EFFECT OF SYNCHRONIZATION OF FOLLICULAR WAVE EMEREGENCE ON SUPEROVULATION AND EMBRYO YIELD IN CROSSBRED COWS

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DPV 05004 (OGY)

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DEPARTMENT OF ANIMAL REPRODUCTION,
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ABSTRACT

Title	:	**EFFECT OF SYNCHRONIZATION OF FOLLICULAR WAVE EMERGENCE ON SUPEROVULATION AND EMBRYO YIELD IN CROSSBRED COWS**
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The present study was aimed at assessing the effect of GnRH analogue in synchronizing the follicular wave emergence in Jersey crossbred cattle and to study the effect of initiating FSH treatment (normal and reduced doses) after synchronizing the emergence of follicular wave on superovulatory response and embryo yield.

Six healthy, non-lactating and regularly cycling Jersey crossbred cows aged between 5-6 yrs were utilized for the study. Initially, as a control study, the normal follicular wave pattern was ultrasonographically investigated in all the six cows. In experimental group, all the animals were injected with GnRH analogue (Buserelin acetate; 10 µg i.m.) on Day 6 of the cycle (Day 0 – oestrum) and follicular wave pattern was studied. All the six animals were subjected for four superovulatory treatments. Treatment1-Conventional: FSH (400 mg - Folltropin-V) treatment was initiated on Day 10; Treatment II – Gn-D8-400: FSH treatment (400 mg) was initiated on the day of GnRH synchronized follicular wave emergence (Day 8); Treatment III – Gn-D10-400: FSH treatment (400 mg) was initiated two days after synchronized follicular wave emergence (Day 10) and Treatment IV- Gn-D10-200: Similar to Gn-D10-400 group, but superstimulated with 200 mg.

Monitoring the normal follicular wave pattern revealed that 77.8, 16.7 and 5.6 per cent of oestrous cycles exhibited three, two and four follicular waves respectively. The first wave dominant follicles (DF) were in a growth phase during Days 0.8 – 6.67 of the oestrous cycle and got deviated from the subordinate follicles by 3.30 – 3.67 days after emergence irrespective of the number of follicular waves succeeding the first one. Thus characteristics of the first wave DF was much predictable than that of the subsequent waves. Three peaks of FSH levels could be appreciated in three-wave

cycles preceding the emergence of each follicular wave.

When GnRH was administered on Day 6 of the cycle, the DF (10.83 ± 0.38 mm) ovulated in all the animals (100 %) in a mean interval of 27.67 ± 0.21 h and a synchronized homogenous group of follicles emerged after two days (Day of 8.00 ± 0.0). With the formation of an additional luteal structure (ACL), the progesterone concentration (10.57 ± 0.61 ng / ml) was significantly ($P<0.01$) increased than normal cycle (5.58 ± 0.45 ng / ml) during the mid luteal phase.

In Conventional group, all the animals (100%) responded for FSH, with 13.67 ± 1.80 CL and 2.00 ± 0.37 AF. However, the superovulatory response varied widely (9 - 21ovulations). In Gn-D10-400 group, the animals responded with 11.00 ± 0.63 (10 – 14) CL and 3.50 ± 0.22 (3 – 4) AF comparable to the Conventional group. In both Conventional and Gn-D10-400 groups, almost all the Class III follicles on the day of superovulatory oestrus ovulated indicating that these follicles were healthy enough to respond to endogenous LH surge. In Gn-D8-400 group, the mean number of CL and AFs were 5.00 ± 1.77 (1 – 11) and 12.83 ± 4.65 (2 - 30) respectively. More number of Class II follicles and suprabasal progesterone levels (due to incomplete luteolysis) on the day of oestrus was correlated positively with increased number of AFs.

The embryo recovery rate (53.89 %) in Gn-D10-400 was comparable to that of Conventional group (55.49 ± 9.70 %), but the recovery rate (36.57 ± 16.44 %) was substantially affected in Gn-D8-400 group. In Gn-D10-400 group, a higher percentage (87.24 %) of transferable quality (Grade 1 and 2) embryos and lower incidence of arrested / degenerated embryos (3.33 %) and UFO (1.85 %) was recorded than the Conventional (79.49%, 6.72 % and 6.25 % respectively) group. However, in Gn-D8-400 group, arrested/degenerated and UFO (62.50%) and Grade 4 embryos (20.84%) constituted the major proportion of ova recovered and transferable quality embryos accounted for only 8.34 per cent. Increased anovulatory follicular population from the day of oestrus to the day of embryo collection and thus an increased concentration of oestradiol would have resulted in a poor embryo recovery / quality in Gn-D8 group.

With the reduced dose of FSH (200 mg) in Gn-D10 group, all the animals responded with a mean superovulatory response of 6.33 ± 0.99 CL (4-11) and an average of 44.24 per cent embryos / ova were recovered. When compared with Gn-D8-400 group, a non-significant increase in superovulatory response and embryo recovery rate was recorded in Gn-D10-200 group.

Key Words: GnRH – Follicular wave synchronization – Superovulation – Embryo yield – crossbred cows

LIST OF ABBREVIATIONS

ACL	:	Accessory corpus luteum
AF	:	Anovulatory follicle
B. indicus	:	*Bos indicus*
B. taurus	:	*Bos taurus*
BSA	:	Bovine serum albumin
cAMP	:	Cyclic adenosine mono phosphate
CIDR	:	Controlled internal drug releasing device
CL	:	corpora lutea/ luteum
DPBS	:	Dulbecco's phosphate buffered saline
DF	:	Dominant follicle
E2	:	Oestradiol
E-17β	:	Oestradiol-17β
EB	:	Oestradiol benzoate
ETT	:	Embryo transfer technology
FSH	:	Follicle stimulating hormone
FSH-R	:	Recombinant FSH
FSH-P	:	Porcine FSH
FWS	:	Follicular wave synchronized
GnRH	:	Gonadotrophin releasing hormone
h	:	hour
hCG	:	Human chorionic gonadotrophin
HF	:	Holstein – Friesian
IGF-I	:	Insulin-like growth factor -I
IGFBP	:	Insulin-like growth factors binding protein
i.m.	:	Intra muscular

IU	:	International Unit
LH	:	Luteinizing hormone
ng	:	Nano gram
mg	:	milli gram
min	:	minutes
ml	:	milli litre
mm	:	milli meter
mRNA	:	Messenger RNA
nmol / l	:	nano mole per litre
NIH	:	National Institute of Health
P4	:	Progesterone
PG	:	Prostaglandin
pg	:	pico gram
PMSG	:	Pregnant mare serum gonadotrophin
RIA	:	Radio immuno assay
SOE	:	Superovulatory oestrus
SOR	:	Superovulatory response
vs	:	versus
µg	:	Micro gram
µl	:	Micro litre

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CHAPTER I

INTRODUCTION

Embryo transfer technology (ETT), first successfully accomplished by Walter Heape in 1890, started as a research tool and became a commercial enterprise in cattle in early 1970s (Hasler, 2004). Considering the reduction in generation interval and production of more number of full and half sibs for genetic evaluation, this technique was considered to be a boon to farms. Though this technology was standardized in India in late 80's, it has not been fully exploited in breeding programme. In spite of high technical specialization of operators working in the embryo transfer field, the results of this technique are still unpredictable (Mollo *et al*., 2007).

Superovulation using gonadotrophin is a common and efficient technique for obtaining multiple embryos from genetically valuable females. However, the superovulatory response varied widely among individuals and treatments, affecting embryo transfer efficiency and limiting its practical use. According to Hahn (1992), superovulation of donors with identical physiologic characteristics and even superovulation of the same donor at different times can be ineffective in about 15 to 20 per cent of the cases. Variability in ovarian response has been related to differences in superovulatory treatments, such as gonadotrophin preparation, batch and total dose, duration and timing of treatment (Gonzalez *et al*., 1990 and Quaresma *et al*., 2003). Additional factors that contribute to this variability and inherent to the animal and its environment include nutritional status, reproductive history, age, season, breed etc., (Lerner *et al*., 1986 and Kafi and McGowan, 1997). While considerable recent progress has been made in the field of bovine reproductive physiology, factors inherent to the donor animal i.e. the status of follicular development at the time of initiation of superstimulatory treatment influence the success of superovulation treatment (Kastelic *et al*., 1990).

The conventional protocol of initiating ovarian superstimulation during mid-cycle was originally based on anecdotal and experimental information in which a greater superovulatory response was reported when gonadotrophin treatments were initiated 8-12 days after oestrus (Bo *et al*., 1995a and Lindsell *et al*., 1986). However,

none of these early studies evaluated the follicular status of the animals when superstimulation treatments were initiated and it was hypothesized that Days 7-11 after ovulation would be the approximate time of emergence of the second follicular wave in cattle (Ginther *et al*., 1989a). In this regard, it has been clearly shown that superovulatory response was higher when gonadotrophin treatments were initiated at the time of wave emergence and asynchrony of one or two days reduced the superovulatory response compared to initiating treatments on the day of wave emergence (Nasser *et al*., 1993).However, there is great individual variation in the precise timing of the second follicular wave emergence as well as the general follicular status in cows that have two- or three-wave cycles (Ginther *et al*., 1989a). Thus, a wide variation in superovulatory response observed with conventional treatment could be attributed to cow-to-cow variation in ovarian status at the onset of superstimulatory treatment.

The advent of real-time ultrasonography has provided a means to follow individual follicles of a cohort from day to day, to determine follicular status prior to gonadotrophin treatments (Griffin and Ginther, 1992). However, it is difficult to precisely determine the status of follicular development by a single ultrasonographic examination and it is often not practical to serially examine individual animals in a field-level setting. To obviate these problems, an alternative approach is to standardize the exogenous control of follicular wave emergence (Bo *et al*., 1995a). Kohram *et al.* (1998b) and Rajamahendran *et al.* (1998) found that GnRH treatment between Days 4 and 7 of the oestrous cycle caused rapid disappearance of large follicles and increase in the number of recruitable follicles (4-6 mm), indicative of the emergence of a new follicular wave. Information obtained from these studies enabled researchers to synchronize follicular wave emergence and to initiate the superstimulation treatments at the most favourable time that is optimal for follicle recruitment and the number of follicles capable of responding to exogenous gonadotrophins is maximal (Bo *et al*., 2002).

Synchronization of follicular wave emergence could be achieved by removing the DF of extant wave and promoting the emergence of next follicular wave. The most promising strategies to synchronize the follicular wave are the use of hormones

(viz., oestrogen esters, progesterone and GnRH) and mechanical ablation of DF before the start of superovulation (Bo *et al*., 1995a). Hormonal approach was preferred over mechanical ablation because the latter technique needs ultrasonographic accessories which will not be available every time under field applications. Unfortunately, oestrogen esters are not commercially available in many countries, but GnRH is available easily in the local market.

Buserelin, a GnRH agonist, is a potential drug for controlling follicular development via induction of ovulation, luteinization or atresia of follicles that are responsive to an induced LH surge (Wolfenson *et al*., 1994). Despite the fact that GnRH was shown to synchronize follicular wave emergence as effectively as oestrogen esters following ovulation of a DF in cows, there have been only few reports on the effect of GnRH pretreatment on the superovulatory response or embryo yield (Kim *et al*., 2005 and Sato *et al*., 2005). Thus, further studies on these aspects of superovulation are warranted, especially in crossbred cattle of our country in which there are no previous studies.

Further, one of the major constraints for the adoption of ETT in developing countries has been its higher cost due to the unavailability of inputs in local market, especially FSH, and the need to import material resulting in increased cost of implementation. Undertaking research, to maximize the yield of transferable embryos per collection by minimizing the dose of gonadotrophin will help to lower the cost of superovulation.

So the objective of the present study was

i) To study the effect of GnRH in synchronizing follicular wave emergence.

ii) To study the effect of initiating FSH treatment after synchronized follicular wave emergence on superovulatory response and embryo yield.

iii) To compare the superovulatory response and embryo yield with regular and reduced doses of FSH administered after follicular wave synchronization.

CHAPTER II

REVIEW OF LITERATURE

The present study was aimed at studying the effect of GnRH in synchronizing follicular wave emergence as a pretreatment for superovulation in crossbred cattle and to study the effect of initiating FSH treatment (normal and reduced dosages) at different stages of synchronized follicular wave emergence on superovulatory response and embryo yield.

2.1 FOLLICULAR AND LUTEAL DYNAMICS

2.1.1 Study of follicular dynamics

Follicular development in monovular species is a wave-like sequence of organized events. The waves consist of the synchronous growth of small (4 to 5 mm) antral follicles, followed by the selection and growth of one DF, which achieves the largest diameter and suppresses the growth of the subordinate follicles. In the absence of luteal regression, the DF eventually regresses (becomes atretic) and a new follicular wave begins (Bo *et al*., 1994). For better understanding of ovarian physiology, various investigative techniques have been used to characterize the follicular development in cattle.

The studies and advancements that have led to our current understanding regarding patterns of follicular development are listed below in chronological order:

1960 The two-wave concept for follicular growth during the bovine oestrous cycle was proposed (Rajakoski, 1960).

1972 and 1981 The lifespan and fate of individual follicles during the oestrous cycle of heifers was directly examined (Dufour *et al.*, 1972 and Matton *et al.*, 1981).

1982-1983 Growth and differentiation of oestrogen-active and oestrogen-inactive follicles during the oestrous cycle were distinguished (Ireland and Roche, 1982 and 1983).

1984 Ultrasound to monitor sizes of follicles during the oestrous cycle of heifers was used (Pierson and Ginther, 1984).

1987 The concept of DFs, as observed in primates, was applied to cattle and the three-wave hypothesis for development of DFs during the oestrous cycle was proposed (Ireland, 1987).

1988 Ultrasound analysis and ovarian maps to track growth and atresia of individual follicles throughout the oestrous cycle of heifers were used (Savio *et al.*, 1988 and Sirois and Fortune, 1988).

1990 The autocrine and paracrine role of intrafollicular factors in the regulation of follicular growth, differentiation and function was studied (Ireland *et al*., 2000).

1992 The radioimmunoassay (RIA) method for determining the transient peak in basal serum concentrations of FSH before each follicular wave was used (Adams *et al*., 1992).

1993 The decreased episodic pattern of secretion of LH associated with termination of a follicular wave was studied (Savio *et al*., 1993).

2.1.2 Ultrasonographic study of ovarian structures

The modern era of study of follicular dynamics in cattle began with the classic publication by Pierson and Ginther (1984). They studied ovarian follicular development in heifers using ultrasonography and concluded that there was 1) growth of a large follicle to an ostensibly ovulatory size followed by regression at approximately mid-cycle, 2) selective accelerated growth of the follicle destined to ovulate approximately three days before ovulation, and 3) a few days before ovulation regression of the larger follicles that were not destined to ovulate.

2.1.3 Number of follicular waves per oestrous cycle

Pursley *et al*. (1993) detected 53.8 and 46.2 per cent of three and four waves in mature lactating Holstein cows. Wolfenson *et al*. (2004) and Burns *et al*. (2005)

studied follicular developmental pattern in Holstein cows and heifers and found that 75 -80 per cent and 20 – 25 per cent of them exhibited two and three follicular waves per oestrous cycle respectively.

Viana *et al*. (2000) observed oestrous cycles with two (6.67%), three (60.0%), four (26.67%) and five (6.67%) follicular waves in Brazilian Gir cows and associated the higher incidence of cycles with 3 or 4 waves with a low persistence of the DF. Gaur and Purohit (2007) and Romano *et al.* (2007) recorded 78.57 – 83.0 per cent and 17 .0 - 21.42 per cent incidence of two and three follicular waves respectively in *B. indicus* cattle. The latter authors also reported occurrence of one cycle of two waves and another of three follicular waves in the same animal.

Gong *et al*. (1995) observed that 85.7 per cent of Hereford cross Friesian beef heifers displayed three waves of follicular development during the oestrous cycle, while the remaining heifers had a two wave pattern. But, Malhi *et al*. (2005) found that the proportion of crossbred Hereford cows with two follicular waves was 60 per cent and the remaining animals (40 %) had three waves of follicular development. Satheshkumar *et al*. (2008a) monitored the follicular wave pattern in Holstein Friesian (HF) crossbred cows and placed on record that all the cows exhibited three waves per cycle.

2.1.4 Emergence of follicular waves

Ginther *et al.* (1989b) opined that the day of emergence of the second follicular wave differed between two-wave cycles and three-wave cycles (1 or 2 days earlier in three-wave cycles), as well as between individual animals.

Pursley *et al.* (1993) recorded the days of wave emergence for three wave cycles as 2.0 $\pm$ 0.8, 12.3 $\pm$ 0.8 and 16.7 $\pm$ 0.8, and for four wave cycles as 2.3 $\pm$ 0.7, 6.7 $\pm$ 0.7, 11.3 $\pm$ 0.7 and 18.7 $\pm$ 0.7 in lactating Holstein cows.

Zeitoun *et al.* (1996) stated that the ovulatory follicle of two, three and four follicular wave cycles emerged on day 14.6 $\pm$ 1.2, 16.2 $\pm$ 0.7 and 21.3 $\pm$ 3.0 respectively in Brahman cows. Gaur and Purohit (2007) observed in Rathi cows that in two wave cycles the first and second waves emerged on day 2.10 ± 0.36 and 10.55

± 0.62 respectively, whereas in three-wave cycle the waves emerged on 0.78 ± 0.44, 7.11 ± 1.05 and 13.22 ± 2.44 respectively.

Rhodes *et al*. (2001) recorded that the mean interval from oestrus (day 0) to follicular wave emergence was 1.5 $\pm$ 0.1 days and from ovulation to emergence was 0.4 $\pm$ 0.1 days in crossbred heifers. Malhi *et al*. (2005) found that the mean days of wave emergence were 0.5 $\pm$ 0.1 and 10.5 $\pm$ 0.4 for two wave cycles and 0.5 $\pm$ 0.2, 9.0 $\pm$ 0.5 and 15.83 $\pm$ 0.7 for three wave cycles in crossbred Hereford cows. Satheshkumar *et al*. (2008a) documented three wave cycles in HF crossbred cows with the mean days of emergence of first, second and third waves on 2.0 $\pm$ 0.82, 8.5 $\pm$ 1.0 and 12.75 $\pm$ 1.26 respectively.

2.1.5 Characteristics of dominant follicle

Pursley *et al.* (1993) stated that the events associated with the first DF were consistent, regardless of the subsequent number of follicular waves in each cycle.

Austin *et al*. (2001) concluded that the earliest intrafollicular changes that distinguish a follicle destined to become dominant from other follicles in a growing cohort were enhanced capacity to produce oestradiol and maintenance of low levels of Insulin-like growth factors binding proteins (IGFBP), whereas the follicles destined to become atretic were characterized by loss of capacity to produce oestradiol and enhanced production of low molecular weight IGFBPs.

Ireland and Roche (1982) reported that granulosa cells in ovarian follicles $\geq$ 10 mm in diameter acquired LH receptors and become oestrogenic. Rhodes *et al*. (2001) also indicated that the difference in size of the DF was associated with differences in responsiveness to LH and in capacity to synthesize oestradiol, as there were significant differences in aromatase activity of granulosa cells between follicles.

Grazul-Bilska *et al*. (2007) highlighted the association of vascularity with angiogenic and other factors, and with follicle dominance in the first follicular wave in cows. The expression of angiogenic factors in follicles probably enhanced the recruitment of vascular supply and contributed to maintenance of these follicles in a non-atretic state and subsequently contributed to their selection to become the DF.

2.1.5.1 Phases of dominant follicle

Rajamahendran *et al.* (1994) opined that each DF has a growing phase and a static phase, each lasting about five to six days. It remained dominant for four to five days and generally by day 11 or 12 of the cycle it lost its dominance and began to regress.

Zeitoun *et al.* (1996) reported that *B. indicus* cows with two waves had extended periods of follicular growth (5.2 days) and regression (7.2 days) than three waves (3.6 and 4.0 days respectively).

Singh *et al*. (1998b) found that, on the basis of diameter profiles, all DFs were in the growing phase on Day 3 of first follicular wave, in early static phase on Day 6 of that wave and in late static phase during the first day of second wave. Mihm *et al*. (2006) also concluded that all DFs of first follicular wave were in a growth phase during Days 3.5 – 6.5 of the oestrous cycle and cessation of growth and reductions in oestrogenic activity of these follicles occur simultaneously with the initial decline in LH pulse frequency on Day 8 of the cycle, which was usually six days after the emergence of the first follicular wave.

Ginther *et al*. (2003) opined that in cattle, 7 – 11 follicles per wave emerge over one to several days and entered the common growth phase of about 3 days, which extended from the beginning of wave to the beginning of deviation. They also stated that the future DF emerged six hours earlier in heifers than other follicles of the wave resulting in size advantage at the end of common growth phase.

Knopf *et al*. (1989) suggested that the DF of the first follicular wave in the oestrous cycle grew at a faster rate than that of the second wave due to lower plasma concentrations of progesterone.

Pursley *et al*. (1993) recorded the growth rate of three DFs of three wave cycles as 1.6$\pm$ 0.3, 1.7 $\pm$ 0.3 and 1.5 $\pm$ 0.3 mm / day respectively. Similarly they recorded 1.5 $\pm$ 0.3, 2.1 $\pm$ 0.3, 1.3 $\pm$ 0.3 and 1.2 $\pm$ 0.3 mm/ day growth rate respectively for four DFs of four wave cycles.

Bo *et al*. (2003) reported that the growth rate of DF in *B. indicus* was slower (1.1 – 1.5 mm / day) than in *B. taurus* cows (1.4 – 1.6 mm / day). Martinez *et al*. (2005) found that growth rate of the DFs in *B. indicus* cows were approximately 1.6 mm per day. Satheshkumar *et al*. (2008a) also observed similar DF growth rate of 1.65 – 1.71 mm / day among various waves in HF crossbred cows.

2.1.5.2 Size of dominant follicle

Pursley *et al.* (1993) recorded the peak diameter of three DFs of three wave cycles as 8.1 $\pm$ 0.5, 8.8 $\pm$ 0.5 and 9.6 $\pm$ 0.5 mm respectively. Likely they recorded 10.3 $\pm$ 0.4, 10.6 $\pm$ 0.4, 10.3 $\pm$ 0.4 and 11.3 $\pm$ 0.4 mm maximum diameter respectively for four DFs of four wave cycles.

Satheshkumar *et al*. (2008a) stated that the mean diameter of third (ovulatory) wave (12.83 $\pm$ 1.37 mm) was non-significantly larger than the first two waves (11.0 $\pm$ 0.71 and 10.25 $\pm$ 1.26 mm respectively). Vinoles *et al.* (1999) have documented the suppressive effects of high progesterone levels and the stimulatory effects of low progesterone levels on the growth of the largest follicle in sheep and hypothesized that the smaller size attained by follicles in mid-cycle waves was the result of progesterone-induced suppression of LH.

Kot and Ginther (1999) and Taponen et al. (2000) documented the mean diameter of the preovulatory follicle as 14.6 $\pm$ 0.5 mm and 15.4 $\pm$ 0.4 mm in Holstein and Finnish Ayrshire breed cattle respectively.

Wolfenson *et al.* (2004) recorded larger diameter of preovulatory follicle in Holstein cows (16.5 mm) than heifers (13.0 mm) and attributed it to the longer duration of dominance in the cows and related the smaller preovulatory follicle in heifers to lower FSH concentration, higher progesterone and low frequency of pulsatile LH secretion.

Malhi *et al.* (2005) placed on record the mean diameter of the DF on the day before ovulation was smaller in old cows (12.3 $\pm$ 0.5 mm) than in their daughters (13.9 $\pm$ 0.5 mm), and also the preovulatory follicle was smaller in three wave cycles (11.9 $\pm$ 0.3 mm) than two wave cycles (13.5 $\pm$ 0.5 mm).

2.1.5.3 Effect of dominant follicle on subordinate follicles

Ginther *et al.* (1989a) concluded that the inhibitory factors produced by the DF suppressed other follicles through systemic endocrine channels rather than through intra ovarian autocrine or paracrine channels.

Fortune (1994) stated that the secretion of feed back regulators, such as oestradiol and inhibin, by the DF would cause a decrease in FSH to levels that would not support the further growth of subordinates. They also supposed that the DF itself escaped from this atretic phenomenon by reaching a stage of differentiation in which it could sustain growth in the presence of low levels of FSH and also might be due to increased blood flow through the follicle.

Ginther *et al.* (1999) and Evans *et al.* (2002) opined that ablation of first largest follicle had a positive effect on the survival and continued growth of second largest follicle, indicating that the intact first follicle had a negative effect on second follicle. Their results also indicated that subordinate follicles often remain viable for about two days after the expected beginning of deviation.

Wiltbank *et al.* (2000) opined that all follicles of a wave had the capacity to become the DF, but in 75 per cent of waves the first follicle to emerge during the wave had slight developmental advantage that allowed it to reach a critical diameter before the other follicles and stated that the largest subordinate follicle was only 6 to 8 h of development behind the DF until the time of deviation.

Evans *et al.* (2004) found that the expression of mRNA for oestradiol receptor, aromatase and the LH receptor was 1.6, 11.6 and 2.8 fold greater respectively in granulosa cells of dominant compared with subordinate follicles, while β-glycan mRNA was 6.9 and 1.6 fold greater in granulosa and theca cells respectively of subordinate compared with DFs.

2.1.5.4 Factors affecting follicular dynamics

Several factors influence the follicular growth such as progesterone levels (Sirois and Fortune, 1988 and Knopf *et al.*, 1989) and season (Badinga *et al.*, 1994).

Henricks *et al.* (1986) and Murphy *et al.* (1991) stated that the beef heifers on low dietary intake tend to have greater proportion of oestrous cycles with three follicular waves than heifers with greater dietary intake, due to reduced clearance of progesterone in underfed animals.

Lucy *et al.* (1990) reported that lactating cows placed in negative energy balance before ovulation had preovulatory follicles that grew more slowly than follicles in cows that were in positive energy balance.

Burns *et al.* (2005) indicated that the number of follicles during each wave of oestrous cycle might be suppressed by various physiological states, such as lactation, heat stress, pregnancy and / or inadequate nutrition.

2.1.6 Characteristics of corpus luteum

Kot and Ginther (1999) stated that the most intensive growth of luteal tissue occurred between Day 3 and 4 of the cycle in Holstein heifers.

Taponen *et al.* (2000) analysed the size of CL in Finnish Ayrshire breed cows and heifers and recorded 22.7 $\pm$ 1.1 mm on Days 11 or 12 and 22.6 $\pm$ 0.7 mm on Days 14 or 15. Wolfenson *et al.* (2004) documented the diameter of CL on Day 15 of the cycle in Holstein heifers and cows as 20.8 $\pm$ 1.9 mm and 23.7 $\pm$ 1.4 mm respectively.

Sianangama and Rajamahendran (1996) recorded the initiation and completion of luteal regression in cows occurred on Day 15.5 $\pm$ 0.8 and 19.0 $\pm$ 1.4 respectively.

2.1.7 Oestrous cycle length

Taylor and Rajamahendran (1991) opined that cattle that experienced three follicular waves had a longer oestrous cycle because oestrus was delayed when the second DF failed to ovulate and a third DF required additional time to complete development before ovulation. Malhi *et al.* (2005) also recorded the inter-ovulatory interval for two-wave and three-wave patterns as 20 and 23 days respectively. On the other hand, Zeitoun *et al.* (1996) stated that two wave cycles (20.3 $\pm$ 0.4 days) have

similar length as three wave cycles (20.8 ± 0.3 days) and attributed it to the extended periods of follicular growth and regression in former cycle pattern.

Pursley *et al.* (1993) stated that the interoestrus intervals were longer for cycles with four waves (25.0 days) than three waves (21.3 days). But, Viana *et al.* (2000) could not observe any significant difference in oestrous cycle length between cycles with 3 or 4 waves (21.11 vs 22.25 days).

2.2 SYNCHRONIZATION OF FOLLICULAR WAVE EMERGENCE

Follicular wave emergence has been altered by mechanical procedures or hormonal treatments. New follicular wave emergence depended on the morphological or functional disappearance of the existing wave and an associated FSH increase (Roche, 1996).

