Effect of Testosterone on Ovarian Function in Beef Heifers

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ABSTRACT

Synchronization of follicular wave emergence forms the basis of many reproductive management techniques used in the cattle industry such as estrus synchronization, fixed-time artificial insemination and embryo transfer. Estrogen in combination with progesterone has been used widely for this purpose due to ease of use and efficient, consistent and quick results, irrespective of the status of the dominant follicle or corpus luteum when the treatment is applied. The recent ban by the European Union on use of estrogens in food-producing animals prompted us to examine if testosterone could be used as an alternative drug for follicular wave synchronization. We tested the hypothesis that the administration of testosterone will shorten the life-span of the extant dominant follicle resulting in early emergence of a new follicular wave.

We tested two forms of testosterone (conjugated form: testosterone enanthate and unconjugated form: non-esterified free testosterone) and determined that intramuscular injection of oil-based preparation of the unconjugated form caused a sharp rise in plasma concentrations of testosterone followed by a less rapid decline. The pharmacokinetics of testosterone in blood plasma of 16 heifers was determined after two intramuscular injections of 200 mg of unconjugated testosterone (in 4 ml canola oil) at 12 hour intervals. Testosterone C_{max} was 13.9 ng/mL and the distribution half-life of testosterone in the bloodstream was 3.2 days. Plasma testosterone concentrations were elevated within 2 hours, maintained for initial 36 hours and declined to baseline over 13 days.

To study the ovarian and endocrine effects of testosterone, heifers (n=6 per group) were given two intramuscular injections of 200 mg of unconjugated testosterone in 4 mL canola oil at 12 hour intervals on Days 1 (T1), 3 (T3) or 6 (T6) of the first follicular wave

(Day 0 = ovulation); the Control group was given 4 mL canola oil. Ovarian structures were monitored daily by transrectal ultrasonography over one interovulatory interval and plasma samples were collected. Following treatment, the dominant follicle grew more slowly for the next 5 days in T1 (P=0.05) and T3 (P=0.06) groups compared to the Control group, while the dominant follicle in the T6 group regressed more slowly (P=0.02) than in the Control group. The diameter profile of the dominant follicle of the post-treatment wave (Wave 2) and the ovulatory wave did not differ between treatment and Control groups. Overall, testosterone treatment (T1, T3 and T6 combined) extended the duration of current (Wave 1; P=<0.001) wave in 8 out of 17 heifers compared with the Control group and emergence of the post-treatment wave was not synchronized. Although the interovulatory interval was not affected by the treatments, the proportion of 2-wave cycles tended to be higher (P=0.08) after treatment (T1, T3 and T6 combined) compared to the Control group. The plasma LH concentrations in T1 and T3 groups decreased after treatment, while it did not change in T6 group compared to Control group. FSH concentrations were not affected by the testosterone treatment.

In conclusion, our hypothesis that exogenous testosterone treatment will hasten the emergence of next follicular wave, was not supported. Furthermore, testosterone treatment did not cause the demise of the dominant follicle. Testosterone treatment resulted in slower growth of the dominant follicle when treatment was initiated at or before the time of dominant follicle selection, and slower regression of the dominant follicle when treatment was initiated during the static phase. These changes appear to be mediated by the decline in systemic concentration of LH. In addition, testosterone

treatment delayed the emergence of the next follicular wave irrespective of the status of the dominant follicle at the time of treatment.

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DEDICATION

I would like to dedicate this thesis to my family

- My parents, for never letting me forget that there are more important things in life than me

- My wife, for her unyielding love and without her support I would not progress

- My Children, Karar and Hiba, for bringing laughter to our lives.

Without all of you, I never would have tried

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LIST OF ABBREVIATIONS

AR	Androgen receptors
CIDR	Controlled internal drug release
CL	Corpus luteum
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotrophin
GnRH	Gonadotrophin releasing hormone
hr	Hour(s)
im	Intramuscular
Kg	Kilogram
LH	Luteinizing hormone
mg	Milligram
mL	Milliliter
mRNA	Messenger ribonucleic acid
ng	Nanogram
vs	Versus

1.0 GENERAL INTROUDUCTION

Basic studies in ovarian function and endocrine control mechanisms have helped researchers to develop many reproductive techniques that improve the reproductive efficiency of farm animals (Adams, 2007; Mapletoft et al., 2009). Two examples of control of ovarian function in cattle are the synchronization of follicle wave emergence and synchronization of ovulation. These techniques are applied widely in the cattle industry for fixed-time artificial insemination and embryo transfer (Martínez et al., 2004). Physical and hormonal methods are available for synchronization of follicular wave emergence: dominant follicle ablation, GnRH or LH, and estradiol alone or in combination with progesterone (Martínez et al., 2004). Estrogen in combination with progesterone has been used widely in the synchronization of follicular waves due to ease of use and efficient, consistent and quick results irrespective of the status of the dominant follicle or corpus luteum when the treatment is applied (Bo et al., 1995b; Bridges et al., 1999). In this procedure, the dominant follicle regresses as a result of systemic effect (feedback) of progesterone and estradiol on the pituitary gland leading to suppression of LH and FSH secretion (Bo et al., 2000). When the suppressive effect of estradiol on FSH decreases, a surge of FSH occurs resulting in emergence of new follicular wave (Adams et al., 1992a).

For decades, estradiol and its analogs have been used as implants to improve the feed efficiency and growth rate of beef cattle (Lee et al., 1990; Preston, 1999). Over the last 30 years, there has been an ongoing debate about the use of growth promoters in cattle due to human health concerns from the residue of hormones in meat or milk

(Andersson and Skakkebaek, 1999). As a result, many countries including those in the European Union and New Zealand, have banned the use of estradiol in cattle (EC,1996).

Testosterone is a steroid hormone that is produced locally by theca cells in the ovary. Testosterone has an important role in follicular development as a substrate for estradiol synthesis and can increase the activity of aromatase in granulosa cells in vitro (Wu et al., 2011). Therefore, testosterone may be an alternative to estradiol for use in synchronization of follicular waves. Clinicians and researchers have used testosterone in farm animals for other purposes, e.g., to androgenize cows for use in detecting estrus (Nix et al., 1998). In pigs, testosterone was used during the follicular phase in postpubertal gilts to increase the ovulation rate (Cardenas and Pope, 2002). One study tested the effect of testosterone in heifers during the follicular phase and found that the treatment induced atresia for ovulatory follicles (Rajamahendran and Manikkam, 1994). In addition, testosterone treatment has been shown to exert negative feedback on gonadotrophins leading to suppression of LH secretion in cows and mares (Thompson et al., 1984). The atretogenic effects of testosterone on follicles have been documented in rodents by in vivo and in vitro methods and most of these studies point to a negative impact of testosterone on granulosa cells (Billig et al., 1993; Daniel and Armstrong, 1986) resuliting in a reduction in ovarian weight (Hillier and Ross, 1979).

Based on the above cited studies, it is reasonable to propose that exogenous testosterone may increase estradiol concentrations by providing readily available substrate for aromatization, and by increasing the aromatase activity which in turn could exert a negative feedback on the hypothalamic-pituitary axis (Gross, 1980; Roos et al., 1980; Toranzo et al., 1989) leading to suppression of gonadotrophin hormone release (LH

and FSH). In addition, testosterone itself has an additive and direct suppressive effect on circulating levels of gonadotrophin via negative feedback on the hypothalamus or pituitary glands (Gross, 1980; Handa et al., 1986; Roselli et al., 1990). As described earlier, increasing estradiol or testosterone will reduce LH pulse amplitude and suppress FSH release (Jorgensen and Nilson, 2001; Martínez et al., 2004). These effects may cause the dominant follicle to regress and result in emergence of a new follicular wave due to a subsequent increase in circulating FSH (as a result of removal of suppressive effect of dominant follicle on FSH and subsequent metabolism of estradiol). The main focus of this thesis is to examine the effects of testosterone on follicular dynamics and endocrine parameters in beef cattle, and to evaluate the feasibility of testosterone as an alternative to estradiol for follicular wave synchronization. The review of literature is divided into three main sections: physiology and dynamics of follicle development in cattle, methods to control the emergence of follicular waves, and effects of testosterone on follicle development.

1.1 Physiology of follicular development in cattle

1.1.1 Ovarian follicles

The follicle is a basic unit in the ovary that maintains, nurtures and releases the oocyte as well as performs a series of endocrine and paracrine functions during its development (produces steroid and non-steroid hormones). Ovarian follicles can be classified based on their degree of development from primordial, primary, secondary, tertiary to preovulatory follicles (Van den Hurk, 2005). Primordial follicles are characterized by a single layer of squamous pre-granulosa cells that surround the oocyte.

These are present throughout the reproductive life of the cow and are considered a source for other types of follicles (Fortune, 2003). When squamous cells begin to develop in cuboidal cells (granulosa cells), the follicle becomes a primary follicle. At this stage, the initiation (activation) of follicular growth starts and the granulosa cells begin to divide (Braw-Tal and Yossefi, 1997). When the oocyte is surrounded by 2-6 layers of granulosa cells, the follicle is called a secondary follicle. Meanwhile, intercellular cavities appear in between the dividing granulosa cells and these are filled with fluid. This structure is called a follicular antrum (Van den Hurk, 2005) and the follicle is termed as a tertiary or antral follicle (Braw-Tal and Yossefi, 1997; Lussier et al., 1987). There are different development stages of antral follicles that end in a fully grown follicle (Graafiian or preovulatory follicle) of around 15 mm in the cow (Fortune, 1994). The duration of follicular development from activated primordial follicle to the preovulatory follicle is around 80 to 100 days in cattle (Britt, 1991).

1.1.2 Follicular dynamics in cattle

In 1960, Rajakoski was the first researcher who indicated that the growth of follicles in cattle occurs in a wave-like manner (Rajakoski, 1960). By using real-time, transrectal ultrasonography for ovarian examinations over days, many important events have been recognized in follicular growth in cattle and other domestic animals and the existence of follicular wave pattern was confirmed (Ginther et al., 1989a; Pierson and Ginther, 1987a; Pierson and Ginther, 1987b; Pierson and Ginther, 1984). In cattle, the estrous cycle consists of two or three follicular waves (Ginther et al., 1989a; Ginther et al., 1989b) and it is still not clear why this variation in number of waves occurs in cattle

(Jaiswal, 2004). Follicular waves exist during different physiological conditions such as the prepuberal period (Adams et al., 1994; Evans et al., 1994), pregnancy (Ginther et al., 1996b; Savio et al., 1990a), and postpartum period (Savio et al., 1990a; Savio et al., 1990b) in cattle. In 2-wave cycles, a follicular waves emerge on Day 0 (day of ovulation) and Day 10, on average, while in 3-wave cycles, wave emergence occurs on Days 0, 9 and 16 (Ginther et al., 1989b). In this regard, the interovulatory interval (that is, the duration between two sequential ovulations) is shorter ($P<0.01$) for 2-wave cycles (19.0±0.20 days) than 3-wave cycles (22.5 ±0.30; Jaiswal et al., 2009).

At the beginning of every wave, a group of small antral follicles (average, 24 follicles), detectable by ultrasonography at a diameter of 3-4 mm, grows synchronously (Adams, 1999). This group of follicles continues growing for 2-3 days when a single dominant follicle (diameter around 8 mm) keeps growing while the others regress (subordinate follicles) in a process called selection (Adams et al., 1993a; Ginther et al., 2000). After selection, an active dominant follicle produces more estradiol that exerts a negative feed back on FSH (review, Fortune, 1994; Mapletoft et al., 2002). As a result, the reduction in plasma FSH will prevent the growth of subordinate follicles and the emergence of a new follicular wave to accrue; this process is called dominance (Adams, 1999; Ireland et al., 2000).

When a dominant follicle grows during the luteal phase, it eventually undergoes atresia due to suppression of LH by progesterone produced from the corpus luteum (CL, Adams, 1999). However, the dominant follicle that grows during luteolysis will ovulate because the decreasing progesterone concentration allows LH to increase and support the dominant follicle growth (no suppressive effect; Adams et al., 1992b). The anovulatory

dominant follicle and all subordinate follicles go through three development phases. The growing phase is the stage during which the follicle grows linearly starting from the first detection to the day when the diameter of follicle stop to increase. In the second stage (static phase), the follicle keeps differentiating without any change in the diameter. When the follicle enters the regressing phase, its diameter begins to decrease until the follicle disappears (Ginther et al., 1989b).

1.1.3 Hormonal regulation of follicular development

The development and maturation of follicles in late growing phase occurs under the influence of growth factors and hormones produced from the hypothalamic-pituitary-ovarian axis (HPO axis). These hormones include hypothalamic gonadotrophin-releasing hormone (GnRH) that acts on the anterior pituitary gland to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Roche et al., 1997). In the ovaries, follicles produce estradiol and inhibin, whereas the CL secretes progesterone. The uterus secretes prostaglandin F2α (PGF) to control the life-span of the CL (Rathbone et al., 2001). Growth factors such as BMP-7, IGF-I and II, TGFβ (Transforming growth factor β), and basic fibroblast growth factor (bFGF) play important roles during different stages of follicle development (Webb et al, 2004; Ginther et al. 2001b; Skinner, 2005). In preantral follicles, TGF β and IGF family growth factors are involved directly in activation of follicles while in antral follicles, IGF-I and IGF-II support the growth and maturation of follicles (Webb et al, 2004).

Many studies have been done to understand the pattern of reproductive hormone secretion during the estrous cycle (Fig1.1). In the following section, these hormones are discussed in detail with reference to folliculogenesis.

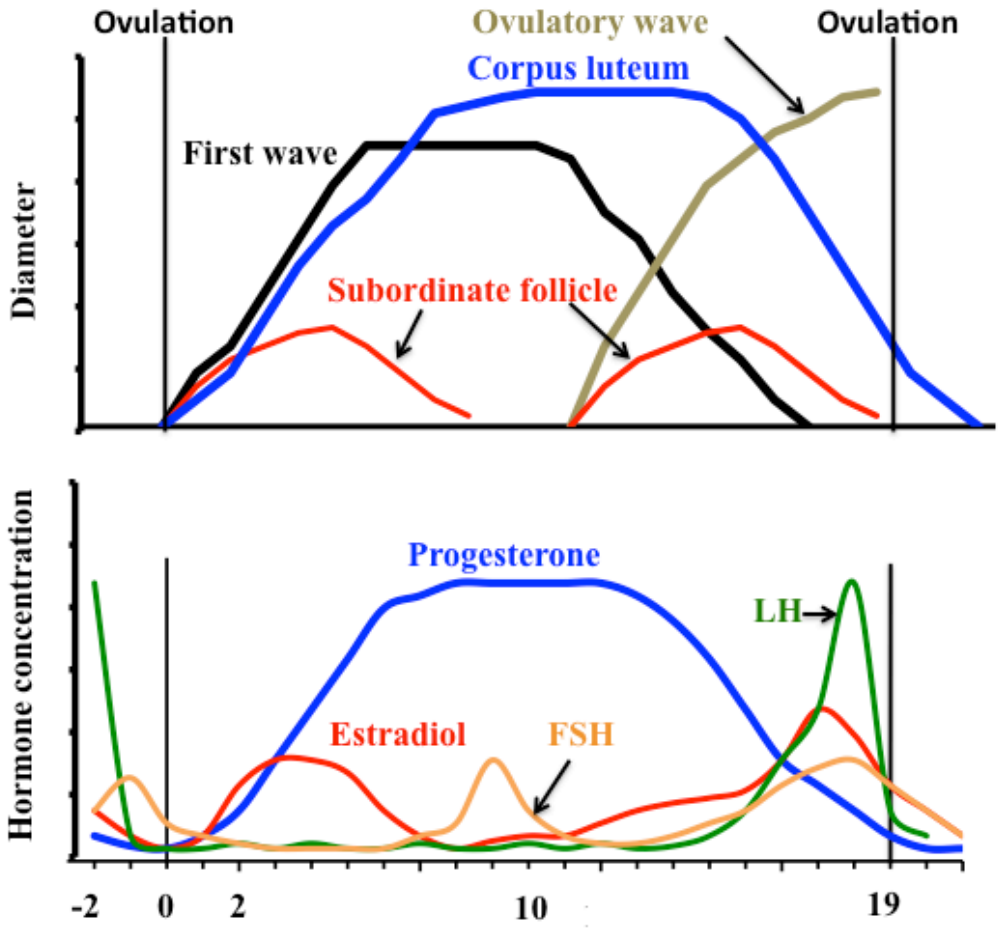

Fig.1.1 Temporal associations between the follicular wave emergence, dominant follicle diameter, corpus luteum diameter, ovulation (Fig A) and plasma concentrations of gonadotropins and steroid hormones (Fig. B) during a 2-wave estrous cycle in cattle.

1.1.3.1 Gonadotrophin-releasing hormone (GnRH)

GnRH is a decapeptide that is synthesized in the cell bodies of neurosecretory neurons of the hypothalamus and modulates gonadotroph activity in the anterior pituitary to secrete FSH and LH (reviwed at Jadav et al., 2010). The GnRH secretion occurs in a pulsatile manner, which results in secretion of LH and FSH in pulses, although only LH pulses tightly follow each and every GnRH pulse (Kaiser, 1997; Vizcarra et al., 1997). Ovarian steroids, estradiol and progesterone, regulate GnRH through feedback mechanisms depending on stage of the estrous cycle (Herbison, 1998; Jadav et al., 2010). Progesterone decreases GnRH pulsatility during the luteal phase (Price and Webb, 1988; Savio et al., 1993), while estradiol increases GnRH pulses during the follicular phase (Karsch and Evans, 1996). A surge in estradiol during estrus also causes surge in GnRH which eventually results in LH surge (Kesner et al., 1981; Martinez et al., 2007

1.1.3.2 Gonadotrophin hormones (FSH, LH)

Both LH and FSH are glycoproteins consisting of two polypeptide subunits, alpha and beta (Baenziger and Green, 1988). The alpha units in both hormones are common, but the beta subunit is different and determines the functionality of gonadotrophin (Norman and Litwack, 1997; Talmadge et al., 1983). The FSH receptors are exclusively present on the granulosa cells of follicles while LH receptors are present on both theca cells and granulosa cells (Xu et al., 1995; Bao and Garverick, 1998) as well as in luteal cells (Niswender,1981). The growth of preantral follicles is gonadotrophin independent and is believed to be regulated by growth factors (Wandji et al., 1996). In early stages of follicle development, the growth factors that are produced from oocytes

and granulosa cells play important roles in initiation of follicles growth (Wandji et al., 1992; Fortune et al., 2000). Some in vitro and in vivo studies suggested that FSH can increase the rate of preantral follicle development (Campbell et al., 2000; Hulshof et al., 1995). Antral follicles at early stages need FSH, for growth (Adams et al., 2008; Jaiswal et al., 2004). An increase in FSH is followed by appearance of 3-4 mm follicles (follicular wave emergence) a day later (Adams et al., 1992b; Jaiswal et al., 2004). Mean FSH concentrations increase 6-8 h before wave emergence and remain elevated for 8 h after wave emergence (Ginther et al., 1998; Jaiswal et al., 2004). Follicles, growing under the FSH support, start producing more estradiol and inhibin which act on the anterior pituitary to inhibit FSH secretion through a negative feedback mechanism (Gibbons et al., 1997). The decline in FSH concentration is closely related with the selection of one follicle as dominant and others as its subordinates (Adams et al., 1992a; 1993a; 1999). During this period, the number of LH receptors on granulosa cells increases (2-4 day after wave emergence), indicating the role of LH in dominant follicle selection (Campbell et al., 1995; Ginther et al., 2001a). After selection, the dominant follicle is dependent on LH for growth; suppression of pulsatile secretion of LH with a GnRH agonist prevented the dominant follicles from growing beyond 7-9 mm (Gong et al., 1996). On the contrary, subordinate follicles undergo atresia due to lack of FSH availability and LH receptors (Ginther et al., 2001a; Ginther et al., 2001b). Progesterone inhibits LH pulsatility, this results in the regression of the dominant follicle during anovulatory waves (Savio et al., 1993). The loss of dominance also results in a decrease in estradiol thus overcoming the negative feedback on FSH secretion from pituitary (Ginther et al., 2003). As a result, FSH concentration rises again and a new follicular wave emerges (Rathbone et al., 2001).

When progesterone concentration declines with the demise of the CL, the dominant follicle continues to grow to produce peak estradiol concentrations which cause an LH surge allowing the dominant follicle to ovulate (Rathbone et al., 2001). Pulsatility of LH also regulates progesterone production from lueal cells and was shown to have an essential role in maintenance of CL function (Niswender, 2000).

1.1.3.3 Ovarian hormones (estradiol, progesterone)

Progesterone and estradiol are steroid hormones derived from cholesterol. Within the follicle, cholesterol is converted to progesterone under influence of LH by stimulating steroidogenic acute regulatory protein (StAR) enzyme in theca and granulosa cells. Progesterone converts to testosterone in the theca cells, the latter is transported to granulosa cells for conversion to estradiol (Norman and Litwack, 1997). Figure 1.1 illustrates the key enzymes required for biosysnthesis of estradiol by the follicular wall. The primary source of circulating concentrations of progesterone is the CL, while that of estradiol is from ovarian follicles. Systemic concentrations of progesterone plays an important role in regulation of LH secretion by exerting negative feedback on hypothalamus-pituitary axis thereby reducing the concentration of GnRH and LH (reviewed in Adams et al., 1992b; Stock & Fortune, 1993; Mihm et al., 2002).

During mid-cycle, high concentrations of progesterone reduce LH pulse frequency and result in regression of the unovulatory follicle (Adams et al., 1992b; Adams, 1999; Savio et al., 1993). During luteolysis, progesterone concentrations decrease, thus removing the suppression on LH pulse frequency and allowing the dominant follicle to grow and the preovulatory surge of LH to accrue (Savio et al., 1993).

A differential effect on LH and FSH is seen during mid-cycle. While FSH increases periodically during high circulating levels of progesterone to induce new wave emergence (Adams et al., 1992b), LH does not increase when the progesterone concentration is high (Mihm et al., 2002).

