Administration of low-dose LH induces ovulation and prevents vascular hyperpermeability and vascular endothelial growth factor expression in superovulated rats

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Abstract

The administration, to rats, of a combination of pregnant mares serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) in high doses induces the ovarian hyperstimulation syndrome (OHSS) which is characterized by increased vascular permeability (VP) and simultaneous overexpression of vascular endothelial growth factor (VEGF) in ovarian cells. hCG has a longer half-life than LH and a greater biological activity, expressed in a higher incidence of complications such as OHSS. Similarly, FSH may also be related to the ovulatory changes within the follicle as there is a simultaneous surge in spontaneous cycles. The aim of this study was to compare the capacity of hCG, FSH and LH to induce ovulation and simultaneously prevent OHSS in the animal model. Immature female rats were treated with 10 IU PMSG for 4 days, and ovulation was triggered with saline, 10 IU hCG, 10 IU FSH, 10 IU LH or 60 IU LH. The number of oocytes ovulated into the tubes, VP and mRNA VEGF expression were evaluated and compared.

All the hormones employed were as effective at triggering ovulation, with similar significant P values when compared with the control for which saline was used. The use of 10 IU LH resulted in significantly lower VP and VEGF expression than that seen in the groups treated with 10 IU hCG, 10 IU FSH or 60 IU LH. In conclusion, FSH and hCG, as well as a sixfold increase in LH, displayed similar biological activities, including increased VP due to excessive VEGF expression. The use of lower doses of LH produced similar rates of ovulation, while preventing the undesired changes in permeability. These experiments should therefore encourage clinicians to determine the optimal dose of LH to be employed in women in order to trigger ovulation and, at the same time, avoid the risk of OHSS.

Introduction

In many mammalian species, including humans, the mid-cycle surge of luteinizing hormone (LH) drives a series of events included under the term ovulation, namely oocyte maturation, follicular rupture and luteinization of granulosa and theca cells (Hoff et al. 1983, Speroff et al. 1999). The LH surge is accompanied by a follicle-stimulating hormone (FSH) surge that might be relevant to these processes. In fact, in addition to ovulation, increased activity of proteolytic enzymes, which degrade the follicular wall, plus luteinization of follicular cells and corpus luteum formation occur following injection of a bolus of recombinant (r) FSH in rats (Galway et al. 1990), mice (Montgomery-Rice et al. 1993) and non-human primates (Zelinski-Wooten et al. 1998).

Due to the inconsistency of spontaneous LH surges and the wide use of gonadotrophin-releasing hormone (GnRH) analogues, human chorionic gonadotrophin (hCG) has been employed clinically for many years in assisted reproduction techniques, since there is a high degree of homology between LH and hCG. However, it is assumed that the biological activity of hCG is sixfold higher than LH, mainly due to its longer half-life and affinity for the common receptor (Yen et al. 1968).

The clinical use of hCG is associated with certain undesirable effects attributed to its biological power. One such example is ovarian hyperstimulation syndrome (OHSS), which is an hCG-dependent phenomenon (Lyons et al. 1994) mediated through the expression, production and secretion of vascular endothelial growth factor (VEGF) in human (Neulen et al. 1995, Wang et al. 2002) and rat (Gomez et al. 2002, 2003) granulosa cells.

Whether rLH and rFSH are responsible for inducing the same phenomenon is not known, but we do know that OHSS is mitigated, in part, in patients in whom the final
preovulatory events have been triggered with rLH (The European Recombinant LH Study Group, 2001), or in whom a bolus of GnRH analogue has induced a spontaneous LH surge (Diedrich et al. 2001). Moreover, Manau et al. (2002) have recently shown that the circula-
tory dysfunction frequently seen in women undergoing controlled ovarian stimulation for assisted reproduction is less intense when rLH is employed instead of hCG.

The aim of the present study was to study the biological activity of hCG, rLH and rFSH in an attempt to determine the minimum effective dose for inducing ovulation and preventing OHSS in the rat model. With this in mind, two aspects were analyzed: their capacity to initiate follicular rupturing and oocyte release, and their ability to simultaneoulsy induce VEGF expression and vascular hyperper-
meability, a phenomenon responsible for the clinical features of OHSS and linked to ovarian VEGF production (Gomez et al. 2002, 2003).

