No-CL Superstimulatory Protocol: Developing a New *Superovulation* Treatment Initiated in the Absence of Corpus Luteum (CL) and Compared with D1 and Traditional Superovulation in Cattle

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Abstract: The objective of this study was to investigate the effectiveness of superovulation under a complete absence of the Corpus luteum (CL) and compare it with D1 protocol and traditional superovulation in cattle. Animals were divided into three groups as following; 1- *D1-protocol*: Animals (n= 7) were leaved to get natural ovulation (D0), then received FSH for the next 4 days of the cycle. GnRH was given 12h after the last dose of FSH. 2- *No-CL superovulation protocol*: Animals (n=10) were synchronized and received PGF2α at D9 or D10 then classified to two subgroups (D9-sub-group and D10-sub-group). After 36 h, all follicles (≥ 5 mm) were aspirated (D0). 3- *Control*: Animals (n=3) were submitted to the conventional superovulation protocol. Blood samples were collected daily for 13 days. Progesterone (P_4) and Estradiol (E_2) in plasma were measured by Enzyme immune assay (EIA).

The results showed that the number of growing follicles was significantly ($P< 0.05$) higher in both D9 and D10 subgroups in comparison to the D1 protocol (25.8 ± 4.3 and 20 ± 1.9 *vs*. 10.9 ± 1.9 respectively). While the number of ovulated follicle was higher in D9 sub-group than D10 sub-group, D1 protocol and control (13.8 ± 4.4 *vs*. 7.6 ± 3.5, 6.8 ± 1.5 and 9.7±0.9).

In conclusion, the superovulation protocol with complete absent of the CL produced high number of growing follicles, decreased variability and considered as a promising superovulation protocol.

Keywords: Cattle, Superovulation, CL, Estradiol, Progesterone, FSH.

INTRODUCTION

The traditional superovulation protocol was designed in the 1980s without the current understanding of follicular dynamics in cattle. However, the using of the transrectal ultrasonography as a tool to study ovarian physiology provided information about the wave like pattern of follicular development in many species [1]. The knowledge regarding follicular wave development has been taken into account when designing regimen to control ovarian activity during superovulation. For instance, the initiation of the FSH treatment at the time of wave emergence elicited a high superovulation response [2]. Furthermore, removal of the dominant follicle by ablation [3,4] or injection of GnRH [5] increased the number of recruited follicles and synchronized the follicular wave emergence [4]. Some researchers have focused on starting the superovulation protocol at the first wave in which the wave emergence occurred at a consistent point (after ovulation) and in the absence of dominant follicle. Comparing the result of superovulation response of the first wave with second wave [6] showed no difference between the two groups. Furthermore, the use of the first wave may be more convenient and time-sparing in superovulation programs.

The traditional superovulation protocol consist of a prolonged progesterone (P_4) priming (12-18d) with FSH initiated approximately 48h before P_4 withdrawal [7]. Although the P_4 is important to synchronize the development of follicles, the high (P_4) level produced by the corpus luteum (CL) has reduced the diameter of dominant follicle [8] and suppressed Estradiol (E_2) secretion [9]. Similarly, the early stage of oestrous cycle (D1-protocol) has a low level of P_4 which increased gradually with the developing of the cyclic CL [10].

In the present study, we developed a new superovulation protocol under a complete absence of CL and compare it with superovulation at the early stage of oestrous cycle and traditional superovulation protocol.

MATERIALS AND METHODS

This experiment was ethically approved and carried out at the Field Center of Animal Science and Agriculture, Obihiro University, Japan. The Holstein non pregnant, non lactating healthy cows were kept under the normal management program receiving maintenance ration by the staffs in this center.

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Experimental Design

1- D1 protocol (n= 7): Animals in this group were leaved to get spontaneous ovulation (D0), then received 28 Armour unit (A.U) FSH on D1 for 4 successive days in the following order (***D1***; 5A.U, ***D2***; 4A.U, ***D3***; 3A.U ***D4***; 2 A.U, twice daily, 12h interval), using (ANTRINR 10 Kawasaki Pharm. Co., Kawasaki, Japan). GnRH, analogue (Fertirelin acetate 100 µg; (Conceral); Nagase Pharm. Co., Osaka, Japan) was administered 12h after the last dose of FSH. *2- No-CL superovulation protocol (n=10)*: Animals were synchronized by two injections of Cloprostenol (ESTRUMATE; 500µg) 11 days apart. The animals received third dose of PGF2α at D9 or D10 and according to the day of injection, this group was classified to two subgroups (D9-sub and D10-sub). After 36 h, all follicles (≥ 5 mm) were aspirated (D0) by transvaginal ultrasound-guided follicle aspiration. For the ultrasound guidance of the aspiration needle, an ultrasound scanner (SSD-5500, ALOKA CO., Ltd., Tokyo, Japan) equipped with a 7.5 MHz transvaginal convex transducer (UST-M15-21079, ALOKA CO., Ltd.) with an attached stainless steel needle guide was used. The FSH treatment started 24 h after aspiration for 4 days as the previous protocols. GnRH was given 12h after the last dose of FSH.

