DEVELOPMENT OF NON-STEROIDAL AROMATASE INHIBITOR-BASED PROTOCOL FOR THE CONTROL OF OVARIAN FUNCTION USING A BOVINE MODEL

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ABSTRACT

Five studies were designed to characterize the effects of a non-steroidal aromatase inhibitor, letrozole, on ovarian function in cattle. The general hypothesis was that non-steroidal aromatase inhibitors have potential as a steroid-free option for the control of ovarian function for the purposes of fixed-time artificial insemination and embryo production. The specific objectives were to determine the effect of route and vehicle, type of aromatase inhibitor, and duration of aromatase inhibitor treatment (short vs prolonged) on ovarian follicles in cattle, and to test the efficacy of an aromatase inhibitor-based protocol to synchronize ovulation in cattle. In the first experiment, heifers were treated with letrozole intravenously (n=10) or intramuscularly (n=10) or allocated in iv and im control groups (n=5/group). During the second experiment, heifers were divided randomly into two groups (n=15/group) and an intravaginal device containing 1 g of letrozole or a blank device (control) was inserted. The third experiment was designed with the goal of formulating and testing an intravaginal device that provides biologically active circulating concentrations of an aromatase inhibitor for a minimum of 4 days. The biological significance of the pharmacokinetic differences between the letrozole intravaginal devices resulting from the third study was evaluated during the fourth study. A final study was designed to determine the effect of stage of the estrous cycle on the proportion of animals that ovulated and the synchrony of ovulation of heifers treated with an aromatase inhibitor-based ovulation-synchronization protocol and to determine subsequent pregnancy outcomes. In all the studies, the effects of aromatase inhibitor on ovarian function were assessed by transrectal ultrasound examination of the ovaries, and blood samples were collected for hormone concentration determination. Results demonstrated that route of administration, or more precisely, the nature of

the vehicle used for the administration of letrozole (intravenous, intramuscular depot, short release intravaginal or prolonged release intravaginal) has an impact on the effects of letrozole on hormonal profiles and ovarian dynamics. The intramuscular route appeared to provide a prolonged release of letrozole from the injection site which had a marked effect on estradiol production, dominant follicle lifespan, and CL form and function. Letrozole treatment during the ovulatory follicle wave by means of a gel-based intravaginal releasing device during the second study resulted in more rapidly growing dominant follicles and larger ovulatory follicles, delayed ovulation (by 24 h) of a single follicle and formation of a CL that secreted higher levels of progesterone. A wax-based vehicle allowed for a steady and continuous delivery of the active compound over the treatment period. During the third study, the addition of a letrozole-containing gel coating increased the rate of initial absorption and hastened the increase on plasma concentrations of the active ingredient, while the letrozole-containing wax-based vehicle prolonged drug-delivery from the intravaginal device. When tested *in vivo* during the fourth study, we confirmed that letrozole-impregnated intravaginal devices formulated with a wax base plus a gel coat vehicle was most suitable for the application of a letrozole-based protocol for the synchronization of ovulation in cattle, since it effectively delivered elevated concentrations of letrozole, and reduced estradiol production resulting in increased follicular growth and lifespan, without adversely affecting progesterone production. The application of a letrozole-impregnated intravaginal device for 4 days, combined with PGF treatment at device removal and GnRH 24 h post-device removal increased the percentage of ovulations and synchrony of ovulation in cattle, regardless the stage of the estrous cycle at initiation of treatment. As observed in previous studies, the effects observed could be associated with an increase in circulating LH

concentrations. However, the effects of treatment on gonadotropin concentrations are inconclusive, possibly due to inadequate sampling frequency. The impact of letrozole treatment of oocyte fertility remains unknown. The results of the five experiments support our general hypothesis that non-steroidal aromatase inhibitors have potential as a steroid-free option for the control of ovarian function in cattle. However, further research is needed in order to elucidate the effects of letrozole treatment during the proestrous on oocyte competence and fertility of the resulting ovulations in cattle.

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DEDICATION

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LIST OF ABBREVIATIONS

µg	micrograms
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
A	adenine
AI	artificial insemination
CIDR	controlled internal drug release
CL	corpus luteum
DES	diethylstilbestrol
EB	estradiol benzoate
EC	estradiol cypionate
E-17β	estradiol-17 beta
ELISA	enzyme-linked immunosorbent assay
FDA	USA Food and Drug Administration
FSH	follicle stimulating hormone
FSHr	follicle stimulating hormone receptor
FTAI	fixed-time artificial insemination
g	gram
G	guanine
GH	growth hormone
GnRH	gonadotropin releasing hormone
h	hours

im	intramuscular
iv	intravenous
kg	kilogram
LCMS/MS	Liquid chromatography tandem mass spectrometry
LH	luteinizing hormone
LHr	luteinizing hormone receptor
mg	milligram
mL	millilitre
mm	millimetre
MOET	multiple ovulation and embryo transfer
mRNA	messenger ribonucleic acid
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ng	nanogram
pg	picogram
PGF	prostaglandin $F_2\alpha$
$P450_{arom}$	P450 aromatase enzyme
$P450_{scc}$	P450 side-chain cleavage enzyme
$P450_{17\text{-}OH}$	P450 17α-hydroxylase
StAR	steroidogenic acute regulatory protein
T	thymine
vs	versus

CHAPTER 1: GENERAL INTRODUCTION

Reproductive efficiency is the single most important factor affecting profitability in the cattle industry. The impact of methods for controlling the estrous cycle in cattle may be illustrated by estimates of the use of artificial insemination (AI) and embryo transfer (ET) in cattle. A conservative estimate of the worldwide use of AI is 83 million cows per year – estimated to represent about 20% of the breedable cattle population (25% in North America) [1]. Worldwide, 53% of cows artificially inseminated are of dairy breeds and 39% are of beef breeds, but in Canada, the gap is much wider: 94% in dairy and 6% in beef. Regarding ET, just over 120,000 donor cows are collected each year worldwide and 800,000 embryos are transferred [2]. In Canada, those number do not exceed 13,500 and 55,000, respectively [3].

Estrogen-based protocols as a treatment for controlling and synchronizing ovulation in cattle, have revolutionized breeding practices and enabled beef producers to make use of AI as never before because labour and resources could now be pre-scheduled (e.g., fixed-time artificial insemination; FTAI). These protocols also allowed for the wider application of superovulation and embryo transfer due to their effectiveness to synchronize follicular wave emergence [4-8]. Steroid-induced wave synchronization is associated with regression of the dominant follicle followed by a surge in circulating FSH and subsequent emergence of a new follicular wave at a consistent interval post-treatment. Steroid-induced regression of the dominant follicle is a result of a systemic alteration in feedback of estradiol and progesterone on pituitary release of LH and FSH [9]. Estradiol suppresses FSH release [10] and has been reported to decrease LH pulse amplitude in sheep [11] and cattle [12]. Progesterone has been reported to decrease LH pulse frequency and suppress maximal diameter of the dominant follicle in a dose-dependent manner

in cattle [13-18]. Once the suppressive effects of estradiol are removed, FSH surges resulting in the emergence of a new wave of follicular development approximately 4 days after estradiol/progesterone treatment, regardless of the stage of development of the dominant follicle at the time of treatment [4, 5, 19].

However, increasing consumer sensitivity to the possible deleterious effects of estrogens in food and in the environment [20] has led to new regulations about the use of estrogenic products in livestock. The European Union has already banned the use of estrogenic products in food producing animals [21-24]. In United States [25] and Canada [26], estrogens cannot be used for synchronization of estrus except by prescription and custom-compounding. In 2007, New Zealand and Australia banned use of estrogens in lactating dairy animals [24]. The ban of the use of estrogens in livestock and lack of commercially availability of estrogenic preparations negatively impacts the implementation of reproductive biotechnologies in cattle production systems, limiting potential reproductive efficiency and genetic improvement provided by the use of AI or MOET [24]. In this context, the development of alternative methods for controlling ovarian function in cattle, with efficacy and predictability comparable with that of estrogen plus progesterone treatments [5], and with no toxic or harmful effects on human and animal health is needed.

Another important factor that negatively impacts reproductive efficiency, mainly in dairy industry, is the decline in fertility observed in high producing cows. Over a period of 15-20 years, the rate of decline in fertility has been 0.5% per annum in the USA [27]; in UK herds, pregnancy at first insemination has gone from 56% to 40%, approaching a decline rate of 1% per annum [28]. The dramatic decline in fertility has been associated with a reduction in expression

of heat, failure to ovulate, and poor luteal function. Regarding luteal function, cows that failed to carry a pregnancy had lower levels of progesterone at days 14 and 21 post-breeding compared to those that remained pregnant [29]. Several research groups have concluded that embryos developing under higher levels of progesterone early in the luteal phase grow more rapidly and were more likely to prevent prostaglandin F2α (PGF) secretion by the uterus; i.e. prevent luteolysis and achieve maternal recognition of pregnancy [30-32]. The development of a treatment to improve early luteal function and therefore support the development of a larger embryo will be of extreme importance not only for AI in dairy industry but also for the success of ET in both beef and dairy herds.

Aromatase inhibitors prevent the body from producing its own estrogens; thus, they could potentially be applied to the management of estrogen-dependent physiologic functions such as reproduction. Letrozole (Femara®, Novartis) is a non-steroidal aromatase inhibitor that inactivates the aromatase enzyme by reversibly binding to the heme group of the P450 subunit of the enzyme. Letrozole is used as an adjuvant treatment for hormone-responsive breast cancer in post-menopausal women [33] and has been used as a fertility therapy for women undergoing assisted reproduction because of its putative effect on FSH secretion through removal of the negative feedback of estradiol [34]. A 5-day regimen of letrozole (2.5 mg/day from 3 to 7 days after the beginning of menses) has been used for ovarian stimulation in women [35], and larger or increasing doses of letrozole have been used to induce ovarian superstimulation [36, 37]. It has also been applied to lower the cost of superstimulatory treatments by reducing the dose of FSH required [38].

We conducted two studies to assess the effects of aromatase inhibitors on ovarian function in cattle. Contrary to the above mentioned hypothesis proposed to explain the effect of aromatase inhibitors in women [34], letrozole treatment of cattle did not induce follicular atresia or hasten emergence of a new follicular wave, whether given as a single intravenous dose on Day 3 post-ovulation [39] or in a 3-day regimen from Days 1-3, 3-5 or 5-7 post-ovulation [40]. Rather, letrozole treatment increased mean plasma LH concentrations resulting in a prolonged period of dominance of the extant DF and delayed emergence of the next follicular wave. In addition, the 3-day letrozole treatment was associated with greater corpus luteum (CL) diameters [40].

Studies done in cattle in our laboratory established a solid foundation for the development of letrozole-based synchronization and fertility treatments:

- Short or prolonged letrozole treatment extends the lifespan of the dominant follicle
- Letrozole treatment can be used to delay follicle wave emergence
- Letrozole treatment induces the formation of a larger dominant follicle
- Letrozole treatment is luteotrophic (larger CL that secretes more progesterone)

The set of studies presented in this dissertation aim to provide convincing data that aromatase inhibitors can be applied as a safe and effective method to control the estrous cycle and improve fertility in cattle.

CHAPTER 2: LITERATURE REVIEW

2.1 Reproductive Physiology in Cattle

2.1.1 Hypothalamus-Pituitary-Ovarian Axis

The hypothalamus-pituitary-ovarian (HPO) axis is comprised by the anatomic and functional relationships existing among the hypothalamus, pituitary gland, and ovaries, thus allowing these endocrine glands to act as a single entity. The hormones involved in the control of this axis include gonadotropin-releasing hormone (GnRH, produced by the hypothalamus), luteinizing hormone and follicle-stimulating hormone (LH and FSH, respectively; produced by the anterior pituitary), and estradiol, inhibin and progesterone (synthetized in the ovaries). However, numerous other factors and hormones have been linked to the control of the HPO axis in mammals. Furthermore, the neuroendocrine nature of the HPO axis integrates external factors such as stress, nutrition, and photoperiod, in the control of the reproductive physiology [41].

The hypothalamus is located in the ventral brain and comprises groups of cell bodies known as hypothalamic nuclei (namely, the paraventricular nucleus, the surge center and the tonic or pulsatile center) [41, 42]. The neurons located in the surge and tonic centers secrete GnRH, while the neurons of the paraventricular nucleus synthetize oxytocin. Anatomic arrangements are of special importance for the communication between the hypothalamus and pituitary gland. GnRH-producing neurons release their secretion through long axons located in vicinity to the pituitary stalk [43]. Within the pituitary stalk there is highly complex capillary network, the hypothalamo-hypophyseal portal system, which allows GnRH to reach the anterior portion of the pituitary, the adenohypophysis. It is believed that the main function of this capillary system is to

prevent the very small amount of GnRH produced by the hypothalamus from getting diluted in the general circulation [42]. Once GnRH binds to its receptors on the gonadotroph cells, LH and FSH are produced and secreted into the blood stream in order to reach their target organ, the ovaries [43].

The gonadotropins (FSH and LH) play an important role in communicating with the ovaries. Gonadotropins activate the ovaries to produce different steroid hormones such as estrogen, androgens and progesterone which are linked to the regulation the estrous cycle. Positive and negative feedback loops are involved in the communication between the ovaries and the hypothalamus and pituitary [10, 44-46]. Estrogen receptors (ER) are present in the hypothalamus, pituitary and in the ovaries of many species, including cattle [47-50]. Hence, alteration in estradiol concentration could have direct and independent effects on all the endocrine glands that comprise the HOP axis. Details of the interactions between gonadotropins and ovarian sex steroids in cattle will be discussed in later sections of this literature review.

Non-steroidal ovarian products have also been related to the control of gonadotropin secretions [51, 52]. Inhibin is a protein produced by the granulosa cell of growing follicles under the influence of FSH. It comprises α and β sub-units and it is known to suppress FSH secretion by acting at hypothalamic and pituitary levels [43, 53-55]. The combination of two β sub-units results in a related protein, known as activin, which counteracts the effect of inhibin by stimulating FSH release [43]. Other factors present in the follicular fluid include follistatin, oxytocin, insulin-like growth factor (IGF), epidermal growth factor (EGF) and plasminogen activator (reviewed in [56]).

2.1.1.1 Kisspeptin and the HPO axis

Kisspeptins (Kps) are a family of peptide hormones which bind to protein G-coupled receptor 54 (GPR54) and integrates nutritional and hormonal information which is critical to metabolism and regulation of reproduction [57]. All Kps are the product of the *KISS-1* gene and contain, at the C-terminal region, a common deca-peptide sequence (Kp-10) that confers to them biological activity [57, 58]. Although Kp receptors (GPR54) have been described in the hypothalamus, pituitary, ovaries and placenta, its major role has been postulated as being the neuroendocrine regulator of GnRH release [58, 59]. Kisspeptin and hypothalamic GPR54 have been associated with the onset of pulsatile GnRH secretion observed at puberty and in seasonal breeders, like sheep [57-59].

Kisspeptin-secreting neurons contain ER and other sex steroid receptors and are located in close vicinity to GnRH-secreting cells in the hypothalamus [59, 60]. Two sub-populations of Kp-secreting cells have been identified: one is located in the arcuate nucleus (ARC) and the other is located in the anteroventral periventricular area (AVPV) [59, 60]. This differentiation of these two sub-populations of cells is relevant since the regulatory effects of estrogens are nucleus-specific. Thus, estrogens inhibit the expression of Kp at the ARC, causing a negative-feedback effect on gonadotropin secretion. As expected, reducing circulating E2 concentrations resulted in higher Kp mRNA levels and increased GnRH output [58]. In contrast, estrogens enhance *Kiss1* expression at the AVPV in rodents mediated by ERα, which suggests that this population of neurons may be involved in the positive-feedback actions of estradiol to generate the preovulatory surge of gonadotropins [57, 59]. In cattle, administration of 100 pmole/kg of Kp during diestrus, proestrus and estrus resulted in increased circulating LH concentrations during diestrous and proestrus, but not during estrous [61].

It is important to highlight that, although estrogen plays a key role in the regulation of GnRH pulsatile secretion pattern, GnRH-releasing neurons seem to lack ER thus making the Kp/GPR54 system pivotal for the normal development of the reproductive functions [59].

2.1.2 Ovarian follicular wave development in cattle

In cattle, ovarian follicles develop in waves [62, 63]. A wave of follicular development begins with the synchronous recruitment of a mean of 24 (range of 8 to 41) small follicles (3 to 4 mm in diameter), an event that is referred to as wave emergence [64, 65]. Additionally, the growth pattern of these follicles at ≤3 mm in diameter has also been describes as occurring in a wave-like manner [66, 67]. In monovular species such as cattle, a single follicle is selected from this cohort of recruited follicles to continue growing (i.e. dominant follicle), while the other follicles in the group undergo atresia (i.e. subordinate follicles) [63, 68-70]. This "selection" process is confirmed by the deviation in diameter between the now dominant follicle and the largest subordinate follicle, which occurs when the dominant follicle is on average 8.5 mm in diameter and about 2.5 days after wave emergence [64]. If the dominant follicle is growing during the early luteal phase, this follicle becomes anovulatory and regresses. Three different phases of follicular growth have been described during anovulatory waves: 1) a growing phase, during which the dominant follicle grows actively; 2) a static phase, during which follicular diameter does not change significantly; and 3) a regressing phase, during which dominant follicle diameter begins to decrease [71]. However, if the dominant follicle is actively growing or in early static phase when regression of the corpus luteum begins, it will become the ovulatory follicle and as such it has the capability of triggering a gonadotropin surge that will lead to ovulation (reviewed in [72]).

Two and three of these waves of follicular development have been most commonly reported during an estrous cycle in cattle [70, 71, 73, 74]. In animals with two or three waves of follicular development, the first wave can be identified, on average, the day of ovulation. The second follicular wave emerges about day 10 and day 9 after ovulation in two-wave and three-wave cycles, respectively [66, 71]. In animals with three follicular waves per cycle, the last and ovulatory wave is detected in average on day 16 post-ovulation [75]. Estrous cycles composed of two waves of follicular development are consistently shorter than 3-wave cycles (19 to 20 days vs 22 to 23 days, respectively) [75].

2.1.3 Gonadotropins and the control of follicular growth

Follicle-stimulating hormone and LH play crucial roles in the regulation of ovarian follicular dynamics. Emergence of a follicular wave and the selection of a dominant follicle have been temporally associated with an increase and decrease, respectively, in FSH levels [76]. A transient increase in plasma LH concentration has been reported in relation to the time of follicular deviation defined as the measured point when a significant difference in diameter between the dominant follicle and the largest subordinate follicle is first identified [77]. Additionally, the peak in LH concentrations that encompasses ovulation has been well documented [78, 79].

As mentioned above, each wave of follicular development is preceded by a surge in FSH concentration [76]. Inhibiting the increase in FSH concentration by administration of a proteinaceous fraction of follicular fluid was followed by a delay in follicular wave emergence. Moreover, promotion of a surge of FSH by ablation of the extant DF resulted in the emergence of a new wave of follicular development within 2 days [76]. These results also provided evidence that intrafollicular factors were, at least in part, responsible for the changes in plasma FSH

concentrations related to follicular dynamics. Further clarification of this concept revealed that increasing plasma concentrations of intrafollicular components (e.g., estradiol, inhibins, IGF, among others), mainly synthesised by the dominant follicle, have a suppressing effect on circulating FSH concentrations and that this decrease in FSH levels was crucial for the process of dominant follicle selection and to ensure monovulation [45, 53, 68, 80].

A FSH-follicle coupling system involving all the follicles in the recruited wave is responsible for the initial drop in FSH concentrations observed after wave emergence. When the dominant follicle reaches a critical stage of development for selection (8.5 mm [81]), it acquires the capability to further suppress FSH concentrations to levels that are not compatible with continuous development of the subordinate follicles [10]. However, a shift from FSH to LH-dependency by the dominant follicle (by acquisition of LH receptors in the thecal and granulosa cells [77]) allows it to continue growing even under very low levels of plasma FSH [53]. A study of the effects of LH suppression by administration of exogenous progesterone before, during and after follicular deviation demonstrated that LH was not indispensable for the initiation of deviation. There was no difference in follicle diameters between progesterone-treated and control animals before and during deviation. However, the dominant follicle was smaller and grew at a slower rate when LH secretion was suppressed after deviation was initiated in the progesterone–treated group [14, 77].

The presence of a healthy dominant follicle at the end of the luteal phase leads to ovulation. The drop on progesterone concentration that takes place during luteolysis induces an increase in LH pulsatility that further feeds the growth of the extant dominant follicle [13]. Additionally, high estradiol production by the dominant follicle exerts positive feedback at hypothalamic level

to increase LH pulsatility. This loop of positive stimulation by high estradiol and low progesterone on GnRH producing cells by estradiol ıeads to the pre-ovulatory surge of LH needed for final oocyte maturation and release [57, 58, 82]. The molecular mechanisms involved in this process have been discussed in previous sections in this review.

The active dominant follicle produces factors that inhibit plasma FSH secretion thus preventing the emergence of a new wave of follicular development during the dominance period [51, 53, 83]. At the end of the static phase (when the dominant follicle starts to regress) or after ovulation (when the source of the follicular components disappears), the negative feedback effect of follicular products (mainly estradiol) on gonadotropin secretion is removed. This results in a surge of FSH secretion followed by emergence of a new follicular wave [15, 46].

In summary, follicle wave emergence is preceded by an increase in plasma FSH concentrations. Follicular selection is related to a decline in circulating FSH concentrations caused mainly by the production of estradiol by the cohort, and in particular by the dominant follicle under the influence of LH. Low FSH concentrations maintained by the dominant follicle ensure that no further waves of follicular development occur during the period of dominance. When LH pulse frequency is low, the dominant follicle losses dominance and regresses, estradiol production declines, FSH surges and a new wave of follicular development is stimulated. However, if the dominant follicle is still viable at the end of the luteal phase, LH pulse frequency increases and stimulates a greater production of estradiol by the dominant follicle that will, in turn, elicit a preovulatory peak of LH and FSH secretion, and ovulation. The disappearance of the dominant follicle after ovulation removes the negative feedback effect of estradiol on FSH

secretion and a post-ovulatory surge of FSH followed by the emergence of a wave of follicular development is observed [84].

2.2 Control of the Estrous Cycle

Based on our knowledge of reproductive physiology gained in the last three or four decades, several strategies and protocols to manipulate phenomena related to the estrous cycle (i.e., wave emergence, selection, luteolysis and ovulation) have been developed. The ideal method of control of the estrous cycle is one that is simple, effective and safe. For the purpose of this review, the rationale and implementation of protocols based on prostaglandin $F_{2\alpha}$ (PGF) and GnRH, and the use of estrogens alone or in combination with progesterone will be briefly discussed.

2.2.1 Prostaglandin and GnRH-based protocols for estrus synchronization

The identification of PGF as the luteolysin responsible for the regression of the CL in cattle provided a new means for controlling the length of the luteal phase and ovulation (reviewed in [85]). Several protocols applying different doses and intervals between doses of prostaglandins have since been designed [86, 87]. One of the main limitations of synchronizing ovulation by a single PGF treatment is that the state of maturity of the dominant follicle at the time of PGF determines the interval to estrus and ovulation. This interval has been described to be, on average, of 3 to 3.4 days. However, if the dominant follicle present at the time of PGF treatment is in the late static or regressing phase, the dominant follicle of the subsequent follicular wave will have to grow in order to reach preovulatory size; in this case the interval from PGF treatment to ovulation may take as much as 6 days [86, 87]. Further, newly formed CL during the

first 3 to 4 days after ovulation are refractory to the luteolytic effects of PGF; responsiveness increases as the CL develops [86]. These important sources of variability in interval from treatment to ovulation limit the use of prostaglandin-based protocols for fixed-time artificial insemination (FTAI). An alternative approach which uses two doses of PGF 11 to 14 days apart is widely accepted and used on many dairy and beef farms. The rationale for this approach is that approximately 67% of the animals (those with a CL ≥5 day-old or those experiencing natural luteal regression) would respond to the first PGF injection by undergoing luteolysis and ovulating. When the second injection of PGF is administered 11 to 14 days later, it is expected that 100% of the animals would have functional and PGF-responsive CLs [69]. However, the use of luteolytic doses of prostaglandin still relies on estrus detection efficiency to provide acceptable outcomes. Consequently, the rate of submission of animals for AI after detected estrus may limit the effectiveness of this protocol. Herd heat detection rates are very variable and have been reported to be ≤ 50% (range from about 30% to 65%) [88-91] in high producing dairy farms and between 53% and 73% in commercial beef farms [90, 92].

The use of fixed-time AI (FTAI) can overcome the negative impact of low estrus detection efficiency. FTAI eliminates the need for estrus detection but requires synchronous growth and ovulation of a viable dominant follicle in order to be successful. Pregnancy rates obtained with FTAI are usually comparable to those obtained after AI with high estrus detection rates, because all animals are inseminated regardless of whether or not they showed estrus [93]. Gonadotropin releasing hormone is commonly used to induce the pituitary release of gonadotropins (LH and FSH) which will induce ovulation and/or luteinisation of the dominant follicle [94, 95]. Emergence of a new wave of follicular development is expected approximately 2 days after

GnRH treatment [94], although its synchrony is highly dependent on the occurrence of ovulation after GnRH [95, 96]. The use of GnRH is usually combined with a luteolytic dose of PGF 7 days later: GnRH is intended to synchronize wave emergence while PGF synchronizes luteolysis. An additional dose of GnRH is used about 48 hours after PGF to induce an LH surge and further synchronize ovulation [93, 97]. Finally, animals are inseminated 16 to 20 hours after the second GnRH dose. This protocol is known as Ovsynch. Several studies in which Ovsynch protocols were used had pregnancy rates similar to those obtained using the two doses of PGF 14 days apart with high estrous detection management in lactating dairy cows (38.9% versus 37.8%, respectively) [93, 94]. However, after the application of Ovsynch to heifers, pregnancy rates were lower than in controls treated with PGF and inseminated after estrus detection (35.1% versus 74.4%, respectively). Poor ovulatory response to the first dose of GnRH and consequently poor synchronization of wave emergence were identified as the causes of the lack of success of the Ovsynch protocols in heifers [96, 98]. Ovulation was induced in 54% of dairy heifers after GnRH treatment [94], and it was reported that 27% to 44% of beef heifers treated with a single GnRH dose failed to ovulate [95, 96].

Alterations to the original Ovsynch protocol were introduced in order to improve the ovulatory response and synchronization. Pre-synchronization of follicular wave emergence using a double PGF treatment 14 days apart followed 12 days later by an Ovsynch protocol (Presynch), or using two consecutives Ovsynch protocol 7 days apart (Double-Ovsynch) improved the ovulatory response to first GnRH and therefore more heifers and cows responded to the second GnRH with synchronous ovulation [99, 100]. Additional modifications include the addition of a progestin device between first GnRH and PGF (to prevent early ovulation, mostly in heifers

[101]) and alterations on the timing of the second GnRH and AI (Cosynch [102]). Specifics of the application and effectiveness of these protocols are beyond the scope of the present thesis and have been reviewed elsewhere [103].

2.2.2 Estradiol and progesterone-based protocols

The interval from PGF-induced luteolysis to ovulation and the variability in the ovulatory response among animals to GnRH treatment depends on the status of the ovarian follicle at the time of treatment. Thus, a method that controls ovarian follicle recruitment would provide the advantage of knowing the stage of follicular development when the ovulatory or luteolytic treatment is given and would improve the ovulatory response and its synchrony.

Combinations of progesterone and estradiol have been used to hormonally induce a new wave of follicular development in a predictable interval of time [5, 12]. This method of synchronizing wave emergence is based on the negative feedback effects that estradiol has on FSH secretion during the luteal phase [104] or under the influence of an exogenous source of progesterone [4, 5, 19, 105]. Additionally, exogenous progesterone suppresses LH secretion leading to the regression of the extant dominant follicle. Reduced secretion of FSH and LH from the pituitary gland terminates the growth of both FSH- and LH-dependent follicles. Reduced circulating estradiol concentration (most likely due to metabolism and clearance) results in a FSH surge and in emergence of a new wave of follicular development about 4 days after estradiol and progesterone treatment [4, 106, 107]. This protocol includes, at the time of estradiol and progesterone treatment, the insertion of a progesterone releasing device for 7 to 9 days and a dose of PGF at the time of progesterone device removal to ensure luteal regression. Animals are inseminated 55 to 60 hours after progesterone withdrawal [107]. Additionally, different forms of

estradiol have been applied in these progestin-based protocols. It has been reported that the use of short-acting preparations such as E-17β or estradiol benzoate (EB) results in more synchronous wave emergence and ovulation than long-acting esters such as estradiol cypionate [8, 108, 109]. When estradiol is added to increase the synchrony of ovulation, it is recommended that it should be administered 24 hours after progesterone device withdrawal and AI should be performed 30-36 hours later [107].

A synchronized ovulatory response is essential for the efficient use of time, resources, labour and the application of reproductive management techniques such as FTAI [5, 109] and multiple ovulation and embryo transfer (MOET) [105, 106, 110]. The outcome of a superstimulation treatment is strongly influenced by the stage of follicular development at initiation of treatment. Optimal ovarian responses were obtained when superstimulatory treatments were initiated about the time of follicular wave emergence (day – 1 or 0 of the follicular wave) [5, 109]. Since progesterone plus estradiol treatments result in precise synchronization of wave emergence (on average 4.3 days after treatment), superstimulatory treatments are initiated on day 4 of the protocol, without the need for estrus detection and the 8 to 12-day waiting period for a natural follicular wave to emerge [4, 111].