Estradiol is synthesized in granulosa cells by aromatization of testosterone and is involved in regulation of gonadotrophin hormones by exerting negative and positive feedback on hypothalamus–pituitary axis (Youngquist and Threlfall, 2007). Estradiol modulates neurotransmitters in hypothalamus that control GnRH pulsatile secretion (reviewed in Smith and Jennes, 2001) and increases the responsiveness of the anterior pituitary gland to GnRH by elevating the density of GnRH receptors (Schoenemann et al., 1985). During the estrous cycle, changes in estradiol concentrations regulate the timing of wave emergence and ovulation through its effect on gonadotrophin hormones (Fortune, 1994; Mihm et al., 2002). Many studies have documented the role of estradiol in dominant follicle selection by suppressing FSH concentration to prevent smaller (subordinate) follicles to grow or a new wave to emerge (Adams and Pierson, 1995; Ginther et al., 1998; Ginther et al., 2000). When progesterone concentrations decline during proestrous, estradiol increases sufficiently to stimulate the hypothalamus to increase the frequency and amplitude of GnRH pulses (Hansel and Echternkamp, 1972). Consequently, LH frequency and amplitude are increased to stimulate follicular maturation and finally results in the LH peak which causes ovulation (Walters and Schallenberger, 1984).

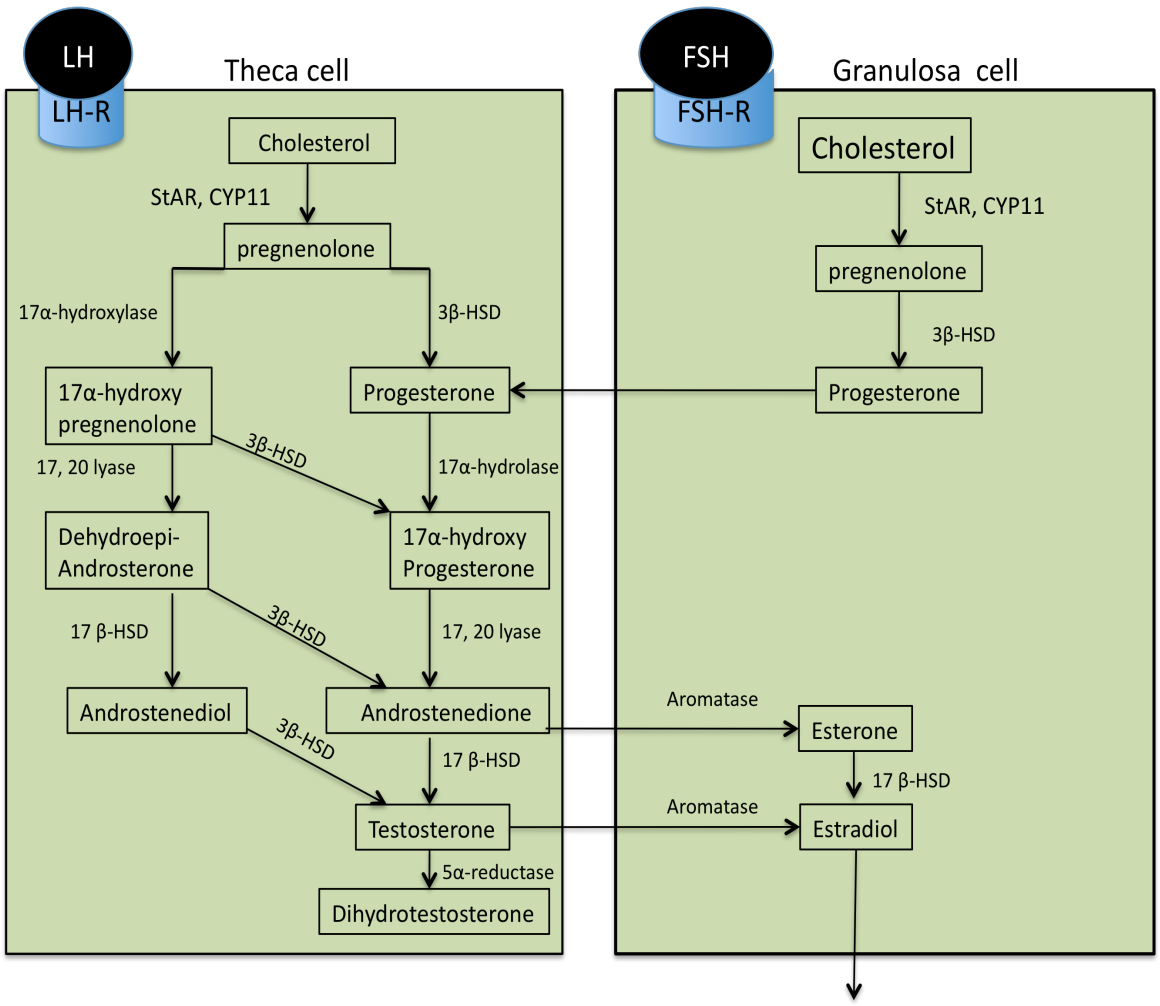

Fig. 1.2 Pathway of estradiol synthesis in ovarian follicles of mammals. Testosterone is the substrate for estradiol that is produced in the theca cell from cholesterol and from progesterone produced in granulosa cells, after a chain of enzymatic reactions. Granulosa cells lack 17α-hydroxylase/17, 20-lyase activity and therefore are unable to convert progesterone to testosterone or androstenedione. In granulosa cell, testosterone or androstenedione is aromatized to estradiol by the P450arom enzyme (figure is based on description from Hanukoglus, 1992).

1.2 Control of follicular wave emergence

Using effective methods to synchronize follicular wave emergence is necessary to increase the degree of synchrony of estrus and ovulation for field application of reproductive management techniques such as fixed-time artificial insemination (Bo et al., 1995b) and embryo transfer (Bo et al., 2002). Many protocols have been applied for this purpose. In the following paragraphs, there is a brief description about different protocols that are used for synchronization of follicular wave emergence in cattle.

1.2.1 Gonadotrophin-releasing hormone-based protocols

Administration of GnRH or its analogues results in LH release that causes ovulation and luteinization of dominant bovine follicle present on the day of treatment (Macmillan and Thatcher, 1991). After the dominant follicle ovulates due to GnRH treatment, a new wave will emerge within 2 days. Based on previous work by Twagiramungu et al (1995), a protocol was developed that involves GnRH and PGF for synchronization of ovulation in lactating dairy cows; this protocol was named Ovsynch (Pursley et al. 1995). It consists of two injections of GnRH (9 days apart) and PGF on Day 7. Fixed-time AI was performed 12-18 hr after the second GnRH injection. GnRH-based protocols appear to result in more synchronous ovulation than PGF alone. In one study, the Ovsynch protocol was compared with other protocols (single PGF injection or two PGF injections); Ovsynch-treated cows had higher pregnancy rates and fewer days open than other groups (33% and 107 d open for Ovsynch vs 12% and 122 d for the single injection, 11% and 129 d for two injections, and 6% and 116 d for untreated controls; Momcilovic et al., 1998). The Ovsynch protocol is used successfully with dairy

cows, but this program did not work very well with heifers (Pursley et al., 1995).

It was observed that the stage of follicle development or estrous cycle is a critical factor for ovulation to occur after the administration of GnRH (Martinez et al., 1999; Vasconcelos et al., 1999). However, confirm that GnRH does not always result in ovulation or luteinization of the extant dominant follicle and, hence, it does not consistently induce the emergence of a new follicular wave (reviewed in Adams 1998). GnRH or LH treatment was given 3, 6, or 9 days after ovulation which correspond to the mid-growing phase, early static phase, or the late static phase of the dominant follicle. An influence on wave emergence was evident - 89 , 67 and 22 % heifers ovulated in Day 3, 6 and 9 , respectively in GnRH. Treatment with GnRH or LH was not associated with a consistent interval to new wave emergence (e.g., 3.1±0.7 days after treatment on Day 3 vs 0.4±0.4 days when treated on Day 9) (Martinez et al., 1999). To improve the ovulation rate after first GnRH treatment, a presynchronization treatment has been suggested that would increase the probability of a dominant follicle capable of ovulating being present at that time; For this purpose, PGF is normally administered 12-14 days before the first GnRH (Moreira et al., 2001; Navanukraw et al., 2004) or a progestin device is placed in the vagina at the time of the first GnRH injection (Colazo et al., 2005a; Leitman et al., 2008).

1.2.2 Estradiol and progesterone

The combination of estradiol and progesterone has been used successfully for synchronization of estrus and ovulation (Bo et al., 1995b). In general, this protocol consists of an injection of estradiol at the time of insertion of a progesterone releasing

device (CIDR) that stays in place for 7-8 days. When the device is removed, a dose of prostaglandin is given to regress the existing CL and followed with AI 55-60 hours later (Martínez et al., 2004). It was observed that estradiol treatment suppresses follicle growth and this effect was profound when given in combination with progesterone (Bo et al., 1994). The treatment worked by inducing the suppression of FSH release (Bo et al., 2000). When estradiol is metabolized and blood levels decrease, FSH release results in emergence of a new follicular wave (Adams et al., 1992a; Bo et al., 1995a). The progesterone treatment during this method prevents ovulation by preventing the LH surge (Ireland and Roche, 1982) which can happen as a result of estradiol treatment. In addition, progestin treatment decreases LH release that assists in regression of the existing dominant follicle growth.

Different forms and esters of estradiol have been used for this purpose such as estradiol benzoate (Baruselli et al., 2004; Butler et al., 2011; Caccia and Bo, 1998; Martinez et al., 2005), estradiol valerate (D'Occhio et al., 1996; Mapletoft et al., 1999; Colazo et al., 2005b), unconjugated estradiol-17ß (Kastelic et al., 1997; Martínez et al., 2005) or estradiol cypionate (Colazo et al., 2002; Sa Filho et al., 2009; Thundathil et al., 1998). The different estradiol preparations have different abilities to synchronize wave emergence based on how long they remain in circulation and suppress FSH. In a comparative study, beef heifers were given 5 mg of estradiol-17ß or 1 mg of esrtadiol benzoate; both groups were treated with 100 mg of progesterone and a CIDR device was placed in the vagina. Estradiol-17ß and estradiol benzoate were equally effective to synchronized the follicular wave emergence and ovulation (Martínez et al., 2005). On the other hand, when estradiol valerate and estradiol cypionate were given as 5 mg and 1 mg

im injections, respectively, they showed lesser degree of synchronization for follicular wave emergence compare to estradiol-17ß (Colazo et al., 2002; Mapletoft et al., 1999). In addition, the ability of estradiol to synchronize the follicular waves can be affected by the dose of estradiol. When estradiol benzoate was given to CIDR–treated beef heifers at different dosages (1, 2.5, or 5 mg); the results indicated that 2.5 mg was more effective to synchronize the follicular wave than 5 mg (Caccia and Bo, 1998). In another study, 1 or 2 mg of estradiol valerate in beef cows at CIDR insertion reduced the period between treatment and next follicular wave emergence compare to treatment with 5 mg (Colazo et al., 2005b).

1.2.3 Follicle Ablation

Based on early studies involving electrocautery of ovarian follicles (Adams et al., 1993b; Ko et al., 1991), physical ablation of follicles ≥ 5mm from both ovaries by transvaginal ultrasound–guided follicle aspiration has been used to synchronize follicular waves (Bergfelt et al., 1994). Destruction of dominant follicle removes the suppressive effect of estradiol on FSH concentration and a new surge of FSH occurs which stimulate a new wave emergence within 2 days (Adams et al., 1992a). Removing the two largest follicles was as efficient to synchronize the wave emergence as ablating all follicles ≥ 5mm (Baracaldo et al. 2000). In another study, the ablation at random stages of estrous cycle followed by PGF 4 days later was used to synchronize ovulation (Bergfelt et al., 1994).

Follicle ablation was also used with superovulation and embryo transfer (Bergfelt et al., 1997). In one study, ablation of the dominant follicle 48 hr prior to start of

superovulation treatment resulted in higher number of ovulations and embryos compared to superstimulation without ablation (Kim et al., 2001). In early lactation high-producing dairy cows, follicle ablation prior to superovulation in combination with progesterone and estradiol treatment resulted in collection of an acceptable number of transferred embryos (Amiridis et al., 2006). Although follicle ablation offers an efficient protocol to synchronize follicular waves, this method needs skill and special equipment making it difficult to apply widely in field conditions.

1.3 Testosterone and follicular development

1.3.1 Synthesis and metabolism

Testosterone is a 19-carbon steroid hormone (Figure 1.3). In the female, the main sources of testosterone are theca cells and the adrenal gland (Vermeulen, 1998). LH controls the biosynthesis of testosterone in theca cells by activating the cyclic AMP pathway that increases transcription of encoding genes of enzymes for conversion of cholesterol to testosterone. Cytochrome P450 cholesterol side-chain cleavage (CYP450scc) is enzyme that converts cholesterol to pregnenolone (Figure 1.2b). Under the influence of two enzymes, 3ß-hydroxysteroid dehydrogenase (3ß-HSD; conversion of pregnenolone to 17α-hydroxypregnenolone) and 17α-hydroxylase 17,20 lyase (CYP17A1; progesterone and 17α-hydroxypregnenolone to androstenedione). Finally, androstenedione is converted to testosterone by 17ß-hydroxysteroid dehydrogenase (17ß-HSD). Testosterone or androstenedione is transport to granulosa cells for conversion to estradiol by aromatase (Craig et al., 2011).

Testosterone is metabolized mainly in the liver. Testosterone reaches the liver through systemic circulation where it undergoes biochemical reactions catalyzed by different enzymes leading to conversion of testosterone to inactive compounds such as androsterone and atiocholanolone (Henry and Norman, 2003).

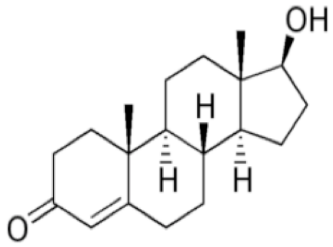

Fig. 1.3 Testosterone (C19 H28 O2) is a cyclo-pentano-phenanthrine structure consisting of 3 hexane rings and 1 pentane ring.

1.3.2 Role of androgen receptors

Although the role of testosterone as a substrate for estradiol in the female is well known, the direct effect of testosterone on the female reproductive system and follicle development is not clear. Androgen receptor (AR) protein exists in oocytes, granulosa cells, and theca cells of rodents, cattle, sheep, and pigs (Juengel et al., 2006; Walters et al., 2008). ARs are also present in luteinizing granulosa cells (Duffy et al., 1999) and increase after stimulation of ovulation (Chaffin et al., 1999). The expression of AR mRNA has been shown to change in granulosa cells depending on the stage of follicle development, e.g., AR mRNA is detected in preantral to early antral follicles in cattle (Hampton et al., 2004) and rats (Tetsuka et al., 1995), preantral to antral follicles in primates (Weil et al., 1998), pigs (Slomczynska and Tabarowski, 2001), and preantral

dominant follicles in humans (Suzuki et al , 1994; Horie et al., 1992). In addition, factors secreted from oocytes may modulate expression of the AR. In the large antral follicles of the rat, a gradient of AR immunostaining was detected - cumulus cells (close to oocyte) exhibited higher expression of AR protein compared with granulosa cells of peripheral layers (Tetsuka et al., 1995). Hormonal treatment has been reported to affect AR expression; in vivo treatment with testosterone resulted in increased AR expression in small antral follicles in primates and was correlated positively with expression of FSH receptors mRNA and proliferation, but negatively correlated with apoptosis (Weil et al., 1998). Furthermore, when granulosa cells from macaque preovulatory follicle were treated with hCG, AR expression increaseed 24 and 36 hr post-treatment (Chaffin et al., 1999) which may imply the role of androgen in preovulatory events. In general, these studies highlight the important role of AR activity in different stages of follicular development in different mammalian species.

1.3.3 Role of testosterone in follicular development

Many researchers have reported that androgens have stimulatory influence on early stages of follicular growth. When cattle preantral follicles were cultured and treated with testosterone, the transition of follicles from primary to secondary follicles was stimulated (Yang and Fortune, 2006). In vivo, when rhesus monkeys were treated with testosterone, the number of small follicles (primary, secondary, and tertiary follicles) was increased (Vendola et al., 1998) and the initiation of primordial follicles was stimulated (Vendola et al., 1999b).

Androgens also promote many ovarian growth factors that regulate the development of follicles. In vitro, testosterone treatment increases the granulosa cells responsiveness to FSH thereby increasing the production of estradiol (Hillier and De Zwart, 1981; Hillier et al., 1977). In vivo treatment of monkeys with testosterone elevated FSH receptor mRNA (FSH-R) expression (Weil et al., 1999) in the ovary; similarly dihydrotesteoterone increased the FSH-R expression in preovulatory follicles in gilts (Cardenas et al., 2002) which may modulate the responsiveness of the follicle to FSH (Drummond, 2006). In addition, testosterone and dihydrotestosterone (DHT) treatment in primate ovaries enhance IGF-1 and IGF-1 receptors mRNA (Vendola et al., 1999a; Vendola et al., 1999b). Based on the above-cited literature, androgens appear to have a direct and crucial role in regulation of follicular growth.

To determine the precise nature of effect of testosterone on follicular growth (i.e., whether observed effects are caused directly by testosterone or indirectly due to increased estradiol as a result of conversion from testosterone), researchers have conducted AR antagonist or aromatase blocker studies. Culturing preantral murine follicles with anti-androgen serum or AR antagonist caused slow development for follicles compare to controls (Murray et al., 1998). In cattle, testosterone increased the transition from primary to secondary follicles and the use of an AR blocker (flutamide) suppressed this transition (Yang and Fortune, 2006). Furthermore, using aromatase blockers in the presence of androgen did not stop follicular growth (Walters et al., 2008) supporting the direct role of androgens in follicular growth during the early stages. During the follicular phase in pigs, testosterone treatment increased the number of preovulatory follicles and subsequent corpora lutea (Cardenas and Pope, 1994; Cardenas and Pope, 1997).

There are also reports that androgens can have an inhibitory effect on follicles. In an in vitro study, treatment of large antral follicles from primate ovaries with testosterone caused a suppression of FSH-stimulated aromatase activity (Harlow et al., 1988). It was also observed that dihydrotestosterone suppressed LH receptor formation on granulosa cells of rats through a post-cAMP mechanism (Jia et al., 1985). The atretogenic effect of testosterone may be mediated through enhanced apoptosis in granulosa cells of antral follicles (Billig et al., 1993; Daniel and Armstrong, 1986; Hillier and Ross, 1979 ; Kaipia and Heueh, 1997; Yuki Okutsu et al., 2010), resulting in suppressed steroidogenesis and follicle development.

1.3.4 Effect of testosterone on gonadotrophin hormones

Many researchers have studied the effect of testosterone on gonadotrophins. When ovariectomized cows and mares were treated with testosterone, LH secretion decreased by 17% to 26% (Thompson et al., 1984). LH secretion was elevated by immununizing ewe (Campbell et al., 1990) or gilts (McKinnie et al., 1988) against androstenedione. Also, using 5 mg of testosterone propionate prevented ovulation due to a block of the LH surge in female rats (Hassani et al., 1978). In contrast, FSH concentrations increased when exogenous testosterone was given to ewe (Radford and Wallace, 1971) and gilts (Jimenez et al., 2008).

In ruminants, LH concentrations in the blood reflects the changing in LH beta subunit mRNAs expression (Aspden et al., 2003; Wise et al., 1985). Studies in a rodent model indicate that testosterone or other androgen treatments modulate LH beta subunit mRNA expression in the pituitary gland and GnRH-mediated LH release (Keri et al.,

1994; Krey et al., 1982; Yasin et al., 1996). These effects may be mediated by androgen receptor interaction with steroidogenic factor-1 (Curtin et al., 2001; Jorgensen and Nilson, 2001). These studies indicate that testosterone can have a direct negative impact on LH secretion through its effects on the pituitary gland.

1.3.5 Testosterone applications in farm animals

Synthetic androgen compounds are used widely in feedlots in North America as growth promotants to increase the productivity of beef cattle, but this application has been banned recently in European countries due to health concern from steroid hormone residues in meat and milk (Holtz, 2009; Lone, 1997). In Canada, many types of implants growth have been approved to use with heifers and steers (Table 1.1). Testosterone and other androgenic compounds were also used to androgenize cows for estrus detection (Kesler et al., 1995; Nix et al., 1998). In pigs, testosterone treatment has been used to increase ovulation rate. Testosterone administration to gilts starting from Day 17 or 18 of cycle resulted in an increased ovulation rate (Cardenas and Pope, 1994). Similarly, treatment from Day 13 of cycle increased ovulation rate and the percentage of blastocysts surviving (Cardenas and Pope, 1997).

Table 1.1. Growth promotant implants approved for use in Canadian beef cattle (based on Agriculture Manitoba website (January 2012):

http://www.gov.mb.ca/agriculture/livestock/beef/baa07s02.html

Implant name	Active elements	Dose (mg)	Anabolic period (Days)	Time of use	Animal use	
					Heifers	Steers
Ralgro	Zeranol	36	70	Birth	✓	✓
Synovex C	Progesterone Estradiol Benzoate	100 10	120	>45 days old	✓	✓
compudose	Estradiol -17	24	168	> 400 Ibs	✓	✓
Synovex S	Progesterone Estradiol Benzoate	200 20	120	> 400 Ibs		✓
Synovex H	Testosterone Estradiol Benzoate	200 20	120	> 400 Ibs	✓	
Synovex Plus	Trebolone acetate Estradiol Benzoate	200 28	120	> 400 Ibs	✓	✓
Revaoler S	Trebolone acetate Estradiol -17	120 24	120	550-990 Ibs		✓
Revaoler H	Trebolone acetate Estradiol -17	140 14	120	660-990 Ibs	✓	

2.0 GENERAL HYPOTHESIS AND OBJECTIVES

2.1 The objectives

The overall objective of this work was to determine the effect of exogenous testosterone treatment on ovarian function and hormone profiles in beef cattle. The specific objectives were:

Objective 1:(Chapter 3) To evaluate plasma pharmacokinetics of testosterone to understand the residual depletion in blood of heifers after two injections of testosterone in an oil-based solution.

Objective2: (Chapter 4) To determine the effect of testosterone administered at different phases of dominant follicle growth on:

i) The growth pattern of the extant dominant follicle, follicle numbers and emergence of subsequent follicular waves.

ii) Synchrony of wave emergence

iii) Systemic concentrations of FSH and LH

iv) Luteal function

2.2 The hypothesis

Administration of testosterone will shorten the life-span of the extant dominant follicle resulting in early and predictable emergence of a new follicular wave.