Materials and Methods

Drugs and reagents

General analytical grade chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) and Merck (Darmstadt, Germany). Pregnant mares serum gonado-
trophin (PMSG) was purchased from Sigma and urinary hCG (Profasi) was obtained from Serono Laboratories (Madrid, Spain). Recombinant FSH and LH (Gonal-F and Luveris respectively) were also purchased from Serono Laboratories. The TRIZOL reagent was obtained from GIBCO (Paisley, Strathclyde, UK). The anaesthetic ketamine (Ketolar) was purchased from Parke-Davis (Barcelona, Spain). Hyaluronidase was purchased from Sigma Chemical Co.

Animals, stimulation protocols and experimental design

Immature female Wistar rats were obtained from Harlam Iberica (Sant Feliu de Codina, Spain) and held in our laboratory for at least 1 week prior to initiating the experiments. They were fed a standard diet and allowed free access to water, and were kept in a 12 h light:12 h darkness schedule (lights on 0700–1900 h). All studies were begun when the animals were 22 days old (42–48 g) and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Protocols for animal handling were approved by the Ethical Animal Committee, School of Medicine of Valencia University.

All the animals employed in this study received 10 IU PMSG/day from day 22 to day 25. On day 26 they were included, depending on the drug and dose administered, in one of the five following groups.

i) PMSG group (n = 30) received 0.1 ml i.p. saline on day 26, thus constituting a control group (non-ovu-
lating, non-OHSS group).

ii) hCG group (n = 30) received 10 IU hCG on day 26, in order to trigger ovulation.

iii) FSH group (n = 30) was given 10 IU rFSH, to induce ovulation.

iv) LH-10 group (n = 30) was given 10 IU rLH on day 26.

v) LH-60 group (n = 30) received 60 IU LH on day 26, in order to trigger ovulation.

Three sets of experiments were performed. Each set contained ten animals per group, and animals were killed at random at two stages: at 17–20 h, following drug administration to trigger ovulation (Pellicer et al. 1988), half were analyzed for ovulation rates and then killed; vascular permeability (VP) and VEGF expression were measured in the remaining half 48 h after hCG, a time at which we had previously determined maximal VP and VEGF expression in hyperstimulated rats (Gomez et al. 2002, 2003). In each set of experiments, one ovary from at least two animals per group was frozen for mRNA analysis. After reverse transcription (RT) of isolated RNA, primers targeting the common region of VEGF isoforms were used in a real-time quantitative flu-
orescent PCR, in order to compare whole mRNA VEGF levels among the five groups and thereby identify differences in VEGF expression.

Induction of ovulation

After they had been killed, the tubes of each animal were isolated under the dissecting microscope and opened with a fine needle to obtain the oocyte–cumulus complexes in drops containing 80 IU/ml hyaluronidase. After 30 min, during which time the granulosa cells dispersed, the number of oocytes released in each tube were counted under the microscope (Pellicer et al. 1988).

VP assays

To measure VP, we employed a previously described method (Ujioka et al. 1997). Rats were anaesthetized with ketamine (100 mg/kg) and kept warm on a thermal blanket to avoid the risk of hypothermia. A fixed volume (0.2 ml) of 5 mM Evans Blue (EB; Sigma) dye diluted in distilled water was injected via the femoral vein. Thirty minutes after injection of the dye, the peritoneal cavity was filled with 5 ml 0.9% saline (21°C, pH 6) and massaged for 30 s. Subsequently, the fluid was carefully extracted with a vascular catheter (Vialon; Becton Dickinson, Madrid, Spain) to prevent tissue or vessel damage. To avoid any protein interference, the peritoneal fluid was recovered in tubes containing 0.05 ml 0.1 M NaOH. After 12 min of centrifugation at 900 g, EB concentration was measured at 600 nm on a Shimadzu 1201 spectrophotometer (Izasa, Madrid, Spain). The level of the extravasated dye in the recovered fluid was expressed as µg EB/100 g body weight.
RNA extraction was performed according to the method of Chomczynski & Sacchi (1987) with minor modifications, namely the use of the TRIZOL reagent. In short, each tissue was weighed and 500 μl TRIZOL reagent/100 mg tissue weight was added. Total RNA was separated from DNA and proteins by adding 250 μl chloroform, and precipitated with isopropanol (overnight at −20°C). The precipitate was washed twice in ethanol, air-dried and resuspended in diethylpyrocarbonate (DEPC)-treated water. The amount of RNA was quantified by spectrophotometry with a SmartSpec 3000 spectrophotometer (BioRad, Barcelona, Spain).

