Impaired final follicular maturation in heifers after superovulation with recombinant human FSH

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The aim of this study was to investigate whether human FSH without contaminating LH can exert a normal superovulation response in cows. One group of heifers ($n = 9$) was stimulated with recombinant human FSH (rhFSH), an FSH source without any LH activity, and another group ($n = 9$) was treated with equine chorionic gonadotrophin (eCG), an FSH source with high LH activity. Daily transrectal ultrasonography showed that eCG- and rhFSH-stimulated heifers ($n = 9$ per group) had the same follicular growth characteristics and equal numbers of follicles > 8 mm in diameter after 3 days of stimulation. The treatment groups differed considerably in steroid production: rhFSH-treated heifers produced much lower oestradiol concentrations than did eCG-stimulated heifers during the first days of stimulation and much lower progesterone concentrations in the period after the LH surge. During the 27–35 h after prostaglandin injection,

rhFSH-treated heifers had fewer LH pulses than did eCG-treated heifers (0.3 versus 3.0 per heifer, respectively; $n = 3$ per group). All rhFSH-treated heifers ($n = 6$) underwent a preovulatory LH surge, but this occurred significantly later than in the eCG-treated heifers ($n = 4$; 39.4 ± 1.9 h versus 47.1 ± 1.5 h in rhFSH- and eCG-treated heifers, respectively). Multiple ovulations occurred in only three of six rhFSH-treated heifers, but in all four eCG-treated heifers with an LH surge. At 24 h after the LH surge, the percentage of metaphase II stage oocytes with cortical granules distributed close to the oolemma was significantly lower in the rhFSH group (7.3%) than in the eCG group (55.9%). In conclusion, final follicular maturation is impaired in heifers treated with rhFSH, which might be due to the combination of a lack of LH activity in the gonadotrophin preparation and the severe suppression of LH pulsatility.

Introduction

Despite the recent development of the technology of *in vitro* production of embryos, superovulation by injection of exogenous gonadotrophin is still the fundamental method to produce *in vivo* derived embryos for embryo transfer in cattle. However, the yield and quality of the embryos raised after superovulation are variable and unpredictable owing to variations in ovarian response, fertilization rate and embryo development (Boland *et al.*, 1991). As well as cow factors, such as genetic background, age, breed, reproductive and lactation status, many other factors are assumed to contribute to this variability (Hasler *et al.*, 1983; Donaldson, 1984a,b; Lerner *et al.*, 1986). An important cause of the relatively low efficiency of superovulation are disorders in preovulatory development (Monniaux *et al.*, 1984; Callesen

et al., 1986). The preovulatory-sized follicles that develop after gonadotrophin stimulation have a high incidence of asynchrony between follicular and oocyte maturation (matured oocytes within unmaturing follicles or vice versa) (Hyttel *et al.*, 1986; Callesen *et al.*, 1987; de Loos *et al.*, 1991). The causes of these disorders in preovulatory development are still unclear. One possibility is that part of the follicles in which growth is stimulated by the gonadotrophin consists of rescued atretic follicles that remain aberrant. Another possibility is related to the relatively short time interval between luteolysis and occurrence of the LH surge in cows stimulated by gonadotrophin compared with unstimulated cows (Bevers and Dieleman, 1987). In addition, several studies have shown that there is a difference in LH pulse frequency between unstimulated and gonadotrophin-stimulated cows (Bevers *et al.*, 1989; Ben Jebara *et al.*, 1994). These events might result in part of the follicles being incompletely matured to respond properly to the LH surge.

The variability in the FSH:LH ratio of gonadotrophin

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preparations is considered to be an additional factor causing variability of superovulatory responses (Murphy *et al.*, 1984; Donaldson and Ward, 1986). As commercially available FSH preparations are isolated from pituitary glands, such variations in LH:FSH ratio are difficult to prevent. One approach used to circumvent this problem has been the isolation of pig FSH to high purity (Donaldson and Ward, 1986; Beckers, 1987; Herrler *et al.*, 1991). However, superovulation of cattle with purified pig FSH led to varied responses from a complete lack of ovulation (Herrler *et al.*, 1991) to a normal ovulation rate but with a small number of embryos (Schmidt *et al.*, 1988) to a normal number of transferable embryos (Donaldson and Ward, 1986). These differences might be due to variations in the content of contaminating LH. More recently, this problem has been resolved by recombinant DNA technology, which enables production of FSH in the complete absence of contaminating LH; this has been achieved for recombinant human FSH (rhFSH; Keene *et al.*, 1989; Van Wezenbeek *et al.*, 1990), recombinant bovine FSH (rbFSH; Wilson *et al.*, 1993) and recombinant pig FSH (rpFSH; Inaba *et al.*, 1997). The biological activities of these recombinant FSH preparations are similar to those of the products extracted from pituitary glands (Mannaerts *et al.*, 1991; Wilson *et al.*, 1993; Inaba *et al.*, 1997).