3.0 Plasma pharmacokinetics of testosterone in beef heifers after intramuscular administration of an oil-based preparation

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3.1 Abstract

Plasma pharmacokinetics of testosterone were examined to understand the residual depletion in blood of heifers after two treatments of unconjugated testosterone in an oil-based solution. Sixteen beef heifers were assigned to 4 groups (n=4 per group). Heifers were given intramuscular doses of 200 mg testosterone (in 4 mL canola oil) 12 hr apart on Day 1, 3 or 6 after ovulation (Day 0). The control group was given canola oil only. Blood samples were collected daily for 5 days and every second day thereafter until 13 days after treatment. From a subset of animals (n=2 per group), blood samples were also collected at 0, 2, 4, 8, 12, 14, 16, 24 and 36 hr after treatment. There were no differences among the testosterone treated groups ($P>0.14$) in plasma testosterone concentrations after treatment, therefore data were combined as a single treatment group (n=16). Plasma testosterone concentrations increased rapidly within 2 hours ($P<0.001$) and then decreased slowly over 13 days. Maximum plasma testosterone concentration was 26.9 ± 4.13 and 30.5 ± 4.07 ng/mL after the first and second doses, respectively. The time of maximum plasma concentration (T_{max}) was 2 h after first and second treatments. The half-life of testosterone in circulation was 3.2 ± 0.6 days. Plasma testosterone concentrations of combined treatment group were no longer different from controls at 13 days (0.5±0.08 and 0.2±0.05 ng/mL, respectively). In summary, intramuscular

administration of unconjugated (free) testosterone resulted in maximal plasma testosterone in within 2 hours, and a declined to pre-treatment concentrations within 13 days.

Keywords: Pharmacokinetics, testosterone, follicle development, cattle, anabolic steroid

3.2 Introduction

Many anabolic steroid hormones and their synthetic analogues have been used in food-producing animals to improve growth rate and fertility (Lone, 1997; Schmidely, 1993). The routes of administration vary depending on the purpose and duration of use, e.g., testosterone can be given by intramuscular injections or subcutaneous implants (Nix et al., 1998) to have an effect over days to months. In addition to wide-spread use of testosterone as a growth promotant, it has been used to androgenize cows for detection of estrus (Nix et al., 1998) or as an atretogenic agent to regress the ovulatory follicle (Rajamahendran and Manikkam, 1994). Recent studies have used relatively high or multiple doses of testosterone (200 - 1500 mg total dose), but the pharmacokinetics of intramuscular administration of oil-based testosterone were not examined (Heekin and Kiracofe, 1983; Nix et al., 1998; Rajamahendran and Manikkam, 1994). Studies to document the dynamics of testosterone absorption and clearance may help to predict and refine appropriate testosterone dosage regimens for use in research and management in cattle. In addition, the pharmacokinetics of testosterone in food animals will facilitate estimation of the rate of testosterone residue depletion in the body and provide an estimate of the appropriate withdrawal periods after testosterone treatment (Lone, 1997). Steroid hormone residues in meat and milk has become a critical issue in many countries

as well as the use such products as performance enhancing substances in animals and humans (EC, 2003).

In this study, we evaluated the plasma pharmacokinetics of testosterone to understand the residue depletion in blood of heifers after two intramuscular treatments of testosterone in an oil-based solution. This study was part of a larger experiment analyzing the effects of testosterone on follicular dynamics in heifers; the dose regimen was selected to provide a period of 24 to 48 hours of elevated testosterone concentrations in plasma.

3.3 Material methods

3.3.1 Experimental design

The experiment was conducted on 16 post-pubertal beef heifers (Hereford cross) between April and August, at the University of Saskatchewan Goodale Research Farm. The heifers were between 12 and 14 months of age and weighed 338 ± 8.1 Kg (range, 274-395 Kg). The heifers were fed alfalfa/grass hay and had water ad libitum during the experimental period. At the start of experiment, ovaries of all heifers were examined by transrectal ultrasonography (B-mode, 7.5 MHz linear-array transducer, MyLab Five, ESAOTE, Genova Italy) to detect the presence of a corpus luteum (CL). Heifers that had a CL, were given prostaglandin F2α im (500 μg of cloprostenol, Estrumate™, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) to cause regression of extant CL (Hafs et al., 1974). Heifers were examined daily by transrectal ultrasonography to detect the day of ovulation (Day 0). Heifers were assigned randomly to one of four groups after ovulation to examine the effects of stage of the estrous cycle on plasma testosterone

kinetics. Heifers were treated with testosterone on Day 1, 3 or 6 after ovulation (n= 4 per group). The remaining heifers (n=4) were placed in a negative control group that received placebo injections. The experiment was approved by the University of Saskatchewan's Animal Research Ethics Board, and adheres to the Canadian Council on Animal Care guidelines for humane animal use.

3.3.2 Testosterone administration

Five gram of unconjugated (i.e., non-esterified or free form) testosterone (Catalog # T-1500; Sigma Chemical Company, St. Louis, MO) was dissolved in 20 mL of benzyl alcohol and mixed with canola oil (No name®, Montreal, Quebec, Canada) to a volume of 100 mL (50 mg testosterone per mL solution). Heifers were given an intramuscular dose of 4 mL im (200 mg testosterone) in the gluteal muscles, for a dose range of 0.50 to 0.73 mg testosterone/Kg body weight. The second dose was given 12 hours after the first. The dose of testosterone was estimated based on the results of previous studies in heifers (Nix et al., 1998; Rajamahendran and Manikkam, 1994). The control group was given 4 mL im of canola oil, twice at an interval of 12 hours.

3.3.3 Blood collection:

Blood samples were collected via jugular venipuncture into heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) from all heifers before the initial testosterone treatment, and at 1, 2, 3, 4, 5, 7, 9, 11, and 13 days after the initial testosterone treatment. In addition, two heifers in each treatment group and 3 control heifers were selected randomly for intensive sampling over the first 24 hr after

testosterone administration. Blood samples were collected at 0, 2, 4, 8, 12, 14, 16, 24, and 36 hr after the first testosterone treatment. The sample at 12 h was collected immediately before the second testosterone treatment). Blood samples were centrifuged at 3000 x g for 10 minutes, and plasma was separated and stored in plastic tubes at -20 °C until assayed.

3.3.4 Plasma testosterone radioimmunoassay (RIA):

A previously-validated solid-phase RIA procedure (Coat-A-Count Total Testosterone, Diagnostic Products, Los Angeles, California) was used to determine total plasma testosterone concentrations (Rawlings and Evans, 1995). The lower and upper limits of quantification were 0.1 ng/mL and 10 ng/mL, respectively. The intra-assay coefficient of variation was 4.8, 8.0, and 12.7% for low (1.61 ng/mL), medium (4.4 ng/mL), and high (9.8 ng/mL) reference samples. The inter-assay coefficient of variation was 12.0, 14.2, 16.1% for low, medium and high reference samples, respectively.

3.3.5 Pharmacokinetic analyses:

The plasma testosterone concentration depletion rate for each heifer was analyzed using linear regression (Microsoft Excel 2007, Microsoft, Redmond, WA). Peak concentrations in plasma (C_{max}) and times to peak concentration (T_{max}) were determined using observed values from the intensive bleed concentration versus time curve. The elimination half life ($T_{1/2elim}$) of testosterone was calculated using the following formula:

$$T_{1/2elim} = \ln(2) / k_{el}$$

where k_{el} is the elimination rate constant (determined as the slope of the natural logarithmic plasma concentration versus time curve). k_{el} was determined using linear

regression of the elimination phase of each individual heifer's plasma testosterone concentration versus time curve. The elimination phase was comprised of plasma samples starting from 24 hours (Day 1) and included the subsequent daily plasma samples (Days 2 – 13). The mean elimination half life of testosterone was calculated from the individual half-lives of all treated heifers.

3.3.6 Statistical analyses:

Plasma testosterone data were expressed as mean ± SEM. A repeated measurement model (Proc Mixed model on SAS, version 9.2) was used to determine whether differences in mean testosterone concentrations between treatment and control groups were different over time ($P < 0.05$). Based on Akaike information criterion, eight covariance matrices (AR, TOEP, ARH (1), TOEPH, CS, HF, SIMPLE, ANTE1) were examined to select the one best suited for final analysis (Littell et al., 1998). In an initial analysis, mean plasma testosterone concentrations over time between the treatment groups were compared (i.e., excluding control group) to determine if the data from these groups could be combined into one (combined) treatment group.

3.4 Results

Mean plasma testosterone concentrations in the three treatment groups did not differ over time ($P = 0.47$ and $P = 0.14$ for daily and hourly plasma samples, respectively), and groups were therefore combined into one treatment group. The mean testosterone concentration was higher testosterone-treated heifers compared to the control group (Fig. 1) for both daily samples ($P < 0.001$) and hourly samples ($P < 0.001$).

In the six intensively-sampled heifers treated with testosterone, the observed maximum plasma testosterone concentration ranged from 18.4 to 41.7 ng/mL (mean ± S.E.M.: 28.7 ± 2.82 ng/mL) after the first treatment and 20.0 to 42.5 ng/mL (30.5 ± 4.07 ng/mL) after the second treatment. Mean plasma testosterone concentrations immediately before the second treatment (12 hr after the initial treatment) was 10.9 ± 1.92 ng/mL (range: 19.2 to 7.2ng/mL) in testosterone-treated heifers, and was higher ($P<0.001$) than the pre-treatment (time 0) sample in control heifers (0.02.± 0.006 ng/mL). The value of plasma testosterone concentrations after the second dose resulted from interaction between the accumulation of testosterone from the first and second injections . Plasma testosterone concentrations within 36 hr after the first testosterone treatment (i.e., 24 hr after the second injection) were 8.1 ± 1.37 ng/mL (range: 4.4 to 10.1 ng/mL)

The time of maximum plasma concentration (T_{max}) was 2 hr in 4 of 6 heifers after the first injection and 4hr in the remaining 2 heifers. The time of maximum plasma (treatment) was 2hr in 5 of heifers after the second injection and 4 hr in the sixth heifer. The other 3 had an observed T_{max} at 4 hours. The mean elimination half-life of testosterone in the combined treatment group was 3.2 ± 0.6 days. On Day 13, there was no significant difference in mean testosterone concentrations between the treatment group and control (0.5±0.08 and 0.2±0.05 ng/mL respectively).

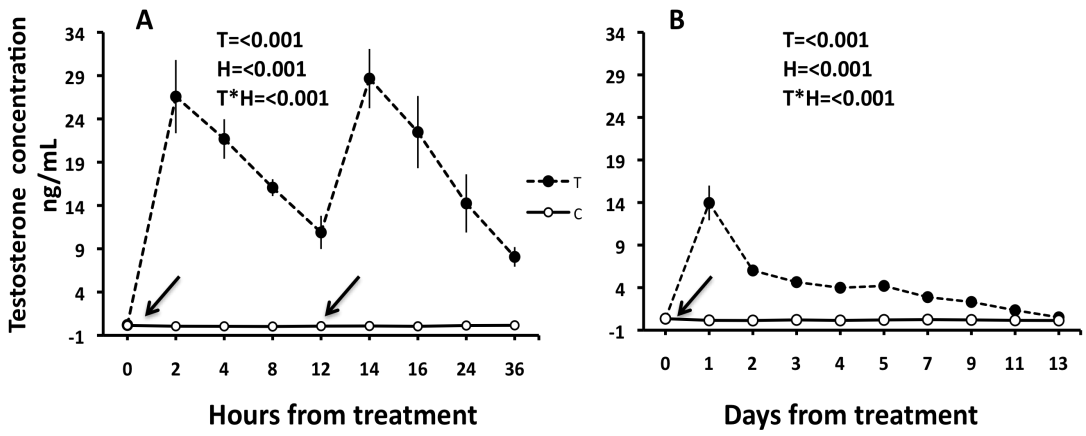

Fig. 3.1 Changes in plasma testosterone concentrations (mean ± SEM) in heifers for the first 36 h (Fig A) and 13 days (Fig. B) after intramuscular administration of unconjugated testosterone in oil (n= 12) or placebo (n=3). Two treatments were given at a 12-hour interval (indicated by arrows). T=Treatment, D=Day, H=hours, T*D and T*H = interaction between time and treatment.

3.5 Discussion

Testosterone has been used in farm animals to improve growth and daily weight gain (anabolic effect), to prevent estrus expression in heifers and to create teaser-cows for estrus detection . For these purposes, long-acting, esterified forms of testosterone have been used either as oil-based injections or subcutaneous implants, and the pharmacokinetics of these forms have been studied (Lone, 1997). The objective of this study was evaluating some of the pharmacodynamic parameters of short-acting, non-esterified testosterone in cattle and provide drug distribution data for study of reproductive functions. To keep testosterone concentration above 5 ng/mL for the first 36 hours followed by decline (to obtain biological effects for the purposes of the follicle dynamics study) in this study, two injections of testosterone were required at 12 hr

intervals. We found that the maximum plasma testosterone concentration (C_{max}) was between 2 and 4 hr after a single intramuscular injection of 200 mg of oil-based unconjugated (free) testosterone and the mean distribution half-life ($t_{½}$) of testosterone was 3.2 ± 0.6 days and the drug depleted to baseline within 2 weeks.

The absorption of esterified testosterone into bloodstream is slow because the ester group lowers water solubility of testosterone leading to prolonged release into circulation. For this reason, most anabolic androgens used as growth promotants in cattle or other farm animals are esters (Nix et al., 1998; Lone, 1997; Pugh et al., 2004; Traish et al., 2009). This study was part of research that focused on the effects of testosterone on ovarian follicular dynamics, dominant follicle regression and synchronization of follicular waves. For that reason, we needed a short-acting testosterone preparation that would provide a rapid increase in the blood within hours, maintain relatively high-levels for 36 hr followed by rapid decrease to baseline. Therefore, unconjugated testosterone was selected and injected twice at 12 hr interval. Testosterone concentrations increased rapidly after injection to reach peak values after 2 hours and plasma levels remained above 17.43 ng/mL for the first 36 hrs in all animals. The pattern of testosterone increase in blood in this study was consistent with results of another study (Rajamahendran and Manikkam, 1994) where the same form testosterone was used in heifers. The elimination half life of testosterone in this study also illustrates the difference in testosterone pharmacokinetics from other studies where ester formulations were used. In this study, the elimination half-life was testosterone was relatively short (3.2 days) compared with testosterone esters e.g., 21.7±1.1 days for testosterone undecanoate (Zhang et al., 1998) and 10.3±1.1 days for testosterone enanthate (Partsch et al., 1995).

The European Union has banned several steroid hormones that could be used as growth promotants in livestock because of concerns of the effects of milk and meat residues on human health (EC, 1996). Many types of growth implants contain ester groups of anabolic steroids (Lone, 1997) that cause release of the hormone slowly over a prolonged period of time; the communication between the tissues and steroid hormones over time increases the residual compounds in the tissues. In our study, injectable unconjugated testosterone, which results in rapid release into the circulation, also resulted in rapid testosterone depletion; testosterone concentrations in treatment groups returned to pretreatment concentrations within 13 days. Although the residual of testosterone in tissues was not examined in this study, the rapid decline of testosterone in plasma indicates that residuals in tissue may also be reduced. Several studies have alluded to the positive relationship between specific compounds in plasma and residuals in tissue. In a study in pigs, researchers investigated the relationship between the level of olaquindox (growth promoter) in plasma and tissue through a marker residue (methyl-3quinoxaline-2-carboxylic acid); they found the correlation coefficient between tissue and plasma concentrations was 0.92 (Yang et al., 2010). In another study, broiler chickens were given moxifloxacin intramuscularly; the drug disappeared from plasma and tissue within 120 hours post-treatment (Goudah, 2009). These studies indicated a positive relationship between the level of drug in the blood and its residual in tissue. In this regard, using unconjugated testosterone as a treatment in cattle may be acceptable if the short duration of drug in plasma is considered and if the withdrawal period is longer than 2 weeks. However, we could not approve same relationship between the residuals of drugs in plasma and tissue; more research is needed to determine the residual of drug in the tissue.

In summary, administration of 200 mg of unconjugated testosterone in oil resulted in elevated plasma concentrations within 2 to 4 hr with a T_{max} of 26.9 ± 4.13 ng/mL; plasma levels decreased to 10.9 ± 1.92 ng/mL by 12 hr. By giving a second injection of 200 mg testosterone 12 hr after first injection, plasma testosterone concentration were maintained above 5 ng/mL for a 48 hour period. Thereafter, plasma testosterone concentrations decreased quickly with depletion half-life of 3.2 days in bloodstream and levels returned to baseline by 13 days. The relatively short period of elevated plasma concentrations of testosterone suggest its possible use in treatment protocols in cattle, but intensive pharmacokinetic studies are needed to determine the fate of drug in tissues.

4.0 Effect of Testosterone on Dominant follicle Growth and Luteal Dynamics in Beef Heifers

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4.1 Abstract:

The study was designed to determine the effects of testosterone on ovarian function in cattle. We tested the hypothesis that administration of testosterone will shorten the life-span of the dominant follicle resulting in early emergence of a new follicular wave. Experiment 1 was conducted to select the testosterone formulation (free or esterified testosterone) that would increase circulating testosterone concentrations within a very short interval and would last for at least 24 hours. Results indicated that the treatment with the unconjugated form of testosterone (free testosterone; n=4) in oil increases the testosterone concentration in blood very rapidly compared to an esterified form (testosterone enanthate; n=4). Therefore, unconjugated testosterone in oil was selected for use in Experiment 2 in which the effect of exogenous testosterone treatment on different days of the first follicular wave on ovarian function in cattle was examined. Beef heifers were assigned at random to four groups (n=6 per group). Heifers were given an intramuscular injection of 200 mg of unconjugated testosterone in 4 mL canola oil twice at 12 h intervals on the Days 1, 3 or 6 after ovulation (Day 0). The control group

was given im canola oil only (n=6; n = 2 on each of the 3 treatment days). Ovarian structures were monitored daily by transrectal ultrasonography for one interovulatory interval. Following treatment, the dominant follicle grew slower for the next 5 days in treatment Day 1(T1; *P*=0.05) and Day 3(T3; *P*=0.06) groups compared to the control group, while the dominant follicle in Day 6 (T6) group regressed more slowly (P=0.02) compared to the control group. The diameter profile of the dominant follicle of the post-treatment wave (Wave 2) and the ovulatory wave did not differ between treatment groups and the control group. Overall, testosterone treatment extended the duration of current wave (Wave 1; *P*=<0.001) and the post-treatment wave (Wave 2; *P*=0.02) compared with the control group. Although the interovulatory interval was not affected by the treatments, the proportion of 2-wave cycles tended to be higher (*P*=0.08) after testosterone treatment compared to the control group. Mean LH concentrations were lower in T1 and T3 groups compared to the control group, while LH concentrations were not affected in the T6 group. Testosterone treatment did not affect circulating FSH concentrations, or the number of follicles (4-5 mm, 6-8 mm or ≥ 9 mm) within the first follicular wave. Corpus luteum diameter during the first 12 days of the treatment cycle did not differ between testosterone-treated and control groups, but rate of regression of corpus luteum during the regression phase (Days 15-18) in treatment groups was faster (*P*<0.05) compare to the control group. In conclusion, our hypothesis that testosterone treatment will shorten the life-span of dominant follicle was not supported. Treatment with testosterone resulted in slower growth of the dominant follicle when treatment was initiated at or before the time of dominant follicle selection, and slower regression of the dominant follicle when treatment was initiated after selection during early static phase. In

addition, treatment with testosterone delayed the emergence of the next follicular wave irrespective of the status of the dominant follicle at the time of treatment.

Keywords: Testosterone, follicle development, cattle, corpus luteum, ultrasonography, follicular and luteal dynamics, FSH, LH,

4.2 Introduction

Synchronization of follicular wave emergence has garnered the interest of researchers because it is a prerequisite in many reproductive techniques such as ovarian superstimulation, and fixed-time artificial insemination. Most methods to control follicular waves depend on removing the effect of the existing dominant follicle by using hormonal treatment (progesterone, estradiol; Martínez et al., 2004), ultrasound-guided follicle ablation (Bergfelt et al., 1994), or inducing ovulation with GnRH (Twagiramungu et al, 1995; Pursley et al., 1995; Martinez et al., 1999). The Ovsynch protocol is one of the most commonly used methods for ovarian synchronization (Pursley et al., 1995; Whisnant et al., 2000) and involves GnRH treatment to induce ovulation of the dominant follicle followed by emergence of a new follicular wave 2 days later (Twagiramungu et al., 1995)., Cows are treated with .prostaglandin on Day 7 after the first GnRH to cause luteolysis. A second GnRH injection is given on Day 9 to induce ovulation for fixed-time artificial insemination. However, the effect of GnRH-based protocols is dependent on the stage of development of the dominant follicle (Martinez et al., 1999) or stage of the estrous cycle (Vasconcelos et al., 1999) when treatment is given. Estradiol alone or in combination with progesterone has been highly effective in synchronizing follicle wave emergence in cattle (Martínez et al., 2004) because it does not depend on the status of the

extant dominant follicle. Exogenous estradiol reduces FSH secretion, and LH pulse frequency and amplitude in cattle (Ginther et al., 2000) leading to regression of antral follicles and emergence of a new wave once the suppressive effect of estradiol on FSH ceases, on average 4 days later (Bo et al., 1995b; Bo et al., 1995a). However, many countries including those in European Union, have banned the use of estradiol in food-producing animals because of potential human health concerns of hormone residues in meat and milk (Lane et al., 2008). As a result, finding alternative treatment that can be used effectively for the synchronization of follicular wave emergence has become a pressing need.

Testosterone may be an acceptable alternative to estradiol. Testosterone is involved in the regulation of ovarian function (Walters et al., 2008) through activation of androgen receptors that are localized in granulosa cells and stromal cells (Drummond, 2006). Further, testosterone increases aromatase enzyme expression in granulosa cells in vitro (Wu et al., 2011). Therefore, exogenous testosterone may increase endogenous estradiol concentration by increasing aromatase activity and by providing readily available substrate for aromatization. This, in turn, would exert a negative feedback effect on the hypothalamic-pituitary axis leading to suppression of gonadotrophin hormone (LH and FSH) release (Ginther et al., 2000; Roos et al., 1980; Toranzo et al., 1989). In addition, testosterone can have additive direct suppressive effect on circulating levels of gonadotrophins via direct negative feedback on the hypothalamus and pituitary glands (Gross, 1980; Handa et al., 1986; Roselli et al., 1990). Since testosterone reduces LH pulse amplitude and suppress FSH release (Jorgensen and Nilson, 2001; Martínez et al., 2004) and may cause the extant dominant follicle to regress.

