Effects of recombinant human follicle-stimulating hormone on embryo development in mice


Research Centre for Reproductive Health, Department of Obstetrics and Gynecology, The University of Adelaide, The Queen Elizabeth Hospital, Woodville, South Australia, Australia

Submitted 26 August 2004; accepted in final form 6 December 2004

Edwards, L. J., K. L. Kind, D. T. Armstrong, and J. G. Thompson. Effects of recombinant human follicle-stimulating hormone on embryo development in mice. Am J Physiol Endocrinol Metab 288: E845–E851, 2005. First published December 14, 2004; doi:10.1152/ajpendo.00398.2004.—We have developed a protocol using recombinant human follicle-stimulating hormone (rhFSH) to induce ovarian stimulation in the mouse to investigate its impact on preimplantation embryo development. Embryos were collected from adult female C57Bl/6 × CBA F1 mice treated with rhFSH (0, 2.5, 5.0, 10.0, or 20.0 IU) or 5 IU equine chorionic gonadotropin (eCG). Embryos were also recovered from nontreated control mice. Embryos were cultured in vitro for 88 h, and the stage of development was morphologically assessed. The allocation of cells to the inner cell mass or trophectoderm of blastocysts was determined by differential nuclear staining. The expression of insulin-like growth factor 2 (IGF-II), the IGF-II receptor, and vascular endothelial growth factor (VEGF) in blastocysts was measured by real-time RT-PCR. Blastocyst development was reduced in the 10 (72.3 ± 5.1%) and 20 (77.3 ± 5.6%) IU rhFSH groups compared with control embryos (96.7 ± 1.0%). The number of inner cell mass cells was reduced (P < 0.001) in the 5, 10, and 20 IU rhFSH groups and the eCG group compared with control embryos. We did not find any effect of rhFSH treatment on IGF-II, IGF-II receptor, or VEGF expression in blastocysts compared with the control group. eCG treatment, however, significantly increased the expression of IGF-II in blastocysts. These results indicate that ovarian stimulation with rhFSH impairs the in vitro development of preimplantation mouse embryos, and these results may have potential implications for clinical ovarian stimulation during infertility treatment and subsequent embryo quality.

blastocyst; blastomere allocation; mouse; ovarian stimulation; recombinant gonadotropin

GONADOTROPIN TREATMENT is routinely used in both animals and humans to induce multiple follicle stimulation thus increasing the number of oocytes or embryos obtained from a single cycle that can potentially be used for techniques such as in vitro fertilization (IVF). The use of exogenous gonadotropins to induce ovarian stimulation, however, has been reported to affect embryo development at a number of different stages. This is particularly well characterized in the mouse, where ovarian stimulation is reported to both decrease (11) and delay (32) in vitro development of one- or two-cell embryos to blastocysts. Ovarian stimulation has also been reported to delay the in vivo development of embryos (11, 32) compared with embryos recovered from naturally cycling control mice, and this has been attributed to an adverse effect of stimulation on the oocytes or embryos as well as the reproductive tract. Murine blastocysts developed in vivo after ovarian stimulation have reduced cell numbers (9); however, it is not known if this reduction in cell number was confined to either the inner cell mass (ICM) and/or the trophectoderm (TE). It has also been reported that ovarian stimulation reduces the number of microvilli on the blastocyst surface compared with blastocysts from nontreated control mice (4). Ovarian stimulation also results in a delay in implantation, a decrease in the expression of the angiogenic factor vascular endothelial growth factor (VEGF) at implantation sites (29), and an increased postimplantation mortality in mice (2, 10–12, 29, 32) and rats (21, 22). Furthermore, ovarian stimulation was associated with a reduction in fetal growth and a prolonged gestation period in mice (10–12, 29, 32). The majority of these studies have used a combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to induce ovarian stimulation. It has previously been reported that eCG can adversely affect embryo development because of its long biological half-life (28, 34) and high luteinizing hormone-like activity (1, 25). Since the introduction of recombinant follicle-stimulating hormone (FSH) in the mid-1990s, many human fertility clinics now use recombinant human (rh) FSH in conjunction with hCG to treat infertility; however, the use of rhFSH in animal models has been limited (23, 31). It is important to assess the impact of rhFSH on preimplantation embryo development to determine if ovarian stimulation affects subsequent embryo development and to determine if gonadotropin-specific effects occur.

