Although coagulation inhibitors AT and PS, but not PC, in our study show a significant decrease after u-hFSH treatment, they remained within the normal range. On the contrary, recombinant FSH does not appear to influence coagulation inhibitors.

It has been demonstrated that reduced sensitivity to activate protein C in the absence of factor V Leiden mutation increases the risk of venous thrombosis (de Visser et al., 1999). Acquired APC resistance has been well documented as developing during pregnancy (Clark et al., 1998; Kjellberg et al., 1999; Benedetto et al., 2002) and OC use (Osterud et al., 1994; Olivieri et al., 1995; Henkens et al., 1995; Rosing et al., 1999; Gris et al., 2001), suggesting that hormonal changes influence response to APC. In our study neither ovarian stimulation regimen appears to significantly modify the response to APC; there was no significant change in nAPC ratio values during gonadotrophin administration and during luteal support. These findings agree with preliminary studies in women treated with hMG or r-hFSH, showing that ovarian stimulation does not cause APC resistance (Biron et al., 1997; Wramsby et al., 2000). However, recently, other authors, using a different test to evaluate APC resistance, Rosing’s test (Rosing et al., 1999; Tans et al., 2003), observed a significant increase in APC sensitivity ratio during r-hFSH administration, and concluded that acquired APC resistance occurred in women undergoing ovarian stimulation (Curvers et al., 2001). These discordant results might be because the APC resistance test used in the latter study had a higher sensitivity to hormonal changes. Alternatively, the significant APC resistance changes associated with ovarian stimulation found might have been amplified by pre-treatment with OC. A third hypothesis is that these changes might simply be a late effect of OC administration and independent of ovarian stimulation. Unfortunately, the study by Curvers et al. (2001) did not comprise statistical comparisons between APC sensitivity ratio values during hyperstimulation and during luteal support with down-regulation values. Therefore, a clear demonstration that ovarian stimulation, without OC pre-treatment, induces APC resistance has not been provided. In the control group no significant changes in APC were observed, in accordance with the results of a previous study (Wramsby et al., 2000).

Tissue-type plasminogen activator decrease during estradiol increase has been observed in women receiving urinary gonadotrophins (Rice et al., 1993; Lox et al., 1995; Hanss et al., 1993). Our results from the u-hFSH group confirm this finding. Furthermore, no significant changes in t-PA levels were found in the luteal phase. Hence, luteal t-PA levels were significantly decreased compared to baseline values. The only study that explored both follicular and luteal t-PA changes
showed a slight, but not significant, decrease at the end of stimulation and no significant differences between luteal values and values before ovarian stimulation (Biron et al., 1997). However, as mentioned earlier, assessment before ovarian suppression was not performed, hence comparisons might be affected by GnRH analogue effect. In fact, in a previous study (Hanss et al., 1993), t-PA was significantly decreased after down-regulation. In the recombinant group a different trend was observed, because a non significant decrease was noted during estradiol increase, whereas a significant luteal increase was observed compared to hCG day values, but no significant differences compared to baseline values was found. This finding may be explained as being due to a lower impact of r-hFSH or as a compensatory response. However, in the control group we observed the same trend as in the u-hFSH group, although the changes were smaller. Therefore, the second hypothesis seems more likely and t-PA decrease may be explained as an estrogen-related effect.

No significant changes in the PAI levels in all study groups were observed. Conflicting results have been reported using urinary gonadotrophins. Some studies showed a decrease in PAI levels as estradiol levels increased (Hanss et al., 1993; Rice et al., 1993; Lox et al., 1995; Magnani et al., 1999). Other studies, evaluating PAI levels also in the luteal phase, found no significant changes, in any phase of treatment (Bremme et al., 1994; Biron et al., 1997), in accordance with our results.

Regarding F1+2, there are no studies available on FSH impact. Using hMG, Bremme et al. (1994) found no changes either at estradiol peak or at progesterone peak. On the contrary, Biron et al. (1997), using GnRH analogue and hMG, observed a highly significant increase during the luteal phase compared to baseline and hyperstimulation values. However, in this study no pre-treatment values were evaluated. In our study, no significant changes were observed in F1+2 levels in women treated with u-hFSH. Recombinant FSH seems to have a different impact compared to u-hFSH. In the recombinant group a significant decrease was noted at the end of ovarian stimulation. No significant differences compared to baseline levels were observed during the luteal phase, suggesting an increase as progesterone levels increase.

In conclusion, during the follicular phase we observed changes in PT, AT, t-PA in the u-hFSH group. However, these changes do not appear to significantly influence haemostasis, as F1+2 levels were unchanged, although the failure to see a rise in F1+2 does not exclude local thrombin generation.

On the contrary, in the women treated with r-hFSH, although we found no significant changes in clotting and fibrinolytic factors, we observed a significant increase in F1+2, suggesting a moderate decrease in clotting activity. No significant changes were observed in the control group.

