Urinary gonadotrophins but not recombinant gonadotrophins reduce expression of VEGF\textsubscript{120} and its receptors flt-1 and flk-1 in the mouse uterus during the peri-implantation period

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BACKGROUND: Ovarian stimulation in humans might affect the perinatal outcome and be considered as a stress factor in the implantation process. In this study we compared the effects of recombinant and urinary gonadotrophins during the mouse peri-implantation period. METHODS: Adult female CD1 mice were treated as follows (a) urinary hFSH and urinary hCG, (b) recombinant hFSH and recombinant hLH and (c) saline. The effects of the gonadotrophins on the expression of vascular endothelial growth factor\textsubscript{120} (VEGF\textsubscript{120}) and its receptors and the corticotrophin releasing hormone (CRH) system during the peri-implantation period were studied. The specific effects of the different gonadotrophins on the onset of implantation were also studied. RESULTS: Urinary gonadotrophin treatment caused lower levels of VEGF\textsubscript{120}, flt-1 and flk-1 mRNA levels, reduced the size of the embryo implantation site, delayed implantation and prolonged the gestational period. Both urinary hFSH and urinary hCG contributed to the adverse effects. Levels of CRH and CRHR1 expression were not influenced. Recombinant gonadotrophin treatment did not alter any of the parameters studied. CONCLUSIONS: Our results show that the VEGF system of the mouse uterus during the peri-implantation period is adversely affected by urinary gonadotrophins but not by recombinant gonadotrophins. The CRH system was not affected by the two types of gonadotrophins.

Key words: CRH/peri-implantation period/recombinant gonadotrophins/urinary gonadotrophins/VEGF\textsubscript{120}

Introduction

Singleton pregnancies from assisted reproduction have a significantly worse perinatal outcome than non-assisted singleton pregnancies (Helmerhorst et al., 2004), although the prevalence of very low birth weight and very preterm birth is low. Factors such as subfertility (Gaudoin et al., 2003) and ovarian stimulation might be involved. Assisted reproduction is nearly always preceded by ovarian stimulation, in contrast to cryopreserved-thawed embryo transfer (ET). Since birth weight after cryopreserved-thawed ET is normal or even increased relative to their controls (Heijnsbroek et al., 1995), one might hypothesize that ovarian stimulation (with or without an in vitro fertilization or intra-cytoplasmic sperm insemination procedure) (Olivennes et al., 1993) is associated with low birth weight and might be considered as a stress factor in the implantation process.

Previously, we have shown that stimulation with urinary gonadotrophins lowered VEGF\textsubscript{120} expression in embryo implantation sites (Sibug et al., 2002). Blastocyst implantation is intimately associated with vascular permeability and angiogenesis. The vascular endothelial growth factor (VEGF) is a potent stimulator of these two processes (Keck et al., 1989). It has four isoforms in rodents, VEGF\textsubscript{115}, VEGF\textsubscript{120}, VEGF\textsubscript{164} and VEGF\textsubscript{188} (Breier et al., 1992; Sugihara et al., 1998), which bind to the two tyrosine-kinase receptors, flt-1 and KDR/flk-1. VEGF\textsubscript{120} and VEGF\textsubscript{164} are highly expressed in the uterus (our unpublished data). VEGF\textsubscript{120}, like VEGF\textsubscript{164} (Halder et al., 2000) showed very distinct transcript signals and also exhibited a spatio-temporal distribution (Sibug et al., 2002). Messenger RNA expression of VEGF, flt-1 and flk-1 correlates spatially and temporally with changes in angiogenesis and vascular
permeability at implantation sites (Jakeman et al., 1993; Chakraborty et al., 1995).

The corticotrophin releasing hormone (CRH) system is another factor hypothesized to play a role systemically and/or locally in blastocyst implantation. Studies in rodents have shown that CRH expression is 3.5-fold higher in the implantation sites than inter-implantation sites (Makrigiannakis et al., 1995) and injection with a CRH receptor 1 (CRHR1) antagonist resulted in a 50–70% reduction in the number of implantation sites (Makrigiannakis et al., 2001). In addition, administration of antibody against CRH inhibits the attachment of the blastocyst to the implantation site (Athanasakis et al., 1999). Activation of CRH receptors during the implantation phase has been demonstrated in the human endometrium (Karteris et al., 2004).