Therefore, in the current study, we have designed a model of rhFSH-induced ovarian stimulation in the mouse and investigated the impact of this stimulation on preimplantation embryo development. The parameters used to assess preimplantation embryo development were in vitro development to blastocyst, blastocyst cell numbers, and cell allocation between the ICM and TE of the blastocyst. Furthermore, because growth factors and their receptors have been demonstrated to play an important role in cell proliferation and differentiation in preimplantation embryos (16, 26, 27), we have also determined the impact of ovarian stimulation on the expression of insulin-like growth factor 2 (IGF-II), the IGF-II receptor, and VEGF in blastocysts.

MATERIALS AND METHODS

Animals

All procedures were approved by The University of Adelaide Animal Ethics Committee and The North Western Adelaide Health Service Animal Ethics Committee. Adult female F1 hybrid CBA × C57Bl/6 F1 mice (8–10 wk) and male C57Bl/6 F1 mice (3–15 mo)

http://www.ajpendo.org | 0193-1849/05 $8.00 Copyright © 2005 the American Physiological Society E845

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were obtained from the Central Animal House at the University of Adelaide. The animals were housed under a 14:10-h light-dark regimen, fed a standard pellet diet ad libitum, and had free access to water.

**Gonadotropin Treatment**

The gonadotropins used in this study were diluted in 0.9% saline, and an injection volume of 0.1 ml was used. The rhFSH treatments were given as two subcutaneous injections 24 h apart, since the activity of rhFSH has a relatively short duration.

**rhFSH treatments.** Female mice were stimulated with a total of 2.5, 5.0, 10.0, or 20.0 IU rhFSH (Gonal-F; Serono). Ovulation was induced after an intraperitoneal injection of 5 IU hCG (Pregnyl, Organon, Holland) 24 h after the second rhFSH injection, and mice were immediately caged overnight with fertile male mice.

**eCG treatment.** Female mice were stimulated with a subcutaneous injection of 5 IU eCG (Folligon serum gonadotropin; Intervet Australia, Victoria, Australia) following 48 h later with an intraperitoneal injection of 5 IU hCG. Immediately after the hCG injection, mice were placed with fertile male mice.

An additional treatment group consisted of female mice injected with 5 IU hCG only before being caged overnight with fertile male mice.

A control group of mice consisted of naturally cycling females that were caged with fertile males at the same time as the experimental groups received their hCG injections.

The presence of a copulation plug the morning after females were caged with males indicated a successful mating, and only females with a copulation plug were included in the study.

**Embryo Recovery and In Vitro Culture**

Female mice were killed by cervical dislocation 22–24 h post-hCG, and cumulus enclosed zygotes were flushed from the oviducts and collected in HEPES-buffered handling media. The embryos were denuded by a short incubation (i.e., <1 min) in ovine hyaluronidase (50 U/ml; Sigma Chemical, St. Louis, MO) in handling media and washed three times in the handling media before being placed in a culture drop. The culture media consisted of potassium simplex optimized media (KSOM) without amino acids (3) but containing L-alanyl-L-glutamine (1.0 mM) (GlutaMax; Invitrogen, Carlsbad, CA) instead of glutamine. Groups of 10–15 morphologically normal pre-ovulatory oocytes (exhibiting a germinal vesicle or metaphase I) were classified.

Presumptive zygotes with fragmentation and unfertilized or immature oocytes (i.e., 2-cell and 4-cell stages) were discarded. Embryos were washed three times in KSOM, trypsinized, and transferred to KSOM containing 10 ng/ml of basic FGF (Sigma) and 200 units Superscript II (Invitrogen). Before addition of the RT enzyme, reactions were incubated at 25°C for 10 min and then 42°C for 50 min. The RT was added, and reactions were incubated at 42°C for 60 min. The reaction was inactivated by incubation at 70°C for 15 min. Embryo cDNAs were stored at −80°C for subsequent RNA extraction.

