The Superovulation of Synchronous Adult Rats Using Follicle-Stimulating Hormone Delivered by Continuous Infusion

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ABSTRACT

The estrous cycles of adult female rats were synchronized with an LHRH agonist on the morning of Day -4 (Day 0 = day of mating). On Day -2, animals received s.c. implants of continuous-infusion osmotic minipumps containing different doses of an FSH preparation (Follitropin) in combination with hCG at various ratios of hCG:FSH or were given single injections of eCG in doses ranging from 15 IU to 60 IU.

Rats infused with the optimal dose (3.4 U/day) of FSH ovulated 44.1 ± 5.4 oocytes/rat while rats treated with the most effective dose (60 IU) of eCG ovulated only 20.5 ± 4.3 oocytes/rat on the morning of Day 1. The inclusion of hCG in pumps at ratios from 0.188:1 to 0.75:1 (hCG:FSH) had no significant effect on ovulation rate. The importance of synchronization of estrus in successful superovulation was demonstrated by the finding that only 70% of the unsynchronized animals ovulated (29.1 ± 4.8 oocytes/rat) whereas 95% of the synchronized animals ovulated (51.0 ± 3.6 oocytes/rat). Oocyte viabilities were assessed by determining fertilization rates and embryonic development in vivo following mating with fertile males. In rats superovulated by use of the FSH regimen, 92% (39.0 ± 4.1) of the recovered embryos were 1-cell zygotes on Day 1, 89% (36.3 ± 5.6) were at the 2-cell embryo stage of development on Day 2, and 88% (28.8 ± 2.2) were at the morula and blastocyst stages on Day 5 following mating on Day 0.

The high ovulation rates and oocyte viability in rats receiving infusions of Follitropin following estrus synchronization offer a reliable method for superovulation of adult rats.

INTRODUCTION

For many years the superovulation of rodents has been used for studying numerous aspects of mammalian reproductive biology. Gonadotropin hormone preparations for this purpose have included anterior pituitary implants [1], eCG [2–4], and FSH preparations of varying purity [5–7]. The regimen used most commonly for superovulation of immature rodents involves a single injection of eCG followed 48–72 h later by a preovulatory hCG injection.

Despite the success in superovulating immature rats and mice, a reliable method for use with adult animals remains to be found. It has been suggested that the reduced ovulatory response in adult rodents may be a consequence of superovulatory treatment during an inappropriate time of the estrous cycle [8–10], since it has been difficult to treat all animals during the same stage of the cycle. This may also explain the observed reduction in frequencies of mating and pregnancy in adult mice [3].

The prospect of finding a regimen for superovulation of adult rats is attractive for two important reasons. First, the ability to produce reliable superovulation in adult animals can yield important information regarding differences between the control of ovarian follicular development and subsequent ovulation in adult and immature animals. Second, when fertilized eggs or embryos are required, it may be difficult to achieve successful mating of immature females with adult males [11, unpublished observation].

The finding that estrous cycles in adult rats can be effectively synchronized by treatment with an LHRH agonist [12] raises the possibility of using this treatment in conjunction with gonadotropin preparations as a new approach to superovulating adult rats. Recent studies have shown that purified FSH, delivered by osmotic minipumps implanted s.c., improves both the yield and viability of oocytes superovulated from immature rats over those obtained with eCG injection [7]. This response is enhanced when the FSH preparations are supplemented with hCG [13]. Therefore, the present study was initiated in an attempt to superovulate adult rats after synchronization with LHRH agonist by infusion of FSH supplemented with various amounts of hCG.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats weighing 200–225 g and adult male Sprague-Dawley rats were obtained from Charles River Canada, Inc., St. Constant, Quebec, Canada. The animals were housed in quarters with controlled temperature and lighting (14L:10D) and given free access to food and water.
Hormones

FSH (Follitropin; Vetepharma Inc., London, Ontario, Canada), eCG (Equinex: Ayerst, Montreal, Quebec, Canada), and hCG (CG-10, Sigma, St. Louis, MO) were used as gonadotropin preparations for the in vivo studies with the LHRH agonist (LHRH-a) des-Gly10,δ-Ala6, L-Pr0NHEt LHRH (L-4513; Sigma) being used to synchronize naturally cycling rats. Purified FSH (USDA-pFSH-I-1) and hCG (batch CR-123) for iodination were kindly supplied by Dr. Douglas Bolt (Beltsville Agricultural Research Center, Beltsville, MD) and Dr. Robert Canfield (Columbia University, New York, NY), respectively. Working standards used in the radioreceptor assays were FSH-P, lot 548C81 (Burns Biotech, Omaha, NE) and hCG (Sigma, Cat #CG-10).