In human infertility clinics, rhFSH is used widely to stimulate multiple follicular development enabling retrieval of oocytes matured *in vivo* that are processed for either IVF or intracytoplasmic sperm injection (ICSI). rhFSH is more effective than urinary-derived FSH with respect to multiple follicular development and yield of oocytes without affecting the developmental competence of the oocytes (Mannaerts *et al.*, 1991; Recombinant Human FSH Product Development Group, 1998; Frydman *et al.*, 2000). Recombinant FSH has also been used for superovulation in cattle; rbFSH resulted in normal production of viable embryos, indicating that exogenous LH activity is not necessary for follicular maturation and ovulation in gonadotrophin-stimulated cattle (Wilson *et al.*, 1993).

In both cows and humans, the information on the effect of recombinant FSH is limited mainly to the yield of embryos and oocytes. Very little is known about the effect of the absence of LH activity in gonadotrophin preparations on endocrinological responses and maturation of oocytes.

The aim of the present study was to acquire more insight into the effects of stimulation of cattle with rhFSH on: (i) follicular growth dynamics; (ii) occurrence of the LH surge; (iii) the pattern of preovulatory LH pulses; (iv) ovulation rate; (v) luteinization of follicles; and (vi) nuclear and cytoplasmic maturation of oocytes. Heifers were treated with rhFSH and a control group was treated with equine chorionic gonadotrophin (eCG), thus enabling a comparison to be made between gonadotrophin preparations without LH activity and those with a relatively high LH activity.

Materials and Methods

Animals

The study was performed from March to November 1998 using Holstein–Friesian heifers ($n = 12$), aged 18–22 months at the beginning of the experiments, which were housed indoors and were fed silage and concentrate. Normal cyclicity was confirmed by measuring the progesterone concentration in peripheral blood samples three times per week during the 3 weeks before the experiments started. The experiments were carried out in three different sessions (1–3). During session 1, the first group of six heifers was assigned randomly to rhFSH ($n = 3$) and eCG ($n = 3$) groups for superovulation to study: (i) follicular development during gonadotrophin stimulation; (ii) ovulation rate; and (iii) plasma progesterone, oestradiol and LH concentrations. This session was repeated (session 2) with the change that the treatments were reversed between the two groups of heifers. Moreover, during session 2, the LH pulse frequency patterns in plasma were measured for each superovulatory treatment. In session 3, the second group of six heifers was stimulated with either rhFSH ($n = 3$) or eCG ($n = 3$) and these heifers were ovariectomized at 24–26 h after the spontaneously induced preovulatory LH surge. The following factors were studied: (i) follicular development during gonadotrophin stimulation; (ii) plasma progesterone, oestradiol and LH concentrations; (iii) the status of oocyte nuclear and cytoplasmic maturation; and (iv) progesterone and oestradiol concentrations in the follicular fluid of large follicles.

Synchronization and removal of dominant follicles

Oestrous cycles were synchronized with Crestar (Intervet International BV, Boxmeer), which includes a 3 mg norgestomet ear implant and an i.m. injection of 5 mg oestradiol valerate and 3 mg norgestomet given at the time of the implant insertion. The implants were removed after 9 days. Two days before removal, the heifers received a single i.m. injection of 10 mg prostaglandin (Prosolvin; Intervet International BV) to ensure complete regression of the corpus luteum. Oestrus (day 0; experimental day 11) occurred a mean 2 days after removal of the implants. From day 3 after oestrus onwards, development of the follicular population was observed every 2 days by ultrasonography for detection of a corpus luteum and a dominant follicle. As the presence of a dominant follicle on the day of superovulation induction could suppress the response of the remaining follicle population (Bungartz and Niemann, 1994), all follicles > 5 mm in diameter, including the dominant follicle, were removed on day 8 by transvaginal ultrasound-guided follicle puncture according to Vos *et al.* (1994).

Superovulation treatment

Superovulation treatment was started at day 10 after oestrus in both the rhFSH and eCG groups. rhFSH (Puregon,

Org 32489; NV Organon, Oss) was supplied as lyophilized powder in vials, each containing 166 iu FSH *in vivo* bioactivity. After reconstitution with solvent, rhFSH was stored at 4°C for the duration of the injection period. In the rhFSH group ($n = 9$; sessions 1, 2 and 3), rhFSH was administered i.m. in decreasing doses according to a schedule reported before for pig pituitary extract (Dieleman *et al.*, 1989). rhFSH was administered twice a day at 07:00 h and 19:00 h in decreasing doses of 600, 500, 300 and 100 iu at days 10, 11, 12 and 13, respectively. As the use of rhFSH for superovulation of cattle has not been reported previously, the initial administration dose of 3 iu rhFSH kg^{-1} per day and the total dose of rhFSH (3000 iu) were based on reported doses of human menopausal gonadotrophin for superovulation of cattle and rhFSH for human treatment (Out *et al.*, 1998). During treatment with rhFSH, 15 mg prostaglandin was injected i.m. on day 12 at 07:00 h, simultaneously with the fifth injection of rhFSH.

In the eCG group ($n = 9$; sessions 1, 2 and 3), 2500 iu eCG (Folligon; Intervet International BV) was administered i.m. to all animals on day 10 at 07:00 h and 15 mg prostaglandin was administered i.m. on day 12 at 07:00 h. The same batch of eCG was used for all heifers. These heifers were injected i.v. with a monoclonal antibody against eCG (Neutra-PMSG; Intervet International BV) 6 h after the peak of the LH surge (Dieleman and Bevers, 1987).

