A Simple Superovulation Method of A Single Injection of Follicle-Stimulating Hormone Combined with Equine Chorionic Gonadotropin for Superovulation of Suffolk Ewes During the Breeding Season: I. Effects of Different Treatments on the Endocrine Profiles

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Abstract. Two trials were conducted to investigate a simple superovulation method for Suffolk ewes during the breeding season. The method involves a single injection of porcine follicle-stimulating hormone (pFSH) combined with equine chorionic gonadotropin (eCG) and was evaluated by measuring the concentrations of plasma hormones (estradiol-17 β (E₂), progesterone (P₄), luteinizing hormone (LH) and follicle-stimulating hormone (FSH)). The ewes were treated with a vaginal sponge containing synthetic steroid with progestative action (P sponge) for 12 days in both trials. In Trial 1 (conducted in 1995), 18 ewes received a single injection (F1) or multiple injections (F6) of a total of 20 mg pFSH, and in combination with an injection of 500 IU eCG (F1+P, F6+P). The injections were conducted on Day - 2 (Day 0 was the day of P sponge removal). In trial 2 (conducted in 1996), 24 ewes were given single injections of pFSH and eCG at different times; (i) both hormones on Day - 2 (F-2/P-2), (ii) pFSH on Day -2 and eCG on Day - 1 (F-2/P-1), and (iii) both hormones on Day - 1 (F-1/P-1). One hundred micrograms of an analogue of gonadotropin-releasing hormone (GnRH) was administered on Day 1 in Trial 1 and at the detection of estrus in Trial 2. Frozen-thawed semen was inseminated in utero using a laparoscope at 30 h and 38 h after P sponge removal in Trial 1 and at 18 hours after the detection of estrus in Trial 2. Blood samples were collected from the jugular vein. E₂, P₄, LH and FSH were assayed by second-antibody enzyme immunoassays. F1+P showed similar endocrine profiles to those of F6 and F6+P with a preovulatory E₂ peak after P sponge removal, but F1 did not show a clear preovulatory E₂ peak. The E₂ concentration in F-2/P-1 rose biphasically; there was a rise just after injection of pFSH and then another rise after P sponge removal. Simultaneous injections of 20 mg pFSH and 500 IU eCG may reduce the E₂ elevation. We concluded that a good ovarian response comparable to the multiple injection method can be achieved by the present simple superovulation method.

Key words: pFSH, eCG, Superovulation, Ewes, Endocrine profiles.

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FSH or eCG are gonadotropins widely utilized for induction of superovulation in ewes. Because of the short half-life (30–110 min) of FSH, it

is necessary to inject it repeatedly [1], and the administration of decreasing doses gave more successful results than that administering of a constant dose [2]. Multiple injections of FSH in decreasing doses resulted in increasing the number of corpora lutea, the number of recovered

oocytes and the pregnancy rate following transfer in cows [3]. In ewes, a single high dosage of eCG (1,000–1,200 IU) could induce a more successful superovulation than multiple injections of FSH [4]. However, the administration of a high dosage of eCG is not recommended owing to its long half-life period, which varies from 50–120 h [1]. Its relatively high LH-like activity may cause abnormal premature activation of sheep oocytes, and the follicular environment would become unsuitable for maturation. Therefore the oocytes would prematurely age before ovulation [5], resulting in decreasing the yield of fertilized eggs [6]. Furthermore, a high dose of eCG induces many anovulatory follicles [7]. Multiple injections of a high dose of FSH combined with a low dose of eCG is now conventionally used in ewes to induce superovulation [2, 4, 8], as it can induce a higher ovulatory response than the multiple injections of FSH alone [9]. However, the use of multiple injections, such as 6 times during 2 or 3 days, increases the workload as well as causes stress in animals.