RT

RT was performed using an Advantage RT-for-PCR KIT (Clontech, Palo Alto, CA, USA). The mastermix per sample was prepared as follows: 4 μl of 5 x reaction buffer, 1 μl dNTPs mix (10 mM each), 0.5 μl recombinant RNase inhibitor and 1 μl Moloney murine leukemia virus reverse transcriptase. One microgram of each sample was diluted to a final volume of 12.5 μl in DEPC-treated water plus 1 μl oligo(dT)18. The mixture was then heated at 70°C for 2 min, and kept on ice until the mastermix (6.5 μl) was added. For each RT, a blank was prepared using all the reagents except the RNA sample, for which an equivalent volume of DEPC-treated water (12.5 μl) was substituted. The RT blank was used to prepare the PCR blank (below). Once all components were mixed, the samples were incubated at 42°C for 1 h, and heated at 94°C for 5 min to stop cDNA synthesis and eliminate DNAse activity. The product was diluted to a final volume of 100 μl with DEPC-treated water and stored at −20°C until PCR analysis.

Real-time PCR

Primers for quantitative PCR were designed using Primers Express Software (Applied Biosystems, Warrington, Cheshire, UK) and synthesized by Applied Biosystems (Barcelona, Spain). The β-actin sense primer was 5′-GGGAAATCGTGCGTGACAT-3′ and the antisense β-actin primer was 5′-GGGAAATCGTGCGTGACAT-3′ (NCBI accession number 55574), giving rise to an expected PCR product of 149 bp. The VEGF primers designed to amplify a region common to all VEGF isoforms were 5′-CAGCATTGCGCTTCAATTAAGA124-3′ for the sense primer and 5′-CAGCATTGCGCTTCAATTAAGA124-3′ for the antisense primer where a 131 bp PCR product was expected (NCBI accession number AF215726).

To amplify cDNA, the RT samples were diluted to a final concentration of 12.5 pg total cDNA/μl. In each reaction, a total of 4 μl (50 pg cDNA) from each RT tube was mixed with 12.5 μl SYBR Green PCR master mix (Applied Biosystems) containing nucleotides, Taq DNA polymerase, MgCl2 and reaction buffer with SYBR green; 1–3 μl 0.25 μM specific primers and double-distilled water were added to a final volume of 25 μl.

Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (Perkin Elmer Corp., Foster City, CA, USA), according to the manufacturer’s instructions, with a heated lid (105°C), and with an initial 10-min denaturation at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each sample was amplified in duplicate for VEGF and β-actin, giving rise to four reactions per sample in each analysis. In parallel, six-fold serial dilutions of known concentrations of VEGF and β-actin cDNA were run as a calibration curve. Quantification data were analyzed with ABI PRISM 1.7 analysis software. Background fluorescence was removed by setting a noise band. Duplicates showing more than a 5% variation were discarded. To validate real-time PCR, standard curves with r > 0.95 and slope values between −3.1 and −3.4 were required.

For each sample, the amounts of VEGF and β-actin cDNA were determined in relation to the standard curves. VEGF/β-actin was used to estimate and compare the relative VEGF expression among samples. The results of each PCR experiment were confirmed in a minimum of three consecutive experiments.

Final PCR products from VEGF and β-actin were subjected to melting analysis to probe the presence of no more than one expected amplified product. A PCR cleanup kit (MO BIO Laboratories, Carlsbad, CA, USA) was used to eliminate primers and the recovered cDNA was sequenced to corroborate that the amplified bands corresponded with VEGF and β-actin cDNA products.

Statistical analysis

Data are expressed as means±s.e.m. An ANOVA test was employed to evaluate the extent of oocyte recovery among groups, while post-hoc Tukey analysis defined significant P values in the hCG group compared with each of the other groups. For the VP and mRNA VEGF expression studies, non-parametrical tests were chosen because of the non-homogeneity and the high level of variance among the groups. In both cases, a Kruskal–Wallis test, used to determine average differences among groups, was employed to evaluate the extent of oocyte recovery among groups, while post-hoc Tukey analysis defined significant P values in the hCG group versus each of the other groups. Significance was defined in all cases as P < 0.05. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA).