Follicular development and hormonal response

Follicular development, and progesterone, oestradiol and LH concentrations in peripheral blood during and after gonadotrophin treatment were compared in heifers treated with either rhFSH ($n = 9$) or eCG ($n = 9$). From day 10 to day 13, individual follicles > 3 mm in diameter were identified on both ovaries using a linear array 7.5 MHz ultrasound scanner (type SSD-210; Aloka, Tokyo). The sizes of the follicles were recorded as the mean of two perpendicular diameters of the antrum of the follicle as measured with built-in callipers. The accuracy between subsequent measurements was ± 1 mm for follicles ≤ 8 mm in diameter. The position of the follicles (> 3 mm in diameter) on the ovaries was registered by photographs taken by video copy (type P60B; Mitsubishi, Tokyo). The follicles were classified into three categories: < 5 mm, 5–8 mm and > 8 mm in diameter; the category > 8 mm was defined as preovulatory-sized follicles (Vos *et al.*, 1994). The same two operators undertook scanning for all days. The ovulation rate was determined for six heifers per group (sessions 1 and 2) on day 7 after oestrus either by palpation per rectum and ultrasonography ($n = 6$; during session 1) by four skilled technicians or after the animals were killed ($n = 6$; during session 2). The numbers of corpora lutea and follicles > 8 mm in diameter on each ovary were recorded. The hormonal response was investigated further by comparing the pulsatile patterns of LH secretion during the follicular phase after prostaglandin injection in three heifers per group (session 2).

Oocyte maturation rate of preovulatory follicles and progesterone or oestradiol dominance of follicular fluids

During session 3, rhFSH- ($n = 3$) and eCG- ($n = 3$) treated heifers were compared for the nuclear and cytoplasmic maturation status of oocytes, and progesterone or oestradiol dominance (progesterone:oestradiol > 1 or progesterone:oestradiol < 1 , respectively) of follicular fluids. The time of the LH surge was determined by measuring the LH concentration in plasma collected at 1 h intervals from 24 h after the prostaglandin injection onwards by a rapid radioimmunoassay. Exactly 24 h after the LH surge (around the expected time of ovulation), the ovaries of the heifers were recovered by ovariectomy as described by de Loos *et al.* (1991). Follicular fluid and oocytes were collected from individual non-atretic preovulatory follicles (> 8 mm in diameter) by aspiration. After retrieval of the oocytes, the follicular fluids were stored at -25°C and progesterone and oestradiol concentrations were assessed by radioimmunoassay. Cumulus-oocyte complexes (COCs) were judged on cumulus expansion, the oocytes were freed subsequently from cumulus cells by vortexing for 3 min and were then fixed for 15 min in 2% (w/v) paraformaldehyde in PBS. As a measure for cytoplasmic maturation, the distribution of cortical granules was assessed using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining as described by Izadyar *et al.* (1998) combined with 4',6-diamidino-2-phenylindole (DAPI; Sigma) staining to determine the stage of nuclear maturation (Izadyar *et al.* 1998). Slides were evaluated under an epifluorescence microscope and the oocytes were classified into three types on the basis of the distribution pattern of the cortical granules: type 1: large aggregates of cortical granules distributed over the entire cytoplasm; type 2: cortical granules localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates; or type 3: cortical granules more or less evenly dispersed in the cortical cytoplasm aligning the oolemma.

In addition, 12 oocytes from the eCG group were investigated by transmission electron microscopy (TEM) to compare the ultrastructural location of cortical granules with the image obtained after FITC-PNA staining. In brief, the oocytes were embedded and cut into semi-thin (2 μm thickness) sections. All semi-thin sections (approximately 60) were stained with toluidine blue and analysed by light microscopy to determine the meiotic stage of each oocyte. Subsequently, the equatorial section of each oocyte was re-embedded, cut into ultrathin sections (70 nm thickness), contrasted by lead citrate and uranyl acetate, and examined in a Philips CM 100 transmission electron microscope for cortical granule morphology. The meiotic stage and cortical granule distribution were evaluated as described by Hyttel *et al.* (1986).

Blood sampling and radioimmunoassay of progesterone, oestradiol and LH

Blood samples were taken from the jugular vein into

10 ml heparinized vacuum tubes, cooled immediately and centrifuged for 10 min at 500 *g* at 4°C. Plasma was either stored at -25°C or used immediately for rapid radioimmunoassay of LH and stored afterwards. Blood samples were taken once a day from day 0 (experimental day 12) of the synchronized cycle until 1 day before injection of rhFSH or eCG, and then at 6 h intervals until 24 h after injection of prostaglandin, subsequently at 1 h intervals until 72 h after prostaglandin treatment for the rapid LH radioimmunoassay to detect the LH surge, then at 6 h intervals until 96 h after prostaglandin treatment and, finally, once a day until day 7 after oestrus. Jugular catheters were used to collect blood samples at 10 min intervals for evaluation of pulsatile LH patterns.