This study tests the hypothesis that treatment with gonadotrophins to induce controlled ovarian stimulation (COS) evokes a stress response, of which the effects persist after conception. Controlled ovarian stimulation, which has been solely performed with urinary gonadotrophins for decades, is currently also performed with recombinant gonadotrophins. Therefore, in this study, we have the following objectives: (a) to determine whether urinary and recombinant gonadotrophins exert similar effects on the expression of VEGF120 during the mouse peri-implantation period; (b) to determine whether the urinary and recombinant gonadotrophins influence the CRH system during the mouse peri-implantation period and (c) to determine the specific effects of the different gonadotrophins on the onset of the implantation process. The results show that urinary gonadotrophins, in contrast to recombinant gonadotrophins, negatively affect angiogenesis, endometrial vascular permeability and blastocyst development.

Materials and methods

Animals and experimental procedure

Adult female CD1 mice (8–10 weeks, Charles River, Germany) were housed five per cage upon arrival and allowed to acclimatize for 1 week. They had free access to food and water with a 12:12 dark:light cycle (lights on at 7.00 hr). All animal experiments were in accordance with the governmental guidelines for care and use of laboratory animals and were approved by the Animal Care Committee of the University of Leiden.

The following groups of mice were used: (a) saline-treated (control); (b) urinary gonadotrophin-treated; and (c) recombinant gonadotrophin-treated mice. The mice were first injected (ip) with either Metrodin (urinary human follicle-stimulating hormone, uhFSH; Serono, Coïssins, Switzerland) or recombinant human follicle-stimulating hormone (rhFSH, Gonal-F; Laboratoires Serono, Aubonne, Switzerland) at 12.00 hr and 47 hr later with Pregnyl (urinary human chorionic gonadotrophin, uhCG; Organon, Oss, The Netherlands), recombinant human luteinizing hormone (rhLH, Lhadi; Laboratoires Serono, Aubonne, Switzerland). Control animals received saline injections. A dose of 5 IU dissolved in 0.1 ml saline for each gonadotrophin was used. Five IU was used since our pilot study showed that this dose of urinary or recombinant gonadotrophins resulted in the same number of eggs ovulated in immature mice. On the first day of injection, the males were removed from their cages, which had not been cleaned for a few days, and the females were transferred into these cages to synchronize their estrus cycle (Whitten effect). In the afternoon of the last injection, males were introduced to the females (one male per two females) and successful mating was confirmed by the presence of vaginal plug on the following early morning. The day of vaginal plug detection was considered as embryonic development day 0 (ED0). Chicago blue dye (0.1 ml 1%; Sigma-Aldrich, Steinheim, Germany) was injected in the jugular vein under isoflurane anesthesia to visualize the implantation sites on embryonic day 5 (ED5) (Psychoyos, 1973). After allowing the dye to circulate for 10 min, the whole uteri with the embryos were dissected out and frozen in dry-ice ethanol-cooled isopentane (AnaLaR, England). The frozen materials were stored at −80°C until processing and 1–2 embryo implantation sites/pregnant mouse were used.

Parallel groups of control, urinary and recombinant gonadotrophin-treated pregnant mice were allowed to give birth. Litter size, birth weight and length of gestation were noted. In addition, resorptions (%) were determined from the difference between implantation sites and litter size. Another set of mice, treated either with urinary gonadotrophins or saline, were used for blood sampling to determine the 17β-estradiol levels from the first day of injection until ED4. For measurement of progesterone levels, trunk blood was collected on ED5 in all the groups.