Evans *et al.* [10] showed that a single injection of a high dose (15 mg) of FSH in combination with a low dose (300 IU) of eCG resulted in a satisfactory superovulation and embryo recovery as compared with multiple injections of FSH combined with a low dose of eCG in ewes, and equally successful results were obtained in goats [11]. Yamada *et al.* have also reported similar results in ewes during the breeding season [12] and anestrus season [13]. However, the endocrinological basis of this simple superovulation method is not well understood. In order to obtain a better understanding of the endocrine profiles around ovulatory periods caused by a simple superovulation method, we compared the endocrine profiles of a single injection of 20 mg pFSH and multiple injections of the same dose of pFSH, and in combination with the administration of 500 IU eCG in Trial 1. In Trial 2, we further observed the endocrine profiles after single injections of 20 mg pFSH and 500 IU eCG at three different times.

Materials and Methods

Animals

Trial 1 was conducted in 1995 (September to November) with 18 parous 2- to 6-year old Suffolk ewes and Trial 2 was conducted in 1996 (October

to December) with 24 non-parous 2- to 3-year old Suffolk ewes. The ewes were fed hay and concentrates with water provided *ad libitum*.

Treatments

To synchronize estrus of ewes, which were at unknown stages of the estrous cycle, intravaginal sponges (P sponge) were inserted for 12 days before experiments. In Trial 1, the sponges which were impregnated with 60 mg 6-methyl-17-acetoxypregnosterone (MAP: The Upjohn International Ltd, Australia) were used and in Trial 2, the sponges which were impregnated with 40 mg Flugestone acetate (FGA, Chrono-gest: Intervet International B.V., Holland) were used.

In Trial 1, ewes were divided into 4 groups to investigate the effects of a single intramuscular injection of 20 mg pFSH (Antorin: Denka Chemical Co., Japan) and the combination with an intramuscular injection of eCG (Serotropin: Teikokuzoki Co., Japan). The first group (F1: N=4) received a single injection of 20 mg pFSH on Day -2 (Day 0 = the day of P sponge removal). The second group received multiple injections (F6: N=4) of the same total dose of pFSH in decreasing doses (5, 5, 3, 3, 2, 2 mg) over Day -2 to Day 0. The third and fourth groups received an additional injection of 500 IU eCG on Day -2 (F1+P: N=6 and F6+P: N=4) to the treatments for the first and second groups, respectively.

Trial 2 was designed to study the optimum timing for the injections of 20 mg pFSH and 500 IU eCG for a simple superovulations method. Ewes divided into 3 groups: one was treated with simultaneous injections of single injections of pFSH and eCG on Day -2 (F-2/P-2: N=9), the second was treated with a single injection of pFSH on Day -2 followed by a single injection of eCG on Day -1 (F-2/P-1: N=7), and the third was treated with simultaneous injections of both hormones on Day -1 (F-1/P-1: N=8).

The ewes in Trial 1 were examined for estrus every 2 h from -12 h to 36 h (0 h: the time of P sponge removal) using an entire ram aproned and equipped with marking harness and crayon. On Day 1, all ewes were injected intramuscularly with 100 µg analogue of gonadotropin releasing hormone (GnRH, Conceral: Takeda Industrial Co., Japan). Thirty h and 38 h after P sponge removal, frozen-thawed semen containing 4.8×10^8 spermatozoa per ewe, were deposited into the uterine

horns with an aid of a laparoscope according to the method of Maxwell *et al.* [14]. In Trial 2, the ewes were observed for estrus every 6 h from 6 h to 30 h, injected with 100 µg GnRH at the time of estrus detection, and inseminated artificially with a laparoscope 18 h after estrus detection with frozen-thawed semen containing 5.3×10^8 spermatozoa per ewe. For each trial, the semen was collected from 6 Suffolk rams by artificial vagina. The semen used in Trial 1 was collected in 1991 to 1994 and the semen used in Trial 2 was collected 1996. The good-quality semen were diluted 1:2 with diluent consisting of 300 mM Tris (hydroxymethyl aminomethane), 127.75 mM glucose, 94.75 mM citric acid, 15% v/v egg yolk and 5% v/v glycerol and were frozen, and thawed according to the method of Salamon and Visser [15] as modified by Fukui [16]. The oocyte recovery was conducted on Day 7 or Day 8 in Trial 1 and on Day 8 in Trial 2.

