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Development of a programmable piggyback syringe pump and four-times-a-day injection regimen for superovulation in non-lactating Holstein cows

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Abstract. The objectives of the present study were to develop a programmable piggyback syringe pump for bovine superovulation and to evaluate the effects of a four-times-a-day injection regimen using the pump. Non-lactating Holstein cows were treated with a total of 30 armour units of porcine FSH by injection four times a day with the pump (study, n = 9) or injection twice a day manually (control, n = 9) for four consecutive days from D10 of the estrous cycle. The pump-driven program successfully induced superovulation in all cows tested. The numbers of small (3– < 5 mm in diameter) and large (≥ 10 mm in diameter) follicles were greater in the study group on D11-13 and D14, respectively. There were fewer unovulated follicles detected on D21 (7 days after estrus) in the study group than in the control group (1.2 ± 0.4 and 3.2 ± 0.6, respectively).
Key words: Cows, Follicles, Piggyback syringe pump, Porcine follicle-stimulating hormone (pFSH), Superovulation
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Superovulation treatment for the production of multiple embryos is a key process of embryo transfer technology in cattle. Bovine superovulation is induced by the administration of either chorionic (equine chorionic gonadotropin, eCG) or pituitary (porcine follicle-stimulating hormone, pFSH) gonadotropin, followed by prostaglandin $F_{2\alpha}$. However, the results of superovulation treatment remain inconsistent among individuals [1–3]. Various factors including hormone formulation, dose and treatment regimen have been suggested to affect the individual variation in the response to superovulation treatment.

Although it had been used to induce superovulation, owing to its long half-life in the circulatory system, eCG is frequently associated with unovulated or sustained growing follicles following ovulation. Furthermore, eCG expresses considerable LH-like activity in non-equine species, and excessive LH-like activity has been suggested to result in abnormal follicular steroidogenesis, leading to detrimental effects on the quality of embryos [4, 5]. In contrast to eCG, pFSH has a relatively short half-life and little LH-like activity in cattle [6]. Therefore, pFSH is widely utilized for induction of superovulation in cows [7]. However, because of its short half-life, pFSH must be injected twice daily over 3 to 4 consecutive days to induce superovulation in cattle [8–10]. Repeated injections are stressful for animals and time-consuming.

To solve this problem, a syringe pump with an indwelling subcutaneous needle could be used for periodic administration of pFSH with programmed dosages and intervals. Several studies have reported utilization of an injection pump for pulsatile injection of FSH to stimulate ovarian follicular development in rabbits [11] and women [12]. Pump-driven protocols for ovarian stimulation have tremendous advantages regarding both mimesis of physiological pulsatility and maintenance of the blood concentrations of injected hormones. To the best of our knowledge, no studies attempting utilization of an injection pump for bovine superovulation have been reported. The pump for bovine superovulation is carried on the back of the cow, driven by long-lasting batteries and operated without disturbing the free movement of the animal. In the present study, therefore, the objectives were to develop a piggyback syringe pump and to evaluate a pump-driven protocol for superovulation in cows.

Four-times-daily treatment with pFSH for four days using the pump successfully induced superovulation in all cows tested. The results in the pump-driven protocol (study group) favorably compare in terms of number of developing follicles with those of the conventional twice-daily protocol (control group). The numbers of small (3– < 5 mm in diameter) follicles from D11 (D0 = day of ovulation) to D13 were significantly greater in the study group than in the control group (Fig. 1). Similarly, the numbers of large (≥ 10 mm in diameter) follicles on D14 were significantly greater in the study group than in the control group. However, the numbers of medium (5– < 10 mm in diameter) follicles were not significantly different between the groups. Prior to the beginning of superovulation treatment, the