Determination of the specific effects of the different gonadotrophins

To determine the specific effects of the different gonadotrophins on the onset of the implantation process, the following groups of mice were used on ED4: (a) saline + saline; (b) uhFSH + uhCG; (c) rhFSH and rhLH; (d) uhFSH + rhLH; (e) rhFSH + uhCG; (f) rhFSH + saline; and (g) rhFSH + rhCG (recombinant chorionic gonadotropin alfa; Ovidrel/Ovidrelle Laboratoires Serono, Aubonne, Switzerland). The recombinant gonadotrophins were injected instead of saline in groups (d) and (e) because treatment with both recombinant gonadotrophins did not delay implantation. Group (f) served as an additional control to validate our assumption that the absence of effects on the implantation process is not due to an interaction between the two recombinant gonadotrophins. The rhLH group (g) was included to determine whether rhCG exerts the same effect as rhLH. The implantation process was assessed based on the number of stained implantation sites and intensity of dye staining.

Riboprobe

The VEGF probe was transcribed from a mouse 451 bp cDNA fragment encoding for the entire sequence of the mouse VEGF120 (courtesy of G. Breier, FRG); the flk-1 probe contained a mouse 800 bp cDNA fragment spanning the trans-membrane domain and part of the extra-cellular ligand-binding domain (courtesy of P. Carmeliet, Belgium). The plasmid for the flt-1 probe was cloned in our laboratory and contained a mouse cDNA fragment of 424 bp covering a part of the extracellular domain inserted in a pGEM-T Easy vector (Promega, USA). The CRH probe contained the rat CRH sequence (courtesy of G. Bent, FRG) and the CRHR1 probe contained 700 bp of the rat (courtesy of J. Preil, FRG).

In vitro transcription for the antisense and sense strands was done using a standard protocol (Boehringer, Mannheim, Germany) and the following enzymes were used: for VEGF120, T3 and T7 RNA polymerase, respectively; for flk-1, SP6 and T7 RNA polymerase, respectively; for flt-1, T7 and SP6 RNA polymerase, respectively; for CRH, SP6 and T7 RNA polymerase, respectively; and for CRHR1, T3 and T7 RNA polymerase, respectively.
Figure 1. Effects of urinary and recombinant gonadotrophins on vascular permeability and expression of the VEGF system in the embryonic implantation site on embryonic day 5. (A) Chicago blue dye penetration along the length of the uterus in the different groups. Arrows indicate implantation sites without dye staining. Lower panels: autoradiograms showing the expression of VEGF$_{120}$, flt-1 and flk-1 in saline (control; B, E, H), urinary (C, F, I) and recombinant gonadotrophin (D, G, J) treated implantation sites. Arrows indicate the site where the blastocyst implants. Note that the uterine epithelium is still relatively intact in the urinary gonadotrophin-treated uterus while it is already degraded in the control and recombinant uteri. Bars: (A) 2.5 mm, (B–J) 0.6 mm; exposure period: (B–D) = 17 days, (E–G) = 50 days, (H–J) = 28 days. Pdz, primary decidual zone; sdz, secondary decidual zone.
**In situ hybridization**

Determination of VEGF₁₂₀, flt-1 and flk-1 expression by *in situ* hybridization is described in detail elsewhere (Sibug et al., 2002). Briefly, pre-hybridized sections of the implantation sites were hybridized with either the antisense or sense probe of the different probes overnight, washed, dehydrated and exposed to Biomax film (Kodak) for 3–12 days. After the development of the films the slides were dipped in KODAK NTB3 photo emulsion and developed after 4–9 weeks.

**Determination of plasma estrogen and progesterone levels**

Total plasma 17β-estradiol and progesterone levels were measured using the radioimmunoassay (RIA) Coat A-Count estradiol and progesterone assays (Diagnostic Products Corporation, Los Angeles, CA). For estrogen, the detection limit of the RIA is 10 pg/ml and the intra- and interassay coefficient variabilities 5.3% and 6.4%, respectively. For progesterone, the detection limit of the RIA is 0.02 pg/ml and the intra- and interassay coefficient variabilities are 4.7% and 6.0%, respectively.

**Densitometric and statistical analyses**

The film autoradiograms were scanned and quantified using the Scion Image for Windows analysis system (Scion Corporation). Comparable sections for each embryo implantation site were assessed and compared. The mRNA levels were measured using 3–5 sections (every 40th micrometre) and levels were expressed as specific optical density (the sum total of gray values minus the background present in the section analyzed). The surface area was measured using the same sections used in quantifying the expression levels of VEGF₁₂₀.