Radioreceptor Assays for FSH and LH Activity

FSH activity was assayed by the method of Cheng [14], with bovine testis membranes as the source of FSH-binding protein, USDA-pFSH-I-1 iodinated with chloramine T as the radioactive ligand, and varying dilutions of the laboratory standard FSH-P which had been characterized against the reference preparation NIH-FSH-S1 [7]. Potencies are expressed in terms of the NIH-FSH-S1 reference preparation, which has been assigned a potency of 1 U/mg.

LH activity was assayed by using rat testicular Leydig cell membranes prepared as described by Katikineni et al. [15] as the source of binding protein, hCG-CR123 iodinated with chloramine T as the radioactive ligand, and varying dilutions of the laboratory standard hCG (Sigma) which had been standardized against the reference preparations NIH-LH-S1. Potencies are expressed in terms of the NIH-LH-S1 reference preparation, which has been assigned a potency of 1 U/mg.

Experiment 1: Superovulation

The treatment protocol for superovulation of mature rats is illustrated in Figure 1. The estrous cycles of adult Sprague-Dawley rats (200–225 g body weight) were synchronized with a 40-μg s.c. injection of LHRH-a at 0800 h on Day −4 (Day 0 = day of mating). Between 0800 and 1000 h on Day −2, the rats were randomly allocated to treatment groups. Rats in groups 1–5 received varying doses of eCG (0, 15, 30, 45, and 60 IU) as single s.c. injections in 0.9% NaCl (saline) vehicle. The remaining 12 treatment groups represented a 3 × 4 factorial design experiment to compare three infusion rates of FSH (0.85, 1.70, 3.40 U/day) in combination with hCG to give 4 FSH : hCG ratios (0, 0.188, 0.375, and 0.750). Hormones were infused via s.c. implanted Alzet osmotic minipumps (model 2001) as described previously [7]. All animals were given 30 IU hCG, administered i.p. between 1500 and 1700 h on Day 0. If zygotes were desired on Day 1, the animals were placed with males of proven fertility after the hCG injection. If secondary oocytes were desired, mating was not permitted. Between 0800 and 1000 h on Day 1, the animals were killed by cervical dislocation and cumulus-enclosed oocytes and zygotes were extruded by puncturing and gently squeezing the ampullae of oviducts. Ovaries were blotted and weighed individually, and the mean of the two ovaries was calculated and recorded.

Experiment 2: The Importance of Synchronized Estrus in Superovulation

Animals were allocated randomly to 4 groups in a 2 × 2 factorial design experiment to compare effect of FSH infusion in synchronized versus nonsynchronized rats. Rats in groups 3 and 4 were synchronized with LHRH-a on Day −4 and group 1 animals were given a saline injection. On Day −2, osmotic minipumps delivering FSH at a rate of 3.4 U/day were implanted in animals of groups 2 and 4. Groups 1 and 3 received no FSH infusion. On Day 0, all animals were injected i.p. with 30 IU hCG, and ovulated eggs were recovered and counted on Day 1.

Experiment 3: Embryo Development in Superovulated Rats

The ovulated oocytes were assessed at 3 stages of embryonic development following mating with males of proven fertility. Animals were synchronized with the LHRH-a, implanted with osmotic minipumps delivering FSH at 3.4 U/day, given a preovulatory hCG injection, and mated as described above. Six animals were killed on Day 1 and oviducts were flushed as described previously [16]. At that time, recovered eggs or zygotes were counted and classified as 1-cell zygotes or unfertilized/degenerated eggs. Unfertilized and degenerated eggs were grouped together for statistical analysis. Eight animals were killed on Day 2 and embryos were counted and classified as 1-cell zygotes, 2-cell embryos, or degenerated embryos. Embryos were considered to be degenerated when irregularities in shape, size, or color were observed.

