Superovulation and Embryo Yield after GNRH Pretreatment in Crossbred Cows

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ABSTRACT

The effect of GnRH pretreatment on superovulatory response (SOR) and embryo yield in crossbred cattle was studied. Six crossbred cows were subjected to three superovulatory treatments: i.Control: Four day FSH schedule was initiated on Day 10 of the cycle ii.Gn-D8: GnRH was administered on Day 6 and FSH schedule was initiated on Day 8 and iii. Gn-D10: GnRH was administered on Day 6 and FSH schedule was initiated on Day 10. In control group, the mean SOR was 13.7 $\pm$ 5.8 CL. In Gn-D8 group, the SOR is inconsistent (5.0 $\pm$ 1.7) with recovery of poor quality embryos, which was attributed to significantly higher progesterone concentration on the day of superovulatory oestrus. In Gn-D10 group, SOR (11.0 $\pm$ 1.6) and embryo yield were comparable to the control group. Transferable quality embryos were significantly higher in Gn-D10 group which could be attributed to the follicular maturation under favourable endocrine environment. Thus, GnRH pretreatment in superovulation protocol ensured consistent SOR and increased yield of transferable quality embryos in crossbred cattle.

Key Words: GnRH pretreatment, superovulatory response, embryo yield, crossbred cows

INTRODUCTION

Gonadotropin induced superovulation is the basic and efficient method of obtaining multiple embryos from the genetically valuable females. However, the superovulatory response (SOR) is highly unpredictable and variable between treatments thus affecting the efficiency of the technology and limiting its practical application (Adams *et al.,* 1993). Variability in ovarian response has been attributed to various exogenous factors such as donor parity and production status, season, hormone preparations and their dose (Lee *et al.,* 2012; Vieira *et al.,* 2015; Abdelatty *et al.,* 2018). Above all, success of ovarian response is dependent on the nature of dominant follicle (DF) and the availability of gonadotropin sensitive follicles at the time of initiation of treatment (Driancourt, 2001). Nasser *et al.* (1993) opined that SOR was found to be higher when gonadotropin treatment was initiated at the time of follicular wave emergence (FWE). However, difficulty in predicting the day of FWE during an oestrous cycle poses a major problem. To obviate this problem, an alternative approach is to control the FWE

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and initiate the superstimulation treatment at the most favourable time that is optimal for recruited follicles to respond for exogenous gonadotropins.

The technique of synchronizing FWE in crossbred cattle was standardized by administering GnRH on Day 6 of the cycle (Satheshkumar *et al.,* 2008 and Satheshkumar *et al.,* 2012). Based on these previous findings, the present research was designed with the objective to study the effect of GnRH pretreatment on SOR and embryo yield in crossbred cattle.

MATERIALS AND METHODS

Experimental animals

Six healthy, pluriparous, non-lactating and regularly cycling crossbred cows aged between 5-6 yrs maintained at the Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, Madras Veterinary College, Chennai were utilized for the study. The cows were housed together and maintained under same conditions throughout the study. All the experimental cows were monitored regularly for their cyclicity and oestrus (Day 0) was confirmed by gynaeco-clinical and ultrasound examination.

Superovulation treatment

All the six animals are subjected for three different superovulatory treatments as mentioned below, with an interval of two months between each treatment.

Treatment 1- Control: Superstimulatory treatment was initiated on Day 10 of the cycle. The schedule included total dose of 400 mg NIH-FSH-P1 (Folltropin-V; Bioniche, USA) administered in equally divided doses (50 mg each; i.m.) twice daily at 12 h interval over a period of four days. Superovulatory oestrus (SO) was induced with two injections of prostaglandin (PG) -Dinoprost tromethamine (Inj. Lutalyse; 25 mg each, i.m.; Pfizer, Belgium) given at 48 and 60 h after first FSH injection. Animals were inseminated during SO thrice at 12 h interval, from 48h post PG administration. To control the potentially confounding effect of handling stress on SOR, cows received an intramuscular injection of saline (2.5 ml) on Day 6, simulating the GnRH administration in other treatment groups.