Testosterone has been used as a treatment in farm animals for different purposes. It has been injected in different forms to produce androgenized cows that can be used for the detection of estrus (Nix et al., 1998). Compared to implants, injectable testosterone was found to be a reliable method to induce "teaser animals" (Heekin, 1983; Kiser et al., 1977). Heifers treated with 200 mg of testosterone per day for 4 days during follicular phase induced preovulatory follicle atresia (Rajamahendran and Manikkam, 1994). In addition, testosterone has been used to increase ovulation rate in postpubertal gilts (Cardenas and Pope, 1994; Cardenas and Pope, 2002). Based on above-cited information, it is reasonable to presume that testosterone may synchronize the emergence of the next follicular wave by causing demise of the dominant follicle present at the time of treatment.

The present study was designed to find alternative protocols to control ovarian follicular wave dynamics in cattle, and in particular, determine the effect of testosterone on follicular dynamics. The objective of this experiment was to determine the effect of exogenous testosterone treatment on different days of the first follicular wave on ovarian function in cattle. We tested the hypothesis that the administration of testosterone will shorten the life-span of the extant dominant follicle resulting in early emergence of a new follicular wave.

4.3 Materials and methods

Two experiments were conducted between April and August, at the University of Saskatchewan Goodale Research Farm. Eight Holstein cows were used in Experiment 1, and 24 post-pubertal beef (Hereford-cross) heifers were used in Experiment 2. The

animals were fed alfalfa/grass hay and had water ad libitum during the experimental period. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adheres to the Canadian Council on Animal Care guidelines for humane animal use.

4.3.1 Hormone preparations:

Five grams of unconjugated (free) testosterone (Catalog # T-1500; Sigma Chemical Company, St. Louis, MO) were dissolved in 20 mL of benzyl alcohol and then mixed with canola oil (No name®, Marques de Commerce de Loblaws INC., Montreal, Quebec, Canada) to make a final volume of 100 mL (50 mg testosterone per mL). Testosterone enanthate was supplied by the manufacturer in liquid form containing 200 mg of testosterone enanthate per mL of sesame oil.

4.3.2 Experiment 1 - Pilot study

The aim of the pilot study was to select a suitable testosterone preparation and dose for Experiment 2. We measured plasma testosterone concentrations after cows were treated with one or two injections of 200 mg of unconjugated testosterone (free-form) or 200 mg of esterified form of testosterone (testosterone enanthate, Delatestryl^{TM}, Theramed Corporation, Mississauga, Canada). On random days of the follicular wave, all follicle ≥5 mm were aspirated using transvaginal ultrasound-guided puncture (ablation). Cows (n=2 per group) were assigned to testosterone or testosterone enanthate groups and were given either a single intramuscular (im) injection 4 days after ablation or two im injections with 24 hours apart starting 3 days after ablation. Blood samples were collected

by jugular venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacationer Systems, Franklin Lakes, NJ, USA) at 0, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 288 hr post-treatment (time of treatment = 0hr). Five additional blood samples were collected 25, 26, 28, and 36 hr post-second treatment for groups that were given two injections. Plasma samples were stored at -20 °C until assayed for testosterone.

4.3.3 Experiment 2 – Effects on ovarian and hormonal dynamics

4.3.3.1 Animals and treatments:

Twenty-four beef heifers (Hereford-cross) between 12 and 14 months of age and average weighing between 273 and 404 Kg (340 ± 0.5Kg) were selected from a herd of 40 heifers following transrectal ovarian ultrasonography (B-mode, 7.5 MHz linear-array transducer, MyLab^{TM}Five, ESAOTE, Genova, Italy) based on the presence of a CL.

All 24 heifers were given prostaglandin (500 μg of cloprostenol, Estrumate^{TM}, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) im to regress the CL (Hafs et al., 1974). Heifers were examined daily by transrectal ultrasonography to detect the day of ovulation (Day 0). After ovulation, heifers were assigned randomly to one of four groups. Heifers in the three treatment groups were given 200 mg of unconjugated testosterone in 4 mL canola oil im twice, at 12 h intervals, on Days 1, 3 or 6 of first follicular wave. The preparation and dose of testosterone was selected based on results from Experiment 1. The control group (n=6) was given 4 mL canola oil im on the corresponding days of treatment, n=2 heifers each on Day 1, Day 3 or Day 6.

4.3.3.2 Ovarian ultrasonography:

Ovarian structures were monitored daily by transrectal ultrasonography during the period between two consecutive ovulations (interovulatory interval). Sketches of both ovaries were made during each examination to record the number, position and diameter of follicles ≥ 4 mm and CL diameter during one interovulatory interval. The total number of follicles ≥ 4 mm in both ovaries was counted starting from wave mergence. Ovulation was determined by the disappearance of a follicle ≥ 8 mm between two consequential examinations, followed by formation of a CL (Pierson and Ginther, 1987b). Wave emergence was determined retrospectively and was defined as the day when the future dominant follicle was first detected between 4 and 5 mm in diameter with the a concurrent increase in the number of 4-5 mm follicles (wave emergence; Ginther et al., 1996a). The dominant follicle was recognized as the largest antral follicle after selection (Ginther et al., 1989b). Day of CL regression was defined as the first day when luteal diameter was seen to decrease constantly over 3 consecutive days (Adams et al., 1993a). The interwave interval of wave 1 and 2 was the interval between the emergences of two consecutive waves while in the ovulatory wave, it was the interval between the wave emergence and ovulation

4.3.3.3 Blood collection:

Blood samples were collected into heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) twice daily (morning and evening) starting from pre-treatment ovulation until post-treatment ovulation (interovulatory interval). Blood samples were also collected at 0, 2, 4, 8, 12, 14, 16, 24, 36, 48, 60, and

72 h post-treatment into vacutainer tubes. All samples were centrifuged at 3000 x g for 10 min; plasma was separated and stored in plastic tubes at -20 °C until assayed.

4.3.4 Hormone radioimmunoassay:

A solid-phase radioimmunoassay (Coat-A-Count Total Testosterone, TKTT2, Diagnostic Products) was used to determine total testosterone concentrations (Rawlings and Evans, 1995). For Experiment 1, all samples were analyzed in a single assay. The range of the standard curve was 0.1 to 10 ng/mL. The intra-assay coefficients of variation were 6.5%, 3.5%, and 13.2% for low (1.25 ng/mL), medium (3.50 ng/mL), and high (7.09 ng/mL) reference samples. Similarly, all samples from Experiment 2 were analyzed in a single assay and the intra-assay coefficients of variation were 4.79%, 8.04%, and 12.65% for low (mean, 1.61 ng/mL), medium (mean, 4.45 ng/mL), and high (mean, 9.80 ng/mL) reference samples.

Plasma FSH concentrations were measured in duplicate using a double-antibody radioimmunoassay (Rawlings et al., 1984). The primary antibody was NIDDK-anti-oFSH-1 and the concentrations were expressed as USDA bovine FSH-Il units. The range of the standard curve was 0.12 to 16 ng/mL. The intra-assay coefficients of variation were 10.7% for low reference samples (mean, 1.61 ng/mL) and 6.8% for high reference samples (mean 3.33 ng/mL).

Plasma LH concentrations were measured in duplicate using a double-antibody radioimmunoassay and were expressed as NIDDK-bLH4 units (Evans et al., 1994a). The range of the standard curve was 0.06 to 8.0 ng/mL. The intra-assay coefficients of variation were 10.9% for low reference samples (mean, 1.16 ng/mL) and 8.1% for high

reference samples (mean, 2.48 ng/mL).

Progesterone concentrations were determined in a single assay using a solid-phase radioimmunoassay (Coat-A-Count®, Catalog number TKPG5, Diagnostics Products Corporation, Los Angeles, USA) with a sample volume of 100 μL (Rawlings et al., 1984). The range of the standard curve was 0.1 to 40.0 ng/mL. The intra-assay coefficients of variation were 9.6% (mean, 1.47 ng/mL), 7.06% (mean, 3.50 ng/mL) and 6.40% (mean, 24.27 ng/mL) for low, medium and high reference samples, respectively.

4.3.5 Statistical analyses:

Data were centralized to wave emergence and analyzed by repeated measures procedures, using the Statistical Analysis System software package (SAS program, version 9.2). Hormone data (LH, FSH, testosterone, progesterone) and follicular data (diameter of dominant follicles and CL, number of follicles within a wave) were analyzed using Proc Mixed repeated measurement for effect of testosterone treatment, day, and the interaction between day and treatment. The concentrations of LH and FSH before (Day -1 and Day 0) and after (Days 1 to 5) treatment were analyzed using one-way ANOVA between treatment combined group (T1, T3 and T6 combined) and the control group. To identify changes in follicle numbers, follicles were categorized according to diameter (4-5 mm, 6-8 mm and ≥ 9 mm). During an interovulatory interval, data for CL diameter were divided into three portions (Days 1-6, 7-12 and 13-18 after ovulation) to represent growing, functional and regression phases. Data for each portion were analyzed separately. Single point measurements (interovulatory interval, interwave interval, number of waves in the interovulatory interval, day of cuprus luteum regression) were

analyzed by one-way analysis of variance and/or two sample t-test analysis. The growth rate of dominant follicle for each day was calculated by subtracting the diameter of the previous day from the current day. Proportions (i.e., proportion of two-wave cycles) were analyzed by Glimmix procedure using binary distribution. When no differences were found among treatment groups, data were combined and re-analyzed as a single treatment group (combined treatment group).

To determine synchrony of emergence of the second wave, data on day of emergence was tested using Bartlett test to check the homogeneity of variance among groups. The deviation on day of wave emergence from the group mean was calculated. In order to understand the variation in time of emergence of the second wave in treatment groups .The heifers were assigned to two groups: One group included heifers in which emergence of the second wave occurred before Day 12 of the interovulatory interval (TN, n= 9) and the other group included heifers in which the second wave emerged after Day 12 (TD, n=8). Data on LH and FSH concentrations were transformed to a percentage of the mean concentration determined in the first two samples (i.e., Days 0 and 1) for each individual heifer before analysis of variance for repeated measures. One heifer was excluded from the T6 group because of very early wave emergence. All values are expressed as mean ± SEM and probability (P) values of ≤ 0.05 were considered statistically significant difference.

4.4 Results

4.4.1 Experiment 1- Pilot Study

Mean plasma concentrations of testosterone across time for Experiment 1 are shown in Figure 1. Plasma testosterone profiles differed between the two types of testosterone when given either as a single (Fig. 5.1A, P=0.006) or two injections (Fig. 5.1B; P= 0.01). Following the first injection of unconjugated (free) testosterone, plasma testosterone levels increased more than 10 fold within 1 h from 1.1 ± 0.39 to 15.7 ± 2.01 ng/mL followed by a decrease to 8.6 ± 0.70 ng/mL by 12 h and to 3.4 ± 0.24 ng/mL within 24 h (combined data from Fig. 1A and 1B). Following the second injection (Fig. 5.1B), testosterone concentrations increased from 3.6 ± 0.45 to 19.6 ± 0.53 ng/mL within 1 h and dropped rapidly as observed following the first injection. In the testosterone enanthate groups, the plasma levels of testosterone increased gradually after treatment from 0.3± 0.18 to reach a maximum of 1.2 ± 0.47 ng/mL 8 h post-treatment (with single injection). After the second injection (Fig. 5.1B), plasma levels of testosterone increased from 1.1 ± 0.04 ng/mL at the time of second injection to reach a maximum of 2.3 ± 0.24 ng/mL at 36 hr. Based on the results obtained in Experiment 1, we decided to treat heifers in Experiment 2 twice at 12 h intervals with unconjugated free testosterone in oil. We predicted that plasma testosterone levels would remain above 8 ng/mL for 24 h followed by a sharp decline

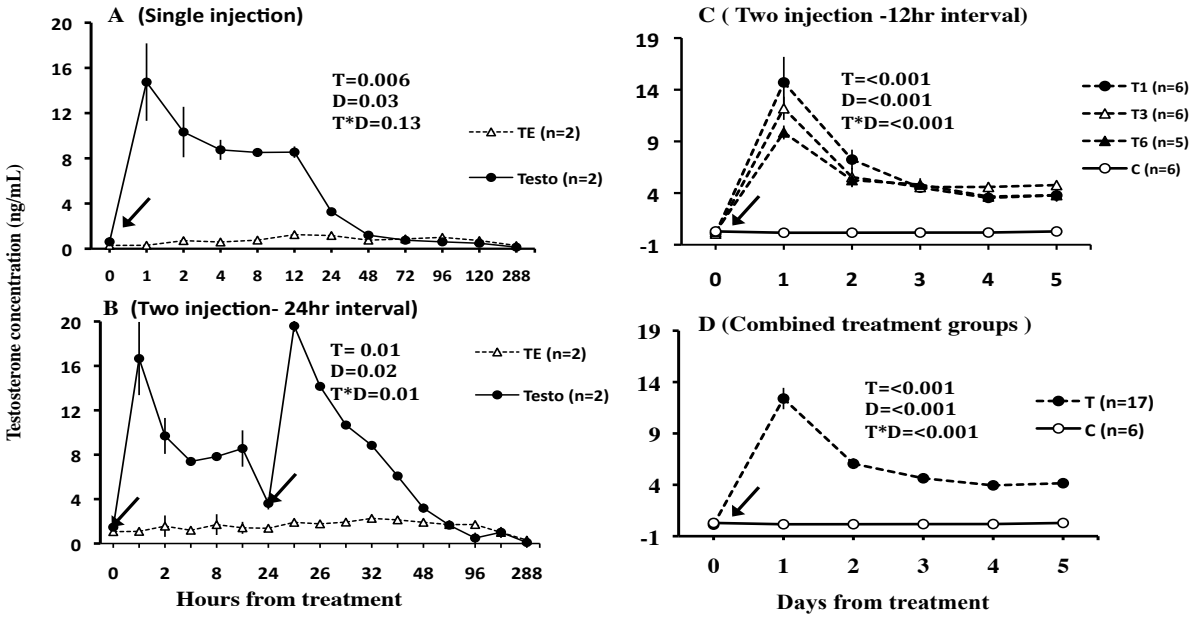

Fig. 4.1. Plasma testosterone concentrations (mean ± SEM) after treatment with testosterone. In In Experiment 1, single im injection (**Fig. A**) or two injections (24 hr apart, **Fig. B**) of 200 mg unconjugated testosterone (testo) or testosterone enanthate (TE) were given to cows. Times of treatment are indicated by obligue arrows. In Experiment 2, (Fig. **C**) heifers (n=6 per goup) on Days 1 (T1), Day 3(T3), and Day 6 (T6) after ovulation were treated with 2 injections of unconjugated testosterone 12 hr apart. Control group heifers were given placebo (4ml canola oil) treatment. There was no difference in plasma testosterone concentration among the three testosterone groups, therefore the combined data from D1, D3 and D6 groups versus control are presented (**Fig D)**. P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on the graphs.

4.4.2 Experiment 2- Effects on ovarian and hormonal dynamics

Results are summarized in Tables 4.1 and 4.2, and Figures 4.2 to 4.9. By design, when there was no difference among testosterone groups, data were combined into a single group (combined treatment group) for analysis.

4.4.2.1Plasma testosterone concentrations

Mean plasma concentrations of testosterone over time are shown in Fig. 4.1C and 4.1D. Mean concentrations of testosterone over the first 5 days following treatment were 6.0 ± 0.93, 5.8 ± 0.67, 4.8 ± 0.58 and 0.2 ± 0.03 ng/mL for T1, T3, T6, and C groups, respectively (P<0.001; Fig.5.1 C). Plasma testosterone concentration were 12.4 ± 0.16 ng/mL 24 h after the first treatment (testosterone treatment groups combined, Fig 1 D), and then decreased rapidly to 6.1 ± 0.17 ng/mL by 48 h and continued to decrease over the next 3 days. Testosterone concentrations in the control group remained at a constant low level during the same period of time.

4.4.2.2 Interwave and interovulatory intervals

No differences were detected between groups for the first interwave interval (Table 4.1), but when combined data from testosterone treatment groups were compared to control (Table 4. 2), a prolonged interval between the emergence of first and second wave (P=0.001) was detected. The second wave interwave interval for T1 was longer (P=0.05) than control, but did not differ from other treatment groups (Table 1). Overall, testosterone treatment (T1, T3 and T6 combined), extended (P=0.02) the second wave interwave interval compared to control (Table 2). The interwave interval of the ovulatory

wave was longer (*P*=0.04) in the T6 group compare to controls (Table1), while T1 and T3 were intermediate and not different from either; however, combined data for all testosterone treatment groups did not differ (*P*=0.10) from the control group (Table 2).

The duration of the interovulatory interval did not differ among groups (Table 1, *P*= 0.23) or when treatment group data were combined and compared to the control group (Table 2, *P*= 0.28). Also, the number of waves in an interovulatory interval did not differ among groups (Table 1, *P*= 0.19) or between the combined treatment group and the control group (Table 2, *P*=0.10). Although the proportion of 2 wave cycles was numerically higher in testosterone treatment groups compared to the control group, the difference was not significant (Table 1, *P*=0.27). However, when testosterone treatment groups were combined (Table 2), there was a tendency for more 2-wave cycles after testosterone treatment compared to control group (*P*=0.08).

Table 4.1. Mean (±SEM) of different ovarian parameters in controls and treatment groups on Day 1 (T1), Day 3 (T3), and Day 6 (T6). n = number of animals in each group

Item	Control	Testosterone treatment		
		Day 1	Day 3	Day 6
Interovulatory interval	21.7±1.35 (n=6)	28.7±4.14 (n=6)	21.4±0.7 (n=5)	21.8±2.84 (n=4)
Interwave interval (Wave 1)	8.7±0.42 (n=6)	12.0±1.32 (n=6)	11.2±1.0 (n=6)	12.2±1.68 (n=5)
Interwave interval (Wave 2)	7.3±0.49^{b} (n=6)	11.7±1.76^{a} (n=6)	7.8±0.75^{ab} (n=6)	9.8±1.16^{ab} (n=5)
Interwave interval (last wave)	6.8±0.40^{a} (n=6)	9.0±0.73^{ab} (n=6)	7.0±0.83^{ab} (n=5)	9.4±0.92^{b} (n=4)
Proportion of 2 wave cycles	1/6 (17%)	4/6 (67%)	3/6 (50%)	4/5 (80%)
CL life-span* (days)	15.9±1.31 (n=6)	14.5±0.96 (n=6)	13.8±1.40 (n=6)	12.6±0.97 (n=5)

^{ab} Within rows, values with no common superscripts are different (P≤0.05)

* Interval from ovulation to the day when CL began to regress

Table 4.2 Mean (±SEM) ovarian parameters of controls and combined testosterone treatment groups, n= number of animals.

Variables	Control	Treatment	P- value
Interovulatory interval	21.6±1.35 (n=6)	24.4±2.07 (n=15)	0.28
Interwave interval (Wave 1)	8.7±0.42 (n=6)	11.8±0.72 (n=17)	0.001
Interwave interval (Wave 2)	7.3±0.49 (n=6)	9.8±0.81 (n=17)	0.02
Interwave interval (last wave)	6.4±0.40 (n=6)	8.3±2.02 (n=15)	0.10
Number of wave in IOI	3.0±0.25 (n=6)	2.4±0.19 (n=15)	0.10
Proportion of 2-wave cycle	1/6 (16.66%)	11/17 (64.70%)	0.08
CL life-span*	15.7±1.3 (n=6)	13.7±0.63 (n=17)	0.14

* Interval from ovulation to the day when CL began to regress

4.4.2.3 Follicular dynamics

The diameter profile of the dominant follicle in different waves is shown in Fig. 4.2 During the first follicular wave (Fig. 4.2A), testosterone treatment affected the diameter of the dominant follicle (P= 0.02) in a phase-specific manner. However, testosterone treatment did not alter the dominant follicle diameter in the next wave (Fig. 4.2B, P=0.83) or the ovulatory wave (Fig. 4.2C, P=0.26). For the first wave dominant follicle, daily growth rate (Fig 4.3A and 4.3B) and mean growth rate over 5 days following treatment on Days 1 or 3, (Fig. 4.3D and 4.3E) were suppressed significantly

compared to the control group. Following testosterone treatment on Day 6, the dominant follicle regressed slower than in the control group (Fig.4.3C and 4.3F; *P*= 0.03).

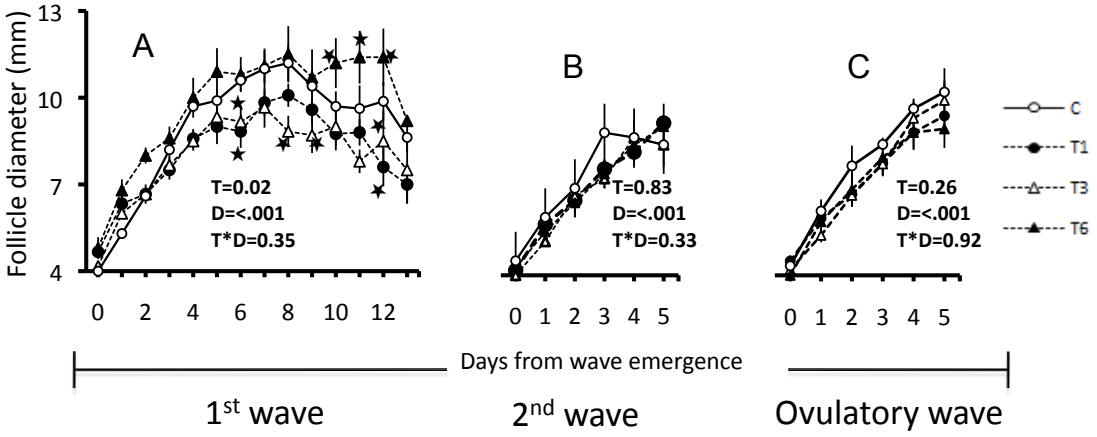

Fig. 4.2. Dominant follicle diameter profile (mean ± SEM) of first, second, and ovulatory waves. Treatment groups T1, T2 and T3 were given 200 mg of free testosterone twice (at 12 hr intervals) on Days 1, 3 or 6 respectively of the first follicular wave, respectively while control (C) group was given placebo (coanola oil) treatment. P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on the graphs. ★ indicates differences between the treatment and control groups ($P<0.05$) for a given day.