2.2.1 Mechanical approach for eliminating dominant follicle

Experiments by Ko *et al.* (1991) have shown that if the DF of the first follicular wave during the bovine oestrous cycle was destroyed during the first few days of the wave, regression of the largest subordinate follicle was significantly delayed, but destruction of the DF later during the wave was followed by early recruitment of the next wave. But, Amiridis *et al.* (2006) have shown that irrespectively of the stage of the cycle, ablation of all visible follicles or even only the DF, caused a rapid decline in oestradiol concentration followed by a strong FSH surge, which induced a new follicle wave emergence.

Adams *et al.* (1992) found that emergence of new follicular wave occurred 2.5 ± 0.2 or 2.0 ± 0.3 days after cauterization of DF on Day 3 or 5 respectively. While, Bodensteiner *et al.* (1996a) stated that aspiration of DF of Wave 1 on Day 5 after ovulation elicited the emergence of Wave 2 by 28.0 ± 5.0 h.

Bergfelt *et al.* (1994) and Bo *et al.* (1995a) reported that mechanical treatments such as ultrasound-guided follicle aspiration of all follicles 25 mm in diameter resulted in synchronous emergence of a new follicular wave in 1.5 days.

Roth *et al.* (2002) and Mussard *et al.* (2007) aspirated all the visible follicles (3 to 8 mm) on Day 4 and 6 of the cycle (Day 0 = day of estrus) and detected new wave of follicular development on Day 1.0 and 1.7 after aspiration respectively.

2.2.2 Hormonal approach for eliminating dominant follicle using Gonadotrophin releasing hormone (GnRH) / Human chorionic gonadotrophin (hCG)

2.2.2.1 Ovulatory response to GnRH/hCG

Macmillan and Thatcher (1991) and Troxel *et al.* (1993) have shown that GnRH administration induced LH release, resulting in ovulation or luteinization of the largest follicle present at the time of GnRH treatment. As suggested by, Spicer and Echternkamp (1986) the final preovulatory maturation of ovarian follicles was associated with the increased numbers of LH receptors, but not FSH receptors, in granulosa cells and hence increased sensitivity of granulosa cells to concentrations of LH in blood. However, the ovulatory response to exogenous hormones was related to various factors.

2.2.2.1.1 Relationship between day of oestrous cycle and ovulatory response

Kim *et al.* (2005) and Saldarriaga *et al.* (2007) recorded 50 and 40 per cent ovulation of DF in response to GnRH, when administered at random stage of oestrous cycle. Atkins *et al.* (2008) also recorded varied ovulatory responses of 0, 92, 31, 69 and 20 per cent, when administered with 100 µg of Cystorelin injection on Days 2, 5 10, 15 and 18 respectively, and they concluded that ovulation of DF in response to GnRH was affected by day of the cycle.

Taponen *et al.* (2000) have shown that administration of GnRH on Days 1 or 2 after ovulation did not induce ovulation of large or small follicles at that time and Kohram *et al.* (1998b) recorded formation of accessory CL (ACL) in all cows treated with GnRH between Days 4 and 7 but only in 33.33 per cent of cows treated with GnRH between days 15 and 18 of the oestrous cycle. Martinez *et al.* (1999) administered 100 µg GnRH on Days 3, 6 or 9 (Day of ovulation - 0) and recorded 89, 56 and 22 per cent ovulation rate respectively.

Gong *et al.* (1995) observed that LH surge provoked by GnRH, at 5 and 10 µg doses, on Day 5 of the cycle induced the DF to ovulate in 11 out of 14 (78.57 %) Hereford cross Friesian beef heifers and related the reduced response to the stage of differentiation of the DF. However, Schmitt *et al.* (1996) and Rajamahendran *et al.* (1998) reported that treatment with buserelin and deslorelin implants on Day 5 of oestrous cycle induced ovulation of DFs in 93 and 100 per cent of the cows and heifers respectively and thus concluded that the first wave DF at Day 5 was responsive for the induction of ACL.

Rusbridge *et al*. (1992) and Sato *et al*. (2005) administered GnRH to heifers and HF cows respectively, on Day 6 of the cycle and found that 75 per cent of the heifers and 100 per cent of cows exhibited induced ovulation and formation of an induced CL. Howard *et al.* (2006) also observed that all Holstein cows administered with 100 µg of GnRH had developed an ACL, indicating that the administration of GnRH on Day 5 or 6 of the oestrous cycle overrode the negative feedback of progesterone on the anterior pituiotary, thereby allowing the secretion of both LH and FSH resulting in ovulation or luteinization of the follicle and subsequent formation of CL.

Sianangama and Rajamahendran (1996) administered 1000 IU hCG on Day 7 of the oestrous cycle and found that all the cows ovulated the first wave DF. Twagiramungu *et al.* (1995) evidenced that treatment with a GnRH agonist induced ovulation and subsequent formation of a new CL in beef cows early during the luteal phase (Day 7) as well as in all cyclic beef cows without a functional CL (P4 < 1 ng / ml) at the time of treatment.

2.2.2.1.2 Relationship between developmental phase of dominant follicle and ovulatory response

Silcox *et al.* (1993) and Gong *et al.* (1995) suggested that the ability of DFs to respond to exogenous GnRH administration was dependent on their stage of development. The former researchers recorded that the ovulatory response of a DF in cattle during the growth phase, plateau phase or atretic phase was 100, 33, and 0 per cent respectively.

Twagiramungu *et al.* (1995) also evidenced that the DF ovulated, in response to GnRH, during its growth or plateau phase but not during its regressing phase. Rhodes *et al.* (2001) detected significant responsiveness to LH on Day 2 after ovulation, equivalent to Day 2.4 after wave emergence and at about the time of deviation of the dominant and subordinate follicles.

2.2.2.1.3 Relationship between size of dominant follicle and ovulatory response

Bartlewski *et al.* (2001) suggested that ovine antral follicles of a similar size and age may vary in their physiological status (degree of follicular maturation) and responsiveness to gonadotrophins.

Perry *et al.* (2007) stated that small follicles were less likely to ovulate in response to exogenous GnRH than larger follicles. Sartori *et al.* (1998) observed that follicles that were 10 mm in diameter rather than 7 or 8.5 mm diameter ovulated in response to 40 mg of purified LH and thus they found the evidence of LH responsiveness in the follicle after deviation. Mussard *et al.* (2007) also documented 100 per cent ovulation, when the largest follicle was determined to be $\geq$ 10 mm on the day (4.8 $\pm$ 0.1) of cystorelin administration. Similarly, Portillo *et al.* (2008) reported that the GnRH administered on Day 6 of the oestrous cycle induced ovulation in 94.4 per cent of *B. indicus* heifers with follicles of 10 – 14 mm size, and the remaining heifers that did not ovulate had a follicle of 5mm size.

Rhodes *et al.* (2001) opined that synthesis of cAMP in response to LH was significantly greater in granulosa cells from the largest follicle compared with cells from smaller follicles at this time, but was only present at concentrations greater than zero responsiveness in cells from follicles >9 mm in diameter.

2.2.2.2 Interval from GnRH to ovulation

Bodensteiner *et al.* (1996a) found that the interval from GnRH treatment to ovulation was 32.0 h in heifers, while Kot and Ginther (1999) observed a mean interval of 26.7 $\pm$ 1.2 h (range: 24.3 – 28.2 h) for ovulation after GnRH injection.

Rajamahendran *et al.* (1998) stated that buserelin injection and deslorelin implants on Day 5 of the cycle induced ovulation of DFs in all the cows with a mean interval of 28.0 $\pm$ 1.2 h from injection/implant to ovulation.

Martinez *et al.* (1999) administered 25 mg of pLH or 100 µg GnRH on Days 3, 6 or 9 and recorded that the mean interval from treatment to ovulation ranged from 1.0 – 1.4 days.

Martinez *et al.* (2003) recorded the interval from GnRH treatment to ovulation as 37.3 h and 36.0 h in animals administered with 100 µg of Cystorelin or Fertagyl respectively on Day 6 or 7 of the cycle.

2.2.2.3 Accessory corpus luteum

Sianangama and Rajamahendran (1996) administered hCG on Day 7 of the cycle and stated that the ACL was detectable on Day 9 and exhibited a shorter lifespan of about 11 to 13 days.

Mussard *et al.* (2007) documented that the cross sectional area of Day 12 spontaneous CL (SCL) was greater (3.6 $\pm$ 0.2 cm^2) than GnRH induced CL (3.0 $\pm$ 0.2 cm^2).

Webb *et al.* (1992) reported that a CL induced with GnRH could develop into a fully functional structure of normal weight and progesterone and oxytocin contents seven days after induction, as compared with spontaneously formed CL of similar age. Twagiramungu *et al.* (1992) suggested that buserelin induced ACL was mature enough to respond to PG six days later.

2.2.2.4 Oestrous cycle pattern in GnRH treated cattle

Macmillan and Thatcher (1991) administered 10 µg buserelin on Day 12 of the cycle in Holstein and Jersey cows and found that the average cycle length was 23.2 days when compared to 24 days in control animals.

Rajamahendran *et al.* (1998) recorded an average cycle length of 28.5 days in normal cycles with three waves and 26.0 days for cycles treated with 8 µg Buserelin on Day 5 of the cycle in Holstein cows.

Taponen *et al.* (2000) indicated that GnRH treatment given on Days 1 or 2 after ovulation did not have any significant effects on inter-oestrus intervals. Likely, Macmillan *et al.* (2003) observed no effect on cycle length if the GnRH was administered in the first half of the cycle, whereas from Day 12 onwards, less than 8 per cent of cows had cycles of < 20 days and an increasing percentage of treated cows had cycles of > 25 days

2.3 FOLLICULAR WAVE SYNCHRONIZATION

2.3.1 Follicular wave synchronization using GnRH

Macmillan and Thatcher (1991) have shown that administration of 10 - 20 µg buserelin on Day 12 of the cycle in Holstein and Jersey cows induced an emergence of a new follicular wave within two days. They observed increased number of Class 2 (medium) follicles between Day 13 and 18 which might be due to recruitment of follicles from Class 1 (small) and classification of Class 3 (large) follicles into Class 2 that appeared smaller due to luteinization induced by GnRH. Kohram *et al.* (1998b) stated that GnRH treatment between Days 4 and 7 or between Days 15 and 18 of the oestrous cycle caused rapid disappearance of large follicles and increase in the number of recruitable follicles (4-6 mm), indicative of the emergence of a new follicular wave within 3 to 4 days of treatment. Sato *et al.* (2005) found that the mean number of small follicles increased from 2.5 days after GnRH until 4.5 days and then decreased when HF cows were administered with 25, 50 and 100 µg of Fertirelin acetate on Day 6 of the cycle.

Martinez *et al.* (1999) and Macmillan *et al.* (2003) confirmed that emergence of a new follicular wave was synchronized only when the GnRH treatment caused ovulation or luteinization of the DF in heifers. They administered 100 µg GnRH on days 3, 6 or 9 and found that the new wave emerged 2.8 $\pm$ 1.4, 2.1 $\pm$ 0.6 and 0.9 $\pm$ 1.3 days respectively after treatment. Martinez *et al.* (2003) administered 100 µg of Cystorelin or Fertagyl on Day 6 or 7 of the cycle and observed that the time interval

from treatment to emergence of a new follicular wave was 1.8 ± 0.1 days and 2.2 ± 0.2 days respectively.

Kim *et al.* (2005) reported that the administration of GnRH (100 mg Fertirelin acetate i.m.) resulted in emergence of a new follicular wave within 2–4 (2.9) days, while Rajamahendran *et al.* (1998) observed that the second follicular wave emerged earlier (Day 9.9) than control animals (Day 12.8) when administered with GnRH agonist on Day 5 of the cycle in Holstein cows. Saldarriaga *et al.* (2007) recorded follicular wave synchronization in 60 per cent of *B. indicus* cows administered with 100µg cystorelin at random stage of cycle and the new wave emerged within 2.5 ± 0.12 days

2.3.2 Follicular wave synchronization using Oestradiol

Bo *et al.* (1994) treated heifers with oestradiol-17β (E-17β) plus progestogen ear implants and found that, the DF ceased to grow 1 day after E-17β treatment and subsequently regressed, resulting in an early emergence of the next follicular wave (Day 5.2 ± 0.2). Conversely, E-17β administration to heifers without progestogen implants did not effectively suppress the DF and emergence of the next wave was delayed (Day 9.8 ± 1. 1).

Bo *et al.* (1995 b) found that the administration of 5 mg E-17β in progestogen-implanted cattle was followed consistently by the emergence of a new follicular wave, on average, 4.3± 0.2 days later regardless of the phase of follicular development at the time of treatment.

Caccia and Bo (1998) and Taniguchi *et al.* (2007) stated that treatment with 2.5 mg oestradiol benzoate (EB) and 50 mg progesterone given at the time of CIDR insertion resulted in synchronous emergence of a new follicular wave 3 to 4 days later, irrespective of the oestrous cycle stage at the start of the treatment.

Kim *et al.* (2005) and Burke *et al.* (2005) recorded a time interval of 4.7 – 4.8 days to wave emergence following EB administration and they stated that follicular wave emergence was relatively asynchronous (2–7 days).

2.4 SUPEROVULATION AND EMBRYO YIELD

2.4.1 Conventional superovulation protocol

Webb *et al.* (1999) opined that FSH was required for follicular growth between 4 to 9 mm in cattle, however, in some pharmacological superovulation treatments, follicles would continue to grow with just FSH, which might have practical implications for more controlled superovulation regimens.

2.4.1.1 Dose of FSH and schedule

Donaldson (1984 a), Echternkamp *et al.* (1989), Goulding *et al.* (1990) and Andrade *et al.* (2003) superovulated donors with 28- 34 mg of FSH-P, starting on various days between 9 and 14 of oestrous cycle, administered twice daily in a declining dose over four days at intervals of 12 h and on the third day they were treated with PGF2α.

Abe *et al.* (2002) initiated superovulatory treatment on Day 10 of the oestrous cycle (oestrus – day 0) with FSH-R at a total dose of 20 mg given twice daily for three days in decreasing doses. Luteolysis was induced with two injections of PG F2α given at the fifth and sixth injections of FSH-R followed by two inseminations at 12 and 24 h after standing oestrus.

Leroy *et al.* (2005) administered twice daily injections of descending doses of pFSH-pLH (5/1) during mid luteal for four consecutive days along with PG analogue and on third day. Inseminations were done at 60 – 72 h after last injection of FSH.

Putney *et al.* (1988) and Simpson *et al.* (1994) administered FSH-P (40 mg total dose) in declining doses as the previous workers, but for a period of five days and dinoprost (40-50 mg total dose) on third day of the superovulation treatment.

Lindsell *et al.* (1986) injected FSH-P subcutaneously at 12 h intervals in decreasing doses for 5 days, however for a reduced total dose of 30 mg (5, 4, 3, 2 and 1 mg) with 500 µg Cloprostenol 72h after initiation of FSH treatment. Takedomi *et al.* (1995) compared single subcutaneous injection of pFSH (30 mg) dissolved in 25 or

50 per cent polyvinylpyrrolidone with normal divided doses (7/7; 5/5 and 3/3 mg) started between days 9 -12 of the cycle.

Nigam *et al.* (2001), Sarvaiya *et al.* (2003) and Patel *et al.* (2007) superovulated donor cows using FSH-P at the dose rate of 400 mg per animal, as total dose given at 12h interval in eight equally divided doses, starting between Days 9 -11 of the oestrous cycle. Superovulatory oestrus was induced with two injections of dinoprost tromethamine (25mg each) given at 48 and 60 h after first FSH injection and inseminated thrice at 12h interval during superovulatory oestrus. The former researchers also tried the superovulation schedule with 200 mg total dose of FSH. Arora *et al.* (1996a), Ansari *et al*. (1998), Murugavel *et al.* (1999) and Arosh *et al.* (2000) also followed the same dose of 400 mg FSH, but in decreasing order in Jersey crossbred cows.

Takagi *et al.* (2001) opined that during superovulation regimen exogenous LH was not necessary for stimulation of the growth of follicles beyond the stage of 8 mm in diameter and that these follicles were competent to induce LH surge.

2.4.1.2 Superovulatory response after conventional superovulation protocol

Boland *et al.* (1991) stated that one third of the donors treated did not respond to superovulation, another third produced an average of one to three embryos and only one third actually superovulated giving a large number of embryos. Shanker *et al.* (1998) observed that 16.12, 18.20, 34.40, 21.50 and 9.67 per cent of donors had 0, 1-2, 3-6, 7-10 and more than 10 CL respectively in response to FSH treatment.

Soboleva *et al.* (2000) developed a model to describe exogenous FSH administration and showed that the ovulation rate response was dependant on both the amount of FSH administered and the time of administration.

2.4.1.2.1 Effect of day of initiation of gonadotrophin treatment on superovulatory response

Lindsell *et al.* (1986) initiated the conventional FSH treatment on Day 3, 6, 9 or 12 and found that the mean number of CL was highest (16.0 $\pm$ 1.2) in Day 9 group

and lowest (9.3 $\pm$ 2.0) in Day 3 group. They suggested that when treatment was initiated on day 3, when the population of responsive follicles was high, numbers of ovulations and embryos were reduced compared to when treatment was initiated on day 9. This raised the possibility that factors other than the responsive follicles may affect the superovulatory response at different stages of cycle.

Bodensteiner *et al.* (1996b) found no differences in the numbers of FSH receptors per granulosa cell between dominant and subordinate follicles on Day 2 or 4 after ovulation, but significantly more receptors per follicle on Day 4.

Rhodes *et al.* (2001) detected responsiveness to FSH in the granulosa cells of all the dissected follicles >4mm in diameter from Day 1 after ovulation, however there was no difference in responsiveness to FSH between granulosa cells from the largest follicle and smaller follicles (Day 1 and 2) or between granulosa cells from dominant and subordinate follicles (Day 3 -5). Thus this study indicated that the ability of the granulosa cells to bind FSH and produce cAMP was not limited to the early stages of follicular wave emergence.

Goulding *et al.* (1990) reported that 20.0 and 3.0 per cent of heifers failed to respond for superovulatory treatment initiated on Day 2 and 10 respectively. They recorded a superovulatory response of 6.7 and 12.9 ovulations in these two groups respectively. Leroy *et al.* (2005) recorded the superovulatory response in lactating and non-lactating HF cows as 10.2 $\pm$ 1.1 and 8.1 $\pm$ 1.1 respectively by administering pFSH-pLH during mid-luteal phase.

Donaldson (1984 a) opined that starting superovulation on Days 9 to 13 had no effect upon embryo production in cows. They were of the opinion that the larger pool of antral follicles reported to be present on Day 9 or 10 might remain on days 11 through 13. However, Arosh *et al.* (2000) stated that superovulation initiated on Day 9 of the oestrous cycle i.e. closer to the emergence of the follicular wave resulted in more recruitment of follicles and more number of ovulations.

2.4.1.2.2 Effect of follicular population and its developmental pattern on superovulatory response

Burns *et al.* (2005) stated that classification of cattle based on the number of antral follicles 3mm or greater in diameter during waves should provide a novel model to examine their relationship to the size of ovarian reserve and superovulatory response.

Initially two groups of workers (Monniaux *et al.*, 1983 and Moor *et al.*, 1984) studied folliculogenesis in an attempt to define the physiology of superovulation. These studies indicated that the degree of superovulation response depended on the number, size, distribution and condition of the antral follicle populations. Exogenous gonadotrophins stimulated mitotic activity in preantral follicles and reduced atresia of antral follicles. The most medium sized antral follicles were found between days 8 and 10 and it was hypothesized that this would be the best time to start cows on superovulation. However, Donaldson (1984a) did not support the hypothesis and stated that antral follicular population was the same on Days 11 through 13 and starting superovulation on Days 9 to 13 had no effect on embryo production in the cow.

Soboleva *et al.* (2000) suggested that the best response occurred when FSH was administered at the time of deviation of dominant and subordinate follicles. Ginther *et al.* (2003) also concluded that all follicles of the common growth phase had the potential for future dominance and administration of FSH early in wave prevented deviation phenomenon and induced several follicles to become dominant in cattle. They also opined that a subordinate follicle might maintain adequate viability for one day or more after the beginning of deviation so that it could convert to the dominant status if the DF failed or ablated.

Lima *et al.* (2007) initiated the gonadotrophin treatment (160-200mg or 240-260 mg NIH-FSH-PI for heifers and cows respectively) at 8-12 days after natural oestrus and opined that the superovulatory response was influenced by ovarian status in terms of total ova/embryo and total viable embryo.

2.4.1.2.3 Effect of dose of FSH on superovulatory response

Alcivar *et al.* (1984), Takedomi *et al.* (1995) and Putney *et al.* (1988) recorded a superovulatory response of 11.6 ± 1.4, 10.5 ± 1.9 and 9.7 ± 1.3 CL in heifers treated with 26 mg, 30 mg and 40 mg of FSH respectively.

Pandit *et al.* (1992) found that the average superovulatory response and number of AFs in cows treated with 32 mg FSH-P was 10.8 and 1.40 respectively, with more number of CL and anovulatory follicles (AFs) in right compared to the left ovaries.

Nigam *et al.* (2001) observed a mean ovulation rate of 12.33 ± 0.49 and no unovulatory follicles in Jersey crossbred cows superovulated with 400 mg FSH. With similar dosage, Ansari *et al.* (1998), Murugavel *et al.* (1999), Arosh *et al.* (2000), Sarvaiya *et al*. (2003) and Rawat *et al.* (2007) recorded a mean of 7.6 ± 1.46 and 0.80 ± 0.37, 7.6 ± 1.1 and 0.33 ± 0.20, 10.88 ± 1.02 and 0.63 ± 0.03, 7.83 ± 2.41and 1.17 ± 0.60 and 9.33 ± 0.72 and 1.5 ± 0.17 superovualtory response and AFs respectively in Jersey crossbred cows. However, Arora *et al.* (1996b) recorded a higher mean number of AFs (4.17 ± 0.87) when similar dose was administered.

Shanker *et al.* (1998) superovulated the crossbred cattle with different total doses of FSH viz., 10, 15 and 20 mg and found that 50.0, 62.5 and 70.83 per cent of animals responded for treatment in respective groups with a mean ovulation rate of 2.83 ± 1.77, 4.37 ± 2.99 and 4.5 ± 2.93 respectively and mean AFs of 2.17 ± 2.61, 2.87 ± 2.37 and 1.58 ± 2.27 respectively. They indicated increase in response of donor and ovulation rate with the increase in dose of gonadotrophin.

Patel *et al.* (2007) found no significant difference in number of ovulations (12.54 vs 10.90) and AFs (1.83 vs 3.07) when superovulated HF x Sahiwal cows by administering either 400 mg or 200 mg total dose of FSH.

2.4.1.3 Embryo/ova recovery after conventional superovulation

2.4.1.3.1 Effect of day of initiation of FSH treatment on embryo/ova recovery

Lindsell *et al.* (1986) initiated the conventional FSH treatment on Day 3, 6, 9 or 12 and found that the mean number total ova or embryos recovered embryos tended to be highest in Day 9 group and lowest in Day 3 group.

Goulding *et al.* (1990) reported a mean embryo recovery percentage of 60.0 $\pm$ 6.0 and 64.0 $\pm$ 4.0 from heifers assigned to either FSH treatment on Day 2 or Day 10 respectively.

Arosh *et al.* (2000) stated that superovulation initiated on Day 9 of the oestrous cycle i.e. closer to the emergence of the follicular wave resulted in more number of ovulations and more number of embryos.

2.4.1.3.2 Effect of dose of FSH on embryo/ova recovery

Alcivar *et al.* (1984) and Putney *et al.* (1988) documented 77.0 per cent and 70.1 per cent embryo recovery rate in heifers treated with 26 mg and 40 mg FSH respectively. Sumretprasong *et al.* (2008) recovered 74.8 per cent of embryos in Thai-Friesian crossbred cattle superstimulated with reduced dose of 260 mg FSH.

Donaldson (1984 b) analysed the relationship between various doses of FSH (20 – 60 mg total dose) on the embryo production in cows and found that the total embryos recovered declined from 14.9 with the dose level of 28 mg to 6.8 with 60 mg.

Shanker *et al.* (1998) suggested that embryo production increased with increase in dose of FSH. They recovered mean numbers of 1.00 $\pm$ 1.15, 0.2 $\pm$ 0.04 and 4.06 $\pm$ 4.20 embryos from crossbred cattle superovulated with 10, 15 and 20 mg total doses of FSH respectively, with a respective embryo recovery rate of 35.29, 34.5 and 72.28 per cent. Irrespective of the dosage, they also observed that 26.08 per cent donors produced no eggs.

With a conventional dose of 400 mg FSH, Arora *et al.* (1996b), Murugavel *et al.* (1999) and Arosh *et al.* (2000) recorded 69.56, 71.43 and 73.5 per cent embryo recovery rate in Jersey crossbred cows. However, Nigam *et al.* (2001) stated that 22.22 per cent of animals did not yield any embryos and the overall recovery rate was 23.42 per cent with a similar dose.

2.4.1.4 Quality of embryo / ova recovered after conventional superovulation

van den Hurk *et al.* (1992) reported that use of FSH decreased or reversed the incidence of atresia and improved oocyte quality in cattle, possibly by altering the follicular microenvironment indirectly, through the IGF-I system, which was known to affect follicular mitogenetic and steroidogenetic activities.

Abe *et al.* (2002) found that number of blastomeres in morulae collected from superovulated cows classified as excellent and good quality was significantly higher than the number in fair and poor quality morulae.

However, Pandit *et al.* (1992) opined that when number of embryos harvested was more from a donor, the occurrence of degenerated embryos or oocytes also increased. Ikeda *et al.* (2006) stated that morphologically poor quality morulae recovered from superovulated cows often showed fragmentation seemingly by asymmetric cell division rather than by the apoptotic process.

2.4.1.4.1 Effect of day of initiation of FSH treatment on embryo/ova quality

Lindsell *et al.* (1986) initiated the conventional FSH treatment on Day 3, 6, 9 or 12 and found that the mean number of fertilized embryos and transferable embryos tended to be highest in Day 9 group and lowest in Day 3 group.

Goulding *et al.* (1990) reported a mean number of freezable (Grades 1 and 2) 1.9 $\pm$ 0.3 and 4.1 $\pm$ 0.6 and transferable (Grade 3) embryos 0.7 $\pm$ 0.2 and 2.1 $\pm$ 0.4 respectively in heifers assigned to either FSH treatment on Day 2 or Day 10 respectively.

2.4.1.4.2 Effect of dose of FSH on embryo/ova quality

Donaldson (1984 b) found that the average number of transferable embryos, declined from 5.9 with the dose level of 28 mg to 2.7 with 60 mg when superovulated with various doses of FSH (20 – 60 mg total dose).

Shanker *et al.* (1998) analysed the relationship between various doses of FSH (10 – 20 mg total dose) on the embryo production in crossbred cows and found that the mean number of transferable embryos increased from 0.17 $\pm$ 0.37 with the dose level of 10 mg to 2.06 $\pm$ 2.72 with 20 mg. They also observed that the number of degenerated embryos increased from 0.67 to 1.44 with these dose ranges.

Murugavel *et al.* (1999), Arosh *et al.* (2000), Sarvaiya *et al.* (2003) and Nigam *et al.* (2001) recorded 60, 73.4, 66.67 and 84.6 per cent of transferable embryos respectively in Jersey crossbred cows superovulated with a total dose of 400 mg FSH. However, Ansari *et al.* (1998) could recover only 23.68 per cent transferable embryos with a similar dose.

Patel *et al.* (2007) superovulated HF x Sahiwal cows by administering either 400 mg or 200 mg total dose of FSH and stated that there was no significant difference in number of viable embryo (5.31 vs 5.37) between them.

2.4.1.4.3 Fertlization rate

Singh *et al.* (1998a) stated that the number of unfertilized eggs recovered per donor increased with the increase in ovulation rate indicating a decrease in fertilization rate with the increase in ovulation rate, when Jersey crossbred cows were superovulated with PMSG.

A higher fertilization rate (82.5 %) and low incidence of unfertilized ova (6.25 %) were recorded by Murugavel *et al.* (1999) and Arosh *et al.* (2000) respectively in Jersey crossbred cattle treated with FSH. Similarly, Arora *et al.* (1996b) and Shanker *et al.* (1998) observed an average 0.5 – 0.56 unfertilized ova per donor out off 5.33 and 4.06 total recovered embryos.