Five animals were killed on the morning of Day 5 and oviducts and uterine horns were removed and flushed. Recovered eggs and embryos were counted and classified as morulae, blastocysts, or degenerated embryos. Embryos regarded in development were classified as degenerated.

Statistical Analysis

Differences in the proportion of animals ovulating were compared using χ² analyses. Mean ovulation rates and mean
numbers of embryos reaching various stages of development were compared using analysis of variance, Bartlett's test for homogeneity of variance, and the Student-Neuman-Keul's test. Factorial analysis was used to assess the relationship between hCG and FSH in producing superovulation [17].

RESULTS

Potencies of Follitropin and eCG

Mean FSH and LH activities of Follitropin and eCG, as determined by radioreceptor assays, are presented in Table 1. Mean activities ± SEM are expressed in terms of the NIH reference preparations, NIH-FSH-S1 and NIH-LH-S1. When the ratio of FSH to LH activity was calculated, Follitropin was found to have more than 70 times the relative FSH activity of eCG.

Ovarian Responses to Increasing Infusion Rates of FSH

The s.c. infusion with FSH at increasing rates in synchronized adult rats over a 3-day period resulted in a dose-dependent increase in both ovulation rate (Fig. 2) and ovarian weight (Fig. 3). FSH doses are expressed in Units (U) where 1 U is equal to 1 mg NIH-FSH-S1 as calculated from Table 1. The maximum superovulatory response was produced with the 3.4 U/day-dose and this was significantly greater ($p < 0.01$) than that of all other treatments except for 1.7

![Number of Oocytes per Rat vs. FSH and eCG](image-url)
U/day FSH. The response at 0.85 U/day was only marginally greater than the ovulation rate observed in control animals (Fig. 2). Of rats receiving the 3.4 U/day, 88.9% yielded >15 ovulations. Several animals were superovulated at a rate of 6.8 U/day (not shown); however, the response was no greater than for the 3.4 U/day dose. Virtually all oocytes recovered from the FSH-infused rats were cumulus-enclosed regardless of infusion rate. The addition of hCG at three different dosage ratios of hCG:FSH (0.188, 0.375, and 0.75) did not significantly influence the ovarian weights or ovulation rates (data not shown).

**Ovarian Responses to eCG Treatment**

Single injections of eCG produced superovulation in occasional animals, but at no eCG dose was mean ovulation rate significantly greater than that of control animals (Fig. 2). In addition, a smaller proportion of rats treated with 30 IU and 45 IU eCG ovulated than of rats in all other treatment groups ($p < 0.01$). Ovarian weights increased with increasing eCG doses, with the 60-IU treatment yielding significantly greater ovarian weights than those observed with the highest FSH dose (Fig. 3). The ovarian weights following treatment with 45 IU and 60 IU eCG were significantly greater than those of all other eCG treatment groups and all FSH treatment groups except for the 3.40 U/day group ($p < 0.01$). Treatment with eCG resulted in the frequent appearance of abnormal or degenerated oocytes, with these aberrant oocytes being most prevalent in the 45-IU and 60-IU treatment groups.

**The Importance of Synchronized Estrus**

Nineteen of the twenty animals synchronized with LHRH-a ovulated on Day 1, whereas only 60% of the unsynchronized animals (12/20) ovulated at the anticipated time (Fig. 4). FSH treatment significantly improved the frequency of unsynchronized animals that ovulated (7/10 vs. 5/10; $p < 0.05$); however, LHRH-a treatment combined with the FSH infusion produced both significantly greater frequencies of animals ovulating (10/10) and significantly greater ovulation rates ($51.0 \pm 3.6$) than all other groups ($p < 0.01$).
Normality of Superovulated Oocytes

When rats were placed with males after a 60-h infusion with FSH and an hCG injection on Day 0, fertilized eggs were found on Day 1 in the oviducts of all rats that had ovulated (Table 2). Of the recovered eggs, 92% (39.0 ± 4.1) were 1-cell zygotes as indicated by the presence of 2 pronuclei and a sperm tail. On Day 2, 89% (36.3 ± 4.7) of the recovered embryos were at the 2-cell stage. At Day 5, 36% (11.9 ± 1.8) and 52% (16.9 ± 2.0) were at the morula and blastocyst stages, respectively, or a total of 28.8 ± 2.2 morulae and blastocysts (Table 2).