3- Control (n=3): Animals were submitted to the conventional superovulation protocol. The animals get synchronized by 2 injection of PGF2α 11 days apart. 5 days later, Controlled internal drug release (CIDR) was inserted with one injection of 1mg estradiol benzoate (EB) (Ginandol. TM., Sankyo Yell. Pharmaceutical, Tokyo, Japan). FSH injection started 5 days later and for 4 days (6,6 5,5 4,4 3,3A.U). At D2, two doses of PGF2α 12h apart were given. CIDR was removed 12h later and 4 ml of GnRH was injected 24h after that (time table for the treatment).

Monitoring of Follicular Development

Ultrasound scanning was performed by the same operator daily until the ovulation then every 48 h starting from D1.

To measure the follicles and CL diameter, color Doppler ultrasonography was used. The examination was performed by using the same ultrasound scanner equipped with a 7.5 MHz convex transducer (UST-995-7.5, Aloka Co.). The diameter of all follicles (≥ 3mm) through each examination was recorded except for the control groups in which the number of CL is only recorded.

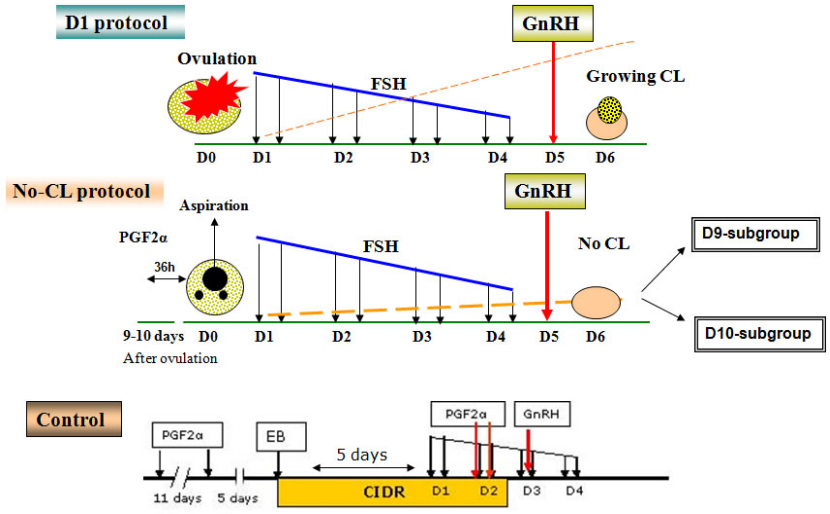
Ovulatory Response

Ovarian response was determined by using the color signals to detect the blood flow with the velocity higher than 2mm/sec of the new CL. Using the color signals showed the blood boundaries of the new CL and so the determination of the CL number became more precise.

Table 1. The Number of Follicles and CL (Mean ±SEM) in the Different Groups

	Group			
	D1 protocol (n=7)	NO-CL protocol (n=10)		Control (n=3)
		D9-sub (n=4)	*D10-sub* (n=6)	
No. of recruited follicles (D1)	9.8 ±1.3	17±3.8	11.2 ±3.2	--------
No. of follicles (D3)	11.0 ± 1.4[b]	24.0 ± 2.3[a]	24.0 ± 2.8[a]	--------
No of large F>8mm at (D5)	10.0 ± 1.9[b]	25.8 ± 4.3[a]	20 ± 1.9[ab]	--------
Number of CL	6.8 ± 1.5	13.8 ± 4.4	7.6 ± 3.5	9.7 ± 0.9

Different superscript within the row denotes significant difference (P<0.05).



Timetable of the treatment in each group

Time table of the treatment in each group.