**Differential Nuclear Staining**

The number of cells within the ICM or TE of expanded and hatching blastocysts was assessed using methods based on HANDSIDE and Hunter (13). Blastocysts were incubated in acidic Tyrode solution (Sigma) at room temperature for up to 3 min to remove the zona pellucida, before being washed in 4 mg/ml polyvinyl alcohol in PBS (PBS-PVA). This was followed by an incubation in ice-cold 10 mM 2,4,6-trinitrobenzenesulfonic acid (Sigma) in PBS-PVA for 10 min, followed by a wash in PBS-PVA. Blastocysts were subsequently incubated in 0.1 mg/ml anti-dinitrophenyl-BSA (Sigma) in protein-free KSOM for 10 min at 37°C. After a PBS-PVA wash, complement mediated lysis was induced by incubating the blastocysts in guinea pig complement (Sigma) diluted 1:5 in PBS with 5 μg/ml propidium iodide (Sigma) for 20 min at 37°C. Blastocysts were then briefly washed in PBS-PVA before being placed in 20 mg bisbenzimide/ml (Hoechst 33342; Sigma) in ethanol overnight at 4°C. The next morning, embryos were mounted in 50% glycerol on a glass slide and overlayed with a coverslip. The differential color of the nuclei was examined under ultraviolet light using an Olympus AH-3 microscope (Olympus Optical) whereby the ICM (stained with Hoechst) appeared blue and the dual-stained TE cells appeared red/pink. Numbers of ICM and TE cells were obtained for each embryo, and ratios of ICM to total number of cells was calculated for individual embryos.

**RNA Extraction**

Total RNA was extracted from blastocysts using Tri Reagent (Sigma) according to the manufacturer’s instructions. The tubes containing Tri Reagent and blastocysts were thawed at room temperature and vortexed briefly before standing for a further 5 min at room temperature. Chloroform (100 μl; Sigma) was added, and samples were vigorously shaken and incubated at room temperature for 15 min. Samples were centrifuged at 13,000 rpm for 15 min at 4°C in an Eppendorf centrifuge 5145C (Eppendorf, Hamburg, Germany), and the aqueous phase was removed. Isopropanol (250 μl; Sigma) and glycogen (20 μg; Invitrogen) were added, and RNA was precipitated by overnight incubation at −80°C, followed by centrifugation at 13,000 rpm for 30 min at 4°C. RNA pellets were washed with 70% ethanol, air-dried, and redissolved in 10 μl sterile water. DNA contamination was excluded by treatment with 2 units DNAse (2 U/μl; DNA-free; Ambion, Austin, TX) for 30 min at 37°C. DNAse was inactivated by addition of 5 μl DNase Inactivation Reagent (DNA-free; Ambion), incubation at room temperature for 2 min, and centrifugation at 13,000 rpm for 1 min. The RNA solution was removed from the pellet and DNAse Inactivation Reagent (DNA-free; Ambion) and used immediately for reverse transcription.

Placental tissue from day 18 pregnant CBA × C57 Bl6 mice was collected and snap-frozen. Total RNA was extracted using Tri Reagent, as described above. Final pellets were redissolved in 40 μl sterile water and treated with DNAse as above. The concentration of RNA extracted from placental tissue was determined by spectrophotometric analysis at 260 nm.

**Reverse Transcription**

Total RNA was reverse transcribed to cDNA using Superscript II RNase H−RT (Invitrogen). For embryos, total RNA from each pool (20–55 blastocysts) was reverse transcribed. For placenta, ten 2-μg aliquots of RNA were reverse transcribed. RNA was initially incubated with random primers (250 ng) at 70°C for 10 min. Reverse transcription was then performed in a final volume of 25 μl containing First-Strand Buffer (50 mM Tris·HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl2), 250 mM of each dNTP (Invitrogen), 0.01 M diithiothreitol, and 200 units Superscript II (Invitrogen). Before addition of the RT enzyme, reactions were incubated at 25°C for 10 min and then 42°C for 2 min. The RT was added, and reactions were incubated for 50 min at 42°C. The reaction was inactivated by incubation at 70°C for 15 min. Embryo cDNAs were stored at −20°C until use. Placental cDNAs were pooled, diluted to a concentration of cDNA equivalent to that generated from 25 ng starting RNA/μl, and stored in aliquots at −20°C.