Concentrations of progesterone and oestradiol were estimated by validated solid phase ¹²⁵I radioimmunoassay methods (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles, CA) as described by Dieleman and Bevers (1987). The sensitivities were 0.05 ng ml⁻¹ and 2 pg ml⁻¹, the interassay coefficients of variation were < 11 and 8.9%, and the intra-assay coefficients of variation were 8 and 9%, for progesterone and oestradiol, respectively.

For the rapid determination of LH concentrations, samples were estimated in triplicate by a rapid solid phase radioimmunoassay using bovine bLH-7981 for iodination and the antiserum of the standard assay (Dieleman *et al.*, 1983). This rapid radioimmunoassay allowed determination of the LH concentration within 4 h after blood sampling. The LH concentration of blood samples during the LH surge was estimated afterwards by a standard assay as well, to validate the timing of the peak of the LH surge. The intra- and interassay coefficients of variation were < 9%. The sensitivity was 0.4 ng ml⁻¹ NIH-LH-B4. Cross-reactivity with eCG (highly purified eCG, PM23-2P; Intervet International BV) was 0.2%.

Data handling and statistical analyses

All data are presented as mean ± SEM. Student's *t* test was used for most comparisons of the mean of two groups. Generalized Estimated Equation analysis (S-plus 2000; MathSoft, Inc., Cambridge, MA) was used for the analysis of time-dependent data, such as: (i) follicular growth; (ii) plasma oestradiol concentrations; and (iii) plasma progesterone concentrations. Data were considered significantly different at *P* < 0.05.

The mean progesterone:oestradiol ratio in follicular fluid per follicle (Table 1) was calculated by taking the mean of the ln values of the progesterone:oestradiol ratios and calculating this back again via e^x.

The patterns of pulses in the 8 h profiles of LH were analysed according to Kooistra *et al.* (1999) by means of the Pulsar program developed by Merriam and Wachter (1982). The program identifies secretory peaks by height and duration from a smoothed baseline using the SD as a scale factor. The cut-off parameters G1–G5 of the Pulsar program

Fig. 1. Follicular response of heifers to stimulation with either equine chorionic gonadotrophin (eCG; *n* = 9) or recombinant human FSH (rhFSH; *n* = 9). Numbers of small (□: < 5 mm in diameter), medium (■: 5–8 mm in diameter) and large (▨: > 8 mm in diameter) follicles are shown from day 0 (at the start of stimulation) to day 3 of superovulation treatment. Values are mean ± SEM.

were set at 3.80, 2.26, 1.56, 1.13 and 0.83 times the assay SD as criteria for accepting peaks 1, 2, 3, 4 and 5 points wide, respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 6.5 h. The splitting cut-off parameter was set at 2.7 and the weight assigned to peaks was 0.01. The A, B and C values of the Pulsar program, used to calculate the variance of the assay, were set at A = 4.81, B = -0.99 and C = 6.16. The values extracted from the Pulsar analyses included the number of peaks, mean peak amplitude, mean of the smoothed baseline, pulse frequency, mean pulse duration and the area under the curve.

Results

Follicular development (sessions 1, 2 and 3)

The number and ratio of small (< 5 mm in diameter), medium (5–8 mm in diameter) and large (> 8 mm in diameter) follicles in heifers were not significantly different between heifers treated with rhFSH (*n* = 9) or eCG (*n* = 9) at each day during superovulation treatment (Fig. 1). The number of large (> 8 mm in diameter) follicles in both groups increased significantly during all days of gonadotrophin treatment (*P* < 0.05). At day 3 of stimulation treatment, the mean numbers of large follicles were 22.0 ± 3.5 and 17.7 ± 2.5 follicles for the rhFSH- and eCG-treated heifers, respectively; these values are not significantly different.

Ovulation rate (sessions 1 and 2)

Although the rhFSH treatment always resulted in occurrence of an LH surge, only three of the six heifers analysed showed multiple ovulations. In contrast, only four of the six eCG-treated heifers underwent a preovulatory LH

Fig. 2. Oestradiol concentrations in heifers during stimulation with either equine chorionic gonadotrophin (eCG; $\blacktriangle$; $n = 7$) or recombinant human FSH (rhFSH; $\blacksquare$; $n = 9$), (a) from the start of stimulation onwards and (b) relative to the time of the preovulatory LH surge. The two eCG heifers without an LH surge are not included. Values are mean $\pm$ SEM.

surge, but these four heifers all showed multiple ovulations. As expected, no ovulations occurred in the two eCG-treated heifers without a preovulatory LH surge. The four eCG-treated heifers with an LH surge had a mean 11.8 ± 2.3 corpora lutea and 4.8 ± 1.5 unruptured follicles > 8 mm in diameter, whereas the six rhFSH-treated heifers had a mean 5.5 ± 2.4 corpora lutea and 12.2 ± 3.6 unruptured follicles. The difference in ovulation rate between the rhFSH- and eCG-treated heifers, although not significantly different, was due entirely to the three rhFSH-treated heifers that did not undergo multiple ovulations.