Blood Collection and Hormonal Determination

Blood was collected daily starting at D1 by caudal venipuncture just after each examination by using 10 ml heparinized tube. All tubes were immediately chilled in ice water for about 15 min and then centrifuged at 3000 rpm for 20 min at 4°C. The obtained plasma was decanted and stored at -30°C until hormonal assay. Following extraction in diethyl ether, the concentration of P_4 was determined by double- antibody enzyme immunoassays (EIA). EIA for P_4 was conducted as described by [11]. The recovery rate was 87%. The standard curve ranged from 0.05 to 50ng/ml, and the ED_{50} of the assay was 7.3ng/ml. Intra- and interassay coefficients of variability (CVs) were 2.9 and 9.3%, respectively. EIA for estradiol-17β (E_2) was conducted as described previously [12]. The recovery rate was 85%. The standard curve ranged from 2 to 2000 pg/ml and ED_{50} of assay was 126.2pg/ml. Intra- and interassay coefficient of variability (CVs) were 10.4 and 15.5%, respectively.

Statistical Analysis

The mean diameter of the ovulated follicles, follicular population at the time of first FSH dose (D1) and at day of GnRH injection (D5) and number of CL were compare between groups using t-test. The percentage data were analyzed using logistic regression.

The plasma concentration of P_4 and E_2 were analyzed by repeated measure ANOVA to determine main effects of group, day and group by day using JMP statistical software (version 5.1; SAS Institute, Cary, NC, USA 2003). The different means were significant at $P<0.05$.

RESULT

The total number of follicles of No-CL protocol at D3 and D5 was significantly ($P<0.05$) higher in both D9 and D10 subgroups in comparison to the D1 protocol (Table **1**).

Table 2. The Ovulatory Response (Mean ±SEM) in the D9 and D10-Sub Groups

	D9-subgroup		D10-subgroup	
	High response (n=3)	**Low response (n=1)**	**High response (n=2)**	**Low response (n=4)**
No. of CL	17 ± 4.2	4	15.5 ± 3.5	2.5 ±1.0

Table 3. Comparison between the Diameter (Mean ± SEM) of the Growing Follicles (mm) During the FSH Treatment between the D9 and D10-Sub Group

	D9-subgroup(n=4)		D10-subgroup(n=6)	
	High response (n=3)	**Low response (n=1)**	**High response (n=2)**	**Low response (n=4)**
D1	4.4 ± 0.1	3.8 ±0.1	3.6 ± 0.1	6.8 ± 2.4
D3	8.6 ± 0.3	**10.6 ± 0.1**	8.6 ± 0.2	**11.2 ± 2.2**
D5	12.4 ± 0.2	15.6 ± 0.4	12.2 ± 0.1	13.2 ± 1.5

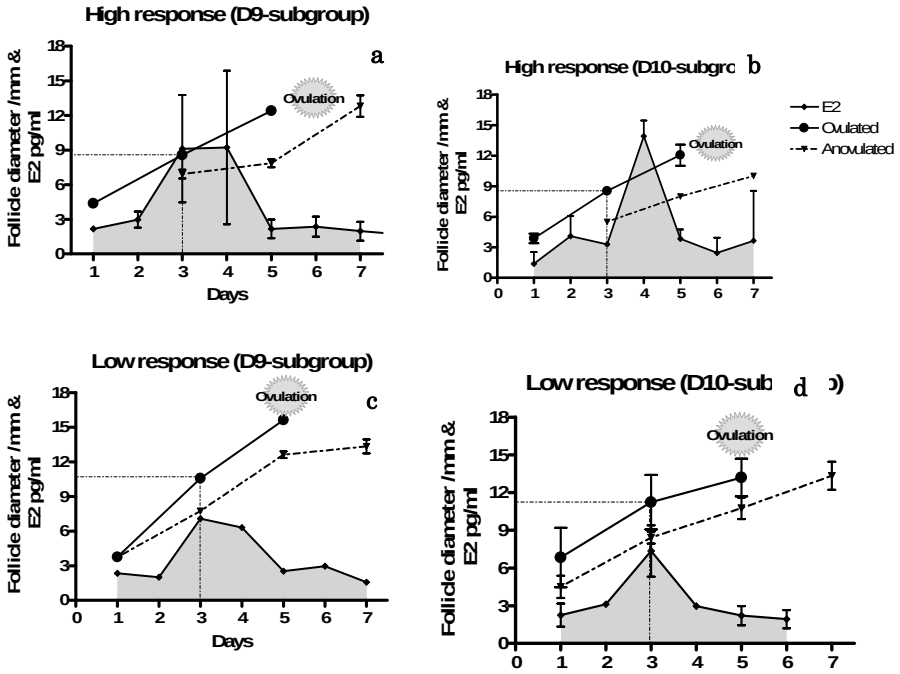


Fig. (1). Follicular growth (mean ±SEM) and E_2 (pg/ml) level in both high (**a,b**) and low (**c,d**) responded cows of D10 and D9 subgroup.

