EFFECT OF INCREASED DOSES OF FINAL GONADOTROPIN-RELEASING HORMONE (GnRH) OF OVSYNCH ON LUTEINIZING HORMONE (LH) PEAK AND CORPUS LUTEUM (CL) FUNCTION IN LACTATING DAIRY COWS AND BUFFALOES

By

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ABSTRACT

EFFECT OF INCREASED DOSES OF FINAL GONADOTROPIN-RELEASING HORMONE (GnRH) OF OVSYNCH ON LUTEINIZING HORMONE (LH) PEAK AND CORPUS LUTEUM (CL) FUNCTION IN LACTATING DAIRY COWS AND BUFFALOES

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Fertility of dairy cows has been decreasing with a corresponding increase in milk productivity. Synchronization protocols using gonadotropin releasing hormone (GnRH) and prostaglandin F2 alpha (PG) have been designed to synchronize ovulation to allow timed artificial insemination (AI). To tackle challenges of lesser concentration of steroid hormones in higher producing cows, studies focused on increasing steroid hormones to imitate that of heifers have been reported. The objective of this thesis was to assess the effect of increased doses of final GnRH of Ovsynch in dairy cows on the LH peak and CL function [CL size and progesterone (P4) secretion]. Dairy cows (N=70) were enrolled in an Ovsynch protocol. Selected cows (n=24) were divided to receive three different doses of final GnRH (100 vs 200 vs 400 µg) of Ovsynch followed by timed artificial insemination (TAI). The LH peak and P4 per CL volume of the three treatment doses were not significantly different ($p > 0.05$) except for P4 per CL volume of 200 vs. 400 µg on day 7 ($p = 0.04$). Similarly, in n=6 buffaloes, two doses (100 vs 400 µg) of final GnRH was used in Ovsynch. There was no significant difference in P4 per CL volume ($p > 0.3$) post treatment between the two treatment doses. Due to unforeseen shortcomings, our data is not adequate to conclude the effect of greater GnRH dose on CL function in cow and buffalo and we suggest further studies on GnRH doses in cow and buffalo synchronization protocols.

This thesis is dedicated to my daddy who had this dream for me. I see him smiling through the stars.

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KEY TO ABBREVIATIONS

AI artificial insemination

CIDR controlled internal drug release device i.e., progesterone intravaginal insert

CL corpus luteum/corpora lutea

CR conception rate

CV coefficient(s) of variation

d day (s)

DF dominant follicle(s)

DIM days in milk

DMI dry matter intake

E2 estradiol

eCG equine chorionic gonadotropin

ELISA enzyme-linked immunosorbent assay

FSH follicle stimulating hormone

FTAI fixed-time artificial insemination

GnRH gonadotropin releasing hormone

h hour (s)

hCG human chorionic gonadotropin

LH luteinizing hormone

LLC large luteal cell (s)

mL milliliter

ng Nano gram

P/AI pregnancy per artificial insemination

P4 progesterone

PG prostaglandin F2α

RIA radioimmunoassay

TAI timed artificial insemination

CHAPTER 1

LITERATURE REVIEW

Section 1: Historical dairy industry production trends in the US

The US cattle population has steadily decreased since 1980 but production of milk during that period increased, an indicator that the milk productivity of dairy cattle is increasing (Figure 1.1).

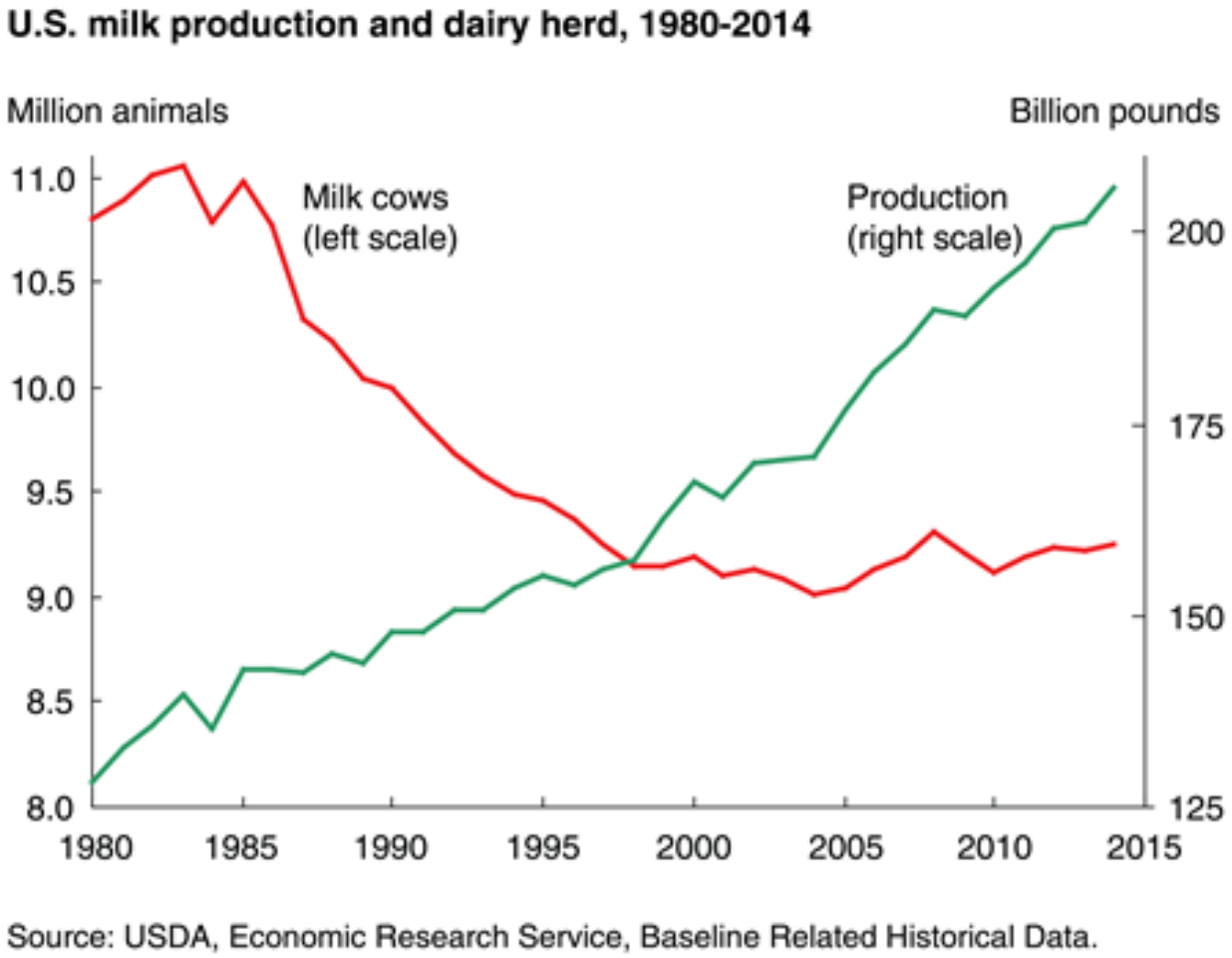

Figure 1.1 US cattle population and milk production trends

Fertility declined over the past 50 years and is directly associated with the increase in milk production per cow. Reduced fertility is marked by increased calving interval, increased duration from calving to conception, and increased number of inseminations required for a conception. A negative genetic correlation between production and reproduction was reported (Lucy, M.C. 2001).

Many researchers have reported this decreasing trend in reproductive efficiency over the last five decades. The pregnancy per artificial insemination (P/AI) of lactating cows decreased from 66% in 1951, to around 50% in 1975 (Spalding et al., 1975). In 1980, P/AI had reportedly gone down to 40% (Butler and Smith, 1989). In 2000, it was shown that P/AI was 45% for cows inseminated at spontaneous estrus and 35% for cows receiving timed artificial insemination (TAI) (Lucy, M. C. 2001). The major cause of this decline is selection of higher producing cows rather than the moderately yielding cows of the 1980s (Diskin et al., 2006). In Holsteins, a calving rate to first service of 55% was reported in 1990 whereas it was reportedly 44% in 2001 (Evans et al., 2007). Reproductive inefficiency in present day cows is marked by decreased expression of estrus, increased diameter of the ovulatory follicle and reduced fertility when cows are inseminated after estrus as well as increased incidence of double ovulation and twinning and increased pregnancy loss.

Dairy cows with greater milk production have lower circulating steroid concentrations compared to heifers although they have larger dominant follicle (DF) and corpus luteum (CL) diameters. There is also decreased expression of estrus in high producing dairy cows, like Holsteins where duration of estrus was inversely proportional to milk production (Lopez et al. 2004). The estrus duration was 6.2 ± 0.5 h in high production Holsteins (>39.5 kg/day) versus 10.9 ± 0.7 h in low milk producing Holsteins (<39.5 kg/day). A possible explanation for this mechanism of lesser steroid concentrations and decreased expression of estrus in higher producing cows is greater blood flow through liver and increased steroid metabolism by the liver (Sartori et al. 2004; Sangsritavong et al. 2002). Greater blood flow is likely the result of increased feed intake in higher producing lactating cows in comparison to nulliparous heifers (Wiltbank et al. 2000; Wolfenson et al., 2004;

Wiltbank et al., 2006). A negative relationship between dry matter intake (DMI) and progesterone (P4) concentrations was reported (Sangsritavong et al., 2002).

Section 2: Estrous cycle in cows

The normal length of an estrous cycle of a cow is of 21 days (17-24 d). Gonadotropin releasing hormone (GnRH) secretion by the hypothalamus is necessary for the normal estrous cycle to occur (Silverman et al., 1987). Synthesis and release of GnRH is controlled by neurons in the hypothalamus, and these are regulated by steroid reproductive hormone concentration in circulation. GnRH and its agonists induce acute secretion of pituitary gonadotropic hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Thatcher et al. 1993). The concentration of FSH and LH in blood remains elevated for 3-5 hours upon injection of exogenous GnRH or its agonists. FSH and LH are required for normal luteal development and function (Juengel et al., 2000).

Estrous cycle in cows occur in wave. Each wave starts with a cohort of follicles recruited and growing in response to FSH. At day 4, one follicle undergoes deviation and other subordinate follicles start to become atretic. The deviated follicle acquires LH receptors and continues to grow as a dominant follicle in response to increase pulses of LH. A dominant follicle will ovulate in response to an LH surge. Cows reportedly have one to four follicular waves in one estrous cycle, where two and three waves are more common (Ireland et al., 2000). Heifers primarily have three follicular waves (Sirois & Fortune, 1988). A strong correlation between fertility and follicle life span and its physiological dominance was reported. The interval from follicle wave emergence to estrus was one day shorter in pregnant cows than in non-pregnant cows, regardless of the number of waves per cycle in cows undergoing spontaneous estrous cycle (Bleach et al., 2004).

FSH is released by the anterior pituitary in response to the effect of either exogenous or endogenous GnRH on the hypothalamic/pituitary axis. FSH is required for the growth of follicles before deviation. Follicles undergo deviation around 2.8 days after a new wave emergence when the future dominant follicle (DF) is around 8.5 mm in diameter (Ginther et al., 1996; Ginther et al., 1998). At the point of deviation, DF starts to dominate with estradiol (E2) production, has accelerated growth (Ginther et al., 1996; Ginther OJ., 2000) and produces inhibin. Inhibin and E2 act on the anterior pituitary to inhibit FSH release and secrete LH. This process is also known as the negative feedback mechanism. Thecal cells of a DF produce androgens, which in turn, are aromatized to E2 by granulosa cells (Niswender et al., 2000). E2 is also required for expression of behavioral estrus in cows. LH pulse frequency helps growth and maintenance of the DF (Hansel and Convey, 1983) whereas other follicles, which are FSH dependent, show growth retardation and finally undergo atresia.

There are no LH receptors expressed by granulosa cells in the first two days of a follicular wave. Follicles acquire LH receptors during the deviation process (Ginther et al., 1996) which are required for ovulation (Xu et al., 1995). A dominant follicle takes seven to ten days to go through emergence, deviation and dominance (Ginther et al., 1989). LH pulses are negatively associated with concentrations of progesterone (P4). At a threshold concentration of E2, with lesser concentrations of P4, GnRH sequentially increases the magnitude and frequency of LH pulse secretion. Increased pulse frequency and magnitude in response to increasing E2 from the DF eventually causes a pre-ovulatory LH surge (Hansel and Convey, 1983). Behavioral estrus is observed during this time due to the effects of E2. LH acts on the ovulatory follicle leading to its ovulation and formation of luteal tissue. The LH surge was observed 43.6 ± 1.6 h (mean ± SEM) after induction of CL regression in heifers (Algire et al., 1992). Induction of ovulation of the DF

is possible before atresia (Silcox et al., 1993) by the use of exogenous GnRH or its agonists and it was reported that ovulation occurred between 24-32 h after GnRH injection in a synchronization protocol, Ovsynch that was developed by Pursley et al. in 1995.

The residual follicular cells differentiate into luteal cells forming corpus luteum (CL). The corpus luteum is comprised of two types of steroidogenic cells. One is large luteal cells (LLC), which are derived from the granulosa cells of the follicle and the second type of cells is small luteal cells (SLC), which are derived from the theca externa of the follicle. SLC and LLC differ in their basal rates of secretion of P4. LLC produce 2-40 fold more P4 than unstimulated SLC. P4 production from LLC accounts for 80% of the total P4 production of the CL in mid-cycle ewes (Niswender et al., 1985). In the case of stimulation of luteal cells by LH, LLC are less responsive to LH stimulation in comparison to SLC (Hansel et al. 1987; Alila et al. 1988). Apart from the steroidogenic cells, the CL also contains endothelial cells, fibroblasts, pericytes, and cells originating from the bloodstream. LH also has a luteotropic effect, acting on steroidogenic luteal cells to cause an increase in P4 concentration in blood.

Steroidogenic cells of a growing CL produce P4, a hormone of pregnancy (Spencer and Bazer, 2002). In Holstein cows, those with greater fertility had 41% greater CL volume and 79% greater mean circulating P4 in comparison to those with lower fertility (Moore et al., 2014).

E2 and LH secretion and concentration are inter-dependent in that the concentration of one affects the secretion of the other. The pulsatile release of LH during the dominance phase of the follicular wave is required for the growth of follicle and E2 production mainly from the granulosa cells of the dominant follicle. In turn, the intensity of luteinizing hormone secretion is dependent on steroid concentrations. P4 and E2 levels regulate LH secretion either by indirect regulation of hypothalamic GnRH secretion or by enhancement or suppression of effect of GnRH on the

pituitary (Rispoli and Nett, 2005). E2 works both ways in regulation of LH. First, it enhances hypothalamic GnRH secretion leading to GnRH surge and finally LH surge. Secondly, E2 secreted from the pre-ovulatory follicle upregulates GnRH receptor mRNA and protein in gonadotrophs which results in greater sensitivity of the anterior pituitary to GnRH (Schoenemann et al., 1985a). However, presence of P4 leads to blockage of stimulatory effects of E2 in the hypothalamus (Schoenemann et al., 1985b; Girmus and Wise, 1992) as well as in the pituitary (Baratta et al., 1994; Rispoli and Nett, 2005).

Failure of ovulation of the dominant follicle may be due to: a- insufficient release of LH in early post-partum stage (Beam and Butler, 1999) or b- downregulation of LH receptors in the follicle and decreased expression of enzymes in the steroidogenic pathway in response to stress hormones. Insufficient LH resulted in insufficient E2 production and subsequent atresia of the DF from the first follicular wave in post-partum beef and dairy cows (Crowe MA, 2008). It was suggested that a GnRH agonist given at the time of the endogenous pre-ovulatory LH surge amplified plasma LH and affected the process of oocyte maturation (Thatcher et al. 1993). A prior exposure to P4 also helps induce the GnRH/ LH surge by E2, triggering ovulation of the DF (Gumen and Wiltbank, 2002).

