Effect of Dosage of Equine Chorionic Gonadotropin Combined with a Single Injection of Porcine Follicle-Stimulating Hormone for Superovulation Treatment in Ewes

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Abstract. The effects of doses of porcine follicle-stimulating hormone (pFSH) and equine chorionic gonadotropin (eCG) for a simple superovulation method in ewes were investigated on ovulation rate, proportions of fertilized oocytes and normal embryos at recovery. The ewes were treated with intravaginal sponges impregnated with fluorogesterone acetate (FGA) for 12 days. For superovulation, an intramuscular injection of pFSH was given at 48 h before sponge removal, and eCG was given at 24 h after the pFSH injection. In Trial 1, the ewes (n=28) were divided into two groups. Group A (n=14) received 20 mg pFSH and 250 IU eCG, and Group B (n=14) was treated with 15 mg pFSH and 500 IU eCG. The effect on different breeds (Suffolk: Sf, Merino $\times$ polled Dorset: MD, South-Down: SD) was also investigated. In Trial 2, the ewes (n=20) were divided into 4 groups with different doses (0: control, 100, 200, 300 IU) of eCG combined with a 20 mg pFSH injection. All ewes were inseminated into the uterus with fresh-diluted or frozen-thawed semen by laparoscope, and the numbers of newly formed and regressed corpora lutea were recorded. Embryos were recovered on Day 6 or 7, and morula and blastocyst stage embryos were observed as being normal. There were no significant differences in the mean ovulation rates between Groups A (23.7 ± 21.5) and B (16.3 ± 9.8), and among the three breeds of ewes. The rates of fertilized oocytes and normal embryos were significantly higher in Group A than Group B (Trial 1). The ewes treated with 200 or 300 IU eCG had significantly shortened time-intervals from sponge removal to the onset of estrus and had higher ovulation rates than controls (Trial 2). The present study indicates that an appropriate eCG dose combined with a single injection of 20 mg pFSH would be 200–300 IU for a simple superovulation method in ewes pre-treated with progestogen-impregnated vaginal sponges.

Key words: Superovulation, FSH, eCG, Breed, Ewe.

(*J. Reprod. Dev.* 45: 307–313, 1999)

Equine chorionic gonadotropin (eCG) [1] or follicle-stimulating hormone (FSH) [2, 3] has been generally used for superovulation in sheep. It has been recognized that multiple injections are necessary to induce superovulation with FSH treatment, but a single injection of FSH combined

with a low dose of eCG increased ovulation rate, as compared with FSH treatment alone [4]. It was demonstrated by our previous studies that superovulation can be induced with a single injection of 20 mg porcine FSH (pFSH) combined with 500 IU eCG in Suffolk (Sf) and Merino $\times$ polled Dorset (MD) ewes [5, 6]. Superovulatory responses in treated ewes has varied with differences of season [1], administration method [4] and breed

[7]. Premature luteal regression before embryo recovery is one of the major drawbacks of superovulatory treatment, and has been reported in superovulated cattle [8, 9], deer [10] and goats [11, 12]. The phenomenon of premature luteal regression is accompanied by a low ova/embryo recovery rate and a high percentage of poor quality embryos recovered [13]. In three different breeds of ewes (Sf, MD and South-Down : SD), Fukui *et al.* [7] found that SD ewes showed the higher rate (70%) of premature CL regression than Sf or MD ewes and concluded that not only parity and season, but also breed of ewe affected the incidence of premature CL regression in superovulated ewes.

The detailed reason for the incidence of the premature CL regression in superovulated ewes has not been clarified yet. Therefore, in this study, two experiments were conducted to determine an appropriate dose of a single injection of pFSH and eCG for maximizing the ovarian response, ovulation rate and the proportion of normal embryos in superovulated ewes. Trial 1 investigated whether or not the decreasing the doses of either pFSH (15 mg) or eCG (250 IU) is possible. In Trial 2, a minimum effective dose of eCG combined with a 20 mg pFSH injection was determined for ovarian response and endocrine profiles during and after superovulation treatment.