**Real-time PCR**

Real-time PCR was performed in an Applied Biosystems 5700 GeneAmp Sequence Detection System (Applied Biosystems, Foster City, CA). Oligonucleotide primers were designed using Primer Express (Applied Biosystems) and were synthesized by GeneWorks (Adelaide, Australia). Primer sequence details are described in Table 1. PCR was performed in 20-μl volumes containing 1× SYBR Green Master Mix (Applied Biosystems) and 500 nM forward and reverse primers in a 50°C pre-denaturation step, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed to confirm the specificity of the amplicons.
VEGF, vascular endothelial growth factor.

with 10.0 IU rhFSH, 20 IU rhFSH, and 5 IU eCG compared with the control group (Table 2).

**In Vitro Embryo Development**

The in vitro development of morphologically normal presumptive zygotes to blastocysts was not affected by ovarian stimulation with 2.5 or 5.0 IU rhFSH or by ovulation induction by hCG compared with the development of embryos in the control group (Table 2). There was a significant reduction in the proportion of embryos to reach the blastocyst stage in embryos from mice treated with 10.0 or 20.0 IU rhFSH compared with embryos recovered from nontreated control mice (Table 2).

**Differential Nuclear Staining**

There was no effect of hCG and 2.5 or 10 IU rhFSH on total blastocyst cell number (Fig. 1A). The total cell number in blastocysts derived from 5.0 and 20.0 IU rhFSH- and eCG-treated mice was significantly reduced compared with blastocysts derived from nontreated control mice (Fig. 1A). The number of TE cells within blastocysts was also significantly reduced in the 20.0 IU rhFSH group (Fig. 1B); however, TE cell number was not altered in the 2.5 IU rhFSH, 5.0 IU rhFSH, 10.0 IU rhFSH eCG and hCG groups (Fig. 1B). There was a significant reduction in the number of ICM cells in the 5.0, 10.0, and 20.0 IU rhFSH groups and the eCG group compared with the untreated control group (Fig. 1C). There was, however, no significant effect of 2.5 IU rhFSH or hCG treatment on the number of ICM cells (Fig. 1C). There was a significant decrease in the ICM-to-total cell ratio in the 10.0 IU rhFSH and

### Table 1. Primer sequences used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank Accession No.</th>
<th>Primer Sequence</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II</td>
<td>U-71085</td>
<td>Forward: AAGAGTTCAGAGAGCACAAGGG</td>
<td>103</td>
</tr>
<tr>
<td>IGF-II receptor</td>
<td>NM_010515</td>
<td>Reverse: CAGCTGATGTTGCGTGGATCAT</td>
<td>88</td>
</tr>
<tr>
<td>VEGF</td>
<td>NM_0095051</td>
<td>Forward: CCAGCTAGAGAGCAACATCAG</td>
<td>75</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>AF176811</td>
<td>Reverse: TCATGATGTTGCGTGGATCAT</td>
<td>91</td>
</tr>
</tbody>
</table>

**Statistics**

All statistical analyses were carried out using SPSS for Windows (SPSS). Data are presented as means ± SE. A minimum of five replicates for each experiment was used. All percentage data were subjected to arcsine transformation. Gene expression data in the placental standard, was calculated from the standard curve. Results were normalized against those obtained for 18S rRNA.