Section 3: Use of GnRH prior to introduction of Ovsynch

GnRH is a deca-peptide which has a half-life of approximately two minutes. It stimulates the release of gonadotropins from the anterior pituitary. This deca-peptide was first isolated from porcine hypothalamus and it was reported to have stimulatory effect on the release of both, luteinizing hormone and follicle-stimulating hormone from the pituitaries of many species. The structure of the deca-peptide has been identified as (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH(2). Synthetic GnRH which have biological properties similar to natural GnRH are

also in use. Synthetic GnRH has a longer half-life in comparison to natural GnRH. Buserelin and desorelin are synthetic GnRH analogues that replace Gly with a hydrophobic amino acid [D-Ser(tertButyl)] to increase stability. The first use of GnRH reported in a study was three repeated doses of 100 µg of GnRH in cows with follicular cysts which resulted in LH surge and initiation of an estrous cycle in the cows (Kittok et al., 1973). Later, 50, 100 and 250 µg doses of GnRH were used in cows with cysts in a study that indicated luteinization of those follicular cysts occurred after GnRH treatment (Cantley et al., 1975; Bierschwal et al., 1975). Cystorelin (Gonadorelin diacetate tetrahydrate) was approved under the Generic Animal Drug and Patent Term Restoration Act 53 FR 50460 on December 15, 1988. It was the first approved GnRH with a single dose of 100 µg indicated for treatment of follicular cysts. Since then, FDA approved the first GnRH product gonabreed (manufactured by Parnell Technologies Pty. Ltd.) to use with PG in synchronization protocols in cows in 2013. Gonabreed contains gonadorelin acetate. Interestingly, very little work was published with respect to the most effective dose of GnRH or its analogues. Chapter 2 of this thesis tests GnRH to determine if post-ovulation P4 is altered by its dose.

Administration of GnRH early post-partum resulted in initiation of ovarian cyclicity and a decrease in the voluntary waiting period (VWP) (Zaied et al. 1980). GnRH administered early post-partum (10 to 18 days) resulted in increased conception rate, decreased interval from calving to conception and fewer services per conception in dairy cows (Lee et al. 1983). Doses of 5, 40 and 320 µg of GnRH administered in heifers resulted in greater LH peak with 320 µg than 5 or 40 µg (Zolman et al., 1974). In repeat-breeding cows, administration of GnRH at estrus helped increase serum LH concentration where LH peak induced by 250 µg was greater than 100 and 50 µg of GnRH. P4 however remained unaffected with increased dose of GnRH (Mee et al., 1993). GnRH has also

been used either at the time of AI or post-AI to try to improve conception rates. In a study with dairy cows, buserelin, a GnRH analogue, given at day 12 post-insemination had no significant effect on conception rates (Drew and Peters, 1994; Thatcher et al., 1993). In another meta-analytical study by Morgan and Lean (1993), a greater (0.25 mg) dose of GnRH at the time of artificial insemination improved fertility in comparison to a lesser (0.125 mg) dose.

Section 4: Studies on strategies to increase fertility

Over time, researchers have come up with different techniques to enhance fertility. The most common markers used to indicate better fertility in a herd are increased conception rates, or reduced rates of pregnancy loss. Management of reproduction in dairy, as well as beef cattle, has been revolutionized with the use of exogenous hormones such as GnRH, PG, E2, P4, hCG, eCG, porcineLH, etc. These hormones are administered to synchronize certain stages of the estrous cycle such as estrus or ovulation. Estrus synchronization techniques rely on the use of PG to control CL development. The Ovsynch synchronization method uses GnRH and PG to control ovulation (Pursley et al. 1995).

Estrus synchronization

One of the most widely used techniques to synchronize estrus is by administering two doses of PG 11-14 days apart. This technique was practiced much earlier than ovulation synchronization in dairy herds. This protocol requires the cows to be observed for signs of heat over a seven-day period to perform AI. This method does not synchronize the growth of follicles, it only regulates the lifespan of a CL (Hafs and Manns, 1975; Larson and Ball, 1992; Lucy and Stevenson, 1986; Momont and Seguin, 1983). This PG-only technique is not useful in anovulatory cows as PG has no role in initiating follicular development and resumption of cyclicity. Apart from this, PG synchronization is a labor intensive and time-consuming technique. Fixed time AI with this

method at 72 to 80 hours after the second PG was tested and it resulted in a lower conception rates in comparison to the P/AI of animals receiving AI after detected estrus (Stevenson et al., 1987).

Ovulation synchronization

Ovulation synchronization is a precise technique designed to synchronize ovulation itself. It has an advantage over estrus synchronization techniques as it eliminates the need of estrus detection and allows AI at a fixed time (Pursley et al., 1995). Ovsynch is, by far, the most widely used synchronization protocol by US dairy producers (USDA 2009) and by cattle producers worldwide. Various studies have been conducted to improve the effectiveness of Ovsynch. In this protocol, GnRH is used in addition to PG, which comprises of 100 µg of 1st GnRH (G1) given at a random stage of the estrous cycle, followed by 25 mg of natural PG (dinoprost) or 500 mcg synthetic PG (cloprostenol) seven days later. Forty-eight hours after PG injection, a final dose of 100 µg GnRH (G2) is given. G2 induces an LH-surge, allowing AI to be performed within 16-20 hours after G2 administration (Figure 1.2). Since ovulation was found to be synchronized in an 8-hour period i.e. 24 to 32 hours after G2 (Pursley et al., 1995; Pursley et al., 1998), AI was timed at 16 to 20 hour after G2. Ovsynch was effective in synchronizing 100 % of cows but only 75 % of heifers. Fertility of lactating dairy cows following Ovsynch varied from 27-39 % P/AI (Pursley et al., 1995; Burke et al., 1996; Pursley et al., 1997a; Pursley et al., 1997b; Pursley et al., 1998).

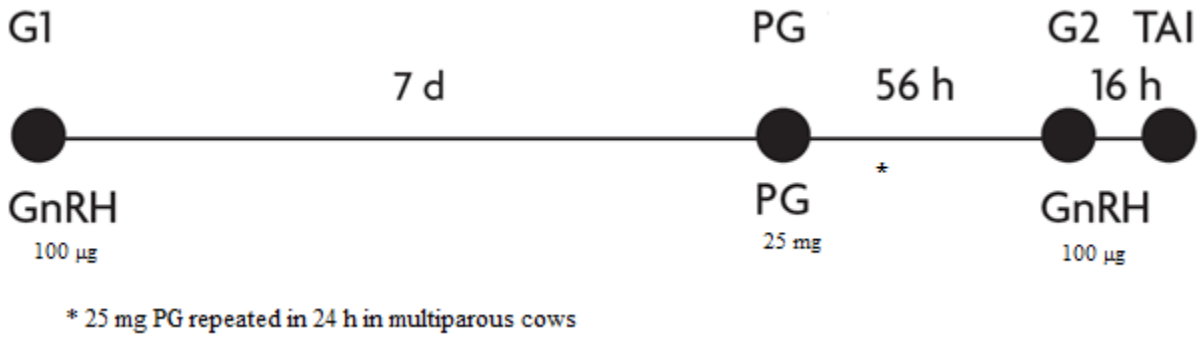

Figure 1.2 Ovulation synchronization to timed artificial insemination protocol

Many factors such as age, lactation and nutrition are responsible, at least in part, for the difference in response to Ovsynch between cows and heifers. Heifers treated with Ovsynch had reduced P/AI in comparison to primiparous and multiparous cows (Pursley et al., 1995). P/AI with Ovsynch was found to be similar to P/AI using estrus synchronization technique with AI done after heat detection in parous cows (Pursley et al., 1997a). Ovsynch resulted in greater pregnancy rates when carried out in animals beyond 60 days postpartum. Ovulation synchronization programs have an advantage in anovular cows as more cows ovulate after Ovsynch in comparison to anovular cows having spontaneous ovulation (Gumen et al., 2003).

Success of this synchronization protocol greatly depends on the response to the first GnRH (G1). A new wave is initiated within 2 days after ovulation to G1 (Pursley et al. 1995). If ovulation occurs in response to G1, then the resulting CL undergoes luteolysis as a result of PG injection given 7 days later, then followed by administration of G2 and fixed-time AI 2 days later, more specifically, 16-20 h after G2. Failure to ovulate in response to G1 resulted in a shorter interval from G2 to ovulation, and fewer synchronized cases, resulting in a smaller ovulatory follicle that, upon ovulation, turns into a small CL (Vasconcelos et al., 2001). Also, greater serum concentrations of E2 at 56 h after PG in Ovsynch was found to be a positive indicator of pregnancy (Martins et al., 2011).

Luteolysis is important to increase conception using FTAI in a synchronization protocol (Brusveen et al, 2009). A negative effect of P4 at time of GnRH treatment was reported on GnRH-induced LH surge (Colazo et al., 2008; Dias et al., 2010). For Ovsynch to be successful, P4 at G1 should be usually above 1 ng/mL (Stevenson et al., 1999; Bello et al., 2006; Giordano et al., 2012) whereas at G2, P4 is lesser than 0.5 ng/mL (Brusveen et al., 2009; Giordano et al., 2012). Lack of complete luteolysis suppresses the GnRH induced LH surge, and reduces fertility in synchronization

protocols (Brusveen, et al. 2009; Martins et al. 2011; Giordano, et al. 2012; Pulley, 2015). A reduced rate of luteolysis in response to a single dose of PG was reported in multiparous lactating cows in comparison to primiparous cows (Giordano et al., 2012; Martins et al., 2011b). Administration of two doses of PG (dinoprost tromethamine) at 24 hour interval in multiparous cows helped achieve complete luteolysis in those cows (Brusveen et al., 2009). It was also reported that a greater dose of PG (cloprostenol; 500 vs. 750 mg) was effective for luteal regression in multiparous cows (Giordano et al., 2013).

The key principle of synchronization by Ovsynch is the precise control of the LH surge and ovulation time. Ovulation was found to be synchronized in an 8 hour period i.e. 24 to 32 hours after G2 (Pursley et al. 1995). Timing for the final GnRH (G2) administration was proposed as 36 to 48 h after PG injection (Peters and Pursley, 2003). Later studies proved that a 56-hour interval was more precise to synchronize ovulation of the DF (Brusveen et al., 2008; unpublished data from Dr Pursley).

Ovulation synchronization rate in Ovsynch was reported as 80-90% (Peters and Pursley, 2002). Ovulation synchronization rate was greatest when Ovsynch was initiated mid-cycle. Ovsynch started at day 5 to 9 of estrous cycle had more cows (>90%) ovulate after the administration of G1. Starting Ovsynch mid-cycle resulted in smaller ovulatory follicles but greater pregnancy rates (Vasconcelos et al., 1999). Bello et al. (2006) found 85% ovulation rate to G1 when Ovsynch was started on day 6. Vasconcelos and associates reported a 91% synchronization rate when Ovsynch was started at day 1-12 of the estrous cycle and 80% when Ovsynch was started at day 13-22. Pre-synchronization before Ovsynch enables Ovsynch be initiated at days 5 to 12 of the cycle.

Different pre-synchronization protocols have been developed and tested over time and all those protocols have increased P/AI in comparison to Ovsynch alone. Presynch/ Ovsynch, Double Ovsynch and G6G are widely used pre-synchronization/ Ovsynch protocols.

Presynch/Ovsynch

Pre-synchronization with two injections of PG 14 d apart done 12 days before Ovsynch increased fertility in cycling cows but no effect was seen in anovulatory cows (Moreira et al., 2001). Greater fertility in response to such pre-synchronization scheme was reported in multiparous but not in primiparous cows (Cartmill et al., 2001). Presynch-10 or 11 are pre-synchronization protocols which use two injections of PG given 14 day apart followed by Ovsynch 10 or 11 days later. This technique is suitable to increase fertility in cycling cows. P/AI in presynch 10 was 45% in comparison of 31% in estrus detection and TAI (Strickland et al., 2010).

Double Ovsynch

Double Ovsynch uses two Ovsynch protocols carried out back to back, with a seven-day interval in between. The first is the pre-synchronization Ovsynch (Ovsynch 1), and the second is the breeding Ovsynch (Ovsynch 2). In comparison to presynch 12-Ovsynch, Double Ovsynch resulted in increased P/AI of 65.2% vs. 45.2% in primiparous (Souza et al., 2008), and induced ovulation in noncyclic cows, and had better synchronization in cyclic cows in comparison to presynch-Ovsynch (Herlihy et al., 2012).

G6G

Cows are treated with a PG injection at a random stage followed by a GnRH injection after two days. Six days later the Ovsynch protocol is carried out. This protocol gave a greater rate of ovulatory and luteolytic response in comparison to simple Ovsynch (Bello et al., 2006). A less than six-day interval between pre-G and G1 of Ovsynch resulted in insufficient time for deviation

and acquisition of LH receptors by the growing follicle. This study also revealed that the concentration of P4 during PG injection and the concentration of E2 and follicle size at G2 of Ovsynch are important indicators of the probability of pregnancy at 35 days post AI.

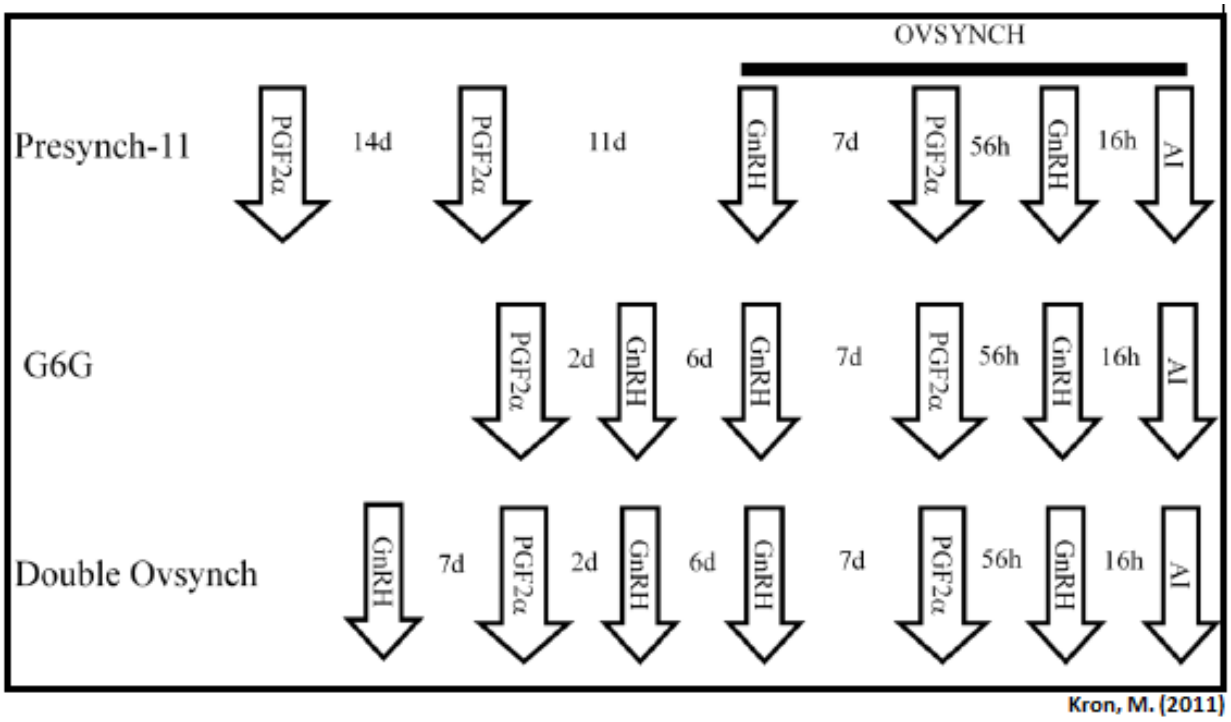

Figure 1.3 The three most common Ovsynch programs currently in use in U.S.