Materials and Methods

Trial 1

A total of 28 mature ewes (9 Sf, 10 MD and 9 SD) were used during February to March, 1998. The ewes were divided into two groups: Group A (n=14) received an intramuscular injection of 20 mg pFSH (Antorin, Denka Pharmaceutical Co. Ltd., Japan) and 250 IU eCG (Serotropin, Teikoku-Zouki Pharmaceutical Co. Ltd., Japan), and Group B (n=14) was treated with 15 mg pFSH and 500 IU eCG. All ewes were pre-treated with intravaginal sponges impregnated with a synthetic progestogen, 40 mg fluorogesterone acetate (FGA: Chronogest, Intervet, the Netherlands) for 12 days. Ewes received a single injection of pFSH 48 h before sponge removal, and eCG was administered 24 h later. Rams aproned and equipped with marking harness and crayon were added to the ewe flock and estrus was detected by continuous observation for estrous behavior at 6 h intervals during the

period 6–30 h after sponge removal. The ewes were given an intramuscular injection of 100 µg gonadotropin releasing hormone (GnRH: Conthelal, Takeda Industrial Co. Ltd., Japan) at estrous detection. At 18 h after the beginning of estrus, artificial insemination (AI) with frozen-thawed semen was performed using a laparoscope by the method of Maxwell and Butler [14]. On Day 7 (Day 0: the day of sponge removal) after sponge removal, mid-ventral laparotomy was carried out under local anaesthesia with 2% xylocaine. Ewes were pre-administered atropine sulfate (Tanabe Chemical Co., Japan), and xylazine (celactal: Bayer, Germany). The numbers of CL, large follicle (≥ 5 mm in diameter) and regressed CL were recorded for all ewes. CL which were small and pale in morphological appearance, were considered as prematurely regressed CL according to the description of Jabbour *et al.* [15]. The oocytes were recovered by flushing the uterus and classified into fertilized oocytes and normal embryos. They were considered as fertilized oocytes when they were cleaved to the 2–16 cells stages, and normal embryos when they were at morula to blastocyst stages.

Trial 2

Trial 2 was designed to determine a minimum dose of eCG combined with a 20 mg pFSH injection using 20 MD ewes during September to November, 1998. All ewes were treated with FGA sponge, a 20 mg pFSH injection 48 h before sponge removal, and a GnRH injection at estrous detection as in Trial 1. The ewes were divided into 4 groups by doses (0: control, 100, 200, 300 IU) of eCG administered 24 h before sponge removal. Estrous detection and recovery of embryos were carried out as in Trial 1. Fresh diluted-semen was used for AI in Trial 2. Semen was collected from a mature ram and diluted 1:2 with a diluent consisting of Tris (300 mM), glucose (127.75 mM), citric acid (94.75 mM) and egg yolk (15%: v/v) [16]. A laparotomy was carried out to obtain embryos at the morula stage on Day 6 after sponge removal. Blood (5 ml) from the jugular vein was collected from all ewes. The blood was centrifuged at 4 C, and plasma was separated and stored at 20 C until assay. Plasma estradiol-17 β (E₂) and luteinizing hormone (LH) concentrations were measured by enzyme-immunoassay (EIA) [17, 18]. The intra- and inter-assay coefficients of variance

(CVs) 5.8% and 7.5% for E₂, and were 4.2% and 13.2% for LH, respectively.

Statistical analysis

Data of the onset time of estrus, E₂ concentration, numbers of large follicles and ovulation rate were analyzed by Fisher's exact probability test. The proportions of ewes with regressed CL, recovery and cleavage rates, and the proportion of normal embryos were compared by a procedure of general linear models (GLM), combined with the Duncan's multiple range test.