Hormonal profiles (sessions 1, 2 and 3)

All nine heifers treated with rhFSH underwent an LH surge that occurred a mean 47.1 ± 1.5 h after prostaglandin injection. In the eCG-treated group, seven of nine heifers underwent an LH surge that occurred a mean 39.4 ± 1.9 h

after prostaglandin injection, which was significantly earlier than in the rhFSH group ($P < 0.01$). The amplitudes of the LH surge in the rhFSH (19.6 ± 1.3 ng ml⁻¹) and eCG (15.9 ± 2.3 ng ml⁻¹) groups were not significantly different. Furthermore, the mean amplitude of the LH surge of the three rhFSH heifers without multiple ovulations (19.4 ± 1.7 ng ml⁻¹) was not significantly different from that of the other six heifers (19.7 ± 1.8 ng ml⁻¹).

During the superovulation treatment, the mean oestradiol concentration of the rhFSH group ($n = 9$) was significantly lower than that of the eCG-treated group from 12 h onwards ($n = 7$; Fig. 2a). The mean oestradiol concentration of the rhFSH-treated group was also significantly lower than that of the eCG-treated group at the days of the LH surge (63.2 ± 10.1 versus 110.8 ± 28.0 pg ml⁻¹, respectively; Fig. 2b). At the day of the LH surge, the oestradiol concentration in blood calculated per follicle of the rhFSH-treated heifers was approximately 50% of that of the eCG-treated heifers (2.9 ± 0.2 versus 5.6 ± 0.6 pg ml⁻¹, respectively). The oestradiol concentration profiles of the two eCG-treated heifers that did undergo an LH surge did not differ from those of the other eCG-treated heifers until the day corresponding to the day of the LH surge. Likewise, there was no significant difference in oestradiol production between the rhFSH-treated heifers with and without ovulations in the period up to the LH surge. In all eCG-treated heifers and the three rhFSH-treated heifers with multiple ovulations, the oestradiol concentration decreased from the day of the LH surge onwards (Fig. 2b; sessions 1 and 2). In contrast, an increase in oestradiol concentration was observed during the days after the LH surge in the three rhFSH-treated heifers without multiple ovulations before the subsequent decrease in oestradiol concentrations (Fig. 3; only rhFSH-treated heifers are shown).

At the start of the gonadotrophin treatment the eCG-treated heifers ($n = 4$) and rhFSH-treated heifers ($n = 6$) had similar progesterone concentrations (4.2 ± 0.4 versus 4.2 ± 0.4 ; sessions 1 and 2, two eCG-treated heifers without an LH surge were not included). eCG but not rhFSH induced a significant increase in progesterone concentration at days 1 and 2 after the start of treatment, which reached 9.1 ± 0.8 ng ml⁻¹ for the eCG-treated heifers versus 5.1 ± 0.3 ng ml⁻¹ for the rhFSH-treated heifers at day 2 (the day of prostaglandin administration). This finding indicates that eCG has a stimulating effect on the corpus luteum. After the LH surge, the newly formed corpora lutea of the eCG-treated heifers also produced significantly higher progesterone concentrations than did the corpora lutea and luteinized follicles of the rhFSH-treated heifers from day 2 after ovulation onwards. The progesterone concentrations of the eCG- and rhFSH-treated heifers were 4.9 ± 1.2 versus 2.6 ± 0.4 ng ml⁻¹, respectively, at day 2 after ovulation, and 42.8 ± 4.0 versus 26.8 ± 4.2 ng ml⁻¹, respectively, at day 6 after ovulation. The rhFSH-treated heifers without multiple ovulations did not differ in progesterone production from the rhFSH-treated heifers that underwent multiple ovulations (results not shown).

Fig. 3. Oestradiol concentrations of individual recombinant human FSH (rhFSH)-stimulated heifers around the time of the preovulatory LH surge (indicated with a vertical line). (a,c,e) Three heifers without multiple ovulations and (b,d,f) three heifers with multiple ovulations.

LH pulsatile patterns (session 2)

The patterns of LH concentrations in three eCG-treated and three rhFSH-treated heifers 27–35 h after prostaglandin injection are shown (Fig. 4). The eCG-stimulated heifers had more LH pulses per heifer (mean 3.0 ± 1.7 per 8 h) than did the rhFSH-stimulated heifers (mean 0.3 ± 0.3 per 8 h). The rhFSH- and eCG-treated heifers had a similar baseline LH concentration (0.51 ± 0.01 and 0.45 ± 0.02 ng ml⁻¹, respectively) and a similar mean LH concentration (4.1 ± 0.1 and 4.3 ± 0.2 , respectively). It should be noted that two of the three eCG-treated heifers were closer to the preovulatory LH surge than the three rhFSH heifers (maximum of the LH surge at 37 and 38 h versus 41, 44 and 49 h after prostaglandin injection, respectively). In these two eCG-treated heifers, the start of the preovulatory LH surge had already occurred during the second half of the sampling period. The third eCG-treated heifer, which had the highest number of LH pulses ($n = 6$) did not undergo an LH surge. The numbers of follicles > 8 mm in diameter on

day 3 of superovulation treatment were 16, 17 and 17 (mean 16.7 follicles) in the eCG-treated group and 22, 14 and 47 (mean 27.7 follicles) in the rhFSH-treated group; these numbers of follicles are not significantly different.