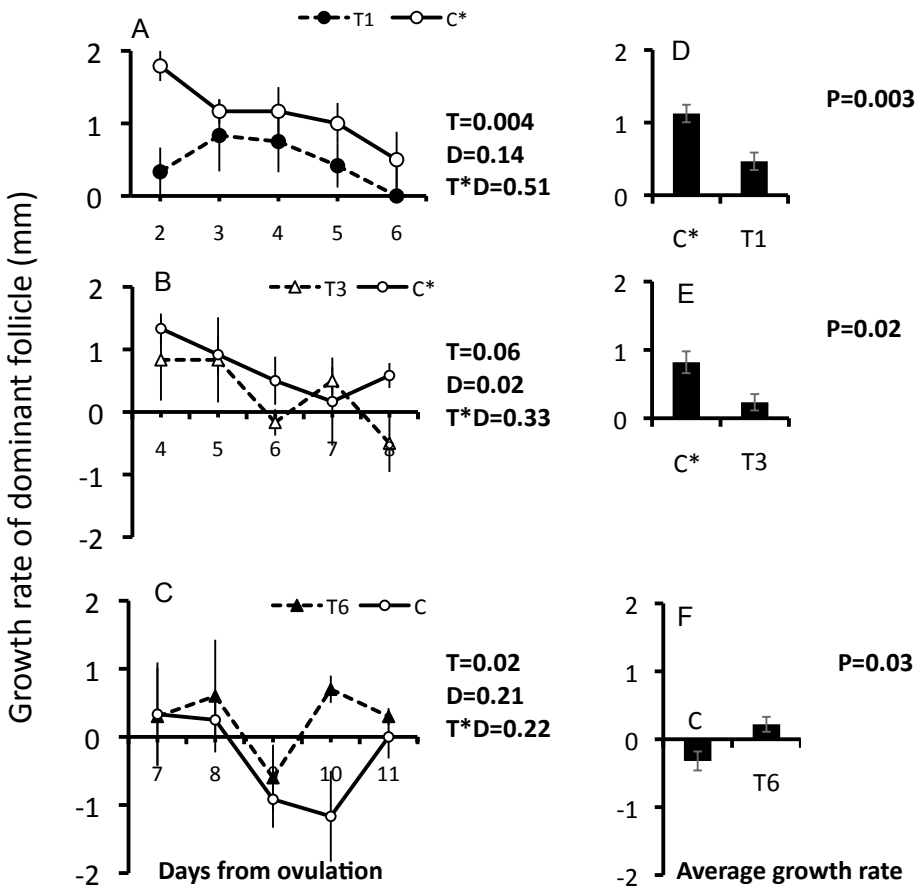

Fig. 4.3. Growth rate of the dominant follicle for the first 5 days post-treatment (Fig. A to C) and the average growth rate (Fig. D to F) for T1 (Fig A and D), T3 (Fig. B and E) and T6 (Fig. C and F) treatment groups compared to the control (C) group within the same period. The number at each day after treatment represents the difference between the diameter of dominant follicle on that day from that of the previous day. For T1 and T3 groups, control group (C* n=12) included all untreated control animals (n=6) combined with those from T6 group (before initiation of treatment). P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on Fig A to C and P-value between treatment and control group is indicated on Fig D to F.

4.4.2.4 Follicle numbers

There were no differences among treatment groups and the control group in the number of follicles in different size categories (4-5, 6-8, and ≥ 9 mm) in the first, second or ovulatory waves (Fig 4.4).

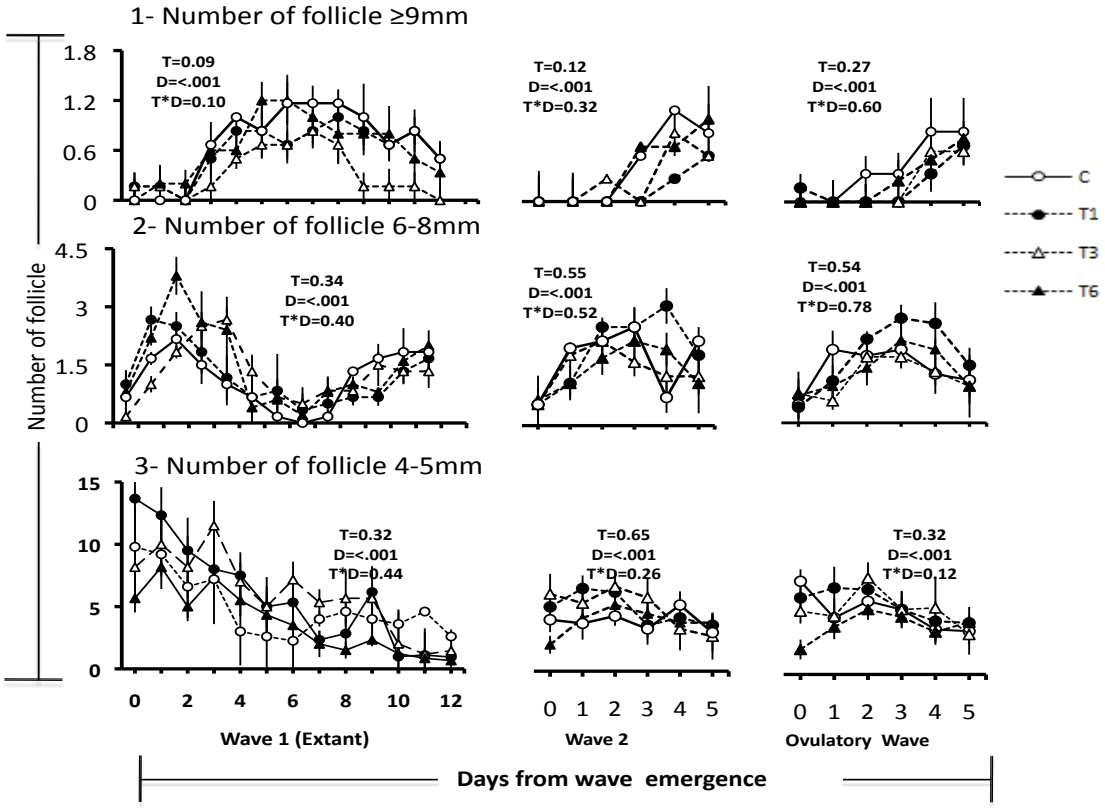

Fig 4.4. Mean number of follicles in different diameter groups (4-5, 6-8, and ≥ 9 mm) during first, second, and ovulatory waves. Treatment groups T1, T3 and T6 were given 200 mg of unconjugated testosterone twice (at 12 hr intervals) on Days 1, 3 or 6 of the first follicular wave, respectively while control (C) group was given placebo (canola oil) treatment. Data were centralized to the mean day of wave emergence (Day 0) for Wave 1 (left graphs), Wave 2 (middle graphs) and Ovulatory Wave (right graphs). P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on the graphs.

4.4.2.5 Corpus luteum diameter and progesterone concentration

No differences were found among treatment groups (data not shown) in CL diameter profiles, therefore, the data from T1, T3 and T6 groups were combined. When combined treatment group was compared to the control group, there was no difference in CL diameter during the first and second portions (P= 0.49 and P=0.58, respectively), but the CL regressed more rapidly (P<0.05) in testosterone treated heifers after Day 15 of cycle (Fig 4.5A). However, the first day of CL regression did not differ among treatment groups (Table 4.1, P=0.35) or between the testosterone treatment (combined treatment group) and the control group (Table 4.2, P=0.14). Testosterone treatment did not affect plasma progesterone concentrations between Days 11-17 of cycle (Fig. 4.5B).

5.4.2.6 Gonadotrophin Hormone Analysis

Heifers treated with testosterone (combined treatment group) had a decrease in LH concentrations post-treatment compared to the control group (Fig. 6A, P=0.01). Mean plasma LH concentrations following treatment were lower than those before testosterone treatment (P=0.04), but did not differ from the control group (Fig 6E). Treatment on Days 1 and 3 suppressed mean plasma LH concentrations significantly (Fig. 6 B and 6C; P=0.03 and P=0.05 respectively), while treatment on Day 6 only numerically reduced plasma LH concentrations (Fig. 6D, P= 0.11). When intensive bleeding data were examined, LH concentrations for the combined treatment group were not affected for the first 24 hr after treatment (Fig.6 F), but declined significantly (P<0.03) at 36 and 48 h after treatment (Fig. 6G).

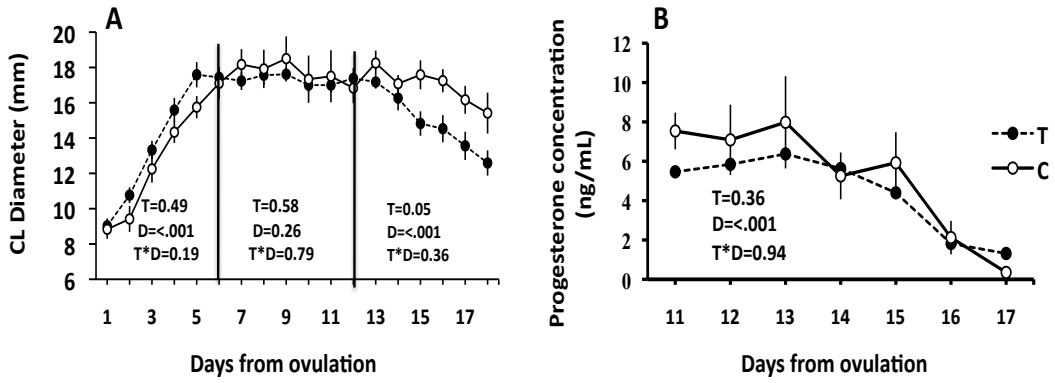

Fig. 4.5. Comparison of corpus luteum diameter (mean ± SEM) profile (Fig. A) and plasma progesterone concentrations (Fig. B) between the combined testosterone treatment groups (T) and the control group (C). Corpus luteum diameter data (Fig. A) were divided into three phases for statistical analysis (Days 1 to 6, 7 to 12, and 13 to 18 from ovulation) and progesterone data are presented for Days 11 to 17 (Fig. B). P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on the graphs

FSH concentration did not differ among treatment groups versus the control group or between the combined treatment group and the control group (Fig. 7). In addition, mean FSH concentrations before or after treatment did not differ among groups (Fig. 7E, *P*=0.12).

4.4.2.7 Comparison of synchrony of wave emergence among treatment groups

The interval from treatment to emergence of the next follicular wave was highly variable among treatment groups compare to controls (Fig 8A, *P*=0.02). The deviation of

day of wave emergence from the group mean was greater in T1 and T6 treatment groups compared to control group (Fig. 8 B, *P*= 0.01

4.4.2. Analysis of data based on first wave interwave interval

The first wave IWI (interval between the day of wave emergence of Wave 1 and 2) for the control group was 8.7±0.42 (Table 4.1, n=6, range 5 to 8 days). When data from testosterone treated heifers were analyzed irrespective of the day of the wave on which treatment was given (i.e. all testosterone treated animal together; n=17), the first wave interwave intervals showed two patterns (Fig. 9A): animals in which interwave interval was similar to control, i.e., ≤11 days (normal group, TN) or ≥12 days (delayed group, TD). The number of treated heifers in TN group was 9 (T1, n=3; T3, n=3; T6, n=3) while the number of heifers in TD was 8 (T1, n=3; T3, n=3; T6, n=2). The interval between day of treatment and emergence of the next wave was longer for TD group (11.6±0.88 day, *P*= < 0.001) compared to TN (6.0±0.71 day) group. This difference in interval to wave emergency was associated with difference in timing of the FSH surge between TN and C verses the TD (Fig 4.9B) group. Plasma progesterone and testosterone concentrations and the diameter of first wave dominant follicle did not differ between the TN and TD groups (P= 0.45, *P*= 0.14, and *P*=0.11 respectively). During treatment, plasma FSH (Fig 4.9C) and LH (Fig 4. 9D) concentrations in TD group were lower (*P*=0.04 and *P*=0.02) than in TN group.

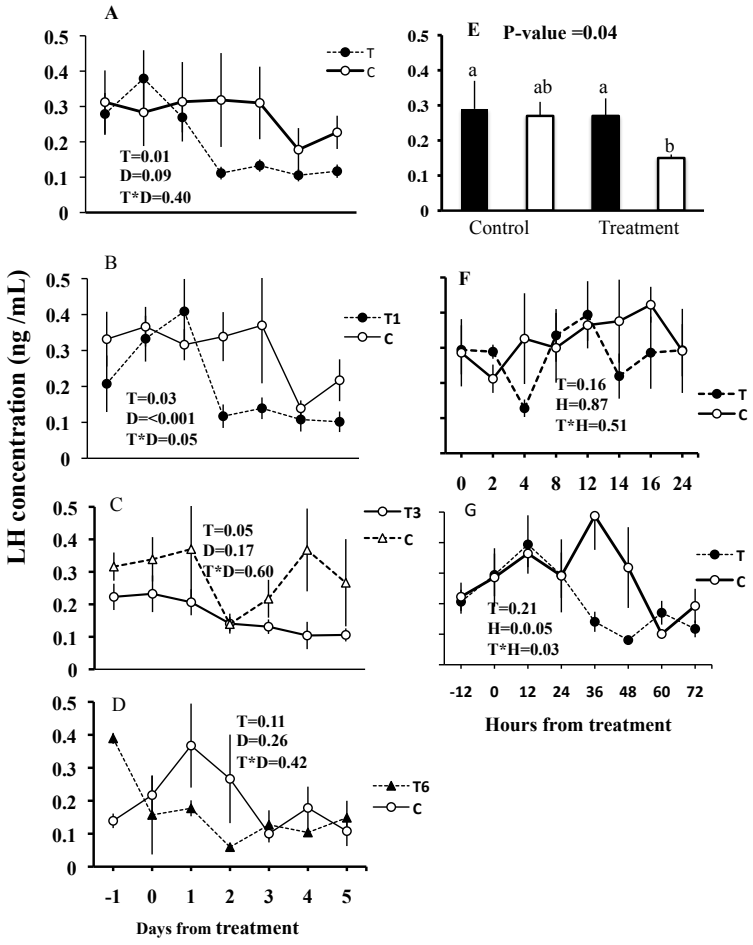

Fig. 4.6. Mean (±SEM) plasma LH concentrations after treatment with 200 mg unconjugated testosterone twice at 12 hr intervals: Combined testosterone treatment group (Fig. A, E, F, G), T1 (Fig. B), T3 (Fig. C), and T6 (Fig. D) groups compared to controls (C). Fig E compares the mean concentration of plasma LH before (Day -1 and 0; black box) and after treatment (Days 1-5; white box) between control and combined treatment group. Fig F and G illustrate the change in plasma LH concentrations from 0 to 24 and -12 to 72 hr, respectively after treatment. P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on the graphs and different letters on top of bars in Fig. E indicate statistical differences ($P < 0.05$). Day 0 = day of treatment.

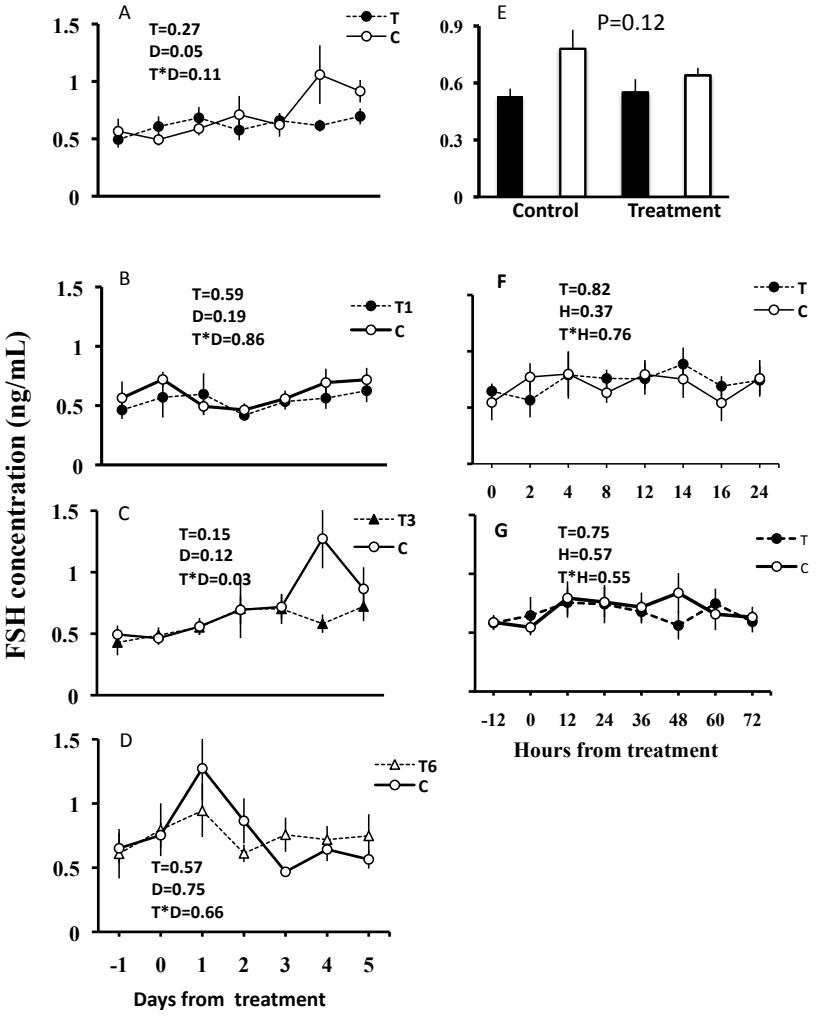

Fig.4.7 Mean (±SEM) plasma FSH concentrations after treatment with 200 mg unconjugated testosterone twice at 12 hr intervals: Combined testosterone treatment group (Fig. A, E, F, G), T1 (Fig. B), T3 (Fig. C), and T6 (Fig. D) groups compared to controls (C). Fig E compares the mean concentration of plasma FSH before (Day -1 and 0; black box) and after treatment (Days 1-5; white box) between control and combined treatment groups. Fig F and G illustrate the change in plasma FSH concentrations from 0 to 24 and -12 to 72 hr after treatment, respectively. P-values for treatment (T), day (D) and treatment*time interaction (T*D) are indicated on the graphs. Day 0 = day of treatment

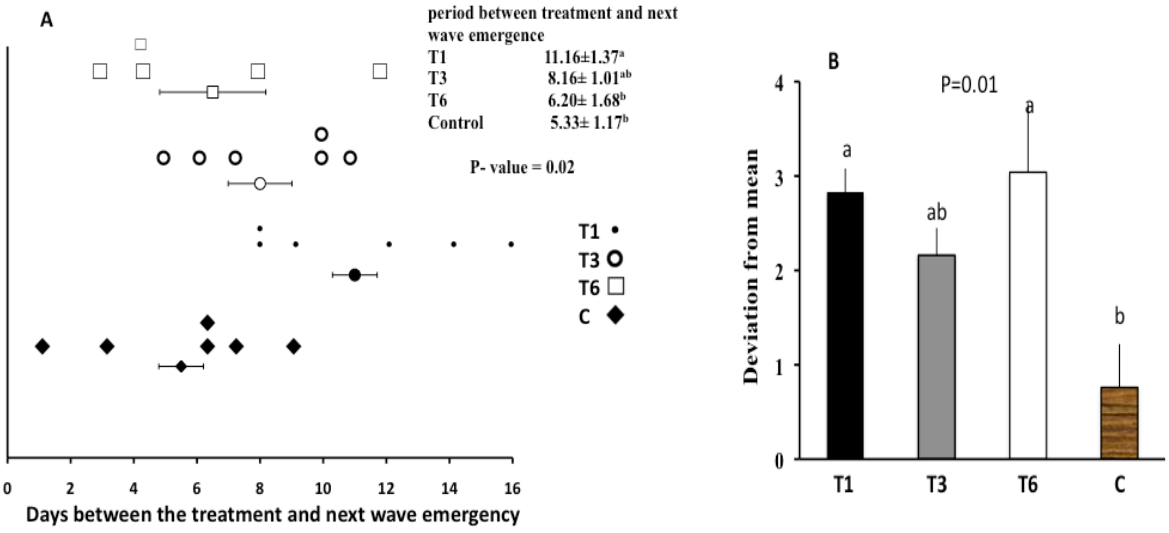

Fig.4.8 Distribution of day of next wave emergence after treatment (Fig A) and deviation among animals from the mean day of wave emergence (Fig. B) among animals treated with testosterone on Days 1 (T1), 3 (T3), 6 (T6) after ovulation and control (C). Individual symbols in Fig A represent each animal; symbols with horizontal bar indicate group mean±SEM. For Fig B, difference between group mean and data point of each animals was defined as "deviation from mean" and analyzed by one-way analysis of variance. Different alphabets on top of bars in Fig. B indicate statistical differences ($P<0.05$). Day 0 = day of treatment.

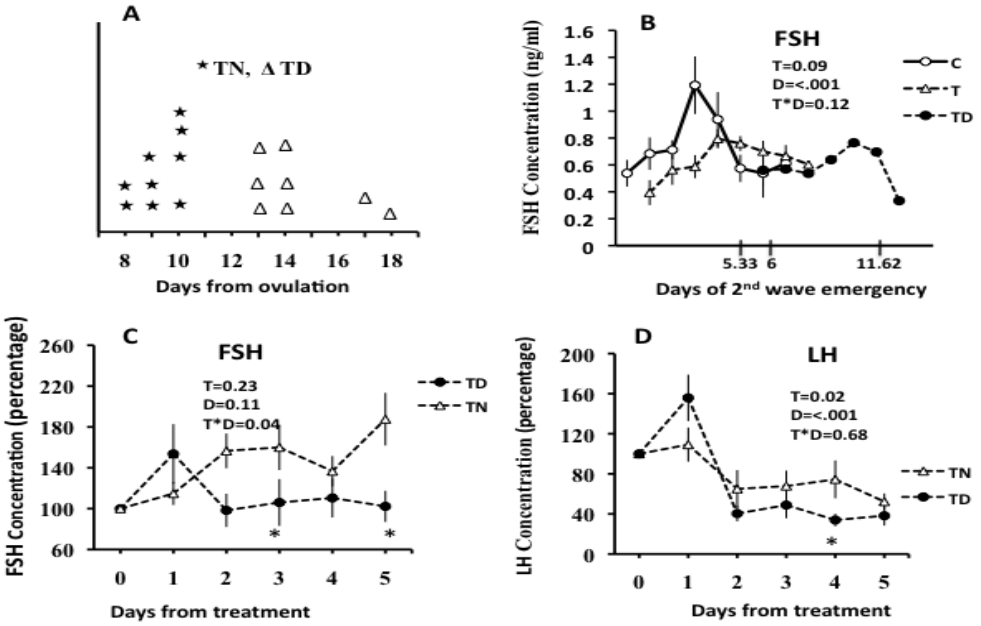

Fig.4.9 Treated heifers were distributed into two groups based on the second wave emergence (before 12 days; TN, or after 12 days; TD). **A**) Day of second wave emergence in TN, TD and control. **B**) The surge of FSH of TD, TN and C (the FSH concentration was measured 4 days before wave emergence and 1 day after). **C**) differences in FSH percentage between TD and TN after testosterone treatment for 5 days. D) The different in LH percentage between TD and TN after testosterone treatment for 5 days after treatment.