Results

Trial 1

Effects of single pFSH and eCG injections on the estrus, ovarian responses and recovered oocytes are shown in Table 1. There were no significant differences in the onset times of estrus between Groups A and B (24.0 ± 6.3 h and 24.0 ± 3.5 h), and among the three breeds of ewes (23.3 ± 5.9 h, 25.2 ± 4.7 h and 23.1 ± 4.1 h for Sf, MD and SD ewes, respectively). In the proportion of the incidence of premature luteal regression, Group B (28.6%) tended to be higher than Group A (14.3%) without a significant difference, but MD ewes had a higher

proportion of CL regression than Sf ewes (40.0% vs 0%: $P < 0.05$). There were no significant interactions in both the ovulation rate and the number of large follicles between the treatments and among the breeds. The recovery rate of oocytes was significantly higher ($P < 0.01$) in Group B (59.2%) than in Group A (43.0%), and in Sf (73.8%) and MD (63.3%) ewes than in SD (25.5%) ewes. However, the rates of fertilized oocytes and normal embryos were significantly higher ($P < 0.01$) in Group A (56.5 and 51.1%) than in Group B (33.8 and 27.0%), but no significant differences were found among the breeds. In the Sf ewes, the rates of fertilized oocytes (22.5% vs 69.4%) and normal embryos (17.5% vs 69.4%) were significantly lower ($P < 0.01$) in Group B than in Group A.

Trial 2

The effects of the various doses of eCG combined with a single pFSH injection on estrus, ovarian responses and recovered oocytes are shown in Table 2. Two out of 4 ewes and 1 out of 4 ewes treated without eCG (control) and with 100 IU eCG, respectively did not show estrus. Time-intervals from sponge removal to the onset of estrus were significantly ($P < 0.01$) shortened in the ewes treated with 200 (17.7 h) or 300 IU (11.5 h) than 0 (30.0 h) or 100 IU (24.7 h) treated ewes. The incidence of

Table 1. Effects of single pFSH and eCG injections on estrus, ovarian responses and recovered oocytes (Trial 1)

Treatment									
Group: pFSH/eCG (mg/IU)	No. of ewes* ¹	No. of estrus ewes (%)	Time of estrus (h)* ²	% of ewes with regressed CL (No.)	Ovulation rate ($\pm$ sem)	No. of large follicles	Recovered oocytes (%)	Fertilized oocytes (%)	Normal embryos (%)
A: 20/250	14	12 (85.7)	24.0 ± 6.3 (n=12)	14.3 (2/14)	23.7 ± 21.5 (n=9)* ³	0.71 ± 1.07	43.0 ^A (92/214)* ⁴	56.5 ^A (52/92)* ⁵	51.1 ^A (47/92)* ⁶
B: 15/500	14	13 (92.9)	24.0 ± 3.5 (n=13)	28.6 (4/14)	16.3 ± 9.8 (n=10)* ³	1.64 ± 2.34	59.2 ^B (74/125)* ⁴	33.8 ^B (25/74)* ⁵	27.0 ^B (20/74)* ⁶
Breed									
Sf	9	8 (88.9)	23.3 ± 5.9 (n=8)	0.0 ^a (0/9)	12.9 ± 6.0 (n=8)* ³	0.86 ± 1.32	73.8 ^A (76/103)* ⁴	44.7 (34/76)* ⁵	42.1 (32/76)* ⁶
MD	10	10 (100)	25.2 ± 4.7 (n=10)	40.0 ^b (4/10)	15.8 ± 7.5 (n=5)* ³	0.90 ± 1.20	63.3 ^A (50/79)* ⁴	52.0 (26/50)* ⁵	46.0 (23/50)* ⁶
SD	9	7 (77.8)	23.1 ± 4.1 (n=7)	22.2 (2/9)	32.5 ± 24.1 (n=6)* ³	2.00 ± 2.65	25.5 ^B (40/157)* ⁴	42.5 (17/40)* ⁵	30.0 (12/40)* ⁶

*¹ Ewes were pretreated with FGA sponge for 12 days.

*² Time after sponge removal (mean $\pm$ SEM).

*³ Number of ewes that showed normal CL.

*⁴ Number of recovered oocytes / total number of CL.