2.3 Decline in Fertility and its Impact on Reproductive Efficiency

Fertility can be defined as the ability of the animal to conceive and maintain pregnancy if served at the appropriate time in relation to ovulation [112]. This definition implies that fertility can be negatively impacted at several different steps in the pathway leading to obtaining a healthy offspring: altered ovarian function and cyclicity, poor estrous behaviour or detection, failure to ovulate, and embryonic or fetal loss. Strategies to control or modify estrous, ovulation, and

ovarian dynamics have been discussed in previous sections of this review. In this section we will briefly discuss aspects related to CL dysfunction and pregnancy failure.

2.3.1 Proposed mechanisms for the decline in fertility

It is now evident that fertility has declined with rising milk yields [113, 114]. In the USA the rate of decline in fertility was 0.5% per annum, over a period of 15-20 years,[27]; in UK herds, pregnancy at first insemination has gone from 56% to 40% between 1975-1982 and 1995-1998, approaching a decline rate of 1% per annum [28]. However, there is recent evidence that the historical decline in fertility has reached a nadir and has now begun to improve, at least in the United States [115].

This dramatic decline in fertility has been associated with a reduction in expression of estrus, failure of ovulation, and poor luteal function [114]. Strategies to control estrus, time to ovulation and even eliminate heat detection have already been discussed.

After ovulation, the CL is formed and secretes progesterone. In a non-bred female, and under the influence of estradiol of follicular origin, the endometrium will start secreting PGF about 16 days post-ovulation, which will induce the luteolytic process. However, if the animal has been bred and a conceptus in present in the uterus by day 16 post-ovulation, the embryo signals its presence and prevents the uterine secretion of PGF and luteolysis. Hence, the period between days 14 and 21 of the bovine estrous cycle are critical for the establishment of pregnancy [116].

From the process of pregnancy recognition and luteolysis, several steps can be identified in which a malfunction could lead to pregnancy failure. In cattle, the natural process of luteolysis is driven by estradiol [116]. Estradiol secreted by the growing dominant follicle of each follicular

wave in the cycle primes the uterine endometrium and induces the expression of oxytocin receptors [117]. Oxytocin, also of follicular origin, acts on the endometrial receptors stimulating the synthesis and secretion of PGF. It has been shown that administration of exogenous estradiol hastens the process of luteolysis in cattle [117].

Luteolysis is a default process in cattle, meaning that it has to be actively blocked in order to sustain a pregnancy. In cattle, the embryo is responsible for signaling its own presence. Embryonic secretion of interferon-τ prevents PGF secretion, although the precise mechanism of its action is still unknown. It has been proposed that interferon-τ blocks the effect of estradiol on the endometrium, hence preventing the acquisition of oxytocin receptors. An embryo that fails to secrete interferon-τ, or that secretes insufficient amounts of the protein, will fail to signal its presence and the process of luteolysis will take place [116].

Progesterone concentrations are high during the luteal phase of the estrous cycle, which is necessary to prepare the uterus for embryonic development. In order to block luteolysis and maintain high levels of progesterone, the embryonic trophoblast has to elongate and occupy the whole uterine lumen, meaning that larger embryos are more likely to succeed in preventing luteolysis. Progesterone levels have been associated with embryonic growth and elongation rate and interferon-τ secretion capabilities [116, 118, 119]. However, progesterone does not seem to act directly on the embryo in order to influence its growth. Rather, the effects that progesterone has on uterine secretion, from which the embryo feeds, are responsible for the increased trophoblastic elongation observed in cows supplemented with progesterone during the first week post-breeding [118, 120]. It has been shown that cows that failed to carry a pregnancy had lower levels of progesterone between days 14 and 21 post-breeding compared to those that remained

pregnant [29]. In dairy cows, low circulating progesterone concentrations have been associated with increases in metabolic rate and liver clearance of steroid hormones due to high dry matter intake [120] and thus, reduced embryonic survival.

2.3.2 Methods to improve fertility post-AI and embryo transfer

Several strategies to improve pregnancy rates post-breeding or embryo transfer have been tested. The main goals of such strategies can be summarized as follows: (1) to increase the antiluteolytic signal from the conceptus (by increasing progesterone concentration post-breeding or embryo transfer), and (2) to decrease the effect of estradiol (from the dominant follicle) on endometrial secretion of PGF [114, 116].

Several research groups have concluded that embryos developing under higher levels of progesterone early in the luteal phase grow more rapidly (i.e. trophoblast expansion) and were more likely to prevent prostaglandin F2α (PGF) secretion by the uterus; i.e. prevent luteolysis and achieve maternal recognition of pregnancy [30-32, 118, 119]. Higher levels of progesterone have been achieved by inducing the ovulation of a larger dominant follicle (which results of a larger CL) e.g. stimulating follicle growth with eCG, by treating with luteotrophic hormones (such as hCG, and GnRH) to induce accessories CLs and stimulate CL growth and progesterone secretion [120, 121], or by supplementation with exogenous progesterone (in most cases by the use of intravaginal devices) [30, 116, 118, 122].

Estradiol of follicular origin has been identified as the stimulus driving the expression of oxytocin receptors in the endometrium and, therefore, triggering the mechanism of luteolysis. It has been shown that reduction of circulating estradiol concentrations by ablation of the dominant follicle delays the occurrence of luteolysis, while estradiol supplementation hastens the initiation

of the luteolytic process [117]. In an attempt to reduce estradiol circulating concentrations during the critical period of maternal recognition of pregnancy (days 15 to 21 post-ovulation), researchers have treated cows with GnRH in order to induce ovulation and remove the estrogenic activity of a dominant follicle. It was concluded that increasing the proportion of cows with 3 waves of follicular development reduces the odds of having a highly estrogenic dominant follicle during such period over 2-wave animals, and therefore improves pregnancy rates [116, 117].

2.4 Estrogens and Other Sex Steroid Hormones

Steroid hormones are cholesterol-derivatives commonly classified, based on their physiological behaviour, in five groups: glucocorticoids, mineralocorticoids, progestins, androgens, and estrogens. The last three groups, also referred to as sex steroid hormones, are associated with the mechanisms that govern the reproductive physiology of mammals.

2.4.1 Steroidogenic pathway for the synthesis of sex steroid hormones

Estrogens are the final product of a complex biosynthetic pathway that, in ruminants, involves five main enzymes: the P450 cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase ($P450_{17\text{-}OH}$), 17β-hydroxysteroid dehydrogenase (17β-HSD), and P450 aromatase (P450arom) [123, 124].

The rate limiting step in the biosynthesis of sex steroid hormones appears to be the incorporation of cholesterol into the mitochondria [125]. Free cholesterol is highly hydrophobic, and although it can cross the cellular membrane by diffusion, this process is extremely slow. Therefore, the incorporation of cholesterol inside the mitochondria depends on an active (energy

dependant) mechanism to transverse the hydrophobic mitochondrial wall. The protein mediating the translocation of cholesterol is the "steroidogenic acute regulatory protein" (StAR) [125].

Once inside the mitochondria, cholesterol is metabolized to pregnenolone by the P450scc enzyme. Pregnenolone is then the substrate for two different enzymes: 3β-HSD and $P450_{17\text{-}OH}$, giving rise to progesterone and 17α-hydroxypregnenolone, respectively. In thecal cells of the ovarian follicle, $P450_{17\text{-}OH}$ is highly expressed and pregnenolone is converted mainly into 17α-hydroxypregnenolone. The 17α-hydroxypregnenolone is processed into androstenedione by the sequential activities of $P450_{17\text{-}OH}$ and 3β-HSD and a small amount of androstenedione is transformed into testosterone by the enzyme 17β-HSD. Both androgens, androstenedione and testosterone, are now available to be either secreted as such or further converted into estrone and estradiol, respectively, by the action of the cytochrome P450 aromatase (P450arom) within the granulosa cells [123, 126, 127]. The major enzymatic pathway leading to the synthesis of estrogens, irrespective of species or tissue, is summarized in Figure 2. 1.

2.4.2 Source and actions of estrogens

Estrogen production has an important role in several system and organs, and it is crucial for the normal development and function of the whole organism. Some illustrative examples of sources and actions of estrogens in mammal species are summarized below.

The placental is an important, though transient, source of estrogens. Placental production of estrogens, mainly during the last portion of gestation, induces the growth of the myometrium, induces the production of oxytocin receptors in the myometrium and promotes mammary gland development, among other actions [42, 128].

During fetal life, estrogens play a key role in sex differentiation in the brain, stimulated by the presence of androgens [124]. Testosterone produced by the fetal testis can cross the blood-brain barrier and be converted into estrogens by the aromatase enzyme present in the brain. This local production of estrogen signals the brain of the presence of a male fetus and inhibits the development of the pulsatile pattern of GnRH secretion [42].

At puberty, estrogens induce bone maturation, linear growth, and epiphyseal fusion. In young adults, estrogens are important for maintenance of bone density, not only in females but also in males [129]. However, estrogens are not as efficient as androgens when it comes to stimulation of protein production and muscular growth [128].

After puberty, estrogens are produced by the ovaries. Several reports indicate that estrogens are in part responsible for the development and maturation of follicles and oocytes in the ovaries. Studies have shown that estradiol induces granulosa cells proliferation and expression of LH receptor in granulosa and thecal cells of the dominant follicle. Estradiol also promotes the formation of gap junctions between granulosa cells to facilitate communication and nutritional/hormonal supply from the basal membrane [130]. In females of all species, estrogen induces the development of external sexual characteristics and it induces oestrous behaviour [128]. Although estrogens were thought as the “female” sex hormone for many years, it is now clear that estradiol synthesis in the testis is necessary for a normal spermatogenesis to occur [131]. Estrogens of testicular origin can contribute to at least with 15 % of total amount of estrogen circulating in blood in the adult male [132].

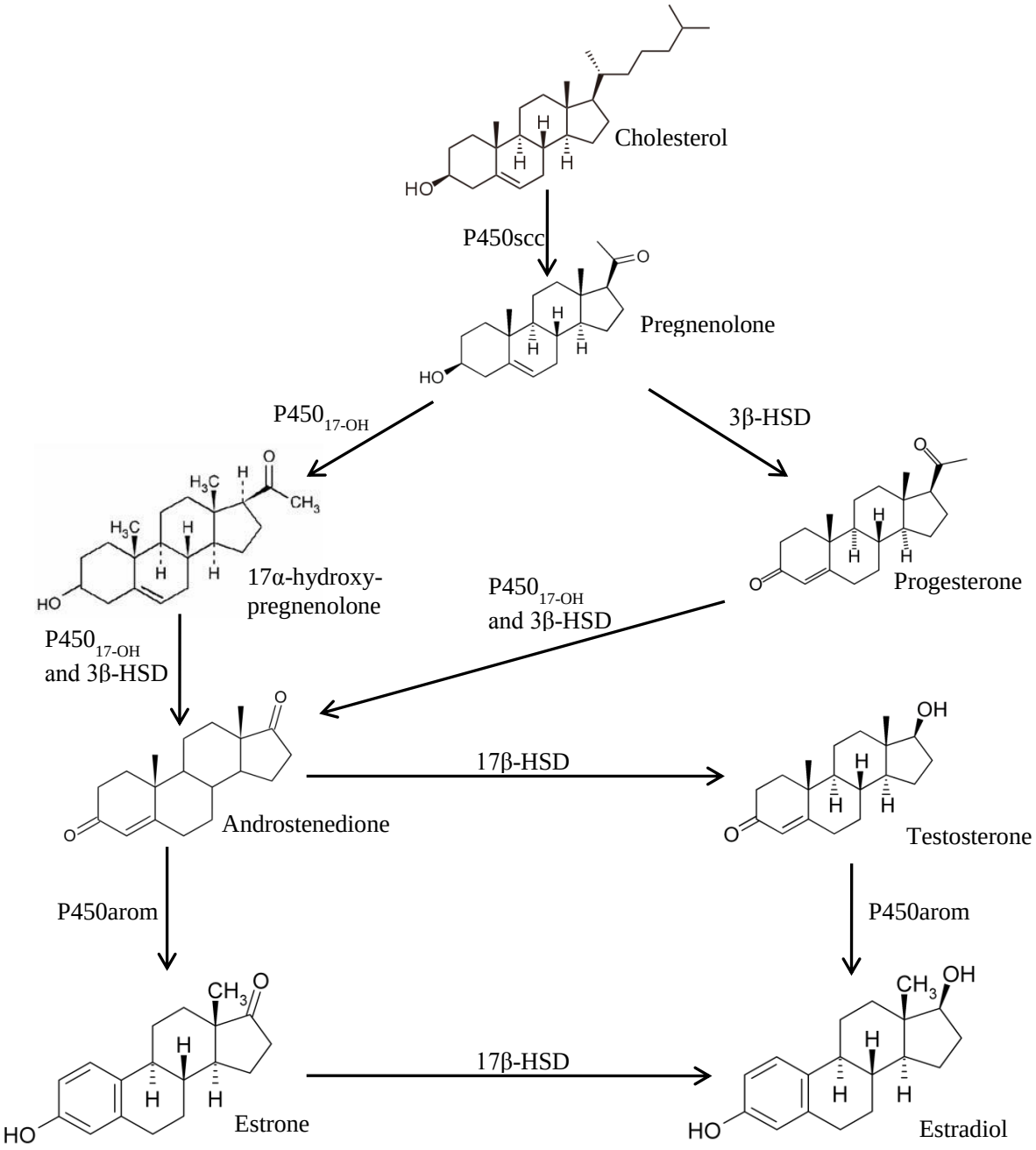


Figure 2. 1. Synthesis of estrogens in ruminants. Five main enzymes (P450 cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase (P450$_{17\text{-}OH}$), 17β-hydroxysteroid dehydrogenase (17β-HSD), and P450 aromatase (P450arom)) are involved in the enzymatic pathway leading to the synthesis of estrogens from cholesterol.

In post-menopausal women and non-human primates, in which ovarian estrogen production is no longer the main source of estrogenic products, peripheral estrogen synthesis (adipose tissue, skin, muscle) becomes responsible for the blood levels of estrogens. Adipose tissue contributes with the largest amount of extragonadal estrogens due to its wide distribution, and these levels of estrogen are necessary to protect cognitive function of the brain and to prevent bone demineralization [129, 133].

Many other actions of estrogens in mammals can be cited: stimulation of hepatic synthesis of lipoprotein binding proteins, increasing of the synthesis of coagulation factor, decreasing levels of low density lipoproteins (LDL) while increasing high density lipoproteins (HDL), among other actions [129, 133, 134].

Estrogens, however, are not only related with normal and physiologic functions. These steroid hormones are strongly related to the development of pathologic conditions such as cardiovascular disease [134], hormone dependant breast cancer, endometrial cancer and endometriosis in women [129]. It has been shown that the adipose tissue located in the vicinity of breast tumours produces higher amounts of estrogen than normal adipose tissue. In the pathologic conditions mentioned above, estrogens exert their actions in a paracrine or autocrine fashion, promoting additional estrogen synthesis which stimulates further tumor growth and metastasis [133, 134].

Estrogens are synthesized in all members of the vertebrate phylum [135, 136]. In more developed species i.e. primates, estrogens are synthesized in many different locations in the body such as the gonads, adipose tissue, placenta, liver, skin and brain. In ungulates, adipose tissue synthesis of estrogens has not been fully demonstrated, and in rats, the placenta does not seem to

have the capability to synthesize estrogens [135]. Another characteristic of estrogen synthesis is that different tissues can secrete different types of estrogens. For example, it has been described that the main estrogen produced in the ovaries is estradiol, while estrone is synthesized in adipose tissue and estriol predominates in the placenta. An interesting observation is that the $P450_{arom}$ catalyzing these reactions across these different tissues is the same, which confirms the notion that different types of estrogens synthesized in different tissues are not the result of the activity of alternative $P450_{arom}$ enzymes, but rather the result of the presence of different precursors in each location [135].

2.4.3 Regulation of steroidogenesis in bovine ovary

Timely variations in the expression of LH receptors (LHr) and FSH receptors (FSHr) as well as differences in cell responsiveness to the activation of such receptors have been described during folliculogenesis and maturation of ovarian follicles in cattle [65]. Researchers have been able to relate the changes in expression and activity of the gonadotropin receptors with the control of steroidogenesis in the bovine ovary, and ultimately, with ovarian follicular dynamics [126, 127]. It was shown that granulosa and cumulus cells of small ovarian follicles ($\leq$ 3 mm in diameter) express FSHr mRNA, although this stage of follicular development has been thought to be gonadotropin-independent. It is now known that these small follicles do respond to surges in FSH concentration [66]. The beginning of recruitment of small antral follicles has been associated with the expression of $P450_{arom}$ and $P450_{scc}$ mRNA in their granulosa cells. It is believed that the surge in FSH related to emergence of each wave [76] could be responsible for the induction of such steroidogenic enzymes in granulosa cells. The thecal cells of pre-antral and small antral follicles expresses LHr mRNA, $P450_{scc}$ mRNA, 3β-HSD mRNA, StAR mRNA and

$P450_{17\text{-}OH}$ mRNA, and androgens are available for the newly recruited follicles to begin synthesizing and secreting estradiol [126, 127].

By the time of follicular selection, several follicles in the recruited cohort begin to express LHr mRNA and 3β-HSD mRNA on their granulosa cells. It has been hypothesized that estradiol and FSH induce these changes, since LHr mRNA and 3β-HSD mRNA are expressed in follicles with higher levels of $P450_{arom}$ and $P450_{scc}$ mRNA in their granulosa cells (FSH-induced enzymes for the synthesis of estrogens). Forty eight hours after wave emergence, LHr mRNA and 3β-HSD mRNA were only found only in granulosa cells of the future dominant follicle [126].

The dominant follicle of the first follicular wave remains viable for 4 to 5 days, after which most mRNAs begin to decline, except for $P450_{arom}$ mRNA which begins to decline 2 days later. If progesterone levels decrease 6 or 7 days after wave emergence, this dominant follicle becomes preovulatory and all mRNAs, except $P450_{arom}$ mRNA, increase [137]. These observations indicate that LH pulsatility may be responsible for the expression of steroidogenic enzymes mRNA in the granulosa and thecal cells. Thus, mRNA expression is suppressed under low pulsatility of LH observed during the luteal phase and is increased during the pre-ovulatory increase in LH pulsatility. These observations also support the notion that $P450_{arom}$ activity does not seem to be the rate limiting step in estradiol production. Conversely, it appears that the availability of androgenic precursors produced by the thecal cells controls the rate of estrogen secretion by granulosa cells of the ovarian follicle (reviewed in [126]).

2.4.4 Local effects of estrogens in the ovary

A local effect of estrogens in ovarian follicles is suggested by the presence of estradiol receptors in granulosa cells of many mammalian species [43, 130, 138]. Studies have shown that estrogens

have paracrine/autocrine effects in the ovary that include prevention of granulosa cell apoptosis, stimulation of granulosa cell growth, induction of steroidogenic enzymes and gonadotropin receptors, among others [43, 130]. Despite the evidence of a local effect of estrogen in the ovary, it is not clear whether estrogen is a major regulator of follicular growth. The use of animal models in which estradiol synthesis at follicular level was compromised or inhibited resulted in increased follicular atresia and apoptosis. However, it was difficult to determine if the observed effects were due to lack of estradiol per se, or to unbalanced gonadotropin secretion cause by estradiol deficiency [43, 139]. These observations contradict those in which extremely low levels of estradiol (achieved by either suppression of estradiol synthesis or mutational alterations of the steroidogenic pathway) did not affect follicular development, oocyte maturation, subsequent luteal development and function, oocyte fertilization and cleavage rates; suggesting the estrogens are not required, or are required in very low concentrations, for normal ovarian function to occur [37, 39, 40, 43, 139-141].

2.4.5 Metabolism and elimination of estrogens

Estrogen bioactivity is regulated through two main mechanisms: metabolism and elimination. Estrogens can be either inactivated or converted into compounds with lower biological activity before excretion. In ruminants, estradiol-17β is in the most part deactivated in the liver by redox reactions (oxidation, reduction and hydroxylation) and it is later conjugated with glucuronic or sulphuric acid prior to bile excretion. A small amount is also converted into estradiol-17α, which has very low estrogenic activity in cattle. In both pathways, fecal elimination is the most important route of excretion in this species, although small amounts of

estradiol-17α, and the glucuronic and sulphate conjugates can be isolated from the urine [142-144].

2.5 Aromatase Enzyme

Aromatization of androgenic precursors represents the very last step of the synthesis of estrogens. This reaction is catalyzed by the P450 aromatase enzyme ($P450_{arom}$); details regarding aromatase classification, gene structure and regulation are presented below.

2.5.1 Classification

The aromatase enzyme, $P450_{arom}$, is an enzymatic complex that belongs to the super-family of P450 proteins, which includes more than 480 members divided in 74 different families. $P450_{arom}$ is the unique member of family 19 [145]. $P450_{arom}$ is located in the endoplasmic reticulum of mammalian cells that express the CYP19 gene, such as adipose tissue, brain, adrenal glands, gonads, liver and placenta [124, 136, 137, 146-148]. $P450_{arom}$ contains a heme group in its structure and is functionally associated with another member of P450 cytochrome family, an NADPH reductase, which acts as donor of reductive equivalents [149].

2.5.2 CYP19 gene: Structure and regulation

The aromatase gene (CYP19 gene) is a special case of tissue specific regulation of transcription by alternative use of different promoters [150]. The gene contains nine exons (from exon II to X) that are translated exons, meaning that the sequence of ribonucleotides is actually translated in a sequence of amino acids in the protein. Exon X has the sequence that encodes the heme binding region and the 3’ untranslated region with alternative poly-adenylation signals. The CYP19 gene contains several forms of a first untranslated exon (exon I) located distally from the encoding

region. Exon I is present in the mRNA transcripts, but does not encode a sequence of amino acids in the aromatase protein [151]. In human, it is estimated that the CYP19 gene has around 120 kb, where 30 kb include the translated exons and their introns and about 90 kb contains the alternative forms of untranslated exons I and their promoters [124, 152]. In cattle, the *Cyp19* gene is estimated to contain at least 56 kb, although the complete length of the gene remains unknown [150].

Exon I is particular in that it can be differentially spliced among several structures located upstream from the translational starting site (exon II). These structures are alternative forms of exon I, which include their own regulatory signals and promoter regions, and that are activated and spliced out in a tissue specific fashion. For instance, in human and bovine ovaries, exon I is spliced out and the transcripts start with exon II [150, 152]. In human adipose tissue, exon I.4 is activated for the synthesis of $P450_{arom}$ [150]. In the placenta, exon I.1 is expressed [150]. In cancer-associated adipose tissue, exon I.3 and II are expressed [133, 136].

After transcription, mRNA undergoes the process of maturation, where introns are spliced out and exons are arranged together prior to the initiation of translation. In the case of aromatase transcripts, this process is crucial to ensure the translation of the same and unique aromatase protein even when the original transcripts differ among tissues [135, 146, 152]. Irrespective of the tissue of origin, every alternative exon I expresses the same splicing donor sequence (GT) at its 3' end. This donor sequence of exon I matches with the splicing donor sequence (AG) present at the 5' end of exon II. This means that during the process of maturation, exon I becomes closely attached to exon II, regardless of the content or the length of the sequence of ribonucleotides that were between them in the heteronuclear mRNA [135, 146, 152]. The mature

aromatase mRNA is then translated into a sequence of amino acids, starting with the translational start signal (ATG) located within exon II. As a result, all the information located upstream from the translational start site (exon II) is not translated into the polypeptide chain and the final protein is the same in all tissues that have the capability to synthesize estrogens [152].

2.6 Estrogen Receptors

2.6.1 Classification and structure

The actions of estrogens are mediated through the estrogen receptor (ER), a member of a large superfamily of intracellular receptors that function as hormone-activated transcription factors [153, 154]. The ER and other steroid hormone receptor proteins have a common structural and functional organization, with three distinct regions responsible for ligand/hormone-binding, DNA-binding, and transcription activation [153].

Specific domains have been identified in the intracellular receptors superfamily. There is an A/B domain, located at the N-terminal region of the protein, which contains factors that interact with elements of the transcription-initiation complex in the target gene. A highly conserved region, domain C, contains two zinc fingers in its structure and is involved in interaction with DNA. At the carboxy-terminal region, another highly conserved region known as domain E/F binds to the hormone and mediates dimerization, nuclear translocation, and transactivation of transcription, being structurally and functionally complex [153, 154].

To-date, two isoforms of ER have been identified, ERα and ERβ. Structurally, ERα and ERβ share high homology on their C domain, suggesting that both isoforms of ER bind to the same elements in the DNA by the zinc finger configuration. Their E/F hormone binding domain has

less homology (53-60%), although researchers have not found significant differences in affinity for E-17β. The main difference between the two known types of ER resides in their A/B domain, suggesting that each isoform interacts with different transcription elements and activation factors to exert their function [48, 154].

The presence of plasma membrane-bound ER has been described more recently [155]. Due to the structural similarity between intracellular and membrane ER, it is thought that translocation of the intracellular form of ER determines their membrane association [155]. These membrane ERs have been associated to E2-induced rapid changes in cellular physiology, as these modifications do not require gene up-regulation or down-regulation to take place. Plasma membrane ER can also be identified in their ERα and ERβ isoforms [155-157].

2.6.2 Mechanism of action

The "classic" ER is an intracellular transcription factor that exists in inactive apoprotein forms either in the cytoplasm or nucleus. Upon binding estrogen, the ER undergoes activation and binds to a unique region of the DNA called estrogen-response-element (ERE), often located in the 5' flanking region of estrogen responsive genes. The estrogen-occupied receptor is then thought to interact with transcription factors and other components of the transcriptional complex to modulate gene transcription [154, 158].

Since the documentation of the existence of plasma membrane ERs, efforts have been directed to describe their mechanism of action. ERs anchored to the cellular membrane are unable to establish direct DNA association in order to alter gene transcription. Plasma membrane ER appears to be coupled to the G protein second messenger pathways. Activation of these pathways may results in calcium influx, cAMP and phosphatidylinositol 3 production, and

protein phosphorylation changes. The activation of membrane ER results in changes in cellular biology mostly related to kinase activation. Membrane ER activation cascade has also been reported to lead to modifications in gene transcriptions mediated by one of the above described second messenger pathways [155, 157].

It has been proposed that membrane and intracellular ERs have complementary functions in order to promote changes in cell biology, due to the nature of the reaction initiated by both ER types (signaling vs transcriptional changes). Hence, it is believed that membrane ERs are responsible for the initial rapid modification in cellular gene transcription, which is then maintained by the activation and long-acting effects of the intracellular ER [155].

2.6.3 Distribution of estrogen receptors

Tissue distribution of intracellular ERα and ERβ is differential in the sense that, even though both ER types may be present in a given tissue, one always predominates over the other in quantity and transcription signal strength. Thus, ERα appears to be the predominant type in most female reproductive tissues (uterus, oviduct, ovaries, mammary gland, and vagina), and the hypothalamus, muscle, and liver in cattle, while ERβ has only been found in significant quantities in the ovaries, jejunum, kidney and regions of the central nervous system [47]. ERβ expression prevails in the lungs, cardiovascular system and male reproductive tissues, such as prostate and testis [48, 158, 159]. These differences in tissue distribution between the ER subtypes could explain the selective action of estradiol as a stimulant or suppressor of physiological activities in different tissues.

Plasma membrane ER have been described in a number of cell types such as cardiomyocytes, endothelial cells, breast cancer cells, osteoblasts and neurons [155, 157]. In all

these tissues, they have been associated with effects such as prevention of cardiac hypertrophy (cardiomyocytes), vasorelaxation (endothelial cells), growth and proliferation (breast cancer cells and osteoblasts) and mood swings (brain nuclei) [156]

2.7 Use of Estrogens in Food Producing Animals: Regulatory Limitations

The use of estrogens and other sex steroids have been developed as a tool for controlling and manipulating reproductive phenomena in cattle and other farm animals. However, steroid hormones have also been extensively applied as growth promotants in food producing animals [160]. The first compound approved by the USA Food and Drug Administration (FDA) to be used as growth promoter in beef cattle and sheep was diethylstilbestrol (DES), which had estrogenic effects. Since then, several compounds have been formulated and approved for use in farm animals including estradiol-17β, estradiol benzoate, progesterone, testosterone, melengestrol acetate and zeranol, among others [160]. Estrogenic products are used to improve the efficiency of meat production as well as the proportion of lean meat. Subcutaneous implants are most commonly used and they may contain single steroid hormones or combinations of steroid hormones [160-163].

However, the potential for residues of exogenous steroid hormones in meat and other foods originating from treated animals have increased biosafety concerns [22, 164-166]. The main issues include the involvement of naturally occurring hormones and synthetic hormonally active compounds on the development of different forms of cancer, abnormal growth of prepubertal children and altered reproductive function [165, 167]. While several researchers have attributed carcinogenic effects to estrogens and estrogenic compounds (i.e., zeranol), other research groups have failed to replicate these results [20, 168]. The acceptable levels of estrogens and other

steroid hormones in food originating from treated animals have been under consideration for many years and consensus has not been reached. The controversy revolves around determining acceptable daily intake (ADI) levels, minimum residual levels (MRL) and no-observed-effect levels (NOEL) for the different hormones under study [169, 170]. Factors such as method of quantification, and age range and physiologic status of the population under consideration have made it difficult to determine a common tolerance level for estrogens. Further, the impact of the natural occurrence of steroid hormones in food of animal origin is not completely understood [170].