4.5 Discussion

Our hypothesis that the administration of testosterone will shorten the life-span of the extant dominant follicle resulting in early emergence of a new follicular wave was not supported. Dominant follicle development after testosterone treatment differed depending on stage of follicle development at the time of treatment. Treatment on Days 1 or 3 resulted in reduced the plasma LH concentrations and a decreased growth rate of the dominant follicle. However, treatment on Day 6 did not affect plasma LH concentration but delayed follicular regression. In all treatment groups emergence of the next follicular wave was delayed, resulting in a tendency for more 2-wave cycles than in control heifers.

In Experiment 1 (pilot study), we tested two formulations of testosterone to determine which formulation would sustain elevated testosterone levels and for how long a period of time. Basal levels of circulating testosterone in cattle are <1 ng/mL (Nix et al., 1998; Wise et al., 1982). Results from our pilot study showed a marked difference in plasma testosterone levels following one or two im treatments with unconjugated (free) testosterone and testosterone enathnate (Fig.4.1) which was consistent with previous reports (Nix et al., 1998; Silvia et al., 1989). Plasma levels of testosterone increased slowly after treatment with testosterone enathnate while testosterone concentration increased more than 10 fold within 1 hr after injection of free testosterone.. Testosterone enathnate contains an ester group that improves the solubility of testosterone in oil (Encyclopedia, 2011). As a result, testosterone enathnate is released slowly over a prolonged period of time from its injection site into the blood stream. On the contrary, unconjugated testosterone would enter the blood stream very quickly. Based on our

knowledge of estradiol formulations (Martinez, 2005; Mapletoft, 1999), we wanted to achieve high circulating concentrations of testosterone for a 24 hr period to cause local (Billig et al., 1993; Jia et al., 1985) and systemic effects (Thompson et al., 1984), followed by a sharp decline in circulating plasma concentration to remove these effects. The pilot study indicated that the unconjugated testosterone formulation would achieve the desired levels of plasma testosterone for hypothesis testing. Our results indicated that 12 h after the testosterone injection (Fig. 4.1C,D), plasma levels dropped to 50% of the peak levels, a second injection was administered at that time to maintain elevated levels of testosterone for at least for first 24 hr.

Depletion half-life of testosterone was estimated to be 3.2 days and testosterone concentrations were maintained above 14 ng/mL (range: 28.7-14.5 ng/ mL) for 36 hr (Chapter 3). By 48 hr after first treatment, plasma concentration of testosterone dropped to 6.1 ± 0.17 ng/mL. Therefore, we were confident that our testosterone preparation and treatment regimen were suitable to cause the physiological effects on follicular and luteal dynamics, and systemic hormone concentrations of other reproductive hormones.

Results document that increasing plasma testosterone concentrations resulted in several changes in follicular wave dynamics. When the testosterone treatment was given during the growing phase of the dominant follicle, before or at selection (Day 1or 3 groups, respectively), it suppressed the growth of dominant follicle. Slower growth rates of the dominant follicle were associated with a concurrent reduction in plasma LH concentrations. LH secretion is required for growth of the dominant follicle, especially after selection when it becomes more LH dependent (Lucy, 2007). Reduction in mean LH concentrations and LH pulse frequency are considered crucial factors that decide the fate

of dominant follicle (Savio et al., 1993). The effect of testosterone on circulating LH concentrations in this study is in agreement with results of a previous study in which of the administration of 200 mg of testosterone per day for 4 days resulted in a reduction in circulating LH concentrations and regression of the preovulatory follicle (Rajamahendran and Manikkam, 1994). Unexpectedly, testosterone treatment during the early static phase (Day 6) resulted in slower regression of the dominant follicle. Although the difference in LH concentrations between the treatment and control groups following treatment on Day 6 was not statistically significant, LH concentrations were numerically lower and followed the same trend as observed following treatment during the growing phase of the dominant follicle. The inability to detect statistical differences in LH concentrations following testosterone treatment on Day 6 may relate to changes in circulating progesterone concentrations. By Day 6 of the cycle, the corpus luteum is fully functional and producing large amounts of progesterone (Adams et al., 1992b; Rhodes et al., 1995) which suppresses LH release, making it difficult to detect a suppressive effect of testosterone treatment.

The effect of testosterone on LH concentration may be a result of a direct effect on the pituitary gland (Yasin et al., 1996) or indirectly through modulation of estradiol production (Abbot et al., 1988). A study in ovariectomized cows and mares revealed a 17% and 34% (respectively) reduction in LH release after GnRH challenge in testosterone treatment animals (Thompson et al., 1984) indicating that observed reduction in LH levels in our study may be partially due to extra-ovarian effects of testosterone. In ruminants, LH concentrations in the circulation reflect changes in LH beta subunit mRNAs expression in adenohypohysis (Aspden et al., 2003; Fetherston and Boime, 1982;

Wise et al., 1985). Studies in a rodent model also indicate that androgen treatments modulate LH beta subunit mRNA expression in the pituitary gland, and GnRH mediated LH release and synthesis of mRNA β-subunit (Keri et al., 1994b; Krey et al., 1982; Yasin et al., 1996). These effects may be mediated by androgen receptor interaction with steroidogenic factor-1 (Curtin et al., 2001; Jorgensen and Nilson, 2001). One study in female rats showed that a high dose of testosterone suppressed LH secretion and LH ß mRNA response to GnRH (Yasin et al., 1996). These studies imply that testosterone can have a direct negative effect on LH secretion through modulate the responsiveness of pituitary gland to GnRH release .

The second possible pathway for the observed testosterone-mediated LH suppression is through altering the follicular products, especially estradiol production. Testosterone is one of the known substrates that can be aromatized to estradiol (Walters et al., 2008), so the high amounts of testosterone that were injected in animals may increase the level of estradiol by aromatase activity of the granulosa cells of growing or early static dominant follicles. To support this notion, testosterone treatment of granulosa cells in vitro, has been shown to stimulate aromatase activity (Wu et al., 2011). Consequently, estradiol concentrations may have increased in the blood leading to suppression of the dominant follicle growth (Bo et al., 2000) by reducing the frequency and the amplitude of LH pulses (Wolfe et al., 1992). Unfortunately, plasma estradiol concentrations were not measured in this study. It is interesting to note that the growing dominant follicle did not undergo complete regression following testosterone treatment and it resumed growth. Although a suppressive effect of testosterone treatment on Days 1 or 3 was observed for 5 days, its magnitude may not be sufficient to cause complete

regression of the dominant follicle or testosterone treatment may have a negative short term effect on LH concentration.

The emergence of the next wave was delayed in half of the testosterone-treated heifers (Fig. 4.9A) and there tended to be more 2-waves cycles in testosterone-treated heifers compared to control group. This may be a result of the effect of converted estradiol that can suppress the FSH surge leading to a delay in emergence of the next follicular wave (Bo et al., 2000). In another study, treatment with estradiol valerate on Day 3 or 6 of the first follicular wave resulted in a delay in emergence of the next wave, presumably by delaying the FSH surge (Bo et al., 1993).

It is noteworthy that number of follicles in the wave did not differ among treatment groups. Compared to very synchronous wave emergence following estradiol treatment (Bo et al., 1993), the interval between testosterone treatment and emergence of the next wave was highly variable, suggesting that testosterone is unlikely to be useful for the synchronization of follicle wave emergence. It has been shown that follicle wave emergence is stimulated by a surge of FSH (Adams et al., 1992a; Kulick et al., 1999). In the present study, the group of heifers in which emergence of the next wave was delayed, the FSH surge was also delayed compared to the control group or those animals in which wave emergence was not delayed (Fig4.9 B,C). These effects are likely due to a direct negative feedback of estradiol on the pituitary gland (Adams et al., 1992a; Roche et al., 1998). The effect of testosterone treatment on timing of next wave emergence in our study was consistent with report in which 5 mg of estradiol valerate in heifers resulted in delayed and more variable intervals to emergence of a new follicular (Bo et al., 1993). In our study, we speculate that the delay of the FSH surge and emergence of the next wave

may either be due to direct suppressive effects of estradiol or testosterone on hypothalamic-pituitary axis or the ability of testosterone to keep the dominant follicle functional for a prolonged period of time. Although follicular growth was initially suppressed due to a decline in LH levels, testosterone treatment may have increased the expression of intrafollicular factors such as estradiol or IGF-1 that kept the dominant follicle functional (Quirk et al., 2004). This notion is supported by the finding that the early static phase dominant follicle (T6 group) maintained its diameter for a longer period. This ability of testosterone treatment to prolong the dominant follicle growth profile may be the result of increasing levels of estradiol in the follicular fluid through an increase in the availability of testosterone in blood and an increase in the expression of aromatase activity in granulosa cells (Wu et al., 2011). Consequently, the follicle that remains estradiol active has the ability to continue growing (Ireland and Roche, 1983). In addition, high estradiol concentration has been shown to increase the secretion of IGF-I by porcine granulosa cell (Hsu and Hammond, 1987). IGF-I has also been shown to suppress follicle apoptosis (Chun et al., 1994) and the increasing IGF-I expression has been shown to enhance the responsiveness of follicular cells to FSH and LH resulting in additional growth and steroidogenesis in dominant follicles (Bao and Garverick, 1998). It may be speculated that testosterone treatment provided a high level of testosterone that was converted to estradiol in the dominant follicle leading to the suppression of the FSH surge and delayed follicle wave emergence.

It is interesting to note that testosterone concentrations increase before CL regression in the cow (Peterson et al., 1978; Wise et al., 1982), goat (Homeida and Cooke, 1984) and sheep (Herriman et al., 1979), which suggests that testosterone might

play an important role in luteolysis (Homeida and Khalafalla, 1990; Tropea et al., 2010). In our study, CL diameter in the combined treatment group regressed more rapidly than in the control group (Table 4.2), however, we could not document any changes in circulating levels of progesterone during this period (11-17 day from ovulation). Our results are consistent with the findings of another study (Silvia et al., 1989) in which no difference in progesterone concentration was found between heifers treated with androgen (testosterone and dihydrotestosterone) versus untreated control animals. In any case, the observed luteal effects appear to be very subtle. It is noteworthy that testosterone concentrations would have returned to normal by the time of luteal regression and any observed effects would be due to direct or indirect uterine influences because of elevated testosterone during first 6 days of the luteal development.

In summary, testosterone treatment during the pre-selection and selection phases of the growing dominant follicle suppressed its growth rate, which was associated with a reduction in circulating LH concentrations. Treatment during the early static phase of the dominant follicle delayed the regression phase. Overall, extant and subsequent follicular wave intervals were longer and testosterone treated animals tended to have more 2-wave cycles than controls. In 8 out of 17 heifers in testosterone treatment groups, emergence of the next wave was delayed due to a delay in the FSH surge. Testosterone treatment failed to synchronize the emergence of next follicular wave, alter the number of follicles in different size categories, and had minimal effect on luteal function. In conclusion, after exogenous administration of testosterone, dominant follicles grew more slowly, but contrary to our hypothesis, treatment did not shorten the life-span of the extant dominant

follicle and did not result in early synchronous emergence of a new follicular wave. Rather, emergence of the next wave was delayed in some but not all animals.

5.0 GENERAL DISCUSSION, CONCLUSIONS AND FUTURE STUDIES

5.1 General Discussion

Reproductive efficiency of farm animals is an important consideration for increasing the productivity of animals for economic benefits and food security. For this reason, many reproductive techniques have been developed and applied in farm animals to increase fertility, to get more offspring from animals with higher genetic value or to simply manage the breeding period. Follicular wave synchronization is one of these techniques that helps to improve the efficiency of artificial insemination and embryo transfer. Many drugs such as estradiol, progesterone, GnRH or LH or physical removal of the dominant follicle (i.e., follicular ablation) have been used for this purpose. Among these hormones, estradiol is most efficacious and practical to use; however, recent bans on use of estrogens in farm animals by the European Union have limited its availability in many countries. In this thesis, we investigated whether testosterone could be used as an alternative to estradiol for follicular wave synchronization.

In this section, the results of two studies (Chapters 3 and 4) are summarized to show the effects of exogenous testosterone treatment on the ovarian function and hormone profiles in beef cattle. These results would be connected with available information related to this subject. To the best of our knowledge, this is the first study in cattle that investigated the effect of testosterone on the growth pattern of dominant follicle and corpus luteum during different phases of development, examined changes in the numbers of small follicles and emergence of next follicular wave, and correlated ovarian changes with systemic concentrations of hormones such as FSH, LH and

progesterone and testosterone. For this reason, we will use other species for comparison with our results.

Testosterone is a steroid hormone that has been shown to play a crucial role in growth and reproductive performance. As an anabolic hormone, testosterone is used widely as growth promotant in farm animals (Lone, 1997). During follicular differentiation, testosterone acts as a substrate for estradiol and therefore plays an indirect but essential role in regulation of follicular development (Drummond, 2006). Testosterone has been shown to exert a negative effect on plasma concentrations of gonadotrophin hormones (LH, FSH) in males and females (Huang et al., 2001; Thompson et al., 1987). In cattle, testosterone has been used to androgenize cows for estrus detection (Nix et al., 1998). Furthermore, testosterone was used in pigs to increase the ovulation rate (Cardenas and Pope, 1994; Cardenas and Pope, 1997).

It was expected that an injected overdose of testosterone would convert to estradiol resulting in increased estradiol concentration in the blood. High estradiol would a exert negative feedback on gonadotrophin hormones (FSH, LH) leading to regression of the existing dominant follicle. In addition, published results indicated that testosterone may have direct negative effects on granulosa cells (Hillier and Ross, 1979). Based on these effects, we predicted that exogenous testosterone treatment would decrease the life-span of extant dominant follicle by direct systemic effects and/or indirect ovarian effects and therefore the suppressive effect of dominant follicle on FSH through estradiol would be removed leading to early emergence of next follicular wave.

At the beginning of this project, we needed to select a testosterone preparation and the dose that would be most likely to cause physiological effects. Based on previous

studies that used estradiol for follicular wave synchronization (Martinez et al., 2005, Colazo et al., 2002, Mapletoft 1999), we predicted that a testosterone preparation that results in sharp increase in plasma testosterone concentration followed by a decline over approximately 36 hr would be most suitable. This pattern of elevation in testosterone concentration was designed to exert high testosterone level for a specific period that may enough to suppress the dominant follicle growth and yet disappear rapidly enough to permit an FSH surge and allow emergence of a new wave. In the pilot study (Chapter 4), we tested testosterone enanthate (conjugated esterified preparation) and unconjugated testosterone (free-form). compared to unconjugated testosterone. Two injections of testosterone enanthate at 24 hr intervals resulted in a very small increase and very slow decline in plasma concentration of testosterone (Fig 4.1). Therefore, we selected unconjugated testosterone for further studies,however, by the end of first 24 hr period (i.e., at the time of second injection), plasma testosterone had decreased markedly. Consequently, we opted to decrease the interval between two injections to 12 hours for the final study. The results of pharmacokinetic study (Chapter4) indicated that plasma testosterone concentration increased quickly (2 hr) after injection of unconjugated testosterone and testosterone concentrations were maintained above 14 ng/mL (28.7-14.5 ng/ mL) for 36 hours (after two injections). Depletion half-life of testosterone was 3.2 days and plasma concentrations returned to baseline within 2 weeks. These levels compare very well those obtained in another study (Rajamahendran and,Manikkam, 1994) where daily injections of 200_mg of testosterone _in oil (the average of the maximum testosterone concentration after first injection was 16 ng /mL) over 4 day period caused atresia of the preovulatory follicle. We were confident that the type of

testosterone (unconjugated free form), dose of testosterone (200 mg) and treatment design (two im injections at 12 hr intervals) were optimal for detecting ovarian and hormonal effects.

Exogenous testosterone treatments were initiated when the dominant follicle was in the preselection growth phase (Day 1 of wave), at the time of selection (Day 3) or post-selection at beginning of the static phase (Day 6) of the first follicular wave (Chapter 4). Testosterone treatment on Days 1 and 3 decreased the growth rate of dominant follicle for next 4 to 5 days while treatment on Day 6 caused the dominant follicle to regress more slowly than controls (Fig 4.3). Testosterone treatment did not cause the complete demise of the dominant follicles at any stage and the next wave emergence did not emerge early or synchronously. Contrary to our hypothesis, the next wave emergence in all treatment groups was delayed (Table 4.1, 4.2).

Underlying causes of altered follicular function appear to be the effects of exogenous testosterone treatment on plasma LH concentrations. Compared to controls, LH concentrations were reduced for 5 days post-treatment (Fig 4.6) on Day 1 and Day 3 of the follicular wave. It is known that LH is required for dominant follicle growth after selection (Lucy, 2007). Observed LH reduction is consistent with results of studies that used testosterone treatment in female rats (Hassani et al., 1978 ; Yasin et al., 1996) and ovariectomized cows and mares (Thompson et al., 1984). In these studies testosterone had an estradiol-independent direct effect on gonadotrophin secretion. It has been observed that testosterone negatively affects the expression of LH β subunit mRNA in the pituitary gland that consequently reduces the amount of LH release (Aspden et al., 2003; Wise et al., 1985). In addition, enhanced conversion of testosterone to estradiol

conversion could modulate the LH secretion (Abbot et al., 1988). It is not yet clear what caused LH suppression, that is, whether it occurred due to a direct action of testosterone on the hypothalamic-pituitary axis, or conversion of testosterone to estradiol or both. Although the experiment was not designed to address this question directly, it may be partly answered by plasma estradiol analysis. Unfortunately, this assay was not available for the present investigation due to technical problems. On Day 6 of follicular wave, the situation was different; our results show that the treatment at this time had no effect on LH concentration or the diameter of the dominant follicle. It may the interaction between the stage of dominant follicle development (fully growing follicle) and hormonal meliu at the time of treatment that played role at this stage causing a different response from other groups.

It has been shown that the effect of testosterone on gonadotrophin hormones requires up to 24 hr before GnRH is suppressed in gonadectomized animals (Gharib et al., 1986; Yasin et al., 1996). In our study, LH concentrations were reduced by 24 hr in testosterone treated animals compared to controls (Fig. 4.6 G), which implies that this effect may be due to a direct action of testosterone on the hypothalamus or pituitary gland. In general, the magnitude of the LH suppression may not be sufficient or the period of low LH was not long enough to cause complete regression of the extant dominant follicle. Although the growth of pre-selection (Day 1) and recently selected (Day 3) dominant follicles was reduced, these follicles kept growing and remained active for a prolonged period of time, thereby delaying the emergence of next wave. Based on the results from Chapter 4 (Fig 4.1), plasma testosterone concentration in all treatment groups remained elevated for several days after treatment and returned to baseline levels

only after 2 weeks. The availability of high concentrations of testosterone may have induced stimulatory effect on aromatase activity (Wu et al., 2011) resulting in increased level of estradiol production by granulosa cells. The high level of estradiol could have modulated the effects of testosterone on LH secretion (Abbot et al., 1988; Bo et al., 2000). It is interesting to note that when high doses of long-acting estradiol esters (e.g. estradiol valerate) were injected (Martinez et al 2005), the FSH surge was delayed and occurred at variable times after treatment resulting in delayed follicle wave emergence and high variability. This situation is analogous to what we observed in our study. Regardless the underlying mechanism; dominance was maintained for a longer period of time leading to delay in the next wave emergence in all treatment groups.

It has been observed that estradiol exerts suppression of FSH release (Bo et al., 2000) and when the estradiol effect was removed, the synchrony of FSH release resulted in the synchronized emergence of a new follicular wave (Adams et al., 1992a; Bo et al., 1995a). In contrast to estradiol, we failed to demonstrate any immediate decrease in plasma FSH concentrations after the testosterone treatments in our study but the delay in wave emergence in treated heifers was associated with delay in FSH surge. In other words, we cannot identify the mechanism by which the FSH surge was delayed; whether it was by converted estradiol or by direct effect of testosterone is not clear. Another notable observation was that different heifers within each treatment group responded differently. Overall, based on interval to emergence of the next wave emergence, heifers were divided into two groups. In one group, time of next wave emergence was not different from that observed for control group (TN), while in the second group there was a clear delay in next wave emergence (TD), although these animals were given same dose

of testosterone as to the first group. The reason of this variation among heifers is not understandable and we could not find an explanation for this biphasic response.

Testosterone treatment did not have an effect on the number of > 4 mm follicles that were recruited into different follicular waves (second and ovulatory waves), or the plasma progesterone concentration during the same period, i.e., 11-17 post ovulation. Therefore, it appears that observed ovarian and endocrine effects of testosterone were limited to a one week period post-treatment . On the other hand, the CL diameter of treatment group between Days 13-17 post-ovulation showed minor differences from the control group, length of the second wave was longer after treatment and testosterone-treated heifers tended to have more 2-wave cycles. The last two effects are inter-related; treated heifers had more two-wave cycles and the length of second (anovulatory) wave in 3-wave cycles is shorter than that the length of second (ovulatory) wave in 2-wave cycles (Jaiswal et al., 2009).

5.2 General conclusions

Based on the research that was conducted for this thesis, we conclude the following points:

- Two intramuscular injections of oil-based unconjugated testosterone (200 mg) at 12 hr intervals can be used effectively to maintain plasma testosterone concentrations above 10.9 ng/mL for 36 hr from start of the treatment. Elimination half-life of this preparation of testosterone in the blood stream was 3.2 days and plasma concentrations returned to baseline within 2 weeks.
- Testosterone treatment did not shorten the life-span of the extant dominant follicle or induce early emergence of next follicular wave; our proposed hypothesis was not supported.
- Testosterone treatment given before or at the time of selection, suppressed plasma LH concentrations and dominant follicle growth rate, while treatment in the early static phase did not affect LH concentration, but led to slow regression of the DF
- Testosterone treatment delayed emergence of the next wave irrespective of the day of treatment. Next wave emergence was not synchronous.
- Heifers treated with testosterone tended to have more 2-wave cycles

5.3 Future studies

The studies reported in this thesis clearly documented the follicular and hormonal effects of testosterone but also raised many questions for future studies:

- One limitation of this study is the lack of estradiol measurements in plasma. Attempts were made, however, estradiol concentrations could not be reliably measured due to lack of a sensitivity and specificity of existing estradiol radioimmunoassay.
- There is need to identify the cause of eduction in plasma LH concentrations. This is important to understand the underlying mechanism of action of testosterone.
- Perform a more elaborate pharmacokinetic study of testosterone in cattle that would report important information missing in our work especially the testosterone residues in tissues.
- Repeat the study after increasing the dose of testosterone to test if greater suppressive effect on LH would cause complete demise of the dominant follicle.
- Are multiple injections of testosterone needed to get effective and consistent results and can a single very high dose cause sufficient LH suppression?
- Will lactating dairy/beef cows respond differently to testosterone compared to heifers in this study?