2.4.1.5 Effect of dominant follicle on superovulation and embryo yield

It has been shown that superstimulation treatment in the presence of a DF resulted in reduced ovarian responses in dairy heifers (Grasso *et al.*, 1989). Therefore, removal of the DF might abolish the inhibition to recruitment and stimulate a pool of follicles responsive to superstimulation with gonadotrophin.

Guilbault *et al.* (1991) reported that in dairy heifers treated with FSH, the presence of a DF before superovulation treatment altered the maturation process of growing follicles and decreased the superovulatory response and reduced the ovulation rate by 40 to 50%.

Bungartz and Niemann (1994) reported that donor cows superovulated in the absence of a DF yielded more corpora lutea (11.7 ± 0.9 vs 4.7 ± 1.1), ova and embryos (8.2 ± 1.2 vs 2.8 ± 1.0) and transferable embryos (5.0 ± 1.0 vs 2.1 ± 0.9) than the donors treated in the presence of a DF.

Rouillier *et al.* (1996) observed that the follicles in the presence of a DF at the beginning of FSH stimulation displayed a higher degree of follicular atresia compared with follicles obtained from cows treated with FSH in the absence of a DF.

Wolfsdorf *et al.* (1997) reported that the presence of DF of the first wave inhibited intraovarian follicular responses to exogenous FSH. They also observed an immediate increase in small follicles after ablation of the dominant follicle and a greater recruitment of medium and large follicles during superovulatory treatment in the absence of a dominant follicle, which substantiated the idea of dominant follicle's inhibitory effect on subordinate follicles.

Kim *et al.* (2001) suggested that a DF secretes increased amounts of E-17β, inhibin and other factors, which causes the subordinate follicles to become atretic and to regress. Thus, the presence of a DF at initiation of gonadotrophin treatment has been known to decrease the superovulatory responses. In other studies by Andrade *et al.* (2003) and Lima *et al.* (2007) it was affirmed that superovulatory treatment which was started at a time when no DF was present resulted in acceptable embryo recovery and significantly more numbers of transferable embryos per donor.

However, Bergfelt *et al.* (1997a) did not find a positive effect of follicle ablation on the superovulatory response and concluded that the exogenous FSH treatment would override any systemic inhibitory effect that a persistent DF might be exerting on the pituitary and the ovaries.

2.4.2 Superovulation and embryo yield after follicular wave synchronization

2.4.2.1 Superovulation protocol after follicular wave synchronization

2.4.2.1.1 Superovulation after mechanical ablation of dominant follicle

Wolfsdorf *et al*. (1997) aspirated the DF on Day 6 in heifers and superovulated on Day 8 by twice daily intramuscular injections of 32 mg of FSH-P given in decreasing doses and $PGF_2\alpha$, at the time of the fifth (35 mg) and sixth (20 mg) injection of FSH-P.

Kim *et al.* (2001) removed the DF by ultrasound-guided follicular aspiration on Day 8 of the oestrous cycle and initiated FSH treatment on Day 10 with daily injections (constant dose; total dose – 400 mg) for four days and 30 and 15 mg PG on sixth and seventh injection of FSH. Similarly, Amiridis *et al*. (2006) ablated all visible follicles with diameter > 3mm by transvaginal ultrasound guided aspiration on day 8 of the oestrous cycle, but initiated the superovulation treatment (porcine FSH 400 mg) on day 9 distributed in eight intra muscular injections in decreasing dose at 12h interval.

Lima *et al*. (2007) assigned the Limousin purebred heifers and cows to various groups at 8-12 days after natural oestrus viz., ablation of palpable follicles at 0, 24 or 48h or non-ablation, prior to superovulation treatment initiation. Gonadotrophin treatment consisted of a total dose of 160-200mg or 240-260 mg NIH-FSH-PI for heifers and cows respectively, given in equal sub doses twice daily for four days.

2.4.2.1.2 Superovulation after oestrogen / progesterone treatment

Bo *et al.* (1995a) tested the hypothesis that E-17β plus progestogen treatment would induce a synchronized crop of follicles as responsive to exogenous gonadotrophins as those of the second (spontaneous) follicular wave of the cycle.

They placed progestogen ear implants on Day 0 (ovulation) plus 5 mg of E-17β i.m. on Day 1 and were superstimulated on Day 5.

Bo *et al.* (1996) preferred to synchronize the follicular wave emergence for superstimulation by treatment with 5 mg E-17 β and 100 mg progesterone at the time of progestogen/progesterone device insertion followed by FSH four days later.

Andrade *et al*. (2003) synchronized emergence of a new follicular wave by inserting intravaginal device containing 1.9 g of progesterone and administered an intramuscular injection of 4mg EB 24 h later. Superovulation protocol was initiated in donors on various days of cycle viz., at day 0 , between days 2 and 6, days 7 and 12, days 13 and 16 as well as between days 17 and 20, with 400 IU of p-FSH administered in eight decreasing doses (80/80; 60/60;40/40; 20/20) at intervals of 12 h. Forty-eight hours after the beginning of FSH treatment, donors were administered with 1 mg Cloprostenol injection and 12 h later CIDR devices were removed from groups

2.4.2.1.3 Superovulation after GnRH

Kohram *et al.* (1998 a and b) administered a single dose of 200 µg GnRH with or without concomitant removal of the largest follicle two days before initiation of superovulation treatment with Folltropin –V.

Sato *et al*. (2005) administered GnRH (Fertirelin acetate 25 – 100 µg) on day 6 of the oestrous cycle (day 0- oestrus) and initiated FSH treatment 2.5 days after GnRH. They superovulated the cows with a total dose of 42 AU porcine FSH twice daily in decreasing doses over 5 days, and PGF2α analogue (750 µg) along with seventh and eighth injections of FSH.

Son *et al*. (2007) began the gonadotrophin treatment three days after GnRH (100 µg Gonadorelin) injection in CIDR pretreated Korean native cows. They superovulated with pFSH, twice daily, with a gradual decrease in dose (total dose – 28 mg) over four days, with 25 mg and 15 mg $PGF_2\alpha$ on 5^{th} and 6^{th} FSH injections. CIDR was withdrawn at the seventh FSH injection and GnRH (200 µg) was

administered 24 h after CIDR removal. Similarly, Farin *et al.* (2008) initiated FSH treatment two days after GnRH in CIDR pretreated cows.

2.4.2.2 Superovulatory response after follicular wave synchronization

Kim *et al.* (2001) concluded that the removal of the DF 48 h before superstimulation promoted follicular growth, and increased ovulation (9.6) in Holstein cows than control animals (6.1) and attributed it to the larger follicle size at the time of LH surge than in the control animals.

Sato *et al*. (2005) divided the animals into three groups and administered 25, 50 or 100 µg of Fertirelin acetate on Day 6 of the cycle, 2.5 days prior to FSH treatment and recorded 20 $\pm$ 1.9, 19.3 $\pm$ 6.9 and 20.0 $\pm$ 1.9 corpora lutea respectively. They suggested that the improvement in superovulatory response of the 25 µg GnRH group was due to a reduction of the negative effects, such as lack of LH and sensitivity to GnRH in the pituitary gland resulting from excessive release of gonadotrophin following high doses of GnRH. Guzeloglu *et al*. (2001) stated that GnRH treatment induced follicle turnover, reduced the pool of damaged follicles and led to the recruitment of healthy follicles.

Kohram *et al.* (1998a) indicated that administration of GnRH two days prior to superstimulation decreased the proportion of cows with a number of large follicles (i.e., $\geq$ 7 mm) on the day of FSH treatment when compared with control group (19.6 vs 48.0 %) and lead to decrease in the ovulatory response (6.2 vs 11.2).

Hill and Kuehner (1996) stated that transvaginal ultrasound-guided follicle ablation of all follicles or just the DF two days prior to superstimulation during mid dioestrus resulted in a higher superovulatory response than cows in which the DF was not ablated. Amiridis *et al*. (2006) superovulated cows with or without ablation of all visible follicles and found that the cows with follicle ablation had higher superovulatory response (10.14 $\pm$ 1.23) in comparison with non-ablated ones (6.96 $\pm$ 0.60) and suggested that elimination of DF prior to superovulation induced new follicular wave emergence and therefore improved the ovarian response to gonadotrophic stimulation. They also recorded more AFs in the ovaries of non-ablated

groups (4.15 ± 2.10) than in the ablated ones (2.30 ± 0.80). However, Bergfelt *et al.* (1997a) found no difference in the superovulatory response between the ablated and non-ablated control groups.

Son *et al*. (2007) evaluated the effectiveness of superovulatory protocols using EB or GnRH induced synchronization of follicular wave emergence in CIDR treated Korean native cows and showed that the superovulatory response was 16.7 ± 1.8 and 15.9 ± 2.0 respectively.

2.4.2.3 Embryo / ova recovery and quality after follicular wave synchronization

Kim *et al.* (2001) observed that removal of the DF 48 h before superstimulation treatment increased both total ova (7.7) and transferable embryos (4.6) than control animals (3.9 and 2.3 respectively). Amiridis *et al*. (2006) superovulated cows with or without ablation of all visible follicles and found that the cows with follicle ablation had higher mean embryo yield (6.57 ± 0.94) and mean transferable embryos (4.43 ± 0.89) in comparison with non-ablated ones (2.46 ± 0.53 and 2.18 ± 0.47 respectively). Lima *et al*. (2007) also opined that the superovulatory response was enhanced in terms of total ova/embryo and total viable embryos when palpable large follicles were ablated before initiating the gonadotrophin treatment.

Shaw and Good (2000) reported that follicle ablation resulted in a significantly higher number of ova/embryos collected but a comparable number of transferable embryos than cows normally superstimulated 7 to 13 days after oestrus. Son *et al*. (2007) evaluated the effectiveness of superovulatory protocols using EB or GnRH induced synchronization of follicular wave emergence in CIDR treated Korean native cows and recorded that the overall ova yield was 10.0 ± 1.4 and 6.7 ± 1.4 respectively, and the mean number of transferable embryos was 4.0 ± 1.0 in both the groups. The corresponding values in conventional superovulation protocol were 9.1 ± 1.4 and 6.0 ± 1.3 respectively.

Andrade *et al*. (2003) opined that the use of steroid hormones (progesterone and oestrogen) prior to superovulation of Nelore donors efficiently promoted the regression of DFs and the start of a new follicle wave, so that the superovulatory

treatment which was started at a time when no DF was present, resulted in acceptable embryo recovery. Based on the pregnancy rates obtained they also supported the hypothesis that the superovulatory protocol using a combination of progesterone and oestrogen, did not interfere with the morphology of the embryo, nor with its developmental capacity *in vivo*.

Sato *et al*. (2005) divided the animals into three groups and administered 25, 50 or 100 μg of fertirelin acetate on Day 6 of the cycle, 2.5 days prior to FSH treatment and recovered 22.3 ± 5.0, 17.5 ± 6.4 and 22.3 ± 2.1 ova/embryos respectively which was significantly greater than the conventional treatment (10.5 ± 1.9).

However, Wolfsdorf *et al*. (1997) reported that removal of the DF before superovulation did not increase the number of ova and freezable embryos, even though it promoted follicular growth and ovulation. Similarly Kohram *et al*. (1998 a) recorded an increase in mean numbers of unfertilized ova and degenerated embryos when GnRH was given shortly (i.e., 2 days) before superstimulation and attributed it to the compromise in oocytes competence. Deyo *et al.* (2001) also reported that GnRH or pLH treatments consistently resulted in a lower number of embryos collected than when follicular wave emergence was synchronized with E-17β and progesterone or by follicular ablation.

2.4.3 Superovulatory oestrus

Alcivar *et al*. (1984) observed the time interval from PG injection to oestrus as 59.2 ± 2.2 h in heifers superovulated with FSH P.

Kathiresan *et al*. (1997) classified the donor cows based on the time of onset of oestrus post prostaglandin injection and found that the animals that exhibited oestrus at 48h post PG produced more embryos (5.1) than animals that showed oestrus before 48h (3.14) and after 48h (1.5). Percentage of transferable embryos was lower (50%) in animals that exhibited oestrus before 48h than other groups (66.67%) and attributed this to high oestrogen levels for a longer period in former animals.

2.5 ENDOCRINOLOGICAL PATTERN IN NORMAL AND FOLLICULAR WAVE SYNCHRONIZED OESTROUS CYCLE

2.5.1 Follicle stimulating hormone (FSH)

2.5.1.1 FSH levels in normal follicular wave emergence

Castilho *et al*. (2007) observed no significant changes in FSH concentrations during the post ovulatory period and recorded the FSH concentrations during first follicular wave as 0.2 – 0.8 ng /ml. In an extensive study by Kaneko *et al*. (1991), it was found that the basal concentrations of FSH decreased gradually from the late luteal phase (15.7 $\pm$ 2.5 ng / ml) to the day before FSH surge i.e. 84 -12 h before LH peak (10.4 $\pm$ 1.5 ng / ml).

Wolfenson *et al*. (2004) correlated the FSH peaks during the cycle with the growth patterns of DFs and stated that the FSH peaks preceded the growth of the DFs by about 1-2 days, except for the FSH peak preceding the second wave in cows with three wave cycles which preceded by about 5 days.

Ginther *et al*. (2003) also observed that the wave stimulating FSH surge occurred close to the time of detection of the wave and reached peak concentrations when the largest follicle was about 5 mm in heifers and the mean concentrations decline with about a 3 day interval, between peak concentrations and the beginning of deviation.

Bodensteiner *et al* (1996b) stated that the mean increase in plasma FSH concentration began 22 to 52 h before wave emergence and the peak levels reached between 8 h before and 8 h after wave emergence.

2.5.1.2 FSH levels in GnRH synchronized follicular wave emergence

A new and synchronized wave of 5- to 10-mm follicles emerged within two days after GnRH treatment (Twagiramungu *et al*., 1994) which was likely due to the short term effect of GnRH-induced release of FSH that occured within 2 to 4 h after treatment (Chenault *et al*., 1990)

Zolman *et al*. (1974) also documented that circulating concentrations of LH and FSH increased within 30 min after GnRH injection and reached peak concentrations at 120-150 min and then decreased to mean basal concentrations between 4 -5 h after injection.

2.5.1.3 FSH levels in superovulatory cycle

Demoustier *et al*. (1988) concluded that the pFSH concentration increased immediately after i.m injection of FSH-P and reached a maximum value (0.51 ng / ml) three hours later, then declined to undetectable levels 12 h after injection based on which they estimated the half life of pFSH in cow to be approximately 5 h.

Takedomi *et al*. (1995) observed that plasma levels of pFSH increased within 3 h after each FSH injection and maintained for 9 h followed by an abrupt decline by 12 h. They also stated that relatively high levels (40-80 ng/ml) were maintained in circulation from 3 – 66 h after the first injection.

2.5.2 Luteinizing Hormone (LH)

2.5.2.1 LH levels during normal preovulatory surge

Spicer and Echternkamp (1986) stated that the preovulatory changes in frequency of the pulsatile LH release were more dramatic, increasing from one or two pulses of LH per six hours during mid luteal phase to three to eight pulses per six hours during late follicular phase.

According to Kaneko *et al*. (1991) plasma concentrations of LH were maintained at low basal levels (0.7 $\pm$ 0.2 ng / ml) during the late luteal phase, but increased from three days before an LH peak until the onset of LH surge (1.2 $\pm$ 0.3 ng / ml). They observed preovulatory surge of LH within 4 – 8h after onset of oestrus with a magnitude varying between 23.3 -82.0 mg / ml in Japanese Brown cows. Wolfenson *et al*. (2004) estimated higher concentration of LH during preovulatory surge in Holstein heifers (20 ng / ml) than cows (9 ng / ml)

During the preovulatory surge a normal period of 10h of LH secretion was observed by Chenault *et al*. (1975) and Rahe *et al*. (1980).

Maquivar *et al.* (2007) recorded that the pre-ovulatory LH-surge in relation to ovulation ranged between 24.0 ± 12.4 h and indicated that LH surge in its relation to the time of ovulation might be used as a good predictor for the time of ovulation in Zebu cows.

Callesen *et al*. (1988) estimated the pre surge concentration of LH as 3.9 ± 0.2 ng / ml in superovulated cows, which reached a peak level of 38.8 ± 1.8 ng / ml during surge and lasted for 15.0 ± 1.0 h.

2.5.2.2 LH levels after GnRH injection

Coleman *et al*. (1988) noted that the concentration of LH increased in Fertirelin acetate treated heifers, averaging 18.6 ng / ml at 120 min while, Atkins *et al* (2008) observed that peak circulating concentrations of LH occurred 90 min after the GnRH injection.

Rajamahendran *et al*. (1998) administered GnRH agonist on Day 5 of the cycle in Holstein cows and observed that the plasma concentrations of LH increased within one hour, reached maximum levels within 3h and remained elevated for 5 -6 h. Martinez *et al*. (2003) and Portillo *et al*. (2008) too observed that the plasma LH concentrations increased within 15 min to 1 or 2 h after GnRH (Day 6 or 7) and remained elevated above the base line for approximately 240 min.

Bage *et al*. (2002) found that the interval from LH rise to ovulation was 21.8 ± 4.3h.

2.5.3 Progesterone (P4)

2.5.3.1 Progesterone levels in normal oestrous cycles

Kaneko *et al*. (1991) found that in normal oestrous cycles, the plasma concentrations of P4 were higher (> 4.5 ng / ml) during the late luteal phase, and a decrease from – 72 h before oestrus was associated with spontaneous luteolysis. After ovulation, plasma P4 began to increase from the basal levels and reached 2.5 ng / ml at 168 h.

Twagiramungu *et al.* (1995) observed that when PG induced luteolysis was incomplete, oestrus did not occur and the DF became persistent. Subluteal concentrations of P4 for prolonged periods of time were associated with increased frequency of LH pulses, inhibition of induction of the LH ovulatory surge and the development of a persistent dominant follicle.

Zeitoun *et al.* (1996) opined that the mean P4 concentrations (5.2 $\pm$ 0.7, 4.9 $\pm$ 0.5 and 5.9 $\pm$ 1.2 ng / ml respectively) were similar for cows with two, three or four follicular waves. They also stated that cows with four follicular waves maintained P4 > 1ng/ml longer (16.0 days) than two (13.0 days) or three (13.4 days) waves.

Taponen *et al.* (2000) evidenced that in normal cycles, the P4 concentration increased continuously from Days 2 to 11, but a slight decline in P4 was noticed between Days 5 and 9 after ovulation in some animals.

Wolfenson *et al*. (2004) reported that the plasma P4 concentrations were higher in heifers than in cows, by about 3 ng /ml and suggested that low progesterone in cows could have resulted from enhanced hepatic metabolism of the circulating hormone.

2.5.3.2 Progesterone levels in GnRH / hCG treated oestrous cycles

Schmitt *et al* (1996) and Rajamahendran *et al*. (1998) administered buserelin or hCG on Day 5 of oestrous cycle and recorded 10.1 and 12.6 ng / ml concentrations of P4 respectively between Day 8 and 16 as against in control animals (8.4 ± 1.1 ng / ml) which might be due to the hypertrophy of the luteal cells in the SCL and/or combined effects of the original and induced CL. In a similar study, Geary *et al*. (2001) found that the serum P4 was higher on Day 7 after hCG treated cows (2.2 ng / ml) than in GnRH treated cows (1.3 ng / ml).

Howard *et al*. (2006) reported that all Holstein cows administered with 100 µg of GnRH on day 5 of the cycle had developed an ACL and the plasma P4 concentrations were 5.2 $\pm$ 0.5 ng / ml and 3.4 $\pm$ 0.5 ng / ml on Day 13 in GnRH treated and control animals respectively. In GnRH treated *B. indicus* heifers, Portillo *et al*. (2008) recorded a range of 8.0 -8.5 ng / ml on Day 13 of the cycle.

Busch *et al.* (2008) observed that in cows that were induced to ovulate a small DF (≤ 11 mm) with GnRH had decreased serum concentrations of P4 from the time of ovulation to plateau and a reduced rate of P4 increase during the same period compared with GnRH induced ovulation of large follicles.

2.5.3.3 Progesterone levels in superovulatory cycles

Callesen *et al*. (1988) found that in superovulated cattle the P4 concentration increased from 4.9 ± 0.2 ng / ml on the day of first FSH to 8.6 ± 0.5 ng / ml on the day of PG injection and after that declined to below 1ng / ml in 24 – 48 h. They also reported that animals with high P4 concentration at the time of superovulatory oestrus lacked proper LH surge. Novotny *et al*. (2005) also affirmed that higher levels of P4 (10.2 ± 4.15 ng / ml) in donors at the time of insemination were associated with lower superovulatory responses, lower embryo yields and decreased number of transferable embryos.

Kim *et al.* (2001) estimated the plasma P4 concentration at the time of embryo recovery as 30.9 ± 5.4 ng /ml in cows superovulated after removal of the DF and 18.6 ± 3.5 ng / ml in normally superovulated cows.

Silva *et al*. (2002) stated that the non-responders to superovulatory treatment had P4 levels significantly lower than superovulatory responders on Days 5 (0.4 vs 9.0 ng / ml) and 7 (1.1 vs 18.3 ng / ml) after superovulatory oestrus and opined that measuring plasma P4 concentration on Day 5 after the superovulatory oestrus could identify donors with poor or no response.

Rawat *et al*. (2007) recorded 3.10 ± 0.44, 3.01 ± 0.31, 1.07 ± 0.09 and 4.84 ± 0.73 ng /ml of plasma P4 concentration on the day of initiation of FSH treatment (Day 10), day of PG (Day 12), day of superovulatory oestrus and the day of embryo collection. They found that there was a significant positive correlation between ovulation rate and the P4 concentration on the day of embryo collection.

Murugavel *et al*. (1999), Arora *et al* (2001) and Silva *et al.* (2002) and evidenced positive correlation between plasma P4 concentration on Day 5 and 7 after superovulatory oestrus with superovulatory response, viable embryos, fertilized ova

and total ova recovered. However, the latter researchers stated that the concentration of progesterone at the initiation of superovulation had no positive correlation with the above parameters.

2.5.4 Oestradiol

Kaneko *et al*. (1991) observed that the concentrations of E- 17β were lower (<2 pg/ml) between – 126 and -84 h and significantly increased at – 48h before oestrus and maximum levels (11.6 $\pm$ 1.5 pg / ml) were observed 4 h before the LH peak.

Wolfenson *et al*. (2004) related the low oestradiol concentration around oestrus in cows to lower steroidogenic capacity of the preovulatory follicle.

Malhi *et al*. (2005) estimated the preovulatory peak oestradiol concentrations in older cows and in their daughters to be 9.78 $\pm$ 1.44 pg /ml and 6.77 $\pm$ 1.24 pg / ml respectively.

CHAPTER III

MATERIALS AND METHODS

The present study was aimed at assessing the effect of GnRH analogue in synchronizing the follicular wave emergence as a pretreatment for superovulation in Jersey crossbred cattle and to study the effect of initiating FSH treatment (normal and reduced doses) after synchronizing the emergence of follicular wave on superovulatory response and embryo yield.

3.1 EXPERIMENTAL ANIMALS

Six healthy, non-lactating and regularly cycling Jersey crossbred cows aged between 5-6 yrs maintained at the Centralised Embryo Biotechnology Unit, Department of Animal Biotechnology, Madhavaram, Chennai- 600 051 were utilized for the study. All the cows were maintained under ideal and identical stall fed conditions through out the study. They were fed daily with adequate concentrates, green fodder and paddy straw and *ad libidum* water. All the experimental cows were monitored regularly for oestrus symptoms and cyclicity of the animals was confirmed by frequent gynaecological examination.

3.2 EXPERIMENTAL DESIGN

3.2.1 Study of Ovarian dynamics

A real time B-mode ultrasound scanner (Sonovet 600, Universal Medical Systems) equipped with a 7.5-MHz rectal probe was used to observe follicular and CL characteristics. The ovaries of each cow were examined every other day throughout an oestrous cycle starting from observed oestrus (Day 0) to subsequent standing oestrus. Ovaries were scanned on multiple planes (Ginther, 1993) to ensure complete and accurate study of follicles and CL. The internal ultrasound caliper was utilized to measure the length and width of these structures and the diameter was determined by taking the mean of their length and width (Zeitoun *et al*., 1996).

3.2.1.1 Follicular dynamics in normal oestrous cycle (Control group)

Initially, as a control study, the normal follicular wave pattern was ultrasonographically investigated in all the six cows. A total of 18 normal cycles (three cycles per cow) were studied.

The data from all the cycles involved the profiling of day-to-day identity of follicles (identity method) as described by Ginther (1993). The number, diameter and relative position of all follicles with a diameter of ≥4 mm in both ovaries were mapped during each examination and on subsequent days the follicles were individually identified with reference to the records of the previous day, based on which, the following parameters were arrived as described by Savio *et al.* (1988) and Tom *et al.* (1998).

Day of wave emergence: The day the DF of a particular wave was retrospectively identified at a diameter of 4–5 mm is known as Day of wave emergence. If the follicle was not detected until it was ≥5 mm, a growth rate of 1.5 mm / 24 h was used to retrospectively determine the first examination when the follicle would have been ≤ 4 mm (Bergfelt *et al*., 2003).

Dominant follicle (DF): Dominant follicle is the follicle that reached the largest diameter among the cohort of follicles which emerged in each wave.

Growth phase and Growth rate of dominant follicle: Growth phase is the period extending from the day of wave emergence to the day that the follicle appears to cease its progressive increase in diameter. For ovulatory follicle the period from wave emergence to ovulation was considered as growing phase. Growth rate was arrived by dividing the maximum size of DF by the duration of growth

Deviation of dominant follicle: Deviation was defined as the time at which growth rate of the largest follicle became greater and the diameter was 2 mm larger than the second largest follicle (subordinate follicle) (Castilho *et al*., 2007).

Day of maximum diameter: The day of maximum diameter is the day at which the maximum diameter was attained by the DF. When the follicle had the same

diameter for more than one day, the first day was taken as the day of maximum diameter.

Static phase of dominant follicle: Static phase of the DF is the period extending from the last day of the growth phase to the first day that the follicle began a progressive decrease in diameter.

Regression phase and Regression rate of dominant follicle: The day that the follicle appeared to begin a progressive decrease in diameter was defined as the onset of regression of the DF (Rajamahendran *et al*., 1994) and the period from the last day of static phase to the day the follicle was no longer detectable or identifiable was referred to as regression phase. The maximum size of DF divided by duration of regression gives the rate of regression.

3.2.1.2 Follicular dynamics in GnRH treated oestrous cycle

All the animals included in the experimental study were injected with GnRH analogue -Inj. Receptal (Buserelin acetate 10 µg i.m.; Intervet International GmbH, Germany) on Day 6 of the cycle and follicular wave pattern was studied as described previously except that ultrasonographic study was conducted daily from Day 6 to Day 9 to assess the fate of first wave DF and the emergence of subsequent wave. The response of the DF to the GnRH administration i.e., its ovulation was monitored ultrasonographically in hourly interval from 24h after the GnRH administration, to assess the time interval from GnRH to ovulation. Ovulation was confirmed when the largest identified follicle was no longer seen and was retrospectively confirmed with the visible recognition of luteal tissue (ACL) in the same location (Kim and Kim, 2007). A total of 12 cycles (two cycles / animal) were studied.

3.2.2 Luteal characteristics in normal and GnRH treated oestrous cycles

As in follicular study, the luteal structures were also mapped during each examination and diameter was arrived. The developmental pattern of the luteal

structures, the day at which they attained the maximum diameter and their regression pattern was recorded (Taponen *et al*., 2000)

3.3 SUPEROVULATION AND EMBRYO YIELD

3.3.1 Treatment groups

All the six animals were subjected for four superovulatory treatments (Conventional and follicular wave synchronized (FWS) groups), as mentioned below with an interval of two months between each treatment.