**RESULTS**

**Gonadotropin Treatment and Embryo Recovery**

There was a significantly greater total number of embryos recovered from mice treated with 5.0, 10.0, or 20 IU rhFSH compared with the nontreated control group and the hCG only treated group (Table 2). The number of embryos recovered from the 5 IU eCG-treated mice was also significantly greater than the control and hCG only groups (Table 2). Furthermore, the number of embryos recovered from the 5 IU eCG-treated mice was the same as the 2.5 and 5.0 IU rhFSH groups but was significantly less than the 10.0 and 20.0 IU rhFSH treatment groups (Table 2). Of the total embryos recovered, those with fragmentation were classified as abnormal. There was a greater proportion of abnormal embryos collected from mice treated

### Table 2. Effect of gonadotropin treatment on embryo recovery and development

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No. of Embryos/Mouse</th>
<th>Abnormal Embryos, %</th>
<th>Development to Blastocyst, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>9.3 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>96.7 ± 1.0</td>
</tr>
<tr>
<td>hCG (5.0 IU)</td>
<td>9.5 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>89.2 ± 3.4</td>
</tr>
<tr>
<td>rhFSH (2.5 IU)</td>
<td>11.3 ± 1.1</td>
<td>1.4 ± 0.9</td>
<td>74.1 ± 2.8</td>
</tr>
<tr>
<td>rhFSH (5.0 IU)</td>
<td>31.3 ± 1.1</td>
<td>9.2 ± 3.1</td>
<td>84.9 ± 5.1</td>
</tr>
<tr>
<td>rhFSH (10.0 IU)</td>
<td>44.5 ± 4.3</td>
<td>13.2 ± 3.7</td>
<td>73.1 ± 5.1</td>
</tr>
<tr>
<td>rhFSH (20.0 IU)</td>
<td>41.1 ± 3.1</td>
<td>11.6 ± 3.6</td>
<td>77.3 ± 5.6</td>
</tr>
<tr>
<td>eCG (5.0 IU)</td>
<td>22.2 ± 1.6</td>
<td>19.8 ± 2.1</td>
<td>83.5 ± 5.1</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. hCG, human chorionic gonadotropin; rhFSH, recombinant human follicle-stimulating hormone; eCG, equine chorionic gonadotropin. A minimum of 16 mice were used for each treatment group. Different superscripts denote a significant difference within columns, P < 0.05.
eCG groups compared with the control group (Fig. 1D). The ICM-to-total cell ratio was not altered in response to 2.5, 5.0, and 20.0 IU rhFSH or hCG (Fig. 1D).

**Blastocyst IGF-II, IGF-II receptor, and VEGF mRNA Expression**

Although there was no significant effect of rhFSH treatment on the expression of the IGF-II gene in blastocysts, 5 IU eCG treatment significantly increased the expression of IGF-II in blastocysts compared with the control group (P < 0.05, Fig. 2A). Furthermore, there was no significant effect of ovarian stimulation on the expression of the IGF-II receptor compared with the nontreated control group (Fig. 2B). There was also no significant effect of ovarian stimulation on VEGF mRNA abundance in blastocysts derived from any ovarian stimulation treatment (Fig. 2C).

**Fig. 1. Effects of gonadotropin treatment on total blastocyst cell number (A), trophectoderm cell number (B), inner mass cell number (C), and the proportion of inner cell mass (ICM) cells (D) relative to total cell number. hCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; eCG, equine chorionic gonadotropin. Data are presented as means ± SE; a minimum of 55 blastocysts were stained for each treatment group. Different superscripts denote a significant difference between treatment groups (P < 0.05).**

**Fig. 2. Effects of gonadotropin treatment on gene expression for IGF-II (A), IGF-II receptor (B), and vascular endothelial growth factor (VEGF; C). All results are normalized for 18S rRNA abundance and expressed as a percentage of the control group. Data are presented as means ± SE from 5 replicates of pooled blastocysts. *Significant effect of eCG treatment compared with the control group (P < 0.05).**

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="" /></td>
<td><img src="image2.png" alt="" /></td>
<td><img src="image3.png" alt="" /></td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, we have demonstrated that ovarian stimulation induced with rhFSH can adversely affect embryo development and quality in the mouse. The adverse effects of recombinant FSH were dose dependent, with doses of 10.0 and 20.0 IU rhFSH reducing in vitro development rates and doses of 5.0 IU and above altering the allocation of cells between the ICM and TE within the blastocyst. We observed that rhFSH treatment had no effect on IGF-II, IGF-II receptor, and VEGF gene expression in resulting blastocysts. We have also described the impact of eCG-induced ovarian stimulation on cell allocation within the blastocyst and on the expression of the IGF-II, the IGF-II receptor, and VEGF genes in blastocysts.