Table 4. Comparison between the Level (Mean ± SEM) of E_2 (pg/ml) During the FSH Treatment between D9 and D10-Sub Group

	D9-subgroup		D10-subgroup	
	High response (n=3)	**Low response (n=1)**	**High response (n=2)**	**Low response (n-4)**
D3	9.1 ± 4.1	7.1	-------	7.4 ± 2.0
D4	9.2 ± 6.7	------	13.9 ± 1.1	------

However, the mean number of ovulated follicles increased in D9 subgroup only.

Two out of six cows (33.3%) in ***D10-subgroup*** and 3 out of 4 cows (75%) of ***D9-subgroup*** had more than 10 CL after treatment Table **2**.

In the animals with weaker response, ovulated follicles became larger than 10 mm at D3 while the diameter of the non-ovulated follicles was still smaller than 8 mm (Table **3**). On the other hand, in the animals with higher response, the follicular diameter was around the 8 mm (Fig. **1**).

Another difference has been noticed, that the E_2 level was higher on D3 or D4 in the animals with higher response compared with the lower response (Fig. **1**, Table **4**).

Comparison of the Hormonal Profiles between the Different Groups

The analysis of P_4 from D0 to D6 between the three groups revealed an effect of group ($P<0.01$) and group by day ($P<0.01$). In D1 protocol, the P_4 level was increased gradually form D3 concomitantly with the growth of cyclic CL. In NO-CL protocol, The P_4 level was lower than 0.29 ± 0.02 ng/ml for 6 days. In the control, the P_4 level was high on D1 and D2 of the FSH treatment then started to decrease after the removal of the CIDR to reach the nadir at day 4. At day 7, P_4 start to increase without significant difference between the groups.

The analysis of E_2 from D0 to D6 revealed an effect of group ($P<0.05$) and an effect of group by day ($P<0.01$). In D1 protocol, the E_2 increased to be above 2 pg/ml from D2 to D4, and then decreased gradually (Fig. **2**). On the other hand, the E_2 of No-CL protocol showed a peak on D3 or D4 (Fig. **2**). Likewise, the E_2 level in control group peaked at day 5 (Fig. **2**).

DISCUSSION

The increase in the number of follicles at D1 in no-CL group, confirmed the hypothesis that follicle aspiration prior to superovulation improves the ovarian response [4,13].