Despite all the uncertainties around the issue of steroid hormone residues in food originating from treated animals, the international situation evolved towards the banning of steroid hormones and their synthetic analogs for use in food producing animals in all member states of the European Union (EU) in January 1989 [21, 171]. The use of E-17β and its ester derivatives for reproductive management was prohibited in the EU on October 14, 2006 [23, 171]. The position adopted by the EU led to the prohibition of the use of estradiol and its esters in New Zealand and Australia in lactating dairy animals in 2007 [24]. In United States and Canada, the use of estradiol and zeranol (an estrogen-like compound) as growth promoters is still permitted [25, 171, 172]. Estrogens can only be applied for the purpose of reproductive management following veterinary prescription and custom compounding. However, compounding has recently come under scrutiny in the USA since the FDA declared that it is illegal to compound analogs of drugs that are banned from use in food producing animals, which would be the case for E-17β and its derivatives [173, 174]. This worldwide situation has created a void in the

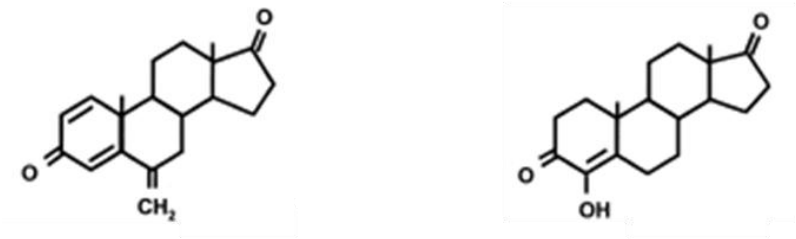
availability of treatments to efficiently control reproductive function and fertility in food producing animals such as cattle.

2.8 Aromatase Inhibitors

2.8.1 Classification

Aromatase inhibitors can be classified based on their chemical structure and mechanism of action:

(1) Steroidal aromatase inhibitors (Type I) are compounds derived from androstenedione (Figure 2. 2). These compounds bind irreversibly to the active site of the $P450_{arom}$ inducing a covalent change on the structure of the enzyme which results in lasting and selective inhibition. Because of their irreversible effects, these compounds are also called "suicide inactivators". Formestane and exemestane are examples of type I inhibitors [175].



Exemestane Formestane

Figure 2. 2. Chemical structure of steroidal aromatase inhibitors Exemestane and Formestane: its chemical structure resembles that of androstenedione (see Figure 2.1).

(2) Non-steroidal aromatase inhibitors (Type II) are compounds that have a hetero-atom (a nitrogen containing heterocyclic moiety, the triazole group; Figure 2. 3) as a common characteristic in their chemical structure. These inhibitors bind to the heme group in the $P450_{arom}$, occupying part of the active binding site of the enzyme, and interfering with the enzyme activity

in a reversible way. All these compounds have been found to be very selective for the estrogen synthetase. Examples of these compounds are letrozole, anastrozole, and fradozole [175].

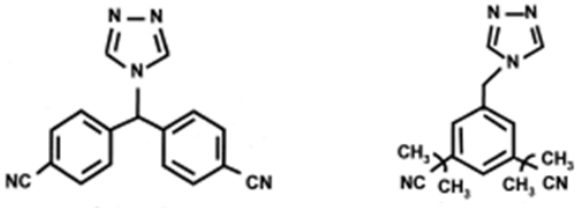


Figure 2. 3. Chemical structure of non-steroidal aromatase inhibitors letrozole and anastrozole: their chemical structures contain a triazole group that selectively binds to the heme group of the aromatase enzyme.

Since steroidal aromatase inhibitors mimic the structure of androstenedione, they have been related with undesired mild androgenic effects. Furthermore, the irreversible nature of the inhibitor-enzyme interaction implies that recovery of estrogen production will be delayed after interruption of treatment, given that *di novo* synthesis of $P450_{arom}$ protein will be needed [176].

Exemestane, letrozole and anastrozole are the most recently developed aromatase inhibitors (known as third-generation aromatase inhibitors). The main difference between second and third-generation aromatase inhibitors is the improved selectivity observed in the third generation, which has reduced the secondary effect associated with non-specific enzymatic inhibition [177].

2.9 Letrozole

Letrozole [4,4'-(1H-1,2,4-triazol- 1-yl-methylene)-bis-benzonitrile] is a type II, very potent non-steroidal aromatase inhibitor. Its chemical structure contains a triazole group that selectively interacts with the heme group of the $P450_{arom}$ enzyme, thereby reversibly inhibiting the bioactivity of the enzyme [178-180]. Letrozole is highly selective for $P450_{arom}$ blocking only

estrogen production without altering progesterone or corticosteroid synthesis [181, 182]. In North America, letrozole is marketed under the name of FEMARA® (Novartis) and it is approved for the treatment of estrogen-dependent breast cancer in postmenopausal women.

2.9.1 Pharmacokinetic parameters of letrozole

The pharmacokinetic parameters of letrozole in humans have been described in studies conducted on healthy post-menopausal women, breast cancer patients, and in healthy male volunteers [182-185]. It was reported that letrozole is rapidly and completely absorbed after oral administration [184], with a half-life is approximately 42 hours in healthy subjects [184, 185] and 82 hours in breast cancer patients [183]. Breast cancer patients also have been shown to have higher area under the curve (AUC) values than healthy individuals which may suggest a reduced metabolic clearance and consequently lower elimination rate [183]. Approximately 60% of letrozole found in circulation is bound to plasma proteins, mainly albumin [184, 185]. Letrozole is eliminated mainly via metabolism by P-450 isozymes (CYP3A4 and CYP2A6) into a pharmacologically inactive alcohol metabolite [183, 184]. The main route of excretion of letrozole is urine, where 6.8% of the given dose of letrozole was found unchanged [183].

A study of the pharmacodynamic characteristics of letrozole in healthy postmenopausal women in which three different doses of letrozole were used revealed maximal suppression of serum estradiol of 76.5%, 78.5% and 78.8% from baseline at 72 hours post-treatment with 0.1, 0.5 and 2.5 mg of letrozole given in a single oral dose, respectively [186]. In postmenopausal breast cancer patients, i*n vivo* aromatization and plasma estrogen levels were measured after 0.5 or 2.5 mg of letrozole given orally and daily for at least 6 weeks. Letrozole inhibited aromatase activity by 98.4% and >98.9%, respectively; resulting in a reduction in plasma estradiol

concentrations of 84.1% and 68.1%, respectively [187]. In a trial conducted in healthy male volunteers, estradiol suppression was about 30% at the lowest dose (0.02 mg) and 80-90% at the highest dose (30mg) tested [182].

2.9.2 The use of letrozole in cancer therapy

Seventy-five percent of women suffering from breast cancer have hormone receptor positive (+) disease [188]. This means that circulating hormones, mainly estrogens and progesterone, stimulate the division and growth of the tumour tissue. Therefore, several systemic methods of treatment have been developed to reduce the effects of estrogens (of any source) on breast cancer growth either by irreversibly binding to tumour-estrogen receptors (anti-estrogens) or inhibiting estrogen synthesis at the enzymatic level (aromatase inhibitors) [189]. In January 2001, letrozole was approved as a first-line treatment for breast cancer in post-menopausal women by the Food and Drug Administration (FDA) [33, 190]. Because of its potent inhibition of estradiol production, high oral bioavailability, selectivity and mild secondary effects, letrozole has become a drug of preference for the treatment of breast cancer, either as a first-line treatment or as a neo-adjuvant therapy [190-192]

Letrozole has also been proposed for the treatment of endometrial cancer and endometriosis [193, 194]. As both pathologic conditions are steroid hormone-responsive, letrozole and other aromatase inhibitors could be beneficial in treatment; promising results have already been reported [177, 195-197].

2.9.3 Letrozole in the treatment of infertility in women

Commercially available letrozole (FEMARA®) is not labelled for use in any pathologic condition other than hormone-dependent breast cancer in post-menopausal women [198, 199]. However, there have been numerous reports on the use of letrozole for the treatment of subfertility or infertility in women undergoing ovarian superstimulation, intra-uterine insemination (IUI) or timed intercourse [194, 200, 201]. Although the effects of estrogen deprivation on the reproductive physiology of premenopausal women are not completely elucidated, letrozole has been applied in assisted reproduction in women based on its apparent effect of removing the negative feedback effects of estradiol on gonadotropin secretion. Theoretically, removal of circulating estradiol by means of aromatase inhibition would elicit a surge in FSH concentration which would in turn induce the recruitment of a new wave of follicular development, drive follicular growth or even trigger the development of more than one ovarian follicle to a pre-ovulatory size [34, 35]. Letrozole has been used alone as a single or a 5-day regimen for ovarian stimulation [35, 202, 203], and in higher or increasing doses to induce ovarian superstimulation in women [36, 37]. Letrozole has also been utilized in combination with the administration of gonadotropins for ovarian superstimulation treatments prior to intra-uterine insemination. Improvements in the ovarian response of gonadotropin-poor responders and a significant decrease in the dose of FSH required to achieve an acceptable stimulatory response have been reported as the main advantage of the application of letrozole in fertility management treatments [38, 204].

2.9.4 Letrozole toxicity

Letrozole has proven to be a well-tolerated drug, practically lacking of toxic effects when used as indicated for breast cancer treatment in post-menopausal women. Toxicity studies performed for the approval of FEMARA® concluded that repeated dosing caused sexual inactivity and atrophy of the reproductive tract in male and female mice, rats and dogs at doses of 0.6, 0.1 and 0.03 mg/kg, respectively. Although all the reported toxic effects of letrozole have been observed during long-term treatment (as used for breast cancer therapy), less is known about its toxicity when used in a short-term treatment, as applied for ovarian stimulation. Letrozole is classified as embryotoxic, and it is contraindicated in premenopausal and pregnant women [198, 199].

In 2005, the safety of letrozole as a treatment for ovulation induction was seriously challenged by an abstract published by Biljan et al. [205]. In that retrospective study, the authors reported an increased risk for congenital cardiac and bone malformations related to the use of letrozole as an infertility treatment. However, the scientific credibility of that study was strongly questioned based on the number of letrozole-treated cases versus control cases reported (150 vs 36,050 babies, respectively) and the inadequacy of the comparison made between these groups. Shortly thereafter, another retrospective study reported the absence of teratogenic effects of letrozole when used as an ovulation induction therapy [206]. A prospective trial was designed to evaluate pregnancy outcomes after ovarian stimulation with aromatase inhibitors, and it further confirmed the safety of aromatase inhibitors (letrozole and anastrozole in particular) as a tool for infertility management in women [207].

2.9.5 Use of letrozole in cattle

Based on the use of aromatase inhibitors, mainly letrozole, for the control of reproduction in women, we hypothesized that non-steroidal aromatase inhibitors could also be applied to the management of ovarian function in cattle, even though the exact mechanism of action of ovarian function by letrozole was not clearly understood.

Two studies were designed to characterize the effects of letrozole on ovarian function in cattle. In a first study, post-pubertal beef heifers were given phosphate-buffered saline (control), or letrozole at a dose of 500, 250, or 125 µg/kg intravenously 4 days after follicular ablation (~2.5 days after wave emergence). In a second study, post-pubertal beef heifers were given no treatment (control) or 85 µg/kg of letrozole per day from Days 1 to 3, Days 3 to 5, or Days 5 to 7 (250 µg/kg total dose, Day 0 = pre-treatment ovulation) corresponding to the periods before, during and after selection of the dominant follicle, respectively. In both studies, the diameter profile of the dominant follicle was larger in heifers treated with letrozole than in control heifers and the intervals to new wave emergence and onset of regression of the extant dominant follicle were longer in heifers treated with letrozole than in controls, regardless of the dose (high, medium, or low; single vs multiple) or the stage of the follicle wave in which treatments were initiated. Furthermore, during the second experiment, the mean CL diameter was larger in letrozole-treated heifers, although there were no differences in plasma progesterone concentrations between treated and control animals. The effects on dominant follicle and CL diameter profiles appeared to be the result of the significantly increased plasma LH concentrations observed in letrozole-treated animals during both treatment approaches (single vs multiple dose). However, it was concluded that the incomplete and inconsistent inhibition of

estradiol production and the lack of a surge on FSH observed in both experiments may be a result of insufficient circulating levels of letrozole during the treatment period [39, 40].

These results suggested that letrozole had potential as a non-steroidal method for controlling ovarian function in cattle, but further studies were needed in order to clarify dosage and timing of treatment to predictably affect follicular wave dynamics in cattle. Furthermore, the prospective use of letrozole as a treatment to improve post-breeding and ET fertility is supported by its luteotrophic effect (more progesterone for embryo support, enhanced trophoblast elongation and successful maternal recognition of pregnancy) and its effectiveness in reducing plasma estradiol concentration (minimizing estradiol-induced expression of endometrial oxytocin receptors).

2.10 The Bovine Model for the Study of Human Reproduction

Similarities between cattle and women regarding ovarian size, follicular size and endocrine regulation of the follicular development, together with the accessibility and adaptability of the bovine species, have validated the bovine model as a tool to assess and further investigate human reproductive physiology [68, 208-212]. The description of a wave-like pattern of follicular growth during the estrous cycle in women, as observed in other mammals (cow, mare, sheep) [68, 212], represented a break-through discovery. Two or three waves of follicular development were observed during the follicular (ovulatory wave) as well as the luteal phase (non-ovulatory waves) of the menstrual cycle in healthy female volunteers [210]. It was also shown that the wave-like pattern observed in humans was controlled by the same endocrine mechanisms as described in other monovulatory species such as cattle [209]. Consequently, the bovine model has been successfully implemented for the study of several features of human reproduction

including ovarian senescence [213], oocyte competence after reproductive aging [214] and the effect of aromatase inhibitor on ovarian function [40].

We anticipate that the data generated and included in the present thesis work will be of great value to improve our understanding of the effects of aromatase inhibitors when used as infertility or subfertility treatment in women.

CHAPTER 3: GENERAL HYPOTHESIS

The overall hypothesis tested in this thesis is that aromatase inhibitors can be used as an effective, safe, and steroid-free method for controlling ovarian function and to improve fertility in mammals, using a bovine model.

CHAPTER 4: GENERAL OBJECTIVES

The overall objectives of the studies reported herein were:

- To determine the effect of aromatase inhibitors on ovarian function in cattle.
- To determine the effect of vehicle and route of administration of letrozole on ovarian function in cattle
- To determine the effect of duration of aromatase inhibitor treatment on ovarian follicle dynamics in cattle
- To develop an aromatase inhibitor-based protocol to synchronize ovulation in cattle.

CHAPTER 5: EFFECT OF VEHICLE AND ROUTE OF ADMINISTRATION OF A NON-STEROIDAL AROMATASE INHIBITOR ON OVARIAN FUNCTION IN A BOVINE MODEL

Relationship of this study to the thesis:

Previous studies of the effect of aromatase inhibitors on ovarian function in cattle used the intravenous route for administration of letrozole treatment. In order to develop a reliable and efficient method to synchronize ovulation in cattle, more practical routes of administration need to be explored. It this chapter, the effect of route and vehicle for the administration of letrozole in ovarian dynamics in cattle are described.

5.1. Abstract

Treatment of cattle with letrozole, a non-steroidal aromatase inhibitor, has been associated with elevated mean plasma LH concentrations, prolonged follicular dominance, delayed emergence of the next follicular wave, and potential luteotrophic effects. The objective of the present study was to determine the effects of vehicle and route of administration of letrozole on ovarian function in sexually mature beef heifers. Ovarian follicular function was synchronized among heifers using transvaginal ultrasound-guided follicular ablation followed by a luteolytic dose of PGF bid 4 days later. The ovaries were examined daily by transrectal ultrasonography until ovulation was detected (Day 0=ovulation). On Day 3, heifers were assigned randomly to four treatment groups and given letrozole at a dose of 1 mg/kg intravenously (iv, in benzyl alcohol, n=10) or intramuscularly (im, in benzyl alcohol plus canola oil 1:1 v/v, n=10), or given a placebo iv (benzyl alcohol, n=5) or im (benzyl alcohol plus canola oil 1:1 v/v, n=5). The ovaries were monitored daily by ultrasonography and blood samples were collected twice daily from pre-treatment to post-treatment ovulations. The interovulatory interval did not differ among groups, nor did the day-to-day diameter profiles of the dominant follicle of Wave 1 (first follicular wave after ovulation). However, the interval between emergence of Waves 1 and 2 was longer in heifers treated with letrozole im (11.7±0.3 days) than in placebo-treated controls (10.0±0.4 and 9.5±0.5 days for iv and im placebo-treated controls, respectively; $P<0.05$), and intermediate in heifers given letrozole iv (10.6±0.30 days). The day-to-day diameter profile of the corpus luteum and plasma progesterone concentrations were greater ($P<0.03$, and $P<0.05$, respectively) in heifers treated with letrozole im vs placebo-treated control im. Plasma estradiol concentrations were lower in both letrozole-treated groups, compared to placebo-treated controls ($P\leq0.03$).

Plasma LH concentrations tended to be elevated at the time of wave emergence in heifers treated with letrozole im compared to other groups (group-by-day interaction, P=0.06), and plasma FSH concentrations tended to be greater (P<0.09) in heifers treated with letrozole by either route compared to corresponding placebo-treated control groups. In summary, letrozole dissolved in benzyl alcohol and given intravenously at a dose of 1 mg/kg on Day 3 did not alter ovarian function in cattle, but the same dose given intramuscularly in canola oil vehicle resulted in a longer inter-wave interval, a greater CL diameter profile, greater plasma progesterone concentrations, and persistent inhibition of estradiol production. We conclude that intramuscular administration of letrozole in oil is a feasible route and vehicle for the development of an aromatase inhibitor-based treatment protocol for herd synchronization in cattle.

5.2. Introduction

Estradiol and its esters, in combination with progesterone, have been used effectively for synchronizing follicle wave emergence and ovulation in cattle [4, 5]. The degree of synchrony achieved with estradiol has made fixed-time artificial insemination feasible [6, 7] and has improved the efficiency of embryo transfer programs [4, 109].

The use of natural or synthetic estrogens, whether for anabolic purposes, therapeutic purposes or for controlling reproductive function, has been the subject of considerable controversy in the cattle industry (reviewed in [20, 167]) due to increasing concern about potential toxic and carcinogenic effects of steroid hormone use in food producing animals [20, 22, 170]. As a result, estradiol and other steroid hormones have been banned for use as growth promotants and for reproductive management in animals designated for human consumption in the European Union [21, 23]. In 2007, New Zealand and Australia have banned the use of

estrogens in lactating dairy animals [24]. The use of estradiol and zeranol (an estrogen-like compound) as growth promotants is still permitted in the United States [25] and Canada [172], but no commercial preparations are available for the purposes of reproductive management. Hence, treatments for estrus synchronization must be custom-compounded and used by prescription only. In this context, the development of alternative methods for controlling ovarian function in cattle is needed.

Letrozole, a non-steroidal aromatase inhibitor, inactivates the aromatase enzyme responsible for the synthesis of estrogens by reversibly binding to the heme group of its P450 subunit. In women, letrozole is indicated as an adjuvant or first-line treatment for hormone-dependent breast cancer during post-menopause [33] and has also been used for ovarian stimulation and ovulation induction in women undergoing assisted reproduction [34, 35, 215]. In women, letrozole is commonly used at a dose of 1 to 5 mg per day for 5 days [35, 216].

It has been hypothesized that letrozole exerts its ovarian stimulatory effect by removing the negative feedback of estradiol on FSH secretion [34]. However, a single intravenous dose of letrozole given to beef heifers on Day 3 post-ovulation or in a 3-day regimen from Days 1-3, 3-5 or 5-7 post-ovulation did not induce an elevation in circulating FSH concentrations and did not induce follicular atresia or hasten emergence of a new follicular wave [39, 40]. Rather, letrozole treatment increased mean plasma LH concentrations resulting in a prolonged period of dominance of the extant dominant follicle and delayed emergence of the next follicular wave. Further, a luteotrophic effect was apparent by the observation of larger diameter corpora lutea in heifers treated with letrozole in a 3-day regimen.

The objectives of the present study were to test the hypothesis that letrozole will terminate dominant follicle growth and result in the emergence of a new follicular waves at predictable intervals thereafter when administered at a higher dose than previously tested (1 mg/kg vs 0.5mg/kg) and to determine the effect of vehicle and route of administration of letrozole on ovarian and endocrine function in cattle.

5.3. Materials and Methods

5.3.1. Cattle

Hereford-cross beef heifers, 14 to 20 months of age and weighing between 340 and 482 kg, were chosen randomly from a herd of 50 heifers maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52° North and 106° West). Heifers were fed alfalfa/grass hay and grain to gain approximately 1.3 Kg per day and had water ad libitum during the experimental period from October to December. Heifers were initially examined by transrectal ultrasonography (7.5 MHz linear-array transducer, Aloka SSD-900; Tokyo, Japan) to confirm that they were post-pubertal by detection of the presence of a CL [217]. Animal procedures were performed in accordance with the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

5.3.2. Treatments and examinations

Heifers in which a CL was detected during the initial examination underwent transvaginal ultrasound-guided follicular ablation of the two largest ovarian follicles to synchronize follicular wave emergence. New wave emergence was expected to occur 1 to 1.5 days later [218, 219]. Four days after follicular ablation, two doses of 500 µg of cloprostenol (PGF, Estrumate,

Schering-Plough Animal Health, Pointe-Claire, QC, Canada) were administered intramuscularly (im) 12 h apart to induce regression of the CL and synchronize ovulation [220]. Heifers were assigned randomly at the time of ovulation (Day 0) to the following groups and given 1 mg/kg of letrozole on Day 3 either intravenously (iv, n=10) or im (n=10), or placebo treatment iv (n=5) or im (n=5). For iv administration, letrozole was prepared in 95% benzyl alcohol to a final concentration of 100 mg/mL resulting in an injection volume of 4-5 mL. For im administration, letrozole was prepared in 95% benzyl alcohol mixed with sesame oil 1:1 v/v to a final concentration of 50 mg/mL resulting in an injection volume of 8-10 mL applied in two injection sites. Intravenous and im placebos were prepared using benzyl alcohol and benzyl alcohol plus sesame oil 1:1 v/v, respectively and the volumes were similar to those injected in the letrozole-treated groups.

5.3.3. Ovarian ultrasonography

The observations from ultrasound examination were recorded on a sketch sheet in which each ovary and its structures (CL and follicles ≥ 4 mm in diameter) were represented in size and location [73]. Ovulation was defined as the disappearance of a follicle ≥8 mm between two consecutive daily examinations and confirmed by subsequent development of a CL [217]. Follicular wave emergence was taken as day of ovulation for Wave 1 (first wave of the interovulatory interval) or defined retrospectively as the day when the dominant follicle was first identified at a diameter of 4 or 5 mm for Wave 2 [45, 64]. If the dominant follicle was not identified until it reached 6 or 7 mm, the previous day was considered day of the follicular wave emergence [87]. The dominant follicle of a wave was defined as the largest antral follicle of that wave [221]. The day of onset of follicular and luteal regression was defined as the first day of an

apparent progressive decrease in diameter that terminated in their disappearance or loss of individual identity [45].

5.3.4. Collection of blood samples

Daily blood samples were collected by jugular or coccygeal venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Additionally, blood samples were collected twice daily from treatment (Day 3) until emergence of the first post-treatment wave was detected, and daily thereafter until ovulation occurred. A subset of heifers in the letrozole treated groups (n=4 for iv and im each) underwent more frequent blood sampling via indwelling jugular catheter, as previously described to determine letrozole concentration and pharmacokinetic parameters [84]. Samples were taken at the time of catheter placement and at 0, 10, 20, 30 min, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours post-treatment. Blood samples were centrifuged at 1500 x g for 20 minutes and plasma was separated and stored in plastic tubes at -20 °C.

5.3.5. Hormone assays

Plasma LH concentrations were determined in duplicate in a single assay using a double-antibody radioimmunoassay (NIDDK-bLH4) [11, 222]. The minimum and maximum values along the standard curve were 0.06 and 8 ng, respectively. The intra-assay coefficient of variation was 9.8% for low reference samples (mean, 1.1 ng/mL) and 14.4% for high reference samples (mean, 2.7 ng/mL).

Plasma FSH concentrations were determined in duplicate with a double-antibody radioimmunoassay using NIDDK-anti-oFSH-1 primary antibody and expressed as USDA bovine

FSH-Il units [11, 222]. The minimum and maximum values along the standard curve were 0.12 and 16 ng, respectively. The intra- and inter-assay coefficients of variation were 10.6% and 10.7%, respectively, for low reference samples (mean, 2.3 ng/mL) and 5.7% and 5.6%, respectively, for high reference samples (mean, 5.5 ng/mL).

Plasma concentrations of estradiol were determined using a commercial radioimmunoassay kit (Double Antibody Estradiol; Diagnostic Products, Los Angeles, CA, USA). The procedure was carried out at the Department of Animal Health and Biomedical Sciences, University of Wisconsin–Madison, as described elsewhere [79, 223] with the following modifications: Standards (0.78–100 pg/mL) were prepared in steroid-free (charcoal-treated) bovine plasma. The standards (250 µL in duplicate) and plasma samples (500 µL in duplicate) were extracted with 3 mL of diethyl ether, frozen in a dry-ice/methanol bath, decanted into assay tubes, and dried overnight under a fume hood. The dried samples and standards were re-suspended with 100 µL of assay buffer (0.1% gelatin in PBS). The intra-assay and inter-assay coefficients of variation were 10.5% and 10.6% for high reference samples (mean, 11.1 pg/mL), and 14.8% and 12.3% for low reference samples (mean, 2.6 pg/mL), respectively. The sensitivity of the assay was 0.1 pg/mL.

Plasma progesterone concentrations were determined in duplicate in a single assay using a commercial solid-phase radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corporation). The range of the standard curve was 0.1 to 40.0 ng. The intra-assay coefficient of variation was 3.9% for low reference samples (mean, 1.4 ng/mL) and 3.9% for high reference samples (mean, 14.6 ng/mL).

5.3.6. Letrozole concentrations

Plasma concentrations of letrozole were determined using high performance liquid chromatography tandem mass spectrometry (LC/MS/MS). Letrozole was extracted from 250 µL of plasma with 250 µL of 0.1 M ammonium acetate followed by the addition of 5 mL of methyl t-butyl ether (MTBE) and vortexed for 15 sec. The organic layer was removed and transferred to a fresh 15 mL plastic tube and dried by gentle nitrogen gas flow. The dried extract was reconstituted in 1 mL of 100% ethanol, sonicated for 5 min and transferred to a labelled vial for further analysis. Separation was accomplished by HPLC (Agilent 1200, Santa Clara, CA, USA) fitted with an analytical column (50x2.1 mm, 3 µm particle size; Thermo Scientific Betasil C18, Waltham, MA, USA) operated at 35ºC. Gradient conditions were used at a flow rate of 250 µL/min, starting at 85% A (0.1% acetic acid) and 15% B (0.1% acetic acid in acetonitrile). Initial conditions were held for 2 min and then ramped to 100% B at 6 min, held until 9 min, decreased to 0% B at 11 min, returned to initial conditions at 13 min, and then held constant until 15 min. Mass spectra were collected using a tandem mass spectrometer (Applied Bioscience SCIEX 3000, Foster City, CA, USA) fitted with an electrospray ionization source, operated in the negative ionization mode. Chromatograms were recorded using multiple reaction monitoring (MRM) mode, where at least two transitions per-analyte were monitored. The following instrument parameters were used: desolvation temperature 450ºC, desolvation (curtain) gas 6.0 arbitrary units (AU), nebulizer gas flow 4 AU, ion spray voltage 4500 V, collision gas 12 AU, collision energy 46 AU, declustering potential 30 AU, and a dwell time of 100 msec. Quantification using these transitions was performed using Analyst 1.4.1 software provided by SCIEX (Applied Bioscience, Foster City, CA, USA). The minimum and maximum values along

the standard curve were 0.25 and 500 ng/mL respectively. The limit of quantification used in this method was 0.25 ng/mL and the mean recovery was 70%.

Plasma concentration-time profiles were generated for letrozole given intravenously and intramuscularly. Concentrations below the limit of quantification were considered non-detectable and were taken as zero. The following pharmacokinetic parameters were determined: C_{max} (maximum observed plasma concentration of letrozole), t_{max} (time to reach C_{max}), $t_{1/2}$ (terminal elimination half-life), *AUC* (area under the plasma letrozole concentration-time curve from zero to infinity calculated as AUC_{last}). The concentration of letrozole in plasma as a function of time (C–t) data for each heifer was analyzed by non-compartmental techniques using a computer modeling program (WinNonLin Standard Edition Version 2.1, Pharsight Corporation, Mountain View, CA, USA). Peak letrozole concentration in plasma (Cmax) and time to peak letrozole concentration (tmax) were determined using observed values. The apparent terminal rate constant (λ) was determined by linear regression of the last 6–8 points on the terminal phase of the logarithmic plasma concentration vs time curve. The area under the C–t curve until the final plasma sample (AUC_{last}) was determined using the linear trapezoidal rule. The total area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by adding the $C_{last\ obs}/\lambda + AUC_{last}$. The terminal half-life ($T_{1/2\lambda}$) was calculated as $\ln 2 / \lambda$. The mean residence time (MRT) was calculated as the area under the moment curve extrapolated to infinity ($AUMC_{0-\infty}$) $/ AUC_{0-\infty}$. Systemic clearance (Cl_S) was determined using the dose divided by AUC_{0-inf}. The apparent volume of distribution (V_λ/f) was calculated by clearance divided by λ.