Greater GnRH doses used in synchronization protocols

The first study that used 50, 100 and 250 µg doses of GnRH in cows with cysts reported luteinization of those follicular cysts after GnRH treatment with no significant difference among the treatments (Cantley et al., 1975; Bierschwal et al., 1975). A higher dosage of GnRH (200 µg vs. 100 µg) resulted in greater plasma luteinizing hormone concentrations in beef heifers (Dias et al. 2010) and beef cows. Giordano et al. (2012) reported a greater LH peak with 200 µg of GnRH in comparison to 100 µg in both, greater and lesser P4 environment. Souza et al. (2009) also reported a greater LH peak with 100 µg, compared to 50 µg, in a study with lactating cows. However, in a study with cyclic dairy heifers, an increase in the dose of gonadorelin from 0.1 to

0.5 mg had no effect on the occurrence of induced short estrous cycles or on the LH response (Rantala et al., 2009). Yamada and colleagues (2002) also reported no significant difference in LH peak, conception rate, ovulation synchronization, and CL function with 50 and 100 μg of GnRH analogue, fertirelin. (Yamada et al., 2002). In a study in llamas, it was reported that reducing the GnRH dose from 50 to 6.25 g resulted in a progressive decrease of LH released from the pituitary gland. However, CL development, or P4 secretion from llamas that ovulated was unaffected (Silva et al, 2012).

Ferguson et al. (2012) reported that increasing the dose of GnRH to 200 μg increased pregnancy rates in *Bos indicus* and *Bos taurus* cows But pregnancy rates were not increased in *Bos taurus* heifers with increased GnRH dose study with co-synch+CIDR. However, Fricke et al., 1998 found that different doses of GnRH (100 μg vs. 50 μg) in lactating dairy cows resulted in similar conception rates.

Another study conducted in lactating dairy cows by Giordano et al. (2013) reported that 200 μg of G1 of Ovsynch resulted in a greater number of ovulations in comparison to 100 μg in cows with greater P4 concentrations at the time of GnRH injection. However, only marginal effects of increased doses on fertility were found.

Section 5: Progesterone and fertility

Progesterone is considered a key component of studies aimed at fertility improvement in cattle. Its concentration both pre and post AI have significant effect on reproductive parameters.

Effect of progesterone pre AI

A greater P4 environment at the time of G2, adversely affected GnRH-induced LH surge (Colazo et al., 2008; Dias et al., 2010, Giordano et al., 2012). It was speculated that P4 at the time of GnRH treatment resulted in a decrease in pituitary GnRH receptors, as occurs during the luteal phase

(Rispoli and Nett, 2005), and that an association exists between LH peak and pituitary GnRH receptors after GnRH treatment (Wise et al., 1984). Suppression of LH, due to greater P4 concentration, might be either due to a decreased number of GnRH receptors, decreased responsiveness within pituitary cells to GnRH receptor activation, or decreased quantity of releasable LH present in the pituitary. However, double doses of exogenous GnRH resulted in greater LH release in high as well as low P4 environment (Giordano et al., 2012). Also, GnRH itself regulates GnRH receptors agonistically (Nett et al., 2002). One study in sheep showed results where LH suppressed by P4 was not associated to GnRH receptors (Girmus et al., 1996). Cows that received CIDR (controlled internal drug release device) in a synchronization protocol had fewer ovulations of the ovulatory follicles in response to GnRH (Galvao et al., 2004). During Ovsynch , P4 concentration at G1 is greater in comparison to P4 concentration at G2 (Stevenson et al., 1999; Stevenson J.S., 2008; Bello et al., 2006; Brusveen et al., 2009; Giordano et al., 2012). P4 supplementation with CIDR insert, which has 1.38 g P4, is commonly used. CIDR is an intravaginal device that releases small amounts of P4 over a period of time. Such P4 supplementation has been demonstrated to regulate LH pulse frequency, and lactating cows treated with CIDR during the interval between G1 and PG injection of Ovsynch, had an increase in DF diameter (Stevenson et al., 2006).

Effect of progesterone post-AI

In 1983, it was first reported that lactating dairy cows with greater P4 in circulation have greater chances of pregnancy (Fonseca et al., 1983), and P4 resulted in an improved conceptus growth and development (Forde et al., 2009). Bisinotto and colleagues (2010) found that P4 concentrations and fertility were positively correlated. Elevated P4 concentration post-AI was proven to accelerate conceptus elongation. This, in turn, resulted in more interferon τ production, known to help in

maternal recognition of pregnancy (Lonergan and Forde, 2015). Beltman et al. (2009a) also reported a correlation between day 5 and 6 P4 concentration and day 16 conceptus size. In the first week after conception, P4 plays an important role in maintaining an optimum uterine environment that supports the process of conceptus elongation, which is around the time of maternal recognition (Clemente et al., 2009). It was also found that the probability of P/AI was greater when P4 was 2.5 - 5.2 ng/mL on day 4 to 7 (Parr et al., 2012).

P4 supplementation during synchronization followed by TAI, had effects on ovulatory follicle size, and periovulatory circulating P4, and E2 concentrations. However, postovulatory P4 or luteal volume remained unaffected (Herlihy et al., 2012). Another study reported that supplementation with exogenous P4 at different stages of embryo development increased ($P < 0.05$) peripheral P4 concentrations, without any effect on embryo survival rate. Also, supplementation failed to have any effect on either CL weight or conceptus size in pregnant animals (Beltman et al., 2009a). In another study, P4 supplementation during early pregnancy resulted in a 5% increase in pregnancy rate, but the supplementation done after day 6 did not have any positive effect (Mann & Lamming, 1999). Thus, timing of exogenous P4 supplementation is also important for the early development of an embryo.

Attempts to supplement P4 by creating accessory CL, either by administration of GnRH analogue or hCG have had mixed results. Buserelin and hCG given on day 5 after insemination resulted in accessory CL formation and increase P4 concentration (Schmitt et al., 1996).

Lower P4 concentrations that are associated with greater milk production during the growth of pre-ovulatory follicle, results in increased chances of double ovulation and twinning, which is associated with pregnancy loss and reduced fertility (Wiltbank et al., 2000; Hayashi et al., 2008).

Serum P4 concentration is determined by rate of P4 secretion and P4 metabolism. Endogenous secretion of P4 depend on various factors which are explained below.

Factors affecting progesterone concentration

Blood supply to CL

It was reported that CL blood supply rather than CL size might be the determining factor for P4 production (Herzog et al., 2010). Greater blood flow delivered a greater amount of steroid precursors and gonadotropins to the ovary in rabbits (Janson et al., 1981). Blood circulation is also the delivery route for biological substances and nutrients necessary for P4 secretion from the corpus luteum (Acosta et al., 2002). Twenty-two percent of the total volume of the corpus luteum is the capillary lumina. The close positioning of the capillaries with the luteal tissues helps in fulfilling the greater metabolic need of the CL. The CL consumes two to six times more oxygen/weight than the liver, kidney or heart (Dharmarajan et al., 1985). Blood flow to the CL and P4 secretion/ concentration have a synergistic effect on each other. While it is believed that a greater blood flow to the CL ensures greater P4 secretion, blood flow to the corpus luteum increases as concentrations of P4 in serum increase. A supporting point for this theory of blood flow is the luteolytic process of the CL. It was reported that luteal blood flow decreases rapidly in response to the luteolytic action of PG produced by the endometrium, which takes about 48 hours (Acosta et al., 2002).

Size of ovulatory follicle

In ruminants, the diameter of the pre-ovulatory follicle had a positive effect on the CL size and P4 secretion (Busch et al., 2008). Similarly, a positive impact of follicle size at the time of G1 and G2 was also seen on the size and function of the CL, seen as significant difference in P4 concentrations and luteal size (Martins et al., 2011). Vasconcelos et al., 1999 reported that smaller follicles

resulted in decreased concentrations of circulating P4. However, pregnancy rate was less for cows expected to ovulate larger follicles (Vasconcelos et al., 1999). A greater pregnancy loss was detected with ovulatory follicles <15 mm diameter in comparison to the ones which were >15 mm (Stevenson et al., 2006). Likewise, follicles <11 mm resulted in reduced conception rates and increased embryonic mortality when those follicles were induced to ovulate (Perry et al., 2005). However, spontaneous ovulation of similar sized follicle did not compromise the conception rate, probably because they are physiologically mature. However, that is not the case when ovulation is induced with exogenous hormones such as GnRH on such follicles. When exogenous E2 is given to ovulate medium follicles (15-19 mm), there is an increased percent of pregnancy in first service cows. A follicle larger than 19 mm would be aged, and granulosa cells would have less steroidogenic capacity, compromising the oocyte quality.

Ovulation of medium size follicles led to a greater pregnancy rate (PR) in beef heifers (Perry et al., 2007). Improved fertility was achieved when the dominance phase of DF was less than 5 days (Unpublished data). A marked decrease in pregnancy rate was seen in a study where the dominance was longer than 9 days and highest fertility was achieved when duration of dominance was less than five days (Austin et al., 1999). Cows on day 5 had greater concentrations of estradiol, a steroid environment similar to heifers, which is related to improved fertility. In ewes, E2 synthesized by young ovulatory follicles contribute to an optimal environment for fertilization in the uterus and oviduct (Sangsritavong et al., 2002; Miller and Moore, 1976)

Effect of luteolysis

Luteolysis occurs in response to endogenous or exogenous PG on the CL. It results in loss of luteal cells and a drop of P4 concentration. E2 then increases pituitary sensitivity to GnRH, which leads to increased LH release. Another study from our lab showed a time gap of 36 h from complete

luteolysis to LH surge. In another study, the interval between PG injection and ovulation was 100 h. So considering those two time frames, a 56 h interval between PG and G2 is used in the experiment described in Chapter 2. Our aim was to let the follicle grow for an optimum time, with P4 concentrations reduced to basal level.

LH peak

Luteinizing hormone is the most important luteotropic agent in different species when compared to prolactin, growth hormone and E2, (Niswender et al., 2000). Different studies that tested the effect of LH on luteal function have reported mixed results. It was reported that an increased LH secretion induced by exogenous GnRH may have a negative effect on the CL function, with a reduced P4 secretion (Colazo et al., 2009; Lucy and Stevenson, 1986; Rodger and Stormshak, 1986). However, other studies reported a positive effect of LH on luteal function (CL size and P4 concentration). Cows treated with 25 mg of porcine luteinizing hormone (pLH) developed a larger CL area, and greater plasma P4 concentration than those that were treated with 8 mg pLH (Ree et al., 2009). This result indicates that LH has a positive impact on P4 secretion. Also, in an in vitro study, treatment of bovine corpora lutea with LH increased the secretion of luteal angiogenic factors (Redmer et al., 1988), which results in greater blood flow in the CL and greater P4 concentration.

LH receptor

LH binds to its specific receptor on steroidogenic cells of the CL, which results in activation of the intracellular events in those cells leading to enhanced P4 secretion. Thus, it is speculated that there is a correlation between the number of LH receptors, and the biological function of the CL.

One study showed that ovine LH treatment in sheep led to a significant increase in the number of LH receptors initially, which then started to decline to pre-injection concentrations by 48 hours

(Suter et al., 1980). A greater degree of positive correlation between the number of receptors occupied by LH at 10 min and the number lost by 24 h was suggested. This observation suggests that loss of LH receptors is due to binding of LH to those receptors. Concentrations of LH do not affect secretion of P4 from human, bovine, or porcine LLC (Alila et al., 1988; Hansel et al., 1990). The fact that LLC do not seem to respond to different concentrations of LH might be due to limited LH receptors in each LLC.

Intracellular transport of cholesterol

It was proposed that LH increases steroid production by facilitating the transport of cholesterol through the cell, by Niswender et al. (2000). Also, the acute stimulation of steroidogenesis by protein kinase A and the acute inhibition by protein kinase C may be primarily due to alterations in intracellular transport of cholesterol (Wiltbank et al., 1993). Cholesterol is the base compound required for P4 secretion. A greater reserve of cholesterol in steroidogenic cells might lead to greater P4 secretion from those cells.

Differentiation of SLC to LLC

Conversely, it was suggested that LH, and/or hCG, can promote differentiation of small steroidogenic luteal cells into large steroidogenic luteal cells in ewe (Gamboni et al., 1984; Farin et al., 1988). LH is the one hypophyseal hormone required for the support of luteal development and function in normal cyclic ewes having an intact uterus. Even when a lesser dose of LH was used, steroidogenic and non-steroidogenic luteal cell types had increased volume and diameter indicating the trophic role LH played affecting all luteal cell types (Farin et al., 1990). Another study demonstrated that treatment with LH resulted in an increased P4 concentration in a study with ewes ($p < .05$). No association was found between the greater P4 concentration and change in mean cell volume or diameter in both small and large luteal cells. Summarizing these data, the

authors proposed that treatment of ewes with LH results in the conversion of small luteal cells to large luteal cells (Farin et al., 1988). However, no additional data is available to support this theory. In fact such cell conversion is highly unlikely when considering the origin of the two steroidogenic cells are different. LLC is derived from granulosa cells and SLC from theca cell.

Section 6: An insight to buffalo reproduction

Buffalo have been domesticated over several centuries, however, genetic manipulation (selection and crossbreeding) has been practiced only in recent decades (Sivarajasingam, S. 1987). Buffalo milk production is constrained by factors such as the animal's late age of sexual maturity and late age at first calving, the seasonal effect on the estrous cycle, poor expression of estrus (Perera, 2011) and longer gestation period. Likewise the conception rate is low, with a long calving interval (values), long dry period and long post-partum anestrous.

The problem of post-partum anestrous is more prevalent in buffalo, which are malnourished and have poor body condition (Baruselli et al., 2001), increased partitioning of energy for milk production (Rhodes et. al., 2003), suckling management (Perera, BM. 1987) and climate (Nanda et al., 2003). Many technologies to induce estrus and ovulation during post-partum anestrous have been used in cattle, but not as much in buffalo. This means that the problem in buffalo could be solved with subsequent research to find the best option to mitigate anestrous, start the estrous cycle and even synchronize ovulation in buffalo. Many aspects of reproduction in buffalo have not been studied much so an intervention in buffalo reproduction maybe able to overcome such drawbacks.

Difference between cattle and buffalo reproduction

Studies involving reproduction improvement have been conducted more elaborately in cows than in buffalo. Nevertheless, the results of those studies can be used as base information in order to design reproductive management programs in buffalo. Cattle (*Bos* spp.) are related in some ways

to buffalo (*Bubalus* spp.). They are both large ruminants raised for mainly milk and meat depending on location. Cattle farming is more widespread than buffalo farming. Buffalo differ from cattle with respect to ovarian structure and follicle number. Buffalo have smaller ovaries than cattle and have fewer primordial follicles (Danell, B. 1987). There were 12,636 primordial follicles in cyclic buffalo heifers, which is less than the 150,000 primordial follicles reported in cattle (Erickson, B.H. 1966) and there are 10,132 primordial follicles in the non-cycling animals, with a range of 1,222 to 40,327 in an ovary pair. Histological evaluation of buffalo ovaries also indicated a small number of follicles (Danell, B. 1987). The percentage of atresia was 66.7% in Buffalo whereas it was 50% in cattle.

Concentrations of steroids is less in buffalo than in cattle owing to the smaller DF and CL. A small ovulatory follicle leads to less estradiol in circulation, and estrus signs are in turn dependent on estradiol concentration (Baruselli, 1997). The mean diameter of ovulatory DF was 10.78 ± 1.8 and 11.24 ± 2.15 mm in the right and left ovary, respectively, in Iraqi buffalo (Azawi et al., 2009); whereas ovulatory DF greater than 16 mm have been reported in cattle. In cattle, a DF size <15 mm has been associated with low fertility. Small follicle size and slow growth rate are the reasons behind silent estrous that compromises fertility in buffalo (Awasthi et al., 2007).

Protocols to increase steroid levels in buffalo are necessary.