3.3.1.1 Treatment 1: Conventional superovulation

Superstimulatory treatments (400 mg NIH-FSH-P1 of Folltropin-V; Bioniche Animal Health Inc., Athens, GE, USA) in twice daily equally divided doses (50 mg each) over a four days period were initiated on the Day 10 (anticipated time of second follicular wave emergence). Superovulatory oestrus was induced with two injections of Inj. Lutalyse (Dinoprost tromethamine: 25mg each, i.m.; Pfizer Manufacturing Belgium NV, Belgium) given at 48 and 60 h after first FSH injection (Patel *et al*., 2007).

To control the potentially confounding effect of handling stress on superovulatory response cows received an intramuscular injection of saline on Day 6, simulating the GnRH administration in other treatment groups.

3.3.1.2 Treatment II: Superovulation initiated on the day of synchronized follicular wave emergence (Day 8) – Gn-D8-400

All the animals were injected with GnRH analogue- Buserelin acetate (Inj. Receptal; 10 µg i.m.) on Day 6 of the cycle and gonadotrophin treatment for superovulation was initiated 48h after GnRH (Day 8) i.e., the day of synchronized follicular wave emergence, as ascertained by the ultrasonographic study of GnRH treated cycle. Superovulation schedule was followed as described in Conventional group.

3.3.1.3 Treatment III: Superovulation initiated two days after synchronized follicular wave emergence (Day 10) – Gn-D10-400

GnRH analogue (Inj. Receptal; 10 µg i.m.) was administered to all the animals on Day 6 of the cycle and FSH treatment for superovulation was initiated 96h after GnRH (Day 10) i.e., two days after the emergence of synchronized follicular wave and before the deviation of DF in synchronized follicular wave, which was ascertained by the ultrasonographic study of GnRH treated cycle. Superovulation and other procedures were followed as described previously.

3.3.1.4 Treatment IV: Superovulation initiated two days after synchronized follicular wave emergence (Day 10) with half dose of FSH– Gn-D10- 200

In this treatment group, the animals were injected with GnRH (Inj. Receptal; 10 µg i.m.) on Day 6 of the cycle and FSH treatment for superovulation was initiated on Day 10 but with reduced dose. Superstimulatory treatment consisted of 200mg NIH-FSH total dose, in twice daily equally divided doses (25mg each) over a period of 4 days and oestrus was induced with two injections of dinoprost tromethamine as described previously (Patel *et al*., 2007).

3.3.2 Assessment of follicular and luteal characteristics during superovulation treatment

As described by Burns *et al*. (2005), number of follicles of various size categories (Class I- $\leq$ 5 mm; Class II - > 5 - < 9 mm; Class III - $\geq$ 9 mm) and the nature of CL on the day of initiation of FSH treatment, on the day of PG and on the day of superovulatory oestrus (SOE) were determined by ultrasound scanning.

3.3.3 Superovulatory oestrus

Ovaries were scanned in all the animals on the day of superovulatory oestrum (Day 0) to assess the number and size of follicles.

3.3.4 Breeding

Animals were inseminated thrice with frozen thawed proven bull semen at 12h interval, starting from 48h after PG $F_{2\alpha}$ injection in all the groups.

3.3.5 Embryo collection

Embryos / ova were recovered non-surgically on Day 7 after the onset of superovulatory oestrum, by adopting 'ebb flow' method described by Greve *et al*. (1995). Embryos were flushed from the uterine horns with Dulbecco's phosphate buffered saline (DPBS) (BIOLIFE Advantage, Agtech Inc., USA) by intermittent gravity flow through two-way, round tip Foley catheter.

3.3.6 Superovulatory response

On the day of embryo collection (Day 7 of the superovulatory cycle), the superovulatory response was assessed by estimating the number of CL and anovulatory follicles (AF) by rectal palpation and confirmed by ultrasound scanning. Based on this, animals were categorized as either responders (animals having more than two CL) or non-responders (animals having two or less than two CL) (Purwantara *et al.*, 1993).

3.3.7 Embryo recovery and embryo quality

The flushing effluent was filtered through 75 µm EmCon embryo filter (Agtech Inc., USA). About 30-50ml of the flushed medium was transferred to Petri dishes and screened under a zoom stereomicroscope (Nikon) for the presence of embryos / ova.

Subsequently ova/embryos were transferred to the holding media containing DPBS + 0.4% Bovine serum albumin (BSA fraction V) and evaluated. Numbers of ova/embryos, unfertilized ova and transferable embryos collected were recorded for each cow. The embryos were morphologically scored for quality, colour and developmental stage. The morphological quality of transferable embryos was graded into four classes (1 = excellent, perfect embryo; 2 = good, trivial imperfections; 3 =

fair, definite but not severe problems such as extruded cells or a small amount of degeneration; 4 = poor, partly degenerated or vesiculated cells) and apart from the unfertilized oocytes, embryos in earlier developmental stages than morulae were categorized as 'arrested or degenerated' based on the method described by Lindner and Wright (1983).

3.4 RADIO IMMUNO ASSAY OF HORMONES

3.4.1 Blood sampling

Blood samples (5 ml) were collected from jugular vein into heparinized tubes and placed on ice immediately after collection. Plasma was separated by centrifuging the blood sample at 3000 rpm (1500 *g*) for 15 min., within 30 min of blood collection. The plasma was transferred into duplicate two-ml vials and stored at -20^{o} C until assayed. Hormone assay was carried out at Animal Physiology Division, National Institute of Animal Nutrition and Physiology (NIANP), Adugodi, Bangalore, Karnataka.

3.4.2 Estimation of Follicle stimulating hormone (FSH)

For estimation of FSH concentrations in normal cycles, blood samples were taken every day throughout the cycle. In GnRH treated cycles, blood was collected on hourly basis from the time of GnRH administration (0h) to six hours (Chenault *et al*., 1990). In superovulated animals blood samples were collected on the day of superovulatory oestrus.

Concentration of plasma FSH was measured by double antibody RIA method as per the procedure provided by Fortune and Hansel (1985). The RIA kit was supplied by The National institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Hormone and Peptide Program (NAPP), Torrance, California, USA. Purified bovine FSH antigen (bFSH, IOD / BIO - AFP 5318C) and ovine FSH antiserum (anti – oFSH-1, A.S., RIA - AFPC 5288113Rb) were used. The sensitivity of the assay was 0.2 ng / ml and intra- and inter assay co-efficient variations were 5.7 to 8.9 and 12.2 respectively.

3.4.3 Estimation of Progesterone

For plasma progesterone estimation in control and GnRH treated animals, blood samples collected from early (Day 6) and mid (Day 11) luteal phases of oestrous cycles. In superovulated animals, blood samples were collected on the day of initiation of FSH treatment, day of PG, day of superovulatory oestrus and day of embryo collection.

Plasma concentration of progesterone was measured with solid-phase radio immuno assay kit (Coat – A – Count, Immunotech SAS, France; supplied by M/S Anand Brothers, New Delhi). The radioactivity was counted in ^{125} I (COBRA-III, PACKARD 5405, USA) gamma counter.

3.5 STATISTICAL ANALYSIS

Data on follicular and luteal characteristics and plasma progesterone concentrations in normal and GnRH treated cycles and superovulatory cycles were analysed by Student's *t*-test and by Analysis of Variance (ANOVA) with completely randomised design. Follicular and hormonal (progesterone) status was correlated with superovulatory response. The values in percentage were converted in to arcsine radianse before they were subjected to one way analysis of variance. SPSS.10.0® software was used for analysis of data. Analysis of data was carried out as per Snedecor and Cochran (1994).

CHAPTER IV

RESULTS

4.1 FOLLICULAR DYNAMICS IN NORMAL OESTROUS CYCLE

Data on the follicular wave pattern in normal oestrous cycles of Jersey crossbred cows were presented in Table 1 and 2 and Figure 1 and 2.

Monitoring the normal follicular wave pattern revealed that, out of eighteen oestrous cycles studied, fourteen cycles (77.8%), three cycles (16.7%) and one cycle (5.6%) exhibited three, two and four follicular waves respectively. The incidence of three-wave cycles was significantly higher ($P<0.01$) than two-wave and four-wave cycles. Among the six animals studied, only two (33.33%) cows exhibited same number of waves consistently for three consecutive cycles, but the remaining cows (67.77%) varied in their follicular wave patterns within consecutive cycles.

One cycle with four waves was not included for further analytical studies. Though the values for two- wave cycles were included, they could not be statistically compared with the three-wave cycles.

4.1.1 Length of oestrous cycle

The mean inter-oestrus intervals for three- and two-wave oestrous cycles were 21.50 $\pm$ 0.27 (20 – 23) and 20.33 $\pm$ 0.33 (20 – 21) days respectively. The three-wave oestrous cyles were longer in duration than two-wave cycles.

4.1.2 Emergence of follicular waves

In three-wave oestrous cycles, the follicular waves emerged on the mean Days of 0.80 $\pm$ 0.28 (0 – 2), 9.40 $\pm$ 0.31 (8 – 11) and 15.60 $\pm$ 0.27 (15 – 17) respectively, whereas for two-wave cycles the follicular waves emerged on mean Days of 1.67 $\pm$ 0.33 (1 – 2) and 9.67 $\pm$ 0.33 (9 – 10) respectively (Day 0 – oestrum).

Figure 1: INCIDENCE OF FOLLICULAR WAVE PATTERNS IN NORMAL OESTROUS CYCLES OF JERSEY CROSSBRED COWS

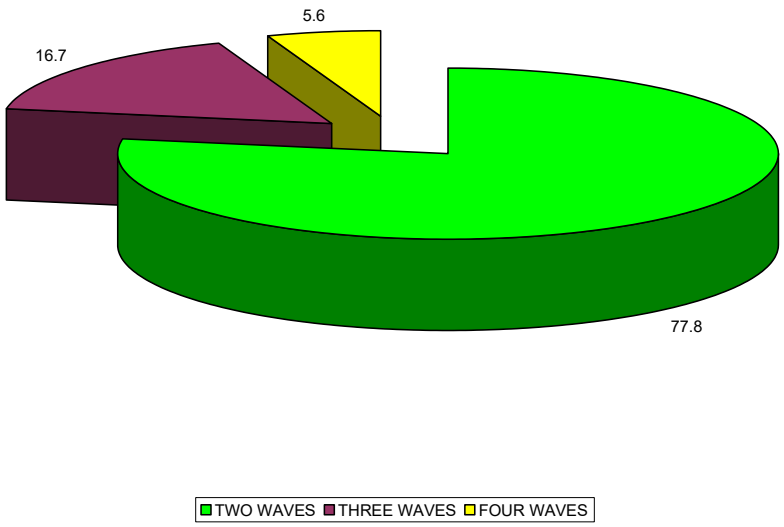
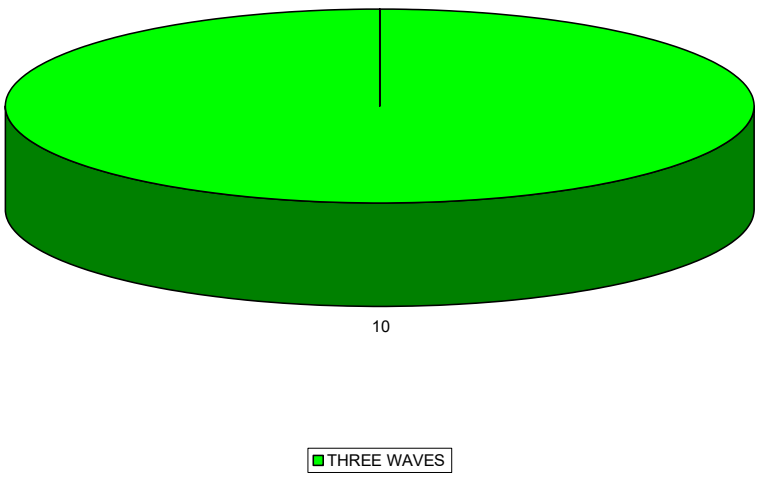


Figure 1a: INCIDENCE OF FOLLICULAR WAVE PATTERNS IN GnRH TREATED OESTROUS CYCLES OF JERSEY CROSSBRED COWS



4.1.3 Characteristics of dominant follicles

Developmental characteristics of dominant follicles (DF) of different follicular waves in normal oestrous cycles of Jersey crossbred cows were presented in Table 1 and 2.

4.1.3.1 Dominant follicle in its growth phase

4.1.3.1.1 Growth phase and growth rate

In three-wave cycles, the DF of first wave attained the mean maximum diameter of 11.05 ± 0.39 mm on the mean Day of 7.30 ± 0.45 of the cycle with a mean growth phase of 7.40 ± 0.49 days at a growth rate of 1.54 ± 0.08 mm/day. Similarly, the DF of the second wave reached the mean maximum diameter of 10.0 ± 0.37 mm on mean Day 15.7 ± 0.63 of the cycle with a growth phase of 7.4 ± 0.45 days at the mean growth rate of 1.40 ± 0.09 mm / day. The corresponding values of the DF in the ovulatory (third) wave were 11.75 ± 0.38 mm, Day 21.50 ± 0.27, 6.90 ± 0.28 days and 1.72 ± 0.08 mm/day respectively. Statistical analysis revealed that the diameter of the second follicular wave DF was non-significantly smaller than the first wave DF and significantly ($P<0.01$) smaller than the third wave DF (ovulatory follicle). The growth rate of third wave DF was non-significantly faster than its counterpart in the first wave and significantly ($P<0.01$) faster than its counterpart in the second follicular wave.

Though statistically incomparable, the first wave DF of two-wave cycles attained a maximum size of 11.67 ± 0.83 mm on the mean Day of 6.67 ± 0.58 with a shorter mean duration (5.33 ± 0.58 days) at an increased mean growth rate (2.09 ± 0.21 mm / day) than the three wave cycles. However the ovulatory follicle reached the maximum size (12.33 ± 1.17 mm) on Day 20.33 ± 0.33 with an increased growth phase period (11.67 ± 0.67 days) at a much slower rate (1.08 ± 0.15 mm/day) than its counterpart in three wave cycles.

4.1.3.1.2 Deviation of dominant follicle during the growth phase

During the growth phase, the DF of the first, second and third waves deviated from the subordinate follicles on the mean Day of 4.10 ± 0.38, 13.10 ± 0.53 and 18.10

± 0.46 respectively with a mean interval of 3.30 ± 0.15, 3.70 ± 0.30 and 2.50 ± 0.22 days respectively from emergence to deviation. The deviation of the third wave DF occurred significantly (P<0.01) earlier than the previous waves. The diameter of the DF at the time of deviation in first, second and third waves of three-wave cycles were 8.40 ± 0.21, 8.05 ± 0.25 and 7.65 ± 0.34 respectively, and the diameter of subordinate follicle at the time of deviation were 6.55± 0.24, 6.25 ± 0.24 and 6.05 ± 0.26 respectively. There was no significant difference between diameters of DFs or subordinate follicles of various waves. The time interval from emergence to deviation of the DF of third wave (ovulatory follicle) in three-wave cycles was significantly (P<0.01) shorter than the DFs of first and second waves.

In two-wave cycles, the days of deviation for first and second waves were 5.33 ± 0.67 and 12.33 ± 0.33 respectively, with an interval of 3.67 ± 0.33 and 3.33 ± 0.33 days from the day of emergence respectively. Unlike the three wave cycles, period for deviation from the day of emergence did not differ much between waves in two-wave cycles.

4.1.3.2 Dominant follicle in its static phase

In three-wave cycles, the static phase of DF of second wave was shorter (0.40 ± 0.25 days) than the first wave (1.80 ± 0.34 days). In two-wave cycles, the first DF was static for a mean period of 1.67 ± 0.67 days.

4.1.3.3 Dominant follicle in its regression phase

The DF of first wave in three-wave cycles started regressing on the Day of 9.10 ± 0.43 and completely regressed in a period of 7.50 ± 0.80 days at the rate of 1.64 ± 0.21 mm/day. The DF of the second wave initiated its regression phase on the Day of 16.10 ± 0.77 and regressed in a significantly (P<0.01) shorter period (4.90 ± 0.43 days) with an increased (though non-significant) regression rate (2.20 ± 0.23 mm / day) than the first wave DF. In two-wave cycles, the corresponding values for first wave DF were Day 8.33 ± 0.33, 7.67 ± 1.33 days and 1.59 ± 0.20 mm/day respectively.

The perusal of data on various characteristics of DF of the first follicular wave, irrespective of two- or three-wave cycles, revealed that the DF emerged between Days 0.8 -1.67, got deviated between Days 3.30 – 3.67 and reached its

TABLE 1: Characteristics of dominant follicles in three- and two-follicular wave oestrous cycles in Jersey crossbred cows

S.No	Characteristics of dominant follicle	THREE-WAVE CYCLE (n=14)			F	Significance	TWO-WAVE CYCLE (n=3)	
		I wave Mean ± SE	II wave Mean ± SE	III wave Mean ± SE			I wave Mean ± SE	II wave Mean ± SE
1	Day of wave emergence	0.80 ± 0.28^{a}	9.40 ± 0.31^{b}	15.6 0 ± 0.27^{c}	665.839	**	1.67 ± 0.33	9.67 ± 0.33
2	Maximum diameter (mm)	11.05 ± 0.39^{a,b}	10.00 ± 0.37^{a}	11.75 ± 0.38^{c}	5.128	**	11.67 ± 0.83	12.33 ± 1.17
3	Day of Max DM	7.30 ± 0.45^{a}	15.70 ± 0.63^{b}	21.50 ± 0.27^{c}	219.502	**	6.67 ± 0.58	20.33 ± 0.33
4	Growth phase (days)	7.40 ± 0.49^{a}	7.40 ± 0.45^{a}	6.90 ± 0.28^{a}	0.453	N.S	5.33 ± 0.58	11.67 ± 0.67
5	Growth rate (mm / day)	1.54 ± 0.08^{a,b}	1.40 ± 0.09^{a}	1.72 ± 0.08^{b}	3.267	*	2.09 ± 0.21	1.08 ± 0.15
6	Static phase (days)	1.80 ± 0.34	0.40 ± 0.25		@	@	1.67 ± 0.67	
7	Day of regression	9.10 ± 0.43^{a}	16.10 ± 0.77^{b}		63.181	**	8.33 ± 0.33	
8	Regression phase (days)	7.50 ± 0.80^{b}	4.90 ± 0.43^{a}		8.069	**	7.67 ± 1.33	
9	Regression rate (mm / day)	1.64 ± 0.21^{a}	2.20 ± 0.23^{a,b}		3.243	N.S	1.59 ± 0.20	
10	Oestrous cycle length (days)	21.50 ± 0.27					20.33 ± 0.33	

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

NS – Not significant ($P > 0.05$); @ - Statistically not comparable

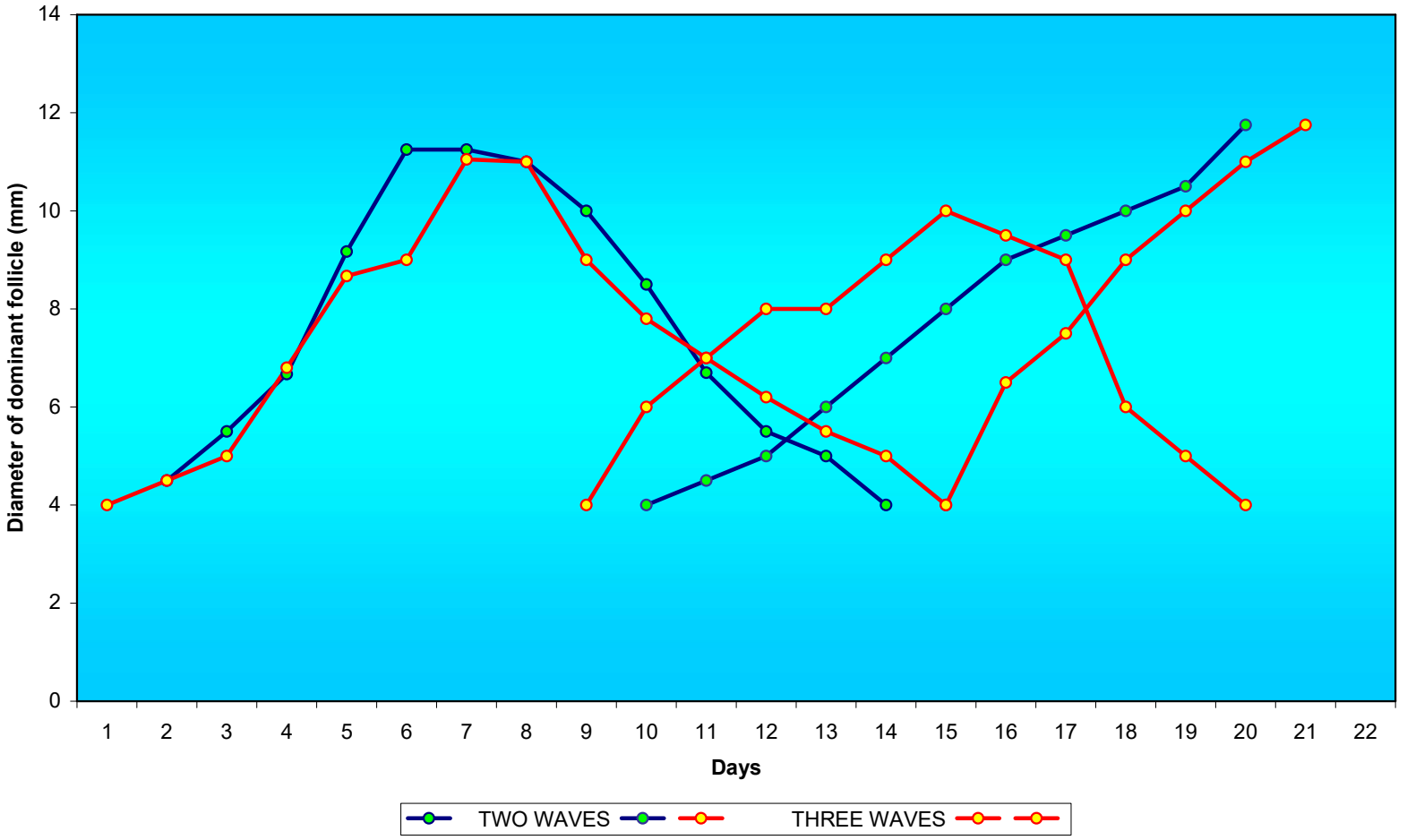
TABLE 2: Deviation of dominant follicles in three- and two-follicular wave oestrous cycles in Jersey crossbred cows

S. No	Characteristics of dominant follicle	THREE-WAVE CYCLE			F	Significance	TWO-WAVE CYCLE	
		I wave	II wave	III wave			I wave	II wave
1	Day of deviation of DF	4.10 ± 0.38 [a]	13.10± 0.53 [b]	18.10 ± 0.46 [c]	239.683	**	5.33 ± 0.67	12.33 ± 0.33
2	Duration between emergence and deviation (days)	3.30 ± 0.15 [b]	3.70 ± 0.30 [b]	2.50 ± 0.22 [a]	6.857	**	3.67 ± 0.33	3.33 ± 0.33
3	DM of DF on day of deviation (mm)	8.40 ± 0.21[a]	8.05 ± 0.25 [a]	7.65 ± 0.34 [a]	1.887	N.S.	9.00 ± 0.0	8.17 ± 0.17
4	DM of largest subordinate follicle on the day of deviation (mm)	6.55± 0.24 [a]	6.25 ± 0.24 [a]	6.05 ± 0.26 [a]	1.032	N.S.	7.00 ± 0.50	6.00 ± 0.0

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

NS – Not significant ($P > 0.05$)

Figure 2: FOLLICULAR DYNAMICS IN NORMAL OESTROUS CYCLE OF JERSEY CROSSBRED COWS



maximum size between Days 6.67 – 7.30. From the data presented, it was evident that the DF of the first wave, irrespective of the wave patterns, got deviated and established its dominance by Day 4 and was in growth phase between Days 4 and 6 of the oestrous cycle.

4.2 FOLLICUAR DYNAMICS IN GnRH TREATED OESTROUS CYCLE

4.2.1 Characteristics of dominant follicles in GnRH treated oestrous cycle

Developmental characteristics of DFs of various waves in GnRH treated oestrous cycle were presented in Table 3 and 4 and Figure 1a and 3.

4.2.1.1 Ovulatory response of dominant follicle after GnRH administration

The diameter of first-wave DF at the time of GnRH administration i.e., on Day 6 of the oestrous cycle was 10.83 $\pm$ 0.38 mm. The DF ovulated in all the animals (100 %) in the mean interval of 27.67 $\pm$ 0.21 h (27 – 28 h) after GnRH administration (Plate1).

4.2.1.2 Characteristics of dominant follicle in GnRH synchronized follicular waves

All the GnRH treated oestrous cycles (100 %) had three follicular waves with a mean length of 20.00 $\pm$ 0.63 days.

After the GnRH induced ovulation of the first wave DF, a synchronized homogenous group of follicles emerged two days after GnRH injection (Day of 8.00 $\pm$ 0.0) of the cycle in all the animals. In the synchronized second wave, the DF reached a maximum diameter of 9.33 $\pm$ 0.11 mm on Day 11.67 $\pm$ 0.42 with a growth phase of 4.67 $\pm$ 0.42 days, at a growth rate of 2.07 $\pm$ 0.16 mm / day, and remained in the static phase for a period of 3.0 $\pm$ 0.31 days. It started its regression on Day 13.67 $\pm$ 0.92 extending to a period of 4.0 $\pm$ 0.63 days at the rate of 2.58 $\pm$ 0.32 mm / day.

The ovulatory follicle emerged on Day 13.67 $\pm$ 0.76 and reached a maximum diameter of 10.50 $\pm$ 0.32 on Day 20.00 $\pm$ 0.63, with a growth phase of 7.33 $\pm$ 0.56 days and at a growth rate of 1.47 $\pm$ 0.12mm / day. Statistical analysis of the DF of the

TABLE 3: Ovulatory response of first wave dominant follicle after GnRH

S.No	Ovulation of dominant follicle	Mean ± SE
1	Diameter of DF on Day 6 (mm)	10.83 ± 0.38
2	Percentage of animals responded with ovulation to GnRH (%)	100
3	Time interval from GnRH injection and ovulation (h)	27.67 ± 0.21

TABLE 4: Characteristics of dominant follicles in GnRH synchronized follicular waves in Jersey crossbred cows

S. No	Characteristics of dominant follicle	GnRH TREATED CYCLE (n=12)		F	Significance
		II wave Mean ± SE	III wave Mean ± SE		
1	Day of wave emergence	8.00 ± 0.0 [a]	13.67 ± 0.76 [b]	209.769	**
2	Maximum diameter (mm)	9.33 ± 0.11[a]	10.50 ± 0.32 [b]	5.524	**
3	Day of maximum diameter	11.67 ± 0.42 [a]	20.00 ± 0.63 [b]	112.479	**
4	Growth phase (days)	4.67 ± 0.42 [a]	7.33 ± 0.56 [b]	4.167	**
5	Growth rate (mm / day)	2.07 ± 0.16[b]	1.47 ± 0.12 [a]	2.508	**
6	Static phase (days)	3.0 ± 0.0			
7	Day of regression	13.67 ± 0.92			
8	Regression phase (days)	4.0 ± 0.63			
9	Regression rate (mm / day)	2.58 ± 0.32			
10	Oestrous cycle length (days)	20.00 ± 0.63			

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

GnRH synchronized second follicular wave revealed that, the diameter was significantly (P< 0.01) smaller, the growth phase was significantly (P<0.01) shorter and the growth rate was significantly (P<0.01) faster than the third wave DF.

4.2.2 Follicular development patterns in normal and GnRH synchronized second follicular wave

Comparative data on the characteristics of second follicular wave DF of normal and GnRH treated oestrous cycles were presented in Table 5.

The day of emergence of GnRH induced synchronized wave (8.00 $\pm$ 0.0) was significantly (P< 0.01) earlier than the emergence of second follicular wave in normal cycles (9.40 $\pm$ 0.31 days).