Blood sampling and P_4 , E_2 and LH assays

Blood samples were collected from all ewes in Trial 1 in EDTA-2Na evacuated tubes by jugular venipuncture. In trial 2, blood was collected from 4 ewes in each of the group. For the results, we used the data from the ewes having normal corpora lutea (Trial 1; F1 N=3, F1+P N=3, F6 N=3, F6+P N=4. Trial 2; F-2/P-2 N=2, F-2/P-1 N=4, F-1/P-1 N=3). Plasma was separated by centrifugation ($1630 \times g$, 15 min) at 4 °C and stored at -30 °C until assayed. The blood samples for measuring E_2 , P_4 and FSH were collected twice a day from Day -5 to Day -3, every 6 h from Day -2 to Day 1, and once a day from day 2 to the day of embryo recovery. The blood samples for the LH assay were collected every 2 h from -18 h to 36 h in Trial 1 and from 6 h to 38 h in Trial 2. Plasma E_2 [17] and P_4 [18] were extracted by diethylether and measured by the second-antibody enzyme immunoassays (EIA). LH [19] was measured by EIA using the biotin-streptavidin amplification technique. Intra- and inter-assay coefficients of variation were within 13%. The cross-reactivities of LH were 0.5% for pFSH and 4.3% for eCG, respectively.

FSH assay

Plasma concentrations of FSH were measured by a modified method for assay of LH by Mutayoba *et al.* [19]. The minimum and maximum

detection limit were 0.4 ng/ml and 25.0 ng/ml, respectively. The intra-assay and inter-assay coefficients of variation for N=106 samples were 13.8% and 16.7%. The correlation between values in EIA and RIA of the 106 samples was 0.86. The cross-reactivities of anti-oFSH were 0.9% for pFSH and 2.0% for eCG, respectively.

Statistical analysis

Data in the endocrine profiles were tested on uniformity of variance and analyzed by Duncan's multiple range test to examine the differences in the values between the groups in Trial 1 and Trial 2, and by a two sample t-test to compare the difference between the F1+P group in Trial 1 and the F-2/P-2 group in Trial 2.

Results

The effects of a single injection and multiple injections of 20 mg pFSH, and in combination with an injection of 500 IU eCG (Trial 1)

The effects of a single injection and multiple injections of 20 mg pFSH, and in combination with an injection of 500 IU eCG on E_2 levels are shown for a representative ewe in Fig. 1.

A high dose of pFSH injection induced an instant E_2 elevation just after the injection in three ewes of F1 group (Fig. 1, top panel). This was only the peak observed and was considered to be the main E_2 peak of the F1 group (Table 1).

In the F1 group, the preovulatory E_2 peak was not observed after P sponge removal. On the other hand, the F1+P, F6 and F6+P groups showed a highly elevated preovulatory E_2 peak that would cause a LH surge after P sponge removal (Fig. 1 and Table 1). Biphasic E_2 secretion was observed in all the F6+P ewes: the first E_2 peak was found just after P sponge removal, and was as high as the second E_2 peak, which was the main preovulatory peak. The main E_2 peak in F1+P was higher ($P<0.05$) and that of F6+P tended to be higher ($P=0.075$) than in the F1 and F6 groups (Table 1). After the preovulatory peak, the E_2 concentrations decreased quickly to basal levels in all groups.

The P_4 level was less than 1 ng/ml during the period from P sponge removal to 36 h in all groups and began to rise over 2 ng/ml from Day 2 to Day 7 (data not shown). GnRH were administered at 24 h, when the main E_2 peak was observed in Trial

Fig. 1. Representative changes of E_2 and LH concentrations in plasma after a single injection (F1 and F1+P) or a multiple injections (F6 and F6+P) of 20 mg pFSH, and in combination with 500 IU eCG (F1+P, F6+P) (Trial 1). #: The number of individual ewes; R and L: number of ovulations from the right and the left ovaries, respectively.