Features and hurdles in buffalo reproduction

Reproduction in buffalo is also controlled by day length. A seasonal pattern of ovarian cyclicity is found among buffalo in subtropical countries whereas equatorial buffalo are capable of breeding throughout the year. The age at which a buffalo attains puberty ranges from 16 to 22 to 36 to 40 months in various countries (Borghese et al., 1994a). Under optimized conditions, the range goes down to 15 to 18 months in river buffalo (Borghese et al., 2005). It is expensive to rear an animal

for 3.5 years before it is ready for production. Cattle attain puberty at 1 year of age in developed countries whereas zebu cattle of Asian countries range from 16 and 40 months to reach puberty with an average of 25 months (Galina and Arthur, 1989). So even cattle production in developing and under-developed countries struggle with low fertility issues.

Estrous cycle in buffalo

Estrous cycles in buffalo range from 17 to 26 days with an average of 21 days. There is a greater variation in the estrous cycle of buffalo in comparison to that of cattle (Jainudeen and Hafez, 1993). The duration of estrus in buffalo ranges from 5 to 27 hours. Ovulation occurs between 24 to 48 hours (mean 34 hours) after onset of estrus; the estrus to ovulation interval is 6 to 72 hours (in short and medium estrus) after the end of estrus. The estrous cycle in buffalo consists of one to three waves, with two waves being the most common (Baruselli, 1997). The number of follicles recruited per wave is less in buffalo than in cattle (Adams et al., 1994; Rajakoski, 1960).

Majority of buffalo population exist in developing countries, where farming practices are conventional and buffalo are malnourished. The body condition score (BCS) adversely affects fertility of an animal (Baruselli, 2001) especially in heifers that have low BCS.

Weak signs of estrus and variability in estrous length makes estrus detection difficult in buffalo. So natural breeding is still preferred to artificial insemination (AI) in many developing countries (Barile, 2004). However, if ovulation is synchronized, greater percent of conception rate (CR) can be achieved in buffalo with timed AI in comparison to AI after heat detection.

Efforts to improve buffalo fertility

Synchronization protocols using hormones have been used to improve fertility in buffalo. Heat or estrus synchronization with two doses of PG given 14 days apart is not an efficient technique as it still requires heat detection for AI and buffalo have minimal heat signs. However, ovulation

synchronization protocols such as Ovsynch followed by timed AI can be an efficient tool in buffalo reproduction. A synchronization protocol like Ovsynch has helped to hasten puberty, increase fertility in low breeding season and allow use of timed-AI in buffalo because of difficulty in estrus detection. Baruselli et al. (2002) suggested that GnRH treatment inhibits estrus symptoms leading to silent estrus cases.

Ovulation synchronization in buffalo

AI to detected estrus is difficult in buffalo due to less pronounced heat signs and silent estrus. Estrus synchronization with timed AI is also not preferred because of variability in time to ovulation. Ovsynch which uses GnRH and PG injections to control follicle and CL development was introduced in cattle by Pursley et al. (1995). The same protocol has been used in buffalo with an advantage of heat detection in reproduction protocol not being required. Seasonal effects have been reported in buffalo fertility with synchronization techniques. Synchronization performed during marginal breeding activity or seasonal anestrous has had less success rate.

Ovsynch is not as effective in inducing estrus in non-cyclic buffalo as in cyclic buffalo, as it was seen to cause earlier onset of ovulation in acyclic buffalo in comparison to cyclic buffalo. The conception rates were found to be less (37.5% vs. 60%) in acyclic vs. cyclic buffalo. The reasons for the lesser conception rate were asynchronous ovulation and sub-luteal function. It was suggested that two inseminations at 0 and 24 hours might increase conception (Ali and Fahmy, 2007). Baruselli et al. (2003) used Ovsynch on buffalo in which he found that 60.6% of the animals ovulated to G1, 78.8% of the animals had synchronized ovulation and the conception rate to timed AI was 45.4%. They concluded that synchronization protocols used in buffalo in greater reproductive season gave satisfactory results. This was also proven in another study done by Barile et al. (2005). They also found that ovulation depends on follicle diameter at the time of GnRH

injection. The interval between GnRH injection and time of ovulation was 32 hours. Primiparous buffalo showed less response to the synchronization protocol than multiparous buffalo.

Modified versions of Ovsynch have also been studied in buffalo. A study on buffalo with Ovsynch vs. presynch-Ovsynch indicated no significant difference in conception rates. Greater concentrations of P4 at AI did tend to decrease chances of pregnancy (Oropeza et al., 2010).

Larger follicles gave rise to greater ovulation and pregnancy rates per AI following timed AI in buffalo synchronized with a progesterone releasing intra vaginal device (PRID; Monteiro et al., 2016).

LH given in place of G2 using Ovsynch-like protocol had similar results (Berber et al. 2002) in terms of ovulation synchronization and conception rates.

In a study comparing PRID with the Ovsynch protocol, no significant difference in fertility rate was reported (47.8% and 42.5% respectively). A greater conception rate was found in buffalo synchronized with PRID compared with Ovsynch, as PRID helped to synchronize acyclic animals in the non-breeding season (Barile et al., 2004).

LH peak-ovulation interval is less variable than estrus-ovulation interval in buffalo so LH peak-ovulation interval is more reliable in fixing time for AI. Ovsynch was efficient in synchronizing buffalo in breeding season, whereas PRID was efficient in all season (Barile, 2015).

Progesterone supplementation in buffalo

In buffalo, embryos arrive earlier in the uterus compared to cattle, resulting in a close relation between early embryonic events and the onset of progesterone secretion in buffalo. This can be an important aspect while determining a protocol to increase pregnancy rate by exogenous hormone supplementation in buffalo (Neglia, 2001).

P4 supplementation using PRID during the follicle development stage in Ovsynch has helped to synchronize acyclic buffalo. P4 supplementation by forming accessory CLs through use of hCG, buserelin and PRID was studied (Campanile et al., 2007). hCG and buserelin given on day 5 post AI helped increase day 15 P4 concentration. AI is favorable at 48 and 72 hrs after PRID removal in greater breeding season; 72 and 96 hours in low breeding season as seasonal variation in time of ovulation exists (Barile et al., 2001a; Barile et al., 2001 b; Barile, V.L. 2012).

Summary

Reproduction management in cows is important to maintain fertility while efforts to increase milk production are under way. Lower fertility has been associated with increased milk production that leads to decreased concentrations of steroid reproductive hormones- E2 and P4. P4 concentration pre- and post-AI are important for improving fertility. Synchronization protocols such as Ovsynch, which uses a combination of GnRH and PG injections, are widely being used to allow FTAI in dairy and beef cows. Methods to maintain or increase pregnancy rate while using synchronization protocols have been studied. Manipulations of the doses and timing of GnRH and PG in such synchronization protocols have been evaluated. GnRH is a preferred hormone in such protocols because of its efficacy, availability and cost effectiveness. Studies that deal with higher doses of GnRH have reported increased LH production and greater conception rate. To our knowledge, effect of a higher final GnRH dose on CL function has not been reported in any synchronization study. CL function is target for improvement as it is an indicator of reproductive potential of a cow. The experiment discussed in Chapter 2 deals with an increased GnRH dose at G2 of Ovsynch and its effect on LH peak and CL function. Chapter 2 discusses use of three different doses of GnRH in Ovsynch and we expect a greater LH peak and increased CL function as an effect of increased dose.

Likewise, field of buffalo reproduction has not been studied as widely as cow. However, study results of cattle reproduction can be used as a base for study to improve buffalo fertility. Fertility issues are more prevalent in buffalo. Apart from physiological factor affecting fertility, other factors such as breed, nutrition, and environment also adversely affect fertility in buffalo population. Hormonal intervention in reproduction management of buffalo is not as widely studied as cattle. Nevertheless, use of hormone as synchronization protocol can be beneficial in reproductive management in buffalo. Chapter 3 deals with use of two different doses of GnRH in Ovsynch in Buffalo to test any effect of increased GnRH dose on CL function.

CHAPTER 2

EFFECT OF INCREASED DOSES OF FINAL GONADOTROPIN RELEASING HORMONE OF OVSYNCH ON LUTEINIZING HORMONE PEAK AND CORPUS LUTEUM FUNCTION IN LACTATING DAIRY COWS

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Introduction

A paucity of information exists on the impact of the LH surge on post-ovulation P4 production of resulting corpora lutea. Most information on the relationship between LH and progesterone was studied during the estrous cycle (Niswender et al. 2000). Clearly, from many studies, LH directly affects production of P4 from small luteal cells (Ree et al., 2009). Seguin et al. (1976) tested increasing doses of GnRH on post-AI magnitude of the LH surge and post-AI progesterone production and found a positive effect of dose on magnitude of the LH surge and an increase of 2 ng/mL P4 in GnRH treated cows compared to saline treated controls. Cantly et al. (1975) tested increasing linear doses of GnRH in cows diagnosed with ovarian cysts and found no difference in post-treatment P4 compared to normally cycling controls. Bierschwal et al. (1975) followed up this study in the same laboratory with greater numbers of cows and determined that GnRH was effective in treating cows with ovarian cysts.

GnRH was first approved for use in the U.S. in 1988 for cattle. The approved dose was 100 µg. GnRH was initially approved to treat cows with cystic follicles based partially on the studies just mentioned (Kittok et al., 1973; Cantley et al., 1975; Bierschwal et al., 1975). Recently, GnRH was approved for use with PG products for synchronization of ovulation programs. The dosage for gonadorelin acetate remained at 100 µg, but gonadorelin hydrochloride was approved for a range of 100 to 200 µg per dose.

In essence, few GnRH dose studies have been published since the original studies in the 1970's. Fricke and Wiltbank (1998) reported no differences in pregnancies per AI in cows treated with 100 or 50 µg of gonadorelin acetate at the final GnRH of Ovsynch, but it was not clear if sufficient numbers of cows were utilized for this study. Giordano et al. (2012) had sufficient numbers of cows to test the effect of 100 vs. 200 µg GnRH and found no effect of dose on fertility.

With the advent of ultrasound it is clear that measurement of CL diameter and calculation of luteal volume could provide a more scientific look at the possibility of an effect of LH surge on post-AI P4 production per unit of luteal mass.

The objective of this study was to measure any effect of 100 vs 200 vs 400 µg of GnRH at final GnRH of Ovsynch on the LH surge, and CL size and progesterone concentration post ovulation. The hypothesis of this study was increasing doses of GnRH will increase LH peak but will not affect post-AI progesterone production of corpora lutea.

Materials and methods

This study was conducted from July – November, 2015 at the Michigan State University Dairy Teaching and Research station with lactating Holstein dairy cows (n=70) with parity ranging from 1st to 6th lactation. Cows were housed either in a free stall barn with individual cow bed and free access to water or in tie-stall barn with free access to water. Cows were fed twice daily with a TMR consisting mainly of corn silage, haylage, soybean meal, ground corn, cottonseed, straw, high moisture corn and vitamin/ mineral added. On the day of treatment cows were 141.5± 14 DIM and ranged from 69 to 286. Average milk production of the cows during the period of the experiment was 85.6 ± 4 lbs./cow/d ranging from 57 to 129 lbs./cow/d. The cows were milked twice daily. The research was conducted with approval from the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Ovsynch was used as synchronization protocol which was started with 100 µg of GnRH (gonadorelin diacetate tetrahydrate) (Cystorelin; Merial, Duluth, GA) followed by 25 mg of PG (Lutalyse; Zoetis, Florham Park, NJ) in 7 days; and 100 µg of GnRH 2 days later. A 56 h interval was maintained between PG and final GnRH (G2) injections. In addition, a CIDR device was inserted into resynch cows 1 d after 1st GnRH of Ovsynch and removed at PG of Ovsynch. Two

PG injections 24 h apart were administered to multiparous cows to ensure complete luteolysis. Only cows that ovulated to the first GnRH (G1) of Ovsynch were selected for this study to ensure all ovulatory follicles had similar antral follicle age at the time of treatment. The study was started with n=70 cows but only n=24 cows were selected and divided into three treatments: 100, 200 or 400 µg of G2 based on their parity and pre-ovulatory follicle diameter. All injections were given intramuscularly using 18-gauge needles for PG and 20-gauge needles for GnRH. All animals received AI 16-20 hours following treatment.

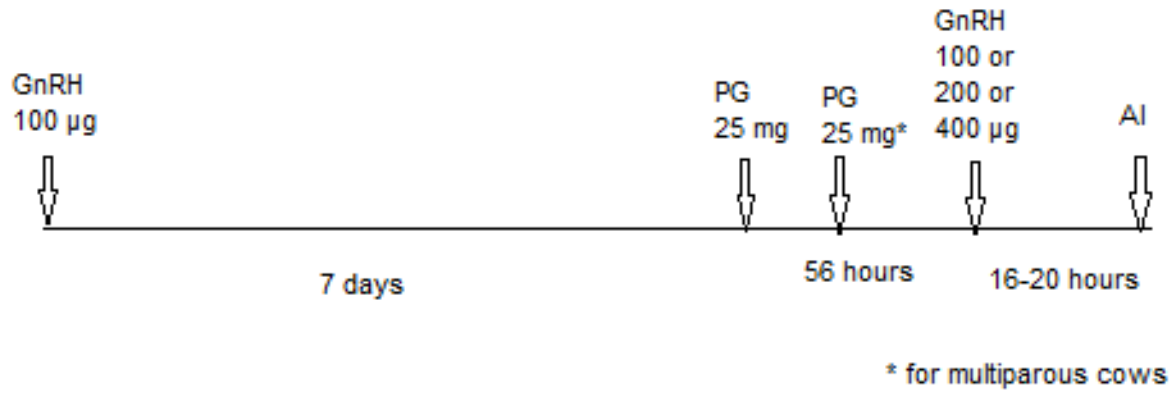

Figure 2.1 Ovsynch protocol used in the study

Blood collection

Blood was collected by coccygeal venipuncture throughout the study. Blood sampling was done using an 18 gauge needle, vacutainer and vacutainer tubes without anticoagulant (BD Vacutainer, Pre analytical Solutions, Franklin Lakes, NJ). The blood sampling days were days 0, 7, 10, 14, 17 where Day 0 is the day of treatment. The blood samples from day 7, 10, 14, 17 were used for analysis of post-AI progesterone concentration. On day 0, serial blood collection was performed in all cows, also by coccygeal venipuncture. Serial blood collection was started with the first blood sampling immediately before injection of G2 and blood was collected every 30 minutes up to 4 hours; a total of 9 samples was collected from each cow. The blood samples from the serial

sampling were used to determine LH surges in each animals. All samples were refrigerated overnight immediately after sampling. On the following day, blood samples were centrifuged at 2000 rpm for 20 minutes and the separated serum was stored at -20˚C until hormone analysis.

Ultrasound examination

MyLab^{TM}One VET ultrasound machine manufactured by Esaote with linear high frequency (up to 12 Mhz) transrectal transducer was used to perform ultrasonography of the ovaries of the cows. Ovaries were scanned to measure the number and size of the follicles and CL on (with respect to day of treatment) days -9, -7, -2, 0, 7, 10, 14, 17. The height and width of the follicle or CL was measured with the in-built calipers and an average of the readings was taken as diameter of the follicle or CL.

Cows were scanned on days -9 to check the presence and sizes of ovulatory follicles before giving G1; on day -7 to check if the ovulatory follicles previously scanned have ovulated in response to the G1; day -2 to check presence and size of CL before giving PG, day 0 to check if the CL have regressed in response to PG and to measure the size of pre ovulatory follicle. The cows were inseminated the following morning (Day 1). Cows were again scanned on days 7, 10, 14 and day 17 to measure the developing CL.