Because rhFSH is not routinely used to induce superovulation in the mouse, we investigated the ovarian response to a range of rhFSH doses. We found that 2.5 IU rhFSH resulted in a basal level of stimulation, that the response to 5.0 IU rhFSH was three times greater than basal stimulation levels, and that a maximum ovarian response resulted from stimulation with 10 and 20 IU rhFSH.

In the present study, although high levels of rhFSH induced ovarian stimulation and produced large numbers of embryos, this was associated with an increased proportion of abnormal zygotes; however, the mechanisms responsible for this are not yet clear. Interestingly, the proportion of abnormal zygotes was not altered in response to 2.5 or 5.0 IU rhFSH, suggesting that there is a threshold in follicle stimulation required to produce this adverse effect. This suggests that the number of embryos produced by the higher levels of ovarian stimulation treatments has an impact on the developmental outcome of embryos, which probably reflects oocytes being recruited that would normally become atretic, as well as oocytes that are forced to mature ahead of time. We also found an increase in the proportion of abnormal zygotes recovered from eCG-stimulated mice compared with nontreated control mice, which is in agreement with previous studies that have recovered one-cell (24) or two-cell embryos (10) after eCG treatment. This has mainly been attributed to the long half-life of eCG and its high level of luteinizing hormone activity, which are thought to prematurely induce meiotic maturation of oocytes, resulting in the fertilization of meiotically aged oocytes (5, 30) that are often fragmentated or abnormal in appearance.

The proportion of embryos developing in vitro was also reduced in embryos recovered from mice treated with 10 or 20 IU rhFSH, suggesting that development competence has been compromised after high levels of follicle stimulation. It has previously been reported that ovarian stimulation with eCG resulted in delayed and/or impaired (10, 11, 32) in vitro development of mouse embryos. Interestingly, we did not find any adverse effects of eCG treatment on in vitro blastocyst development, which conflicts with previous reports. The present study, however, is the first study to our knowledge to report that the in vitro development of mouse embryos is impaired after ovarian stimulation with recombinant FSH. It has been reported that exogenous gonadotropin treatment increases the frequency of chromosomal abnormalities (6, 7, 33), and it is possible that this underlies the impaired in vitro embryo development after rhFSH and eCG treatments.

Embryos recovered from mice treated with 5.0 and 20 IU rhFSH and eCG developed into blastocysts with significantly reduced cell numbers. The reduction in total cell number in the 20 IU rhFSH group appeared to be because of a decrease in both the number of ICM cells and TE cells, since the proportion of ICM cells to total cell number was not altered. In the 5.0 IU rhFSH and eCG groups, there was a reduction in the number of ICM cells only. These results indicate that different mechanisms may be responsible for the observed decrease in blastocyst cell numbers in the 20.0 IU rhFSH group compared with the 5.0 IU rhFSH and eCG groups, which may also explain why there was no observed decrease in blastocyst cell numbers in the 10 IU rhFSH group. It has previously been reported that the total blastocyst cell number and the number of ICM cells are positively correlated with fetal development after embryo transfer (18). The results of the present study suggest, therefore, that treatment with 5.0, 10.0, and 20.0 IU rhFSH, or eCG, results in embryos that may have a reduced developmental capacity posttransfer.