5.3.7. Statistical analyses

Statistical analyses were done using the Statistical Analysis System software package (SAS Learning Edition 9.1, 2006; SAS Institute Inc., Cary, NC, USA). Time-series hormone data and follicular and CL diameter profiles were analyzed by repeated measures using the PROC MIXED procedure. The main effects were treatment (letrozole im, letrozole iv, placebo-treated control im and placebo-treated control iv), time, and their interactions. Data were combined and re-analyzed as a single letrozole treatment group and/or a single placebo-treated control group when no differences were detected between routes of administration. Single point measurements (inter-wave interval, interovulatory interval, dominant follicle diameter at treatment, and treatment to onset of CL regression) were analyzed by one-way analysis of variance. Gonadotropin data were centralized to the day of post-treatment wave emergence to adjust for a delay in new wave emergence caused by letrozole treatment. Estradiol concentrations between two given points in time within each treatment group were assessed using paired t-tests. Individual time point comparisons between treatment groups were performed using least significant difference (LSD) test.

5.4. Results

The mean diameter of the dominant follicle at the time of treatment (Day 3; Day 0 = ovulation), did not differ among groups (Table 5. 1). No effect of route of treatment (im vs iv) was detected on the day-to-day dominant follicle diameter profiles in either the letrozole-treated or placebo-treated control groups (Figure 5. 1); therefore, data were combined into a single letrozole group for comparison with the single placebo-treated control group. No differences in day-to-day

dominant follicle diameter profiles were observed in letrozole-treated heifers compared to placebo-treated controls from Days 0 to 13 (Figure 5. 1).

Table 5. 1. Follicle dynamics (mean ± SEM) in heifers treated intravenously (iv) or intramuscularly with letrozole (1mg/kg of body weight) or placebo (control). Data are presented as mean ± SEM.

End point	Control im (n=5)	Letrozole im (n=10)	Control iv (n=5)	Letrozole iv (n=10)
Interwave interval (days)	9.5 ± 0.50 [a]	11.7 ± 0.34 [b]	10.0 ± 0.43 [a]	10.6 ± 0.30 [a,b]
Interovulatory interval (days)	18.2 ± 1.10 [a]	19.7 ± 0.83 [a]	17.5 ± 1.10 [a]	18.9 ± 0.70 [a]
Dominant follicle at treatment (mm)	11.0 ± 0.86 [a]	10.2 ± 0.61 [a]	11.2 ± 0.86 [a]	10.9 ± 0.61 [a]

[ab] Within rows, values with different superscripts are different ($P<0.05$)

Dominant follicle diameter profiles did not differ between letrozole-treated and placebo-treated control heifers. However, the interval from treatment to emergence of a new follicular wave was significantly longer in heifers treated with letrozole im compared with either of the placebo-treated control groups while the letrozole iv group was intermediate (Table 5. 1). The inter-ovulatory interval did not differ among groups nor did the variability in interwave interval (i.e., degree of synchrony; Table 5. 1).

Corpus luteum diameter profiles were larger in heifers treated with letrozole im than in the respective control group ($P=0.04$) while that of letrozole iv and placebo-treated control iv groups did not differ and were intermediate (Figure 5. 2). There was a tendency for a treatment effect on plasma progesterone concentration ($P=0.06$) that was determined to be a reflection of numerically higher concentrations in the letrozole im group on all but two days from Day 5 onward (Figure 5. 2). The day-to-day CL diameter and progesterone profiles were significantly

larger in letrozole-treated heifers when data were combined into a single letrozole-treated group and a single placebo-treated control group (Figure 5. 3).

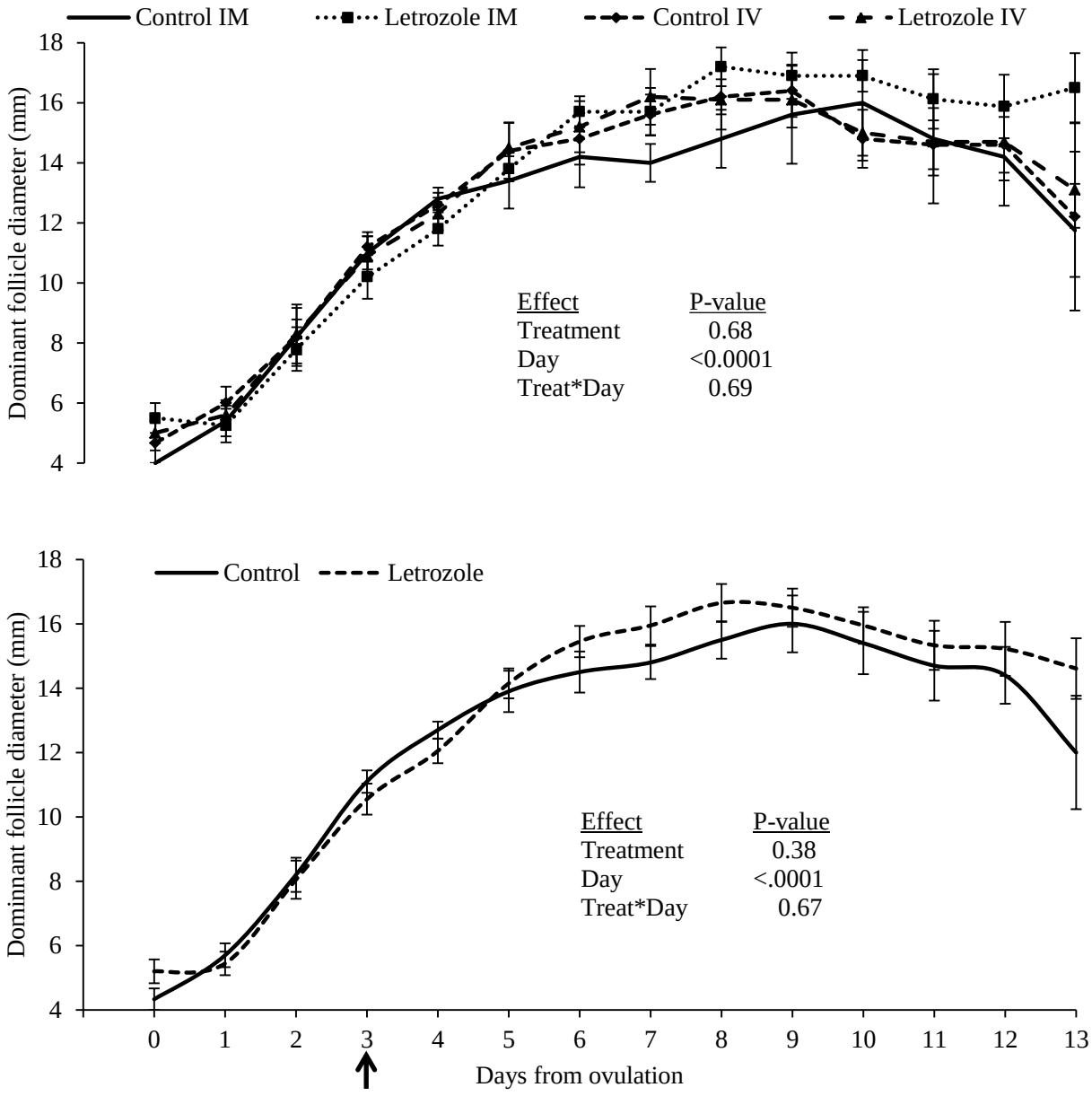


Figure 5. 1. Dominant follicle diameter profile (mean ± SEM) in heifers treated with letrozole intramuscularly (im, n=10) or intravenously (iv, n=10), and their respective placebo-treated

controls (n=5 per group). In the bottom panel, im and iv routes were combined within letrozole and control groups. The arrow indicates the day of treatment.

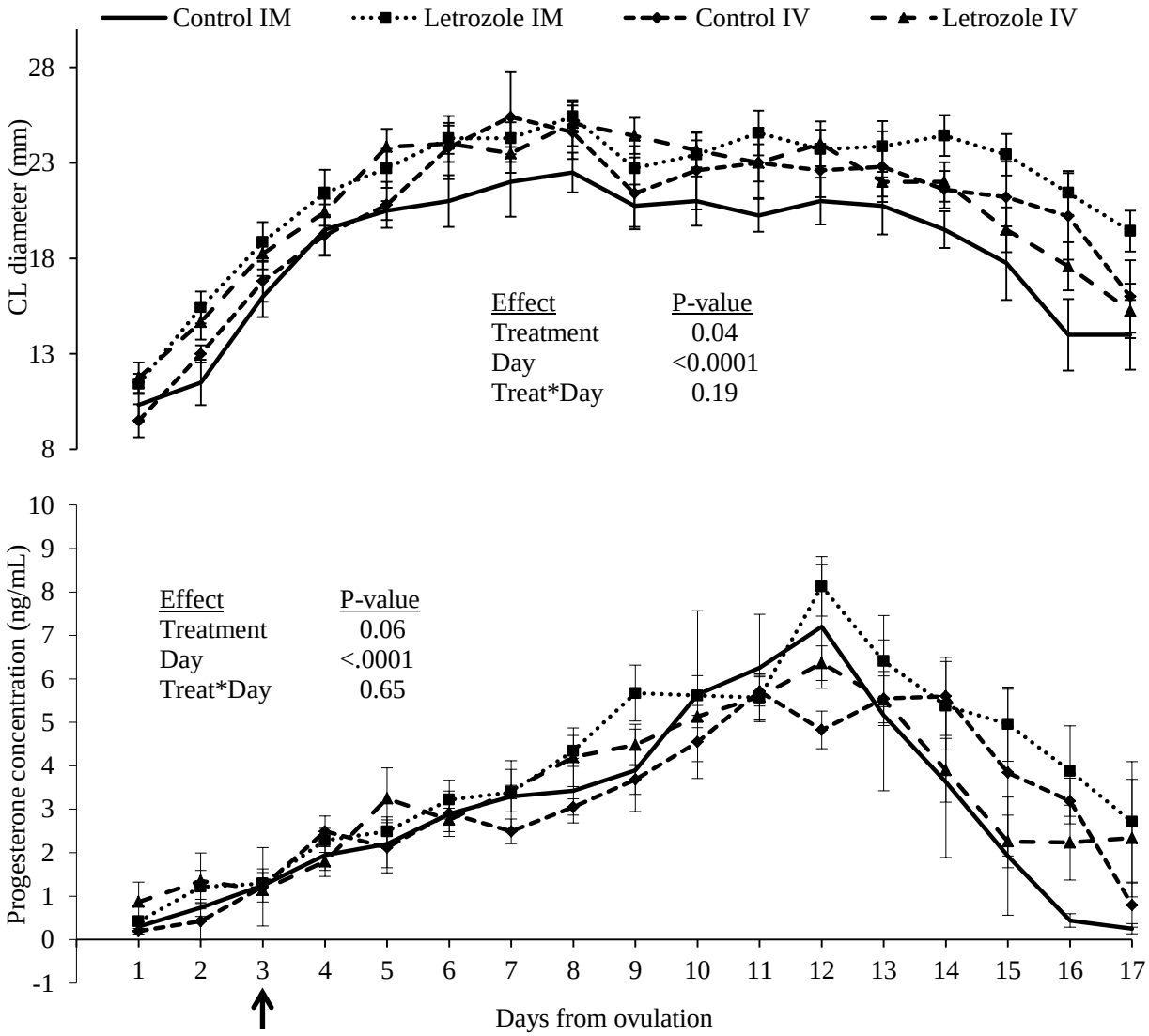


Figure 5. 2. Corpus luteum diameter and plasma progesterone concentration (mean±SEM) in heifers treated with letrozole intramuscularly (im, n=10) or intravenously (iv, n=10), and their respective placebo-treated controls (n=5 per group). The arrow indicates the day of treatment.

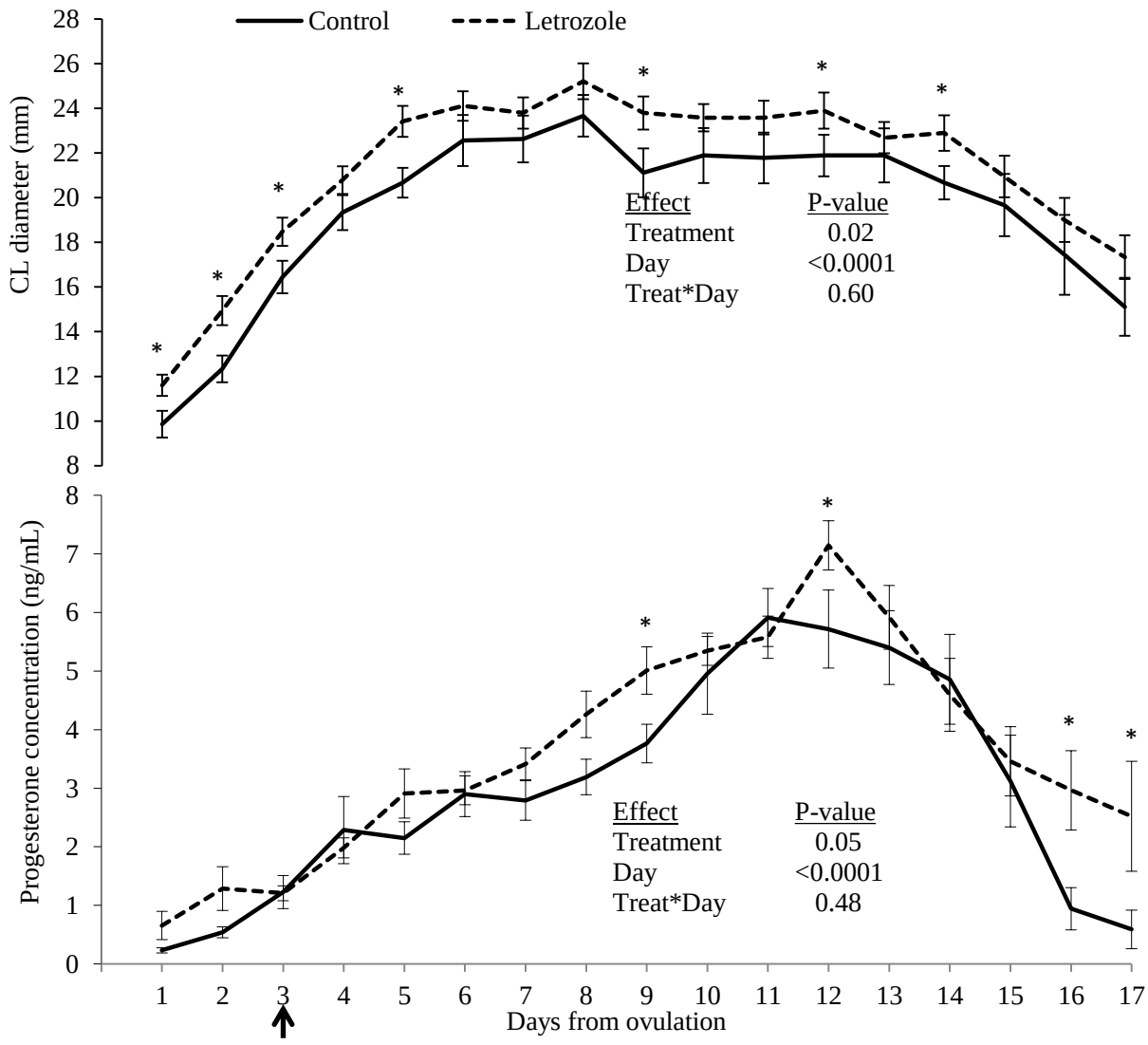


Figure 5. 3. Corpus luteum diameter and plasma progesterone concentration (mean ± SEM) in heifers treated with letrozole (intravenous and intramuscular routes combined, n=20) or a placebo (intravenous and intramuscular routes combined, n=10). (*) indicates P≤0.05 between groups for that specific time point (analyzed by LSD). The arrow indicates the day of treatment.

Plasma estradiol concentrations were not different among groups at the time of treatment (P = 0.45, mean 1.7 ± 0.14 pg/mL). No differences were observed in estradiol concentration

between the placebo-treated control groups hence data were combined. In the letrozole im group, plasma estradiol concentrations started to decline immediately after treatment and became significantly lower on Day 5 (P = 0.013, Figure 5. 4). Estradiol concentration remained low until the end of the observational period (Day 9). In the letrozole iv group, estradiol concentration dropped rapidly and were significantly lower by 24 h after treatment (P = 0.03, Figure 5. 5). However, estradiol concentration returned to pre-treatment levels within 3 days of treatment and did not differ from the placebo-treated control iv group by Day 6 (P = 0.64, Figure 5. 5).

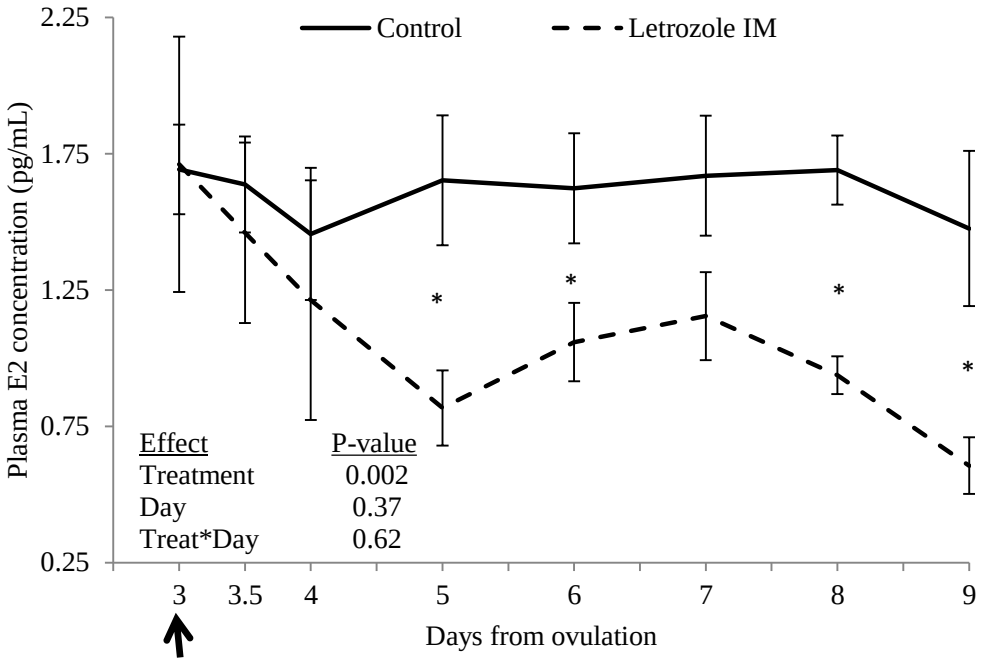


Figure 5. 4. Plasma estradiol concentration (mean ± SEM) in heifers treated with letrozole intramuscularly (im, n=10) compared to placebo-treated controls (iv and im combined; n=10). (*) indicates P≤0.05 between groups for that specific time point (analyzed by LSD test). The arrow indicates the day of treatment.

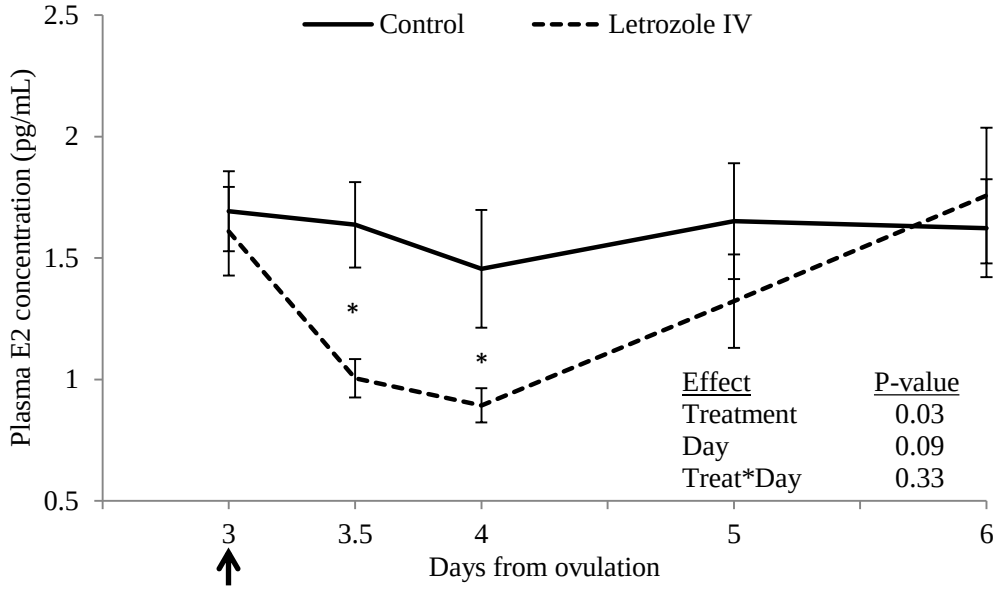


Figure 5. 5. Plasma estradiol concentration (mean ± SEM) in heifers treated with letrozole intravenously (iv, n=10) compared to placebo-treated controls (iv and im combined; n=10). (*) indicates P≤0.05 between groups for that specific time point (analyzed by LSD test). The arrow indicates the day of treatment.

Plasma FSH concentrations were centralized to the day of post-treatment wave emergence. No differences were detected for route of administration so data were combined in the respective treatment groups. Plasma FSH concentrations increased as the day of wave emergence neared and tended to be higher in the letrozole group than in the placebo-treated control group (P = 0.09, Figure 5. 6).

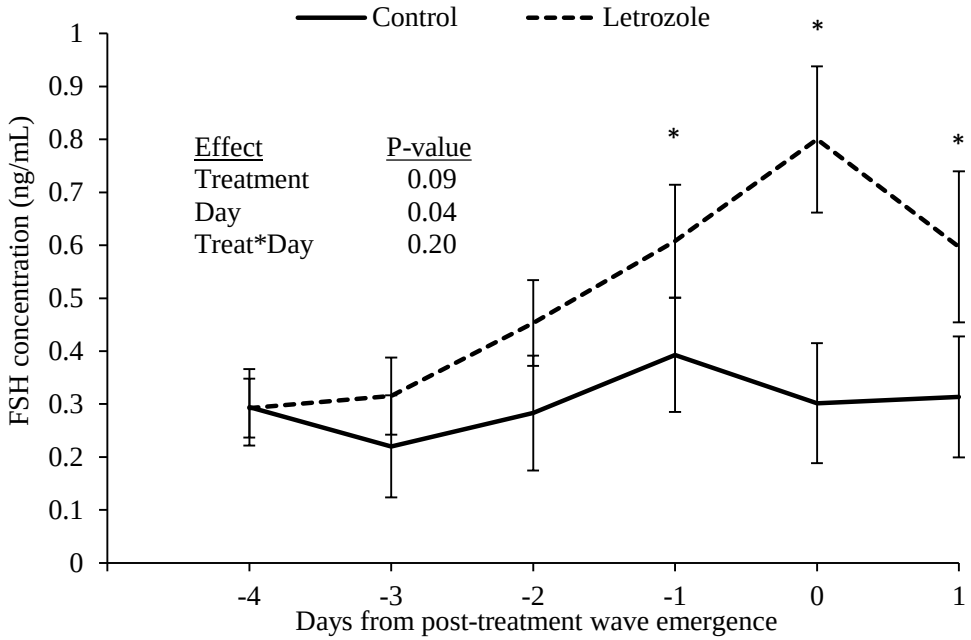


Figure 5. 6. Plasma FSH concentration (mean ± sem) in heifers treated with letrozole (intravenous and intramuscular routes combined; n=20) compared to pacebo-treated controls (intravenous and intramuscular routes combined; n=10). Data were centralized to the day of post-treatment wave emergence. (*) indicates P≤0.05 between groups for that specific time point (analyzed by LSD test).

There was a tendency for a treatment-by-day interaction on plasma LH concentrations (P<0.06) as a result of higher concentrations in the letrozole im group than other groups on the day of new wave emergence (Figure 5. 7).

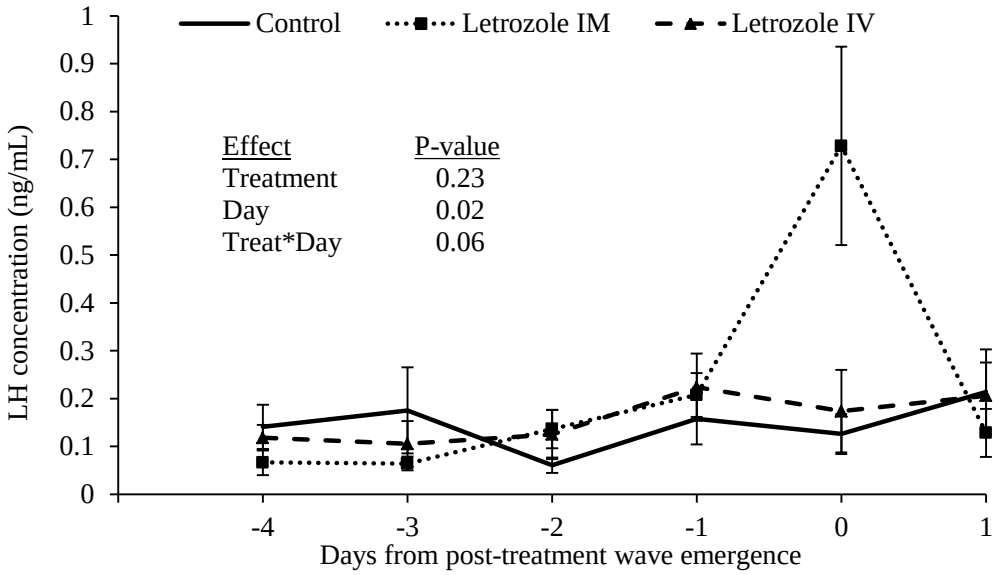


Figure 5. 7. Plasma LH concentration (mean ± SEM) in heifers treated with letrozole intravenously (n=10) and intramuscularly (n10) compared to placebo-treated controls (intravenous and intramuscular routes combined; n=10). Data were centralized to the day of post-treatment wave emergence.

No adverse effects were observed in any heifers after iv or im letrozole administration. The mean plasma letrozole concentration vs time curves for both letrozole groups are depicted in Figure 5. 8. The pharmacokinetic parameters of letrozole are presented in

Table 5. 2. The mean bioavailability of letrozole after im administration was 54%.

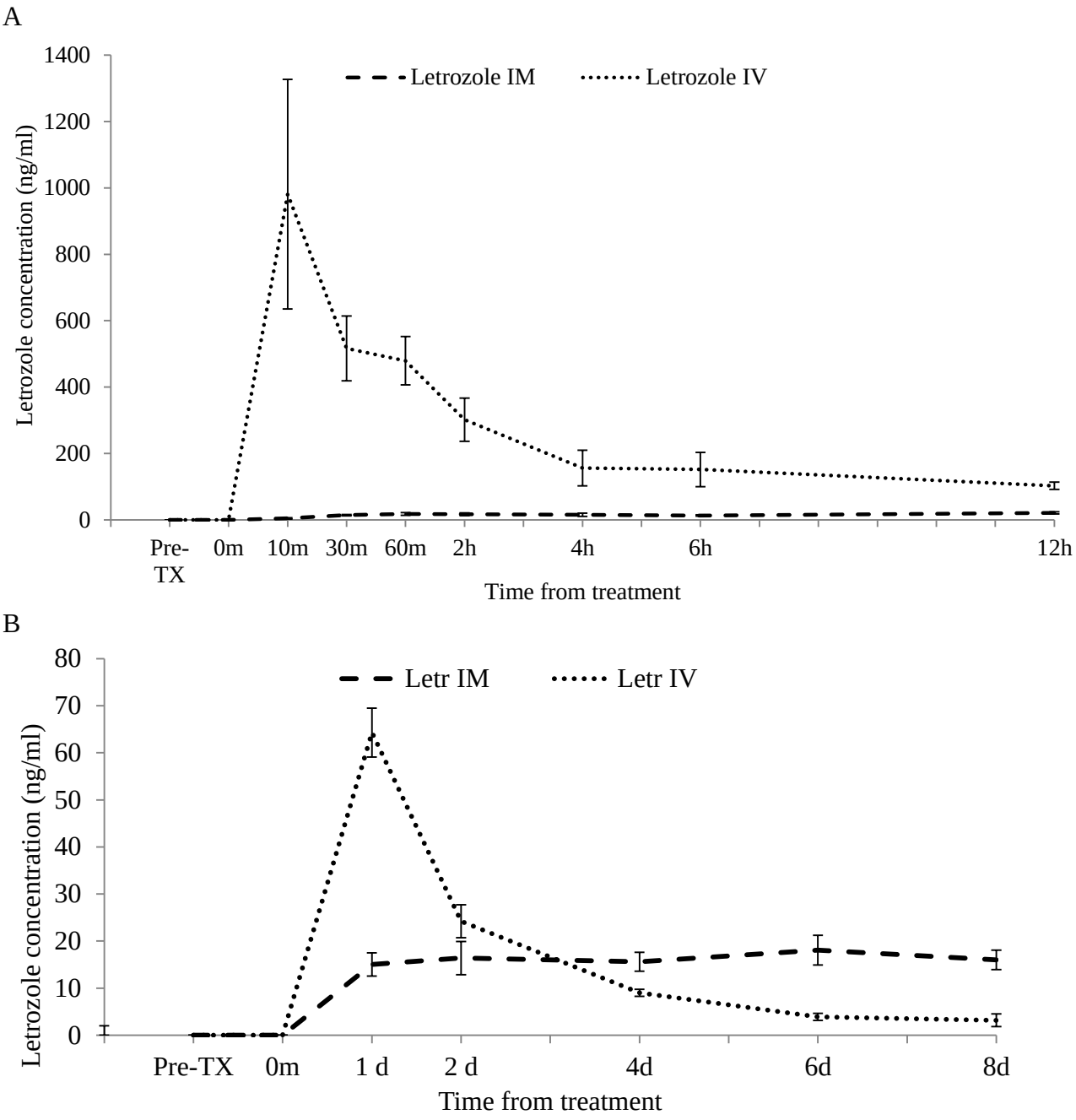


Figure 5. 8. Plasma letrozole concentration (mean ± SEM) during the first 12 h (A) and 8 d (B) in heifers after a single administration of 1 mg/kg of body weight intravenously (iv, n=4) or intramuscularly (im, n=4).