6.0 REFERENCES

Abbot S.D., Docherty K., Clayton R.N. (1988) Regulation of LH subunit mRNA levels by gonadal hormones in female rats. J . Mol. Endocr 1:49-60.

Adams G.P., Kot K., Ginther O. (1993a) Selection of a dominant follicle and suppression of follicular growth in heifers. Animal Reproduction Science 30:259-271.

Adams G.P., Evans A., Rawlings N. (1994) Follicular waves and circulating gonadotrophins in 8-month-old prepubertal heifers. J Reprod FertiI 100:27-33.

Adams G.P., Jaiswal R., Singh J., Malhi P. (2008) Progress in understanding ovarian follicular dynamics in cattle. Theriogenology 69:72-80.

Adams G.P., Matteri R.L., Kastelic J.P., KO J., Ginther O.J. (1992a) Association between surges of follicle-stimulating hormone and the emergence of follicle waves in heifers. J. Reprod. Fertil. 94:177-188.

Adams G.P. (1999) Comparative patterns of follicle development and selection in ruminants. J Reprod Fertil Suppl 54:17-32.

Adams G.P. (2007) Application of the Bovine Model for Study of Ovirian Function in Other species Arch. Latinoam. Prod. Anim. 15(supl.1):7-19.

Adams G.P., Matteri R.L., Ginther O.L. (1992b) Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. J Reprod Fertil. 96:627-640.

Adams GP, Pierson RA (1995) Bovine model for study of ovarian follicular dynamics in humans. Theriogenology 43: 113-120.

Adams GP (1998) Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization & superstimulation. Proceedings of the XX Congress of the World Association for Buiatrics, Sydney, Australia, 2: 595-605.

Adams GP, Kot K, Smith CA, Ginther OJ (1993b) Effect of the dominant follicle on regression of its subordinates in heifers. Canadian Journal of Animal Science 73: 267-275.

Amiridis G.S., Tsiligianni T., Vainas E. (2006) Follicle ablation improves the ovarian response and the number of collected embryos in superovulated cows during the early stages of lactation. Reprod Domest Anim 41:402-407.

Andersson A., Skakkebaek N. (1999) Exposure to exogenous estrogens in food: possible impact on human development and health. Eur J Endocrinol 140l 477-485.

Araki K., Arai K.Y., Watanabe G., Taya K. (2000) Involvement of inhibin in the regulation of follicle-stimulating hormone secretion in the young adult male Shiba goat. J Androl 21:558-565.

Aspden W.J., Jackson A., Trigg T.E. (2003) Pituitary expression of LH beta- and FSH beta-subunit mRNA, cellular distribution of LH beta-subunit mRNA and LH and FSH synthesis during and after treatment with a gonadotrophin-releasing hormone agonist in heifers Reproduction Fertility and Development 15:149-156.

Baenziger J., Green E. (1988) Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. Biochim Biophys Acta 947:287-306.

Bao B., Garverick H.A. (1998) Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J Anim Sci 76:1903-1921.

Baracaldo M.I., Martinez M.F., Adams G.P., Mapletoft R.J. (2000) Superovulatory response following transvaginal follicle ablation in cattle. Theriogenology 53:1239-50.

Baruselli P.S., Reis E.L., Marques M.O., Nasser L.F., Bo G.A. (2004) The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. Anim Reprod Sci 82-83:479-486.

Bergfelt D.R., Lightfoot K.C., Adams G.P. (1994) Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 42:895-907.

Bergfelt D.R., Bo G.A., Mapletoft R.J., Adams G.P. (1997) Superovulatory response following ablation-induced follicular wave emergence at random stages of the oestrous cycle in cattle. Anim Reprod Sci 49:1-12.

Billig H., Furuta I., Hsueh J.W. (1993) Estogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 133:2204–2212.

Bo G.A., Adams G.P., Pierson R.A., ́Trıbulo H.E.T., Caccia M., Mapletoft R.J. (1994) Follicular wave dynamics after estradiol- 17β treatment of heifers with or without a progestin implant. Theriogenology 41:1555-1559.

Bo G.A., Adams G., Pierson R., Mapletoft R. (1995a) Exogenous control of follicular wave emergence in cattle. Theriogenology 43:31-40.

Bo G.A., Adams G., Caccia M, Martinez M., Pierson R., RJ. M. (1995b) Ovarian follicular wave emergence after treatment with progestogen and estradiol in cattle. Anim Reprod Sci 39:193-204.

Bo G.A., Adams G.P., Nasser L.F., Pierson R.A., Mapletoft R.J. (1993) Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 40:225-239.

Bo G.A., Baruselli P.S., Moreno D., Cutaia L., Caccia M., Tribulo R., Tribulo H., Mapletoft R.J. (2002) The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology 57:53-72.

Bo G.A., Bergfelt D.R., Brogliatti G.M., Pierson R.A., Adams G.P., Mapletoft R.J. (2000) Local versus systemic effects of exogenous estradiol-17 beta on ovarian follicular dynamics in heifers with progestogen implants. Anim Reprod Sci 59:141-57.

Braw-Tal R., Yossefi S. (1997) Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. J Reprod Fertil. 109:165-171.

Bridges P., Lewis P., Wagner W., Inskeep E. (1999) Follicular growth, estrus and pregnancy after fixed-time insemination in beef cows treated with intravaginal progesterone inserts and estradiol benzoate. Theriogenology 52:573-583.

Britt J.H. (1991) Impacts of early postpartum metabolism on follicular development and fertility. Dairy Session 1: 39-43.

Brogliatti G. M., Lagomarsino H., Adams G.P.(1998) Follicular ablation for ovulation synchronization in cattle. Theriogenology 49:339(abstr).

Burke C.R., Mussard M.L., Gasser C.L., Grum D.E., Dayemail M.L. (2003) Estradiol benzoate delays new follicular wave emergence in a dose-dependent manner after ablation of the dominant ovarian follicle in cattle. Theriogenology 60:647-658.

Butler S.A., Atkinson P.C., Boe-Hansen G.B., Burns B.M., Dawson K., Bo G.A., McGowan M.R. (2011) Pregnancy rates after fixed-time artificial insemination of

Brahman heifers treated to synchronize ovulation with low-dose intravaginal progesterone releasing devices, with or without eCG. Theriogenology 76:1416-1423.

Caccia M., Bo G.A. (1998) Follicle wave emergence following treatment of CIDR-B-implanted beef heifers with estradiol ben- zoate and progesterone. Theriogenology 49:341(abstr).

Campbell b., Scaramuzzi R., Webb R. (1995) Control of antral follicle development and selection in sheep and cattle. Reprod Fertil 49:335-350.

Campbell B.K., Scaramuzzi R.J., Downing J.A., Evans G. (1990) Steroid secretion rates and plasma binding activity in androstenedione-immune ewes with an autotransplanted ovary. J Reprod Fertil 89:485-496.

Campbell B.K., Telfer E.E., Webb R., Baird D.T. (2000) Ovarian autografts in sheep as a model for studying folliculogenesis. Mol. Cell. Endocrinol. 163:137-139.

Cardenas H., Pope W.F. (1994) Administration of testosterone during the follicular phase increased the number of corpora lutea in gilts. J Anim Sci 72:2930-2935.

Cardenas H., Pope W.F. (1997) Administration of testosterone from day 13 of the estrous cycle to estrus increased the number of corpora lutea and conceptus survival in gilts. J Anim Sci 75:202-207.

Cardenas H., Pope W.F. (2002) Control of ovulation rate in swine. J. Anim. Sci. 80 (E. Suppl. 1):E36–E46.

Cardenas H., Herrick J.R., Pope W.F. (2002) Increased ovulation rate in gilts treated with dihydrotestosterone. Reproduction 123:527-33.

Chaffin C.L., Stouffer R.L., Duffy D.M. (1999) Gonadotropin and steroid regulation of steroid receptor and aryl hydrocarbon receptor messenger ribonucleic acid in macaque granulosa cells during the periovulatory interval. Endocrinology 140:4753-4760.

Chun S.Y., Billig H., Tilly J.L., Furuta I., Tsafriri A., Hsueh A.J. (1994) Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. Endocrinology 135:1845-1853.

Colazo M., Rutledge M., Small J., Kastelic J., Siqueira L., Ward D., Mapletoft R. (2005a) Effects of presynchronization with a used-CIDR and treatment with eCG on fertility in lactating cows subjected to a Cosynch protocol. Reprod Fertil Dev 17: 156(abstr)..

Colazo M.G., ́ınez M.F.M., P.R.Whittaker, J.P.Kastelic, Mapletoft R.J. (2002) Estradiol cypionate (ECP) in CIDR-B based programs for fixed-time AI in beef heifers. Theriogenology 57:371 Abstr.

Colazo M.G., Martinez M.F., Small J.A., Kastelic J.P., Burnley C.A., Ward D.R., Mapletoft R.J. (2005b) Effect of estradiol valerate on ovarian follicle dynamics and superovulatory response in progestin-treated cattle. Theriogenology 63:1454-68.

Craig Z.R., Wang W., Flaws J.A. (2011) Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signaling. Reproduction 142:633-46.

Curtin D., Jenkins S., Farmer N., Anderson A.C., Haisenleder D.J., Rissman E., Wilson E.M., Shupnik M.A. (2001) Androgen suppression of GnRH-stimulated rat LH

bategene transcrip- tion occurs through Sp1 sites in the distal GnRH-respon- sive promoter region. Mol Endocrinol 15:1906-1917.
D'Occhio M., Niasari-Naslaji A., O'Niell C., JE F., Aspden, Whyte T., A D. (1996) Oestrous behaviour and fertility after synchronisation treatment using oestradiol-progestogen for ten days or hCG-progestogen for eight days in beef heifers. Proc Int Cong Anim Reprod 4:18(abstr.).
Daniel S.A., Armstrong D.T. (1986) Androgens in the ovarian microenvironment. Semin Reprod Endocrinol. 4.
Downey B.R. (1980) Regulation of the estrous cycle in domestic animals-- a review. Can Vet J 21:301-306.
Drummond A.E. (2006) The role of steroids in follicular growth. Reproductive Biology and Endocrinology 4:16-26.
EC (1996) Council directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing directives 81/602/EEC, 88/146/EEC and 88/299/EEC, Official Journal of the European Communities.
EC (2003) L 262, 14/10/2003. Directive 2003/74/EC of the European Parliament and of the Council on 22 September 2003 amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyristatic action and of beta-agonist. Official Journal of the European Union :17-21.
Encyclopedia,A.D.A.M Medical (2011) Hudson's FTM Resource Guide: Bioidentical Testosterone www.ftmguide.org/bioidenticalt.html.
Evans A., Adams, G.P. , Rawlings N. C.(1994) Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. J Reprod Fert 100:187-194.
Fetherston J., Boime I. (1982) Synthesis of bovine lutropin in cell-free lysates containing pituitary microsomes. Journal of Biological Chemistry 257:143-147.
Fortune J.E. (1993) Follicular dynamics during the bovine estrous cycle: a limiting factor in improvement of fertility? Anim. Reprod. Sci. 33:111-125.
Fortune J.E. (1994) Ovarian Follicular Growth and Development in Mammals. Bio Repr 50:225-232.
Fortune J.E. (2003) The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Animal Reproduction Science 78:135-163.
Fortune J.E., Cushman R.A., Wahl C.M., Kito W.S. (2000) The primordial to primary follicle transition. Mol. Cell. Endocrinol. 163:53-60.
Gharib S.D., Bowers S.M., Need L.R., Chin W.W. (1986) Regulation of rat luteinizing hormone subunit messenger ribonucleic acids by gonadal steroid hormones. J Clin Invest 77:582-589.
Gibbons J., Wiltbank M., Ginther O. (1997) Functional interrelationships between follicles greater than 4 mm and the follicle-stimulating hormone surge in heifers. Biology of Reproduction 57:1066-1073.
Ginther O., Knopf L., Kastelic J. (1989a) Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil. 87:223-230.

Ginther O., Kastelic J., Knopf L. (1989b) Composition and characteristics of follicular waves during the bovine estrous cycle. Anim Reprod Sci 20:187-200.
Ginther O., Bergfelt D., Kulick L., Kot K. (1998) Pulsatility of systemic FSH and LH concentrations during follicular wave development in cattle. Theriogenology 50:507-519.
Ginther O., Bergfelt D., Beg M., Kot K. (2001a) Follicle selection in cattle: role of luteinizing hormone. BioI Reprod 64:197-205.
Ginther O., Beg M., Bergfelt D., Donadeu F., Kot K. (2001b) Follicle selection in monovular species. BioI Reprod 65:638-647.
Ginther O., Beg M., Kot K., Meira C., Bergfelt D. (2003) Associated and Independent Comparisons Between the Two Largest Follicles Preceding Follicle Deviation in Cattle. Biol Reprod 68:524-529.
Ginther O., Bergfelt D.R., Kulick L.J., Kot K. (2000) Selection of the dominant follicle in cattle: Role of estradiol. Biol Reprod 63:383-389.
Ginther O.J., Witbank M.C., Fricke P.M., Gibbons J.R., Kot K. (1996a) Selection of dominant follicle in cattle. Biol. Reprod. 55:1187-1194.
Ginther O.J., Kot K., Kulick L., Martin S., Wiltbank M. (1996b) Relationships between FSH and follicular waves during the last six months of pregnancy in cattle. J Reprod Fert 108:271-279.
Gong J., Campbell B., Bramley T., Gutierrez C., Peters A., Webb R. (1996) Suppression in the Secretion of Follicle-Stimulating Hormone and Luteinizing Hormone, and Ovarian Follicle Development in Heifers Continuously Infused with a Gonadotropin-Releasing Hormone Agonist. Biol Repod 55:68-74.
Goudah A. (2009) Pharmacokinetics and tissue residues of moxifloxacin in broiler chickens. Br Poult Sci 50:251-258.
Gross D. (1980) Effect of castration and steroid replace- ment on immunoreactive gonadotropin-releasing hor- mone in hypothalamus and preoptic area. Endocrinology 106:1442-1450.
Hafs H.D., LouisT.M., Noden P.A., Oxender W.D. (1974) Control of the estrous cycle with prostaglandin F2{alpha} in cattle and horses 38:10-21.
Hampton J.H., Manikkam M., Lubahn D.B., Smith M.F., Garverick H.A. (2004) Androgen receptor mRNA expression in the bovine ovary. Domest Anim Endocrinol 27:81-8.
Handa R.J., Reid D.L., Resko J.A. (1986) Androgen recepters in brain and pituitary of female: Cycle changes and comparisons with male. Biol Reprod 34:293-303.
Hansel W., Echternkamp S. (1972) Control of ovarian function in domestic animals. Am Zoologist 12:225-243.
Hanukoglu I. (1992) Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. J Steroid Biochem Mol Biol 43:779-804.
Harlow C.R., Shaw H.J., Hillier S.G., Hodges J.K. (1988) Factors influencing follicle-stimulating hormone-responsive steroidogenesis in marmoset granulosa cells: effects of androgens and the stage of follicular maturity. Endocrinology 122:2780-2787.
Hassani M., Plas-Roser S., Roos J., Aron C. (1978) Action of testosterone propionate on the gonadotrophic function of the pituitary gland in the cyclic female rat. Acta Endocrinol (Copenh) 89:551-556.

Heekin M. (1983) Comparison oftwo testosterone treatments for heat detector cows. Calif Vet 3:11-12.
Heekin M.D., Kiracofe H.G. (1983) Comparison of two testosterone treatments for heat detector cows Calif Vet 3:11-12.
Henry H.L., Norman A.W. (2003) Encyclopedia of Hormones: Androgen effects in mammals. 1 ed. Acdemic Press, San Diego.
Herbison A. (1998) Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrine Reviews 19:302-320.
Herriman I.D., Harwood D.J., Mallinson C.B., Heitzman R.J. (1979) Plasma concentrations of ovarian hormones during the oestrous cycle of the sheep and cow. J Endocrinol 81:61-4.
Hillier S., Ross G. (1979) Effects of exogenous testosterone on ovarian weight, follicular mophology and intraovarian progesterone concentration in estrogen-primed hypophysectomized immature female rats. Biol of Reprod 20: 261-268.
Hillier S.G., De Zwart F.A. (1981) Evidence that granulosa cell aromatase induction/activation by follicle-stimulating hormone is an androgen receptor-regulated process in-vitro. Endocrinology 109:1303-5.
Hillier S.G., Knazek R.A., Ross G.T. (1977) Androgenic stimulation of progesterone production by granulosa cells from preantral ovarian follicles: further in vitro studies using replicate cell cultures. Endocrinology 100:1539-49.
Hodgen G.D. (1982) The dominant ovarian follicle. Fertil Steril 38:281-300.
Holtz S. (2009) Reducing and Phasing Out the Use of Antibiotics and Hormone Growth Promoters in Canadian Agriculture, Canadian Institute for Environmental Law and Policy.
Homeida A.M., Cooke R.G. (1984) Plasma concentration of testosterone and 5 - dihydrotestosteron around luteolysis in goats and their behavioural effects after ovariectomy Journal of Steroid Biochemistry 20:1357-1359
Homeida A.M., Khalafalla A.E. (1990) Effects of oxytocin and oxytocin antagonist on testosterone secretion during the oestrous cycle of the goat (Capra Hircus). J.Reprod Fertil 89:347-350.
Horie K., Takakura K., Fujiwara H., Suginami H., Liao S., Mori T. (1992) Immunohistochemical localization of androgen receptor in the human ovary throughout the menstrual cycle in relation to oestrogen and progesterone receptor expression. Hum Reprod 7:184-190.
Hsu C.J., Hammond J.M. (1987) Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells in vitro. Endocrinology 120:198-207.
Huang B.M., Leu S.F., Yang H.Y., Norman R.L. (2001) Testosterone effects on luteinizing hormone and follicle-stimulating hormone responses to gonadotropin-releasing hormone in the mouse. J Androl 22:507-513.
Hulshof S.C.J., Figueiredo J.R., Becker J.F., Severs M.M., Van den Donk J.A., Van den Hurk R. (1995) Effects of fetal bovine serum, FSH and 17β-estradiol on the culture of bovine preantral follicles. Theriogenology 44:217-226
Ireland J.J., Roche J.F. (1982) Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. J Reprod Fertil 64:295-302.

Ireland J.J., Roche J.F. (1983) Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinology 112:150-156.

Ireland J., Mihm M., Austin E., Diskin M., Roche J. (2000) Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements, and terms. J Dairy Sci. 83:1648-1658.

Jadav P., Patel D., Kavani F., Dhami A. (2010) GnRH and its applications in bovine reproduction. J.Adv.Dev.Res. 1:74-80.

Jaiswal RS, Singh J**,** Adams GP (2004) Developmental pattern of small antral follicles in the bovine ovary. Biology of Reproduction 71:1244-51.

Jaiswal R.S., Singh J., Marshall L., Adams G.P. (2009) Repeatability of 2-wave and 3-wave patterns of ovarian follicular development during the bovine estrous cycle. Theriogenology 72:81–90.

Jia X.C., Kessel B., Welsh T.H., Jr., Hsueh A.J. (1985) Androgen inhibition of follicle-stimulating hormone-stimulated luteinizing hormone receptor formation in cultured rat granulosa cells. Endocrinology 117:13-22.

Jimenez E., Cardenas H., Pope W.F. (2008) Effects of Androgens on Serum Concentrations of Gonadotropins and Ovarian Steroids in Gilts. Biol Repeod 79:1148-1152.

Jorgensen J.S., Nilson J.H. (2001) AR suppresses transcription of the LH ß subunit by Interacting with steroidogenic factor-1. Molecular Endocrinology 15:1505-1516.

Juengel J.L., Heath D.A., Quirke L.D., McNatty K.P. (2006) Oestrogen receptor alpha and beta, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep. Reproduction 131:81-92.

Kaipia A., Heueh A.J. (1997) Regulation of ovarian follicle atresia. Ann. Rev. Physiol. 59:349-363.

Kaiser U.B. (1997) Molecular mechanisms of the regulation of gonadotropin gene expression by gonadotropin-releasing hormone. Mol Cells. 8:647-656.

Karsch F.J., Evans N.P. (1996) Feedback actions of estradiol on GnRH secretion during the follicular phase of the estrous cycle. Acta Neurobiol Exp (Wars) 56:715-25.

Kastelic J., McCartney D., Olson W., Barth A., Mapletoft R. (1997) Melengestrol acetate and estrdiol 17 for synchronizing estrus and improving reproductive perfonnance in beef cattle. Can J Anim Sci 77:683-688.

Keri R.A., Wolfe M.W., Saunders T.L., Anderson L., Kendall S.K., Wagner T., Yeung J., Gorski J., Nett T.M., Camper S.A., Nilson J.H. (1994) The proximal promoter of the bovine luteinizing hormone , subunit gene confers gonadotrope-specific expression and regulation by gonadotropin-releasing hormone, testosterone, and 17P-estradiol in transgenic mice. Mol Endocrinol 8:1807-1816.

Kesler D.J., Favero R.J., Esarey J.C., Berger L.L. (1995) Controlled delivery of testosterone propionate suppresses fertility in treated females and induces prenatal androgenization in female offspring without phenotypic masculinization. Drug Development and Industrial Pharmacy 21:1513-1527.

Kesner J.S., Convey E.M., Anderson C.R. (1981) Evidence that estradiol induces the preovulatory LH surge in cattle by increasing pituitary sensitivity to LHRH and then increasing LHRH release. Endocrinology 108:1386-1391.

Kim I.H., Son D.S., Yeon S.H., Choi S.H., Park S.B., Ryu I.S., Suh G.H., Lee D.W., Lee C.S., Lee H.J., Yoon J.T. (2001) Effect of dominant follicle removal before superstimulation on follicular growth, ovulation and embryo production in Holstein cows. Theriogenology 55:937-945.

Kiser T., Britt J., Ritchie H. (1977) Testosterone treatment of cows for use in detection of estrus. J Anim Sci 44:1030-1035.

Ko J.C., Kastelic J.P., Del Campo M.R., Ginther O.J. (1991) Effects of a dominant follicle on ovarian follicular dynamics during the oestrous cycle in heifers. J Reprod Fertil 91:511-519.