Lab assay for hormones

Concentrations of LH and P4 in serum were quantified by radioimmunoassay (RIA). Luteinizing hormone concentrations were determined in duplicate samples by RIA as described in Palhao et al. (2009) and modified as described by Hannan et al. (2010). The standard curve ranged from 0.08 to 10 ng/mL of LH. Therefore, samples with percent binding less than 10% due to high LH concentrations were diluted either 1:4 or 1:20 and reanalyzed to obtain percent binding in the 30 to 60% range which corresponded to 1.25 and 0.625 ng/mL standards. To assess the precision of

the 5 separate LH assays run, control samples with a low (0.3 ng/mL) and high (1.3 ng/mL) concentration of LH were evaluated in each assay. Average sensitivity of the assays was 0.12 ng/mL. For the low sample, the average intraassay CV was 5.5% (range = 1.34 to 9.09) whereas the interassay CV was 7.5%. For the high LH sample the average intraassay CV was 3.2% (range = 1.45 to 4.30) and the interassay CV was 5.8%.

Statistical analysis

SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used for analysis of data. Data were tested for normality. Normality was not met while considering all data, and since we had actual figures as data, no transformation was done. Rather, data were analyzed individually for specific days post treatment.

Proc mixed procedure was run separately for each data set to test any significant difference in effect of different doses of GnRH on CL volume, P4 level, P4 per unit CL volume and LH peak for different days post treatment. The area under the curve (AUC) of all treatments were measured by SAS AUC calculation by trapezoidal rule (Shiang, K. D. 2004).

Results

Out of n=70 parous cows enrolled in initial phase of treatment, only n=33 cows responded to G1 and PG and were divided into 3 treatments. However, data from one cow having an LH surge earlier than treatment, five cows with double ovulations and three cows with early CL regression were removed from the data set as values from those cows confounded the data set. Ultimately, just n=24 cow data were used for analysis and interpretation. Cows (n=24) were divided as 9 vs 7 vs 8 cows into treatments 100 µg vs 200 µg vs 400 µg of G2.

Mean LH peak was 10.76 for 100, 11.01 for 200 and 13.25 for 400 µg group. When the effect of treatment on LH peak was tested statistically with SAS, no significant difference between

treatments was found. There was also no significant difference between effect of treatments on AUCs and on time to LH peak. However, we found that LH in all cows peaked within a one hour period in the study ranging from 1-2 h.

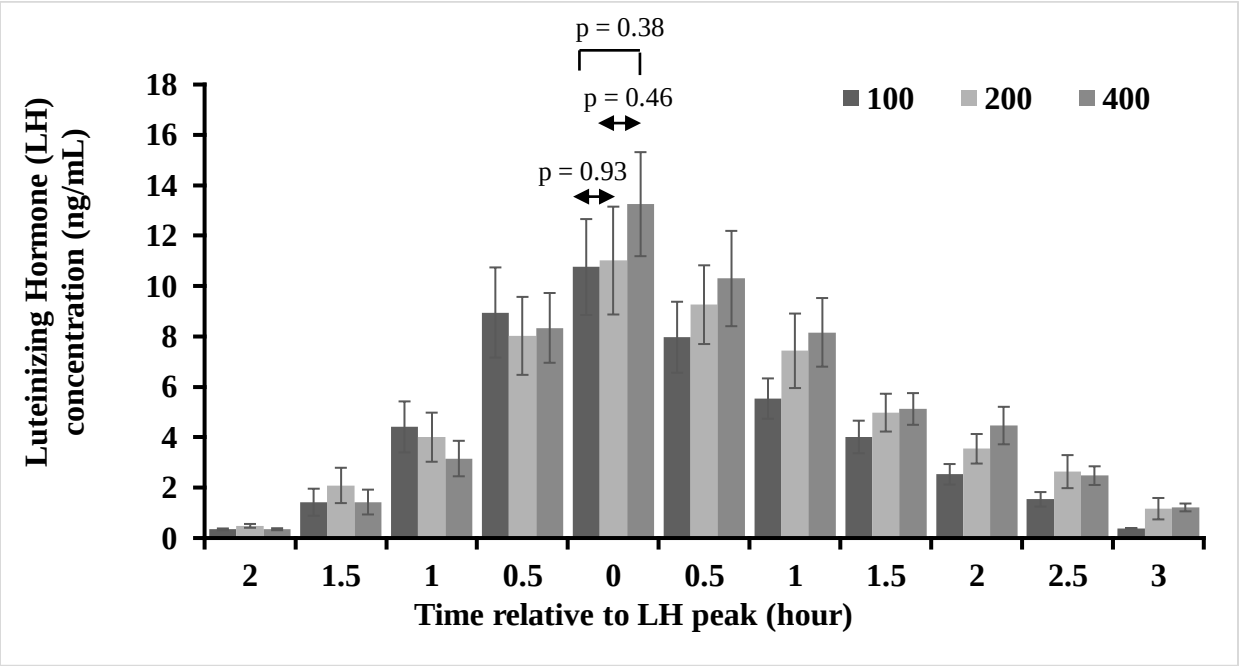

Figure 2.2 LH surge curve plotted as time relative to LH peak where 0 hour coincides with the time of LH peak, with 9 LH readings taken every 30 minutes within a 4 hour period. n=24, n= (9 vs 7 vs 8) in 100 µg vs 200 µg vs 400 µg treatment groups

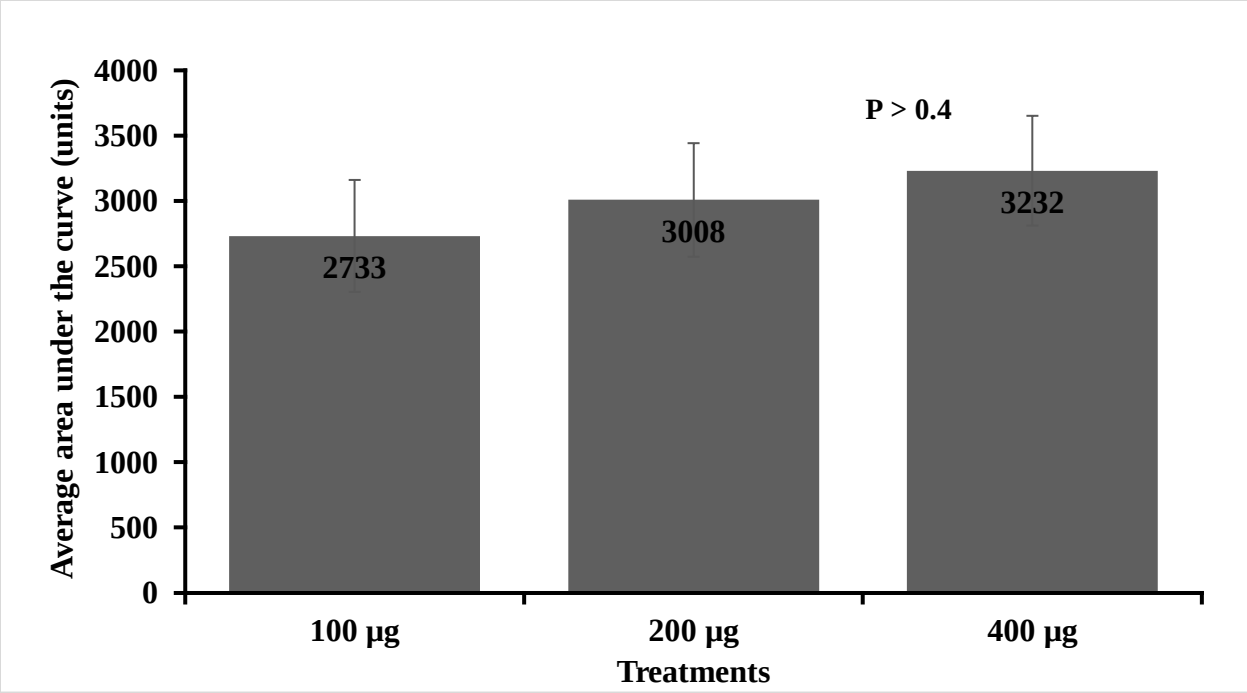

Figure 2.3 Area under the curve (AUC) of n=24, n= (9 vs 7 vs 8) in 100 µg vs 200 µg vs 400 µg treatment groups. Each curves were plotted using the LH values over a 4 hour period and readings plotted according to time relative to LH peak

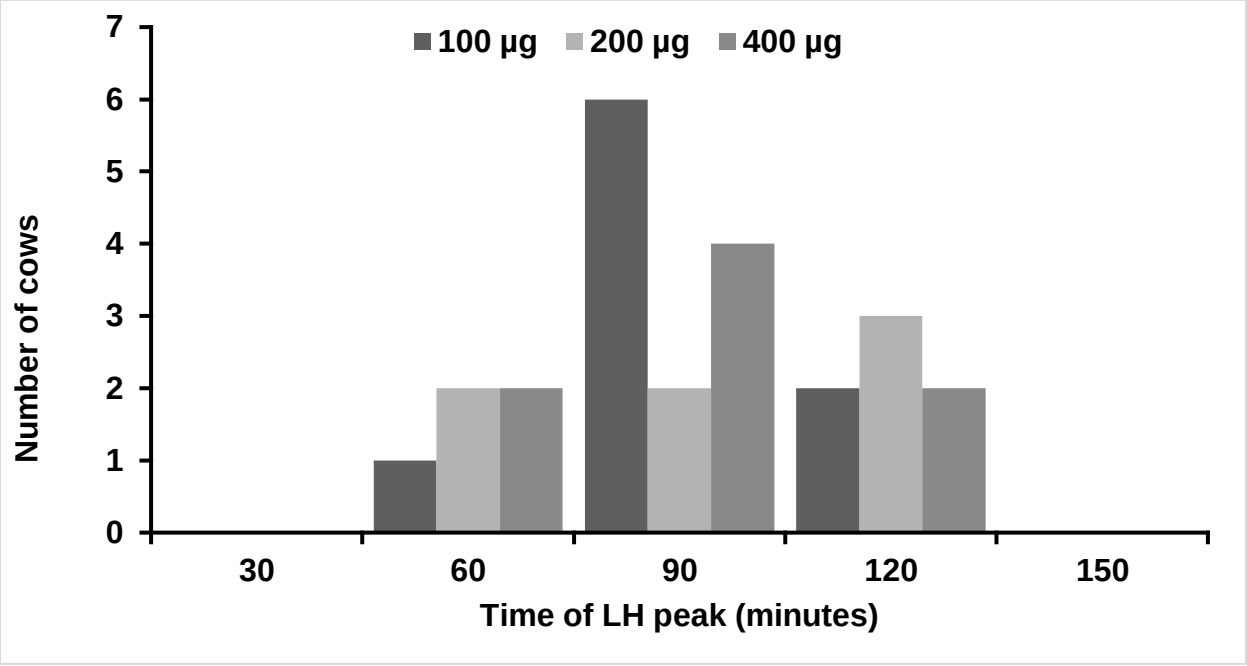

Figure 2.4 Time to LH peak of n=24, n= (9 vs 7 vs 8) in 100 µg vs 200 µg vs 400 µg treatment groups.

The CL volume was calculated using the formula: CL volume =4/3*Π*(r^3) where r (radius) = d/2, d (diameter) =average of height and width of the CL.

When the CL volume data was run on SAS by proc mixed procedure, no significant difference was found between the treatments.

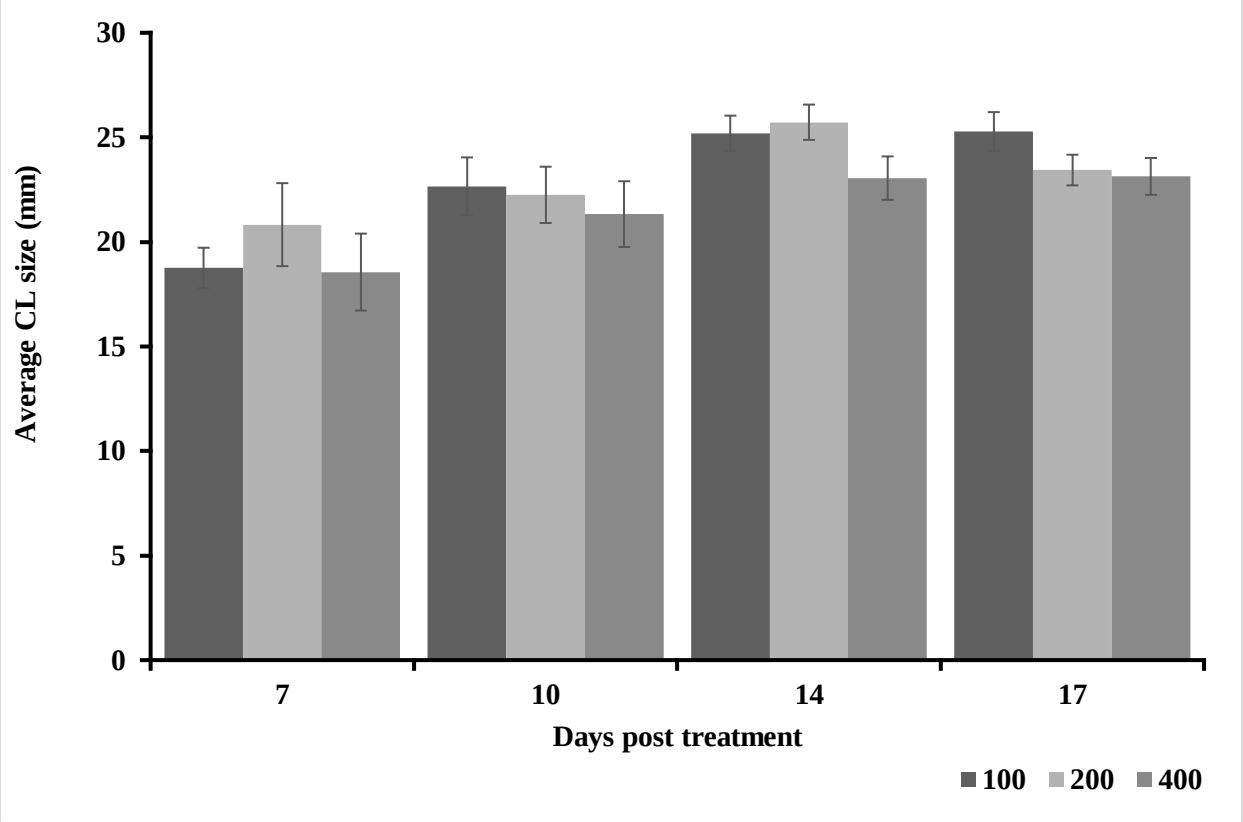

Figure 2.5 Average size of corpus luteum (CL) post treatment of N=24, n=9 vs n=7 vs n=8 in 100 µg vs 200 µg vs 400 µg treatment groups. Average CL size expressed as millimeter (mm) was calculated as average of height and width of the CL measured by the in-built calipers of the ultrasound machine.