Table 5. 2. Pharmacokinetics of letrozole after intravenous administration and AUC_{last} after intravenous and intramuscular administration of 1mg/kg in cattle.

Parameter	Heifers 1	Heifers 2	Heifers 3	Heifers 4	Mean	SEM
Maximal concentration (C_{max}) (ng/mL)	542.6	412.9	1938.5	1030.9	981.2	345.73
Half-life ($T_{1/2}$) (hours)	33.6	37.4	30.2	32.0	33.3	1.52
Volume of distribution (V_z/f) (L/kg)	9.4	9.7	6.4	6.8	8.1	0.84
Systemic clearance (Cl_S) (L/hour/kg)	0.2	0.2	0.1	0.1	0.2	0.01
iv - area under the curve (AUC_{last}) (hours x ng/mL)	4896.5	5441.0	6716.0	6522.0	5893.9	434.96
im - area under the curve (AUC_{last}) (hours x ng/mL)	2776.0	2371.7	4361.3	3191.1	3175.0	429.34
Bioavailability after im treatment					54%	

5.5. Discussion

Previous studies on the effects of letrozole on ovarian function in women have been interpreted to mean that aromatase inhibitor treatment stimulated follicular growth by removing the negative feedback effect of estradiol on FSH secretion [34, 141]. However, results of the present study and studies previously conducted by our group [39, 40] support the hypothesis that the hormone responsible for the effects of letrozole on ovarian function in cattle is LH rather than FSH.

Two previous studies conducted in cattle showed that letrozole has a stimulatory effect on the growth and lifespan of the dominant follicle [39, 40]. Similarly, women treated with letrozole from Days 3 to 7 of the menstrual cycle had larger dominant follicles than untreated controls [224]. Although an effect on dominant follicle diameter was not evident in the present study, the prolonged interval from treatment to new wave emergence in both letrozole-treated groups suggests that the functionality of the dominant follicle was indeed prolonged. However, this effect on dominant follicle lifespan was more evident in heifers given letrozole intramuscularly

than in those treated intravenously. This observation may be related to the longer residence time of letrozole in circulation in the im group, given that the oil-based formulation probably served as a depot, delaying and prolonging drug release from the injection site.

In the present study, letrozole treatment had a luteotrophic effect. The effect was evidenced by larger CL diameter profiles and increased plasma progesterone concentrations in the letrozole-treated heifers. The effect seemed to be more evident in the letrozole im group. Similar findings have been reported in women, where enhanced luteal function was associated with increased circulating LH concentrations but no difference in FSH concentrations [225]. Our present results are consistent with those of previous studies in cattle in which luteotrophic effects were observed after treatment with a single or a 3-day regimen of letrozole; circulating LH was increased and FSH remained unchanged [39, 40].

Plasma estradiol concentrations were significantly reduced by letrozole treatment and the magnitude and duration of estradiol reduction was significantly greater in heifers treated intramuscularly than in those treated intravenously. The vehicle formulated for intramuscular administration of letrozole appeared to result in a steady and prolonged released of letrozole from the injection site. Plasma estradiol concentrations in heifers treated intramuscularly took 24 h longer than in those treated intravenously to reach minimum and remained low until the end of the observational period (6 days after treatment). In the letrozole iv group, estradiol levels reached the minimum concentration between 12 and 24 h after treatment but returned to placebo-treated control levels within 2 days. Given that the half-life of letrozole in cattle is about 32 h, the differences observed between routes of administration may be attributable to a function of the absorption pattern after intramuscular administration.

The pharmacokinetic parameters presented in the present study are in accordance with those reported previously [40]. As anticipated, the intramuscular vehicle acted as a depot, releasing letrozole from the injection site at a slow and steady rate. Although the effects of the im letrozole treatment on ovarian function were more consistent and reliable that those obtained with the iv route, the fact that withdrawal of the circulating concentration of letrozole depends entirely on absorption and clearance, making it is difficult to manipulate the length of the treatment, remains to be a strong limitation for this route and vehicle of administration. It is important to note the limited information provided by the AUC_{last} and the bioavailability data for the letrozole im group since the observational period was not prolonged enough as to determine volume of distribution and systemic clearance for letrozole im formulation.

Gonadotropin concentration data were centralized to day of post-treatment wave emergence to permit direct comparison among groups since letrozole treatment was associated with delayed wave emergence. Plasma FSH concentrations tended to be higher around the time of wave emergence in the letrozole-treated group. A surge on LH concentration was observed on the day of wave emergence on the letrozole im group while no such increase was detected in the letrozole iv and combined placebo-treated control groups. It is unclear as to what led to the LH surge although we speculate that it may be related to the suppressed circulating concentrations of estradiol in letrozole-treated heifers.

In summary, our results demonstrate that route of administration, or more precisely, the nature of the vehicle used for the administration of letrozole (depot vs intravenous) has an impact on the effects of letrozole on ovarian dynamics and hormonal profiles. The intramuscular route provided a prolonged release of letrozole from the injection site which had a marked effect on

estradiol production, dominant follicle lifespan, and CL form and function. As observed in previous studies [39, 40], the effects observed were associated with an increase in circulating LH concentrations. We concluded that letrozole treatment given intramuscularly on Day 3 post wave emergence lengthens the lifespan of the dominant follicle, delays post-treatment wave emergence, and has a luteotrophic effect as evidenced by a larger CL and higher circulating progesterone concentrations. However, the same dose of letrozole given intravenously was less effective in altering ovarian function. Our results further support the working hypothesis that letrozole has potential as a steroid-free option for the control of ovarian function for the purposes of fixed-time artificial insemination and embryo production.

5.6. Acknowledgements:

We thank Brad Blackmore and the staff at the Goodale Research Farm for assistance with handling and managing the cattle. We also thank Dr Jonathan Naile and Dr John Giesy for assistance in letrozole measurement, and Dr Alan Chicoine for assistance in pharmacokinetic parameter calculations. The authors are thankful to the Natural Sciences and Engineering Research Council of Canada and Bioniche Animal Health Inc. for financial support.

CHAPTER 6: NON-STEROIDAL AROMATASE INHIBITOR TREATMENT WITH AN INTRAVAGINAL DEVICE AND ITS EFFECT ON PRE-OVULATORY OVARIAN FOLLICLES IN A BOVINE MODEL

Relationship of this study to the thesis:

In chapter 5, we demonstrated that intramuscular administration of aromatase inhibitors is a feasible route for the control of ovarian function in cattle and allows for extended letrozole release from the injection site when a depot (oil based) vehicle was employed. However, one of the main limitations of the intramuscular route is the difficult control of the length of treatment, which is crucial to minimize variability and to control timing during an ovulation synchronization protocol. An alternative technology that allows not only for prolonged treatments but also for controlled treatment termination or withdrawal is the use of intravaginal devices. Additionally, past experiments have only assessed the effect of aromatase inhibitor on ovarian activities when given during non-ovulatory follicular waves. In this chapter, we described the effects of a prototype letrozole-containing intravaginal device formulated for cattle on pre-ovulatory ovarian follicles.

6.1. Abstract

Letrozole, a non-steroidal aromatase inhibitor, prevents the body from producing its own estrogen. The objectives of the present study were to test the hypotheses that letrozole treatment, initiated prior to selection of the preovulatory dominant follicle, will induce the growth of more than one follicle to a pre-ovulatory size, and will delay ovulation. Post-pubertal beef heifers were given two luteolytic doses of PGF (12 h apart) and monitored by ultrasonography for ovulation. Five to eight days later, ovarian follicular wave emergence was synchronized by ultrasound-guided transvaginal follicular ablation (Day 0=wave emergence) and a luteolytic dose of PGF was given 60 and 72 h later. On Day 1, heifers were divided randomly into two groups (n=15/group) and an intravaginal device containing 1 g of letrozole or a blank device (control) was inserted. The intravaginal devices were removed on Day 7, or at the time of ovulation, whichever occurred first. Transrectal ultrasonography and blood sample collection was performed daily from the day of ablation to 12 days after subsequent ovulation. The mean ($\pm$SEM) interval from device placement to ovulation was longer in letrozole-treated animals compared to controls (6.1±0.25 vs 5.1±0.26 days, respectively; $P<0.01$). Single dominant follicles were present in both groups. The day-to-day diameter profiles of the dominant follicles of the ovulatory wave were larger ($P<0.05$) and the maximum diameters greater in letrozole-treated heifers (14.6±0.51 vs 12.4±0.53 mm, respectively; $P<0.01$). The diameter profile of the corpus luteum (CL) that formed after treatment did not differ between groups; however, plasma progesterone concentrations were higher ($P<0.01$) in heifers treated with letrozole. Estradiol concentrations were reduced following letrozole treatment ($P<0.05$), although a preovulatory rise of estradiol occurred in both groups. Administration of letrozole with an intravaginal device

during growth of the ovulatory follicle delayed ovulation by 24 h and resulted in the formation of a CL that secreted higher levels of progesterone. A sustained-release intravaginal device may be useful for the development of an aromatase inhibitor-based protocol to control ovulation for herd synchronization and to enhance fertility by increasing circulating progesterone concentrations during the first 7 days post-ovulation in cattle.

6.2. Background

Estrogen-based protocols, as a treatment for synchronizing ovulation in cattle, have modernized breeding practices and allowed producers to reliably control the timing of ovulation, thus enabling efficient use of time, labour and resources by allowing pre-scheduled insemination. Estradiol-based protocols also allow wider application of superovulation and embryo transfer by enabling effective synchronization of follicular wave emergence [4-8]. However, increasing consumer sensitivity to the possible deleterious effects of estrogens in food and in the environment [20] has led to new regulations about the use of estrogenic products in livestock. The European Union has already banned the use of estrogenic products in food producing animals [21-24]. In the United States [25] and Canada [26], estrogens cannot be used for synchronization of estrus except by prescription and custom-compounding. In 2007, New Zealand and Australia banned use of estrogens in lactating dairy animals [24]. These policies have created a void in methods to control reproductive function for breeding management in cattle.

Non-steroidal aromatase inhibitors prevent the body from producing its own estrogen. Letrozole, a non-steroidal aromatase inhibitor, is used as an adjuvant treatment for hormone-responsive breast cancer in post-menopausal women [33] and has been used as a fertility

treatment for women undergoing assisted reproduction [34]. The putative effect of letrozole on ovarian function in women is through elevated FSH secretion by removal of the negative feedback of estradiol [34]. Tests of this hypothesis in a bovine model, however, were not supportive of an effect on FSH. In cattle, a single intravenous dose given on Day 3 post-ovulation, or a 3-day regimen given on Days 1-3, 3-5 or 5-7 post-ovulation did not induce an elevation in circulating FSH concentration but did increase mean plasma LH concentrations. The effect on LH secretion resulted in a prolonged period of dominance of the extant dominant follicle and delayed emergence of the next follicular wave [39, 40]. Further, a luteotrophic effect was inferred from the observation that heifers treated with letrozole for 3 days had larger corpora lutea following treatment. Similar results were observed when letrozole was prepared in an oil-based vehicle and administered intramuscularly (Chapter 5).

To date, studies on the effects of letrozole on ovarian function in cattle have been focused on non-ovulatory follicular waves [23-25]. The present study was designed to determine the effect of an extended period of treatment with letrozole on the pre-ovulatory follicle in cattle. We hypothesized that letrozole treatment initiated before selection of the dominant ovulatory follicle and extended over the follicle growing phase will induce the development of more than one follicle to a preovulatory size, and delay ovulation. Additionally, we hypothesized that the CL resulting from ovulations after letrozole treatment will be larger and secrete more progesterone than those from control heifers.

6.3. Methods

6.3.1. Cattle

Hereford-cross beef heifers (n=30), 15 to 20 months of age and weighing between 235 and 405 kg (average 336 kg), were chosen from a herd of 51 heifers maintained in outdoor pens at the University of Saskatchewan Goodale Research Farm (52° North and 106° West). Heifers were fed alfalfa/grass hay and concentrate to gain approximately 1.3 Kg per day and had water ad libitum during the experimental period from October to December. Heifers were initially examined by transrectal ultrasonography (MyLab5 VET, Canadian Veterinary Imaging, Georgetown, Ontario Canada) to detect the presence of a CL (i.e., confirm post-pubertal status; [217]. Animal procedures were performed in accordance with the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

6.3.2. Treatments and examinations

Heifers in which a CL was detected were given two luteolytic doses of PGF (12 h apart) and monitored by ultrasonography for ovulation. Five to eight days later, the two largest ovarian follicles were ablated by transvaginal ultrasound-guided follicular aspiration to synchronize follicular wave emergence which was expected to occur 1 to 1.5 days later [218, 219]. Prostaglandin (500 μg cloprostenol, Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) was given intramuscularly at 60 and 72 h after follicular ablation to induce regression of the CL and shift from a non-ovulatory to an ovulatory follicular wave [87]. At the time of follicular wave emergence (Day 0; i.e., 1.5 days after follicle ablation), heifers were assigned randomly to two groups and given an intravaginal device containing 1 g of letrozole

(letrozole group, n=15) or a placebo (letrozole-free) intravaginal device (control group, n=15). Devices were inserted on Day 1 and were kept in place until Day 7 or until ovulation was detected, which ever occurred first.

Intravaginal devices were prepared using a Cue-Mate spine (Bioniche Animal Health, Bellville, ON, Canada) assembled with two blank (progesterone-free) silicone pods that were coated with a gel-based vehicle containing letrozole or vehicle only (control). The vehicle contained the following (all ingredients % w/w): letrozole 10%, gelatin 20% (Gelatin type B, Fisher Scientific, Pittsburgh, PA, USA), polymer 65% (prepared by mixing distilled water 68%, Poloxamer 188 12% and Poloxamer 407 20%, both from Spectrum Chemical, New Brunswick, NJ, USA) and distilled water 5%.

6.3.3. Ovarian ultrasonography

The observations from ultrasound examinations were recorded on a sketch sheet in which each ovary and its structures (CL and follicles ≥ 4 mm in diameter) were represented by size and location [73]. Ovulation was defined as the disappearance of any follicle ≥8 mm between two consecutive daily examinations and was confirmed by the subsequent development of a CL [217]. Follicular wave emergence was defined as occurring 1.5 days after follicular ablation [218]. The dominant follicle of a wave was defined as the largest antral follicle of that wave [45].

6.3.4. Collection of blood samples

Blood samples were collected by coccygeal venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Samples were collected daily from pre-treatment follicular wave emergence (Day 0) to 12 days after the subsequent ovulation.

In a subset of letrozole-treated animals (n=4), frequent blood samples were collected using an in-dwelling jugular catheter, as previously described [84], at the time of catheter placement and 0, 10, 20, 30 min, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after treatment for measurement of plasma letrozole concentration. Blood samples were centrifuged at 1500 x g for 20 min and plasma was separated and stored in plastic tubes at -20°C.

6.3.5. Hormone assays

Plasma LH concentrations were determined in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) [11, 222]. The minimum and maximum values along the standard curve were 0.06 and 8 ng/mL, respectively. All samples were analyzed in a single assay; the intra-assay coefficient of variation was 11.4% for low reference samples (mean, 0.9 ng/mL) and 12.2% for high reference samples (mean, 2.1 ng/mL).

Plasma FSH concentrations were determined in duplicate using a double-antibody radioimmunoassay using NIDDK-anti-oFSH-1 primary antibody and expressed as USDA bovine FSH-Il units [11, 222]. The minimum and maximum values along the standard curve were 0.12 and 16 ng/mL, respectively. All samples were analyzed in a single assay; the intra-assay coefficients of variation were 7.9 and 6.5%, for low (mean, 2.4 ng/mL) and high reference samples (mean 4.9 ng/mL), respectively.

Plasma concentrations of estradiol were determined using a commercial radioimmunoassay kit (Double Antibody Estradiol; Diagnostic Products Corp., Los Angeles, CA, USA). The procedure was carried out at the Department of Animal Health and Biomedical Sciences, University of Wisconsin–Madison, as previously described [79, 223], with the following modifications: Standards (0.78–100 pg/mL) were prepared in steroid-free (charcoal-treated)

bovine plasma. The standards (250 μL in duplicate) and plasma samples (500 μL in duplicate) were extracted with 3 mL of diethyl ether, frozen in a dry-ice/methanol bath, decanted into assay tubes, and dried overnight under a fume hood. The dried samples and standards were re-suspended with 100 μL of assay buffer (0.1% gelatin in PBS). The intra- and inter-assay coefficients of variation were 10.5 and 10.6% for high reference samples (mean 11.1 pg/mL), and 14.8 and 12.3% for low reference samples (mean 2.6 pg/mL), respectively. The sensitivity of the assay was 0.1 pg/mL.

Plasma progesterone concentrations were determined in duplicate using a commercial solid-phase radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/mL. All samples were analyzed in a single assay; the intra-assay coefficients of variation were 9.7% and 5.8 % for low- (mean, 1.7 ng/mL) and high-reference samples (mean, 18.7 ng/mL), respectively.

6.3.6. Letrozole concentrations

Plasma letrozole concentrations were quantified using liquid chromatography tandem mass spectrometry (LCMS/MS), as described [39]. Briefly, letrozole was extracted from 250 mL plasma with 250 mL of 0.1M ammonium acetate followed by the addition of 5 mL methyl t-butyl ether (MTBE) and vortexed for 15 s. The organic layer was removed and transferred to a fresh 15-mL plastic tube and dried by gentle nitrogen gas flow. The dried extract was reconstituted in 1 mL of 100% ethanol. Separation was accomplished by HPLC (Agilent 1200; Agilent, Santa Clara, CA, USA) fitted with an analytical column (50 x 2.1 mm, 3 mm particle size; Betasil C18; Thermo Scientific, Waltham, MA, USA) operated at 35ºC. Mass spectra were collected using a tandem mass spectrometer (SCIEX 3000; Applied Bioscience, Foster City, CA,

USA) fitted with an electrospray ionisation source, operated in the negative ionisation mode. Quantification was performed using Analyst 1.4.1 software provided by SCIEX (Applied Bioscience). The minimum and maximum values along the standard curve were 0.25 and 500 ng/mL, respectively. The limit of quantification used in this method was 0.25 ng/mL and the mean recovery was 70%.

The following pharmacokinetic parameters were determined: C_{max} (maximum observed plasma concentration of letrozole), t_{max} (time to reach C_{max}), $t_{1/2}$ (terminal elimination half-life), *AUC* (area under the plasma letrozole concentration-time curve from zero to infinity calculated as AUC_{last}). The concentration of letrozole in plasma as a function of time (C–t) data for each heifer was analyzed by non-compartmental techniques using a computer modeling program (WinNonLin Standard Edition Version 2.1, Pharsight Corporation, Mountain View, CA, USA). Peak letrozole concentration in plasma (C_{max}) and time to peak letrozole concentration (t_{max}) were determined using observed values. The apparent terminal rate constant (λ) was determined by linear regression of the last 6–8 points on the terminal phase of the logarithmic plasma concentration vs time curve. The area under the C_{-t} curve until the final plasma sample (AUC_{last}) was determined using the linear trapezoidal rule. The total area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by adding the $C_{last\ obs}/\lambda + AUC_{last}$. The terminal half-life ($T_{1/2\lambda}$) was calculated as $\ln_2 /\lambda$. The mean residence time (MRT) was calculated as the area under the moment curve extrapolated to infinity ($AUMC_{0-\infty}$) /$AUC_{0-\infty}$. Systemic clearance (Cl_S) was determined using the dose divided by $AUC_{0\text{-inf}}$. The apparent volume of distribution (V_λ/f) was calculated by clearance divided by λ. Absolute bioavailability was calculated by comparing letrozole AUC_{last} obtained using intravaginal devices to the AUC_{last} obtained after a single iv

injection of letrozole (Chapter 5), corrected by dose (Bioavailability = ($AUC_{last\ intravag}$/$Dose_{intravag}$)/($AUC_{last\ iv}$/$Dose_{iv}$) x 100).

6.3.7. Statistical analyses

Statistical analyses were done using the Statistical Analysis System software package (SAS Learning Edition 9.1, 2006; SAS Institute Inc., Cary, NC, USA). Time-series data (hormone concentrations, follicle and CL diameter profiles) were analyzed by repeated measures using the PROC MIXED procedure. The main effects were treatment (letrozole and control), time, and their interactions. Single-point measurements (dominant follicle diameter at device placement, maximum diameter of extant dominant follicle, intervals from ablation to wave emergence, and from device placement to ovulation) were analyzed by t-tests. Individual time point comparisons between treatment groups were performed using least significant difference (LSD) test. Significance was defined as $P \leq 0.05$.

6.4. Results

The diameter of the dominant follicle at the time of intravaginal device placement on Day 1 (Day 0 = wave emergence) did not differ between groups (Table 6.1). The day-to-day diameter profile of the dominant follicle during treatment and the maximum diameter of the ovulatory follicle were larger in the letrozole-treated group (P=0.05 and P=0.01; respectively; Figure 6. 1 and Table 6.1; respectively). The interval from device placement to ovulation was longer in heifers treated with letrozole than in controls (P=0.01, Table 6.1). Single ovulation occurred in all heifers, regardless of treatment.

Table 6. 1. Effects of a letrozole-containing intravaginal device on ovarian function in heifers (mean±SEM).

End point	Control (n=15)	Letrozole (n=15)	P-value
Device placement to ovulation (days)	5.1±0.26	6.1±0.25	<0.01
Max. diameter of extant dominant follicle (mm)	12.4±0.53	14.6±0.51	<0.01
Dominant follicle diameter at device placement (mm)	3.9±0.47	4.2±0.46	0.68

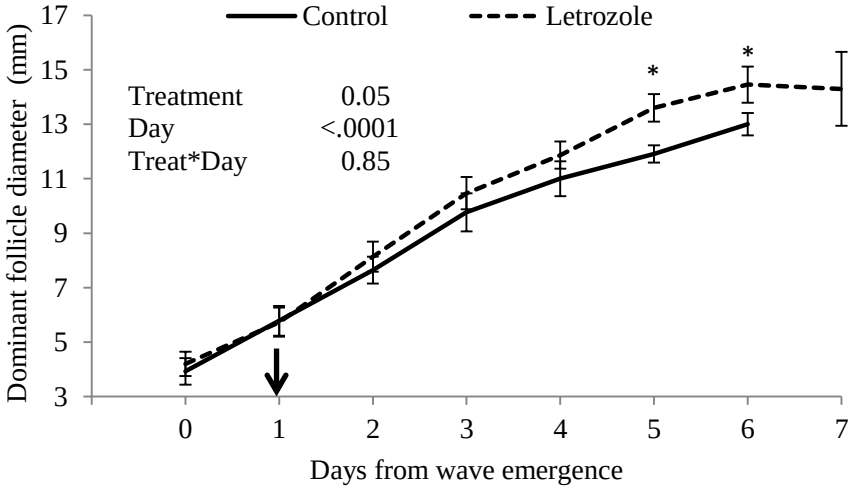


Figure 6. 1. Dominant follicle diameter (mean±SEM) in heifers treated with a blank (control, n=15) or a letrozole-containing intravaginal device (letrozole, n=15). Devices were inserted on Day 1, indicated by the arrow (Day 0 = wave emergence). * On indicated days, values differed between groups (P≤0.05).

Corpus luteum diameter profiles were not different between letrozole and control groups (P=0.82, Figure 6. 2). However, progesterone concentrations were higher during the observational period (first 12 days post-ovulation) in the letrozole-treated heifers compared to control heifers (P=0.01, Figure 6. 3).

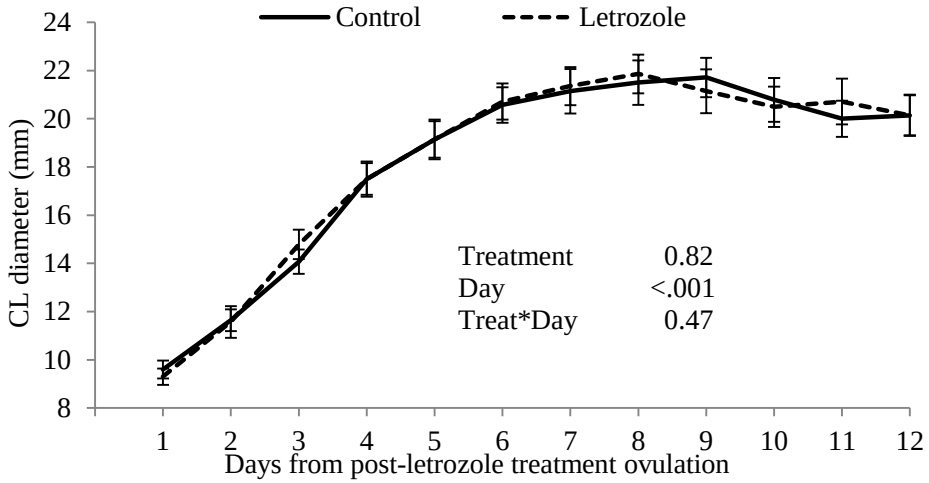


Figure 6. 2. Corpus luteum diameter (mean±SEM) following post-treatment ovulation in heifers treated with a blank (control, n=15) or a letrozole-containing intravaginal device (letrozole, n=15).

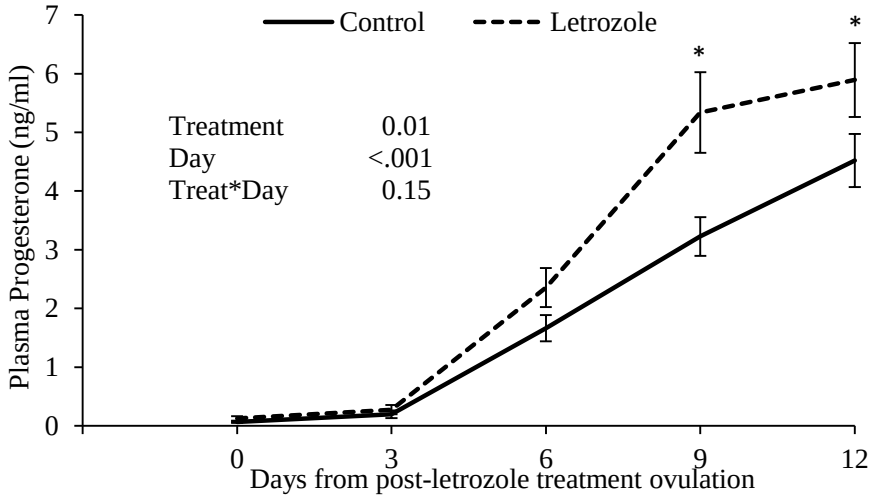


Figure 6. 3. Plasma progesterone concentrations (mean±SEM) following post-treatment ovulation in heifers treated with a blank (control, n=15) or a letrozole-containing intravaginal device (letrozole, n=15). * On indicated days, values differed between groups (P≤0.05).

Plasma estradiol concentrations were lower in the letrozole-treated group than in the control group (P=0.04, Figure 6. 4). Treatment with letrozole did not prevent a pre-ovulatory rise in estradiol but the rise was delayed in the letrozole group (Figure 6. 4).

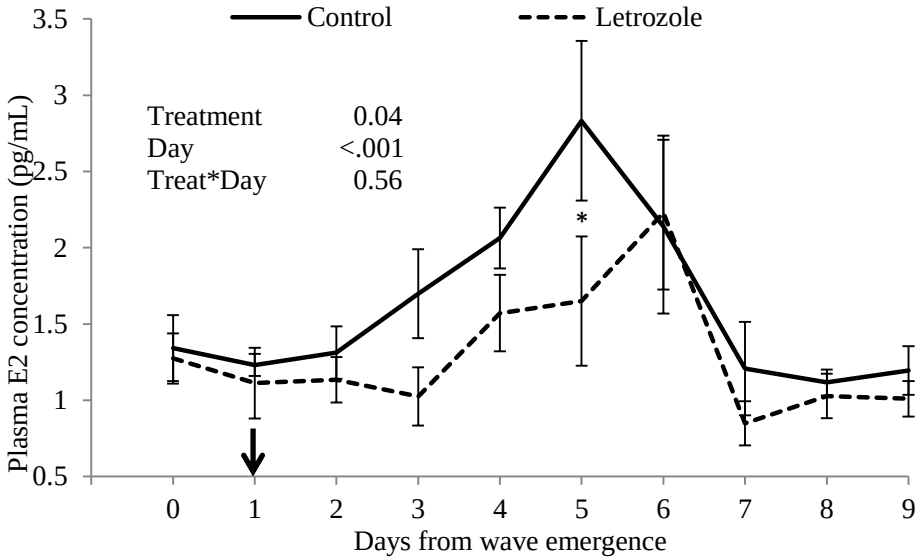


Figure 6. 4. Plasma estradiol concentrations (mean ±SEM) in heifers treated with a blank (control, n=15) or a letrozole-containing intravaginal device (letrozole, n=15). Devices were given on Day 1, indicated by the arrow (Day 0 = wave emergence) of the ovulatory wave. * On indicated days, values differed between groups (P≤0.05).

There was a tendency for lower plasma FSH concentrations in the letrozole group compared to the control group (P = 0.1; Figure 6. 5). Mean plasma LH concentrations did not differ between groups (P = 0.61; Figure 6. 6).