Eventhough, the maximum diameter of DF in GnRH synchronized second wave (10.10 $\pm$ 0.31 mm) was smaller there was no significant difference when compared with the DF of second wave (13.10 $\pm$ 0.53 mm) in normal oestrous cycle. However, the difference in the day of maximum size and growth phase of the DF of synchronized second wave in GnRH treatment group was highly significant (P<0.01) than its counterpart in normal cycles. The growth rate of DF was significantly (P<0.05) higher in GnRH treatment group than the control group. The day of deviation and interval from emergence to deviation of the DF of GnRH synchronized second wave were significantly (P<0.01) earlier (10.10 $\pm$ 0.31 and 2.70 $\pm$ 0.26 respectively) than the second wave of normal cycles (13.10 $\pm$ 0.53 and 3.70 $\pm$ 0.30 respectively).

The static phase of DF in second wave of normal cycle (0.40 $\pm$ 0.25) was shorter than the treatment group (3.0 $\pm$ 0.0). In treatment group, the day of regression (13.67 $\pm$ 0.92) was significantly (P<0.01) earlier than the control group. However there was no significant difference in the regression phase and regression rate among treatment and control groups.

The length of oestrous cycle was shorter in GnRH treated group (20.00 $\pm$ 0.63) when compared with normal cycle (21.50 $\pm$ 0.27) but it showed no significant difference between them.

TABLE 5: Characteristics of dominant follicles in normal and GnRH synchronized second follicular wave

S.No	Characteristics of dominant follicle	NORMAL OESTROUS CYCLE	GnRH TREATED CYCLE	F	Significance
		II wave Mean ± SE	II wave Mean ± SE		
1	Day of wave emergence	9.40 ± 0.31 [b]	8.00 ± 0.0 [a]	89.858	**
2	Day of deviation of DF	13.10± 0.53 [b]	10.10 ± 0.31 [a]	187.957	**
3	Duration between emergence and deviation (days)	3.70 ± 0.30 [b]	2.70 ± 0.26 [a]	5.250	**
4	Maximum diameter (mm)	10.00 ± 0.37 [a]	9.33 ± 0.11 [a]	8.471	N.S
5	Day of maximum diameter	15.70 ± 0.63 [b]	11.67 ± 0.42 [a]	67.577	**
6	Growth phase (days)	7.40 ± 0.45 [b]	4.67 ± 0.42 [a]	7.590	**
7	Growth rate (mm / day)	1.40 ± 0.09 [a]	2.07 ± 0.16 [b]	7.004	*
8	Static phase (days)	0.40 ± 0.25	3.00 ± 0.00	@	@
9	Day of regression	16.10 ± 0.77 [b]	13.67 ± 0.92 [a]	25.464	**
10	Regression phase (days)	4.90 ± 0.43 [a]	4.00 ± 0.63 [a]	5.611	N.S
11	Regression rate (mm / day)	2.20 ± 0.23 [a,b]	2.58 ± 0.32 [b]	2.375	N.S
12	Oestrous cycle length (days)	21.50 ± 0.27 [a]	20.00 ± 0.63 [a]	67.577	N.S

Values within the row with different superscripts differ significantly ** ($P < 0.01$) * ($P < 0.05$)
NS – Not significant ($P > 0.05$); @ - Statistically not comparable

4.3 LUTEAL DYNAMICS

4.3.1 Luteal dynamics in normal oestrous cycle

Developmental patterns of corpus luteum (CL) during normal oestrous cycle were presented in Table 6 and Figure 4.

In normal cycles, the CL grew to a mean maximum diameter of 22.06 $\pm$ 0.43 mm on the mean Day of 9.0 $\pm$ 0.47 and remained fluctuating around this size before it started regressing constantly from the mean Day of 15.89 $\pm$ 0.68.

4.3.2 Luteal dynamics in GnRH treated oestrous cycle

The characteristics of spontaneous corpus luteum (SCL) and induced accessory corpus luteum (ACL) of GnRH treated oestrous cycles were presented in Table 6 and Figure 5.

In GnRH treated cycles, the SCL developed to a maximum diameter of 22.55 $\pm$ 0.78 mm on the Day of 9.60 $\pm$ 0.78 and started regressing constantly on the Day of 16.80 $\pm$ 0.70. The ACL formed due to the GnRH induced ovulation of first wave DF (Plate 2), attained a maximum diameter of 18.45 $\pm$ 0.58 mm on the Day of 14.60 $\pm$ 0.40 and started its constant regression on Day 17.40 $\pm$ 0.78. The maximum size attained by ACL was significantly ($P<0.01$) smaller when compared with the SCL. However, there was no significant difference with respect to the day of initiation of regression between them.

4.3.3 Luteal dynamics in normal vs GnRH treated oestrous cycle

The CL of normal oestrous cycles attained the maximum diameter and started regressing non-significantly earlier than the SCL of GnRH treated cycles. The maximum diameter of ACL was significantly ($P<0.01$) smaller than CL of normal cycle. However, the ACL started regressing non-significantly later than the CL of normal cycle.

4.4 ENDOCRINOLOGICAL PATTERN IN NORMAL AND GnRH TREATED CYCLES

4.4.1 Plasma FSH concentrations in normal oestrous cycle

The pre-follicular wave FSH levels during three-wave cycles of normal oestrous cycle were presented in Table 7.

The mean concentration of FSH during the oestrous cycle was 0.25 ± 0.03 ng / ml. In three wave cycles, three peaks of FSH levels could be appreciated and each starting 1 – 2 days before the emergence of the follicular wave. These FSH peaks started increasing from basal levels on Days 0.0, 7.17 ± 0.30 and 13.17 ± 0.48 respectively and reached the peak concentrations of 0.42 ± 0.03, 0.42 ± 0.02 and 0.45 ± 0.02 ng / ml respectively on Days 1.83 ± 0.31, 9.67 ± 0.42 and 15.33 ± 0.49 respectively. The FSH concentrations returned to basal levels on the mean Days of 4.17± 0.31, 12.00 ± 0.36 and 17.5 ± 0.56 respectively.

4.4.2 Plasma FSH concentrations after GnRH administration

The FSH surge in response to GnRH injection on Day 6 of the cycle was represented in Figure 6.

The mean basal concentration of 0.25 ± 0.03 ng / ml at the time (0 h) of GnRH administration peaked to height of 0.67 ± 0.07 ng / ml within two hours and decreased to the basal concentration of 0.24 ± 0.04 by six hours after injection.

4.4.3 Plasma progesterone concentrations in normal and GnRH treated cycles

The plasma concentrations of progesterone in normal and GnRH treated oestrous cycles were presented in Table 6.

The mean plasma progesterone cocncentration during the early luteal phase (Day 6) ranged between 3.78 ± 0.18 and 3.83 ± 0.17 ng / ml. In normal oestrous cycle, the progesterone increased to 5.58 ± 0.45 ng / ml during the mid luteal phase (Day 11). In GnRH treated cycle, with an additional luteal structure to contribute, the

TABLE 6: Characteristics of corpus luteum and plasma Progesterone concentration in normal and GnRH treated oestrous cycle of Jersey crossbred cows

S.No	Characteristics of corpus luteum	NORMAL OESTROUS CYCLE	GnRH TREATED OESTROUS CYCLE		F	Significance
		CL Mean ± SE	SCL Mean ± SE	ACL Mean ± SE		
1	Day of maximum diameter	9.00 ± 0.47 [a]	9.60 ± 0.78 [c]	14.60 ± 0.40 [b]	116.532	**
2	Maximum diameter (mm)	22.06 ± 0.43 [a]	22.55 ± 0.78 [a]	18.45 ± 0.58 [b]	117.315	**
3	Day of constant regression	15.89 ± 0.68 [a]	16.80 ± 0.70 [a]	17.40 ± 0.78 [a]	1.083	N.S
4	Progesterone concentration during early phase (ng / ml)	3.78 ± 0.18 [a]	3.83 ± 0.17 [a]		1.380	N.S
5	Progesterone concentration during mid phase (ng / ml)	5.58 ± 0.45 [b]	10.57 ± 0.61 [c]		91.380	**

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

NS- Not significant ($P > 0.05$)

Figure 4: LUTEAL DYNAMICS IN NORMAL OESTROUS CYCLE OF JERSEY CROSSBRED COWS

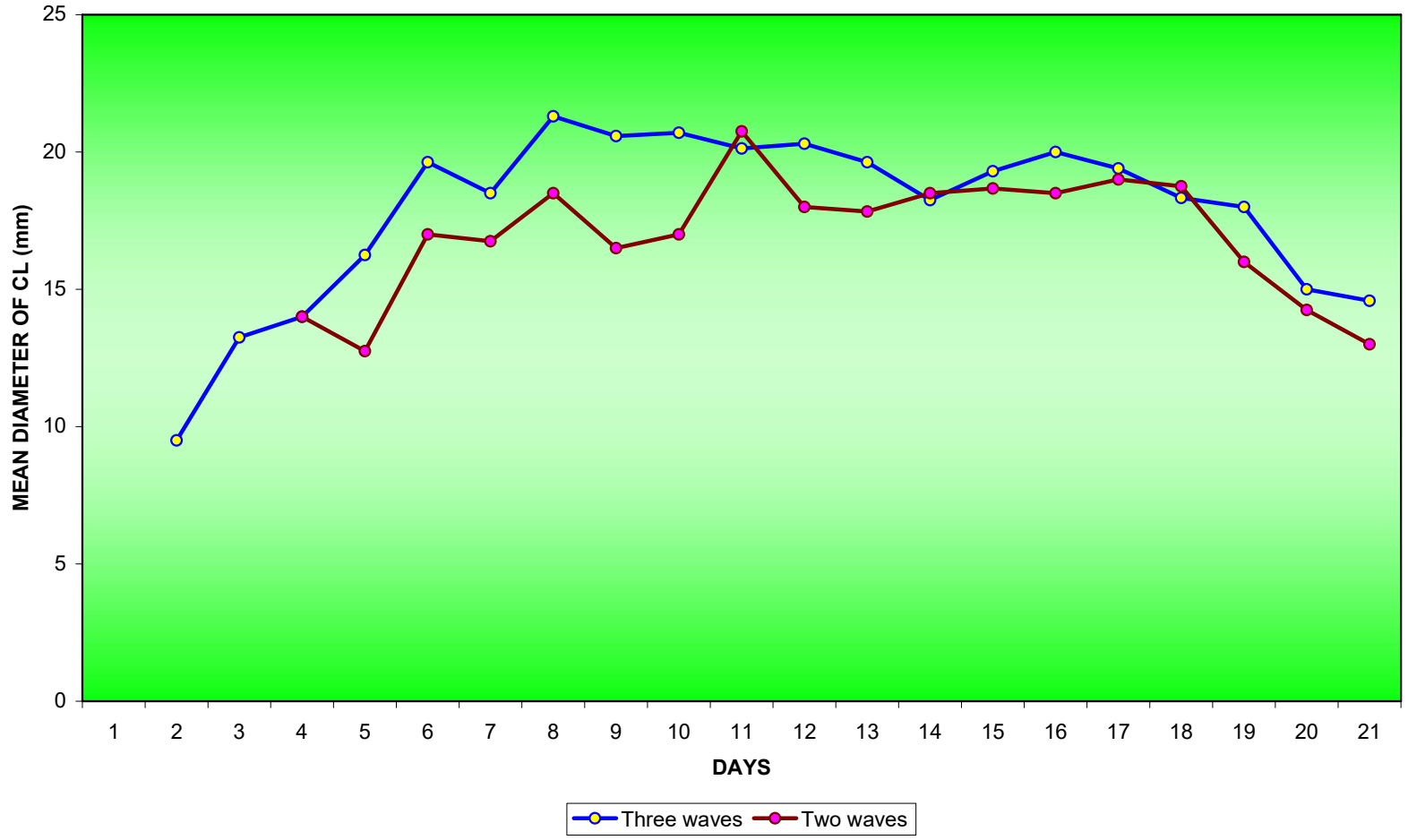


Figure 5: LUTEAL DYNAMICS IN GnRH TREATED OESTROUS CYCLE OF JERSEY CROSSBRED COWS

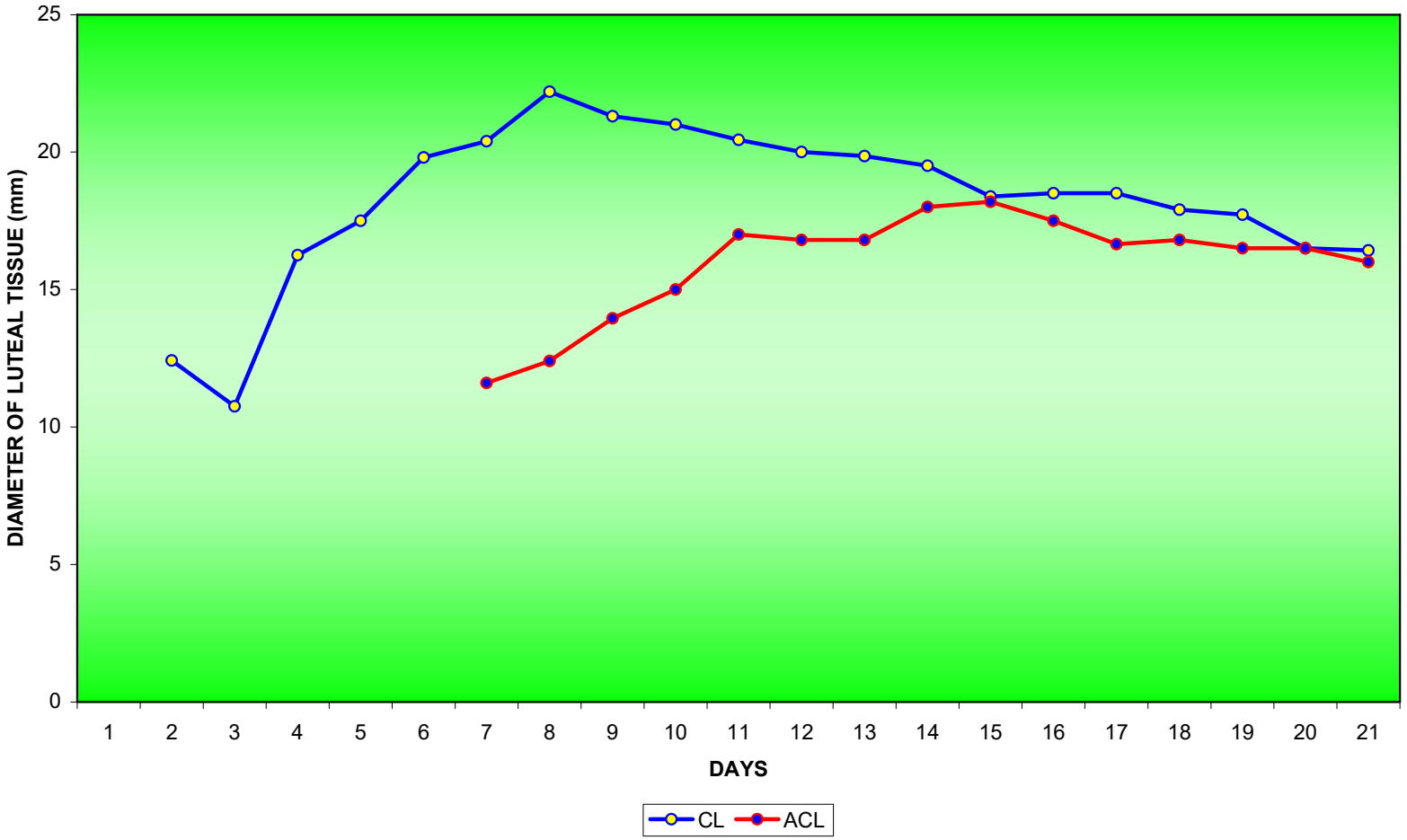
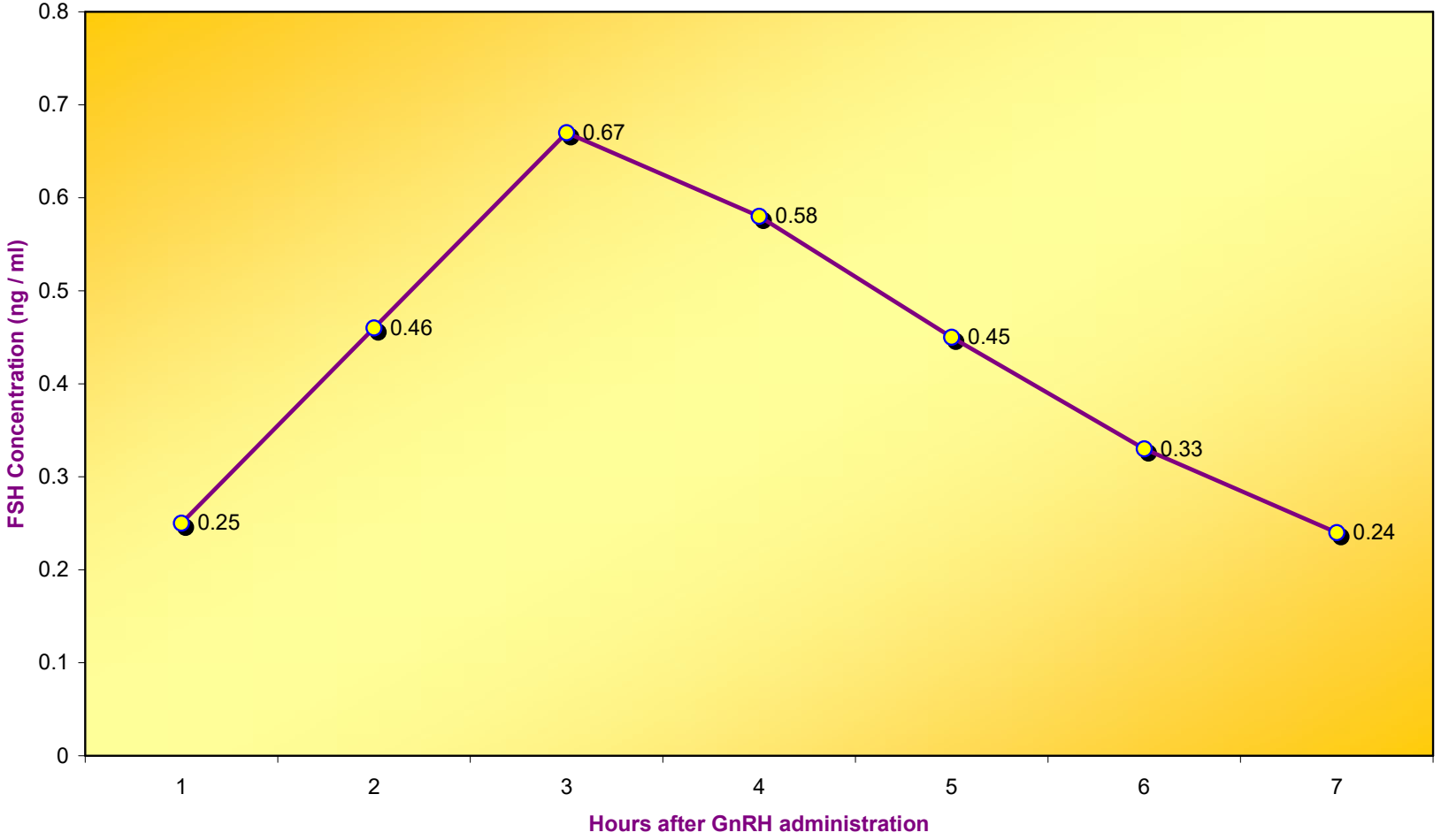


TABLE 7: Pre-follicular wave FSH levels in normal oestrous cycles of Jersey crossbred cows

S.No	Pre – follicular wave FSH	First wave	Second wave	Third wave
1	Day of start of FSH increase (Day)	0.0	7.17 ± 0.30	13.17 ± 0.48
2	Day of wave emergence (Day)	0.80 ± 0.28	9.40 ± 0.31	15.60 ± 0.27
3	Day of FSH peak (Day)	1.83 ± 0.31	9.67 ± 0.42	15.33 ± 0.49
4	Peak FSH concentration (ng/ml)	0.42 ± 0.03	0.42 ± 0.02	0.45 ± 0.02
5	Day of return to basal levels	4.17± 0.31	12.00 ± 0.36	17.5 ± 0.56
6	Basal level of FSH (ng/ml)	0.25 ± 0.03		

Figure 6: FSH SURGE AFTER GnRH ADMINISTRATION ON DAY 6 OF THE OESTROUS CYCLE IN JERSEY CROSSBRED COWS



progesterone level was significantly ($P<0.01$) more (10.57 $\pm$ 0.61 ng / ml) than the normal cycle by Day 11 of the cycle.

4.5 SUPEROVULATION

4.5.1 Follicular recruitment and developmental pattern in superovulatory cycle

Various categories of follicles viz., Class I- $\leq$ 5 mm; Class II - > 5 - < 9 mm and Class III - $\geq$ 9 mm, during the day of GnRH injection, initiation of gonadotrophin treatment and superovulatory oestrus in various treatment groups viz., Conventional and follicular wave synchronized (FWS) groups, were presented in Table 8.

4.5.1.1 Follicular population in conventional superovulatory cycle

The follicular development in Conventional superovulatory cycle was represented in Plate 3.

The mean number of Class I, II and III follicles on Day 6 of the cycle in the Conventional group were 0.89 $\pm$ 0.20, 0.78 $\pm$ 0.15 and 1.00 $\pm$ 0.0 respectively. On the day of first FSH injection (Day 10), all the classes of follicles were present with numbers of follicles in each category accounting for 5.50 $\pm$ 0.99, 0.67 $\pm$ 0.33 and 0.50 $\pm$ 0.22 respectively. The number of Class I follicles were significantly more than the Class II and III follicles on the first day of superovulation treatment. On the day of superovulatory oestrus, there were no Class I follicles and the mean numbers of Class II and III follicles were 5.17 $\pm$ 0.83 and 9.83 $\pm$ 1.17 respectively. There was a distinct increase in the number of Class II and III follicles (average total: 15.0) on the day of superovulatory oestrus when compared with the day of initiation of FSH administration.

4.5.1.2 Follicular population in Gn-D8-400 group superovulatory cycle

The recruitment of homogenous group of follicles on the day of synchronized wave emergence and their developmental pattern in response to superovulation treatment initiated on the day of synchronized follicular wave emergence (Day 8) was depicted in Plate 4 and 5.

On the day of GnRH injection (Day 6), the number of Class I, II and III follicles were 0.33 ± 0.33, 0.33 ± 0.21 and 1.00 ± 0.0 respectively. When FSH injection was initiated on the Day 8 of cycle (day of synchronized follicular wave emergence), there were a mean number of 6.50 ± 0.56 and 0.83 ± 0.31 Class I and II follicles respectively, with no Class III follicles. On the day of superovulatory oestrus, the total number of follicles (17.5) drastically increased when compared to the first day of gonadotrophin administration. There were no Class I follicles and the numbers of Class II follicles (9.17 ± 1.35) were more when compared to Class III follicles (8.33 ± 2.88).

4.5.1.3 Follicular population in Gn-D10-400 group superovulatory cycle

Follicular status two after emergence of GnRH synchronized wave i.e., the day of initiation of FSH treatment (Day 10) and further development in response to FSH was shown in Plate 6.

When GnRH was administered on Day 6 of the cycle there were 0.50 ± 0.22, 0.83 ± 0.30 and 1.00 ± 0.0 numbers of Class I, II and III follicles respectively. On Day 10 i.e. two days after the day of synchronized follicular wave emergence, when FSH was initiated the mean number of corresponding classes of follicles was 3.17 ± 0.48, 6.50 ± 0.56 and 0.0. The number of Class II and III follicles on the day of superovulatory oestrus were 3.67 ± 0.42 and 11.00 ± 0.93 respectively, with no Class I follicles accounted for a total of 14.67 follicles.

4.5.1.4 Follicular population in Gn-D10-200 group superovulatory cycle

Follicular developmental pattern with reduced dose (200mg) of FSH initiated two days after GnRH induced follicular wave emergence was presented in Plate 7.

The mean number of Class I, II and III follicles on the day of GnRH, FSH and superovulatory oestrus were 0.33 ± 0.33, 0.50 ± 0.22 and 1.67 ± 0.17, 2.67 ± 0.33, 6.50± 0.34 and 0.0 and 0.67 ± 0.67, 3.50 ± 1.72 and 6.33 ± 1.17 respectively. There was an increase in the total number of follicles (10.5) on the day of superovulatory oestrus.

TABLE 8: Follicular recruitment and developmental pattern in superovulatory cycles of Jersey crossbred cows

S. No	Treatment Groups	FOLLICULAR POPULATION								
		Day 6			Day of I FSH			Day of superovulatory oestrus		
		Class I Mean ± SE	Class II Mean ± SE	Class III Mean ± SE	Class I Mean ± SE	Class II Mean ± SE	Class III Mean ± SE	Class I Mean ± SE	Class II Mean ± SE	Class III Mean ± SE
1	Conventional	0.89 ± 0.20	0.78 ± 0.15	1.00 ± 0.0	5.50 ± 0.99 ^{c}	0.67 ± 0.33 ^{a}	0.50 ± 0.22	0.0	5.17 ± 0.83 ^{a}	9.83 ± 1.17 ^{a,b}
2	Gn-D8-400	0.33 ± 0.33	0.33 ± 0.21	1.00 ± 0.0	6.50 ± 0.56 ^{c}	0.83 ± 0.31 ^{a}	0.0	0.0	9.17 ± 1.35 ^{b}	8.33 ± 2.88 ^{a}
3	Gn-D10-400	0.50 ± 0.22	0.83 ± 0.30	1.00 ± 0.0	3.17 ± 0.48 ^{b}	6.50 ± 0.56 ^{c}	0.0	0.0	3.67 ± 0.42 ^{a}	11.00 ± 0.93 ^{b}
4	Gn-D10-200	0.33 ± 0.33	0.5 ± 0.22	1.67 ± 0.17	2.67 ± 0.33 ^{b}	6.50 ± 0.34 ^{c}	0.0	0.67 ± 0.67	3.50 ± 1.72 ^{a}	6.33 ± 1.17 ^{a}
	F	@	@	@	16.514	47.116	@	@	2.358	2.954
	Significance	@	@	@	**	**	@	@	*	*

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

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

NS – Not significant ($P > 0.05$) ; @ - Statistically not comparable

4.5.1.5 Follicular population among various treatment groups

The number of Class I and II follicles were more on the day of GnRH synchronized wave emergence (Day 8) than on day of the normal wave emergence (Day 10), but statistically there was no significant difference between them. However, there was a significant ($P< 0.01$) decrease of Class I follicles and significant ($P<0.01$) increase of Class II follicles on Day 10 of synchronized follicular wave than the former groups (Conventional groups and Gn-D8), when the gonadotrophin treatment was initiated.

On the day of superovulatory oestrus, the Class II follicular population was significantly ($P<0.05$) more in Gn-D8 treatment group than the other groups. However, in Gn-D10-400 group the number of Class III follicles were non-significantly more than Conventional and significantly ($P<0.05$) more when compared with Gn-D8 and Gn-D10-200 group.

Interestingly, a prominent luteal tissue, probably that of ACL, could be ultrasonographically detected on the day of superovulatory oestrus in four animals of Gn-D8 group, indicating incomplete luteolysis (Plate 5).

4.5.2 Superovulatory response

The superovulatory response as indicated by number of corpora lutea (CL) and anovulatory follicles (AF) in Conventional and FWS group animals were presented in Table 9.

4.5.2.1 Superovulatory response in Conventional group

The mean number of CL in the right and left ovaries were 8.00 ± 0.89 and 5.67 ± 1.28 respectively, accounting for an overall mean of 13.67 ± 1.80 ovulations per animal. There was a wide variation in number of ovulations between individual animals ranging from 9 – 21 ovulations per animal. The number of AFs in the right and left ovaries were 1.33 ± 0.21 and 0.67 ± 0.21 respectively, with a total of 2.00 ± 0.37 per animal (Plate 3).

4.5.2.2 Superovulatory response in Gn-D8-400 group

When FSH was initiated on the day of GnRH synchronized follicular wave emergence (Day 8), the mean number of total ovulations was 5.00 ± 1.77 (3 - 11), with right and left ovaries contributing 2.83 ± 0.87 and 2.17 ± 0.91 ovulations respectively. The number of AFs found in right and left ovaries were 7.00 ± 2.74 and 5.83 ± 1.94 respectively, and the overall mean was 12.83 ± 4.65 anovulations per animal (Plate 4 and 5).