Treatment II - Gn-D8: The animals were injected with GnRH analogue- Buserelin acetate (Inj. Receptal; 10 µg i.m.; Intervet International, GmbH, Germany) on Day 6 of the cycle and FSH schedule for superovulation (as mentioned in control group) was initiated 48 h after GnRH i.e., on Day 8, the day of synchronized FWE (Satheshkumar *et al.,* 2012).

Treatment III - Gn-D10: GnRH analogue was administered on Day 6 of the cycle and superovulation schedule was initiated 96 h after GnRH i.e., on Day 10, two days after the synchronized FWE and before the deviation of DF (Satheshkumar *et al.,* 2012).

Assessment of follicular characteristics during superovulation treatment

Number of follicles of various size categories (Class I - $\leq$ 5 mm, Class II - > 5 -

< 9 mm and Class III - ≥ 9 mm) on the day of initiation of FSH treatment and on the day of SO were determined by ultrasound scanning using a real time B-mode ultrasound scanner (Sonovet 600, Universal Medical Systems) equipped with a 7.5 MHz rectal probe.

Superovulatory response and embryo yield

On the day of embryo collection (Day 7 post SO), the SOR was assessed by estimating the number of corpus luteum (CL) and anovulatory follicles (AF) by rectal palpation and confirmed by ultrasound scanning. Based on which animals were categorized as either responders (animals having > 2 CL) or non-responders (animals having ≤ 2 CL) (Vieira et al., 2015). Embryos / ova were recovered non-surgically by flushing the uterine horns using two-way Foley's catheter as described by Kathiresan *et al.* (1997). The embryos were morphologically scored for quality, colour and developmental stage (Lindner and Wright, 1983). The grade 1 (Excellent) and 2 (Good) embryos were considered as transferable, while grade 3 (Fair) and 4 (Poor) were non-transferable quality embryos. Apart from the unfertilized oocytes (UFO), embryos in earlier developmental stages than morulae were categorized as 'arrested or degenerated'.

Plasma progesterone (P_4) concentration

Blood samples were collected on the day of initiation of FSH treatment, day of PG, day of SO and day of embryo collection in all the experimental groups. Plasma was separated by centrifuging the blood sample and stored in duplicate vials at -20^{O}C until assayed. The P_4 concentration was measured with solid-phase radio immuno assay kit (Coat – A – Count, Immunotech SAS, France) and the radioactivity was counted in I^{125} gamma counter (STRATEC, Germany). The sensitivity of the P_4 assay was 0.05 ng / ml and intra-assay co-efficient variation was 6.5. Hormone assay was carried out at Department of Veterinary Physiology, Veterinary College and Research Institute, Namakkal, Tamilnadu.

Statistical analysis

Data on follicular characteristics, SOR, embryo yield and plasma P_4 concentrations in superovulatory cycles were analyzed by Student's *t*-test and by Analysis of Variance (ANOVA) with completely randomized design. SPSS.10.0® software was used for analysis of data. Analysis of data was carried out as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Various categories of follicles during the course of superovulatory protocol in control and GnRH treated groups are presented in Table 1. Perusal of the data revealed that GnRH pretreatment favoured the availability of more number of FSH-responsive follicles at onset of protocol than the control group in concurrence with the earlier findings of Satheshkumar *et al.* (2008) and Satheshkumar *et al.* 2012).

Table – 1. Mean ± SE of follicular numbers during and recruitment and development in superovulatory cycles of crossbred cows