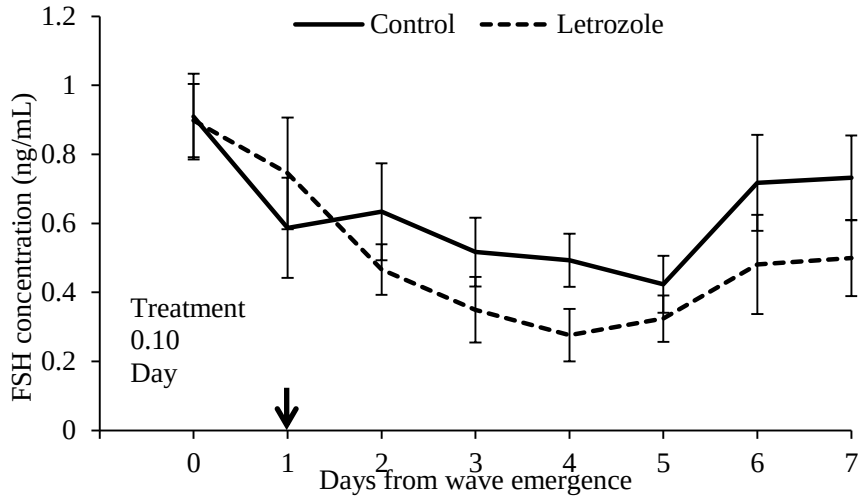


Figure 6. 5. Plasma FSH concentrations (mean ±SEM) in heifers treated with a blank (control, n=15) or a letrozole-containing intravaginal device (letrozole, n=15). Devices were given on Day 1 (Day 0 = wave emergence) of the ovulatory wave.

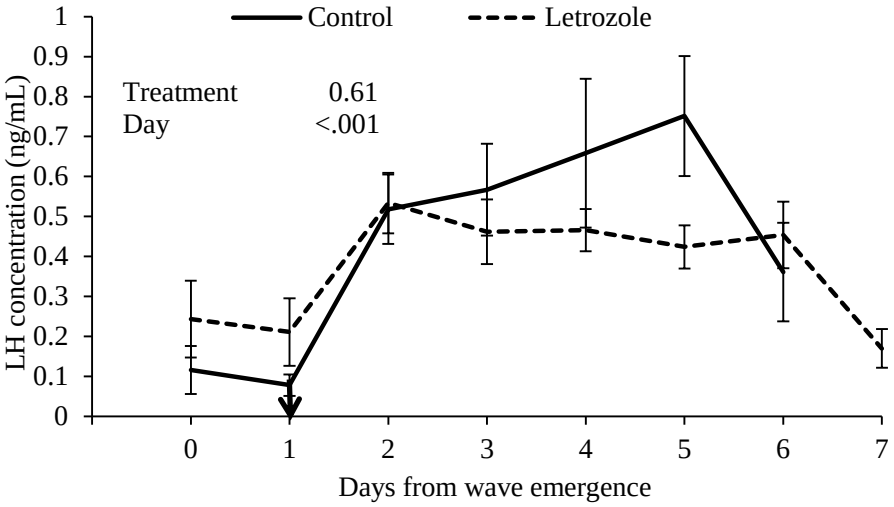


Figure 6. 6. Plasma LH concentrations (mean ±SEM) in heifers treated with a blank (control, n=15) or a letrozole-containing intravaginal device (letrozole, n=15). Devices were given on Day 1, indicated by the arrow (Day 0 = wave emergence) of the ovulatory wave.

Plasma letrozole concentrations are shown in Figure 6. 7. The half-life of letrozole in plasma was 33.3±4.56 h. Maximal concentrations in plasma (C_{max} 31.7±1.65 ng/mL) occurred at 24 h post-device insertion (Table 6. 2). Additional letrozole pharmacokinetic parameters are summarized in Table 6. 2.

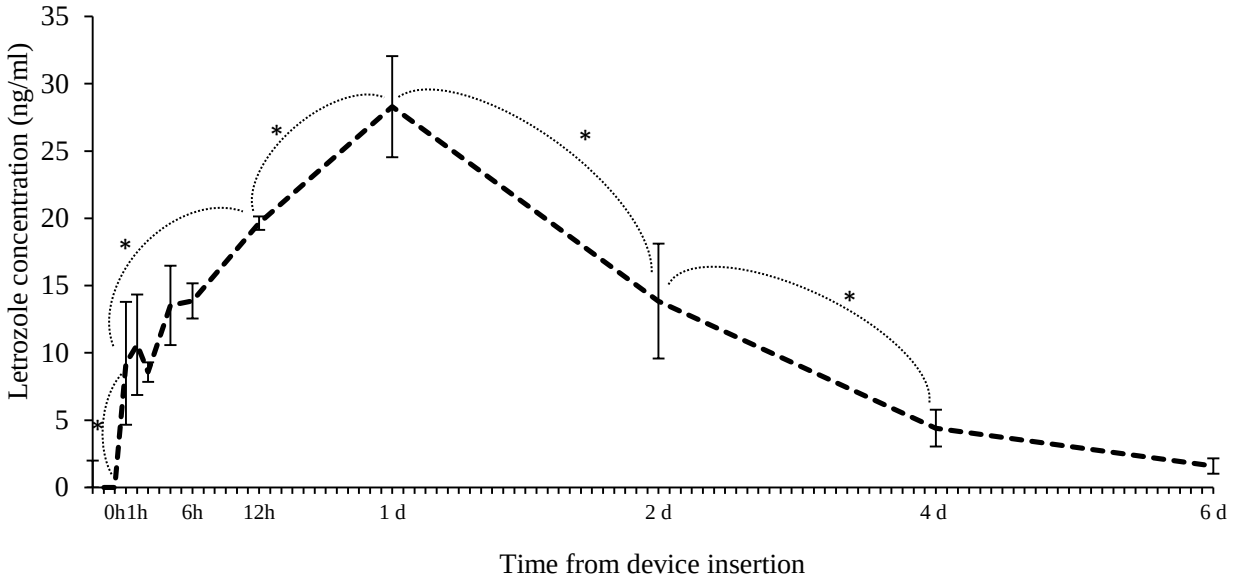


Figure 6. 7. Plasma letrozole concentration (mean±SEM) in heifers (n=4) given an intravaginal letrozole-releasing device for 6 days. * Between indicated time points, values differed (P≤0.05).

Table 6. 2. Pharmacokinetics of a letrozole-containing intravaginal device in heifers.

Parameter	Heifer 1	Heifer 2	Heifer 3	Heifer 4	Mean	SEM
Maximal concentration (C_{max}) (ng/mL)	32.4	35.3	27.3	31.7	31.7	1.65
Half-life ($T_{1/2}$) (hours)	35.1	20.4	41.9	35.7	33.3	4.56
Area under the curve (AUC_{last}) (hours x ng/mL)	3538.1	2697.7	2583.1	1698.0	2629.2	376.39
Volume of distribution (V_z/f) (L/kg)	6.2	4.9	9.9	13.2	8.5	1.87
Systemic clearance (Cl_S) (L/hour/kg)	0.1	0.2	0.2	0.3	0.2	0.03
Mean residence time (MRT) (hours)	71.5	56.2	65.5	48.7	60.5	5.03
Bioavailability					16%	

6.5. Discussion

Previous studies of the effects of letrozole on ovarian function in cattle were focused on the non-ovulatory portion of the estrous cycle in cattle [39, 40]; hence, the effect of letrozole treatment on pre-ovulatory follicles was the subject of the present study. Letrozole treatment during the pre-ovulatory follicular wave resulted in a greater diameter of the ovulatory follicle. This observation is consistent with the results of previous studies in which larger dominant follicles were observed when letrozole treatment was administered during non-ovulatory follicular waves [39, 40]. The hypothesis that letrozole treatment initiated prior to the onset dominant follicle selection would result in multiple ovulatory follicle development [40] was not supported by the results of the present study; single ovulations were detected in both groups. However, ovulation was delayed by 24 h in the letrozole-treated group. The larger ovulatory follicle diameter observed in the letrozole-treated heifers may have been affected by the length of the growing phase of these follicles due to delayed ovulation. However, ovulatory dominant follicle diameters in the letrozole-treated group were already larger than the control group when compared 5 days after initiation of treatment (Day 6 post-wave emergence). The stimulus driving the accentuated follicular growth is unclear. However, daily sampling has limitations when attempting to interpret gonadotropin concentrations and its correlation to ovarian dynamics. Perhaps changes in gonadotropin pulse-frequency would have been detected using more frequent sampling.

Estradiol concentrations were reduced following treatment with letrozole-impregnated intravaginal devices, and the preovulatory rise in estradiol concentrations occurred 24 h later than in the control group. However, the follicles maintained ovulatory capability. We infer that the delay in estradiol rise observed in the letrozole-treated group is responsible for the delay in

ovulation in this group. The notion is further supported by plasma LH concentrations which, although not significantly different between groups, appeared to increase 24 h later in the letrozole-treated group than in the control group.

In the present study, a letrozole-impregnated intravaginal device was used to provide extended estradiol suppression during the ovulatory wave. The intravaginal route of administration provides the advantage of reducing animal handling and distress caused by daily injections [226]. The duration of estradiol inhibition was influenced by the pharmacokinetic characteristics of the intravaginal device, and accounts for the occurrence and timing of the estradiol rise observed in the letrozole-treated group. The half-life of letrozole observed following administration via an intravaginal device (33 h) corresponded to that reported previously after single intravenous administration in beef heifers (Chapter 5). Hence, the profile of letrozole concentration over time obtained in the present study was affected primarily by the absorption characteristics of the formulation used in the intravaginal devices. Based on the plasma letrozole concentration profile, the intravaginal devices released letrozole for only 24 h post-insertion, and elimination and plasma clearance took place thereafter. Therefore, letrozole concentrations may have dropped below a critical level relatively rapidly, allowing for the pre-ovulatory estradiol rise to occur after only a 24 h delay. Bioavailability has been defined as the amount of a drug given by any route, other than intravenously, that reaches general circulation and is available at the site of action [227]. The low bioavailability observed with the intravaginal devices (16%) may be explained in part by the melting point of the gel-vehicle used. This gel-based vehicle is commonly used for intravaginal suppositories for women, in which body temperature is lower than that of cattle (37° vs 39° C) [228, 229]. Rapid liquefaction of the

letrozole-containing gel resulted in loss of the preparation through the vulvar opening during micturition, defecation, or ultrasound examinations (the latter was observed by the author).

Letrozole treatment during the growing phase of the ovulatory follicle resulted in the ovulation of a larger follicle. Although larger follicles did not result in larger CL, elevated plasma progesterone profiles were observed over the first 12 days post-ovulation in the letrozole-treated group. Preovulatory letrozole treatment may have affected the number or proportion of large luteal cells (granulosa cell origin) and small luteal cell (thecal cell origin) contained within the CL [230], resulting in an increase in progesterone production per CL volume. Small and large luteal cells are present in the bovine CL in a ratio of 7.6:1 [231]. Small luteal cells respond directly to LH stimulus to secrete progesterone [232, 233], while large luteal cells appear to be responsible for sustained secretion of progesterone in the absence of a stimulus [233]. Treatment with letrozole may have resulted in an increase in luteal cell numbers or an alteration in the proportion of small and large luteal cells within the CL and an increase in progesterone-producing capability per CL volume. In this regard, treatment of cows with equine chorionic gonadotropin resulted in increased density and number of large luteal cells which increased the capacity of the CL to produce progesterone [234]. Although we were unable to document the effect of letrozole treatment on gonadotropin secretion in the present study, previous studies have shown an increased in gonadotropin secretion after single or 3-day letrozole regimen [39, 40].

In summary, letrozole treatment during the ovulatory follicle wave resulted in more rapidly growing dominant follicles and larger ovulatory follicles, delayed ovulation (by 24 h) of a single follicle and formation of a CL that secreted higher levels of progesterone. The effects of treatment on gonadotropin concentrations are inconclusive, possibly due to inadequate sampling

frequency. However, results confirmed that letrozole treatment effectively reduces estradiol production in cattle. Finally, the formulation used for the development of an intravaginal device containing letrozole impacts on the effect of treatment on ovarian function. Based on these observations, we hypothesize that a letrozole-releasing device capable of a more sustained drug release may delay ovulation even further, while allowing more than one follicle to develop to a pre-ovulatory size when treatment is initiated prior to dominant follicle selection.

We conclude that a sustained-release intravaginal device has potential in the development of an aromatase inhibitor-based protocol for control of ovulation for herd synchronization. The enhanced effects of letrozole treatment on CL function has the potential of enhancing fertility by increasing circulating progesterone concentrations during the first 7 days post-ovulation in cattle.

6.6. Acknowledgements:

We thank Dr. Ildiko Badea for assistance in intravaginal device formulations and Dr. Al Chicoine for his help in interpreting plasma letrozole concentrations. We also thank Brad Blackmore and the staff at the Goodale Research Farm for assistance with handling and managing the cattle. The authors are thankful to the Natural Sciences and Engineering Research Council of Canada and Bioniche Animal Health Inc. for financial support.

CHAPTER 7: FORMULATION AND PHARMACOKINETIC CHARACTERISTICS OF AN INTRAVAGINAL DEVICE FOR AROMATASE INHIBITOR DELIVERY IN CATTLE

Relationship of this study to the thesis:

Chapters 5 and 6 provide evidence that extended aromatase inhibitor treatment predictably affects ovarian function and has potential as a method to synchronize ovulation in cattle. However, the advantages of intravaginal administration over im treatments (reduce animal distress, user friendly, no negative impact in meat quality, controlled length of treatment) led us to pursue this route of administration further. During chapter 6, we identified the short-comings of the intravaginal vehicle tested, namely low melting point and loss of the formulation through the vulvar opening. We concluded that prior to successfully applying aromatase inhibitors to the synchronization of ovulation, formulation adjustments are needed in order to provide biologically relevant levels of aromatase inhibitor during the desired period of time. In chapter 7 we describe the steps taken towards the development of an aromatase inhibitor-releasing intravaginal device to be applied in the development of an aromatase inhibitor-based method for the synchronization of ovulation in cattle.

7.1. Abstract:

The goal of this study was to formulate and test an intravaginal device that provides biologically active circulating concentrations of an aromatase inhibitor for a minimum of 4 days. Three compounds with estradiol inhibitory capability (letrozole, anastrozole and fenbendazole) were tested in vitro using a bovine granulosa cell culture. Letrozole was found to be the most efficient and potent estradiol inhibitor. Liposome-based and a wax-based formulations were used to assess letrozole diffusion through bovine vaginal mucosa in a diffusion chamber study. Samples were collected over a 24 h period. The wax-based vehicle was selected for further development of a letrozole intravaginal device based on its steady release rate. In an *in vivo* study in cattle, three different intravaginal devices containing 3 g of letrozole were tested: Wax (with 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine, DOPE) + gel coat (n=2), Wax + gel coat (n=4) and Wax (n=4). Blood samples were collected serially from 0 to 120 h, and daily thereafter to measure circulating concentrations of letrozole by LC/MS/MS. The addition of a letrozole-containing gel coating improved initial absorption and hastened the increase on plasma concentrations of the active ingredient, while the letrozole-containing wax-based vehicle maintained prolonged delivery from the intravaginal device.

7.2. Introduction

Control of the estrous cycle in animal species of commercial interest, such as cattle, has a great impact on the efficiency of meat and milk production. Numerous treatments and protocols have been successful in achieving the goal of timely control of the occurrence of several reproductive events such as luteolysis, estrus and ovulation [107, 235, 236]. However, the application of many

of these protocols involves the administration of hormonal combinations in food producing animals, many of which are perceived as having a negative impact on consumer health [22, 237, 238]. The situation has led to the legal banning of steroid hormones within particular geographic locations (i.e., European Union, New Zealand, and Australia) or physiological categories of animals (i.e., lactating dairy cattle; [23, 24]).

Non-steroidal aromatase inhibitors are compounds that prevent the body from producing estradiol by inhibiting the activity of the aromatase enzyme that is responsible for the conversion of androgens into estrogens [239, 240]. Aromatase inhibitors are used widely for the treatment of estrogen-responsive breast cancer in postmenopausal women [241, 242]. However, the potential benefits of these drugs for the treatment of other pathological conditions, such as endometriosis or unexplained infertility, has gained attention [194, 197].

Recent studies have revealed the potential of aromatase inhibitors as a tool to control the estrous cycle in cattle [39, 40]. These studies have focused on the use of letrozole, which has been the compound of choice for treatment of sub-fertility or infertility in women [34]. Letrozole treatment in cattle extended the lifespan of the dominant follicle, delayed the emergence of the next ovarian follicular wave, and altered the timing of ovulation. Letrozole treatment also had a consistent luteotrophic effect; i.e., development of a larger corpus luteum that produced more progesterone. This latter effect was unexpected and may be of particular interest for the purposes of enhancing embryo development and reducing embryonic loss. Effective circulating concentrations of letrozole in cattle have been achieved by intravenous, intramuscular or intravaginal administration. Collectively, these results provide impetus for the development of an aromatase inhibitor-based synchronization and fertility treatment in cattle.

The intravaginal route of administration of letrozole is of particular interest because it allows for extended treatment protocols, it is minimally invasive for the animal, it reduces animal handling and treatment-associated stress, and is most likely to be accepted by practitioners and producers [243]. In a previous study (Chapter 6), we tested a prototype of an intravaginal device for providing extended treatment with letrozole. However, it appeared that the formulation was released too rapidly and plasma levels of letrozole declined significantly within 24 h after device insertion. We determined that adjustments in vehicle formulation were needed to provide more extended and uniform release of aromatase inhibitor for controlling ovarian function in cattle.

The objective of the present study was to formulate and test an intravaginal device that provides biologically active circulating concentrations of an aromatase inhibitor for a minimum of 4 days.

7.3. Materials and Methods

The development of an effective formulation for intravaginal administration of an inhibitor of estradiol production in cattle in the present study involved three stages. Firstly, the inhibitory effects of three different compounds on estradiol production were examined using bovine granulosa cells in culture. Secondly, the absorbability of the selected aromatase inhibitor prepared in two different vehicles was tested in diffusion chamber studies of bovine vaginal mucosa. Thirdly, an *in vivo* study was done in cattle to determine the pharmacokinetic characteristics of the respective formulations developed in the diffusion chamber studies.

7.3.1. In vitro testing of different inhibitors of estradiol production

Inhibition of estradiol production by two aromatase inhibitors (letrozole and anastrozole) and a benzimidazole (fenbendazole) was tested *in vitro* using a bovine granulosa cell culture. The commonly used anthelmintic, fenbendazole, was added as the third possible estradiol inhibitor based on reports that benzimidazole drugs have mild anti-estrogenic effects in mammals and may disturb reproductive events [244, 245], and that deworming treatment with another benzimidazole (albendazole) in ewes resulted in decreased estradiol concentrations in follicular fluid [246].

The granulosa cell culture protocol has been described previously [247]. Briefly, bovine ovaries were obtained at a local abattoir and transported to the lab where granulosa cells of antral follicles were collected by rinsing the follicle wall with Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12, Invitrogen Life Technologies, Burlington, ON, Canada). Granulosa cells were maintained in culture for 6 days at 37°C in 5% CO_2, in 700 uL of culture medium [247] which was replaced every 2 days. Cells were maintained in culture from Days 0 to 2, followed by the addition of 1 ng/mL of FSH from Days 2 to 4. On Day 4, depending on the treatment group, the culture medium was supplemented with 1 ng/mL FSH plus the estrogen inhibitor of interest, with FSH alone (positive control) or no FSH (negative control). On Day 6, the culture medium was collected and total protein was measured by the Bradford method (Bio-Rad, Mississauga, ON, Canada). Estradiol levels were determined by radioimmunoassay and expressed as total pg of estradiol per ug of protein.

Three different levels of each compound were tested. The standard concentration used for letrozole was 20 ng/mL, based on previously determined plasma concentrations observed after

placement of an intravaginal device containing 1 gram of letrozole in cattle (Chapter 6). The half maximal inhibitory concentration (IC_{50}) values in MCF-7 cancer cell preparations have been reported to be 0.07 and 0.82 ng/mL for letrozole and anastrozole, respectively [192, 248]. Based on this, we considered letrozole to be approximately 11 times more potent than anastrozole; therefore, the standard dose of anastrozole was set at 200 ng/mL. The standard dose of fenbendazole was based on a reported maximum concentration 160 ng/mL fenbendazole in plasma after a single oral administration of 7.5 mg/kg of body weight in cattle [249]. Low and high doses of each aromatase inhibitor were arbitrarily set at standard concentration x 1/10 and standard concentration x10, respectively (Table 7. 1).

Table 7. 1. Treatment groups tested for estradiol inhibitory capability using an in vitro bovine granulosa cell culture.

Treatment	Low dose (1/10 x standard)	Standard dose	High dose (10 x standard)
Letrozole	2 ng/mL	20 ng/mL	200 ng/mL
Anastrozole	20 ng/mL	200 ng/mL	2000 ng/mL
Fenbendazole	15 ng/mL	150 ng/mL	1500 ng/mL
Positive Control	FSH + no estradiol inhibitor		
Negative Control	No FSH + no estradiol inhibitor		

7.3.2. In vitro diffusion chamber studies

Based on the results of *in vitro* testing of different aromatase inhibitors on granulosa cells, letrozole was chosen for further testing and development. Letrozole was prepared in two different formulations (liposome and wax-based) for testing in *in vitro* diffusion chamber studies. The liposome formulation contained the following ingredients (% w/w): 10% letrozole (Xian

Huayang Biological Science and Technology; Xian, China); 10% hydrogenated soy phosphatidylcholine (Phospholipon 90H; American Lecithin Company, Oxford, CT, USA); 5% cholesterol (Spectrum Chemical and Laboratory Products, New Brunswick, NJ, USA); 2% 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE, Avanti Polar Lipids Inc., Alabaster, AL, USA); 20% propylene glycol and water qs to 100% [250]. The wax-based formulation contained the following ingredients (% w/w): 10% letrozole; 10% Phospholipon 90H; 5% cholesterol; 2% DOPE; and Suppocire D (Gattefosse, Paris, France) q.s. to 100%. Both preparations were heated to 65°C and vortexed to obtain a uniform mixture.

Bovine vaginal mucosa samples were collected from cows within 3 h of slaughter at a local abattoir. *In vitro* absorption studies were performed in flow-through diffusion chambers [251]. Full-thickness bovine vaginal mucosa was set up in the diffusion chambers (9 mm diameter) and maintained at 39.0°C. The perfusion buffer (0.01 M Na-phosphate buffer) was circulated under the mucosa at a flow rate of 112 uL/min, at 39°C. The mucosal samples were treated with 100 uL of liposome formulation (n=3) or wax-based formulation (n=3) for 24 h. The vaginal mucosa in one chamber remained untreated to serve as a negative control. The perfusion fluid was collected from each chamber into a single container, and 500 uL samples were collected from this container at 0, 1, 2, 3, 4, 5, 6, 8, 12, and 24 h after initiation of treatment to determine cumulative concentration. Samples were stored at -20ºC until extraction for LC/MS/MS quantification of letrozole concentration.

7.3.3. Preparation of letrozole-impregnated intravaginal devices

Based on the results from diffusion chamber studies, the wax-based formulation of letrozole was selected for the formulation of the intravaginal devices. Three different devices containing a total

of 3 grams of letrozole were tested in vivo: Wax (DOPE) + gel coat, Wax + gel coat, and Wax. The Wax (DOPE) + gel coat device was formulated with 2 grams of letrozole contained in the wax-based vehicle covered by a gel coat containing 1 gram of letrozole per device. The wax-based vehicle contained the following (all ingredients % w/w): 10% letrozole; 10% Phospholipon 90H; 5% cholesterol NF; 2% DOPE; and Suppocire D q.s. to 100%. The gel coat contained the following (all ingredients % w/w): 10% letrozole, 20% gelatin (Gelatin type B, Fisher Scientific, Pittsburgh, PA, USA), 65% polymer (prepared by hydrating 12% Poloxamer 188 and 20% Poloxamer 407, both from Spectrum Chemical, New Brunswick, NJ, USA, with 68% distilled water), distilled water qs to 100%. The Wax + gel coat device was formulated similarly, except that DOPE was excluded from the formulation. The Wax device was 100% wax-based, and contained the following ingredients (% w/w): 10% letrozole; 10% Phospholipon 90H; 5% cholesterol; and Suppocire D q.s. to 100%.

7.3.4. In vivo testing of letrozole intravaginal devices

The spine of a Cue-Mate (Bioniche Animal Health, Belleville, ON, Canada) with blank (progesterone-free) intravaginal pods was used as a support structure for the letrozole devices. Beef heifers were given intravaginal devices as follows: blank devices (control, n = 4), Wax (DOPE) + gel coat devices (n = 2), Wax + gel coat devices (n = 4), and Wax devices (no gel coat, n = 4). To determine the pharmacokinetics of the respective formulations, blood samples were taken at 0, 10, 20, 30 min, 1, 2, 3, 4, 6, 8, 12 and 24 h, twice daily until Day 4, and daily thereafter until Day 12 after device placement. Frequent sampling was performed using an indwelling jugular catheter as described [84]. Daily blood samples were collected by jugular or

coccygeal venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA).

Plasma samples were analyzed by LCMS/MS for letrozole concentration. Peak concentration in plasma (C_{max}) and time to peak concentration (t_{max}) were determined using observed values. The area under the concentration-time curve until the final plasma sample (AUC_{last}) was determined using the linear trapezoidal rule. Bioavailability was determined by comparing the respective AUCs of letrozole given intravaginally and intravenously (Chapter 5), corrected by dose. Relative bioavailability refers to the availability of one letrozole formulation as compared to another formulation. These measurements determine the effects of formulation differences on drug absorption [252].

7.3.5. Measurement of plasma letrozole concentration

Letrozole concentration was determined using high performance liquid chromatography tandem mass spectrometry (LCMS/MS), as described previously [253]. Partial validation of the method was conducted as recommended when the same matrix but from different species is being analyzed (human plasma versus bovine plasma;[254]). Partial validation parameters included linearity, selectivity, accuracy and precision.

Linearity was tested by running six standard curves independently. Ratio counts versus concentration were plotted and R-square values were calculated. The mean (± SEM) R-square value was 0.998 ± 0.0009. The calibration curve had to have a correlation coefficient (r^2) ≥ 0.99. The acceptance criterion for each calculated standard concentration was 15% deviation from the nominal value except lower limit of quantification (LLOQ) samples, which was set at 20% (Table 7. 2).

Selectivity, defined as the degree to which the response is unaffected by contributions from the matrix, was tested in six independent bovine plasma lots, spiked with one single value within the standard curve (30 ng/mL) and plotted against a non-extracted standard curve made on mobile phase. A pooled plasma sample was also included. The results (30.6 ± 0.19 ng/mL) showed that there was no significant difference for letrozole concentration among plasma sources.

Table 7. 2. Precision and accuracy data of calculated concentrations of calibration samples for letrozole in bovine plasma (n=6).

Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.2	0.75 ± 0.035	10.49	378.5
0.4	0.93 ± 0.039	9.45	241.8
2	2.28 ± 0.033	3.24	114.0
8	7.96 ± 0.045	1.26	99.5
25	22.92 ± 0.114	1.11	91.7
50	48.80 ± 0.167	0.77	97.6
75	77.50 ± 0.324	0.93	103.3
100	99.25 ± 0.258	0.58	99.3

Accuracy and precision were calculated by running four different concentrations of quality control (QC) samples (0.2, 0.5, 30, and 70 ng/mL) six times. Accuracy was calculated as percentage of the true concentration of letrozole recovered by the assay. Precision, expressed as the relative standard deviation was assessed using the following formula: %RSD = (STD DEV x 100) / mean. The acceptance criteria of precision were ≤ 20% LLOQ and ≤ 15% for the remaining concentrations and for accuracy were 100 ± ≤ 20% for LLOQ and 100 ± ≤ 15% or higher for the remaining concentrations (Table 7.3).

Blank bovine plasma standards spiked with 0.2 and 0.4 ng/mL of letrozole did not meet the acceptance criteria for accuracy; therefore the lower limit of quantification was set at 2 ng/mL. Samples below 2 ng/mL were considered as 0 (zero).

Table 7. 3. Precision and accuracy of the LCMS/MS method for determining letrozole concentrations in plasma samples

Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.2	0.8 ± 0.04	12.1	398.2
0.5	1.6 ± 0.05	6.8	325.7
30	28.0 ± 0.16	1.3	93.4
70	72.3 ± 0.24	0.7	103.3

7.3.6. Statistical analyses

Statistical analyses were done using the Statistical Analysis System software package (SAS Learning Edition 9.1, 2006; SAS Institute Inc., Cary, NC, USA). Time-series data (letrozole concentration) were analyzed by repeated measures, using the PROC MIXED procedure. The main effects were formulation, time, and their interactions. Single-point measurements (estradiol concentrations) were analyzed by ANOVA, and differences among more than two means were further analyzed by Tukey's post-hoc test for multiple comparisons. Individual time point comparisons between treatment groups were performed using least significant difference (LSD) test. A probability of $P \leq 0.05$ was used to indicate significance and probabilities between $P > 0.05$ and $P < 0.10$ indicated that a difference approached significance. Data are presented as the mean ± SEM.

7.4. Results

7.4.1. In vitro testing of different inhibitors of estradiol production:

Aromatase activity in bovine granulosa cell culture after estradiol synthesis inhibitor treatment was determined in three replicates. Results are shown in Figure 7. 1. Letrozole and anastrozole were the most effective in reducing estradiol secretion by granulosa cells *in vitro.* Concentration of estradiol following 20 and 200 ng/mL of letrozole, and 200 and 2000 ng/mL of anastrozole did not differ from that of the negative control (no FSH stimulation). Febendazole, at the levels tested did not reduce estradiol secretion significantly by FSH-stimulated granulosa cells.