The optical density values of 3–5 sections for each embryo were pooled and were analyzed for the different treatment groups. One way analysis of variance (ANOVA) and post hoc comparisons were performed. The expression of VEGF₁₂₀, CRH and their receptors, surface area of the embryo implantation site and length of gestation of the different groups were compared using the Tukey’s test and significance was accepted at *P* ≤ 0.05. Correlation analysis was done with Pearson correlation test and significance was accepted at *P* ≤ 0.05.

**Results**

The fertilization rate in the three groups was not significantly different: 42% in the urinary gonadotrophin group, 43% in the recombinant group and 48.5% in the control.

**Implantation sites**

The embryo implantation sites were distinct and larger in the control and recombinant groups in comparison with the urinary group (Figure 1A). Measurements of the surface area of the implantation sites showed that the urinary gonadotrophin-treated implantation sites were smaller in comparison with the recombinant gonadotrophin-treated and control implantation sites (2.0 ± 0.4 mm², 3.5 ± 0.1 mm² and 3.7 ± 0.2 mm², respectively; Figure 2A). The intensity of dye staining of the implantation sites (index of vascular permeability) of the control and recombinant groups was darker than that of the urinary group. In the latter group, dye staining varied from very light to moderate. There were implantation sites, indicated as knob-like swellings along the length of the uterus, which were not stained (Figure 1A). The uterine epithelium of the implantation site was relatively intact in the urinary gonadotrophin group while it is already degraded in the recombinant gonadotrophin and control groups (Figure 1B–J). This showed unequivocally that urinary gonadotrophins caused a delay in the implantation of the blastocyst.

**Expression of VEGF 120, flt-1, flk-1, CRH and CRHR1**

There was a significant difference in the VEGF₁₂₀ expression in the embryo implantation sites on ED5 (Figures 1B–D and 2B). The urinary gonadotrophin-treated group showed significantly lower VEGF₁₂₀ mRNA levels than the recombinant gonadotrophin-treated and control groups (1.4 ± 0.4, 4.8 ± 0.7 and 4.9 ± 0.7, respectively). However, the topographical distribution of the expression pattern was the same...
for all the groups. Signal labelling was highest in the primary decidual area, moderate to high in the luminal epithelial lining and endometrial glands and light to moderate in the endothelial cells. VEGF120 expression was also present in the implantation sites without dye staining. In general, the level of expression positively correlated with the intensity of dye staining.

The expression of flt-1 (Figures 1E–G and 2C) and flk-1 (Figures 1H–J and 2D) receptors was distinct in the primary and secondary decidual zone. However, the expression of flt-1 in the secondary decidual area is less than that of flk-1. The intensity of the labelling was high in the endothelial cells of the endometrium and myometrium while it was light to moderate in the uterine lining and endometrial glands. The urinary gonadotrophin group showed a lower expression of flt-1 and flk-1 in comparison with recombinant gonadotrophin and control groups (Figure 2C and D; for flt-1: 1.8 ± 0.4, 7.6 ± 0.7 and 5.5 ± 1.6, respectively; for flk-1: 7.8 ± 1.0, 16.5 ± 2.0 and 17.5 ± 2.0, respectively). Overall, the flk-1 mRNA levels were 2–3 fold greater than those of flt-1.

Treatment with urinary and recombinant gonadotrophins did not influence CRH and CRHR1 expression. All the groups showed homogeneous labelling distribution in the luminal epithelia, glandular epithelia, stroma and myometrium and comparable levels of expression (CRH: 2.7 ± 0.3, 2.5 ± 0.4, 2.1 ± 0.2; CRHR1: 1 ± 0.2, 1.5 ± 0.4, 1.6 ± 0.4; for control, urinary and recombinant, respectively). The blastocyst exhibited light but distinct CRH and CRHR1 signals. No specific signal was observed using the sense probes in all the transcripts studied.