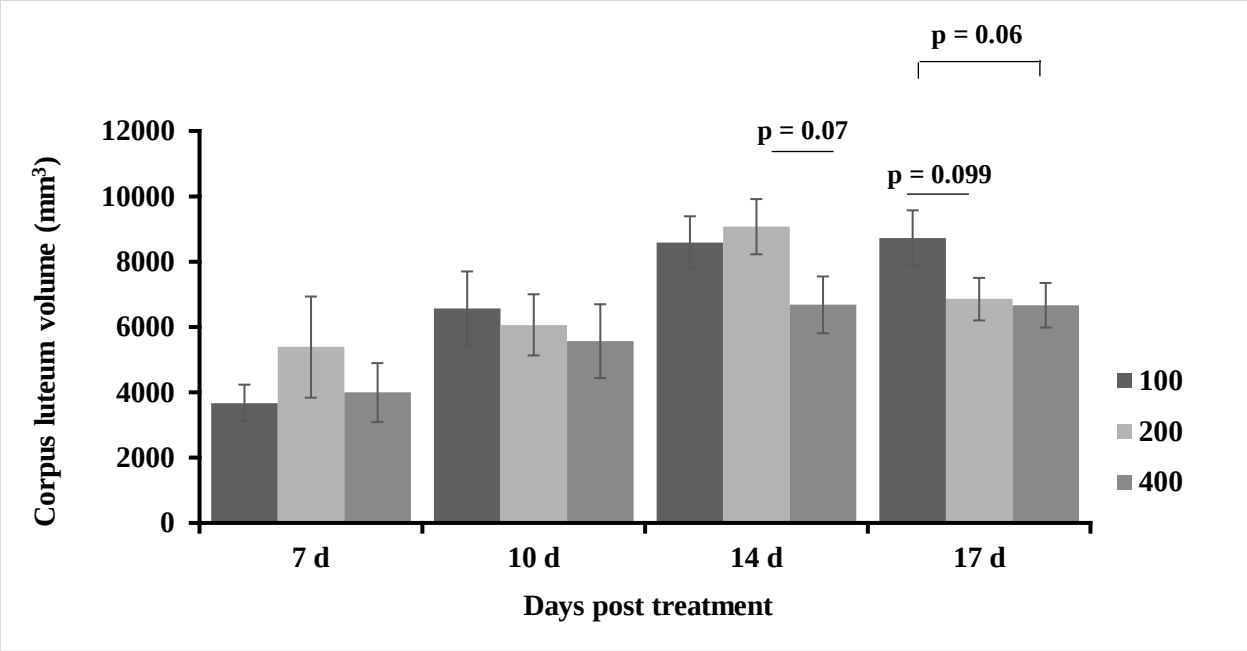

*Figure 2.6 Corpus luteum (CL) volume post treatment of N=24, n=9 vs n=7 vs n=8 in 100 µg vs 200 µg vs 400 µg treatment groups denoted as mm^3, CL volume was calculated using the formula: CL volume =4/3*Π*(r^3) where r (radius) = d/2, d (diameter) =average of height*

The effect of treatment on P4 concentration was tested by proc mixed procedure of SAS and no significant difference was found between treatments.

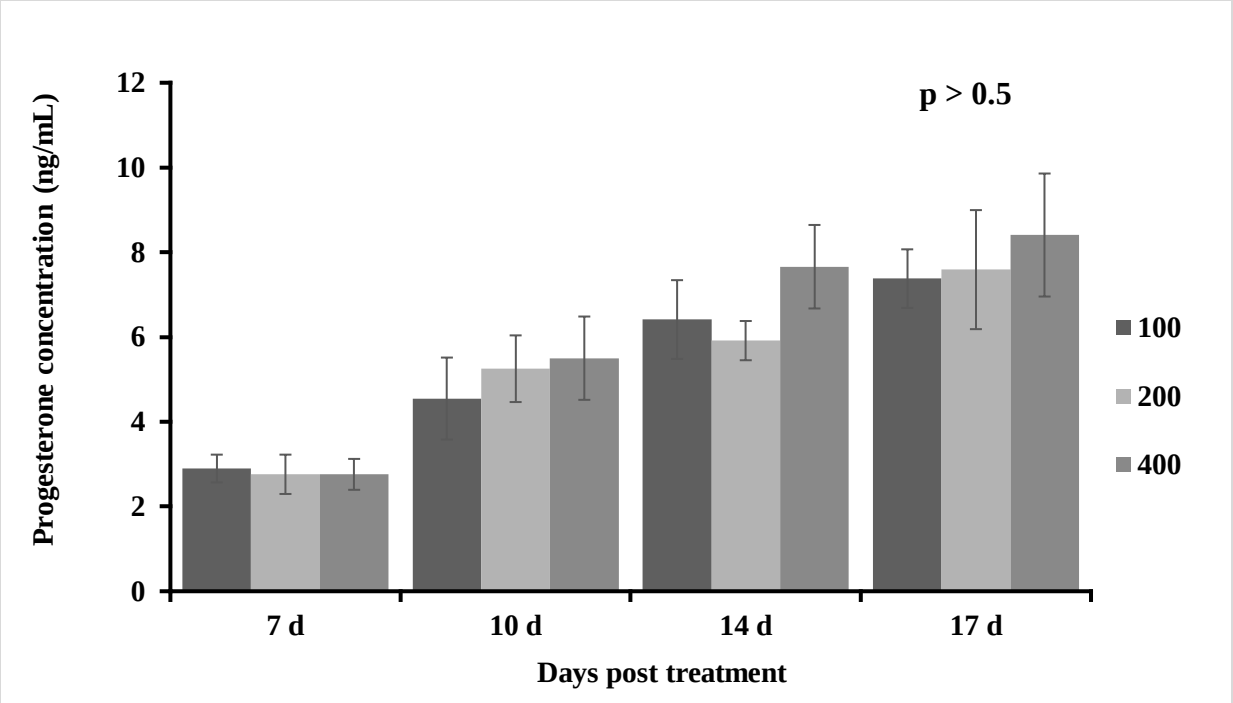

Figure 2.7 Progesterone concentration post treatment of N=24, n=9 vs n=7 vs n=8 in 100 µg vs 200 µg vs 400 µg treatment groups. Progesterone concentration is expressed as ng/mL

The progesterone per unit CL volume was calculated by dividing P4 values by the CL volume on each day. The data were run in SAS proc mixed procedure, no significant difference was found between treatments.

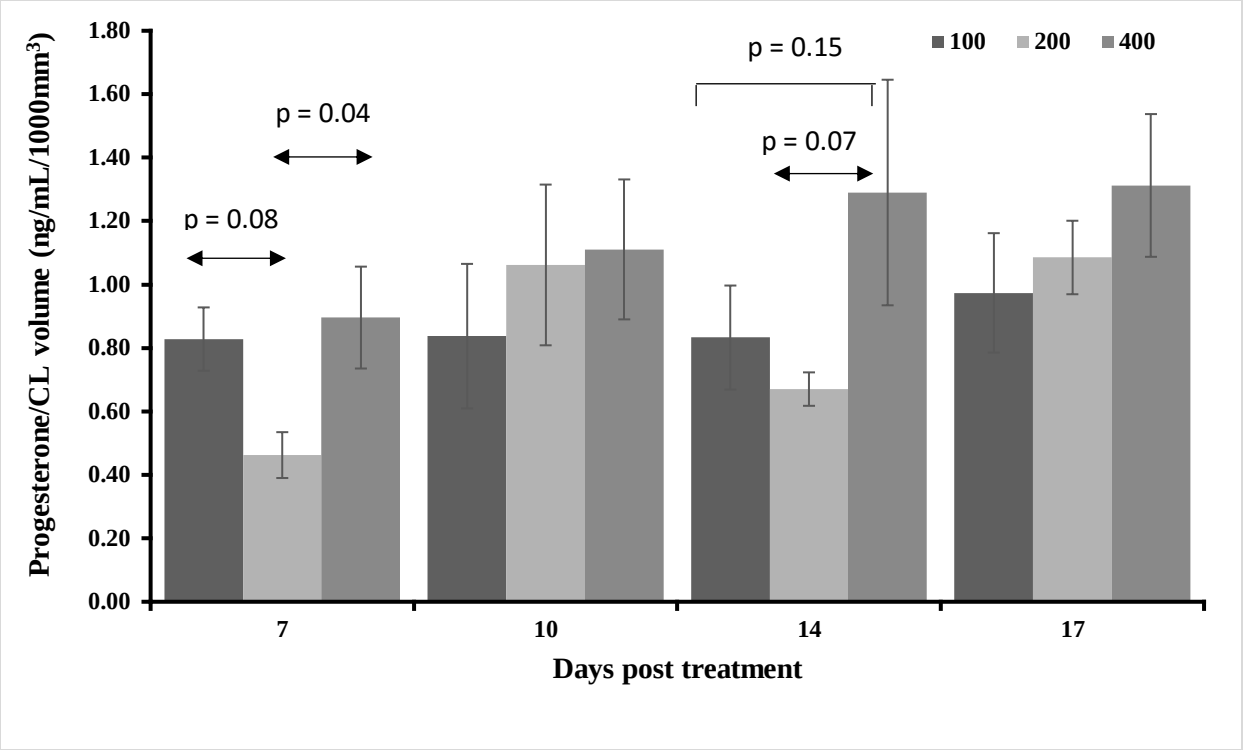

Figure 2.8 Progesterone per unit CL volume post treatment of N=24, n=9 vs n=7 vs n=8 in 100 µg vs 200 µg vs 400 µg treatment groups, expressed as ng/mL/1000 mm^3.

Discussion

It has been proven that animals which respond to G1 of Ovsynch respond better to PG and G2 of Ovsynch and have better synchronization and fertility (Bello et al., 2006). Cows ovulate within a 24-32 h window post GnRH injection (Pursley et al., 1995) but heifers have a variable time of ovulation post GnRH treatment. Among n=33 cows that responded to G1, all but one cow ovulated in the window of 24-32 h post GnRH injection.

Synchronization rate in this study is less than synchronization rate that was reported in the pioneer study of Ovsynch (20/20 cows and 18/24 heifers) (Pursley et al., 1995). Peters and Pursley (2002) had indicated that synchronized ovulation percentage following Ovsynch was 80-90%. The stage of estrous cycle at the start of Ovsynch is important for synchronization. A 91% synchronization

rate was reported when Ovsynch was started at day 1-12 of the estrous cycle and 80% when Ovsynch was started at day 13-22 in lactating dairy cows (Vasconcelos et al, 1999).

It is believed that hormonal management protocols involving use of exogenous hormones have resulted in greater incidences of double ovulation. In women, it was reported that use of hormones and IVF technique resulted in increased frequency of twins (Imaizumi, Y., 2003). Greater producing cows with short estrus duration and anovular cows with less P4 concentration at onset of Ovsynch are also at risk of double ovulation and twinning. Double ovulation was increased in lactating Holstein cows with less P4 in comparison to greater P4 concentration during the development phase of the follicle (Cerri et al., 2011). Higher P4 concentrations during the growth of the pre-ovulatory follicle have been reported to decrease chances of double ovulation (Wiltbank et al., 2000; Hayashi et al., 2008). Double Ovsynch technique was found to increase P4 during follicle growth and decrease double ovulation and twinning (Wiltbank et al., 2014). This technique also helped to increase fertility and decrease chances of pregnancy loss (Cunha et al., 2008). There were five cows that had double ovulations in this study.

Mean LH peak tended to increase with increased dose of GnRH with 400 µg resulting in the greatest LH peak. However, the differences in LH peaks of the treatments were not statistically significant. Mean AUC of the LH curves of the treatments were ranked as 400>200>100 in this study. Study on the effect of 50 µg vs. 100 µg on LH surge reported 100 µg of GnRH being more effective than 50 µg (Jensen et al., 1983; Souza et al., 2009). Likewise, 200 µg of GnRH resulted in greater plasma LH concentrations in comparison to 100 µg in a study with beef heifers (Dias et al. 2010) and beef cows. A similar result was reported in dairy cows in both higher and lower P4 environment (Giordano et al., 2012) though other studies reported no effect of GnRH above 100 µg (Rantala et al. 2009; Yamada et al., 2002). Positive results of greater ovulation in response to

G1 reported has led to greater synchronization at G2. However, in our study we selected only cows responding to G1 and used a higher dose of GnRH at G2 to see its effect on LH and CL function. The higher dose of G2 showed an increase in progesterone per CL volume on certain days only. However, progesterone concentration on different days was not significantly different between treatments. Progesterone per CL volume of 200 and 400 µg groups on day 7 was found to be significantly different ($p=0.04$). Also a tendency toward significant difference in progesterone per unit CL volume was found between 100 and 200 on day 7 ($p=0.08$) and 200 and 400 on day 14 ($p=0.07$). Since effect of greater G2 on P4 did not coincide with CL volume values, here P4 was not proportional to CL volume. This finding is supported by papers which stated that CL blood supply and not its size determine the P4 production concentration (Herzog et al. 2010; Wise et al. 1982). This is a likely explanation for difference found in CL and P4 results. We are concerned about greater P4 concentration post treatment until day 17 in the study. Greater P4 concentration during early luteal phase (3-7 d) helps in elongation of embryo and improves fertility.

No studies have reported any effect of greater dose of GnRH in a synchronization protocol on CL function. Increased P/AI has been reported by some researchers (Ferguson et al., 2012; Morgan and Lean, 1993). Other studies reported no effect of greater GnRH on CR in lactating dairy cows (Fricke et al., 1998) or marginal effects on fertility (Giordano et al., 2013). Mechanism by which greater dose have effects on CL function can be by inducing a greater LH peak. Though LH peaks in our study did not differ significantly, we still believe that a greater LH peak result due to a greater GnRH dose. One study reported P4 concentrations 7 days after estrus for cows with multiple ovulations being less than that of cows with single ovulations though the CL volume was greater (Lopez et al., 2005).

We attempted to nullify other factors that may affect the P4 concentration such as pre- ovulatory follicle size that have reportedly had a positive effect on the CL size and P4 secretion (Binelli et al 2009; Busch et al. 2008; Martins et al. 2011). In our study, cows were blocked according to parity and we maintained a balance in follicle size in all treatments. The aim of this method of blocking was to nullify the effect of unequal follicle size on P4 concentrations. In addition, the age of pre-ovulatory follicles was same for all cows as only the cows responding to G1 were enrolled in the study. When pre-ovulatory follicle sizes were plotted against CL volume and P4, the curve did not show a positive linear relationship between them. Factors other than pre-ovulatory follicle size probably have more effect on CL size and P4 production.

Our data had much variability within a treatment which we think has confounded the results. We do not have adequate evidence to conclude that there is a significant effect of greater dose of G2 of Ovsynch on CL volume and P4 concentration post treatment. However, we still cannot rule out the possibility of effect of higher dose of GnRH as we found some tendency of significant difference between the doses on progesterone concentration per unit CL volume. Further studies using similar doses need to be carried out to determine the dose that optimizes the P4 concentration. GnRH is an important tool for reproductive management because of its efficacy, availability and low price. Cost of a 100 µg dose of GnRH (Cystorelin) is $2.40 so a double dose will be $4.80 and a dose four times the labeled dose will be $9.60. GnRH is less costly in comparison to other hormones used in reproductive management. If further studies do show an effect of greater dose of GnRH, dairy farmers can benefit from new dosage being administered in reproductive protocols.

CHAPTER 3

EFFECT OF DIFFERENT DOSES OF FINAL GONADOTROPIN RELEASING HORMONE OF OVSYNCH ON CORPUS LUTEUM FUNCTION IN BUFFALO

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Introduction

Buffalo have an economic importance in a developing country like Nepal because they are better at converting poor-quality roughage into milk and meat. Milk with greater fat and protein percentage is produced with a lesser nutrition level. Buffalo are reported to have a 5% greater digestibility of crude fiber than greater-yielding cattle; and a 4 to 5% greater efficiency of utilization of metabolic energy for milk production (Mudgal, V.D. 1988).

Reproductive management is a tool to increase productivity in any dairy animal. It is more important in buffalo because buffalo reproduction has always faced problems of longer gestation period, silent estrous, and heat stress affecting estrus behavior (Perera, BM. 2011) A wide variability in estrus length and ovulation time makes it difficult to predict timing of AI in buffalo (Campanile et al., 1988). Poor expression of estrus is another hurdle in buffalo reproduction that requires investment of time and labor to determine the correct timing of AI. Conventional heat synchronization did not result in good CR after timed AI and so AI to heat detection is still preferred by buffalo farmers (Barile et al., 2004). Precise breeding technique such as synchronization of ovulation (Ovsynch) and use of fixed timed AI has a good scope in buffalo.