7.4.2. Diffusion chamber study results:

Letrozole concentrations obtained with each formulation on the vaginal mucosa in diffusion chamber studies are shown in Figure 7. 2. Although not statistically different, the wax-based formulation released letrozole at a consistent rate as indicated by the minimum changes in letrozole concentration over time. On the contrary, the sudden increase followed by a decrease in letrozole concentration observed in the liposome-based vehicle indicates that by the 12 h point most letrozole had been released and absorbed through the vaginal mucosa, after which letrozole concentrations dropped due to dilution.

The absorbability of the gel coating had been tested previously using diffusion chamber trials for a prototype intravaginal device (Chapter 6). The procedure was performed as described herein; except that temperature setting of the chambers was 38.0°C, and samples were collected hourly for 12 h. Results from that study are presented in Figure 7. 3 to illustrate the rapid release of letrozole from the gel formulation.

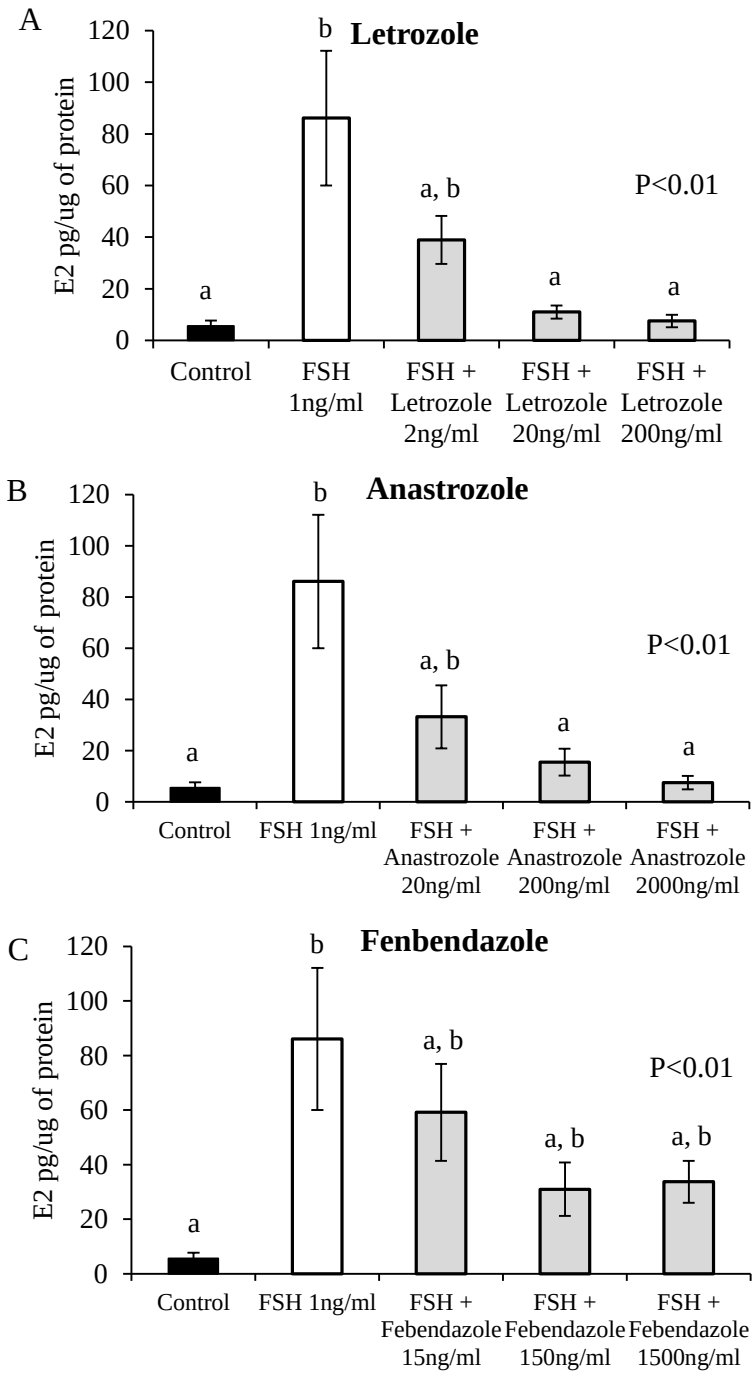


Figure 7. 1. Effect of three different inhibitors of estradiol production on estradiol secretion by bovine granulosa cells in culture. Cells were cultured in vitro for 6 days under non-luteinising

conditions without treatment (negative control), or treatment with FSH alone (positive control) or with letrozole (A), anastrozole (B) or fenbendazole (C) at 1/10 x standard, standard or 10 x standard doses Data are presented as the mean ± SEM estradiol concentrations in three independent replicate cultures for each inhibitor. [ab] Values with no common superscript are different (P<0.05).

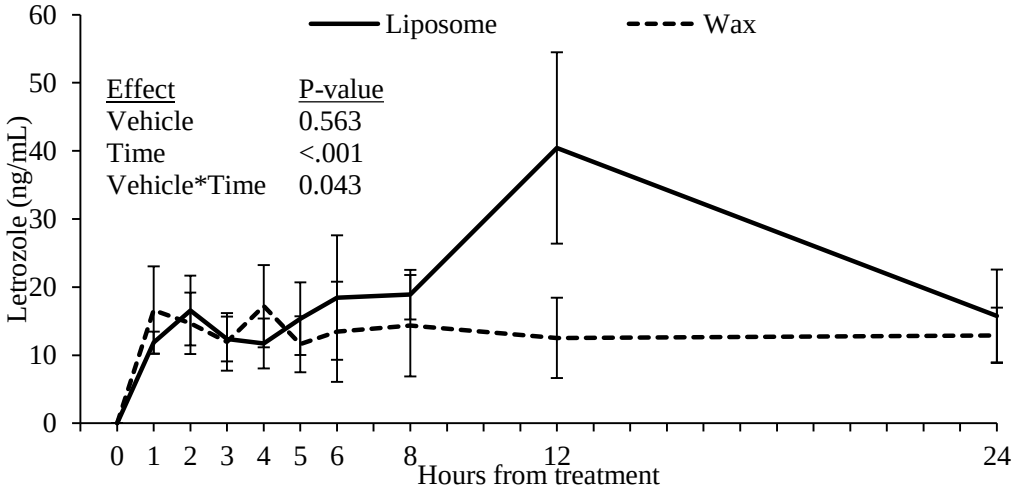


Figure 7. 2. Letrozole concentrations in saline during diffusion chamber trial for 24 hours. Letrozole was prepared in a liposome- or a wax-based vehicle and its diffusion through bovine vaginal mucosa was tested in diffusion chambers using phosphate buffered saline as perfusion buffer. Data from three diffusion chambers per formulation are presented as mean ± SEM.

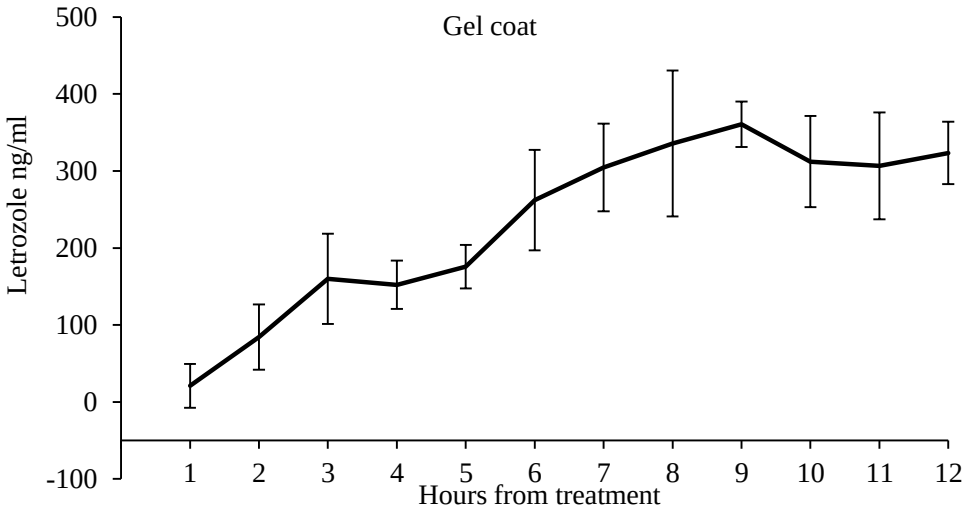


Figure 7. 3. Letrozole concentrations in saline during diffusion chamber trial for 12 hours. Letrozole was prepared in a gel vehicle and its diffusion through bovine vaginal mucosa was tested in diffusion chambers using phosphate buffered saline as perfusion buffer. Data from two diffusion chambers per formulation are presented as mean ± SD.

7.4.3. In vivo testing of letrozole intravaginal devices:

All devices remained in place for 8 days. Although a mild vaginitis was observed at the time of device removal, general health of the heifers was not compromised and was considered to be optimal by an attending veterinarian (JY).

The formulations coated with gel delivered letrozole more rapidly than that without a gel coating. There was no difference among formulations in mean letrozole concentrations over the first 12 h or during the 12 day observational period. However, when multiple comparisons among groups were performed by hour during the first 12 h, devices containing gel coat had significantly higher concentration of letrozole in plasma by 3 h post-device insertion compared to the wax alone group (Figure 7. 4). Letrozole concentration profiles over 12 days following device insertion are showed in Figure 7. 5. Wax alone group reached concentration similar to the

gel coating devices (wax (DOPE) + gel coat and wax + gel coat) by 60 h, although concentration achieved with the wax (DOPE) + gel coat devices were significantly lower than the other two formulations after 4 days from device insertion. Pharmacokinetic parameters for the three formulation tested are summarized on Table 7. 4 and Table 7.5.

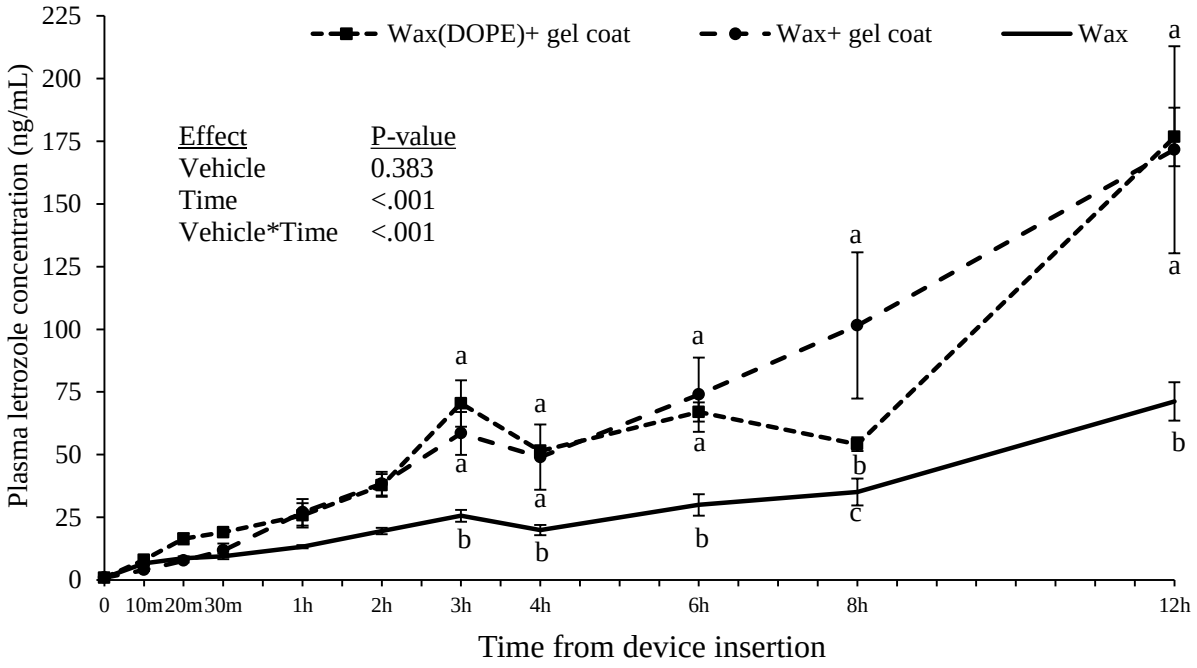


Figure 7. 4. Letrozole concentrations in plasma (mean±SEM) during the first 12 h following treatment with a letrozole-containing intravaginal device in heifers. Letrozole devices were prepared in three formulations: Wax (DOPE) + gel coat (n=2), Wax + gel coat (n=4), Wax only (n=4). [a b c] On indicated days, values differed among groups (P≤0.05).

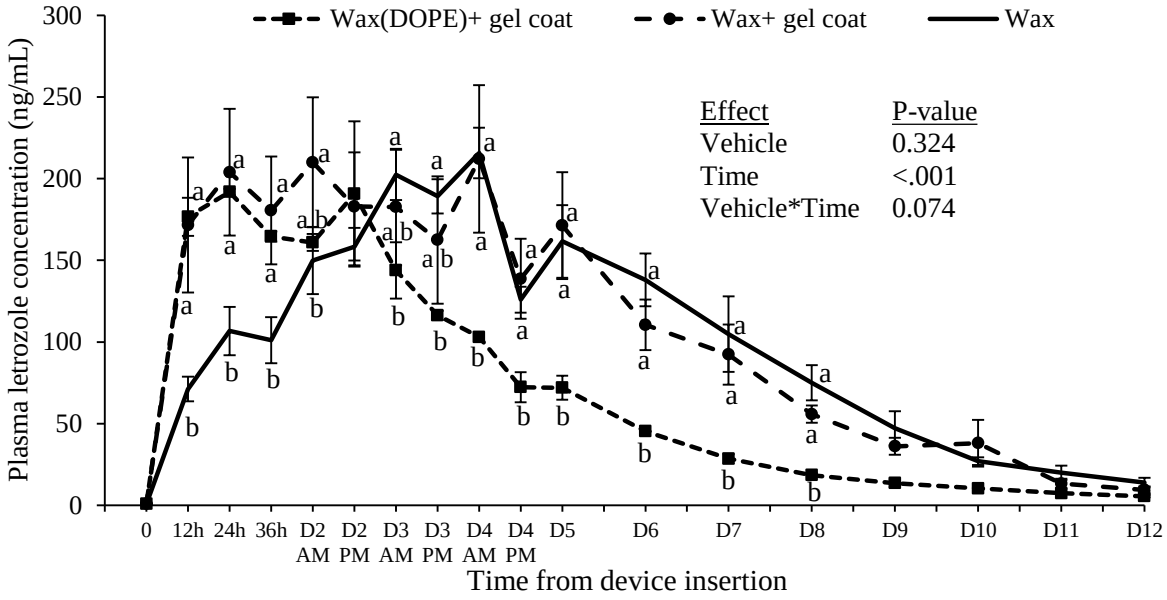


Figure 7. 5. Plasma letrozole concentrations in heifers (mean ± SEM) over 12 days following treatment with a letrozole-containing intravaginal device. Letrozole devices were prepared in three formulations: Wax (DOPE) + gel coat (n=2), Wax + gel coat (n=4), Wax only (n=4). [ab] On indicated days, values differed among groups ($P \leq 0.05$).

Table 7. 4. C_{max} and t_{max} (mean±SD) in cattle after treatment with different letrozole-containing intravaginal devices: Wax (DOPE) + gel coat (n=2), Wax + gel coat (n=4) and Wax (n=4).

Parameter	Wax (DOPE) + gel coat	Wax + gel coat	Wax
C_{max} (ng/mL)	214 ± 29.3	240 ± 91.4	225 ± 25.7
t_{max} (h)	42 ± 25.5	72 ± 27.7	90 ± 12.0

Table 7. 5. Blood plasma letrozole content (AUC_{last}) in cattle after treatment with different letrozole-containing intravaginal devices: Wax (DOPE) + gel coat (n=2), Wax + gel coat (n=4) and Wax (n=4).

	Area under the curve (AUC_{last}) (hours x ng/mL)		
	Wax (DOPE) + gel coat	Wax + gel coat	Wax
	18424	36485	24783
	19453	20151	20693
	-	35397	31519
	-	18815	24816
Mean	18938	27712.0	25452.9
SD	727.5	9528.2	4483.5
Bioavailability	43%	63%	58%
Relative Bioavailability	68%	100%	92%

7.5. Discussion:

We tested the efficacy of three compounds (letrozole, anastrozole and fenbendazole) for inhibiting estradiol production by bovine granulosa cell cultures *in vitro*. Results showed that letrozole and anastrozole were efficacious in reducing estradiol production to levels found in non-FSH-stimulated granulosa cells. Fenbendazole, however, did not reduce estradiol concentrations significantly. This is consistent with reports that letrozole and anastrozole are very specific inhibitors of the aromatase enzyme [181, 255], while the mechanism by which fenbendazole impairs estrogen synthesis in mammals remains unclear. However, it has been reported that albendazole, a benzimidazole anthelmintic drug closely related to fenbendazole, can inhibit the activity of cytochrome P450 enzymes (CYP enzymes) *in vitro* in rat and mouflon sheep [256, 257], a family of enzymes of which aromatase is a member [258]. It is possible that fenbendazole may have had a direct, although mild, effect on P450 aromatase activity in the

bovine granulosa cells *in vitro*. In comparing relative potencies, letrozole was more potent than anastrozole; estradiol synthesis was reduced to that of non-FSH-stimulated cells at a lower dose for letrozole than anastrozole (20 ng/mL vs 200 ng/mL, respectively). This observation is in agreement with a previous report that letrozole was several orders of magnitude more potent than anastrozole *in vitro* using cell culture from tissues of different origins [259].

Results obtained from the diffusion chamber trials indicated that a wax-based formulation was the most suitable for the development of a prolonged-release vehicle for administration of letrozole. The wax-based vehicle released letrozole at a steady rate. The profile (letrozole concentration over time) from the liposome-treated chambers indicated rapid liquefaction and absorption of the letrozole-containing vehicle through the vaginal mucosa. Letrozole concentrations peaked at 12 h and began to decrease by 24 h post-treatment. This pattern of rapid release of letrozole from the vehicle was also observed in a previous study in which a prototype intravaginal device with a polymer-based vehicle was tested in cattle (Chapter 6). In the prototype study, plasma letrozole concentrations increased rapidly by 24 h after device insertion, but declined rapidly thereafter, likely as a result of rapid and complete liquefaction of the vehicle and loss via vulvar discharge.

An increased interest in aromatase inhibitor-based protocols to control ovarian function in mammals creates the need for the development of effective routes and vehicles of administration to ensure the desired biological effects are achieved. Regarding prolonged treatment in farm animals, the intravaginal route is preferable for the administration of aromatase inhibitor because it is well-tolerated by the animals, it reduces handling and stress, it is user-friendly, there is a

high retention rate (which varies with device design), it is easy applied, and it enables controlled withdrawal [243].

After selection of the wax-based vehicle for further development, three different formulations of intravaginal devices were tested. The addition of DOPE to the wax-based mixture was to determine whether it would enhance absorption through the vaginal mucosa by enhancing the fusion of the liposomes to the cellular membrane [260]. However, the addition of DOPE to the formulation greatly increased the cost of the devices and hastened the elimination of letrozole (2 days after device insertion vs. 4 days in the other groups). Compared to the wax-only device, the addition of the gel coat hastened the initial increase in plasma letrozole concentrations (maximum concentration at 12 h vs. 3 days).

Bioavailability was calculated using historical AUC_{last} data obtained in earlier studies using an intravenous route of administration (Chapter 5). We concluded that the Wax + gel coat device provided the highest bioavailability, followed closely by the Wax-only device. Although the total amount of letrozole delivered (AUC_{last}) did not differ between the Wax + gel coat device and Wax-only device, the characteristics of the delivery during both the first 12 hours and 12 days did differ.

Partial validation of the LCMS/MS method to measure letrozole levels in bovine plasma was required. The results of validation indicated that samples below 2 ng/mL could not be quantified by the method employed in this study given that accuracy did not meet the required criteria for blank bovine plasma standards spiked with 0.2 and 0.4 ng/mL of letrozole. However, all the other parameters investigated during partial validation (precision, linearity, and selectivity) fell within acceptable ranges.

In summary, among the aromatase inhibitors tested, letrozole was the one that had greatest potency, as determined by inhibition of estradiol production *in vitro*. Furthermore, a wax-based vehicle, with a higher melting point than the polymer-based vehicle used in a previous study, provided steady and continuous delivery of the active compound over the treatment period. Finally, the addition of a letrozole-containing gel coating increased initial absorption and hastened the increase on plasma concentrations of the active ingredient, while the letrozole-containing wax-based vehicle prolonged drug-delivery from the intravaginal device. The biological response to these pharmacokinetic differences remains to be tested *in vivo* by assessment of estradiol inhibition and ovarian function.

7.6. Acknowledgement:

We thank Dr. Jane Alcorn for help in interpreting plasma letrozole concentrations and Deborah Michel for technical assistance. We are thankful to Gattefosse for kindly providing Suppocire D for the formulation of the intravaginal devices. We also thank Brad Blackmore and the staff at the Goodale Research Farm for assistance with handling and managing the cattle. The authors are thankful to the Natural Sciences and Engineering Research Council of Canada and Bioniche Animal Health Inc. for financial support.

CHAPTER 8: EFFECT OF AROMATASE INHIBITOR INTRAVAGINAL DEVICES ON OVARIAN FUNCTION IN CATTLE

Relationship of this study to the thesis:

To develop an efficient non-steroidal aromatase inhibitor-based method to synchronize ovulation in the bovine model, we need to be able to manipulate the duration of exposure of the animals to the active ingredient (letrozole). To this end, prolonged-release letrozole-containing intravaginal devices were formulated (Chapter 7). The biological response to these devices is assessed in the present chapter with the aim of determining the most suitable formulation to be applied in a letrozole-based protocol for synchronization of ovulation in cattle.

8.1 Abstract

A study was designed to determine the biological response to two letrozole intravaginal device formulations by assessment of estradiol inhibition and changes in ovarian function in vivo. Heifers in which a CL was detected during an initial examination were treated intramuscularly (im) with 500 µg of cloprostenol to synchronize ovulation. At the time of ovulation (Day 0), heifers were assigned randomly to three groups and given an intravaginal device containing wax + gel formulation (3 g of letrozole per device, n=4), wax formulation (3 g of letrozole per device, n=4), or a blank device (control, n=4). Intravaginal devices were inserted on Day 3 and kept in place for 8 days. Transrectal ultrasound examinations were done and blood samples were taken daily. The dominant follicle diameter profile was larger in heifers treated with the wax + gel coat letrozole-containing intravaginal device, and the interwave interval was prolonged in heifers in both letrozole-treated groups ($P<0.001$). Plasma estradiol concentrations were reduced significantly in the letrozole-treated groups. Although no differences were observed in corpus luteum diameters were detected among treatment groups, plasma progesterone concentrations were lower ($P<0.02$) in heifers given the wax formulation. We concluded that letrozole-impregnated intravaginal devices formulated with a wax base plus gel coat vehicle is most suitable for the application of a letrozole-based protocol for the synchronization of ovulation in cattle. It effectively reduced estradiol production resulting in prolonged dominant follicle growth and lifespan, without adversely affecting progesterone production.

8.2 Introduction

Control of the estrous cycle in animal species of commercial interest, such as cattle, impacts the efficiency and economy of meat and milk production. Numerous treatments and protocols have

been used to control reproductive events [107, 235, 236]; however, many of these protocols involve the administration of steroid hormones in food producing animals. The reproductively active steroid hormones, such as estradiol, are perceived as having a particularly negative impact on consumer health [22, 237, 238]. This internationally shared opinion has led to a ban on the use of steroid hormones in food producing animals and in specific categories of animals (e.g., lactating dairy cattle) in many countries (i.e., European Union, New Zealand, and Australia; [23, 24]).

Recently, non-steroidal aromatase inhibitors have been investigated as an alternative for controlling ovarian function using a bovine model [39, 40]. The studies have focused on the use of letrozole, a non-steroidal aromatase inhibitor that inactivates the aromatase enzyme responsible for the synthesis of estrogens by reversibly binding to the "heme" group of the P450 subunit [192]. Letrozole is used as an adjuvant or first-line treatment for hormone-dependent breast cancer in post-menopausal women [33]. It has also been used in women because of its potential for removing the negative feedback effect of estradiol on FSH secretion [34, 35, 215]. For ovarian stimulation in women, letrozole is commonly used at a dose of 1 to 5 mg per day for 5 days [35, 216], and has been used in higher or increasing doses for ovarian superstimulation [36, 37].

Using the bovine model, we have found that letrozole treatment extends the lifespan of the dominant follicle and thereby delays emergence of the next follicle wave and/or ovulation (Chapter 6). Letrozole treatment also had a luteotrophic effect; that is, larger CL and/or higher concentrations of progesterone were detected in letrozole-treated heifers (Chapter 6). The luteotrophic effect of letrozole treatment has important implications for the purposes of

enhancing embryo development and reducing embryonic loss [39, 40]. Finally, biologically active concentrations of letrozole have been achieved via intravenous, intramuscular or intravaginal administration ([39, 40] Chapters 5 and 6). Collectively, these results provide the impetus for the development of aromatase inhibitor-based synchronization and fertility treatments in cattle.

The intravaginal route of administration of letrozole is of particular interest because it permits extended treatment periods, is minimally invasive for the animal, reduces animal handling and associated stress, and is most likely to be accepted by practitioners and producers [243]. In a previous study (Chapter 6), we tested a prototype of an intravaginal letrozole-releasing device, but circulating concentrations of letrozole declined within 24 h after device insertion as a result of rapid release from the formulation (Chapter 6). In a follow-up study, two intravaginal devices were developed (wax + gel coat and wax alone) and tested in cattle to determine their pharmacokinetic characteristics. The wax-based vehicle resulted in steady and continuous delivery of letrozole over the treatment period in heifers. Further, the addition of a letrozole-containing gel coating increased initial absorption and hastened the increase of plasma concentrations of the active ingredient while the letrozole-containing wax-based vehicle maintained the prolonged delivery from the intravaginal device (Chapter 7).

The present study was designed to determine the effects of recently developed letrozole intravaginal device on circulating estradiol concentrations and ovarian function. We hypothesized that differences in the release and absorption characteristics of the intravaginal formulations would induce differential effects on ovarian function in cattle.

8.3 Materials and Methods

8.3.1 Cattle

Hereford-cross beef heifers (n=12), 15 to 20 months of age and weighing between 342 and 592 kg (457 ± 17.7 kg), were chosen from a herd of 50 heifers maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52° North and 106° West). Heifers were fed alfalfa/grass hay and grain to gain approximately 1.3 Kg per day and had water available ad libitum during the experimental period from August to September. Heifers were initially examined by transrectal ultrasonography (MyLab5, Canadian Veterinary Imaging, Georgetown, Ontario Canada) to confirm that they were post-pubertal as indicated by the presence of a CL [217]. Animal procedures were performed in accordance with the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

8.3.2 Treatments and examinations

The spine and blank pods (progesterone-free) of a commercially available intravaginal device for cattle (Cue-Mate, Bioniche Animal Health, Belleville, ON, Canada) were used as a support structure for the administration of letrozole. Device formulations and pharmacokinetic parameters have been described elsewhere (Chapter 7). Briefly, one device type (wax + gel coat) was prepared using a wax-based vehicle containing 2 g of letrozole per device, coated with a gel-based formulation containing 1 g of letrozole (total of 3 g of letrozole per device). The second device type (wax) was compounded entirely of the wax-based formulation so as to contain 3 g of letrozole per device.

Heifers in which a CL was detected during the initial examination were treated intramuscularly (im) with 500 µg of cloprostenol (PGF, Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) to synchronize ovulation [220]. On Day 0 (ovulation), heifers were assigned randomly to three groups (n=4 per group) and given an intravaginal device containing the wax + gel formulation, wax formulation, or no letrozole (blank intravaginal device, control) starting at Day 3. The intravaginal device was left in place for 8 days.

8.3.3 Ovarian ultrasonography

Observations from daily ultrasound examinations were recorded on a sketch sheet in which each ovary and its structures (CL and follicles ≥ 4 mm in diameter) were represented by size and location [73]. Ovulation was defined as the disappearance of any follicle ≥8 mm between consecutive examinations, and was confirmed by the subsequent development of a CL [217]. Follicular wave emergence was taken as the day of ovulation, or determined retrospectively as the day when the dominant follicle was first identified at a diameter of 4 or 5 mm [45, 64]. If the dominant follicle was not identified until it reached 6 or 7 mm, the previous day was considered the day of the follicular wave emergence [87]. The dominant follicle of a wave was defined as the largest follicle of the wave [221]. The day of onset of follicular or luteal regression was defined, in retrospect, as the first day of an apparent progressive decrease in diameter [45].

8.3.4 Collection of blood samples

Blood samples were collected by coccygeal venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Samples were collected daily

from pre-treatment ovulation (Day 0) to Day 15. Blood samples were centrifuged at 1500 x g for 20 min, and plasma was separated and stored in plastic tubes at -20 °C.

8.3.5 Hormone assays

Plasma concentrations of estradiol were determined in duplicate using a commercial RIA kit (Double Antibody Estradiol; Diagnostic Products, Los Angeles, CA, USA). The procedure was carried out at the Department of Animal Health and Biomedical Sciences, University of Wisconsin – Madison, as described elsewhere [79, 223], with the following modifications: Standards (0.78–100 pg/mL) were prepared in steroid-free (charcoal-treated) bovine plasma. The standards (250 µL in duplicate) and plasma samples (500 µL in duplicate) were extracted with 3 mL of diethyl ether, frozen in a dry-ice/methanol bath, decanted into assay tubes, and dried overnight under a fume hood. The dried samples and standards were re-suspended with 100 µL of assay buffer (0.1% gelatin in PBS). The intra-assay and inter-assay coefficients of variation were 10.5% and 10.6% for high reference samples (mean 11.1 pg/mL), and 14.8% and 12.3% for low reference samples (mean 2.6 pg/mL), respectively. The sensitivity of the assay was 0.1 pg/mL.