Results

Ovulation induction

Figure 1 shows the efficacy of all the hormones and doses tested in inducing follicular rupture. As expected, multiple administration of PMSG did not, in itself, induce follicular...

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rupture, as revealed by the absence of oocytes recovered in the PMSG group. Because of the higher activity of hCG vs LH (sixfold), the administration of 10 IU hCG (hCG group) was as effective as the injection of 60 IU LH (LH-60 group) in inducing ovulatory processes. Surprisingly, FSH was demonstrated not only to induce ovulation, but also to have a similar biological activity to that of hCG when a 10 IU dose was tested (FSH group). Despite its lower biological activity, a 10 IU dose of LH (LH-10 group) was as effective in inducing ovulation (20.0 $\pm$ 1.8 oocytes/rat) as hCG (27.2 $\pm$ 2.5), LH-60 (26.7 $\pm$ 4.1) and FSH (30.4 $\pm$ 3.2). Not unexpectedly, significant differences ($P < 0.05$) were found when oocyte recovery in the PMSG group (0.7 $\pm$ 0.1) was compared with that in the hCG group, these differences being more extensive than any other group treated with drugs to trigger ovulation.

**VP**

The presence of EB in the peritoneal cavity was used as a quantitative method with which to evaluate VP 48 h after the drugs (rFSH, rLH or hCG) were administered. Levels of VP in the hCG group were used as a reference for the onset of OHSS. As shown in Fig. 2, FSH and sixfold LH (FSH and LH-60 groups) displayed similar biological activities to that of the hCG group, inducing increased VP, as shown by the absence of statistical differences when compared with the hCG group. In fact, the level of extravasated EB in the hCG group (30.7 $\pm$ 4.1 $\mu$g EB/100 g body weight), which was stimulated to provoke OHSS symptoms, was similar to those of the FSH (28.2 $\pm$ 3.0 $\mu$g EB/100 g body weight) and LH-60 (21.1 $\pm$ 3.7 $\mu$g EB/100 g body weight) groups. The onset of OHSS was also revealed by the appearance of ascites in approximately the same (80%) percentage of animals in each of the FSH, LH-60 and hCG groups. Surprisingly, a lower 10 IU dose of LH (LH-10 group), which had been equally effective in triggering ovulation, did not produce the symptoms of OHSS, as indicated by the absence of ascites in all animals, as occurred in the control PMSG group. The absence of OHSS symptoms following a 10 IU dose of LH was confirmed by the quantification of extravasated dye levels in the LH-10 group (12.5 $\pm$ 2.3 $\mu$g EB/100 g body weight), which was significantly ($P = 0.006$) lower than those in the hCG group and in the range of those of the non-ovulating, PMSG group (10.8 $\pm$ 1.9 $\mu$g EB/100 g body weight), which were significantly ($P = 0.03$) lower than those in the hCG group.

**VEGF expression**

VEGF expression did not differ among the hCG, LH-60 and FSH groups, revealing the expected similar biological activities of these drugs and doses with respect to increasing ovarian VEGF expression. As seen in Fig. 3, both the PMSG and LH-10 groups had similar VEGF levels, which were statistically lower than those of the hCG group, known to provoke OHSS symptoms. VEGF expression was associated with increased VP, shown by the expected similar values found in the hCG (1.9 $\pm$ 0.2), LH-60 (1.4 $\pm$ 0.3) and FSH (1.7 $\pm$ 0.3) groups. The said expression was significantly reduced in the LH-10 (1.2 $\pm$ 0.1, $P = 0.02$) and PMSG (1.0 $\pm$ 0.02, $P = 0.03$) groups when compared with that of the hCG (OHSS) group.

**Discussion**

The results of the present study have shown that all three hormones tested at different doses were capable of inducing follicular rupture and ovulation. Moreover, hCG,
leads to the initial rise in progesterone 12 h before the LH surge, the complete maturation of the oocyte 32 h after the surge and follicular rupture 36 h after the LH surge (Hoff et al. 1983, Speroff et al. 1999, Yeh & Adashi 1999). Thus, it would be logical to determine the optimal rLH dose to induce ovulation and avoid OHSS.