1, except for the F1 group. No preovulatory E_2 peak was observed in the F1 group. LH surges induced by GnRH injection were found around 26 h (Fig. 1).

The effects of single and multiple injections of pFSH on FSH levels are shown in Fig. 2. In this Figure, FSH data from the F1 and F1+P groups are pooled (upper panel) and the data from the F6 and F6+P groups are pooled (lower panel), since the changes in FSH concentration of the F1 and F1+P, and of the F6 and F6+P were similar, respectively. After a single injection of 20 mg pFSH, plasma concentrations of FSH peaked quickly, then decreased moderately with a slight oscillation. The FSH surge was induced by the GnRH injection concomitantly with the LH surge (Fig. 2). The FSH levels were maintained high by the multiple administration of pFSH for a longer period than by the single injection of pFSH (Fig. 2).

The effects of timing to inject 20 mg pFSH and 500 IU eCG (Trial 2)

The effects of timing to inject 20 mg pFSH and eCG on E_2 from the beginning of the treatment to P sponge removal are shown in Fig. 3. In this figure, the E_2 concentrations were averaged in each group. In contrast to the F-2/P-1 group, both the F-2/P-2 and F-1/P-1 groups which received simultaneous injections of pFSH and eCG did not show an E_2 rise just after the injections (Fig. 3). The E_2 levels at P sponge removal were higher in the groups receiving the pFSH injection on Day - 2 (F-2/P-2 and F-2/P-1) than that received the injection on Day - 1 (F-1/P-1) ($P<0.05$, Table 2).

The effects of the timing to inject 20 mg pFSH and 500 IU eCG on concentration of E_2 in plasma of a representative ewe are shown in Fig. 4. The E_2 profiles varied widely among the ewes. The E_2 profile in the F-2/P-1 group was biphasic. The

Table 1. The effects of a single (F1) or multiple injections (F6) of 20 mg pFSH, and in combination with 500 IU eCG (F1+P, F6+P) on the E_2 profiles

Treatment	F1 (N=3)	F1+P (N=3)	F6 (N=3)	F6+P (N=4)
Time of main E_2 peak (h)	-12.0 $\pm$ 3.5 ^a	20.0 $\pm$ 0.0 ^b	18.0 $\pm$ 0.0 ^b	22.5 $\pm$ 1.5 ^b
Conc. of main E_2 peak (pg/ml)	6.4 $\pm$ 2.0 ^c	28.7 $\pm$ 7.6 ^d	7.3 $\pm$ 4.0 ^c	22.3 $\pm$ 5.2 ^{cd}

Data are expressed as mean $\pm$ SEM. The times (h) are hours from P sponge removal. a, b) Values with different superscripts are significantly different ($P<0.01$). c, d) Values with different superscripts are significantly different ($P<0.05$).

Fig. 2. The effects of a single injection (F1) or multiple injections (F6) of 20 mg pFSH on the FSH concentrations in plasma (mean $\pm$ SEM). The upper panel shows pooled data of the F1 and F1+P groups and data in the lower panel shows pooled data of the F6 and F6+P groups.

Fig. 3. The effects of timing to inject a single dose of pFSH and eCG on the endocrine profiles of E_2 from the beginning of the treatment to P sponge removal. Ewes received a single injection of 20 mg pFSH and 500 IU eCG. (Δ), both hormones on Day - 2 (F-2/P-2 N=2); ($\square$), pFSH on Day - 2 and eCG on Day - 1 (F-2/P-1 N=4); ($\diamond$), both hormones on Day - 1 (F-1/P-1 N=3). *, values differ significantly from the basal concentration of E_2 ($P < 0.05$).