Nuclear and cytoplasmic maturation of oocytes 24 h after the LH surge (session 3)

At the time of ovariectomy (24 h after the preovulatory LH surge), no follicles had undergone ovulation in the rhFSH ($n = 3$) and eCG ($n = 3$) groups. The mean number of preovulatory sized follicles of rhFSH- (25.7 ± 8.7) and eCG- (30.3 ± 4.4) treated heifers was not significantly different. A total of 66 and 82 preovulatory follicles of rhFSH and eCG groups, respectively, were aspirated, from which 56 (84.8%) and 65 (79.3%) COCs were recovered, respectively. Oocytes for which the nuclear stage could not be detected due to technical reasons were excluded from the results (rhFSH group: two oocytes; eCG group: one oocyte).

Fig. 4. LH pulsatile secretion patterns from blood samples collected at 10 min intervals during an 8 h period from 27 to 35 h after prostaglandin injection. Heifers were stimulated with either equine chorionic gonadotrophin (a,c,e: heifers 1, 2 and 3, respectively) or recombinant human FSH (b,d,f: heifers 4, 5 and 6, respectively). Arrows indicate LH pulses.

Large variation of cumulus expansion rates was observed for both the rhFSH-treated heifers (range 25–95%) and the eCG-treated heifers (range 45–100%) (Table 1). The relationships between stage of nuclear maturation and distribution patterns of cortical granules of the oocytes are summarized (Table 1). The rate of nuclear maturation was not significantly different between the rhFSH (63%, $n = 53$) and eCG (67%, $n = 33$) groups. However, the percentage of metaphase II stage oocytes with type 3 cortical granule distribution was much lower in the rhFSH group (7%) than in the eCG group (58%). There was a correlation between the percentage of oocytes with an expanded cumulus and the percentage of oocytes in metaphase II for both the rhFSH and eCG groups. For example, for the rhFSH group, heifer 3, in which 95% of the COCs had an expanded cumulus, had a metaphase II rate of 95%, whereas heifer 1, in which the cumulus expansion rate was 25%, had a metaphase II rate of 29%.

The transmission electron microscopy (TEM) study of 12

oocytes with an expanded cumulus from eCG-treated heifers confirmed the reliability of the FITC–PNA staining technique for assessing the distribution of the cortical granules. Eight of the twelve oocytes were in metaphase II, and these all showed a type 3-like distribution of the cortical granules. The other four oocytes were in metaphase I and had either a type 1 ($n = 2$) or type 3 ($n = 2$) distribution of the cortical granules. Therefore, the TEM observations were consistent with those after FITC–PNA staining.

Progesterone and oestradiol concentrations of follicular fluids (session 3)

The mean progesterone:oestradiol ratio per heifer is shown in combination with the oocyte maturation states (Table 1). All 66 follicles of the three rhFSH-treated heifers and all 52 follicles of two eCG-treated heifers (heifers 5 and 6) were progesterone-dominated (progesterone:oestradiol > 1). However, in one eCG-treated heifer (heifer 4), 90% of

Table 1. Maturation status of oocytes and steroid hormone concentrations of follicular fluid from individual heifers stimulated with recombinant human FSH (rhFSH) or equine chorionic gonadotrophin (eCG)

Heifer number	Total number of oocytes	Cumulus expansion (%)	Number of oocytes at metaphase I				Number of oocytes at metaphase II				Number of degenerated oocytes				Mean progesterone (ng ml ⁻¹)	Mean oestradiol (ng ml ⁻¹)	Progesterone: oestradiol				
			T1		T2		T3		nd		T1		T2					T3		nd	
rhFSH																					
Heifer 1	24	25	0	4	0	0	0	7	0	0	0	13	0	0	214	48	4.1				
Heifer 2	8	75	0	0	0	0	3	3	1	0	1	0	0	0	308	26	11				
Heifer 3	21	95	0	0	0	0	5	8	3	4	1	0	0	0	582	5.3	122				
Total	53		0	4	0	0	8	18	4	4	2	13	0	0							
(%)			(7)				(15)	(34)	(7)	(7)	(4)	(25)									
eCG																					
Heifer 4	10	45 ^a	5	0	0	2	0	1	2	0	0	0	0	0	88	150	0.5				
Heifer 5	10	83 ^a	0	0	2	0	0	1	7	0	0	0	0	0	210	47	3.8				
Heifer 6	13	100 ^a	1	1	0	0	0	1	10	0	0	0	0	0	196	62	3.3				
Total	33		6	1	2	2	0	3	19	0	0	0	0	0							
(%)			(18)	(3)	(6)	(6)		(9)	(58)												

T1: cortical granules as large aggregates located over the entire cytoplasm; T2: cortical granules localized in the periphery as small aggregates and as individual particles; T3: cortical granules dispersed evenly as individual particles in the cortical cytoplasm aligning the oolemma. nd: oocytes from which the cortical granule distribution pattern could not be determined due to technical failure; degenerated oocytes: oocytes with degenerated chromatin.

Progesterone: oestradiol: mean of the progesterone: oestradiol ratios of the individual follicles and, therefore, is different from the mean progesterone ratio divided by the mean oestradiol ratio.