4.5.2.3 Superovulatory response in Gn-D10-400 group

When gonadotrophin treatment was started two days after the synchronized follicular wave emergence (Day 10), the number of CL in the right and left ovaries were found to be 6.50 ± 0.50 and 4.50 ± 0.62, with an overall mean of 11.00 ± 0.63 (10 – 14) ovulations. The corresponding values for AFs were 1.50 ± 0.34, 2.00 ± 0.26 and 3.50 ± 0.22 respectively (Plate 6).

4.5.2.4 Superovulatory response in Gn-D10-200 group

When superovulated with reduced dose (200 mg) of FSH, a mean number of 3.83 ± 0.70 and 2.50 ± 0.67 ovulations were found in right and left ovaries respectively. The total number of CL ranged from 4 – 11 with a mean of 6.33 ± 0.99. The number of AFs found in right and left ovaries were 1.00 ± 0.26 and 1.67 ± 0.31 respectively, and the overall mean was 2.17 ± 0.48 anovulations per animal (Plate 7).

4.5.2.5 Superovulatory response among various treatment groups

The percentage of superovulatory responders was presented in Figure 7. All the animals (100%) subjected for superovulatory treatment in Conventional group and Gn-D10 groups responded with more than two ovulations. But, in Gn-D8-400 group, only four (66.67%) animals responded to the superovulation treatment with more than two ovulations. Among these four responders only two animals had more than 10 ovulations and the remaining two animals had three ovulations each.

TABLE 9: Superovulatory response in Conventional and Follicular wave synchronized groups of Jersey crossbred cows

S.No		Conventional Mean ± SE	Follicular wave synchronized groups			F	Significance
			Gn- D8-400 Mean ± SE	Gn- D10-400 Mean ± SE	Gn- D10-200 Mean ± SE		
1	Corpus luteum						
	Right ovary	8.00 ± 0.89^{b} (6 – 12)	2.83 ± 0.87^{a} (1 – 6)	6.50 ± 0.50^{b} (6 – 8)	3.83 ± 0.70^{a} (1 – 6)	9.811	**
	Left ovary	5.67 ± 1.28^{b} (1 – 9)	2.17 ± 0.91^{a} (0 – 5)	$4.50 \pm 0.62^{a,b}$ (2 – 6)	2.50 ± 0.67^{a} (1 – 5)	3.347	*
	Total	13.67 ± 1.80^{b} (9 – 21)	5.00 ± 1.77^{a} (3 – 11)	11.00 ± 0.63^{b} (10 – 14)	6.33 ± 0.99^{a} (4 – 11)	8.405	**
2	Anovulatory follicle						
	Right ovary	1.33 ± 0.21^{a} (1-2)	7.00 ± 2.74^{b} (1 – 18)	1.50 ± 0.34^{a} (0 – 2)	1.00 ± 0.26^{a} (0 – 2)	4.241	**
	Left ovary	0.67 ± 0.21^{a} (0 – 1)	5.83 ± 1.94^{b} (1 – 12)	2.00 ± 0.26^{a} (1 – 3)	1.67 ± 0.31^{a} (0 – 2)	5.537	**
	Total	2.00 ± 0.37^{a} (1 – 3)	12.83 ± 4.65^{b} (2 – 30)	3.50 ± 0.22^{a} (3 – 4)	2.17 ± 0.48^{a} (1 – 4)	4.661	**

Range within parenthesis

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

The superovulatory response in Gn-D10-400 group was comparable to Conventional group, but had significantly ($P < 0.01$) higher number of ovulations than Gn-D8 and Gn-D10-200 groups. The Gn-D8-400 group had the lowest ovulation rate and a significantly ($P < 0.01$) increased number of AFs when compared with other treatment groups.

4.5.3 Correlation between follicular population and superovulatory response

Correlation between follicular population in various stages of superovulatory cycle and the superovulatory response was presented in Table 10.

Statistical analysis revealed that, the follicular population on the Day of superovulatory oestrus had a better correlation with the superovulatory response than the follicular status on the day of initiation of FSH. In Conventional and Gn-D10 groups, the Class III follicles on the day of superovulatory oestrus had a significant ($P<0.05$) correlation with the superovulatory response i.e the number of CL. In Gn-D8-400 group, the number of Class II follicles on the day of oestrus negatively correlated ($P<0.01$) with superovulatory response and positively correlated ($P < 0.01$) with the number of AFs.

4.6 EMBRYO / OVA RECOVERY

The number of embryos recovered and the embryo recovery rate in Conventional and FWS groups were presented in Table 11.

4.6.1 Embryo / ova recovery in Conventional group

A mean number of 7.67 ± 2.01embryos / ova were recovered out of an average of 13.67 ovulations with a recovery rate of 55.49 ± 9.70 per cent.

4.6.2 Embryo / ova recovery in Gn-D8-400 group

From an average of 5.00 ovulations, 3.00 ± 1.61 embryos / ova were recovered with a mean recovery rate of 36.57 ± 16.44 per cent.

TABLE 10: Correlation between follicular population and superovulatory response in Jersey crossbred cows

S.No	Treatment groups	Correlation between groups	Correlation Coefficient	Significance
1	CONVENTIONAL			
	Day of I FSH	Foll I x CL	0.578522	N.S
		Foll II x CL	0.351611	N.S
		Foll III x CL	-0.16552	N.S
	Day of SOE	Foll I x CL	--	
		Foll II x CL	0.740233	N.S
		Foll III x CL	0.851268	*
2	Gn-D8-400			
	Day of I FSH	Foll I x CL	-0.43503	N.S
		Foll II x CL	-0.36765	N.S
		Foll III x CL	--	
	Day of SOE	Foll I x CL	--	
		Foll II x CL	-0.91196	**
		Foll II x AF	0.968648	**
		Foll III x CL	0.429838	N.S
3	Gn-D10-400			
	Day of I FSH	Foll I x CL	0.578522	N.S
		Foll II x CL	0.351611	N.S
		Foll II x AF	0.821584	*
		Foll III x CL	-0.16552	N.S
	Day of SOE	Foll I x CL	--	
		Foll II x CL	0.740233	N.S
		Foll III x CL	0.851268	*
4	Gn-D10-200			
	Day of I FSH	Foll I x CL	0.06742	N.S
		Foll II x CL	0.409852	N.S
		Foll III x CL	0.641391	N.S
	Day of SOE	Foll I x CL	--	
		Foll II x CL	0.49364	N.S
		Foll III x CL	0.87646	*

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
N.S Non-significant

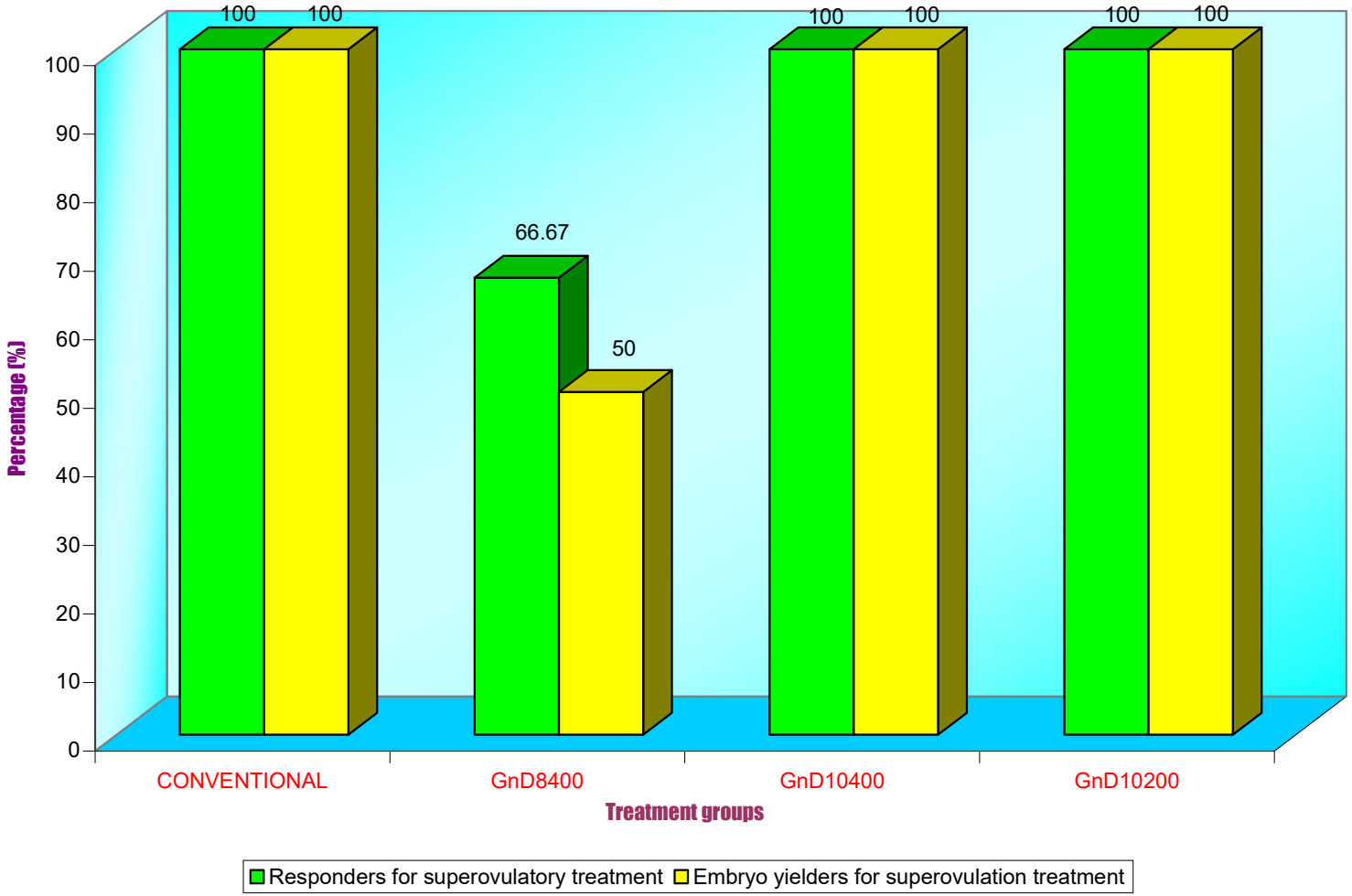
TABLE 11: Embryo/ova recovery in superovulated Jersey crossbred cows

S.No	Treatment groups	EMBRYO RECOVERY	
		Number of embryo/ova recovered Mean ± SE	Embryo/ova recovery rate (%) Mean ± SE
1	Conventional	7.67 ± 2.01 ^{b}	55.49 ± 9.70 ^{a}
2	Gn-D8-400	3.00 ± 1.61 ^{a}	36.57 ± 16.44 ^{a}
3	Gn-D10-400	6.00 ± 0.73 ^{a,b}	53.89 ± 4.16 ^{a}
4	Gn-D10-200	2.33 ± 0.42 ^{a}	44.24 ± 6.53 ^{a}
	F	3.102	0.989
	Significance	*	N.S

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

NS – Not significant ($P > 0.05$)

Figure 7: SUPEROVULATORY RESPONDERS AND EMBRYO YIELDERS IN SUPEROVULATED JERSEY CROSSBRED COWS



4.6.3 Embryo / ova recovery in Gn-D10-400 group

The number of embryos / ova recovered from an average of 11.00 ovulations was 6.0 ± 0.73 and the embryo recovery rate was 53.89 ± 4.16 per cent.

4.6.4 Embryo / ova recovery in Gn-D10-200 group

A mean number of 2.33 ± 0.42 embryos / ova were recovered out of an average of 6.33 ovulations with an embryo recovery rate of 44.24 ± 6.53 per cent.

4.6.5 Embryo recovery among various treatment groups

Embryos could be recovered from all the animals in Conventional and Gn-D10 groups (100%), but in Gn-D8-400 group embryos were recovered from only three (50%) out of six animals (Figure 7).

The number of embryos recovered in Conventional group was non-significantly higher than Gn-D10-400 group, but significantly (P>0.05) higher than other FWS groups i.e. Gn-D8-400 and Gn-D10-200 groups. Eventhough, the embryo recovery rate was considerably low in Gn-D8-400 group, there was no statistical difference when compared with other groups.

4.7 QUALITY OF RECOVERED EMBRYOS

The various grades of embryos / ova recovered from superovulated animals of Conventional and FWS groups were presented in Table 12 and Plate 8.

4.7.1 Quality assessment based on morphological criteria

4.7.1.1 Embryo quality in Conventional group

In conventional group, the mean numbers of Grade 1, 2, 3 and 4 embryos were 3.67 ± 0.92, 2.00 ± 0.37, 0.33 ± 0.33 and 0.67 ± 0.33 respectively, with the corresponding percentages of 49.72 ± 4.52, 29.77 ± 4.87, 2.08 ± 2.08 and 6.38 ± 3.01 respectively. A mean number of 0.50 ± 0.22 (6.72 ± 3.98 %) embryos were arrested / degenerated and unfertilized ova (UFO) accounted for a percentage of 4.35 ± 2.30.

The values could not be statistically assessed, however, the percentage of transferable quality i.e., Grade 1 and 2 embryos (79.49 %) were considerably more than other grades of embryos.

4.7.1.2 Embryo quality in Gn-D8-400 group

In Gn-D8-400 group, the numbers of Grade 1, 2, 3 and 4 quality embryos were 0.17 ± 0.17, 0.17 ± 0.17, 0.33 ± 0.33 and 0.33 ± 0.21 respectively and their respective percentages were 4.17 ± 4.17, 4.17 ± 4.17, 8.34 ± 8.34 and 20.84 ± 16.34. The percentage of arrested / degenerated embryos and UFO were 16.66 ± 12.34 and 45.84 ± 22.74 respectively. The yield of arrested/ degenerated embryos and UFO were considerably higher (62.5 %) in this group, leaving the transferable quality embryos contributing lower (8.34 %) than the other grades of embryos.

4.7.1.3 Embryo quality in Gn-D10-400 group

In this group, the numbers of 3.33 ± 0.42, 1.83 ± 0.48, 0.33 ± 0.21 and 0.17 ± 0.17 embryos were classified as Grade 1, 2, 3 and 4 respectively, with the corresponding percentages of 56.83 ± 5.49, 30.41 ± 7.37, 5.19 ± 3.47 and 5.16 ± 3.28 respectively. A mean number of 0.17 ± 0.17 (3.33 ± 3.33 %) and 0.17± 0.17 (1.85 ± 1.85 %) recovered ova were arrested/ degenerated and unfertilized respectively. The transferable quality embryos (Grade 1 and 2) accounted for major proportion (87.24 %) of recovered embryos.

4.7.1.4 Embryo quality in Gn-D10-200 group

In Gn-D10-200 group, the numbers of Grade 1, 2, 3 and 4 quality embryos were 1.00 ± 0.26, 0.67 ± 0.21, 0.33 ± 0.21 and 0.17 ± 0.17 respectively and their respective percentages were 48.61 ± 14.01, 26.39 ± 9.23, 16.67 ± 10.54 and 4.17 ± 4.17. The number of arrested/ degenerated embryos was 0.17 ± 0.17 (4.17 ± 4.17 %) and there was no UFO in this group.

4.7.2 Quality assessment based on developmental stages

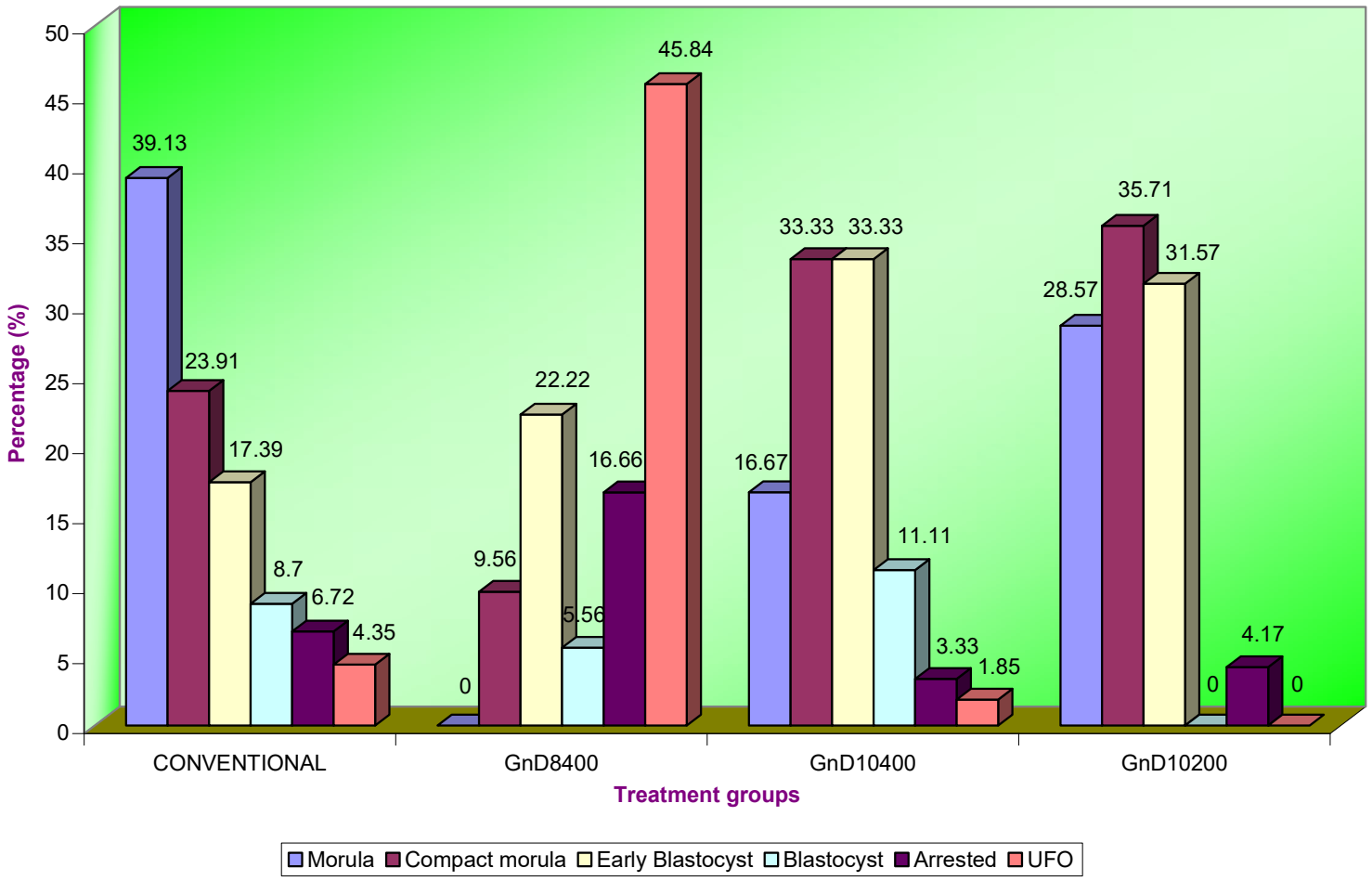
As represented in Figure 8, the percentages of various developmental stages of recovered embryos viz., Blastocyst (B), early blastocyst (EB), compact morulae (CM)

TABLE 12: Quality of embryos recovered from superovulated Jersey crossbred cows

S.No	Treatment groups	Number of embryos/ova recovered	QUALITY OF EMBRYOS					
			Grade 1 Mean ± SE	Grade 2 Mean ± SE	Grade 3 Mean ± SE	Grade 4 Mean ± SE	Arrested / Degenerated Mean ± SE	UFO Mean ± SE
1	Conventional	7.67 ± 2.01	3.67 ± 0.92 (49.72 ± 4.52)	2.00 ± 0.37 (29.77 ± 4.87)	0.33 ± 0.33 (2.08 ± 2.08)	0.67 ± 0.33 (6.38 ± 3.01)	0.50 ± 0.22 (6.72 ± 3.98)	0.33 ± 0.21 (4.35 ± 2.30)
2	Gn-D8-400	3.00 ± 1.61	0.17 ± 0.17 (4.17 ± 4.17)	0.17 ± 0.17 (4.17 ± 4.17)	0.33 ± 0.33 (8.34 ± 8.34)	0.33 ± 0.21 (20.84 ± 16.34)	0.67 ± 0.49 (16.66 ± 12.34)	1.33 ± 0.80 (45.84 ± 22.74)
3	Gn-D10-400	6.17 ± 0.87	3.33 ± 0.42 (56.83 ± 5.49)	1.83 ± 0.48 (30.41 ± 7.37)	0.33 ± 0.21 (5.19 ± 3.47)	0.17 ± 0.17 (5.16 ± 3.28)	0.17 ± 0.17 (3.33 ± 3.33)	0.17 ± 0.17 (1.85 ± 1.85)
4	Gn-D10-200	3.17 ± 0.48	1.00 ± 0.26 (48.61 ± 14.01)	0.67 ± 0.21 (26.39 ± 9.23)	0.33 ± 0.21 (16.67 ± 10.54)	0.17 ± 0.17 (4.17 ± 4.17)	0.17 ± 0.17 (4.17 ± 4.17)	0.0

Mean percentage in parenthesis

Figure 8: DEVELOPMENTAL STAGES OF EMBRYOS / OVA RECOVERED FROM SUPEROVULATED JERSEY CROSSBRED COWS



and morulae (M) accounted for 8.7, 17.39, 23.91 and 39.13 in Conventional group, 5.56, 22.22, 9.56 and 0 in Gn-D8-400 group, 11.11, 33.33, 33.33 and 16.67 in Gn-D10-400 group and 0, 31.57, 35.71 and 28.57 in Gn-D10-200 group.

4.7.3 Quality of recovered embryos among various treatment groups

Though statistically could not be analysed, it was very evident that the percentage of transferable quality (Grade 1 and 2) embryos in Gn-D10-400 group was higher (87.24%) than Conventional group (79.49%) and in Gn-D10-200 group (75.0%). The lowest percentage (8.34%) of transferable quality embryos and highest percentage (20.84%) of Grade 4 embryos were recorded in Gn-D8-400 group than the Conventional and other FWS groups. It was also found that the percentage of arrested/ degenerated embryos and UFOs (62.5 %) were very much higher in Gn-D8-400 group than other treatment groups. Based on the developmental stages, embryos recovered from Gn-D10 groups were in better advanced stages (compact morulae and early blastocyst) than Conventional and Gn-D8 groups.

4.8 ENDOCRINOLOGICAL PATTERN IN SUPEROVULATED ANIMALS

4.8.1 Plasma FSH concentrations in various treatment groups

The plasma FSH concentrations on the day of superovulatory oestrus were presented in Figure 9.

On the day of superovulatory oestrus, the FSH concentration was lower (1.99 $\pm$ 0.11 ng / ml) in Gn-D10-400 group than Conventional (2.14 $\pm$ 0.14 ng / ml), Gn-D8-400 (2.82 $\pm$ 0.50 ng / ml) and Gn-D10-200 (2.10 $\pm$ 0.19 ng / ml) groups, though there was no significant difference between them.

4.8.2 Plasma Progesterone concentrations in various treatment groups

The plasma progesterone concentrations on the day of first FSH, day of PG, day of superovulatory oestrus (SOE) and day of embryo collection (SOR) were presented in Table 13.

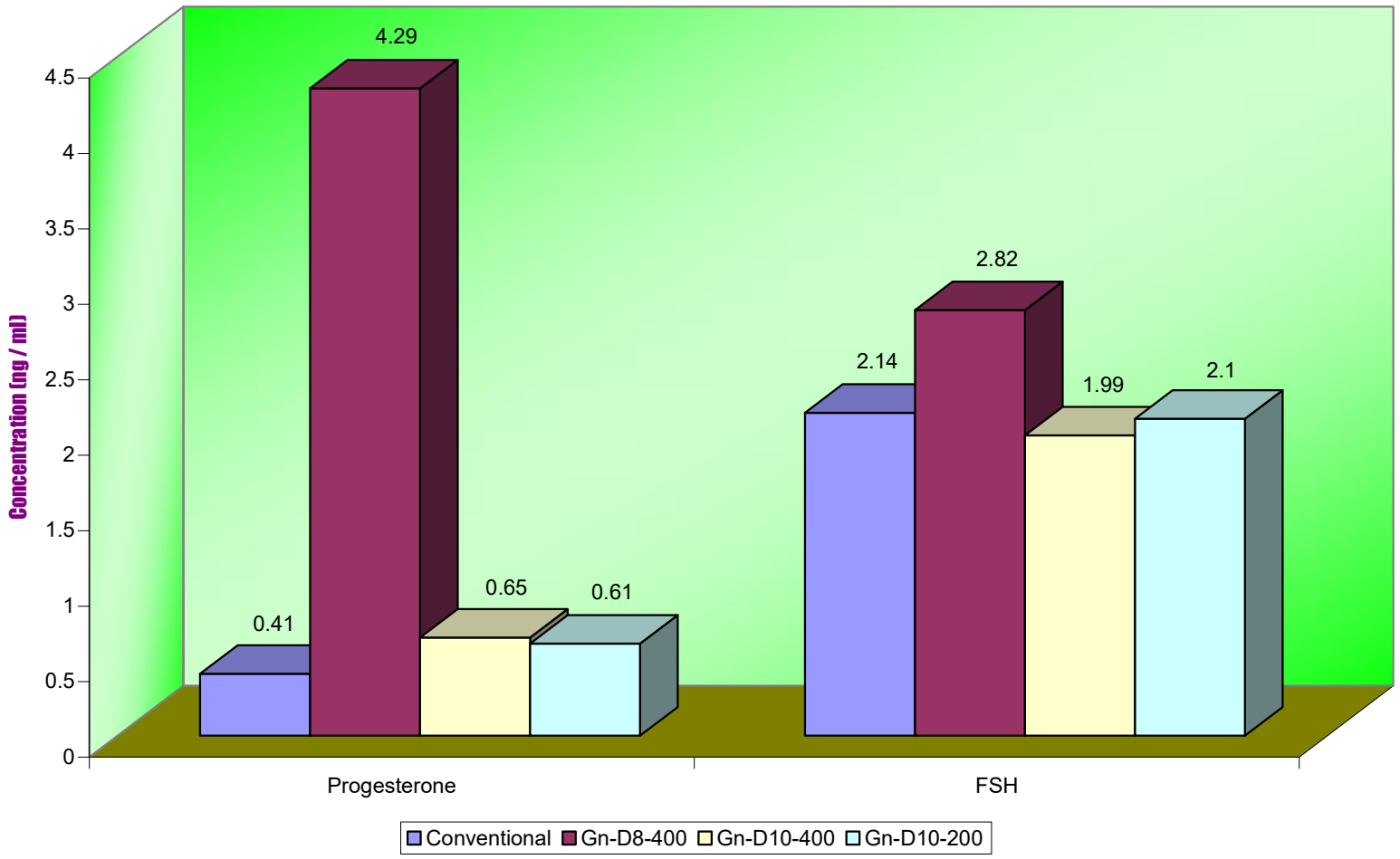
TABLE 13: Plasma progesterone concentrations in superovulated Jersey crossbred cows

S.No	TREATMENT GROUPS	PROGESTERONE CONCENTRATION (ng / ml)			
		Day of I FSH	Day of PG	Day of SOE (Day 0)	Day of SOR (Day 7)
1	Conventional	5.34 ± 0.86 [a]	8.50 ± 0.27 [a]	0.41 ± 0.03 [a]	18.76 ± 1.24 [c]
2	Gn-D8-400	6.67 ± 0.58 [a,b]	9.38 ± 1.11 [a]	4.29 ± 0.95 [b]	6.21 ± 1.38 [a]
3	Gn-D10-400	8.84 ± 0.84 [b]	10.01 ± 0.86 [a]	0.65 ± 0.08 [a]	17.03 ± 1.03 [c]
4	Gn-D10-200	8.84 ± 0.72 [b]	9.64 ± 0.70 [a]	0.61 ± 0.08 [a]	11.13 ± 0.94 [b]
	F	5.185	0.646	15.197	21.576
	Significance	**	N.S	**	**

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

N.S – Not significant ($P > 0.05$)

Figure 9: PLASMA PROGESTERONE AND FSH CONCENTRATIONS ON THE DAY OF SUPEROVULATORY OESTRUS IN VARIOUS TREATMENT GROUPS



On the day of initiation of FSH treatment in Gn-D10 groups, the average plasma progesterone concentration was 8.84 ng / ml, which was non-significantly higher than the Gn-D8 (6.67 $\pm$ 0.58 ng / ml) and significantly ($P<0.01$) higher than the Conventional (5.34 $\pm$ 0.86 ng / ml) groups. When progesterone concentrations were compared on the Day of prostaglandin administration, though the levels were higher in Gn-D10-400 group, there was no significant difference between the groups. On the day of superovulatory oestrus, Gn-D8 group was having significantly ($P<0.01$) higher level of progesterone (4.29 $\pm$ 0.95 ng / ml) when compared with Conventional (0.41 $\pm$ 0.03 ng / ml), Gn-D10-400 group (0.65 $\pm$ 0.08 ng / ml) and Gn-D10-200 group (0.61 $\pm$ 0.08 ng / ml). On the day of embryo collection (Day 7), the plasma progesterone concentration was significantly ($P<0.01$) lower (6.21 $\pm$ 1.38) in Gn-D8-400 group than other groups. The progesterone levels in Gn-D10-400 group (17.03 $\pm$ 1.03 ng / ml) were comparable to the Conventional group (18.76 $\pm$ 1.24 ng / ml), but significantly ($P<0.01$) higher than Gn-D10-200 group (11.13 $\pm$ 0.94 ng / ml).