In the luteal phase we found a significant increase in FBG levels in all groups. However, a significant difference between the two FSH preparations was observed. The use of r-hFSH resulted in a more pronounced increase in FBG levels. No other significant changes were noted in the u-hFSH group, compared to pre-ovulatory values. However, during the luteal phase, PT INR values decreased, as no significant differences were detectable at the midluteal phase in comparison with pre-treatment values and PS values decreased, as a significant difference was appreciable in comparison with baseline values. Antithrombin III and t-PA were not significantly changed at the midluteal phase compared to pre-ovulatory values, thus their levels remained significant lower than baseline values. In the r-hFSH group during the luteal phase PT INR significantly decreased, but did not differ from baseline levels. Other parameters such as FBG, FVIII, t-PA, rose significantly. However, only FVIII and FBG midluteal values resulted in being significantly higher than baseline levels.

Unlike u-hFSH, r-hFSH does not appear to significantly influence coagulation inhibitors and t-PA levels, hence its impact on haemostasis should be slighter. However, r-hFSH induces a significant increase in FVIII levels that have been shown to predict thromboembolic events. On the other hand, although the effects of u-hFSH and r-hFSH were different, all the changes in the haemostasis parameters were still within normal ranges. Furthermore, the total effect of both treatments was limited, because at end of treatment F1+2 had not increased significantly.

A previous report suggested that activation of coagulation can persist several weeks after ovarian stimulation (Belaen et al., 2001). In our study, if no pregnancy occurred, after 4 weeks no significant differences were found in comparison with pre-treatment values, suggesting that the effect of ovarian stimulation with both u-hFSH and r-hFSH was transitory and the 4-week washout period was sufficient to eliminate any residual drug influence.

In order to investigate the effect of pregnancy on haemostasis parameters, we separately pooled the women who became pregnant and the women who did not conceive, without distinguishing by treatment type. This was necessary because there were only a small number of pregnant women in each treatment group.

At the end of stimulation all of the haemostasis parameters showed the same trend in both groups. Hence we can suppose that the two groups were quite comparable, even if we cannot exclude intrinsic differences due to the different treatment used.

At the midluteal phase no differences were observed between the pregnant and non-pregnant women except for the trend of t-PA and PS. These parameters were unchanged in non pregnant women compared to baseline and follicular stimulation and no pre-treatment values were evaluated. In our study, if no pregnancy occurred, after 4 weeks no significant differences were found in comparison with pre-treatment values, suggesting that the effect of ovarian stimulation with both u-hFSH and r-hFSH was transitory and the 4-week washout period was sufficient to eliminate any residual drug influence.

The significance of PS and t-PA changes during implantation phase are not clear. A PS decrease throughout pregnancy has been well documented (Malm et al., 1988). We showed that this decrease is very precocious (Cerneca et al., 1997). Our data disagree with Curvers’ study that did not find luteal PS levels decrease in women who became pregnant (Curvers et al., 2001). However, in this study, pre-treatment with OC containing levonorgestrel was used. The same authors, in a previous study, showed that OC with levonorgestrel induces a modest increase in PS values (Tans et al., 2000). We hypothesise that in this study pre-treatment with OC might have masked luteal PS decrease in women who conceived. We are unable to
explain the causes of pregnancy-associated PS decrease. Protein S exists in two forms: as a free protein (~40%) and as part of a high molecular weight complex, the other component being C4b-binding protein (C4BP) (Dahlback, 1984). C4b-binding protein is a regulatory protein of the classical pathway of the complement system. PS decrease during pregnancy has been correlated with C4BP increase (Malm et al., 1988). No data are currently available as to the significance of C4BP changes during pregnancy, but it is known that complement regulation plays an important role in the early stage of the gestation (Fenichel et al., 1995; Taylor and Johnson, 1996; Duc-Goiran et al., 1999).

During embryo implantation in humans, trophoblast cells invade the endometrium, and plasminogen activators (PA) are involved in this tissue invasion process (Lockwood et al., 1999). Studies on surplus embryos from patients undergoing in vitro fertilization treatments showed that PA activity was present in secretions of blastocysts, but not of embryos at the 2–4-cell stage, suggesting that PA may play a significant role in early human development and embryo implantation (Khamsi et al., 1996).

In pregnant women at eighth week no significant differences were found compared to baseline values, except for FVIII and PS that were significantly higher and lower respectively in comparison with pre-treatment values. Elevation of FVIII throughout pregnancy has been well documented (Clark et al., 1998). There are no available studies on FVIII changes before and after conceiving. It is interesting to note that FBG and FVIII, although they are both acute phase reactants, show an opposite trend during early gestation stages. In fact, although fibrinogen increases during pregnancy, in our study we observed a return to pre-treatment values at 8 weeks of gestation. Hence, it is likely that early gestational FVIII elevation is multifactorial. PS decrease at 8 weeks is consistent with the findings of previous works (Cerneca et al., 1997). We failed to confirm the large increase in APC resistance in early pregnancy as described by other authors (Curvers et al., 2001). We observed a slight but significant increase in APC resistance after 8 weeks of pregnancy, but the nAPC ratio values did not differ from the pre-treatment values. However, we used, as previously mentioned, both different APC resistance tests and different ovarian stimulation protocols.

In summary, our results indicate that ovarian stimulation by recombinant FSH does not influence coagulation and fibrinolysis parameters significantly, as already reported for urinary gonadotrophins. Several differences between r-hFSH and u-hFSH impact on haemostasis can be appreciated, but they are probably of no clinical importance. The changes in haemostatic parameters induced by both treatments are slight and they are no longer detectable after 4 weeks.

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