Krey L., MacLusky N., Davis P., Lieberburg D., Roy E. (1982) Different intracellular mechanisms underlie testosterone's suppression of basal and stimulation of cyclic luteinizing hormone release in male and female rats. Endocrinology 110:2156-2167.

Kulick L., Kot K., Wiltbank M., Ginther' O. (1999) Folliculiar and hormonal dynamics during the first follicular wave in hifers Theriogenology 52:913-921.

Lane E., Austin E., Crowe M.A. (2008) Oestrous synchronisation in cattle-Current options following the EU regulations restricting use of oestrogenic compounds in food-producing animals: A review. Anim Reprod Sci 109:1-16.

Lee C., Henricks D., Skelley G., Grimes L. (1990) Growth and hormonal response of intact and castrate male cattle to trenbolone acetate and estradiol. J Anim Sci 68:2682-89.

Leitman N.R., Busch D.C., Bader J.F., Mallory D.A., Wilson D.J., Lucy M.C., Ellersieck M.R., Smith M.F., Patterson D.J. (2008) Comparison of protocols to synchronize estrus and ovulation in estrous-cycling and prepubertal beef heifers. J Anim Sci 86:1808-1818.

Littell R.C., Henry P.R., Ammerman C.B. (1998) Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 76:1216-1231.

Lone K.P. (1997) Natural sex steroids and their xenobiotic analogs in animal production: growth, carcass quality, pharmacokinetics, metabolism, mode of action, residues, methods, and epidemiology. Crit Rev Food Sci Nutr 37:93-209.

Lucy M.C. (2007) The bovine dominant ovarian follicle J. Anim. Sci. 85:E89- E99.

Lussier J.G., Matton P., Dufour J.J. (1987) Growth rates of follicles in the ovary of the cow. J Reprod Fertil 81:301-307.

Macmillan K.L., Thatcher W.W. (1991) Effects of an agonist of gonadotropin-releasing hormone on ovarian follicles in cattle. Biol Reprod 45:883-889.

Mapletoft R.J., Bo G.A., Baruselli P.S. (2009) Control of ovarian function for assisted reproductive technologies in cattle. Anim. Reprod. 6:114-124.

Mapletoft R.J., Mart´ınez M.F., Adams G.P., Kastelic J.P., Burnley C.A. (1999) The effect of estradiol preparation on follicular wave emergence and superovulatory response in norgestomet- implanted cattle. Theriogenology 51:411. Abstr.

Mapletoft R., Steward K., Adams G. (2002) Recent advances in the superovulation in cattle. Reprod Nutr Dev. 42:601-611.

Martinez M., Kastelic J., Bó G., Caccia M., Mapletoft R. (2005) Effects of oestradiol and some of its esters on gonadotrophin release and ovarian follicular dynamics in CIDR-treated beef cattle. Anim Reprod Sci 68:37-52.

Martinez M.F., Kastelic J.P., Colazo M.G., Mapletoft R.J. (2007) Effects of estradiol on gonadotrophin release, estrus and ovulation in CIDR-treated beef cattle. Domest Anim Endocrinol 33:77-90.

Martinez M.F., Adams G.P., Bergfelt D.R., Kastelic J.P., Mapletoft R.J. (1999) Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. Anim Reprod Sci 57:23-33.

Marínez M.F., Bó G.A., Mapletoft R.J. (2004) Synchroinzation of follicular wave emergence and ovulation for reprodactive biotechnology. 1°Simposio Internacional De Reproducao Animal Aplicada : 26-53.

McKinnie M.R., Britt J.H., Esbenshade K.L. (1988) Ovarian Function and Hormone Secretion of Gilts Actively Immunized against Androstenedione . J Anim Sci 66:3131- 3143.

Mihm M., Crowe M.A., Knight P.G., Austin E.J. (2002) Follicle wave growth in cattle. Reprod Domest Anim 37:191-200.

Mihm M., Good T.E.M., Ireland J.L.H., Ireland J.J., Knight P.G., Roche J.F. (1997) Decline in serum follicle stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. Biol. Reprod. 57:1328–1337.

Momcilovic D., L F Archbald, Waiters A., Tran T., Kelbert D., Risco C., WW Thatcher. (1998) Reproductive performance of lactating dairy cows treated with gonadotropin releasing hormone (GnRH) and/or prostaglandin F2 alpha for synchronization of estrus and ovulation. Theriogenology 50:1131- 1139.

Moreira F., Orlandi C., Risco C.A., Mattos R., Lopes F., Thatcher W.W. (2001) Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J Dairy Sci 84:1646-1659.

Murray A.A., Gosden R.G., Allison V., Spears N. (1998) Effect of androgens on the development of mouse follicles growing in vitro. J Reprod Fertil 113:27-33.

Navanukraw C., Redmer D.A., Reynolds L.P., Kirsch J.D., Grazul-Bilska A.T., Fricke P.M. (2004) A modified presynchronization protocol improves fertility to timed artificial insemination in lactating dairy cows. J Dairy Sci 87:1551-1557.

Nix J.P., Spitzer J.C., Chenoweth P.J. (1998) Serum testosterone concentration, efficiency of estrus detection and libido expression in androgenized beef cows. Theriogenology 49:1195-207.

Norman A.W., Litwack G. (1997) Hormones (2nd ed)San Diego, California:Academic Press; 1997.

Niswender G.D. (1981) Response of the corpus luteum to luteinizing hormone. Environmental Health Perspectives 38:47-50.

Niswender G.D., Juengel J.L., Silva P.J., Rollyson M.K., McIntush E.W. (2000) Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 80:1-29.

Partsch C.J., Weinbauer G.F., Fang R., Nieschlag E. (1995) Injectable testosterone undecanoate has more favourable pharmacokinetics and pharmacodynamics than testosterone enanthate. Eur J Endocrinol 132:514-519.

Peterson A.J., Fairclough R.J., Smith S.T. (1978) Radioimmunoassay of androstenedione and testosterone in cow plasma at the time of luteolysis and oestrus. J. Reprod. Fert. 52:127-129.

Pierson R., Ginthe O. J. (1987a) Ultrasonic imaging of the ovaries and uterus in cattle. Theriogenology 29:21-37.
Pierson R.A., Ginther O.J. (1984) Ultrasonography of the bovine ovary. Theriogenology 21:495-504.
Pierson R.A., Ginther O.J. (1987b) Reliability of diagnostic ultrasonography for identification and measurement of follicles and detecting the corpus luteum in heifers. Theriogenology 28:929-936.
Preston R. (1999) Hormone containing growth promoting implants in farmed livestock. Adv Drug Deliv Rev 38:123-138.
Price C.A., Webb R. (1988) Steroid control of gonadotropin secretion and ovarian function in heifers. Endocrinology 122:2222-2231.
Pugh P.J., Jones R.D., West J.N., Jones T.H., Channer K.S. (2004) Testosterone treatment for men with chronic heart failure. Heart 90:446-7.
Pursley J.R., Mee M.O., Wiltbank M.C. (1995) Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology 44:915-923. DOI: 0093-691X(95)00279-H [pii].
Quirk S.M., Cowan R.G., Harman R.M., Hu C.-L., Porter D.A. (2004) Ovarian follicular growth and atresia: The relationship between cell proliferation and survival. J. Anim. Sci. 82:E40-E52.
Radford H., Wallace A. (1971) The effect of testosterone propionate on ovarian activity in sheep. J Reprod Fertil 24:439-440.
Rajakoski E. (1960) The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, end left-right variations. Acta Endocrinol. Suppl. (Copenh.) 34:1–68.
Rajamahendran R., Manikkam M. (1994) Effects of exogenous steroid hormones on the dominant follicle maintained by a Norgestomet implant in heifers. Canadian Journal of Animal Science 74:457-464.
Rathbone M., Kinder J., Fike K., Kojima F., Clopton D., Ogle C., Bunt C. (2001) Recent advances in bovine reproductive endocrinology and physiology and their impact on drug delivery system design for the control of the estrous cycle in cattle. Adv Drug Deliv Rev 50:277-320.
Rawlings N., Evans A. (1995) Androgen feedback during the early rise in LH secretion in bull calves Journal of Endocrinology 145:243-249.
Rawlings N., Jeffcoate I.A., Rieger D.L. (1984) he influence of estradiol-17beta and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized ewe. Theriogenology 22:473-488.
Rhodes F.M., Fitzpatrick L.A., Entwistle K.W., Kinder J.E. (1995) Hormone concentrations in the caudal vena cava during the first ovarian follicular wave of the oestrous cycle in heifers. J Reprod Fertil 104:33-39.
Roche J.F., Mihm M., Diskin M. (1997) Physiology and practice of induction and control of oestrus in cattle. Bov Pract 31:4-10.
Roche J.F., Mihn M., Diskin M.G., Ireland J.J. (1998) A Review of Regulation of Follicle Growth in Cattle. J Anim Sci 76:16-29.

Roos J., Plas-Roser S., Aron C. (1980) Mechanisms of action of testosterone propionate on LH and FSH release by the pituitery gland in cyclic female rats Acta Endocrinol (Copenh) 95:158-165.

Roselli C., Kelly M., Ronnekleiv O. (1990) Testosterone regulates progonadotropin-releasing hormone levels in the preoptic area and basal hypothalamus of the male rat. Endocrinology 126:1080-1086.

Sa Filho O.G., Patterson D.J., Vasconcelos J.L. (2009) Development of estrous synchronization protocols using melengestrol acetate in Bos indicus cattle. J Anim Sci 87:1981-90.

Savio J.D., Boland M.P., Roche I.F. (1990a) Development of dominant follicles and length of ovarian cycles in postpartum dairy cows. Reprod Fertil 88:581-591.

Savio J.D., Boland M., Hynes N., Roche I.F. (1990b) Resumption of follicular activity in the early postpartum period ofdairy cows. Reprod Fertil 88:569-579.

Savio J.D., Thatcher W.W., Badinga L., Sota R.L.d.l., Wolfenson D. (1993) Regulation of dominant follicle turnover during the oestrous cycle in cows. J. Reprod. Fertil. 97:197-203.

Schmidely P. (1993) Quantitative bibliographic review on the use of anabolic hormones with steroidogenic action in ruminants for meat production. II. Principal mode of action]. Reprod Nutr Dev 33:297-323.

Schoenemann H.M., Humphrey W.D., Crowder M.E., Nett T.M., Reeves J.J. (1985) Pituitary luteinizing hormone-releasing hormone receptors in ovariectomized cows after challenge with ovarian steroids. Biol Reprod 32:574-83.

Silvia W.J., Jacobs A.L., SH. S.H.H. (1989) Effects of testosterone and 5alpha-dihydrotestosterone on luteal lifespan in dairy heifers. Theriogenology 32:871-876.

Skinner M.K. (2005) Regulation of primordial follicle assembly and development. Hum. Reprod. Update 11:461-471.

Slomczynska M., Tabarowski Z. (2001) Localization of androgen receptor and cytochrome P450 aromatase in the follicle and corpus luteum of the porcine ovary. Anim Reprod Sci 65:127-134.

Smith M.J., Jennes L. (2001) Neural signals that regulate GnRH neurones directly during the oestrous cycle. Reproduction 122:1-10.

Suzuki T., Sasano H., Kimura N., Tamura M., Fukaya T., Yajima A., Nagura H. (1994) Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. Hum Reprod 9:1589-1595.

Stock A.E., Fortune J.E. (1993) Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endocrinology 132:1108-1114.

Talmadge K., Boorstein W., Fiddes L.C. (1983) The humen genome contains 7 genes for thebeta sub unite of chorionic gonadotropin but only one gene for the beta subunite of luteinizing hormone. DNA 2:279-287.

Tetsuka M., Whitelaw P.F., Bremner W.J., Millar M.R., Smyth C.D., Hillier S.G. (1995) Developmental regulation of androgen receptor in rat ovary. J Endocrinol 145:535-543.

Thompson D.L., Jr., Voelkel S.A., Reville-Moroz S.I., Godke R.A., Derrick D.J. (1984) Testosterone effects on gonadotropin respone to GnRH: Cows and pony mares J. Anim. Sci. 58:409- 415.

Thompson D.L., Jr., Wiest J.J., Garza W.F., Jr., Ashley K.B., McNeill D.R. (1987) Passive immunization of cyclic mares against androgen: gonadotropin and progesterone concentrations and estrous characteristics. J Anim Sci 64:797-804.

Thundathil J., Kastelic J.P., Mapletoft R.J. (1998) The effect of estradiol cypionate (ECP) on ovarian follicular development and ovulation in dairy cattle. Can J Vet Res 62:314-6.

Toranzo D., Dupont E., Simard J., Labrie C., Couet J., Labrie F., Pelletier G. (1989) Regulation of pro-gonadotropin-releasing hormone gene expression by sex steroids in the brain of male and female rats. Mol Endocrinol. 3:1748-1756.

Traish A.M., Feeley R.J., Guay A.T. (2009) Testosterone therapy in women with gynecological and sexual disorders: a triumph of clinical endocrinology from 1938 to 2008. J Sex Med 6:334-51.

Tropea A., Lanzone A., Tiberi F., Romani F., Catino S., R R.A. (2010) Estrogens and androgens affect human luteal cell function. Fertil Steril. 94:2257-2263.

Twagiramungu H., Guilbault L.A., Dufour J. (1995) Synchronization of ovarian follicular waves with a Gonadotropin-Releasing Hormone agonist to increase the precision of estrus in cattle: a review. J Anim Sci 73:3141-3151.

Van den Hurk R., Zhao J. (2005) Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology 63:1717-1751.

Vasconcelos J.L., Silcox R.W., Rosa G.J., Pursley J.R., Wiltbank M.C. (1999) Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-78.

Vendola K., Zhou J., Wang J., Bondy C. (1999a) Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. Hum Reprod 14:2328–2332.

Vendola K., Zhou J., Wang J., Famuyiwa O.A., Bievre M., Bondy C.A. (1999b) Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol Reprod 61:353-357.

Vendola K.A., Zhou J., Adesanya O.O., Weil S.J., Bondy C.A. (1998) Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest 101:2622-9.

Vermeulen A. (1998) Plasma androgens in women. J Reprod Med 43:725-733.

Vizcarra J.A., Wettemann R.P., Braden T.D., Turzillo A.M., Nett T.M. (1997) Effect of gonadotropin-releasing hormone (GnRH) pulse frequency on serum and pituitary concentrations of luteinizing hormone and follicle-stimulating hormone, GnRH receptors, and messenger ribonucleic acid for gonadotropin subunits in cows. Endocrinology 138:594-601.

Walters D., Schallenberger E. (1984) Pulsatile secretion of gonadotropins, ovarian steroids and ovarian oxytocin during the periovulatory phase of the oestrous cycle in the cow. J Reprod Fertil 71:503-512.

Walters K.A., Allan C.M., Handelsman D.J. (2008) Androgen actions and the ovary. Biol Reprod 78:380-389.

Wandji S., Fortier M., Sirard M. (1992) Differential response to gonadotrophins and prostaglandin E2 in ovarian tissue during prenatal and postnatal development in cattle. Biology of Reproduction 46:1034-1041.

Wandji S., Srsen V., Voss A., Eppig J., Fortune J. (1996) Initiation in vitro of growth of bovine primordial follicles. Biology of Reproduction. Biology of Reproduction 55:942-948.

Webb R., Garnsworthy P.C., Gong J.G., Armstrong D.G. (2004) Control of follicular growth: local interactions and nutritional influences. J Anim Sci 82 E-Suppl:E63-74. DOI: 82/13_suppl/E63 [pii].

Weil S., Vendola K., Zhou J., Bondy C.A. (1999) Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J Clin Endocrinol Metab 84:2951-2956.

Weil S.J., Vendola K., Zhou J., Adesanya O.O., Wang J., Okafor J., Bondy C.A. (1998) Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J Clin Endocrinol Metab 83:2479-85.

Whisnant C.S., Washburn S.P., Farin P.W. (2000) Current concepts in synchronization of estrus and ovulation of dairy cows. J Anim Sci 77:1-8.

Wise M., Nilson J., Nejedlik M., Nett T. (1985) Measurement of messenger RNA for luteinizing hormone beta-subunit and alpha-subunit during gestation and the postpartum period in ewes. Biol Reprod 33:1009-1015.

Wise T., Caton D., Thatcher W., Lehrer A., Fields M. (1982) Androstenedione, dehydroepiandrosterone and testosterone in ovarian vein plasma and androstenedione in peripheral arterial plasma during the bovine oestrous cycle. JReprod Fert 66:513-518.

Wolfe M.W., Roberson M.S., Stumpf T.T., Kittok R.J., Kinder J.E. (1992) Circulating concentrations and pattern of lu- teinizing hormone and follicle-stimulating hormone in circula- tion are changed by the circulating concentration of 17b-es-tradiol in the bovine male and female. J. Anim. Sci. 70:248- 453.

Wu Y., Bennett J., Talla D., Stocco C. (2011) Testosterone, not 5-Dihydrotestosterone, Stimulates LRH-1 Leading to FSH-Independent Expression of Cyp19 and P450scc in Granulosa Cells. Mol. Endocrinol. 25:656-668.

Xu Z., Garverick H.A., Smith G.W., Smith M.F., Hamilton S.A., Youngquist R.S. (1995) Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod 53:951-957.

Yang B., Huang L., Wang Y., Liu Y., Tao Y., Chen D., Liu Z., Fang K., Chen Y., Yuan Z. (2010) Residue depletion and tissue-plasma correlation of methyl-3-quinoxaline-2-carboxylic acid after dietary administration of olaquindox in pigs. J Agric Food Chem 58:937-942.

Yang M.Y., Fortune J.E. (2006) Testosterone Stimulates the Primary to Secondary Follicle Transition in Bovine Follicles In Vitro. Biol Reprod 75:924-932.

Yasin M., Dalkin A.C., Haisenleder D.J., Marshall J.C. (1996) Testosterone is required for gonadotropin-releasing hormone stimulation of luteinizing hormone-beta

messenger ribonucleic acid expression in female rats. Endocrinology 137:1265-1271.

Youngquist R., Threlfall W. (2007) Current Therapy in Large Animal Theriogenology. Philadelphia: Saunders Elsevier; 2007.

Yuki Okutsu M.D., Itoh M.T., Takahashi N., Ishizuka B. (2010) Exogenous androstenedione induces formation of follicular cysts and premature luteinization of granulosa cells in the ovary. Fertility and Sterility 93:927-935.

Zhang G.Y., Gu Y.Q., Wang X.H., Cui Y.G., Bremner W.J. (1998) A pharmacokinetic study of injectable testosterone undecanoate in hypogonadal men. J Androl 19:761-768.

Appendix 1
Statistical analysis model for single-point measurements

1-Mixed model procedure using CRD (Complete randomize design)

```
Data hayder ;
Input ID TRT$ X;
Cards;
72      T1      24
198     T1      40
156     T1      24
176     T1      18
1       T1      45
65      T1      21
.         .
.         .
.         .
.         .
.         .
;
PROC UNIVARIATE DATA=Hayder NORMAL PLOT;
VAR X;
BY TRT;
RUN;
PROC MIXED DATA=hayder;
CLASS TRT;
MODEL X=TRT/DDFM=SATTERTH;
RUN;
```

To test the normality of data

If the model was significant, we tested the different among the treatments over the time by using LSD as following way:

```
Data hayder ;
Input ID TRT$ X;
Cards;
72      T1      24
198     T1      40
156     T1      24
176     T1      18
1       T1      45
65      T1      21
```

;
PROC MIXED DATA=hayder;
CLASS TRT;
MODEL X=TRT/DDFM=SATTERTH;
Lsmeans TRT/pdiff;
RUN;

2-Mixed model procedure using t-test (comparing between tow combined treatment vs control groups)

Data hayder ;
Input TRT$ X;
Cards;
T 17
T 13
T 14
T 9
T 10
T 9
T 8
T 14
T 10
T 13
T 13
T 9
T 14
T 10
T 10
T 18
T 9
T 14
C 10
C 9
C 8
C 9
C 9
C 7
;
proc ttest data=hayder h0=**0**;
class trt;
var x ;
run;

Appendix 2
Statistical analysis model for serial data

1- Mixed model procedure (Repeated Measures)

```
Options nocenter;
Data hayder ;
Input ID TRT$  Day X
Cards;
72      T1      0       1.08
72      T1      1       0.67
72      T1      2       0.49
72      T1      3       0.61
72      T1      4       0.70
72      T1      5       0.60
72      T1      6       0.99
198     T1      0       0.16
198     T1      1       0.42
198     T1      2       0.38
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;
proc sort data=hayder;
by trt;
run;
Proc means n nminss min mean max std stderr data=hayder;
Var X
By TRT;
Run;
Title'AR';
```

X= Parameters (eg. FSH, Dominant follicle diameter)
TRT= Treatments
Day= Time of samples
ID= Animals number

This syntax gave us some parameters of our data such as mean , minimum or maximum value of treatments over the time

```
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = AR r rcorr;
Run;

Title'AR(1)';
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = AR(1) r rcorr;
Run;
Run;
Title 'TOEP' ;
 Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = TOEP r rcorr;
Run;
Title’ARH(1)’ ;
 Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = ARH(1) r rcorr;
Run;
Title' TOEPH';
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type= TOEPH r rcorr;
Run;
Title' CS';
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = CS r rcorr;
Run;
Title' HF';
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = HF r rcorr;
Run;
Title' simple';
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
```

```
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = SIMPLE r rcorr;
Run;

Title' ANTE1';
Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = ANTE(1) r rcorr;
run;
```

To fined the best covariance strictures model, it was tested different types from it and the covariance stricture that had lowest AIC and BIC (AIC= Akaike's Information Criterion; BIC= Bayesian Information Criterion) was considered the best model to analyze the data. If the model was significant, we tested the different among the treatments over the time by using LSD as following way:

```
Options nocenter;
Data hayder ;
Input ID TRT$  Day X
Cards;
72      T1      0       1.08
72      T1      1       0.67
72      T1      2       0.49
72      T1      3       0.61
72      T1      4       0.70
72      T1      5       0.60
72      T1      6       0.99
198     T1      0       0.16
198     T1      1       0.42
198     T1      2       0.38
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;
```

```
Title 'TOEP' ;
 Proc mixed data=hayder covtest cl;
Class ID  TRT DAY;
model X=TRT DAY TRT*DAY/DDFM=KR htype=3;
Repeated DAY/subject=ID type = TOEP r rcorr;
lsmeans trt|day/pdiff ; ……
Run;
```

This syntax used to find the different among treatments over the time of the study for specific parameters