In the present study, we did not find any effects of rhFSH treatment on the expression of the IGF-II, IGF-II receptor, or VEGF genes in blastocysts compared with the control group. Therefore, it appears that, although some markers of embryo quality, such as blastocyst cell number, are altered in response to ovarian stimulation with rhFSH, other outcome measures, such as the expression of genes involved in developmental signaling pathways, such as IGF-II and its receptor, are largely unaltered. In contrast, ovarian stimulation induced by eCG increased IGF-II gene expression in blastocysts. It has previously been demonstrated, using antisense IGF-II oligonucleotides, that IGF-II plays an important role in cell number determination and the rate of progression to blastocyst in vitro (27). Because there is a reduction in cell number and a decreased in vitro blastocyst development in the eCG treatment group, the increased IGF-II expression in these blastocysts may be a compensatory mechanism by which embryos of suboptimal quality attempt to maintain appropriate levels of development. It has previously been reported that the gene expression of insulin-like growth factors in human embryos may be an indicator of embryonic growth potential and embryo quality (19). Furthermore, it has been demonstrated that in vitro culture of mouse embryos in different culture media affects the expression of IGF-II in blastocysts (14, 15). Interestingly, these studies found that suboptimal conditions were associated with a decrease in IGF-II expression, which contrasts with the present study, where an increase in IGF-II expression was found in the eCG treatment group. It is interesting to note that, in the studies by Ho et al. (14, 15), the in vivo control embryos were obtained from ovarian-stimulated mice treated with a combination of eCG and hCG. The decreased IGF-II expression in these studies was therefore in response to in vitro culture conditions. In contrast, in the present study, an increase in IGF-II expression was found in the eCG treatment group compared with control embryos. In this study, all embryos were exposed to the same in vitro culture conditions from the one-cell stage; however, the control embryos were obtained from natural ovulations in untreated control mice. Therefore, it appears that ovarian stimulation does alter embryo development, whereby treatment with eCG increases the expression of IGF-II in blastocysts. The relevance of these differences in gene expression in response to different stimuli, in terms of
subsequent development, however, requires further investigation. It is interesting to speculate that, since IGF-II is an imprinted gene, eCG treatment may alter the methylation status or epigenetic regulation of imprinted genes in the oocyte, leading to alterations in subsequent embryo development. Because the parent of origin methylation marks are expressed in the genome of the mature oocyte, they may be susceptible to changes within the ovarian environment, such as ovarian stimulation. It has been demonstrated that manipulations of preimplantation embryos, such as in vitro culture, can alter the methylation status of imprinted genes and subsequently impact on fetal growth and development (8, 17, 20, 35). In future studies, therefore, it is important to determine if the alteration of imprinted genes by ovarian stimulation is a pathway by which oocyte and embryo quality is adversely affected.

It has previously been reported that ovarian stimulation with urinary human FSH decreased the mRNA levels of a specific VEGF isoform, VEGF120, at embryo implantation sites on embryonic days 5 and 6 and caused a delay in implantation (29). Although in the present study we measured total VEGF expression, we did not find any effect of the ovarian stimulation treatments on VEGF expression in blastocysts. These data suggest that ovarian stimulation may have a greater impact on VEGF expression in the endometrium and that the specific isoform VEGF120 may be more susceptible to ovarian stimulation compared with the total VEGF expression.

hCG was used to induce ovulation in the rhFSH and eCG groups, since it mimics the human situation where ovulation is routinely stimulated by hCG during IVF treatment. In the present study, we found that the proportion of abnormal zygotes in response to hCG was not different from the nontreated control group; therefore, it appears that the hCG treatment did not contribute to the increased proportion of abnormal embryos in the 10.0 and 20.0 IU rhFSH and eCG groups. There was also no difference in the in vitro development rates and blastocyst cell numbers between the hCG group and the control group; however, the hCG treatment group was also not different from the other gonadotropin treatment groups. The contribution of hCG to the adverse effects of rhFSH and eCG on preimplantation embryo development therefore cannot be ruled out. In conclusion, ovarian stimulation with rhFSH and eCG impairs the in vitro development of preimplantation mouse embryos. We have found that both the developmental capacity and blastocyst cell numbers and allocation are altered by gonadotropin treatment. Ovarian stimulation induced by rhFSH did not significantly alter the expression of the genes for IGF-II, IGF-II receptor, or VEGF in blastocysts, indicating that the adverse effects of ovarian stimulation on embryo development were not as a result of differences in gene expression of the selected genes. IGF-II gene expression was increased, however, in the eCG treatment group. Further studies are required to determine if recombinant FSH has similar detrimental effects on embryo quality in species other than the mouse.

REFERENCES


GRANTS

We are grateful to The University of Adelaide Faculty of Health Sciences B3 Scheme for financial support of this work. L Edwards was supported by an National Health and Medical Research Council Peter Doherty Fellowship (ID 219401).