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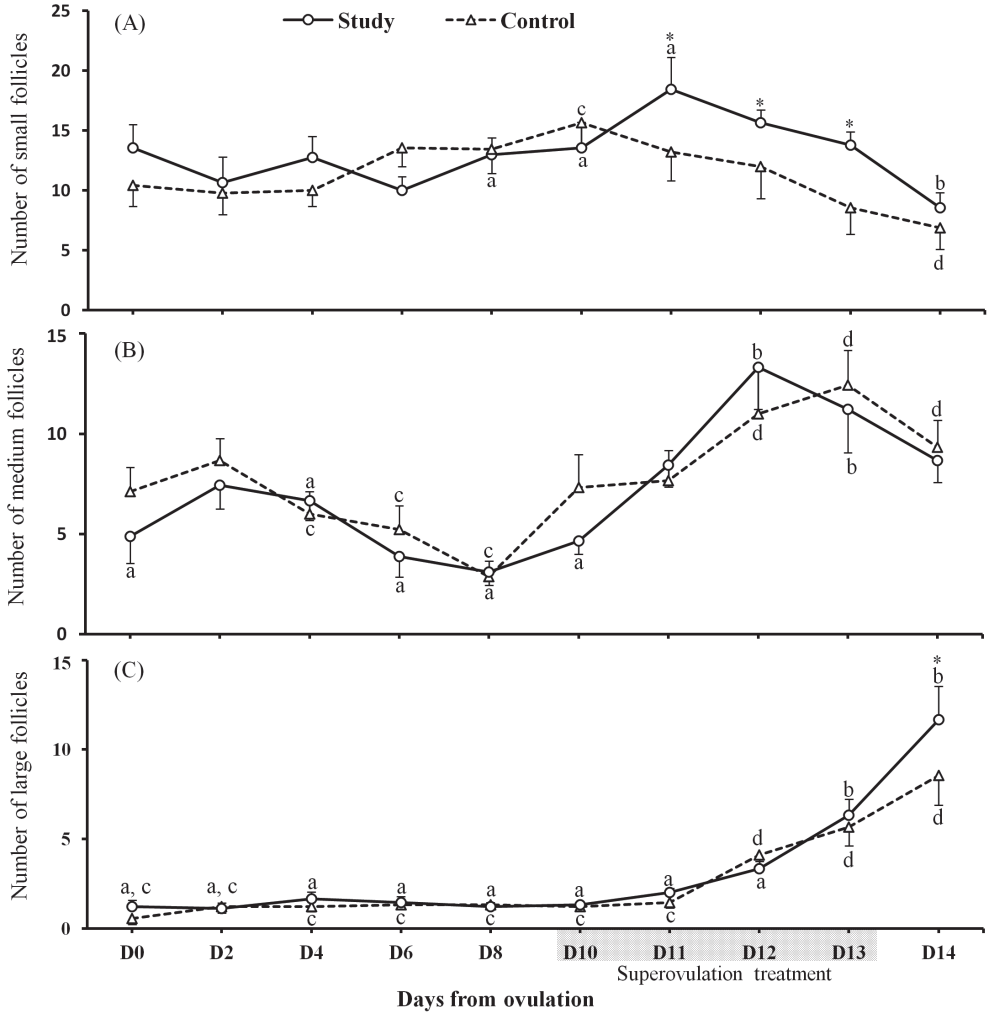

Fig. 1. The daily changes in the mean (± SEM) number of small (A; 3– < 5 mm in diameter), medium (B; 5– < 10 mm in diameter) and large (C; ≥ 10 mm in diameter) follicles in the study and control groups from D0 to D14. Letters indicate values with significant differences ($P < 0.01$) within the study (a and b) and control (c and d) groups. * There is a significant difference between the study and control groups ($P < 0.05$).

distributions of small, medium and large follicles were not different between the groups. These results suggest that four-times-daily treatment more effectively stimulated the transition of the follicular growth than the conventional twice-daily protocol dose, even though the total dosages of pFSH were the same in the two groups. Since the presence of an optimal pFSH dose in superovulation is generally accepted, it is plausible that both the higher and lower doses result in insufficient production of transferable embryos. The current results suggest that four-times-daily treatment improves the efficiency of superovulation without increasing the total dose of pFSH.

Plasma progesterone (P_4) concentrations (Fig. 2) increased significantly ($P < 0.01$) following superovulation treatment in the study and control groups and reached 8.4 and 6.6 ng/ml on D21, respectively. The fluctuation in the plasma P_4 concentrations was not statistically different between the groups. As shown in Table 1, the mean number of corpora lutea (CLs) and cross-sectional diameter of the corpus luteum (CL) on D21 were not statistically significant between the groups. There was a positive relationship between the plasma P_4 concentration and the number of CLs in both groups ($r = 0.55$, $P < 0.05$) on D21.

The numbers of unovulated follicles in the study and control groups were 1.2 ± 0.4 and 3.2 ± 0.6, respectively. The significant difference ($P < 0.01$) in the numbers of unovulated follicles between the groups suggests that the pump-driven and/or four-times-daily procedures improved the ovulation rate significantly ($P < 0.01$) in the study group. Intensive handling of animals including frequent venipuncture and transrectal ultrasonography has been reported to worsen the association with unovulated follicles in superovulated cows [13]. In the present study, the lower stress for pFSH administration in the study group probably caused the decrease in the number of unovulated follicles.