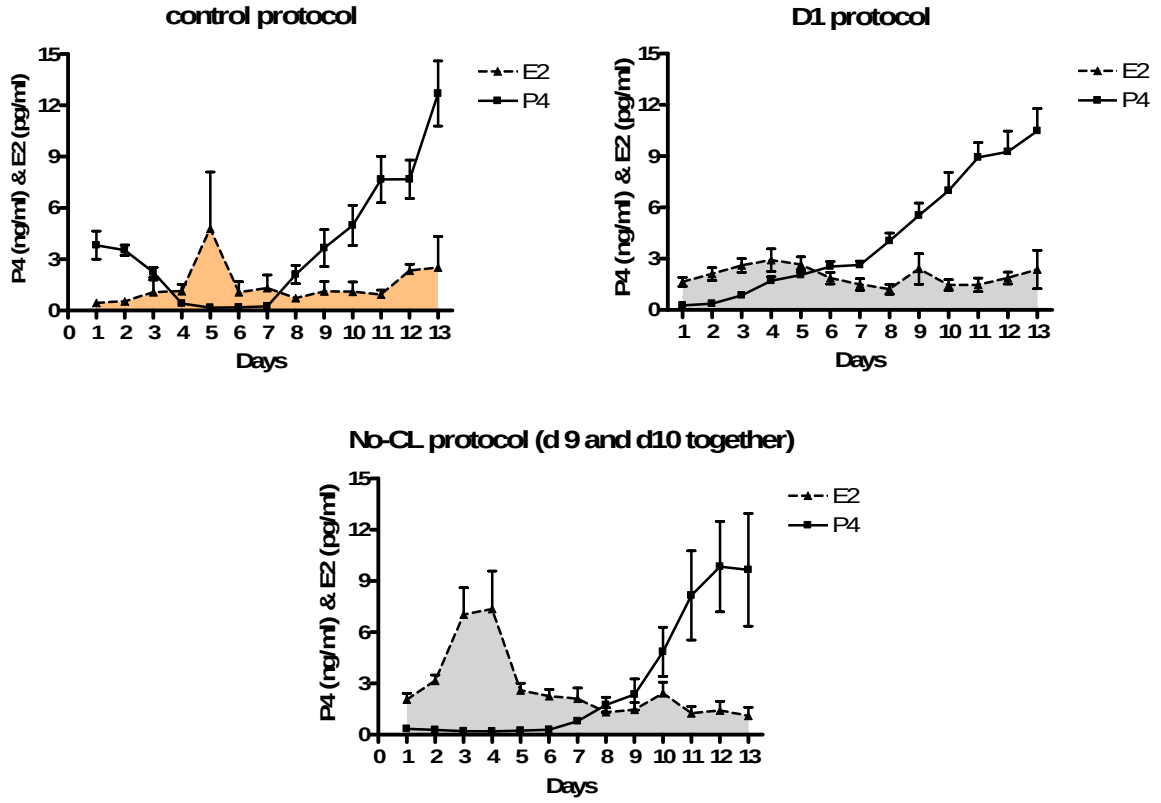


Fig. (2). Comparative changes in the plasma concentration of P4 (ng/ml), E2 (pg/ml) in the 3 groups. Blood sample were collected daily from D1 to D13. Data shown as the mean ± SEM of each time period.

Empirical evidence suggested that elimination of the dominant follicle prior to superovulation induced a new follicular wave emergence which improved the ovarian response to gonadotrophic stimulation [3,4,13-15]. However, D1 protocol had the same merit of absence of the dominant follicle (D.F) after spontaneous ovulation however; the number of emerged follicles was significantly lower than the no-CL protocol. Such increase in recruited follicles at no-CL groups may be attributed to the difference of FSH secretion pattern in the 2 groups. In D1 protocol, FSH had 2 surges; preovulatory surge which associated with the ovulatory LH surge and periovulatory surge which associated with the follicle emergence [16]. In contrast, no-CL group and because of the follicle aspiration, FSH probably had one surge only, which may be higher than that of the D1 protocol.

The FSH can stimulate the follicular growth under the level of P_4 but the GnRH was very important for ovulation [17]. The obtained result revealed that injection of GnRH at D5 induced a successful ovulation. This ovulation ability proved that the growing follicles of the 1st wave had sufficient LH receptors. In the contrary, Calder, *et al.,* [18] hypothesized that the insufficient LH receptors was responsible for the failure of ovulation of the first wave follicles even after injection of hCG.

In no-CL group, the aspiration of all follicles ≥ 5mm 36 h after the injection of the $PGF_{2\alpha}$ and before the LH surge which estimated to be 56 h [19] produced no luteal tissue and subluteal P_4 level. The same finding was supported by Hayashi, *et al*., [20] who aspirated the dominant follicle 42 h after the $PGF_{2\alpha}$ injection during the mid luteal phase and got no luteal tissue. The same authors added that if aspiration of the dominant follicle was post-LH surge, the aspirated follicle would grow to make a functional CL with a visible blood flow.

The ovulatory response was less variable in D9-subgroup (75%) in comparison to D10-subgroup (33%) and the number of ovulated follicles was increased in D9-subgroup (13.8±4.4) in comparison to the other groups. In the present study, the time of $PGF_{2\alpha}$ injection and the time of follicles aspiration played a very important role in the success of the protocol. In D10-subgroup the follicle aspiration occurred at D12 (36h after $PGF_{2\alpha}$ injection) at which the dominant follicle has already lost its activity leading to the emergence of a new wave. At that time, the aspiration was difficult because many follicles were larger than 5mm. One of those follicles escaped from aspiration and became a dominant follicle. This escaped follicle suppressed the growth or ovulation of the other follicles. Supporting this hypothesis, it was reported that the future dominant follicle has an early development advantage than the other recruited follicles during the process of selection [21]. On the other hand, in D9-subgroup, the aspiration of the follicles occurred at D11 at which the dominant follicle was active. So it was easier to remove the dominant follicle and subordinate only. As a result, the follicles of the new wave had the same diameter.

In animals with lower response, there was a co-ordination failure of the follicular growth; one or more follicles became larger than 10 mm at D3. Those follicles had a negative influence on the other follicles [22] and ovulated earlier than those follicles at D4 or D5. Supporting this, animals with higher response in both D9 and D10 subgroup produced high E_2 peak (≥ 9 pg/ml) in comparison to animals with lower response. This result showed that the growing follicles in animals with higher response were more active than those in animals with lower response. The result was supported by Ali *et al*., [23] who observed that the dominant follicles in growing or in early static growth phase were always E_2 - dominant.

In conclusion, the superovulation protocol with complete absent of the CL produced high number of growing follicles, decreased variability and considered as a promising superovulation protocol.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENT

None declared.

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