Treatment Groups	FOLLICULAR POPULATION (Range within parenthesis)								
	Day 6			Day of I FSH			Day of SO		
	Class I	Class II	Class III	Class I	Class II	Class III	Class I	Class II	Class III
Control (n = 6)	0.5 ± 0.2 (0 – 2)	0.8 ± 0.1 (0 – 2)	1.0 ± 0.0 (1)	6.5 ± 0.9 [a] (3 – 9)	0.7 ± 0.3 [a] (0 – 2)	0.5 ± 0.2 (0 – 1)	0.0 (0)	5.1 ± 0.8 [a] (2 – 8)	9.8 ± 1.2 [a,b] (7 – 15)
Gn-D8 (n = 6)	0.4 ± 0.3 (0 -2)	0.7 ± 0.2 (0 – 1)	1.0 ± 0.0 (1)	11.5 ± 0.6 [b] (8 – 14)	0.8 ± 0.3 [a] (0 – 2)	0.0 (0)	0.0 (0)	9.2 ± 1.3 [b] (0 - 18)	8.3 ± 2.8 [a] (5 – 13)
Gn-D10 (n = 6)	0.5 ± 0.2 (0 – 2)	0.8 ± 0.3 (0 – 2)	1.0 ± 0.0 (1)	5.2 ± 0.5 [a] (4 – 8)	7.5 ± 0.6 [c] (5 – 8)	0.0 (0)	0.0 (0)	3.7 ± 0.4 [a] (2 – 5)	11.0 ± 0.9 [b] (9 – 15)
Significance	#	#	#	**	**	#	#	*	*

Class I - ≤ 5 mm; Class II - > 5 - < 9 mm; Class III - ≥ 9 mm

Values within the column with different superscripts differ significantly ** (P < 0.01) * (P < 0.05)

 # - Statistically not comparable

The plasma P_4 concentrations during various stages of the superovulation programme are presented in Table 2. On the day of initiation of FSH treatment, the mean plasma P_4 concentration in Gn-D10 group was significantly (P < 0.01) higher than the control group and non-significantly higher than Gn-D8 group. The increased P_4 concentrations in Gn-D10 group could be attributed to the presence of developing GnRH induced accessory corpus luteum (ACL). Mapletoft *et al.* (2009) indicated the importance of a functional CL with sufficient P_4 concentrations at the time of initiation of gonadotropin treatment in achieving a better SOR.

Table – 2. Mean ± SE of Plasma progesterone concentrations in superovulated crossbred cows

Treatment groups	PROGESTERONE CONCENTRATION (ng / ml)			
	Day of I FSH	Day of PG	Day of SO	Day of collection
Control	5.3 ± 0.9 [a]	8.5 ± 0.3 [a]	0.4 ± 0.0 [a]	18.7 ± 1.2 [b]
Gn-D8	6.7 ± 0.5 [a,b]	9.4 ± 1.1 [a]	4.3 ± 0.9 [b]	6.2 ± 1.4 [a]
Gn-D10	8.8 ± 0.8 [b]	10.0 ± 0.9 [a]	0.6 ± 0.1 [a]	17.0 ± 1.0 [b]
Significance	**	N.S	**	**

Values within the column with different superscripts differ significantly ** (P < 0.01) N.S – Not significant (P > 0.05)

In Gn-D8 group, the P_4 concentration was significantly (P < 0.01) higher on the day of SO than the other groups. Interestingly, a prominent luteal tissue of ACL could be detected on the day of SO in four animals (66.7%) of this group which was also supported by suprabasal levels of P_4 (4.3 ng / ml). It was reported that the immature CL (< 5 days old) was not consistently responsive to $PGF_2\alpha$ due to

luteal insensitivity or insufficient numbers of PG receptors (Duchens *et al.,* 1994). In the present study, the ACL was immature (4 days old) at the time of PG administration and hence would not have responded resulting in incomplete luteolysis. On the contrary in Gn-D10 group, the ACL was mature enough (6 days old) to respond to PG and hence complete lysis could be accomplished as indicated by low P_4 concentration (0.6 ng / ml) on the day of SO.