**Effects on litter size, birth weight and gestational length (Table I)**

The gestation period in the urinary gonadotrophin group was significantly longer by one day in comparison with the control and recombinant gonadotrophin groups and was independent of litter size. Litter size, on the other hand, did not significantly differ among the three groups. However, its distribution range in the urinary group was very variable (data not shown). Birth weight did not also significantly differ but there was a positive correlation between birth weight and gestation period ($r = 0.38; P \leq 0.05$) regardless of treatment.

**Effects of urinary gonadotrophins on 17β-estradiol and progesterone levels**

The treatment with urinary gonadotrophins did not show a clear effect on the 17β-estradiol levels during the estrus cycle and peri-implantation period (data not shown). The uhFSH and uhCG treated-mice appeared to have a slightly higher estrogen levels (≥10 pg/ml) than the control mice. However, the difference between the two groups could not be statistically tested since the estradiol levels of the control animals were beyond the detection limit (≤10 pg/ml). Regarding progesterone, significantly higher levels were only observed in the urinary gonadotrophin group (24.9 ± 1.9 ng/ml) in comparison with the control (18.2 ± 1.1 ng/ml) but not in the recombinant group (19.3 ± 1.8 ng/ml).

**Determination of the specific effects of the different gonadotrophins on ED4 (Table II)**

All mice treated with saline + saline exhibited the highest dye staining intensity and number of stained implantation sites while the mice treated with uhFSH + uhCG did not or barely exhibited stained implantation sites. The rhFSH + rhCG and uhFSH + rhLH treated mice showed an almost similar number of implantation sites and dye staining intensity. The overall results showed that treatment with uhFSH + uhCG exerted the most negative effects while the presence of either only one of them caused lesser effects. RHFSHA in combination with rhLH showed more implantation

<p>| Table I. Effects of urinary human follicle-stimulating hormone and urinary human chorionic gonadotrophin on litter size, birth weight and gestational length |
|---------------------------------|-----------------|------------------|--------------------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Litter size ± SEM</th>
<th>Birth weight, g ± SEM</th>
<th>Gestational length, days ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) control</td>
<td>12.9 ± 0.5 (n = 10)</td>
<td>1.64 ± 0.03 (n = 129)</td>
<td>19.0 ± 0.0</td>
</tr>
<tr>
<td>(b) urinary gonadotrophins</td>
<td>11.6 ± 2.0 (n = 7)</td>
<td>1.61 ± 0.08 (n = 81)</td>
<td>20.2 ± 0.5*</td>
</tr>
<tr>
<td>(c) recombinant gonadotrophins</td>
<td>12.6 ± 0.9 (n = 10)</td>
<td>1.61 ± 0.05 (n = 126)</td>
<td>18.9 ± 0.1</td>
</tr>
</tbody>
</table>
* Significantly different against control and recombinant gonadotrophins ($P < 0.05$).

<p>| Table II. Specific effects of the different gonadotrophins on the number of implantation sites and vascular permeability on embryonic day 4 |
|-----------------------------------------------|----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of implantation sites</th>
<th>Intensity of staining</th>
<th>Low</th>
<th>Moderate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline + saline (n = 6)</td>
<td>–</td>
<td>–</td>
<td>100%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>uhFSH + uhCG (n = 5)</td>
<td>60%</td>
<td>40%</td>
<td>–</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>rhFSH + rhLH (n = 5)</td>
<td>–</td>
<td>20%</td>
<td>80%</td>
<td>–</td>
<td>20%</td>
</tr>
<tr>
<td>uhFSH + rhLH (n = 9)</td>
<td>11%</td>
<td>33%</td>
<td>56%</td>
<td>25%</td>
<td>12.5%</td>
</tr>
<tr>
<td>rhFSH + uhCG (n = 7)</td>
<td>14%</td>
<td>57%</td>
<td>29%</td>
<td>57%</td>
<td>43%</td>
</tr>
<tr>
<td>rhFSH + saline (n = 6)</td>
<td>–</td>
<td>–</td>
<td>100%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>rhFSH + rhCG (n = 6)</td>
<td>–</td>
<td>83%</td>
<td>17%</td>
<td>50%</td>
<td>50%</td>
</tr>
</tbody>
</table>

$n$, number of mice; –, none.
sites and higher intensity of dye staining than in combination with rhCG. RfFSH in combination with saline exhibited the same effects as the saline control.