The progesterone concentration on various stages of superovulatory cycle was correlated with superovulatory response in various treatment groups and presented in Table 14.

In Gn-D8-400 group, the plasma progesterone concentrations on the day of superovulatory oestrus and day of embryo collection were significantly ($P<0.05$) and negatively correlated with CL and positively correlated ($P<0.01$) with AFs. In all the other treatment groups viz., Conventional, Gn-D10-400 and Gn-D10-200 groups, the progesterone concentration on the day of embryo collection was positively correlated significantly ($P<0.05$) with the superovulatory response (CL). There was no significant correlation between progesterone concentrations on the day of initiation of FSH and superovulatory response in any treatment group.

TABLE 14: Correlation between plasma progesterone concentration and superovulatory response in Jersey crossbred cows

S.No	Treatment groups	Correlation between groups	Correlation Coefficient	Significance
1	CONVENTIONAL			
	Day of I FSH	P4 x CL	- 0.583	N.S
		P4 x AF	0.358	N.S
	Day of SOE	P4 x CL	- 0.623	N.S
		P4 x AF	0.463	N.S
	Day of SOR	P4 x CL	0.570	*
		P4 x AF	0.221	N.S
2	Gn-D8-400			
	Day of I FSH	P4 x CL	- 0.083	N.S
		P4 x AF	- 0.083	N.S
	Day of SOE	P4 x CL	- 0.862	*
		P4 x AF	0.901	**
	Day of SOR	P4 x CL	- 0.810	*
		P4 x AF	0.965	**
3	Gn-D10-400			
	Day of I FSH	P4 x CL	0.511	N.S
		P4 x AF	0.609	N.S
	Day of SOE	P4 x CL	- 0.687	N.S
		P4 x AF	0.218	N.S
	Day of SOR	P4 x CL	0.693	*
		P4 x AF	0.195	N.S
4	Gn-D10-200			
	Day of I FSH	P4 x CL	0.475	N.S
		P4 x AF	0.588	N.S
	Day of SOE	P4 x CL	- 0.599	N.S
		P4 x AF	- 0.304	N.S
	Day of SOR	P4 x CL	0.479	*
		P4 x AF	0.635	N.S

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
N.S Non-significant

CHAPTER V

DISCUSSION

The wide variation in superovulatory response observed with conventional treatment limited the efficacy of superovulation technique, which could be attributed to the variations in the status of follicular wave development at the time of initiation of superstimulatory treatment. Hence, the present study was aimed at assessing the effect of GnRH analogue on synchronization of follicular wave emergence and to study the effect of initiating FSH treatment at different stages of synchronized follicular wave on superovulatory response and embryo yield.

5.1 FOLLICULAR DYNAMICS AND CHARACTERISTICS OF DOMINANT FOLLICLE IN DIFFERENT WAVES OF NORMAL OESTROUS CYCLE

5.1.1 Follicular wave pattern and oestrous cycle length

The normal follicular wave pattern monitored in six Jersey crossbred cows revealed that, out of eighteen oestrous cycles studied, fourteen cycles (77.8%), three cycles (16.7%) and one cycle (5.6%) had three, two and four follicular waves respectively. In the present study, the incidence of three-follicular wave cycles was significantly higher ($P<0.01$) than two-wave and four-wave cycles. Similarly, Gong *et al.* (1995) and Satheshkumar *et al.* (2008a) observed that 85.7 per cent of Hereford cross Friesian beef heifers and 100 per cent of HF crossbred cows, respectively, displayed three waves of follicular development during the oestrous cycle. Contrary to these findings, Malhi *et al.* (2005) found that a higher proportion (60.0 %) of crossbred Hereford cows had two follicular waves, while the remaining animals (40.0 %) had three waves of follicular development.

Perusal of the previous reports showed that there was a great variation in the proportion of animals exhibiting two- or three- wave cycle. It was obvious that *B. indicus* cows and heifers exhibited higher incidence (54.5 – 60.0 %) of three waves (Zeitoun *et al.*, 1996 and Viana *et al.*, 2000), while *B. taurus* cows and heifers had

higher incidence (75.0 – 83.0 %) of two-wave cycles (Wolfenson *et al.*, 2004 and Burns *et al.*, 2005). The lesser incidence of four wave cycles reported by the former researchers in *B. indicus* cattle was in concurrence with our findings. Hence, the follicular wave patterns in our study might be attributed to the genetic make-up due to crossbreeding (*B. indicus* x *B. taurus*) of cows.

Circulating FSH concentration was also quoted as a reason for change in follicular wave pattern by Adams *et al.* (1992) in heifers. In concurrence with our findings, they were of the opinion that the heifers with two wave cycles have two FSH surges and three wave cycles have three FSH surges. In the present study, we found that the mean FSH concentrations (0.25 ng / ml) increased one or two days before each wave emergence, reached peak concentrations (0.42 ng / ml) at the time of emergence and decreased to basal levels two to three days after emergence as reported by Bergfelt *et al.* (1997b) and Wolfenson *et al.* (2004).

The mean inter-oestrus intervals for three- and two-wave oestrous cycles were 21.50 $\pm$ 0.27 (20 – 23) and 20.33 $\pm$ 0.33 (20 – 21) days respectively. The three-wave oestrous cyles were slightly lengthier than two-wave cycles. Malhi *et al.* (2005) observed that the duration of the inter-oestrus interval for two- and three-wave patterns was 20 and 23 days respectively. Taylor and Rajamahendran (1991) opined that cattle that experienced three follicular waves had a longer oestrous cycle because oestrus was delayed when the second dominant follicle failed to ovulate and a third dominant follicle required additional time to complete development before ovulation.

5.1.2 Characteristics of first wave dominant follicle

5.1.2.1 Growth phase of dominant follicle

The first wave DF of either three- or two-wave cycles emerged between Days 0.8 -1.67 and reached its maximum size between Days 6.67 – 7.30, which was usually 5-6 days after the emergence of the wave. Perusal of the data revealed that, the mean maximum diameter achieved by the first wave DF was comparable to that of ovulatory follicle. These observations were in concurrence with the findings of Mihm *et al.* (2006).

5.1.2.1.1 Deviation of dominant follicle

During the growth phase, the first wave DF deviated from the subordinate follicles by 3.30 – 3.67 days after emergence, when their diameters ranged between 8.40 – 9.00 mm. At the time of deviation of DF, the diameter of the subordinate follicle was recorded as 6.55 – 7.00 mm. The interval between wave emergence and follicular deviation observed in the present study was consistent to that observed in *B. taurus* and *B. indicus* heifers by Kulick *et al.* (2001) and Castilho *et al.* (2007) respectively.

Ginther *et al*. (1999) and Ginther (2000) observed that the mean diameters of the DF and the largest subordinate follicle in cattle at the beginning of deviation were 8.5 mm and 7.7 mm respectively, with deviation beginning a mean 2.5 days after emergence of the wave. Ginther *et al.* (2003) opined that in cattle, 7 – 11 follicles per wave emerged over one to several days and entered the common growth phase of about 3 days, which extended from the beginning of wave to the beginning of deviation. According to Evans and Fortune (1997), till the time of deviation, the future dominant and subordinate follicles were similar in size, LH receptors on theca cells, FSH receptors on granulosa cells and in their lack of LH receptors on granulosa cells. They also stated that the divergent pattern of growth between dominant and subordinate follicles, after follicle selection (third day of follicular wave), was associated with an increase in mRNA for FSH receptor in granulosa cells of DF and a decrease in mRNA for LH receptor in the theca cells of subordinate follicles.

In the present study, the first wave DFs were in a growth phase during Days 0.8 – 6.67 of the oestrous cycle irrespective of the number of follicular waves succeeding the first one. Similarly, Ginther *et al*. (2003) in their study described that regardless of the number of follicular waves per cycle, first follicular wave had more consistent characteristics than second wave which could be either ovulatory or anovulatory in nature and thus characteristics of the first wave DF was much predictable than that of the subsequent waves.

5.1.3 Characteristics of second wave dominant follicle

5.1.3.1 Emergence of second follicular wave

In three- and two-wave oestrous cycles, the second follicular wave emerged on the mean days of 9.4 ± 0.29 (8 – 11) and 9.67 ± 0.33 (9 – 10) respectively. Similar to our findings, Malhi *et al.* (2005) and Gaur and Purohit (2007) also observed similar days of wave emergence for two-wave cycles and three-wave cycles in crossbred Hereford and Rathi cows respectively. In contrast, Ginther *et al.* (1989b) found that there was a great individual variation in the precise timing of the second follicular wave emergence in cows that had varied follicular wave patterns as it emerged over a wide range of period between Day 8 – 11 of the cycle and they further observed that the second follicular wave emerged earlier in three-wave cycle when compared with two-wave cycles.

In normal three-wave cycles, the second wave DF did not differ significantly in their size with first wave DF, but it was significantly smaller in size when compared to DF (ovulatory follicle) of the third wave, similar to the observations of Viana *et al.* (2000) and Satheshkumar *et al.* (2008a) in *B. indicus* and HF crossbred cows respectively. The former researchers suggested that the smaller diameter of second wave DF might be due to the fact that the second wave emerged during the period of higher progesterone production by the corpus luteum. In the present study, the progesterone concentration observed during the development of second follicular wave DF was 5.57 ± 0.45 ng / ml, which could be a contributing factor for the smaller size of the DF of the second wave as suggested by Vinoles *et al.* (1999).

5.1.4 Characteristics of ovulatory follicle

Perusal of the data revealed that, the mean maximum diameter of the ovulatory follicle was significantly larger than the diameter of the DF of previous waves as reported by Mihm *et al.* (2006). The growth rate of the ovulatory follicle in three-wave cycles (1.72 ± 0.08 mm/day) was comparable with the report (1.29 ± 0.11 mm/day) of Mann *et al*. (2007), however the diameter of ovulatory follicle in their study (15.9 mm) was much bigger than the present findings.

In the present study, the ovulatory follicle of the two-wave cycles, reached the maximum size (12.33 $\pm$ 1.17 mm) with an increased growth phase period (11.67 $\pm$ 0.67 days) at a much slower rate (1.08 $\pm$ 0.15 mm/day) than its counterpart in first wave. As indicated by Kulick *et al.* (2001), the slower average growth rate of the DF of second wave in two-wave cycles was compensated by taking a longer period for reaching maximum diameter and ovulation.

5.2 ROLE OF GnRH IN SYNCHRONIZATION OF FOLLICULAR WAVE EMERGENCE AND FOLLICULAR CHARACTERIZATION

5.2.1 Ovulatory response of first wave dominant follicle after GnRH administration

Different ovulation rates, varying from 44.3 – 85.0 per cent after GnRH treatment at random stages of the oestrous cycle have been reported (Pursley *et al.*, 1993; Martinez *et al*., 1999 and Colazo *et al*., 2007) and these variations in ovulation rate in response to GnRH were attributed to the physiological basis of DF.

Ovulatory response of a dominant follicle in cattle during the growth phase, plateau phase or atretic phase was reported to be 100, 33 and 0 per cent respectively (Silcox *et al*., 1993). When the largest follicle was in the plateau or regression phase, the process of atresia was initiated, LH receptors are decreased (Bodensteiner *et al.,* 1996b) and GnRH did not rescue these large follicles from atresia (Twagiramungu *et al*., 1995). Atkins *et al.* (2008) recorded that, in heifers, when GnRH was administered on Day 2 of the cycle, there was no DF to respond and when administered on Day 10, growth of the DF was ceased in most of the heifers and hence the ovulatory response to GnRH was less, while heifers in the Day 5 and 15 groups had DFs that were growing, and therefore the ovulatory response to GnRH was greater. Macmillan *et al.* (2003) also stated that induction of ovulation using GnRH had been most successful only when injected after follicle deviation and the establishment of dominance. Moreover, LH receptor mRNA was expressed on granulosa cells when the DF was greater than 9mm in diameter and was first observed on Day 4 of the follicular wave, equivalent to Day 5 – 6 of the cycle (Xu *et al*., 1995). Mihm *et al.* (2006) also stated that mRNA expression for the LH receptor, a marker

for differentiation of granulosa cells, was enhanced during the growth of DF (Days 3.5–6.5 of the estrous cycle) and not detected in regressing DF.

In addition to the physiological status of a DF, the magnitude of the GnRH-induced LH release might also play a role in the ovulatory response to GnRH. Ireland and Roche (1982) reported that granulosa cells in ovarian follicles $\geq$ 10 mm in diameter acquire LH receptors and become oestrogenic. This steroid environment i.e. elevated circulating concentrations of E2 was known to influence the magnitude and duration of the GnRH-induced LH release (Price and Webb, 1988). Though these control mechanisms account for the variation in ovulatory response to a GnRH treatment given at different stages of the oestrous cycles (Martinez *et al*., 2003), the physiological status of the dominant follicle, therefore, was considered more important for GnRH induced ovulatory response. In the present study, the first wave DF got deviated between Days 4.10 – 5.33 and was in growth phase with a diameter of 10.83 $\pm$ 0.38 mm on Day 6 of the cycle when GnRH was administered. Thus the 100 per cent ovulation rate achieved in the experimental group of animals might be attributed to the physiological status of the DF at the time (Day 6) of administration of GnRH and it was much in corroboration with the findings of Kohram *et al.* (1998b).

5.2.1.1 Time interval between GnRH administration and ovulation of dominant follicle

In our study, the mean interval for ovulation of DF after GnRH administration was 27.67 $\pm$ 0.21 h, which was in concurrence with the observations of Rajamahendran *et al.* (1998) who reported that buserelin injection on Day 5 of the cycle in Holstein cows induced ovulation of the DF in a mean interval of 28.0 $\pm$ 1.2 h. Similarly, an average of 26.7h, ranging 24.3 – 28.2 h, for induced ovulation after GnRH injection was recorded by Kot and Ginther (1999). But Martinez *et al.* (2003) recorded an increased interval of 37.3 h and 36.0 h in animals administered with 100 µg of Cystorelin or Fertagyl respectively on Day 6 or 7 of the cycle.

5.2.2 GnRH synchronized second follicular wave emergence

In the present study, when GnRH was administered on Day 6, of the cycle the first wave DF ovulated and a new synchronized follicular wave emerged within 2

days of GnRH administration (Day 8) in all the animals (100%). Twagiramungu *et al.* (1994), Pursely *et al.* (1995), Diskin *et al.* (2002) and Kim and Kim (2007) made similar observations that synchronized follicular wave emergence was achieved 2.0 – 2.1 days after GnRH administration. However, an average interval of 2.9 and 1.8 days for follicular wave emergence from GnRH injection was reported by Wolfenson *et al.* (1994) and Bo *et al.* (2003) in *B. taurus* and *B. indicus* cattle respectively. The variations in timing to new follicular wave emergence among the experiments of the present study and to those in the literature might be associated with the differences in breed type, age and lactation status.

Removal of the large follicle by ovulation or atresia in GnRH-treated cows subsequently stimulated the growth of medium sized follicles (Twagiramungu *et al.,* 1995). The follicular stimulation was likely due to the short term effect of GnRH-induced release of FSH that occured within 2 to 4 h after treatment (Chenault *et al*., 1990) and /or to the effect of the delayed increase in FSH concentrations known to occur 1 to 2 days after removal/disappearance of the large DF (Ko *et al*., 1991 and Adams *et al*., 1992) that contains FSH inhibiting factors such as inhibin (Guilbault *et al*., 1991). As documented by Zolman *et al*. (1974) and Gong *et al.* (1995), in the present study it was observed that the circulating concentrations of FSH (0.25 ng / ml) increased after GnRH injection and reached peak concentrations (0.67 ng / ml) at 2 – 3 hours and then decreased to mean basal concentrations (0.24 ng / ml) by six hours after injection, which would have effected the synchronized emergence of second follicular wave two days later.

Macmillan *et al.* (2003) stated that manipulation of follicular development by synchronizing new wave emergence has been most successful using GnRH only when injected after follicle deviation and the establishment of dominance, which would induce their ovulation. Similarly, Bo *et al.* (2002) in their study, confirmed that the emergence of a new follicular wave was synchronized only when the GnRH treatment caused ovulation of the DF. In accordance with these reports, 100 per cent ovulation in response to administration of GnRH on Day 6 of the cycle was also recorded in the present study thus enabling successful synchronization of emergence of second follicular wave that ultimately resulted in a homogenous population of follicles.

5.2.2.1 Characteristics of dominant follicle of synchronized second follicular wave

In the present study, the day of deviation and interval from emergence to deviation of the DF of GnRH synchronized second wave were significantly ($P<0.01$) earlier (10.10 ± 0.31 and 2.70 ± 0.26 respectively) than the second wave of normal cycles (13.10 ± 0.53 and 3.70 ± 0.30 respectively). These characteristics of the DF of the synchronized wave could be attributed to its early emergence than its counterpart in normal cycles.

The DF diameter in GnRH synchronized wave was found to be smaller in the present study similar to the observations of Satheshkumar *et al.* (2008b) in Jersey crossbred cows. As it was explained by Viana *et al.* (2000), the DF that developed during the period of progesterone dominant status will always be smaller in size. Since, the GnRH treatment effected the ovulation and formation of an additional luteal structure (ACL), the progesterone concentration (10.57 ± 0.61 ng / ml) was significantly increased during the mid cycle. As the result of progesterone-induced suppression of LH (Vinoles *et al.,* 1999) the DF of the synchronized wave should also be bound to be smaller in size.

5.2.3 Follicular wave pattern and length of oestrous cycle

Interestingly, all the cows subjected for GnRH treatment on Day 6 of the cycle had three-wave cycles. Removal of the suppressive effect of the DF by way of induced ovulations created a permissive environment, thus allowing for a new wave of follicles to emerge earlier (Rajamahendran *et al.,* 1998). This earlier emerged DF of the new wave subsequent to GnRH injection started regressing much earlier than in normal cycles, which might be due to the suppressive effect of high progesterone secreted by both CL and ACL as reported by Viana *et al.* (2000). Thus early emergence and short period of dominance of second follicular wave dominant follicle would have resulted in the emergence of third follicular wave in all of the GnRH treated animals.

There was no significant difference in the inter-oestrus length among the normal three-wave cycles (21.50 ± 0.27) and GnRH treated cows (20.00 ± 0.63 days). The present finding was in accordance with Macmillan *et al.* (2003) who also reported that there was no effect on cycle length if the GnRH injection was administered in the first half of the cycle.

5.3 LUTEAL DYNAMICS IN NORMAL AND GnRH TREATED CYCLES

5.3.1 Luteal developmental pattern in normal cycle

In the present study, in normal cycles, the CL grew to a mean maximum diameter of 22.06 ± 0.43 mm on the mean Day of 9.0 ± 0.47 and remained fluctuating around this diameter till Days 14 or 15. Taponen *et al.* (2000) also recorded a similar luteal developmental pattern with a mean maximum diameter of 22.7 ± 1.1 mm in Finnish Ayrshire breed cows and heifers, but the size was attained in a later stage of cycle (Days 11 or 12). In the present study, a mean progesterone concentration of 5.58 ± 0.45 ng / ml during the mid cycle (Day 11) was observed. Similarly, Malhi *et al*. (2005) observed that the plasma progesterone concentrations ranged 4.16 – 5.31 ng / ml during the luteal phase (Days 8-15) with a peak concentration of 5.50 – 6.97 ng / ml on Days 13-14. Initiation of luteal regression from the mean Day of 15.89 ± 0.68 recorded in our study corroborated with the observations of Sianangama and Rajamahendran (1996).

5.3.2 Luteal developmental pattern in GnRH treated cycle

The maximum size attained by GnRH induced ACL was smaller when compared with the SCL and this might be due to the smaller diameter of the follicle from which they were derived. Eventhough the origin of ACL was much later than SCL, with respect to the day of initiation of constant regression there was no significant difference between them as mentioned by Gong *et al.* (1995) who also observed that both induced and endogenous CL in GnRH treated heifers regressed at the same time as CL in control animals. So the GnRH induced ACL exhibited a shorter lifespan lasting for about 11 -13 days as reported by Sianangama and Rajamahendran (1996). They suggested that this phenomenon might be due to the

increased proportion of large luteal cells which possess high affinity receptors to the luteolysin PGF2α.

5.3.3 Plasma progesterone concentrations in normal and GnRH treated cycles

In the present study, the plasma progesterone concentration during the early luteal phase (Day 5 – 7) ranged between 3.78 $\pm$ 0.18 - 3.83 $\pm$ 0.17 ng / ml. However, Mann *et al.* (2007) recorded a comparatively low mean plasma progesterone concentration of 2.0 $\pm$ 0.2 ng / ml on Day 5 of the cycle in HF cows. When GnRH was administered on Day 6 of the cycle in the present study, there was a significant increase in the plasma progesterone concentration (10.57 $\pm$ 0.61 ng / ml) during the mid luteal phase (Day 10 – 13) when compared to that of normal cycle (5.58 $\pm$ 0.45 ng / ml). Similarly, Gandy *et al.* (2002), by administering GnRH on Day 5 of the cycle, observed an increase in mean serum progesterone concentration (4.7 $\pm$ 0.1 ng / ml) during the mid luteal phase than in control animals (3.7 $\pm$ 0.1 ng / ml).

5.4 SUPEROVULATION

5.4.1 Follicular population in superovulatory cycle

Singh *et al.* (2004) developed a simple test to predict the superstimulatory response in cattle. They stated that the superstimulatory response was related to the intrinsic number of follicles recruited into a follicular wave and concluded that approximately 71.0 per cent of the number of follicles $\geq$ 2 mm at the time of wave emergence could be expected to constitute the number of follicles $\geq$ 5 mm after superstimulation.

5.4.1.1 Follicular population on the day of initiation of FSH treatment

Monniaux *et al.* (1983) have studied folliculogenesis in superovulatory cycles and opined that the most receptive antral follicles were found between Days 8 and 10 and it was hypothesized that this would be the best time to start cows on superovulation. Similarly, Moor *et al.* (1984) also observed that when compared with Days 7 to 9 and Days 13 to 14, the presence of healthy follicles (2 – 9 mm) was more on Days 10 – 12 and they found that these follicles on Day 10 and 11 must be at

stages of development that allowed them to be stimulated and maintained by exogenous FSH. However, this hypothesis was not supported by Donaldson (1984a) who stated that antral follicular population was the same on days 11 through 13 and starting superovulation on Days 9 to 13 had no effect on embryo production in the cow. However, in the present study the distribution of all the three classes of follicles varying in their responsiveness to the FSH were present in the Conventional group, while a more homogenous group of Class I and II (4 – 9 mm) follicles alone were present in the synchronized group which would respond to FSH treatment uniformly.

By Day 6 of the oestrous cycle, in control and GnRH administered cycle, all the animals had atleast one Class III follicle and varied population of Class I and II follicles indicating that the development of the first wave DF was highly predictable and less variable than the growth and development of DFs of subsequent waves.

The effect of GnRH (Day 6) induced ovulation of first wave DF was detected by the increased number of Class I and II follicles on Day 8 of the cycle. This status was attributed to the synchronized follicular recruitment for the second wave (Rajamahendran *et al.,* 1998). Buserelin had the ability to release not only LH but also FSH (Chenault *et al.*, 1990), and it has been reported that the depletion of the pool of large follicles after buserelin injection was associated with a transient increase in the number of 4- to 6- mm and 7- to 9- mm follicles (Guilbault *et al.,* 1990). As observed in the present study, it was therefore possible that the combination of buserelin induced depletion of large follicles and associated recruitment of medium sized follicles might have led to a greater homogeneity of ovarian follicular inventories among animals at the time of initiation of superstimulation.

Among the GnRH treated groups (Gn-D8 and Gn-D10) it was found that Class II follicles, which was more responsive to FSH treatment, was found to be more on Day 10 group which was in accordance with the findings of Kohram *et al.* (1998a) who observed that the Class I (4-6 mm) follicles decreased and more number of Class II ($\geq$ 7 mm) were present four days after the GnRH treatment. These Class II follicles were in common growth phase and had not yet reached the stage of deviation on Day 10 of the synchronized cycle. Ginther *et al.* (2003) stated that all growing follicles early in the wave have the potential for future dominance and administration of FSH

at this stage prevented the deviation phenomenon and induced several follicles to become dominant. Thus more number of FSH responsive follicles were available on day of initiation of FSH in Gn-D10 group animals.

5.4.1.2 Follicular population on the day of superovulatory oestrus

With the conventional superovulation protocol, there was a significant increase in the number of Class II and III follicles on the day of superovulatory oestrus when compared with the day of initiation of FSH administration. Savio *et al.* (1991) when initiated their superovulation schedule on Day 10.5 of the cycle observed that the number of $\geq$ 10 mm follicles on the final day of FSH treatment was more than that were present on the first day of FSH. . Pierson and Ginther (1984) opined that those follicles (< 2 mm) which were not detected at the time of initiation of FSH treatment were found to be growing and present in larger pools, whether or not they were destined to ovulate at the time of superovulatory oestrus. They emphasized from their finding that after initiating the FSH treatment on Day 10 of the cycle the average numbers of small (2-3 mm), medium (4-10 mm) and large (>10 mm) follicles were 10.5, 8.0 and 1.5 (total: 20.0 follicles) respectively and on the day of superovulatory oestrus the corresponding numbers were 0.0, 7.5 and 19.0 (total: 26.5 follicles) respectively. In our study, though the number of follicles on the day of superovulatory oestrus was less (total: 15.0 follicles), similar pattern was observed.

By initiating the FSH treatment on the day of GnRH synchronized follicular wave emergence (Day 8), there was a hyperstimulatory response in the total number of Class II and III follicles (total: 17.5 follicles) on the day of superovulatory oestrus. Sato *et al.* (2005) in a similar study administered 25-100 µg of GnRH on Day 6 of the cycle and started the FSH treatment after 2.5 days. They found that the mean number of small follicles (4-7mm) increased from 1.5 to 2.5 days, the number of medium follicles (7-10mm) increased from 3.5 days until 5.5 days (during FSH treatment) and the number of large follicles (>10 mm) increased from Day 4.5 until Day 7.5 (day of superovulatory oestrus). The findings of the present study also corroborated with that of Farin *et al.* (2008) who recorded a mean number of 12.4 $\pm$ 1.6 follicles (5-10 mm) after FSH treatment when gonadotrophin treatment was initiated two days after GnRH.

Kohram *et al.* (1998a) initiated the superovulation treatment six days after GnRH and found that the number of $\geq$ 7 mm follicles increased in response to FSH and at oestrus a mean number of 11.5 $\pm$ 3.0 follicles were present. Similarly, in the present study, an average of 14.67 follicles on the day of superovulatory oestrus was recorded in Gn-D10-400 group which was comparable to that of conventional protocol.

On perusal of the results of the present study, it was inferred that the Day 10 treatment group was comparable with that of Conventional group. Though Day 8 group was not statistically different with Conventional and Gn-D10 groups, they showed higher number of follicular availability at the time of superovulatory oestrus.