The SOR as indicated by number of CL and AF in various experimental groups are presented in Table 3. All the animals (100%) subjected for superovulatory treatment in control group and Gn-D10 groups responded with more than two ovulations, but in Gn-D8 group, only four (66.7%) animals responded to the superovulation treatment. Among these four responders only two animals had more than 10 ovulations and the remaining two animals had three ovulations each. Eventhough more numbers of FSH responsive follicles were present initially in Gn-D8 group than the control group, majority of them failed to ovulate reducing the overall SOR. Similarly, Rajamahendran and Calder (1993) and Farin *et al.* (2008) also failed to show improvement in the SOR when the cows were superovulated two days after hCG or GnRH treatment. Significantly more numbers of Class II follicles were recorded than Class III follicles in this group on the day of SO, which might be an indication that follicles have not matured sufficiently to complete the ovulation process, as suggested by D'Occhio *et al.* (1997). Supra-basal levels of P_4 on the day of SO would have inhibited the LH surge and prevented the ovulation (Duchens *et al.,* 1994). Thus the highly inconsistent SOR in Gn-D8 group could be attributed to the aberrant endocrine milieu during the course of superovulatory cycle.

Table – 3. Mean ± SE of superovulatory response and embryo yield in superovulated crossbred cows

Treatment groups	**No. of animals responded** (% in parenthesis)	**Superovulatory response** (Range within parenthesis)		**No. of animals yielded embryos / oocytes** (% in parenthesis)	**No. of embryos / oocytes recovered** (Mean % - in parenthesis)
		CL	**AF**		
Control (n = 6)	6 (100.0)	13.7 ± 5.8 [b] (9 – 21)	2.0 ± 0.4 [a] (1 – 3)	6 (100.0)	8.7 ± 2.0 (63.4 ± 9.7) [a]
Gn-D8 (n = 6)	4 (66.7)	5.0 ± 1.7 [a] (3 – 11)	12.8 ± 4.6 [b] (2 – 23)	3 (50.0)	3.0 ± 1.6 (36.6 ± 16.4) [b]
Gn-D10 (n = 6)	6 (100.0)	11.0 ± 1.6 [b] (10 – 14)	3.5 ± 0.2 [a] (3 – 4)	6 (100.0)	6.8 ± 0.87 (61.8 ± 4.2) [a]
Significance		**	**		**

Values within the columns with different superscripts differ significantly ** ($P < 0.01$)

The SOR in Gn-D10 group was comparable to control group, but had significantly higher number of ovulations than Gn-D8 group. Fortune *et al.* (2001) stated that till the point of deviation, the future DF and subordinate follicles were similar in FSH receptors on their granulosa cells. Ginther *et al.* (2003) also concluded that all follicles in the common growth phase have the potential for future dominance and administration of FSH early in wave prevented deviation phenomenon and induced several follicles to become dominant in cattle. Contrary to the Gn-D8 group, decreased P_4 levels on the day of SO would have favoured the follicular maturation (as indicated by increased numbers of Class III follicles) and ovulation in Gn-D10 group. Thus it could be determined that majority of the recruited follicles of synchronized wave could be rescued from atresia and incorporated into the cohort that attains ovulatory capability when treatment was initiated before the deviation process.

The data on embryo recovery in control and GnRH treated groups are presented in Table 3. Embryos could be recovered from all the animals (100%) in control and Gn-D10 groups, while only three (50%) animals yielded embryos in Gn-D8 group. There was no significant difference in the embryo recovery rate between the control and Gn-D10 groups but the recovery rate was drastically reduced (36.6%) in the Gn-D8 group, which was in corroboration with the previous findings of Kohram *et al.* (1998) and Deyo *et al.* (2001). The Gn-D8 group had a significantly increased number of AFs on the day of SO when compared with other groups. As suggested by Sato *et al.* (2005), abnormally high levels of oestrodiol secreted by the persistent AFs might have hindered the ova transport in the oviducts adversely affecting their recovery in Gn-D8 group.

Various grades of embryos / ova recovered from superovulated animals are presented in Table 4. The percentage of transferable embryos in Gn-D10 group was significantly ($P < 0.01$) higher than Gn-D8 group and non-significantly ($P > 0.05$) higher than control group. The increased concentrations of P_4 at the onset of treatment would have enhanced the quality of follicular inventories and embryo quality thereon. On the other hand, non-transferrable embryos and UFO constituted the major proportion of ova recovered in Gn-D8 group. High levels of E_2 secreted by AFs might have affected the fertilization and subsequent embryo development in this group (Sato *et al.,* 2005).