Concentrations of estradiol and progesterone is less in buffalo than in cattle (Baruselli et al., 1997) due to smaller ovaries, fewer primordial follicles and smaller dominant follicle (Danell, B. 1987). Ali and Fahmy, 2007 reported CR as 60% in cyclic and 37.5% in non-cyclic buffalo following Ovsynch. Baruselli et al., 2003 reported CR as 45.4% in buffalo following Ovsynch. Due to earlier onset of ovulation in non-cyclic buffalo following Ovsynch, two inseminations at 0 and 24 h was suggested to increase CR (Ali and Fahmy, 2007) Study that compared Ovsynch with pre synchronization-Ovsynch showed no significant difference in ovulation and conception rate (Oropeza et al., 2010). Ovsynch in buffalo is reported to give improved fertility in favorable

reproductive season (Barile et al., 2005). As an effort to overcome reduced steroid concentration in buffalo, we used two different doses of GnRH (100 µg vs. 400 µg) in the final GnRH of Ovsynch in this study. 100 µg is the dose of GnRH which was started as treatment of cystic ovary in cows (Kittok et al., 1973; Cantley et al., 1975; Bierschwal et al., 1975) as well as the dose used in most synchronization protocols (Pursley et al., 1995; Bello et al., 2006). Some studies on greater dose of GnRH done on cows have not been successful in determining an optimum dose to improve CL function. Likewise, in buffalo, 100 µg of GnRH is used in synchronization programs (Ali and Fahmy, 2007) and no studies on greater doses of GnRH have been reported to our knowledge.

The objective of the study was to determine whether a greater dose of GnRH (100 vs. 400 µg) using Ovsynch results in improved CL function marked by increased CL size and greater concentration of progesterone. We hypothesized that increased dosage of final GnRH of Ovsynch would not affect CL function.

Materials and methods

Animals

This study was conducted from October 2015 to January 2016 at the Agriculture and Forestry University Livestock Farm, Rampur, Chitwan, Nepal. Ten parous Murrah cross buffalo and four heifers were used in the study. Parity of the buffalo used ranged from 0 to 6. Buffalo were housed in a tie stall barn except when allowed to freely graze in the farm's grasslands from 7 am to 3 pm. They were fed hay straw and grass in the evening. Only a few buffalo in the group were lactating. Lactating females were milked twice daily. Milk yield ranged from 2 to 4 liters per day in the lactating females. The buffalo used in the study were at random stages of estrous cycle. Of the n=10 parous buffalo, n=1 was a first lactation buffalo and n=9 were 2+ lactation buffaloes. All injections were given intramuscularly in neck region with an 18-gauge needle.

All animals received 100 μg of GnRH intramuscularly at a random stage of the estrous cycle (OvaCyst®, gonadorelin diacetate tetrahydrate, Bayer Health Care). A week after the first GnRH (G1), 25 mg PG was administered. In multiparous buffalos, 25 mg PG was repeated 24 hours after the first injection of PG.

Ultrasonography was performed to evaluate diameter of pre-ovulatory follicles at time of treatment (Honda Electronics HS- 1500 Vet with 7.5 MHz transducer, Japan). Only buffalo with a growing pre-ovulatory follicle were enrolled for treatment. Buffalo were blocked by parity and pre-ovulatory follicle diameter and assigned randomly to one of the two treatments. Control buffalo received 100 μg GnRH. Treated buffalo received 400 μg of GnRH. A 56-hour interval was maintained between the PG injection and treatment. All buffalo received AI in the morning of the day following the treatment GnRH, 16 to 20 hour after treatment.

Blood collection

Blood was collected by jugular venipuncture using an 18-gauge needle, Vacutainer and Vacutainer tubes without anticoagulant (BD Vacutainer, Preanalytical Solutions, Franklin Lakes, NJ). Blood was collected on d 0, 7, 14, 21 and 28 following treatment. The blood samples were intended for analysis of progesterone concentration. Blood samples were stored overnight and centrifuged at 3400 rpm for 15 minutes to harvest serum the next day. Serum was stored at -20˚C until the time of assay except for the time when it was shipped from the research site in Chitwan to Kathmandu in an ice box.

Ultrasound examination

Ultrasonography was used to scan ovaries and measure the number and size of the follicles and CL on days -9, -2, 0, 7, 14, 21 and 28. Buffalo were scanned on day -9 to check for the presence and measure sizes of ovulatory follicles before giving G1; day-2 to check the presence and measure

size of CL before giving PG; day 0 to ascertain CL regression in response to PG and to measure the size of the pre ovulatory dominant follicle (DF). After the buffalo were bred on day 1, they were scanned on days 7, 14, 21 and 28 to follow the development of the CL. Day 28 ultrasonography was the first day for pregnancy diagnosis of those buffalo.

Progesterone Assay

The Abraxis Progesterone (bovine) ELISA kit (Abraxis USA) was used to determine serum concentrations of progesterone. The intra-assay coefficient of variation (CV) % was 7.6, 5.4 and 4.9 for low, medium and greater P4 respectively. The inter-assay CV % was 6.8, 8.3 and 2.7 for low, medium and high P4. Forty-two samples were run in duplicate in one plate along with six standards in duplicate. The optical density (OD) of each sample was measured by a spectrophotometer at a wavelength of 450 nm (with a reference filter of 650 nm). The measuring range of the assay is 0 to 20 ng/mL with an analytical sensitivity of 0.06 ng/mL.

Statistical analysis

SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used for analysis of data. Proc Means was used to calculate means and SE. Proc Mixed procedure was used to test the effect of treatment on variables. The effect of treatment was considered significantly different when $p < 0.05$ at the 95% confidence level.

Results

Table 1 The pre-treatment results in buffaloes in Nepal

Buffalo ID	Parity	Rx	Ovulation_G1	CL size	Luteolysis_P4	DF	Side_DF	Ovulation_G2	CL 1	cavity 1	CL 2	cavity 2	CL 3	cavity 3	CL 4	cavity 4	D45 USG
1255	2+	**100**	0	15	0	11	R	**1**	17	5	19	-	19	-	19	-	Pregnant-RH
1426	1	**400**	1	19	1	10	R	**0**	-	-	-	-	-	-	-	-	Open
1286	2+	**400**	1	20	1	13	R	**1**	19	4	24	-	17	-	20	-	Pregnant-RH
1302	2+	**100**	0	-	-	11	R	**0**	-	-	-	-	-	-	-	-	Open
1252	2+	**100**	0	-	-	9	R	**1**	14.5	2	14		14		9		Open
1191	2+	**400**	0	10	1	9	L	**1**	15	-	21	-	16	-	19	-	Pregnant LH
1531	2+	**400**	1	8	1	14	R	**0**			14	-	12	-	-	-	Open
1186	2+	**400**	0	-	-	9	L	**1**	13	-	14	-	13	2	17	-	Open
1296	2+	**400**	1	35	1	10.5	L	**1**	21	17	21	17	19	-	21	-	Open
1243	2+	**100**	1	20	1	9	L	**1**	16	-	17.5	-	17	-	13	2	Open
Heifer1	0	**100**	0	-	-	7.5	L	**0**	-	-	-	-	-	-	-	-	Open
Heifer2	0	**100**	0	-	-	11	L	**1**	19	4	19.5	-	19	-	21	-	Pregnant-LH
Heifer3	0	**400**	1	20	1	8	R	**0**	-	-	-	-	-	-	-	-	Open
Heifer4	0	**100**	0	-	-	8	R	**0**	-	-	-	-	-	-	12	-	Open

Among the n=14 buffaloes, n=6 buffaloes that responded to G2 were 3 from each dose group. The CL sizes determined by ultrasonography every 7 days starting from the day of treatment (day of G2 of Ovsynch) up to 28 days was used to calculate the CL volume by the formula:

CL volume =4/3*Π*(r^3) where r (radius) = d/2, d (diameter) =average of height and width of the CL.

4/14 buffaloes were diagnosed pregnant at the day 45 ultrasonography. Of the 4 animals that were pregnant, there were two from each treatment.

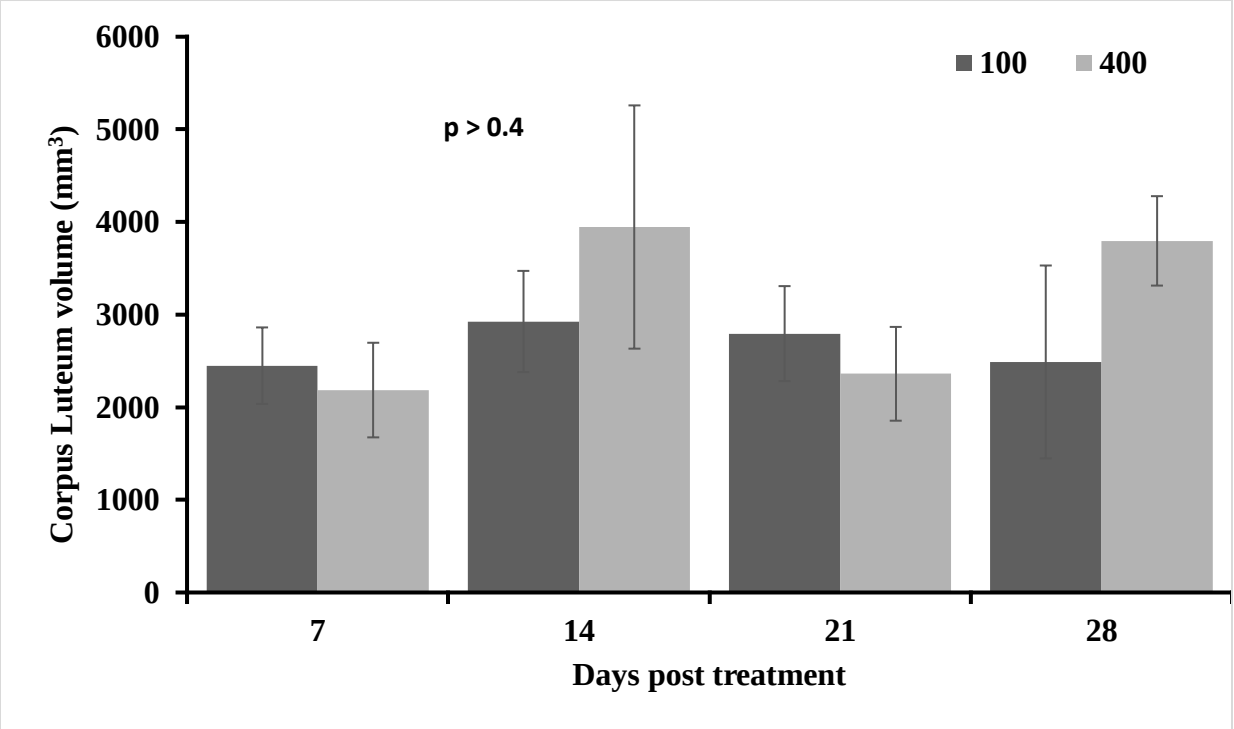

*Figure 3.1 Corpus luteum (CL) volume post treatment of n=6 (n=3 vs. 3) in 100 µg vs 400 µg treatment groups denoted as mm^3, CL volume was calculated using the formula: CL volume =4/3*Π*(r^3) where r (radius) = d/2, d (diameter) =average of height and width of the CL.*

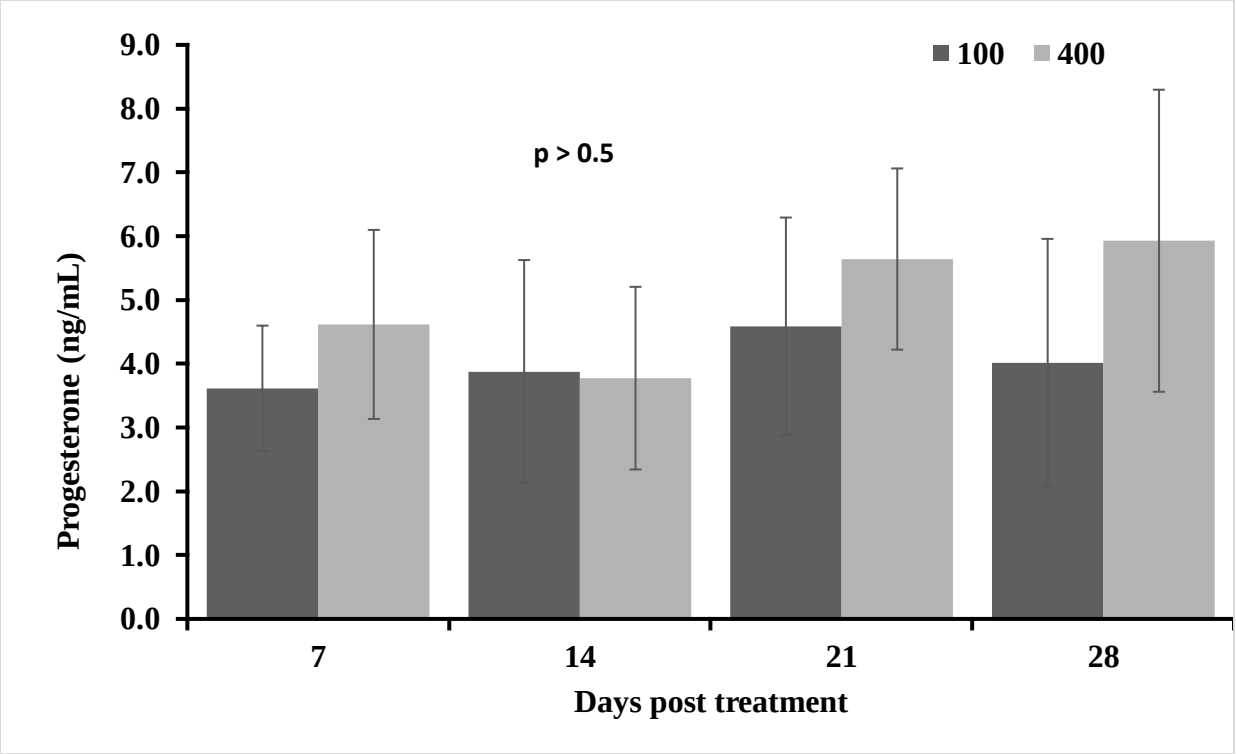

Figure 3.2 Progesterone concentration post treatment of n=6 (n=3 vs. 3) in 100 µg vs 400 µg treatment groups. Progesterone concentration is expressed as ng/mL

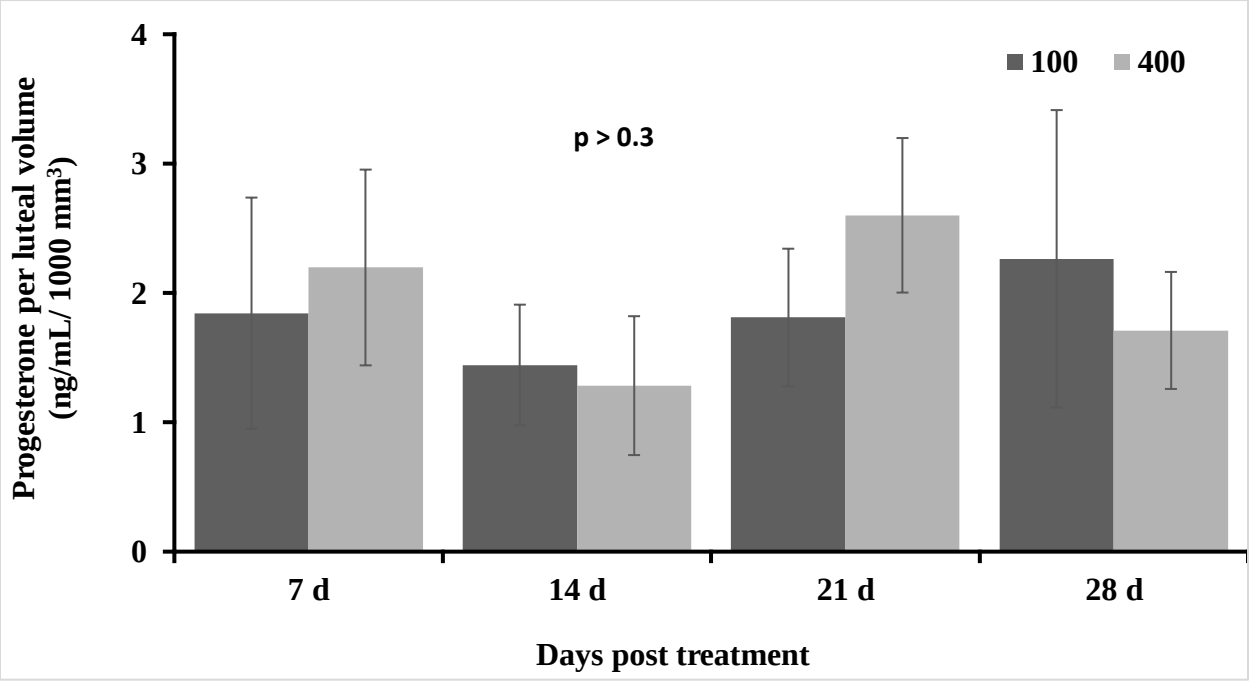

Figure 3.3 Progesterone concentration post treatment of n=6 (n=3 vs. 3) in 100 µg vs 400 µg treatment groups. Progesterone concentration is expressed as ng/mL/1000 mm^3

CL volume ($p > 0.4$), and P4 concentration ($p > 0.5$) and P4 per CL volume ($p > 0.3$) of buffaloes in treatment groups were not significantly different on different days post AI.