*⁵ Number of fertilized (cleaved) oocytes / number of recovered oocytes.

*⁶ Number of normal embryos (morula and blastocyst)/number of recovered oocytes.

A-B: $P < 0.01$, a-b: $P < 0.05$.

Table 2. Effects of various doses of eCG combined with a single pFSH injection on estrus, ovarian response and recovered oocytes in Merino-Dorset (MD) ewes (Trial 2)

Doses of eCG (IU)	No. of ewes ^{*1}	No. of estrous ewes (%)	Time of estrus (h) ^{*2}	Time of LH surge (h) ^{*2}	% of ewes with regressed CL	Ovulation rate ($\pm$ sem)	No. of large follicles	Recovered oocytes (%)	Fertilized oocytes (%)	Normal embryos (%)
0	4	2 (50.0)	30.0 $\pm$ 2.8 ^A (n=2)	26.0 $\pm$ 0.0 ^A	0.0 (0/4)	2.0 $\pm$ 1.6 ^A (n=4) ^{*3}	0.75 $\pm$ 0.95	0.0 ^A (0/8) ^{*4}	—	—
100	4	3 (75.0)	24.7 $\pm$ 4.6 ^A (n=3)	26.0 $\pm$ 0.0 ^A	0.0 (0/4)	2.3 $\pm$ 2.1 ^A (n=4) ^{*3}	1.50 $\pm$ 1.73	77.8 ^B (7/9) ^{*4}	100 (7/7) ^{*5}	85.7 ^a (6/7) ^{*6}
200	6	6 (100)	17.7 $\pm$ 4.5 ^B (n=6)	22.3 $\pm$ 3.7	33.3 (2/6)	13.7 $\pm$ 1.5 ^B (n=3) ^{*3}	0.33 $\pm$ 0.82	56.1 ^B (23/41) ^{*4}	69.6 (14/23) ^{*5}	39.1 ^b (9/23) ^{*6}
300	6	6 (100)	11.5 $\pm$ 1.7 ^B (n=6)	21.3 $\pm$ 1.6 ^B	0.0 (0/6)	12.0 $\pm$ 4.6 ^B (n=6) ^{*3}	1.83 $\pm$ 1.83	66.7 ^B (46/69) ^{*4}	63.0 (29/46) ^{*5}	32.6 ^b (15/46) ^{*6}

^{*1} Ewes were pretreated with FGA sponge for 12 days.

^{*2} Time after sponge removal (mean $\pm$ SEM).

^{*3} Number of ewes that showed normal CL.

^{*4} Number of recovered oocytes / total number of CL.

^{*5} Number of fertilized (cleaved) oocytes/number of recovered oocytes.

^{*6} Number of normal embryos (morula and blastocyst)/number of recovered oocytes.

A-B: $P < 0.01$, a-b: $P < 0.05$.

luteal regression was only observed in the ewes treated with 200 IU eCG. There were no significant differences in the number of large follicles. The ewes treated with 200 or 300 IU eCG had significantly higher ($P < 0.01$) ovulation rates than those with or without 100 IU eCG. There was a significant ($P < 0.05$) difference in the rate of normal embryos between 100 IU (85.7%) eCG and other doses.

The mean E_2 concentration at 18 h after sponge removal in the ewes treated with 300 IU eCG was significantly higher ($P < 0.05$) than the control (Fig. 1). No other significant differences were observed in the E_2 concentration. Typical hormonal profiles in each treatment group are shown in Fig. 2. Control (0 IU) ewes had no estrus with a low level of E_2 . In the ewes treated with 100, 200 and 300 IU eCG, E_2 concentrations were gradually increased following the administration eCG. Estrus was induced around the time of the E_2 peak. All ewes showed LH peaks at 2 h after administration of GnRH. The time to LH surge in the ewes treated with 300 IU eCG was significantly ($P < 0.01$) earlier than in those treated with 0 IU (control) or 100 IU eCG (Table 2).