Table - 4. Mean ± SE of quality of embryos recovered from superovulated crossbred cows

Treatment groups (Number of ova/embryos in parenthesis)	QUALITY OF EMBRYOS (%) (Number of ova / embryos in parenthesis)							
	Grade 1	Grade 2	Transferable (Gr 1+ Gr 2)	Grade 3	Grade 4	Non-transferable (Gr 3 + Gr 4)	Arrested / Degenerated	UFO
Control (52)	49.7 ± 4.5 [a] (26)	29.7 ± 4.8 [a] (15)	79.4 ± 4.7 [b] (41)	2.1 ± 2.1 [a] (1)	6.4 ± 3.0 [a] (4)	8.4 ± 2.6 [a] (5)	6.7 ± 3.9 [b] (4)	4.4 ± 2.3 [a] (2)
Gn-D8 (18)	4.2 ± 2.2 [#] (1)	4.2 ± 2.2 [#] (1)	8.3 ± 2.2 [a] (2)	8.3 ± 3.3 [#] (2)	20.8 ± 6.3 [#] (3)	29.1 ± 4.8 [b] (5)	16.6 ± 12.3 [c] (3)	45.8 ± 22.7 [b] (8)
Gn-D10 (41)	56.8 ± 5.4 [a] (23)	30.4 ± 7.4 [a] (11)	87.2 ± 6.2 [b] (34)	5.2 ± 3.4 [b] (2)	5.2 ± 3.3 [a] (2)	10.3 ± 3.3 [a] (4)	3.3 ± 1.3 [a] (2)	1.8 ± 0.8 [#] (1)
Significance	*	N.S	**	*	N.S	**	*	**

Values within the columns with different superscripts differ significantly ** ($P < 0.01$) * ($P < 0.05$)

N.S – Not significant ($P > 0.05$); # - Statistically not comparable

Based on the developmental stages, Gn-D10 group recorded increased percentage of early blastocysts and blastocysts than the control group (Table 5). Thus homogenous recruitment and development of healthy follicles under an appropriate endocrine milieu would have contributed for the better developmental quality of embryos in Gn-D10 group as suggested by Yadav *et al.* (1986).

Table – 5. Percentage of different developmental stage embryos recovered from superovulated crossbred cows

Treatment groups (Number of embryos in parenthesis)	DEVELOPMENTAL STAGES OF EMBRYOS (%) (Number of embryos in parenthesis)			
	Morula	Compact morula	Early Blastocyst	Blastocyst
Control (46)	34.8% (16)	36.9% (17)	23.9% (11)	4.3% (2)
Gn-D8 (7)	14.3% (1)	42.9% (3)	28.6% (2)	14.3% (1)
Gn-D10 (38)	15.8% (6)	36.8% (14)	42.1% (16)	5.3% (2)

The study proved the hypothesis that GnRH pretreatment ensured increased availability of gonadotropin responsive follicles on the day of initiation of superstimulation treatment. However, initiating the gonadotropin treatment two days post synchronized FWE (before the deviation) had the advantage of proper follicular maturation and favourable endocrine environment in achieving satisfactory SOR and embryo yield, rather than initiating the treatment on the day of synchronized FWE . The problems recorded in Gn-D8 group during the study might be the reasons for limited success by the previous researchers (Kohram et al.,1998 and Deyo *et al.* 2001) when GnRH pretreatment was included in superovulation schedule.

From This study, it was concluded that administration of GnRH on Day 6 of the cycle and initiation of FSH treatment on Day 10 resulted in homogenous recruitment of healthy follicular inventories. Under a favourable endocrine environment satisfactory SOR and embryo yield could be achieved. With the recovery of increased percentage of transferable quality and better developed embryos than the conventional method, it is suggested that GnRH pretreatment could be successfully incorporated in the superstimulation protocol of cattle.

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