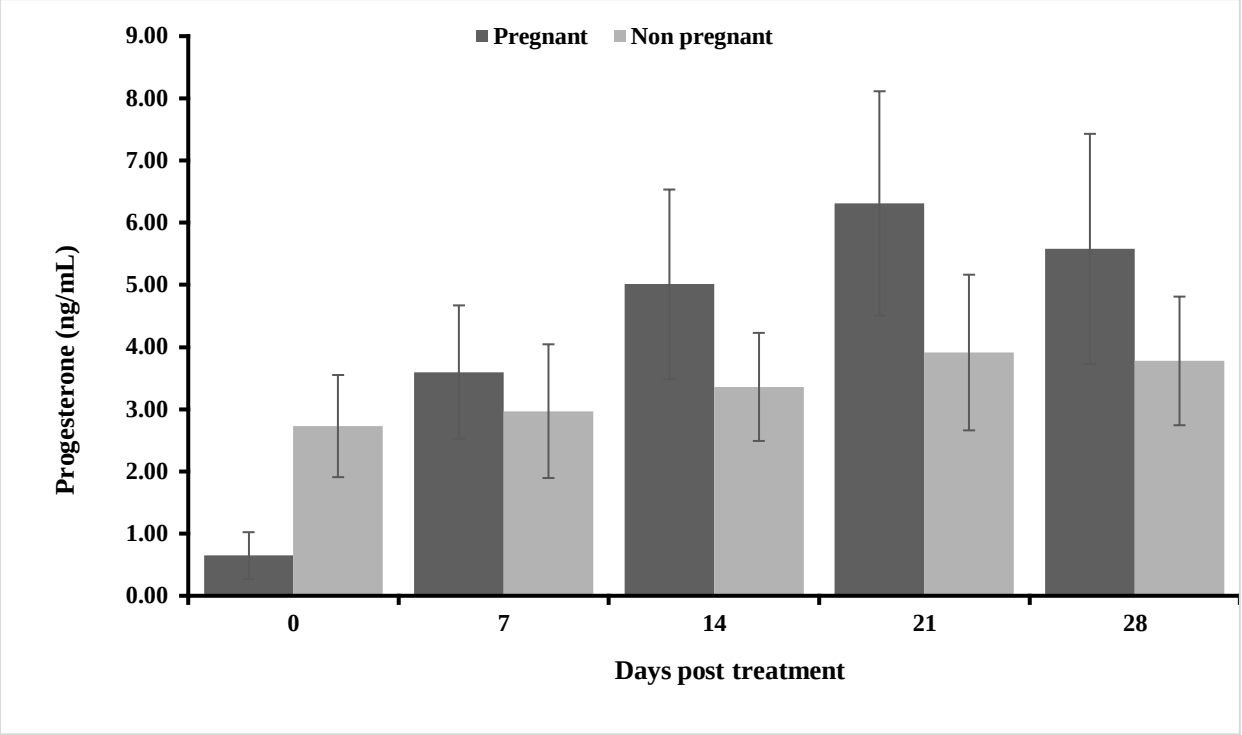

Figure 3.4 Progesterone concentration comparison of pregnant (n=4) and non-pregnant buffalo (n=2).

The P4 concentration of non-pregnant buffalo was greater compared to pregnant buffalo at the time of treatment. P4 concentrations post-treatment were greater for pregnant buffalo in comparison to non-pregnant animals.

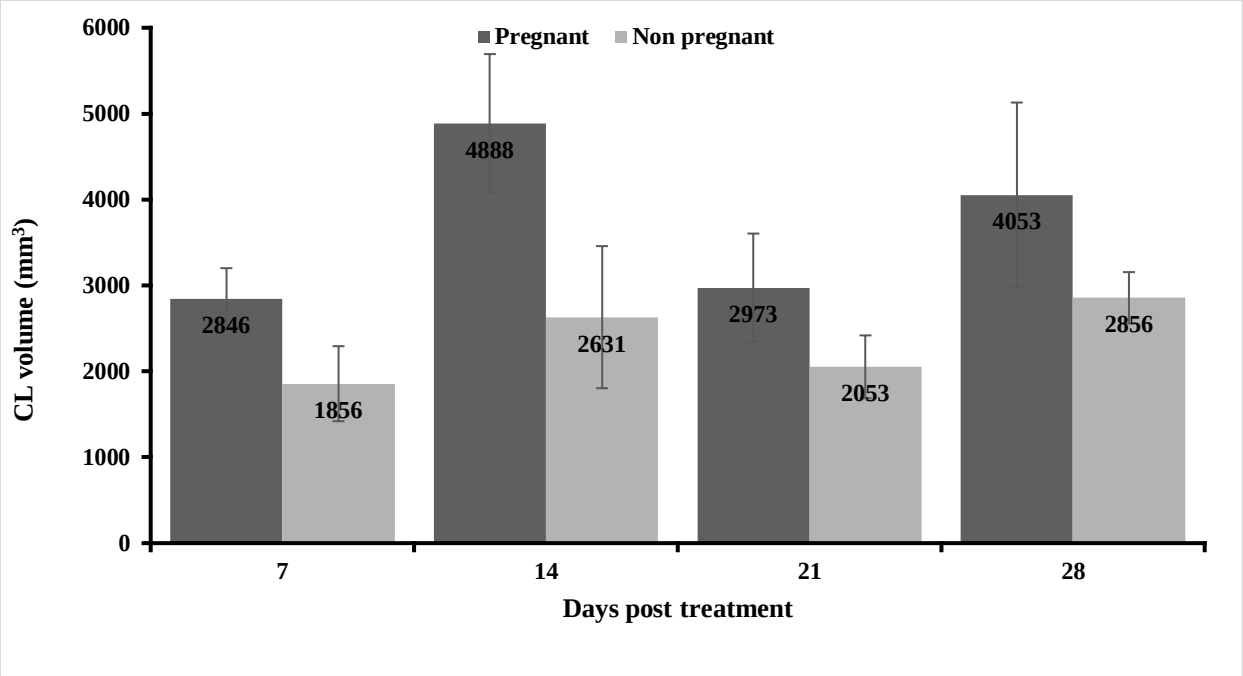

Figure 3.5 Corpus luteum (CL) volume (mm3) comparison of pregnant (n=4) and non-pregnant (n=2) buffalo.

Discussion

While it is well known that ovulation in response to G1 is a prerequisite for success of Ovsynch, our data had a few exceptions. Only 6/14 animals responded to G1 given at random stages of estrous cycle. In cattle, when Ovsynch was used for the first time, 18 of 20 lactating cows and 13 of 24 heifers had responded to G1 (Pursley et al., 1995). G1 resulted in ovulation in 90% of cyclic and 62.5% of non-cyclic buffalo. PG induced luteolysis in 80 and 87.5% for cyclic and non-cyclic buffalo. And 80% of cyclic and 100% of non-cyclic buffalo responded to G2 (Ali and Fahmy, 2007). The animals that did not respond to G1 in this study were possibly acyclic. In our study, two animals that responded to G1 did not respond to G2. Both of those animals failed to regress CL in response to PG as seen in the ultrasonography at G2. However, Baruselli (1997) did not find any relationship between dominant follicle development and presence of a CL or follicle from a previous wave. Out of the six animals that responded to G2, four animals were ones that had also

responded to G1, which is in agreement with data that show response to G1 increases the chances of response to G2. A synchronization rate of 78.8 % was reported in a synchronization protocol in buffalo (Baruselli et al., 2003). This study had 6 of 14 animals synchronized by Ovsynch.

Size of the ovulatory follicle

The ovulatory follicles of buffalo are smaller than those of cattle. Our data shows that the minimum size of pre ovulatory follicle is 8 mm. The maximum size observed was 14 mm in a multiparous animal, but she did not ovulate (even in response to 400 µg of GnRH). Another buffalo with a follicle of 13 mm did ovulate in response to G2. It can be speculated that an ovulatory follicle size of 9 to 13 mm is more responsive to GnRH in buffalo. This finding is supported by a study which reported size of the dominant follicle as 9.82 ± 1.23 and 11.96 ± 2.15 mm for heifer and parous buffalo at the time of final GnRH injection. The ovulation rate was 100% for heifer and 88.8% for parous (Derar et al., 2012). Ovulation time averaged 22.6 h (range 16-36 h) and 10.4 h (range 6-24 h) in heifer and parous buffalo, respectively (P = 0.01). The mean diameter of the CL developed at Day 7 of the protocol was 15.45 ± 0.8 and 19.7 ± 1.3 mm in heifer and parous buffalo, respectively (P = 0.03).

In cattle DF size had a positive effect on the CL function (Pandey et al., 2011; Binelli et al., 2009; Busch et al., 2008). Also greater pregnancy loss was detected with ovulatory follicles <15 mm diameter in comparison to the ones that were >15 mm (Stevenson et al., 2006). Follicles <11 mm when induced to ovulate led to reduced conception rates and increased embryonic mortality (Perry et al., 2005) as GnRH can induce LH surge and ovulation in follicles >9 mm diameter in cattle (Bodensteiner et al., 1996).

Although no significant effect of increased dose of G2 was seen on CL volume or progesterone level, we still suggest that more studies should be carried out to determine an effective

synchronization protocol and doses of GnRH that can help to optimize fertility in buffalo. Buffalo reproductive physiology is different from that of cattle. Ovsynch has been widely studied in cattle but more studies are required in buffalo. Studies involving doses of the hormones used in synchronization protocols are important to optimize reproductive potential in buffalo.

This study has a small data set and is not suitable for a binomial result expression. We had 4/14 buffaloes pregnant at the day 45 ultrasonography.

Greater P4 concentration at the time of G2 of Ovsynch is indicative of incomplete luteolysis. Lack of complete luteolysis are proven to reduce fertility in synchronization protocols (Brusveen et al., 2009; Giordano et al., 2012; Martins et al., 2011). In the present study, the mean P4 concentration of non-pregnant buffalo was found to be greater than that of pregnant buffalo at the time of treatment. In a study with synchronization protocol in buffalo, Ovsynch resulted in pregnancy of 12 of 30 nulliparous and 6 of 14 pluriparous buffalo (Presicce et al., 2005). PRID-PMSG protocol resulted in pregnancy of 12 of 17 buffalo. It was concluded that the efficacy of Ovsynch was the same in nulliparous and pluriparous buffalo. It was also suggested that progestogen treatment along with Ovsynch increases PR in acyclic buffalo. Rensis et al. (2005) also found that supplementation with progesterone along with Ovsynch helped to increase conception rates in non-cyclic buffalo.

Out of the 4 pregnant buffalo, 3 were parous and 1 was nulliparous. Acyclic condition was found to be more prevalent in primiparous and old buffalo when compared to heifers. Also, two types of anestrus have been reported - superficial and deep. Superficial acyclic buffalo have been found to respond to adequate synchronization protocol (Zicarelli, 1998). In our study, buffalo that were acyclic showed some follicular or luteal activity. Those animals might have been the superficial anestrus buffalo. However, adequacy of synchronization might have hindered the response. We

used simple Ovsynch in this study. Pre synchronization followed by Ovsynch can increase the synchronization rate in acyclic animals.

Buffalo have a wider variable time interval between LH surge and ovulation in the natural estrous cycle as well as in hormone-induced ovulations in comparison to cattle (Seren et al., 1995; Zicarelli, 2003). Because of this fact, it was suggested that performing 2 to 3 insemination can help to increase pregnancy rates (Barile et al. 1997; Zicarelli et al. 1997). Single AI was done after synchronization in the present study and we speculate that more than one AI might have helped to increase pregnancy in those animals.

There is not enough evidence to prove positive effect of greater dose of GnRH on luteal function in buffaloes. More studies to explore various aspects of reproductive management is necessary to better understand buffalo reproduction. GnRH is an efficient, readily available and cheap hormone that can be used to control follicular development in cattle as well as buffalo. Mostly result from cattle synchronization studies have been used in buffaloes. However, due to the difference in cattle and buffalo reproductive physiology, studies with buffalo need to be carried out. This will help to determine an optimum dose of hormones in synchronization protocol specific for buffaloes so that farmers can tackle with problems in buffalo reproduction.

CHAPTER 4 SUMMARY

History indicates that the fertility of dairy cattle is in decreasing trend with increase in milk productivity. Fertility is compromised in greater producing dairy cows because of the less concentration of steroid hormones in circulation. Heifers, however, have a greater level of steroid hormone concentration and greater fertility in comparison to lactating dairy cows. For this reason studies that target to imitate the steroid hormone concentration of heifers in lactating dairy animals have been carried out. Also steroid concentration in buffalo are much lesser than that of cows.

Reproductive management techniques have been studied more elaborately in cows over time than buffalo. From the times of traditional natural heat detection-AI to estrus synchronization AI and finally ovulation synchronization coupled with timed AI, this discipline has come a long way. Synchronization protocols are beneficial to dairy and beef farmers as cost saving and efficient tool. The main focus of synchronization protocols is to synchronize ovulation of the preovulatory follicle at a known time and perform TAI. GnRH and PG are the common hormones that are dosed and timed in most synchronization protocols. After the introduction of Ovsynch, many aspects of reproduction has been understood. To further increase P/AI in cows under synchronization protocol, pre-synchronization of the cows before start of Ovsynch have been studied and proven to be effective. Pre-synchronization helps to start Ovsynch at a preferred day of the cycle.

The goal of synchronization protocol in reproductive management is to increase fertility. The concentration of P4 both pre and post AI affect fertility. Various methods to increase P4 during those phases have been studied. Our protocol aims to increase P4 concentration by use of greater G2 of Ovsynch and result in greater CL volume and P4 concentrations. Dose of GnRH used in synchronization protocols have remained unchanged from the first time it was studied as 100 µg indicated for treatment of cystic ovary for more than four decades. Few researchers have studied

greater dose in G1 of Ovsynch in cows and have had both positive and neutral effects. This study is focused on greater dose of G2 of Ovsynch but the result is not sufficient to conclude that greater dose have positive effects on CL function post insemination. However, we found a tendency of difference between treatments on certain days post treatment and so we recommend further study on those doses to determine an optimum dose of GnRH that helps to optimize P4 concentration post AI.

Likewise, modifications in dosing and timing of hormones in current synchronization protocols are necessary to increase fertility in buffalo. Our study result is not adequate to say that dose greater than the traditional 100 µg is beneficial to improve CL function. More studies should be carried out in buffalo to determine the optimum dose of hormones. Majority of such studies were carried out with cow and dose recommendation of cow is also used in buffalo. However, buffalo reproductive physiology is different than cow in many aspects. Apart from this, studies have also proven that multiple inseminations help increase pregnancy rates in synchronized and timed AI buffalo and this can be used in fertility programs in buffalos.

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