Discussion

The administration of exogenous progesterone or progestogen combined with eCG or FSH for superovulatory treatment affects the endocrine responses, and showed a great variation in the

Fig. 1. Plasma estradiol-17 β concentrations of the different eCG treatments combined with a 20 mg pFSH injection in Merino-Dorset (MD) ewes (Trial 2).

onset time of estrus [19], ovarian response [20], recovered oocytes [21] and premature luteal regression [4, 15]. In Trial 1, there were 3 SD ewes that had remarkably multiple ovulations in Group A (n=2: 37 and 76) and in Group B (n=1: 38). The ovulation rate in the SD ewes was high, compared with other breeds of ewes, but there was no significant difference which is probably due to the small number of ewes used in this study (Table 1). In the recovery rate, there were significant differences between Groups A and B, and among breeds in Trial 1. This may relate to the fact that the 3 SD ewes had higher ovulations, as described above. In the rates of fertilized oocytes and normal embryos, Group A was higher than Group B. The difference in the half-life of exogenous gonadotropins (pFSH and eCG) may be a cause of

Fig. 2. The typical hormone profiles in each treatment group with the data of ovarian response and recovered embryos (Trial 2).

the present result. In the ewes treated with eCG, it appears that the higher incidence of large follicles, by increasing eCG doses, may have been derived

from the high circulating E₂ levels causing continued recruitment of follicles. It has been suggested that continued follicular growth results

in an increased level of E_2 during the early luteal phase which may create a hostile uterine environment for sperm, oocytes or embryos [22]. Therefore, it appears from the present results that Group A which had a decreased dose of eCG, a better superovulation treatment with higher the rates of fertilized oocytes and normal embryos than Group B which had a decreased the pFSH dose.

Estrous incidence is influenced by progesterone treatment [23], age [24] and season [23, 25]. It has been reported that some ewes ovulate without estrous behavior by the hormonal treatment in the breeding season [26]. Treatment with progesterone alone does not adequately synchronize estrus and ovulation in cyclic ewes due to the different patterns of follicular growth dependent on the stage of the estrous cycle at the time of sponge insertion [27]. In Trial 1, all the treated ewes showed estrus and ovulated. In Trial 2, one of 4 ewes treated with 100 IU eCG did not show estrus, but ovulated. All the ewes treated with 200 or 300 IU eCG showed estrus, and the estrous onset was significantly earlier than those of ewes treated without or with 100 IU eCG. This suggests that estrous incidence was affected by the dose of exogenous gonadotropin.

In the proportion of the incidence of premature luteal regression, MD ewes showed higher incidence than Sf ewes in Trial 1. Our previous study [7] also showed that SD ewes showed a higher rate of corpus luteum (CL) regression than Sf and MD ewes treated with a 20 mg pFSH injection combined with 500 IU eCG. There is no

report on superovulatory response in SD ewes compared with other breeds of ewes. However, the ovarian response was greater in Merino ewes carrying Booroola gene than those with non-carriers [28]. This suggests that breed differences significantly affect the incidence of premature luteal regression [7]. It has been considered that endogenous $PGF_{2\alpha}$ and/or oxytocin arising from the response to prolonged stimulation by elevated circulating levels of E_2 may be causative factors in premature luteal regression [26]. However, it is uncertain whether the observed, shortened luteal phase resulted from premature luteolysis, or the failure of CL function following the superovulation treatment with a single injection of pFSH and eCG. One possible explanation might be that a higher number of persistent follicles occurred during autumn than in spring [14]. A nutritional status on the incidence of ewes with regressed CL was also suggested by the reports of other workers showing that the mean concentration of plasma glucose was lower in ewes with regressed CL than in ewes with normal CL [4, 15], and stress may play a role [14].

In conclusion, the present study indicates that an appropriate eCG dose combined with a single injection of 20 mg FSH would be 200–300 IU for a simple superovulation method in ewes pre-treated with progestogen-impregnated vaginal sponges. However, the breed of ewes should be taken into account as one of the factors influencing the ovarian response and the subsequent embryo yield.

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