Discussion

The present investigation shows the differential effects of urinary and recombinant gonadotrophins during the mouse peri-implantation period. Controlled ovarian stimulation of sexually mature mice with urinary but not recombinant gonadotrophins adversely affected the peri-implantation processes. Treatment with urinary gonadotrophins led to a delay in embryo implantation, smaller size of the implantation site and prolonged gestational period. These were accompanied by reduced expression of VEGF, flt-1 and flk-1 and increased levels of progesterone.

UhFSH and uhCG exerted independent effects and both contributed to the adverse effects. The uhFSH preparations that we used still contained some uhLH present in a ratio of 60:1 activity, respectively. RfFSH and rhLH mimic the biological activity of their respective endogenous gonadotrophins because no difference was observed among the rhFSH+rhLH, rhFSH+saline and control groups. However, the effects of rhLH and rhCG were not exactly similar. Chorionic gonadotrophin is normally used to mimic the effects of pituitary LH because they have a high degree of homology and bind to the same membrane receptors. Their molecular composition differs however (Wehmann and Nisula, 1980; Stokman et al., 1993), indicating that their biological actions may not be identical (Niswender et al., 1985). Our data agree with reports showing that treatment of New Zealand rabbits with rhLH produces better quality embryos with a higher implantation rate than rhCG (Peinado et al., 1995; Romeu et al., 1995).

Mechanism

The delayed blastocyst implantation suggests a desynchronization of the pre-implantation embryo–maternal endometrium interaction and is a possible mechanism through which the urinary gonadotrophins operate on the implantation process. Signals coming from the developing oocyte/embryo, uterine receptivity factors (Ertzeid et al., 1995; Romeu et al., 1995), indicating that their biological actions may not be identical (Niswender et al., 1985). Our data agree with reports showing that treatment of New Zealand rabbits with rhLH produces better quality embryos with a higher implantation rate than rhCG (Peinado et al., 1995; Romeu et al., 1995).

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prepubertal mice. The fact that the number of resorption sites in urinary gonadotrophin-treated mice is significantly higher in comparison with the other two groups might explain why, at least in the urinary gonadotrophin group, the increase in oocyte number is not reflected in the litter size.

Moreover, the administration of the gonadotrophins did not take place on a set day of the estrus cycle period. At the beginning, we attempted to use mice on a specific day of the cycle. But unlike rats, mice have very irregular cycles and it was not possible to get a sufficient number to perform an experiment. To circumvent this problem, mice were synchronized by using the Whitten’s effect and used blindly. We assumed that females which were fertilized overnight with the males were in the same stage of the cycle because it is known that females show lordosis behaviour and are receptive only during the day of proestrus.

Furthermore, it is possible that factors other than the gonadotrophins themselves could also have contributed to the observed effects. For example, degradation products have been detected in uhCG (Wehmann and Nisula, 1980). Therefore, degradation products and/or other (non-)hormonal contaminating proteins may have interfered with biological actions of the active molecules.

Although our findings described here need to be independently confirmed, strain specificity for our observations seems unlikely as impaired implantation and prolonged gestation effects of urinary gonadotrophins have been observed in other strains of mice (Ertzeid and Storeng, 1992; Ertzeid et al., 1993; Van der Auwera and D’Hooghe, 2001).

In conclusion, our study shows that urinary but not recombinant gonadotrophins exert adverse effects on the implantation process and expression of VEGF 120, flt-1 and flk-1 in mice. CRH and CRH-R1 expressions were not altered, suggesting that the CRH system is not involved in mediating the adverse effects of urinary gonadotrophins on the implantation process. If our data are confirmed, our findings might have implications for transgenesis, since urinary gonadotrophins have been used in other strains of mice (Ertzeid and Storeng, 1992; Ertzeid et al., 1993; Van der Auwera and D’Hooghe, 2001).

References