^aPart of the oocytes of the eCG-treated heifers were used for electron microscopic study. The incidence of cumulus expansion is given for the entire group of oocytes ($n = 20, 23$ and 22 for heifers 4, 5 and 6, respectively).

the follicles were oestradiol-dominated. In comparison to the other eCG heifers, heifer 4 had the lowest incidence of both cumulus expansion (45%) and oocytes in metaphase II (three of ten oocytes). The mean oestradiol concentration of the follicular fluids of the rhFSH group (28.1 ± 3.5 ng ml⁻¹; $n = 66$) was significantly lower than that of the eCG group (89.4 ± 7.2 ng ml⁻¹; $n = 82$). In contrast, the mean progesterone concentration of the follicular fluids of the rhFSH group (369.1 ± 34.0 ng ml⁻¹; $n = 66$) was significantly higher than that of the eCG group (160.7 ± 17.7 ng ml⁻¹; $n = 82$).

Discussion

The main aim of the present study was to investigate whether human FSH without contaminating LH can exert a normal superovulation response in cows. It was found that exogenous LH is not necessary for stimulation of growth of follicles beyond the stage of 8 mm in diameter and that these follicles are competent to induce an LH surge. However, the rhFSH-treated heifers had a higher incidence of aberrations in final follicular maturation. Three of six rhFSH-treated heifers did not undergo multiple ovulations and oocytes collected 24 h after the LH surge had a lower rate of cortical granule distribution at the periphery, which is indicative of less optimal cytoplasmic maturation.

The follicular response to eCG observed in the present study was similar to that reported previously (Bever and Dieleman, 1987; Dieleman *et al.*, 1989). Despite the equal sizes of the follicles in the eCG and rhFSH groups, the eCG-stimulated follicles produced much higher amounts of oestradiol than did the rhFSH-stimulated follicles. Oestradiol production in the eCG-treated heifers was a mean 4.5 times higher at 72 h after the start of stimulation and two times higher at the time of the LH surge than that of rhFSH-treated heifers. This finding is in line with earlier findings on comparisons between gonadotrophin preparations differing in LH activity (Aoyagi *et al.*, 1987; Ben Jebara *et al.*, 1994) and confirms the stimulating effect of exogenous LH activity on oestradiol production in bovine follicles (Webb *et al.*, 1999). As oestradiol is the key factor for inducing the LH surge (Karsch *et al.*, 1992), the earlier occurrence of the LH surge in the eCG group (39 ± 2 h after prostaglandin injection) compared with the rhFSH group (47 ± 2 h after prostaglandin injection) is probably a direct consequence of the higher oestradiol concentration in the eCG-treated heifers. The increase in progesterone concentration on days 1 and 2 after eCG stimulation but not after rhFSH stimulation is also probably due to the LH activity, which stimulates follicles to produce more oestradiol and cyclic corpora lutea to produce more progesterone. This effect of LH activity in gonadotrophin preparations has been described previously by Aoyagi *et al.* (1987) and Ben Jebara *et al.* (1994). It is remarkable that after ovulation the newly formed corpora lutea in the eCG-treated heifers have a higher progesterone-producing activity than the corpora lutea in the rhFSH-treated heifers.

This finding indicates that the eCG-stimulated follicles either contained more follicular cells that could luteinize or that the corpora lutea have a higher progesterone production per luteinized cell.

Gonadotrophin stimulation is known to result in a higher incidence of asynchrony of: (i) the maturation state of the follicle versus its oocyte (de Loos *et al.*, 1991); and (ii) the maturation state of the nucleus versus that of the cytoplasm of the oocyte (Hyttel *et al.*, 1986; Assey *et al.*, 1994). The relatively high variations in steroid concentrations in follicular fluid and maturation status of the oocytes observed in the present study are in agreement with this. The finding that only 7% of the oocytes of the rhFSH-treated heifers had a type III cortical granule distribution points clearly to a less optimal cytoplasmic maturation in most of the follicles in this treatment group. The less optimal oocyte maturation in the rhFSH group was not related to an aberrant ratio of progesterone:oestradiol in the follicular fluid, which was > 1 in all large follicles; in addition, it was not related to the proportion of oocytes with an expanded cumulus.

Experiments in non-stimulated cows have demonstrated that LH is essential for follicular growth beyond 8 mm in diameter (Gong *et al.*, 1996). Obviously, the rhFSH-stimulated heifers had sufficient endogenous LH activity to sustain the growth of a large number of follicles beyond 8 mm in diameter. However, the frequency of LH pulses was very low in the rhFSH-treated heifers and lower than that of the eCG-treated heifers. In the present study, the LH pulses were measured in a fixed time period after prostaglandin administration, which happened to be closer to the LH surge in two of the three eCG-treated heifers than in the three rhFSH-treated heifers. Therefore, the possibility that the LH pulsatility increased in the rhFSH-treated heifers in the period closer to the LH surge cannot be excluded totally. However, the highest number of LH pulses was found in the eCG-treated heifer that did not undergo an LH surge. Downregulation of the preovulatory LH pulses by gonadotrophin stimulation has been demonstrated in several studies (Bever *et al.*, 1989; Ben Jebara *et al.*, 1994; Roberge *et al.*, 1995; Price *et al.*, 1999; Gosselin *et al.*, 2000). Consistent with our results, this downregulation was found more apparent when relatively pure FSH preparations were used instead of eCG (Ben Jebara *et al.*, 1994). At least in unstimulated cows, downregulation of the preovulatory LH pulses has been correlated with failures in ovulation (Roche *et al.*, 2000). The preovulatory LH pulses have also been proposed to induce the maturation-like changes in the COC that already start in the period preceding the LH surge (Hyttel *et al.*, 1997). In gonadotrophin-stimulated cows, the LH pulse frequency during preovulatory follicular development is positively correlated with the number of corpora lutea and total number of embryos (Roberge *et al.*, 1995). The mechanism by which gonadotrophin stimulation suppresses the LH pulsatility is still not known. Our results confirm those of Gosselin *et al.* (2000) that if an ovarian factor is involved in the downregulation of LH