The results of the present study indicate that the piggyback syringe pump can be successfully utilized for bovine superovulation in non-lactating Holstein cows, and the results of four-times-daily treatment with the pump compare favorably with those of conventional twice-daily treatment by manual administration.

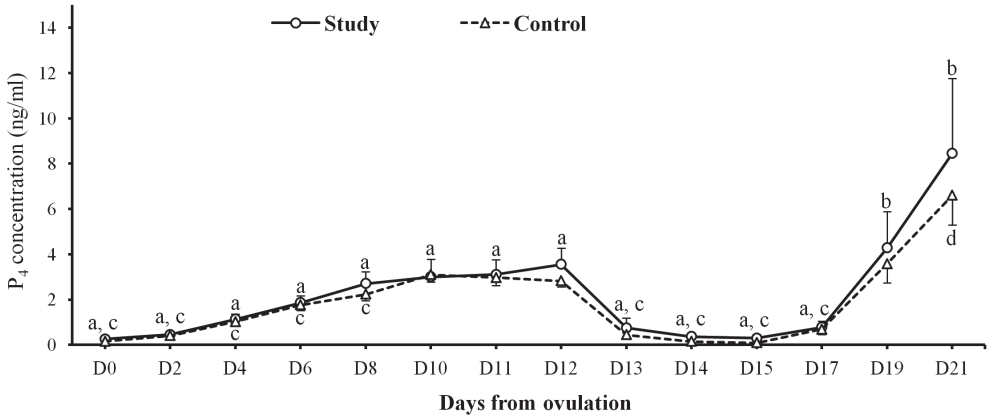

Fig. 2. The fluctuations in the mean (± SEM) plasma progesterone (P_4) concentrations from D0 to D21 in the study and control groups. Letters indicate values with significant difference ($P < 0.01$) within the study (a and b) and control (c and d) groups.

Table 1. The number of CLs, cross-sectional diameter of the CL, number of large unovulated follicles and ovulation rate after four-times-a-day or twice-daily injections of pFSH on D21 in superovulated Holstein cows

	Experimental groups		P-value
	Study	Control	
No. of cows	9	9	
No. of CLs	12.3 ± 1.8	9.2 ± 2.0	0.27
Cross-sectional diameter of CL (mm)	15.0 ± 0.5	14.9 ± 0.7	0.94
No. of unovulated follicles	1.2 ± 0.4	3.2 ± 0.6	0.01
Ovulation rate (%)*	87.6 ± 3.8	70.1 ± 3.5	0.01

The results are expressed as the mean (± SEM). * The ovulation rate was calculated as follows: (No. CLs/ No. CLs + No. unovulated follicles) × 100.

Materials and Methods

Experimental design, farms and features of the pump

This experiment was designed as a 2 × 2 factorial study. Cows were first allocated randomly into two groups. After 4 to 6 months, reciprocal treatment was performed in order to reduce the individual affect of the cows. This experiment was conducted from October 2011 to October 2014 and in accordance with Animal Welfare Guideline No. A201316 of Iwate University. A total of 18 non-lactating Holstein cows (mean age of 5.0 ± 0.4 years old and live body weight of 629.6 ± 22.3 kg) with estrous cycles at regular intervals were used in this experiment. All cows were fed twice daily at 0800 h and 1600 h and had free access to clean water.

The focus of the study was on a new treatment regimen with a programmable microsyringe pump (Icomes Lab, Morioka, Japan, Fig. 3). This pump can be programmed with certain injection intervals without side effects, and manual operation is also possible be pressing a button on the microsyringe pump. The height, width and length of the microsyringe pump are 4, 7 and 10 cm, respectively, and the weight and life of the battery are 190 g and over 96 h, respectively. The pump is able to hold up to 5 ml of injection solution, which was 30 AU of pFSH dissolved in 5 ml saline in the present study.

Synchronization and superovulation treatment

The estrous cycles of the cows were synchronized using a controlled internal drug releasing device (CIDR; Zoetis Japan, containing 1.9 g of P_4) and was left for 7 to 10 days, and at the time of CIDR removal, an intramuscular injection of 0.15 mg per cow of cloprostenol (Dalmazin, Kyoritsu Seiyaku, Tokyo, Japan) was administered. Cows in both groups were treated to superstimulate ovarian follicular development with a pFSH (Antrin-R10, Kyoritsu Pharmacy, Tokyo, Japan) subcutaneously. Superovulation treatment was initiated on D10 of the estrous cycle, and cows received two injections of cloprostenol to induce corpus luteum regression on D12; cows exhibited estrus (based on mounting or standing to be mounted, discharge of clear mucus, bellowing) in the morning on D14. The treatment protocols and frequency injections of pFSH in the two groups are shown in Table 2.