Indeed, the experience accumulated in humans is contradictory. Initial multicentric studies have shown that a single dose of 15 000 IU rLH, comparable with 5000 IU hCG, is necessary to achieve optimal oocyte maturation in IVF and is more efficient than 5000 IU rLH (The European Recombinant LH Study Group 2001). These studies showed a reduced incidence of OHSS when 15 000–30 000 IU rLH was employed as compared with hCG. A more recent publication, however, obtained the same number of mature oocytes and similar implantation rates in embryos derived from women treated with 5000 IU hCG or rLH (Manau et al. 2002), suggesting that the dose of rLH necessary to trigger oocyte maturation and avoid OHSS might be lower than initially expected. Moreover, the same authors showed that the haemodynamic changes associated with controlled ovarian stimulation in IVF are lower when rLH is employed, and that the overall incidence of OHSS is reduced (Manau et al. 2002).

The different drug doses were selected according to the minimal effective dose of hCG necessary for triggering optimal ovulation (Galway et al. 1990), lower doses of hCG having been found to be insufficient for these purposes (Chandrasekhar & Armstrong 1988, Dekel et al. 1995). The same dose was therefore employed for the other hormones tested (LH and FSH). Based on the biological activity of hCG being six times greater than that of LH, due to its longer half-life and affinity for the common receptor (Yen et al. 1968), a sixfold dose of LH was also administered.

The origin of the drugs used to induce ovulation, whether urinary or recombinant, may not have influenced the results of the study, since previous analyses in which hCG of both origins have been compared have provided similar biological responses (The European Recombinant Human Gonadotrophin Study Group 2000, Chang et al. 2001, Trinchard-Lugan et al. 2002).

A further interesting finding of the present study was the ability of rFSH to induce increased VP in hyperstimulated animals, a finding not previously reported. However, a very recent publication has described a familial mutation in the FSH receptor which allowed the binding of hCG and the development of spontaneous OHSS in pregnant women (Vasseur et al. 2003), raising interesting questions about the involvement of FSH in OHSS. Indeed, in our model, FSH also simultaneously induced VEGF expression, which has been reported in non-human primates (Christenson & Stouffer 1997) and human granulosa cells (Lahtinen et al. 1997). Moreover, in this respect, FSH has been shown to be more potent than PMSG (Dissen et al. 1994) and LH (Wang et al. 2002). We know that, in animals, FSH drives many events at mid-cycle, such as

Figure 3 β-Actin mRNA levels in the ovaries of each sample were used as the housekeeping gene to normalize VEGF expression between them. The average VEGF/β-actin ratio in each group was used to compare mRNA VEGF levels among the groups. In order to normalize VEGF expression levels, the VEGF/β-actin ratio in the control PMSG group was assigned the value 1 and used to normalize VEGF expression in the other groups. The VEGF/β-actin ratios of the FSH, LH-10, LH-60 and the PMSG control groups were compared with that of the hCG group, the latter being known to develop OHSS symptoms. After a positive Kruskal–Wallis, a non-parametric Mann–Whitney test revealed that only the PMSG control and the lower 10 IU LH dose showed significant differences when compared with the hCG (OHSS) group. *P < 0.05 compared with hCG group (OHSS symptoms). Values are the means ± S.E.M.
oocyte maturation (Pellicer et al. 1989), luteinization, corpus luteum formation and follicular rupture (Galway et al. 1990, Montogomeri-Rice et al. 1993, Zelinski-Wooten et al. 1998). We have shown here that a dose of 10 IU rFSH has a biological activity similar to that of 10 IU hCG in inducing follicular rupture and increasing VP and VEGF expression. Thus, although the relevance of the mid-cycle surge of FSH in women with normal cycles is still a matter of speculation, the accumulated knowledge suggests two relevant physiological points. First, it is important in all the processes included in ovulation, which is reinforced by the finding of FSH receptors in human oocytes (Meduri et al. 2002). Secondly, the lower absolute serum levels described for FSH than for LH (Hoff et al. 1983, Speroff et al. 1999, Yeh & Adashi 1999) may reflect the different specific activity that both hormones display with regard to ovulation. In other words, lower FSH should be equally effective, a pathway that should be further explored in humans. Alternatively, the viability of a combination of FSH and LH should also be considered.

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