DISCUSSION

The results of the present study clearly show that adult rats can be reliably superovulated to yield more than a 3-fold increase in ovulation rate over control animals. This represents a significant improvement over previous attempts to superovulate adult rats [2, 6] and mice [9]. In addition, high mating frequencies were encountered, and superovulated oocytes demonstrated good viability resulting in high percentages of fertilization and progression to the morula and blastocyst stages.

One previous study [18] reported ovulation rates in response to eCG injection that approached those of the present study; however, maximum ovulation was observed 3 days after eCG treatment, administered during estrus. This would be 1 day before the anticipated endogenous LH surge that normally induces ovulation. Furthermore, no attempt was made to assess the viability of superovulated oocytes. In contrast, we found eCG to be much less effective than FSH at inducing superovulation. The two different responses to eCG may have been due to a difference in the strain of rat used or to the use of a different eCG preparation.

The observed success of superovulation of adult rats in the present study may be attributable to the synchronization of estrous cycles in the animals used, the use of osmotic minipumps to deliver FSH at a constant rate, the FSH preparation itself, or to any combination of these factors. The LHRH-a proved to be very effective in cycle synchronization since more than 90% of the control animals ovulated at the anticipated time, 4 days after treatment. Only 70% of the unsynchronized animals ovulated at the anticipated time, even when treated with FSH. Previous studies using FSH alone have been unsuccessful [5, 6], and the need for proper cycle synchronization has been alluded to previously [9, 10]. However, the fact that low ovulation rates were encountered with LHRH-a-synchronized, eCG-treated rats indicates that estrus synchronization is not the only contributor to the improved superovulatory response.

Recent work with immature rats [7] has demonstrated improved ovulation rates and ovocyte viability when FSH is used instead of eCG. In further studies, the addition of small doses of hCG to a more highly purified FSH preparation was found to improve ovulation rates, while large doses of hCG or LH inhibited the superovulatory effects of FSH [7, 13]. Therefore, the long biological half-life of eCG and its high LH activity [19] probably play a role in the inferior ovarulatory response following eCG treatment. In contrast, radioreceptor assays have shown that the FSH preparation (Foll-
tropin) used in the present study possesses modest LH activity but much less than that of eCG (Table 1). The superovulation studies showed that the inherent LH activity of Foll-tropin is sufficient for inducing optimal superovulation since further hCG supplementation failed to augment the response.

Although we report a reliable method for superovulation of adult rats, the ovulation rate still falls short of that attainable with immature rats [7]. This finding is consistent with previous research that showed a post-puberal fall in superovulation potential for both rats and mice [20]. The decrease may be due to a reduction in the presence of follicles capable of responding to gonadotropins [21], since the rate at which follicles begin to develop is proportional to the number of follicles in the ovary [22] and since many follicles are lost through atresia during prepubertal life. Alternatively, it may result from a change in the sensitivity of follicles to gonadotropins [21]. The slightly lower superovulation potential of adult rats may be offset by the improved mating success rate when embryos are desired. In addition, certain experimental settings require the use of adult animals, for example, the establishment of a new strain of rat whose desired characteristics cannot be tested until adulthood. In these situations, the availability of a reliable superovulation regimen for adult rats would be beneficial.

In conclusion, the results of this study demonstrate that FSH delivered by continuous infusion over a 60-h period is an effective means of superovulating adult rats in which estrus has been synchronized with LHRH-a with significantly higher ovulation rates than those obtained following eCG treatment. With this method, ovulation rates more than three times those of synchronized and nonsynchronized control animals can be achieved. A high percentage of these ova, when fertilized, are capable of reaching at least the blastocyst stage of embryonic development.

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REFERENCES