Ultrasound examination

Ovarian follicular development was monitored by transrectal ultrasonography (7.5 MHz probe, GE Medical Systems, Jiangsu, P.R. China) according to the following schedule: D0, 2, 4, 6, 8, 10–14, 17 and 21. All visible follicles were counted and classified as previously [14] reported according to diameter as small (3– < 5 mm), medium (5– < 10 mm) and large (≥ 10 mm) follicles. On D21, the cross-sectional diameter of an ultrasonic section of the CL was calculated by the square root of the longest × widest point.

Blood sampling and hormonal analyses

Blood samples were collected at approximately 0800 h on D0, 2, 4, 6, 8, 10–14, 15, 17, 19 and 21 into 10 ml heparin tubes by puncture of coccygeal vessels. After collection, samples were immediately stored on ice for at least 30 min and then transported to the laboratory and centrifuged at 3,000 × *g* for 15 min at 4 C. The plasma was stored in tubes at –30 C. Plasma P_4 concentration was measured using a reagents kit (DELFIA Progesterone kit, Wallac Oy, Turku, Finland) according to the manufacturer's instructions [15] by using a multi-plate reader (ARVO MX Wallac 1420 Multilabel Counter, PerkinElmer Life and Analytical Sciences). The inter- and intra-assay coefficients of variation for P_4 were 8.7% and 3.8%, respectively.

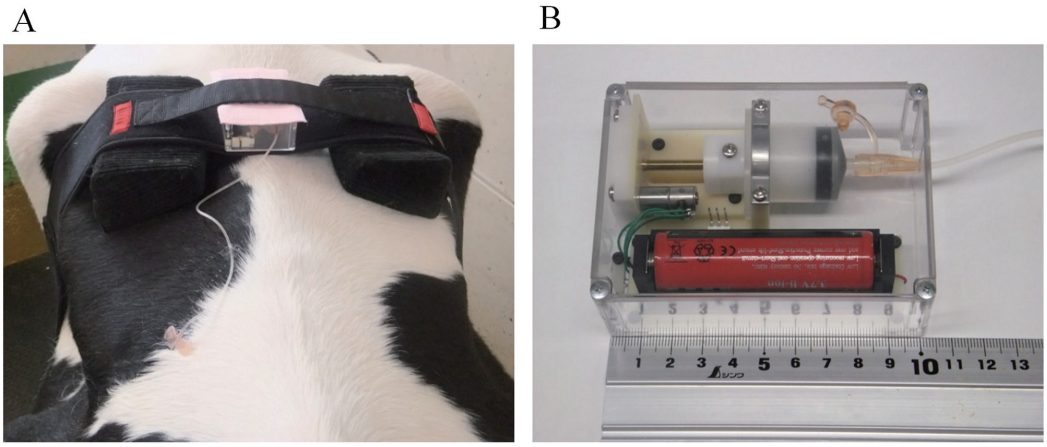

Fig. 3. (A) The tightness of the microsyringe pump attached to the back of the cow with a belt during the superstimulation treatment regimen. (B) The microsyringe pump during operation and connected to an extension butterfly tube.

Table 2. The schedule of superstimulation treatment in non-lactating Holstein cows with a total 30 AU of pFSH over 4 days in the two groups

Experimental group		Date				
		D10	D11	D12	D13	D14
	Time	Dose of pFSH (AU)				
Study	0300		2.0	1.0	1.0	0.5
	0900	4.0	3.0	2.0 + PG	1.5	
	1500	2.0	1.0	1.0	0.5	
	2100	4.0	3.0	2.0 + PG	1.5	
Control	0800	6.0	4.0	3.0 + PG	2.0	
	2000	6.0	4.0	3.0 + PG	2.0	

PG, Prostaglandin $F_{2\alpha}$ (cloprostenol).

Statistical analysis

Statistically significant differences in the 3 sizes of follicles from D0 to D14 and plasma P_4 concentration from D0 to D21 between the study and control groups were analyzed by two-way ANOVA (with treatment and date as the main effect), followed the Tukey Multiple Comparison test (Excel-Toukei 2012 software, Social Survey Research International). The number of CLs and number of unovulated follicles on D21 were analyzed by Student's *t*-test using the GraphPad Prism 6 software for Windows (version 6.03; GraphPad Software, La Jolla, CA, USA). Correlations between P_4 concentrations and the number of CLs on D21 were also analyzed. A value of $P < 0.05$ was considered statistically significant.

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