Table 2. The effects of timing of a single injection of 20 mg pFSH and 500 IU eCG on E_2 , F-2/P-2, both hormones given on Day - 2; F-2/P-1, pFSH given on Day - 2 and eCG given on Day - 1; F-1/P-1, both hormones on Day - 1

Treatment	F-2/P-2 (N=2)	F-2/P-1 (N=4)	F-1/P-1 (N=3)
E_2 conc. at sponge removal (pg/ml)	(8.3, 18.8) ^c	10.4 ± 1.5^d	2.1 ± 0.5^d
Time of main E_2 peak (h)	(18.0, 0.0)	4.5 ± 7.9	24.0 ± 0.0
Conc. of main E_2 peak (pg/ml)	(13.5, 18.8)	26.2 ± 10.0	12.1 ± 5.9
ΔE_2 , increment of E_2 after P sponge removal (pg/ml)	(5.2, 0.0)	15.8 ± 9.1	10.0 ± 5.4

Data are expressed as mean $\pm$ SEM. The times (h) are hours from P sponge removal. c, d) Values with different superscripts are significantly different ($P < 0.05$).

administration of pFSH alone on Day - 2 greatly increased E_2 concentrations just after the injection as same as in the F1 group, and then E_2 levels decreased, followed by a rise after P sponge removal as shown in Fig. 4. In the F-2/P-2 and F-1/P-1 groups, E_2 did not increase immediately after the injection of pFSH (Fig. 4, top and bottom panels, and Table 2).

The increment of E_2 after P sponge removal tended to be larger in the groups that received the eCG treatment on Day - 1 (F-2/P-1 and F-1/P-1) than

on Day - 2 (F-2/P-2, Table 2). The main E_2 peaks of F-2/P-1 tended to be the highest of all three treatments (Table 2). After the main peak, the E_2 levels decreased and became low in all groups.

The P_4 levels were less than 1 ng/ml during period from removal of the P sponge to 42 h in all the groups, and increased up to 2 ng/ml on Day 2 to Day 5 (data not shown).

Although we administrated GnRH at the detection of estrus in Trial 2, there was an inconsistency between timing of the main E_2 peak and the LH

Fig. 4. The effects of timing to inject a single dose of pFSH and eCG on the E₂ and LH concentrations in plasma. Ewes received a single injection of 20 mg pFSH and 500 IU eCG with following schedule. Top, both hormones were given on Day -2 (F-2/P-2). Middle, pFSH were injected on Day -2 and eCG on Day -1 (F-2/P-1). Bottom, both hormones were given on Day -1 (F-1/P-1). #: The number of individual ewes; R and L: number of ovulations from the right and the left ovaries, respectively.

peak. Two out of the four ewes in F-2/P-2 and two out of the four ewes in F-1/P-1 had a spontaneous LH surge before the GnRH injection, and one out of the four ewes in F-2/P-1 and one out of the four ewes in F-1/P-1 had a LH surge after the GnRH injection.

Discussion

The plasma E₂ profiles induced by a high dose of pFSH in combination with a low dose of eCG were almost the same as those of multiple injections with a high dose of pFSH. The present findings are comparable with those of Yamada *et al.* [12]. If the E₂ concentrations represent the number of maturing follicles [20], we can expect this simple superovulation method to result in ovulation and embryo recovery that are equal to those

obtained with commonly used method with the multiple administration of pFSH combined with eCG.

Twenty mg pFSH is divided into multiple injections (5, 5, 3, 3, 2, 2 mg) over 2 or 3 days in general superovulation. It must be injected repeatedly and at short intervals because of its short half-life [1]. A single injection of 500 IU of eCG is generally used for inducing multiple ovulations (3 ovulations) for ewes [21], and the effect continues longer than pFSH owing to eCG's long half-life [1]. Considering the high concentrations of E₂ obtained by the single injection of pFSH combined with eCG (Fig. 1, second panel), there appears to be a synergistic effect of a low dose of eCG and a high dose of pFSH on E₂ production in this simple superovulation method. FSH appeared to play a main role in the recruitment of follicles in the group of potentially ovulatory follicles by increasing aromatase activity [22]. ECG may not only increase the number of follicles due to its FSH-like activity, but also prevent the process of atresia [23] and can contribute to the production of more maturing follicles and to a greater production of E₂ by increasing production of androgen in the theca cells by its rather strong LH-like activity [24]. ECG can also help to prolong the stimulation to follicles by its long half-life.