5.4.2 Superovulatory response

Soboleva *et al.* (2000) developed a model to describe exogenous FSH administration and showed that the ovulation rate response was dependant on both the day of administration and the amount of FSH administered.

5.4.2.1 Superovulatory response in relation to day of initiation of FSH treatment

In the present study by the conventional FSH protocol, all the animals (100%) responded with more than two ovulations as reported previously by Pawshe *et al.* (1992) in crossbred cattle. But Shanker *et al.* (1998) and Mathur *et al.* (2006) observed that only 65.6 and 75.0 per cent of cows responded with more than two ovulations with the same regimen. The mean numbers of CL (13.67 $\pm$ 1.80) and AF (2.00 $\pm$ 0.37) in the present study were in concurrence with the superovulatory response achieved by Nigam *et al.* (2001) and Mathur *et al.* (2006) in Jersey crossbred and Frieswal cows respectively.

Arosh *et al.* (2000) stated that superovulation initiated on Day 9 of the oestrous cycle *i.e.* closer to the emergence of the follicular wave resulted in more recruitment of follicles and more number of ovulations. Moor *et al.* (1984) found that the follicles that ovulated after gonadotrophin treatment were generally medium sized, non-atretic follicles, which were most abundant on Days 9-13 of the cycle. One may therefore anticipate a greater degree of superovulation when gonadotrophin treatments

were initiated in these days. However in our study, the superovulatory response varied widely from nine to twenty one ovulations per animal in conventional treatment initiated on Day 10 of the cycle, which was in concurrence with Adams *et al.* (1994) who also recorded wide variations in the superovulatory response (5 to 18 ovulations) with the similar dose of FSH initiated on Day 10 of the cycle. This disparity in response prompted a retrospective analysis of the interval from wave emergence to initiation of treatment.

When FSH was initiated on the day of synchronized follicular wave emergence (Day 8) *i.e.* two days after GnRH, the mean number of total ovulations and AFs were 5.00 $\pm$ 1.77 (1 – 11) and 12.83 $\pm$ 4.65 (2 - 30) respectively. In this group, only four (66.67%) animals responded to the superovulation treatment with more than two ovulations. Among these four responders only two animals had more than 10 ovulations and the remaining two animals had three ovulations each. In a typical response to ovarian superstimulation with FSH, the number of follicles that ovulated was lower than the number of follicles that were stimulated to grow to a relatively large size. Farin *et al.* (2008) also reported only 77.8 per cent response to superovulation treatment initiated two days after GnRH in cows with a mean ovulation rate of 6.4 $\pm$ 1.5 per animal.

Similarly, Boland *et al.* (1991) and Rajamahendran and Calder (1993) failed to show improvement in the superovulatory response when the cows were superovulated on Day 2 of the oestrous cycle or two days after hCG treatment i.e. approximately two days after the LH surge compared with that obtained at mid cycle. It has been suggested that follicles that did not ovulate after FSH stimulation have not matured sufficiently to respond to the preovulatory surge release of LH (D'Occhio *et al*., 1997).Collectively these results suggested that the occurrence of a LH surge of either endogenous or exogenous source two days prior to initiation of superovulation might compromise the ability of follicles to ovulate and to produce viable embryos. Such negative effects of GnRH on ovulation and embryo production might be due to incomplete luteal regression, since luteolysis was induced only four days after GnRH treatment and / or to luteinization of follicles in GnRH treated group (Stock *et al.,* 1996). It was reported that the CL was not consistently responsive to PGF2α until $\geq$ 5

days after oestrus (Twagiramungu *et al.,* 1992; Nasser *et al.*, 1993 and Wolfenson *et al*., 1994). Callesen *et al.* (1988) elaborated that incomplete luteolysis could be due to insensitivity of the CL to prostaglandins or insufficient number of prostaglandin receptors on CL due to faulty preovulatory development of follicle. In complete agreement with this fact, a prominent luteal tissue, probably that of ACL (3 days old), could be ultrasonographically detected on the day of superovulatory oestrus in four animals of this group, indicating incomplete luteolysis, which was very well supported by the findings of the present study that Gn-D8 group was having significantly higher level of progesterone (4.29 $\pm$ 0.95 ng / ml) when compared with Conventional (0.41 $\pm$ 0.03 ng / ml) and Gn-D10 group (0.65 $\pm$ 0.08 ng / ml). According to Duchens *et al*. (1994) the high progesterone concentrations on the day of oestrum inhibited the oestradiol induced LH surge and thus the ovulation was prevented. However, Adams *et al.* (1994) stated that two doses of cloprostenol effectively induced luteolysis when CL was immature (3-4 days old) and induced ovulation in 58 of 60 heifers which was in concurrence with our study where complete luteolysis and good superovulatory response could be achieved in two animals. Thus the inconsistency in the superovulatory response among the animals treated with FSH on the day of GnRH induced follicular wave emergence (Day 8) could be attributed to the inconsistent luteolytic response and high progesterone level at superovulatory oestrum.

Initiating the superovulation schedule two days after synchronized follicular wave emergence (Day 10) and before the establishment of dominance, the animals responded with 11.00 $\pm$ 0.63 (10 – 14) CL and 3.50 $\pm$ 0.22 (3 – 4) AFs comparable to the Conventional group. Evans and Fortune (1997) stated that till the point of deviation, the future DF and subordinate follicles were similar in FSH receptors on their granulosa cells. According to Xu *et al.* (1995) and Rhodes *et al.* (2001) there was no difference in responsiveness to FSH between granulosa cells from the largest follicle and smaller follicles or between granulosa cells from dominant and subordinate follicles (Days 3-5). Rhodes *et al.* (1997) could observe responsiveness to FSH in granulosa cells from all follicles $\geq$ 5 mm in diameter and no significant differences between DF and subordinate follicles on any day between 0-6 after wave emergence. Besides the small difference in follicular diameter and capacity to produce

E2, Fortune *et al.* (2001) could not observe any other difference between DF and subordinate follicles on Day 2 of the follicular wave. Soboleva *et al.* (2000) suggested that the best response occurred when FSH was administered at the time of deviation of dominant and subordinate follicles which in our study eventually coincided with the Gn-D10-400 group. Ginther *et al.* (2003) also concluded that all follicles of 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. They also opined that a subordinate follicle might maintain adequate viability for one day or more after the beginning of deviation. Adams *et al*. (1994) also stated that irrevocable suppression of all subordinates was not apparent until approximately five days after emergence. It was thus concluded that the superstimulatory response was achieved by preventing atresia and promoting continued development of subordinate follicles of the extant wave when the FSH treatment was initiated before they go for deviation and establishment of dominance.

5.4.2.2 Superovulatory response in relation to follicular population

The effect of first day of treatment with FSH likely reflected differences among populations of follicles from Days 7 to 14 of the oestrous cycle. Perhaps a less synchronous emergence of second wave (within and among animals) resulted in greater variation in the status of follicles at the time FSH treatment was initiated. In the present study too, the wide variation in superovulatory response observed in Conventional group could be attributed to cow-to-cow variation in ovarian status at the onset of superstimulatory treatment. Because of variations in the day of wave emergence among animals, in Conventional group it was not possible to generalize the exact follicular status on the particular day of the cycle, leading to variations in superovulatory response.

Among the treatment groups, animals in the Gn-D8-400 group experienced a significantly low superovulatory response of 5.00 $\pm$ 1.77 CL per animal inspite of having highest number (though not statistically significant) of follicles on the day of oestrus than any other groups. As large numbers of follicles are stimulated, physical limitations within the ovary (e.g., blood supply to individual follicles) or disruptions of normal endocrine mechanisms (e.g., excessive production of ovarian steroids)

could interfere with proper follicular development or ovulation (Lerner *et al.*, 1986). Likely, the more number of Class II follicles on the day of superovulatory oestrus correlated positively with increased number of anovulatory follicles on Day 7 of superovulatory cycle in Gn – D8 group. LH receptor mRNA was expressed on granulosa cells when the DF was greater than 9mm in diameter and the cAMP response to LH was generally low or not detected in granulosa cells from 4-8 mm follicles (Xu *et al*., 1995). Altered receptor-hormone interactions due to larger pool of Class II follicles, as it was observed in the present study in Gn-D8 group, might also be involved in the decrease in superovulatory response as reported by Lerner *et al*. (1986).

There was an interesting positive correlation between Class III follicles on the day of superovulatory oestrus and the number of CL on Day 7 of superovulatory cycle in the present study. In both Conventional and Gn-D10-400 groups, almost all the Class III follicles on the day of superovulatory oestrus ovulated indicating that these follicles were healthy enough to respond to endogenous LH surge. Purwantara *et al*. (1993) also noted that the number of large follicles and the sum of large and medium follicles at the day of insemination were positively correlated with the number of CL palpated on the day of recovery. Sartori *et al.* (2004) also affirmed that the number of follicles $\geq$ 8.5 mm present at the time of superovulatory oestrus and the number of CL at the time of flushing were not different.

In the present study when correlation was established with the number of follicles at the initiation of the FSH treatment and at the time of superovulatory oestrus, it was found that a positive correlation existed between the follicles at superovulatory oestrus and ovulatory response.

5.5 EMBRYO RECOVERY

5.5.1 Embryo recovery in relation to day of initiation of FSH treatment

Boland *et al.* (1991) stated that one third of the donors treated do not respond to superovulation, another third produced an average of one to three embryos and only one third actually superovulated giving a large number of embryos. They also suggested that the day of the oestrous cycle on which treatment with FSH was started

affected number of ova plus embryos recovered in a non-linear manner which was consistent with previous reports of the effect of timing of treatment. The individual mean numbers of embryos recovered for FSH started on Day 7 through 14 of the oestrous cycle by Lerner *et al.* (1986) were 5.8 ± 1.7, 2.5 ± 1.6, 6.8 ± 1.1, 10.1± 1.1, 10.0 ± 0.9, 7.3 ± 1.0, 7.7 ± 1.0 and 8.1 ±1.2 respectively. Lindsell *et al.* (1986) and Mollo *et al*. (2007) also reported a greater yield of embryos when treatment was started on Day 9 as compared with Days 3, 6 or 11, 12 and 13 of the oestrous cycle and established that the day of cycle when the superovulatory treatment was started had a significant effect on the number of viable embryos recovered from flushing.

In the present study when conventional protocol was initiated on Day 10 of the cycle mean recovery rate of 55.49 ± 9.70 per cent was recorded which was comparable with the recovery rate of 59.50 per cent reported by Bo *et al.* (1996). But, Nigam *et al.* (2001) and Mathur *et al.* (2006) experienced a very low overall recovery rate of 23.42 and 37.06 per cent respectively, while Arora *et al.* (1996b), Murugavel *et al.* (1999) and Arosh *et al.* (2000) recorded a higher recovery rate of 69.56, 71.43 and 73.5 per cent respectively in Jersey crossbred cows with a similar schedule. In the present study also it was observed that a wide variation in recovery rate between animals with highest and lowest values being 88.89 and 28.57 per cent respectively with the conventional protocol of initiating the FSH treatment on Day 10 of the cycle.

With the superovulation schedule of initiating FSH treatment on the day of GnRH induced follicular wave emergence (Gn-D8-400), only three (50%) animals yielded embryos with their recovery rate ranging from 66.70 to 80.00 per cent. With no embryos being recovered from the rest of the three animals the overall mean recovery rate was drastically reduced to 36.57 ± 16.44 per cent. But Sato *et al.* (2005) and Farin *et al*. (2008) recovered 87.5 per cent of embryos from cows by initiating the FSH treatment 2 - 2.5 days after administration of GnRH on Day 6 of the cycle. In support to the present study, Deyo *et al.* (2001) also recorded a lower embryo recovery rate when they superovulated after the GnRH or pLH synchronized follicular wave emergence.

In the present study, in Gn-D10-400 group, an average of 6.00 (4 – 9) embryos / ova were recovered out of 11.0 ovulations (53.89 %) when FSH treatment was

initiated two days after synchronized wave emergence. However Son *et al*. (2007) recorded 42.14 per cent recovery rate when superstimulation was induced three days after GnRH in CIDR- pretreated Korean native cows. Park *et al*. (2007) also recorded 33.33 – 43.15 per cent of embryo recovery rate in Hanmoo and Holstein heifers when superstimulated about two days after EB induced follicular wave emergence. Andrade *et al.* (2003) opined that the use of steroid hormones (progesterone and estrogen) prior to superovulation of Nelore donors efficiently promoted the regression of DFs and the start of a new follicle wave, so that the superovulatory treatment which was started at a time when no DF was present, resulted in acceptable embryo recovery.

A predominant population of Class III follicles on the day of superovulatory oestrus in Gn-D10-400 group could be attributed for the increased ovulation rate in this group. Further, the day of luteolysis was effected late in this group when the CL was in a stage, responsive for prostaglandin, which was depicted by lower progesterone concentration (0.65 $\pm$ 0.08 ng / ml) on the day of oestrus providing a conducive environment for the ovulation of the follicular population. But in Gn-D8-400 group, more number of Class II follicles and suprabasal levels of progesterone (4.29 $\pm$ 0.95 ng / ml) on the day of oestrus increased the anovulatory follicular population considerably on the day of embryo collection. These increased numbers of AFs in Gn-D8 group might have resulted in increased concentration of E2 as observed by Saumande *et al.* (1984) which would have resulted in a poor recovery rate. Thus the embryo recovery rate in cows superovulated two days after synchronized wave emergence (Day 10) was comparable to that of conventional protocol, but the recovery rate was substantially affected by initiating the superstimulatory treatment on the day of GnRH synchronized wave emergence (Day 8).

5.6 EMBRYO QUALITY

In conventional group, an average of 79.49% (5.67) embryos was of transferable quality (Grade 1 and 2) which was in concurrence with Mathur *et al.* (2006) who recovered 87.14 per cent good and fair quality embryos with the same protocol. But Detterer *et al.* (1997), Assumpcao *et al.* (1997), Chandra *et al.* (1997) recovered a lower average of 52.38, 35.98 and 68.85 per cent transferable embryos in German Holstein, Zebu and Indian crossbred cows respectively with a similar

protocol. In the present study, a mean number of 0.50 ± 0.22 (6.72 ± 3.98 %) embryos were arrested / degenerated, while unfertilized ova (UFO) accounted for a mean percentage of 3.50 ± 2.30. Similarly, a higher fertilization rate (82.5 %) and low incidence of unfertilized ova (6.25 %) were recorded by Murugavel *et al.* (1999) and Arosh *et al.* (2000) respectively in Jersey crossbred cattle treated with FSH. Arora *et al.* (1996b) and Shanker *et al.* (1998) also observed an average 0.5 – 0.56 unfertilized ova per donor out off 5.33 and 4.06 total recovered embryos. Chandra *et al.* (1997) and Andrade *et al.* (2003) reported 71.0 and 74.7 per cent of fertilization rate in superovulated crossbred and *B. indicus* cows respectively. As reported by van den Hurk *et al*. (1992) the use of FSH decreased or reversed the incidence of atresia and improved oocyte quality in cattle, possibly by altering the follicular microenvironment indirectly, through the IGF-I system, which was known to affect follicular mitogenic and steroidogenic activities.

In the present study, arrested/degenerated and UFO (62.50%) and Grade 4 embryos (20.84%) constituted the major proportion of ova recovered when FSH was initiated on Day 8 of GnRH treated cycle (Gn-D8-400). The transferable quality embryos accounted for only 8.34 per cent. Thus, a total degradation of ova quality was registered when gonadotrophin treatment was started two days after GnRH which was in corroboration with the findings of Kohram *et al.* (1998 a) and Sato *et al.* (2005). Duchens *et al*. (1994) opined that plasma progesterone concentration exceeding 1.2 nmol/ lit on the day of oestrus, as observed in the present study (4.29 ± 0.95 ng / ml), would induce persistence of the ovulatory follicle and thus cause damage to the oocyte. Desaulniers *et al.* (1995) also observed that such disturbed endocrine events in cows could lead to follicles of low steroidogenic capacity and low competence of oocytes.

Sato *et al*. (2005) recorded an increase in mean numbers of unfertilized ova and degenerated embryos when GnRH was given shortly (i.e., 2-2.5 days) before superstimulation and suggested a possibility that a high concentration of E2 in the peripheral circulation from many growing follicles was detrimental to the fertilization of ova and / or subsequent embryo development. Duchens *et al*. (1994) and Kafi and McGowan (1997) also suggested that the endocrine disturbance might lead to altered

gamete or embryo transport in the oviducts and thus a lower fertilization rate or abnormal embryonic development.

Further, Saumande *et al.* (1984) opined that an abnormally high levels of E2 and P4 secreted by the unovulated follicles which were found persisting from the day of superovulatory oestrus to the day of embryo collection, as observed in the present study (Gn-D8 group), might have adverse effects on embryo development.

Thus in the present study, though elimination of DF promoted follicular growth, it resulted in poor quality embryos when superovulatoty treatment was started on the day of synchronized wave emergence (Day 8).

When FSH was initiated two days after synchronized wave emergence (Gn-D10-400) in the present study, an higher percentage (87.24 %) of transferable quality (Grade 1 and 2) embryos and lower incidence of arrested / degenerated embryos (3.33 %) and UFO (1.85 %) than the Conventional and Gn-D8 groups was recorded. Park *et al*. (2007) recovered 70.37 per cent Grade 1 embryos from Hanwoo heifers when FSH was initiated six days after EB which was comparatively equivalent to our schedule. Andrade *et al.* (2003) opined that the steroid hormones efficiently synchronized the start of a new follicle wave and superovulatory treatment which was started at a time when no DF was present did not interfere with the morphology of the embryo or with its developmental capacity *in-vivo*. Further, in the present study, a low concentration (1.99 $\pm$ 0.11 ng / ml) of FSH (though not significant) could be observed on the day of superovulatory oestrus in Gn-D10 group, when compared to Conventional (2.14 $\pm$ 0.14 ng / ml) and Gn-D8 (2.82 $\pm$ 0.50 ng / ml) groups. Fortune *et al*. (2004) opined that FSH induced IGFBP protease degrade the IGFBPs and caused an increase in free IGF which in turn synergized with FSH to increase follicular E2. These oestrogenic follicles exerted negative feedback to decrease the peripheral FSH secretion. As suggested by these researchers, the present finding of low levels of FSH on the day of superovulatory oestrus in Gn-D10 group could be attributed to healthy oestrogenic follicles.

Callesen *et al.* (1995) opined that embryos in later stages (compact morulae / blastocyst) of development had a better average quality grade than those less

developed, just as embryos of the poorest quality grade tended to produce lower pregnancy rates. Based on the developmental stages it was obvious that a higher proportion of compact morulae / early blastocycst could be recovered on Day 7 of the superovulatory cycle in Gn-D10-400 group (66.67%) than Conventional or Gn-D8 groups. Soom *et al*. (1997) in their study to determine the timing of compaction in *in-vivo* produced superovulated bovine embryos found that compaction started on Day 5 post ovulation (equivalent to Day 6 of superovulatory cycle in our study) and 88.89 per cent of the embryos recovered on Day 6 post ovulation (equivalent to Day 7 of our study) were in compact morulae and early blastocyst stage. Thus a better developmental quality of embryos was evident in our study in Gn-D10 group could be attributed to the homogenous recruitment of healthy follicles as against the conventional group.

5.7 EFFECT OF REDUCED DOSE OF FSH ON SUPEROVULATORY RESPONSE AND EMBRYO YIELD WHEN INITIATING THE SUPEROVULATION TREATMENT ON DAY 10 OF THE GnRH TREATED CYCLE

Lerner *et al*. (1986) stated that higher doses of FSH were associated with stimulation, recruitment and maintenance of a greater proportion of antral follicles resulting in increased embryo recovery rate and percentages of transferable embryos in cows. However, the dose rate for superovulation protocol could be reduced without affecting the embryo production (Ireland *et al.*, 2007). Patel *et al*. (2007) followed conventional superovulatory protocol with reduced (200 mg) dose of FSH and found that about 84.0 per cent animals responded to the treatment with an average of 10.90 ovulations per animal comparable to the superovulatory treatment with 400 mg (12.54 ovulations)

In the present study, with the reduced dose of FSH (200 mg) in Gn-D10 group, all the animals responded with a mean superovulatory response of 6.33 $\pm$ 0.99 CL (range: 4-11 CL). The ovarian response was comparable to that of previous experiments by Adams *et al.* (1994) who initiated the treatment one day after wave emergence of Wave 2 using a half-dose of Folltropin-V. In contrary, Sumretprasong *et al*. (2008) recorded a higher (15.4) ovulation rate with 260 mg FSH in Thai-

Friesian crossbred cows which were subjected for follicular wave synchronization prior to superovulation. However, the present observations with reduced dosages was better than the reports of Ansari *et al.* (1998), Murugavel *et al.* (1999) and Sarvaiya *et al.* (2003) who have recorded a comparatively lesser ovulation rate of 7.6 - 7.8 in Jersey crossbred cows with conventional protocol using 400mg of FSH.

In the present study, an average of 44.24 per cent (2.33) embryos / ova were recovered out of 6.33 ovulations in Gn-D10-200 group. However, after synchronizing follicular wave emergence, Sumretprasong *et al*. (2008) recovered very high percentage (74.8 %) of embryos in Thai-Friesian cross-breed cattle superstimulated with 260 mg FSH. Shanker *et al.* (1998) and Nigam *et al.* (2001) stated that irrespective of the dosage 22.22 - 26.08 per cent of donors produced no eggs. But embryos were obtained from all the animals (100%) with the reduced dosage in the present study.

When compared to Conventional and Gn-D10-400 group, a significantly lower number of Class III follicles on the day of oestrus could be attributed for the lower superovulatory response in Gn-D10-200 group. Though the percentage of embryos recovered and transferable embryos were lower than the Conventional and Gn-D10-400 group, there was no statistical significance. But when compared with Gn-D8-400 group, a non-significant increase in superovulatory response and embryo recovery rate was recorded in Gn-D10-200 group, inspite of non-significantly lower number of follicles on the day of superovulatory oestrus. Ireland *et al.* (2007) suggested that the proportion of follicles with high-quality oocytes may be greater for cattle with low follicle numbers than with more number of follicles. This fact could be attributed for higher number of transferable quality embryos (75.0 %) in Gn-D10-200 group than the Gn-D8 group in the present study. Thus, better superovulation results could be achieved by initiating the superovulatory treatment two days after synchronized follicular wave emergence (Day 10) even with a reduced dose of FSH (200 mg) than with a conventional dose (400 mg) on the day of wave emergence (Day 8). However further studies with more number of animals were required to establish the potential of low dose (200 mg) of FSH in inducing superovulation after synchronized follicular wave emergence.

From the foregoing analysis of results it was evident that

i) There was a great variation in the proportion of animals exhibiting two- or three- wave cycle.

ii) There was a great individual variation in the precise timing of the second follicular wave emergence in cows that have two- or three-wave cycles.

iii) The growth and development of the first DF is highly predictable and less variable than the growth and development of subsequent DFs in cattle because of the variable number of DFs occurring during the oestrous cycle.

Hence, GnRH treatment by Day 6 of the cycle caused ovulation of DF and induced a new wave of follicles two days later and consequently a more uniform group of viable follicles was present at the time of superstimulation which was evident by

i) rapid disappearance of large follicles and

ii) increase in the number of recruitable follicles (< 5 mm) indicative of emergence of new follicular wave within two days (Day 8).

Initiating the FSH schedule by the day of GnRH synchronized wave emergence (Day 8) resulted in inconsistent superovulatory response and recovery of poor quality embryos, which might be attributed to

i) Altered receptor-hormone interactions due to larger pool of Class II follicles on the day of superovulatory oestrus

ii) incomplete luteolysis and suprabasal levels of plasma progesterone concentration on the day of superovulatory oestrus (Duchens *et al*., 1994) and

iii) probable reduced storage of LH in the pituitary gland and sensitivity to itself by decreasing the number of receptors on pituitary cells in

response to GnRH administration and subsequently suppressed gonadotrophin secretion from the pituitary gland (Ulker *et al.*, 2001).

In conclusion, initiation of the FSH treatment two days after the synchronized wave emergence i.e. before the establishment of dominance (Day 10) resulted in a superovulatory response and embryo yields comparable to those observed with the conventional method of superstimulation, but with a lesser degree of variation between animals. Further, recovery of an increased percentage of transferable quality and better developed embryos than the conventional method was evident. Based on these findings, it could be suggested that initiating the FSH treatment two days after the GnRH induced follicular wave emergence will help to reduce the variability and unpredictability of results inherent in the superovulation of cattle by

i) recruitment of medium sized follicles and in turn might have led to a greater homogeneity of healthy oestrogenic follicular inventories among animals at the time of induction of luteolysis and

ii) complete luteolysis and conducive endocrine environment supporting ovulation of healthy follicles.

CHAPTER VI

SUMMARY

The present study was aimed at assessing the effect of GnRH analogue in synchronizing the follicular wave emergence as a pretreatment for superovulation in Jersey crossbred cattle and to study the effect of initiating FSH treatment (normal and reduced doses) after synchronizing the emergence of follicular wave on superovulatory response and embryo yield.

Six healthy, non-lactating and regularly cycling Jersey crossbred cows aged between 5-6 yrs were utilized for the study. Ultrasonographic monitoring of the normal follicular wave pattern (n=18 cycles) revealed that, fourteen cycles (77.8%), three cycles (16.7%) and one cycle (5.6%) exhibited three, two and four follicular waves respectively. There was a great variation in the proportion of animals exhibiting two- or three- wave cycle. Irrespective of the follicular wave pattern the first wave DF was in a growth phase during Days 0.8 – 6.67 of the oestrous cycle and got deviated from the subordinate follicles by 3.30 – 3.67 days after emergence. Thus, the growth and development of the first wave DF was highly predictable and less variable than the growth and development of subsequent DFs and there was a great individual variation in the precise timing of the second follicular wave emergence in cows that have two- or three-wave cycles.

When GnRH analogue (Buserelin acetate – 10 µg) was administered on Day 6 of the cycle, the DF (10.83 $\pm$ 0.38 mm) ovulated in all the animals (100 %) in a mean interval of 27.67 $\pm$ 0.21 h and a synchronized homogenous group of follicles emerged after two days (Day of 8.00 $\pm$ 0.0) and consequently a more uniform group of viable follicles was present at the time of superstimulation.

In the Conventional group of superovulation treatment, when FSH treatment was initiated on Day 10 of the cycle, all the animals (100%) responded with 13.67 $\pm$ 1.80 CL. However, the superovulatory response ranged widely from nine to twenty one ovulations per animal, perhaps due to a less synchronous emergence of second

wave and thus a greater variation in the status of follicles at the time FSH treatment was initiated.

Initiating the FSH schedule by the day of synchronized wave emergence (Day 8) resulted in inconsistent superovulatory response (5.00 $\pm$ 1.77 CL) and recovery of poor quality embryos, which might be attributed to more number of Class II follicles, incomplete luteolysis and suprabasal levels of plasma progesterone concentration (4.29 $\pm$ 0.95 ng / ml) on the day of superovulatory oestrus.

Initiating the FSH treatment two days after the synchronized wave emergence i.e. before the establishment of dominance (Day 10) resulted in a superovulatory response (11.00 $\pm$ 0.63 CL) and embryo yields comparable to the conventional method of superstimulation, but with a lesser degree of variation between animals. Further, recovery of an increased percentage of transferable quality and better developed embryos than the conventional method was evident. This led us to suggest that the variability and unpredictability of results inherent in the superovulation of cattle would be reduced by initiating the FSH treatment two days after the GnRH induced follicular wave emergence which might be attributed to a greater homogeneity of healthy oestrogenic follicles, complete luteolysis and conducive endocrine environment at the time of superovulatory oestrus supporting ovulation of healthy follicles.

With the reduced dose of FSH (200 mg) in Gn-D10 group, all the animals responded with a mean superovulatory response of 6.33 $\pm$ 0.99 CL (4-11) and an average of 44.24 per cent embryos / ova were recovered. When compared with Gn-D8-400 group, a non-significant increase in superovulatory response and embryo recovery rate was recorded in Gn-D10-200 group. Further studies are required with more number of animals to establish the potential of reduced dose of FSH in inducing superovulation after synchronized follicular wave emergence.

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