pulsatility, it is a compound other than oestradiol. The exact role of the preovulatory LH pulses for preovulatory follicular development is poorly understood and it is also unclear whether this role can be replaced by administration of a constant level of extraneously supplied LH.

The lack of ovulations in three of six rhFSH-treated heifers and the aberrant oocyte maturation in all rhFSH-treated heifers might also be related to an aberrant expression of follicular LH receptors. An indication for this is that all three rhFSH heifers without multiple ovulations reacted with a transient increase in the oestradiol concentration during the days after the LH surge, although the oestradiol concentration normally decreases rapidly after the LH surge (Bever and Dieleman, 1987). However, these heifers underwent a similar progesterone production in response to the LH surge as the rhFSH heifers with multiple ovulations, indicating that LH receptors were present within the large follicles. However, the increase in oestradiol concentration indicates that at least some of the granulosa cells of some follicles did not luteinize, which might point to an absence of LH receptors on these aberrantly reacting granulosa cells. LH receptors are normally first expressed in granulosa cells in follicles reaching a size of 8 mm in diameter (Bao *et al.*, 1997). This process might be disturbed in rhFSH-stimulated heifers.

Using recombinant FSH of bovine origin for stimulation of cattle, Wilson *et al.* (1993) found an embryo yield similar to that of pituitary extracts. It can be questioned whether a suboptimal dose of rhFSH was used in the present study. Owing to the limited amounts of rhFSH available, dose-response studies could not be applied. The different ways of calculation of the FSH units do not allow comparison of the dose of FSH used in the present study with that of Wilson *et al.* (1993). However, the good follicular response of the rhFSH heifers without symptoms of hyperstimulation gives a strong indication that the chosen dose was appropriate. Furthermore, the risk of hyperstimulation has been reported to be much lower for FSH preparations with a low LH activity (McGowan *et al.*, 1985; Pawlyshyn *et al.*, 1986; Gonzalez *et al.*, 1990). Wilson *et al.* (1993) collected more than two ova or embryos per cow after stimulation with doses of rbFSH ranging from 4 to 64 mg. In concordance with our data, highly purified pig FSH isolated from pituitary glands has also been reported to exert poor superovulation responses, which could be overcome by adding a minimal amount of LH (Schmidt *et al.*, 1988; Herrler *et al.*, 1991). The different superovulation responses of rbFSH and rhFSH compared with highly purified pig FSH raise the interesting question that this might be due to differences among species. Bovine, human and pig FSH might differ in binding affinity or activation of bovine FSH and LH receptors or in degradation time in cows.

In conclusion, the suboptimal results after rhFSH stimulation might be best explained with the following model. The rhFSH-stimulated heifers have endogenous LH activity to sustain the growth of a large number of follicles

beyond 8 mm in diameter. However, the combination of a complete lack of exogenous LH with a severe suppression of preovulatory LH pulses leads to suboptimal follicular development so that the follicles do not respond optimally to the LH surge. This process might be mediated by impaired expression of LH receptors on the granulosa cells. Finally, this process results in a disturbed cytoplasmic maturation of the oocytes in the main part of the follicles and an impaired ovulation process in some of the heifers. In other gonadotrophin preparations the downregulation of the LH pulsatility might be compensated for by administration of extraneous LH activity which is lacking in rhFSH. These results lead to the question of whether pure FSH of human, bovine and pig origin exerts different effects in cattle. In addition, the stimulation of cattle with rhFSH might provide a useful model for further basic research on follicular development, oocyte maturation and ovulation in cattle, which might lead to more insight into ovarian dysfunction of humans, such as luteinized unruptured follicles.

M. Takagi was supported financially by a grant from the Ministry of Education, Science and Culture of Japan. P. J. M. Hendriksen was supported by Holland Genetics (Arnhem, The Netherlands) and the European Union. Human recombinant FSH was kindly supplied by R. G. Hanssen, Organon, Oss, The Netherlands. The authors would like to thank D. M. Blankenstein for assistance and performance of the radioimmunoassay hormone analyses, H. T. M. van Tol and E. Zeinstra for assistance during oocyte collection and J. C. M. Vernooy for the statistical analyses. The authors would also like to thank the animal caretakers and surgery assistants for management and surgical preparation of the animals.

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Received 1 December 2000.

First decision 22 January 2001.

Accepted 26 February 2001.