Biphasic E₂ secretion was observed when pFSH was given on Day -2 and eCG was given on Day -1 (Fig. 4, middle panel), but not when both hormones were given on the same day (Day -2 or Day -1) (Fig. 4, top and bottom panels). Injection of a high dose of pFSH alone seemed to induce a rapid increase in E₂, as shown in the example in Fig. 1 (top panel). The simultaneous injections of a low dose of eCG with a high dose of pFSH might have prevented the subsequent elevation of E₂. It is not known why this E₂ reduction occurred by the simultaneous injections, but not by the separated injections of pFSH and eCG. A combined treatment with 20 mg pFSH and 500 IU eCG might be an overdose for the Suffolk ewe which is a non-prolific breed. The administration of a high dose of gonadotropin accentuates the tendency of luteinization (production of progesterone and oxytocin) by theca interna and granulosa cells, and also inhibits the E₂ production by granulosa cells [25]. Premature luteinization of follicles and premature activation of oocytes may occur not only with eCG but also with FSH when high doses are adminis-

tered [5]. We observed new corpora lutea just after ovulation on the ovary on Day - 1 by laparotomy, which showed the premature ovulation of follicles after the single injection of pFSH combined with eCG. More detailed investigations on the doses of pFSH and eCG and their effects in different breeds will be required in future studies.

The highly elevated E_2 levels that occurred before P sponge removal as a result of the injections of pFSH on Day - 2 and eCG on Day - 1 might have a deleterious effect on oocyte development and fertilizability due to the exposure to high intrafollicular levels of E_2 for a prolonged period [5]. However, considering the continuous E_2 elevation after the injections of pFSH on Day - 2 and eCG on Day - 1, new follicles recruitment, growth and development might have occurred without interruption from Day - 2 to the main E_2 peak.

For hormonal analysis, we used the data from 22 ewes having normal corpora lutea. We also obtained the hormonal profiles from 7 ewes without normal corpora lutea but with many small corpora albicantia (data not shown). Their P_4 profiles showed that corpora lutea might have grown normally before laparotomy, but the P_4 concentrations were low during on Day 4–7. The ewes without normal corpora lutea had normal E_2 , LH and FSH profiles during superovulation, except for one ewe which received a single injection of pFSH only. It can be assumed from this fact that these 6 ewes with the normal endocrine profiles of superovulation might have responded normally by the present superovulation treatment, but were suffered from premature regression of corpora lutea when the P_4 levels decreased on Day 4–7. The premature regression of corpora lutea is thought to occur by the involvement of the uterus [26] and is suspected to occur by the secretion of prostag-

landin F-2 α from the uterus due to the E_2 elevation after the LH surge [27]. An E_2 elevation might have occurred after observation period in these ewes.

In conclusion, the simple superovulation method, i.e. the administration of a single injection of a high dose (20 mg) of pFSH combined with a low dose (500 IU) of eCG, showed comparable endocrine profiles to those obtained by multiple injections of a high dose of pFSH, and in combination with a low dose of eCG. The injection of a low dose of eCG may prolong the stimulatory effect of gonadotropin and maintained the E_2 concentrations for an extended period. In particular, the injections of pFSH on Day - 2 and eCG on Day - 1 caused prolonged E_2 secretion from Day - 2. In addition to the endocrine profiles reported here, further studies will be needed to fully evaluate this method of superovulation. These will include investigations of the ovulatory responses and the embryo quality recovered from ewes.

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