Plasma progesterone concentrations were determined in duplicate using a commercial solid-phase kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/mL. All samples were analyzed in a single assay; the intra-assay coefficients of variation for samples assayed in duplicates were 4.0% for low reference samples (mean, 0.74 ng/mL) and 1.1% for high reference samples (mean, 8.57 ng/mL).

8.3.6 Statistical analyses

Statistical analyses were done using the Statistical Analysis System software package (SAS Learning Edition 9.1, 2006; SAS Institute Inc., Cary, NC, USA). Serial data (hormone and follicle profiles) were compared by analysis of variance for repeated measures using the PROC MIXED procedure to determine the effects of device formulation (wax + gel coat, wax, or control), time, and their interactions. When a main effect or interaction was detected, individual comparisons among groups and days were performed using least significant differences (LSD). Single-point measurements (inter-wave interval and dominant follicle diameter at treatment) were compared among groups by analysis of variance. Significance was defined as $P \leq 0.05$.

8.4 Results

The intravaginal devices were inserted on Day 3 (Day 0 = ovulation), and the mean diameter of the dominant follicle at the time of placement did not differ among groups (

Table 8. 1). The day-to-day diameter profile of the dominant follicle during treatment was greatest ($P<0.05$) in the wax + gel coat group, intermediate ($P<0.05$) in the wax group, and smallest ($P<0.05$) in the control group (Figure 8. 1). The inter-wave interval was longest in the wax group, intermediate in the wax + gel coat group, and shortest in the control group ($P<0.001$,

Table 8. 1).

Table 8. 1. Effects of letrozole-containing intravaginal devices on ovarian function in heifers (mean±SEM).

	Control (n=4)	Wax+gel (n=4)	Wax (n=4)
Dominant follicle diameter (mm) at device insertion (Day 3)	9.7 ±0.5	11.2 ± 0.5	9.2 ± 0.1
Inter-wave interval (days)	10.2 ± 0.4^{a}	12.2 ± 0.4^{b}	14.3 ± 0.4^{c}

[abc] Within rows, values with no common superscript are different (P<0.05)

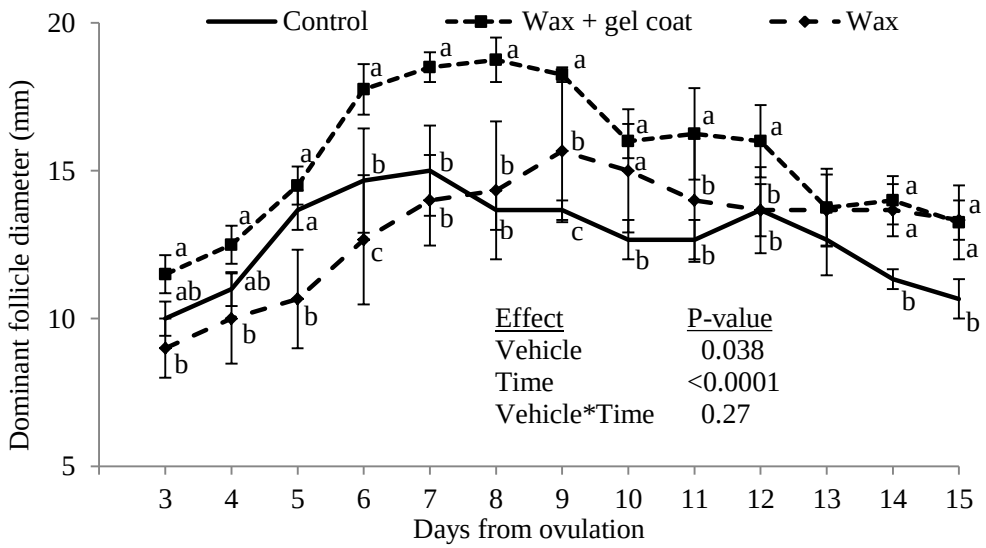


Figure 8. 1. Dominant follicle diameter profiles (mean±SEM) in heifers treated with a blank intravaginal device (control, n=4), or a letrozole-containing device with wax + gel coat (n=4) or wax only (n=4). Devices were inserted on Day 3. [abc] On indicated days, values with no common subscripts are different (P≤0.05).

Plasma estradiol concentrations in the letrozole-treated groups (wax + gel coat and wax) were lower than in the control group from the first post-treatment sample (P=0.002, Figure 8. 2). No difference was detected in estradiol concentrations between the wax + gel coat and wax groups.

Corpus luteum diameter profiles were not different among groups (P=0.36, Figure 8. 3). However, progesterone concentrations were lower in the wax group than in the other groups from Day 9 to Day 12 (P<0.05, Figure 8. 4).

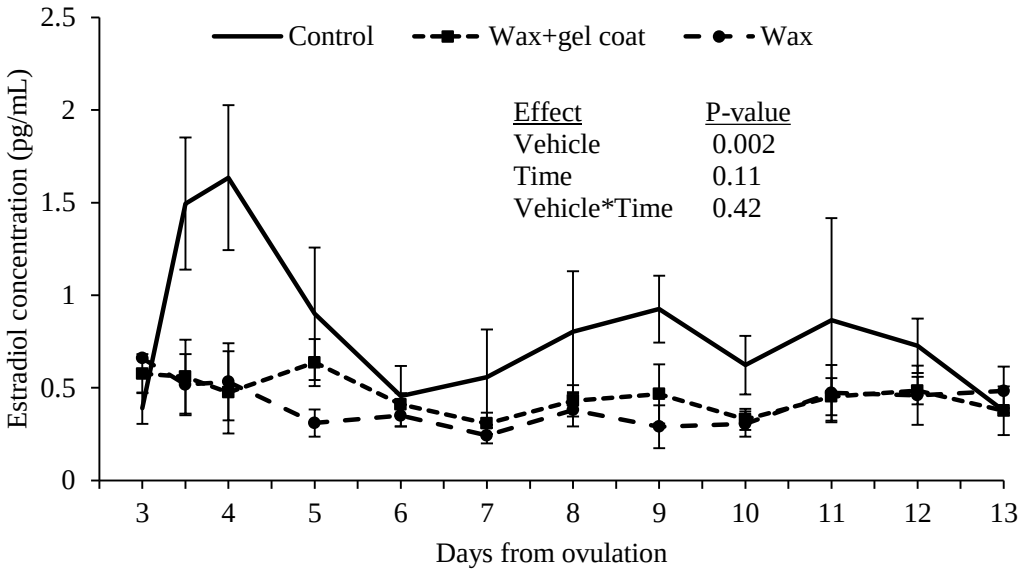


Figure 8. 2. Plasma estradiol concentrations (mean±SEM) in heifers treated with a blank intravaginal device (control, n=4), or a letrozole-containing device with wax + gel coat (n=4) or wax only (n=4). Devices were inserted on Day 3.

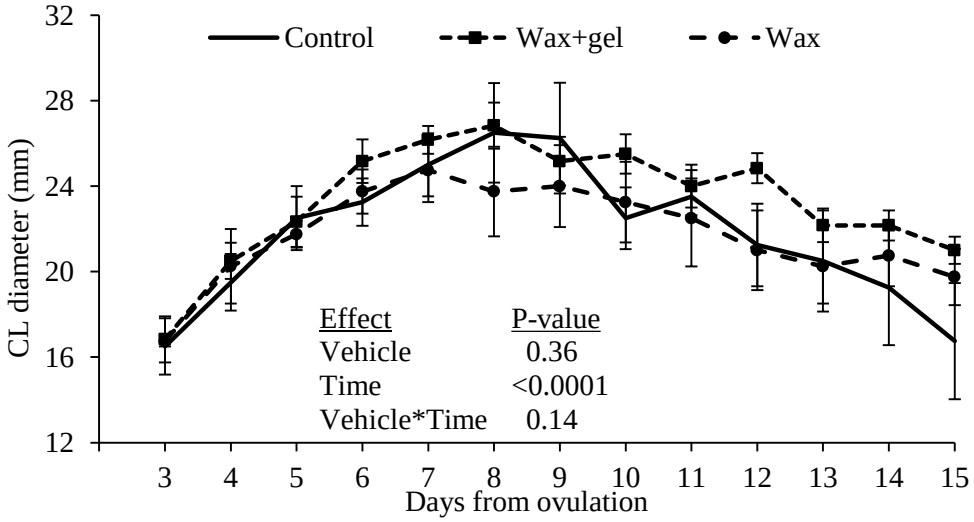


Figure 8. 3. Corpus luteum diameter profiles (mean±SEM) in heifers treated with a blank intravaginal device (control, n=4), or a letrozole-containing device with wax + gel coat (n=4) or wax only (n=4). Devices were inserted on Day 3.

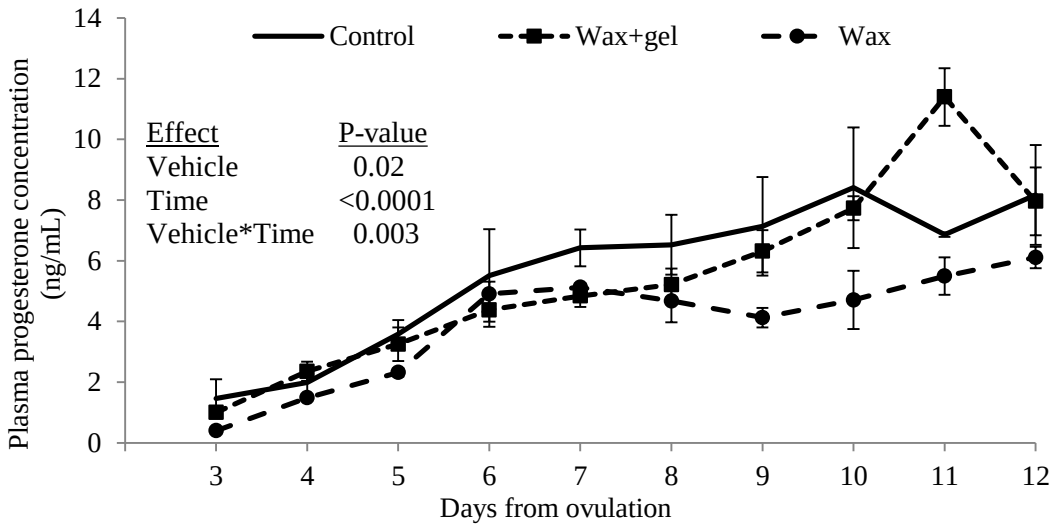


Figure 8. 4. Plasma progesterone concentrations (mean±SEM) in heifers treated with a blank intravaginal device (control, n=4), or a letrozole-containing device with wax + gel coat (n=4) or wax only (n=4). Devices were inserted on Day 3.

8.5 Discussion

Two letrozole-containing formulations were tested in the present study. Our hypothesis that differences in the release and absorption characteristics of the intravaginal formulations would induce differential effects on ovarian function in cattle was supported. The only difference between the two letrozole preparations was the presence of a gel coating on one; the total amount of letrozole (3 g) in each device did not differ (Chapter 7). The letrozole-containing gel coating has a lower melting point than the wax-based vehicle, allowing for rapid liquefaction and absorption of the formulation once the device was inserted into the vagina (Chapters 6). The wax plus gel coat formulation resulted in an early rise in plasma letrozole concentrations and a steady concentration by 24 h after device insertion. However, following insertion of wax-based intravaginal devices, steady plasma letrozole concentrations were reached 60 h after treatment

(Chapter 7). The biological response detected in the present study was consistent with pharmacokinetic differences detected in earlier studies,

Increased LH secretion and larger dominant follicle profiles have been observed following the administration of letrozole in previous studies [39, 40]. Although circulating gonadotropin concentrations were not measured in the present study, the differences in the pharmacokinetic characteristics of the formulations tested likely resulted in differences in gonadotropin secretion (i.e., earlier increase of LH secretion in the wax + gel coat group), and thus, differences in dominant follicle profiles. This is consistent with larger dominant follicle diameter profiles in letrozole-treated heifers, and the progressively longer interwave interval in letrozole-treated heifers given wax plus gel coat and those given the wax-only formulation.

The two letrozole formulations reduced plasma estradiol concentrations significantly and to a similar extent; estradiol concentrations differed from the control group by 12 h after device insertion. The plasma letrozole concentrations 12 h after device insertion, measured in the previous study to be 176.7 ± 11.70 ng/mL in the wax plus gel coat group and 71.2 ± 7.61 ng/mL in the wax group (Chapter 7), were both adequate to suppress estradiol production. Perhaps the amount of letrozole contained in the intravaginal devices may be reduced without compromising the efficacy in reducing estradiol concentrations. However, the lesser efficacy of the wax only formulation in affecting ovarian function suggests that the amount of letrozole required to elicit a detectable reduction in estradiol concentration may not be the same as that required to affect ovarian function, especially during the first hours after device insertion.

Letrozole treatment did not affect CL diameter profiles in the present study, regardless of the formulation used. The significantly lower plasma concentrations of progesterone observed in the

wax-based letrozole impregnated device group were unexpected. Based on the results of previous studies [39, 40], we expected letrozole treatment to suppress estradiol secretion and increase LH release, which in turn resulted in increased CL diameter profiles and/or increased progesterone secretion. Further research is needed in to clarify this finding.

Letrozole is a potent inhibitor of estradiol production in the bovine species (Chapters 5 and 6). Hence, there is increased interest in the development of letrozole-based protocols for the control of ovarian activities in cattle. Based on previous studies, we concluded that the lengthened duration of letrozole treatment would have impact on ovarian function ([39, 40], Chapters 5 and 6). Therefore, it is not surprising that formulation and pharmacokinetic characteristics played a key role in the success of a letrozole-based regimen for the control of reproductive phenomena in the present study. In summary, we observed that the vehicle used to deliver letrozole with an intravaginal device (wax + gel coat vs wax alone) differentially affected ovarian function in cattle. We concluded that letrozole-impregnated intravaginal devices formulated with a wax base plus a gel coat vehicle effectively delivered elevated concentrations of letrozole to the animal and reduced estradiol production resulting in increased follicular growth and lifespan, without adversely affecting progesterone production.

8.6 Acknowledgement

We thank Brad Blackmore and the staff at the Goodale Research Farm for assistance with handling and managing the cattle. The authors are thankful to the Natural Sciences and Engineering Research Council of Canada and Bioniche Animal Health Inc. for financial support.

CHAPTER 9: SYNCHRONIZATION OF OVULATION IN CATTLE WITH AN NON-STEROIDAL AROMATASE INHIBITOR-BASED PROTOCOL: A PILOT STUDY

Relationship of this study to the thesis:

Based on the results of experiments included in Chapters 5, 6, 7 and 8, a non-steroidal aromatase inhibitor-based protocol for the control of ovulation in cattle is presented in Chapter 9. The improved understanding of the effects of route of administration, vehicle, and the type and duration of aromatase inhibitor treatment on ovarian function permitted evidence-based estimation of the timing of letrozole treatment, in combination with other treatments (PGF and GnRH), to synchronize ovulation using the bovine model.

9.1. Abstract

The present study was designed to test the hypothesis that the addition of a letrozole-impregnated intravaginal device in a PGF / GnRH-based protocol will induce ovulation synchrony in cattle regardless of the stage of the estrous cycle in which treatment is given. Forty-eight heifers were treated intramuscularly (im) with 500 µg of cloprostenol (PGF) followed by 100 µg of GnRH 24 h later to synchronize ovulation. Daily ultrasound examinations determined the interval to ovulation which served as control data for the synchronizing effect of aromatase inhibitor treatment in the same animals. At the time of ovulation (Day 0), heifers were assigned randomly and given an intravaginal device containing 3 g of letrozole for 4 days starting on Days 0, 4, 8, 12, or 16. At the time of device removal, heifers were given PGF followed by GnRH 24 h later. Ultrasound examinations were performed daily, from two days prior to device insertion, continuing for 9 days after the post-treatment ovulation. The pre-ovulatory follicle diameters after letrozole treatment were larger in Day 4 group compared to Day 0 and Day 16 groups, and intermediate in Day 8 and Day 12 groups ($P<0.0006$). The percentage of heifers that ovulated (87.1% vs 69.4%, respectively) and synchrony of ovulation was greater following letrozole treatment compared to letrozole-free control data. No effect of group (day of treatment) on ovulation rate was detected. Although estradiol levels did not differ among the letrozole-treated groups, estradiol levels in Groups 0 and 4 were lower than their respective controls, while Groups 8 and 12 did not differ from their respective controls, mostly due to low estradiol concentration in the control samples. Corpus luteum diameter profiles and progesterone production were not affected by group. A small breeding trial was done using the letrozole-PGF-GnRH protocol; from the 43 heifers that were fixed-time inseminated 24 h after the

administration of GnRH, 3 were confirmed pregnant 35 days post-AI. In summary, the addition of a letrozole-impregnated intravaginal device for 4 days, combined with PGF treatment at device removal and GnRH 24 later resulted in a greater proportion of heifers ovulating more synchronously. Results suggest that treatment can be initiated effectively at random stages of the estrous cycle. However, the effects of letrozole treatment on CL lifespan and subsequent fertility were unexpected and must be studied in more detail.

9.2 Introduction

A conservative estimate of the worldwide use of AI is 83 million cows per year – estimated to represent about 20% of the breedable cattle population (25% in North America) [1]. Worldwide, 53% of cows artificially inseminated are of dairy breeds and 39% are of beef breeds, but in Canada, the gap is much wider: 94% in dairy and 6% in beef. Regarding ET, just over 120,000 donor cows are collected each year worldwide and 800,000 embryos are transferred [261]. In Canada, those numbers do not exceed 13,500 and 55,000, respectively [3]. Estrogen-based protocols for controlling follicle development and synchronizing ovulation in cattle have been instrumental in modern breeding practices. These protocols enabled producers to control the timing of ovulation reliably, enabling efficient use of time, labour and resources by allowing pre-scheduled artificial insemination. Estradiol-based protocols optimized the productivity of superovulation and embryo transfer programs due to their effectiveness in synchronizing follicular wave emergence prior to the initiation of superstimulation treatment [4-8]. However, the use of estradiol has been banned in many countries (i.e., European Union, New Zealand, and Australia) [23, 24]. This situation has created a void in treatments that efficiently control ovarian dynamics for the purpose of fixed-time insemination and embryo transfer in cattle.

Aromatase inhibitors have been investigated as a tool with which the estrous cycle in cattle might be controlled with the same precision as the older estradiol-based protocols but with the advantage of being a steroid-free alternative. Studies conducted in cattle have focused on the use of letrozole, a non-steroidal aromatase inhibitor that inactivates the aromatase enzyme responsible for the synthesis of estrogens by reversibly binding to the "heme" group of the P450 subunit. Letrozole is used as an adjuvant or first-line treatment for hormone-dependent breast cancer in post-menopausal women [33], and has been used in assisted reproduction in women because of its potential effect on removing the negative feedback of estradiol on FSH secretion [34, 35, 215].

The intravaginal route of administration of letrozole is of particular interest because it enables extended treatment periods, is minimally invasive, reduces animal handling and stress, and is most likely to be accepted by practitioners and producers [243]. In an earlier study (Chapter 6), we tested a prototype of an intravaginal device for providing an extended treatment with letrozole. However, letrozole was released too rapidly with the formulation used, and plasma concentrations were near base-line within 24 h after device insertion (Chapter 6). We modified the vehicle formulation and two variant intravaginal devices were developed and tested in cattle to determine their pharmacokinetic characteristics (Chapter 7). A wax-based vehicle with a higher melting point than the gel-based vehicle previously tested provided a steady and continuous delivery of letrozole over a 5-day treatment period in heifers. The addition of a letrozole-containing gel coating to the wax vehicle resulted in more rapid initial absorption and hastened the increase in plasma concentrations of the active ingredient (Chapter 7). In a following study (Chapter 8), the wax-based plus gel coat letrozole-containing device suppressed

estradiol production resulting in increased follicular growth and lifespan without adversely affecting progesterone production in the extant CL. We concluded that letrozole-impregnated intravaginal devices formulated with a wax base plus a gel coat vehicle was most suitable for the application of a letrozole-based protocol for the synchronization of ovulation in cattle.

To date, the main findings of studies in cattle are that letrozole treatment 1) extends the lifespan of the dominant follicle, 2) delays emergence of the next follicle wave and/or ovulation, and 3) is luteotrophic (i.e., larger CL and/or more progesterone) ([39, 40], Chapters 5 and 6). Collectively, these results provide compelling justification for the development of aromatase inhibitor-based synchronization and fertility treatments in cattle.

The present study was designed to test the hypothesis that the addition of a letrozole-impregnated intravaginal device in a PGF / GnRH-based protocol will induce ovulation synchrony in cattle regardless of the stage of the estrous cycle in which treatment is given. The principal objective of the study was to determine the ovulation rate and synchrony in heifers treated with a letrozole-based synchronization protocol at different stages of the estrous cycle (Experiment 1). A follow-up experiment was done as a pilot study to determine the pregnancy rate in heifers artificially inseminated following synchronization with the letrozole-based protocol initiated at random stages of their estrous cycle, would increase the percentage and synchrony of ovulations without adversely affecting pregnancy rates.the estrous cycle (Experiment 2).

9.3 Materials and Methods

9.3.1 Experiment 1

9.3.1.1 Cattle

Hereford-cross beef heifers (n=49), 15 to 20 months of age and weighing between 379 and 667 kg (mean of 505 ± 8.6 kg), were chosen from a group of 51 heifers maintained in outdoor pens at the University of Saskatchewan Goodale Research Farm (52° North and 106° West). Heifers were fed alfalfa/grass hay and grain to gain approximately 1.3 Kg per day and had water available ad libitum during the experimental period from December to February. Heifers were initially examined by transrectal ultrasonography (MyLab5 VET, Canadian Veterinary Imaging, Georgetown, Ontario Canada) to confirm that they were post-pubertal, as indicated by detection of a CL [217]. Animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

9.3.1.2 Treatments and examinations

Heifers in which a CL was detected during the initial examination (n=49) were treated intramuscularly (im) with a luteolytic dose of prostaglandin (500 µg cloprostenol, Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) followed by 100 µg of GnRH (Fertiline, Vetoquinol, Lavaltrie, QC, Canada) 24 h later to induce ovulation [220]. Daily ultrasound examinations were performed to determine the interval from GnRH treatment to ovulation to use as control data for the synchronizing effect of letrozole in the same animals. At the time of ovulation (Day 0), heifers were assigned randomly to the following five groups (n=8-

10/group) and given an intravaginal device with a wax-based plus gel coat formulation containing 3 g of letrozole (Chapter 7) for 4 days starting on Day 0, 4, 8, 12, or 16. At the time of device removal, heifers were given 500 µg PGF followed by 100 µg of GnRH 24 h later. The treatment schedule is summarized in Figure 9. 1.

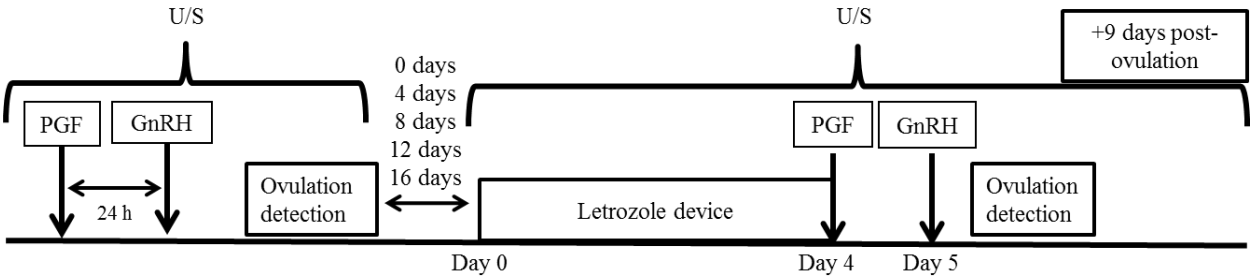


Figure 9. 1. Synchronization treatment schedule for Experiment 1. Heifers (48) were treated im with PGF followed by GnRH 24 h later to synchronize ovulation. Ultrasound examinations (U/S) were done daily to detect ovulation,(Day 0) and heifers were given an intravaginal device containing 3 g of letrozole for 4 days starting on Days 0, 4, 8, 12, or 16. At the time of device removal, heifers were given PGF followed by GnRH 24 h later. Ultrasound examinations were performed daily, from two days before device insertion to 9 days after the post-treatment ovulation.

Ultrasound examinations were performed daily from two days before device insertion to 9 days after post-treatment ovulation. Observations from ultrasound examinations were recorded on a sketch sheet in which each ovary and its structures (CL and follicles ≥ 4 mm in diameter) were represented by size and location [73]. Ovulation was defined as the disappearance of a follicle ≥8 mm between successive examinations after GnRH treatment and was confirmed by the subsequent detection of a CL [217]. The time of follicular wave emergence was defined as the day of ovulation or retrospectively as the day when the dominant follicle was first identified

at a diameter of 4 to 5 mm [45, 64]. If the dominant follicle was not identified until it reached 6 or 7 mm, the previous day was considered day of the follicular wave emergence [87]. The dominant follicle of a wave was defined as the largest antral follicle of the wave [221]. The day of onset of follicular and luteal regression was defined as the first day of an apparent progressive decrease in follicular and luteal diameters, respectively [45].

9.3.1.3 Collection of blood samples

Blood samples were collected by coccygeal venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Samples were collected at the time of device removal for estradiol measurement, and at 3, 6 and 9 days following the post-treatment ovulation to determine progesterone concentrations. Additionally, blood samples were collected on Days 4, 8, 12, and 16 from heifers in the Day 16 group (Day 0 = ovulation) to serve as control data for comparison of estradiol concentrations at the time of device removal in other treatment groups. As such, the Day 16 group did not have a control sample for comparison of estradiol concentrations at the time of device removal. Blood samples were centrifuged at 1500 x g for 20 min and plasma was separated and stored in plastic tubes at -20 °C until assayed.

9.3.1.4 Hormone assays

Plasma concentrations of estradiol were determined using a commercial radioimmunoassay kit (Double Antibody Estradiol; Diagnostic Products, Los Angeles, CA, USA). The procedure was carried out in the Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, as described [79, 223], with the following modifications: Standards (0.78–100 pg/mL) were prepared in steroid-free (charcoal-treated) bovine plasma. The standards (250

μL in duplicate) and plasma samples (500 μL in duplicate) were extracted with 3 mL of diethyl ether, frozen in a dry-ice/methanol bath, decanted into assay tubes, and dried overnight under a fume hood. The dried samples and standards were re-suspended with 100 μL of assay buffer (0.1% gelatin in PBS). The intra- and inter-assay coefficients of variation were 10.5 and 10.6%, respectively for high reference samples (mean 11.1 pg/mL), and 14.8 and 12.3%, respectively for low reference samples (mean 2.6 pg/mL). The sensitivity of the assay was 0.1 pg/mL.

Plasma progesterone concentrations were determined using a commercial solid-phase kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/mL. Samples were analyzed in a single assay. The intra-assay coefficient of variation for samples assayed in duplicates was 4.0% for low reference samples (mean, 0.74 ng/mL) and 1.1% for high reference samples (mean, 8.57 ng/mL).

9.3.2 Experiment 2 - Artificial insemination pilot study

Forty five heifers were selected from the group used previously for the artificial insemination (AI) trial. Three heifers were excluded due to their low body condition. Two weeks after the end of the first experiment, the protocol described previously was applied as follows: a new letrozole-releasing intravaginal device was placed in all heifers on random days of the estrous cycles for 4 days. At device removal, PGF was given followed by GnRH 24 h later. Heifers were artificially inseminated 24 h after GnRH treatment. The semen used for the artificial inseminations was collected from five beef bulls located at a community pasture during their annual breeding soundness evaluation. Fresh semen was classified as good based on breeding soundness exam standards [262] with more than 400 million sperms/mL and more than 70%

individual progressive motility (Dr Anzar, personal communication). Semen from the five bulls was pooled prior to freezing. The straws contained a total of 25 million sperm /mL and Computer Assisted Semen Analysis (CASA) resulted in post-thaw cell motility and progressive motility of 49% and 44%, respectively. Pregnancy was diagnosed by transrectal ultrasonography 35 days after insemination.

9.3.3 Statistical analyses

Statistical analyses were done using the Statistical Analysis System software package (SAS Learning Edition 9.1, 2006; SAS Institute Inc., Cary, NC, USA). Time-series hormone data and follicular diameter profiles were analyzed by repeated measures, using the PROC MIXED procedure to determine the main effects of treatment and time, and their interactions. Individual time-point comparisons were made using the least significant difference (LSD) test. Single-point measurements (dominant follicle diameter at device insertion, pre-ovulatory follicle diameter, CL diameter 9 days after the post-treatment ovulation and interval from GnRH treatment to ovulation) were compared among groups by one-way analysis of variance. The ovulation rate was compared among groups by Chi-Square test. The degree of synchrony between groups was calculated by analysis of variance of the residuals of the interval from GnRH to ovulation. Significance was defined as $P \leq 0.05$.

9.4 Results

9.4.1 Experiment 1

As expected, the diameter of the dominant follicle at the time of device placement was larger in the Day 4 and 12 groups compared to the Day 0 and 16 groups, and intermediate in the Day 8

group (Table 9. 1). Similarly, the maximum diameter of the ovulatory follicle after letrozole treatment was greater in Day 4 group compared to the Day 0 and 16 groups, and intermediate in Day 8 and 12 groups (Table 9. 1). The day-to-day dominant follicle diameter profiles during treatment were larger in the Day 4 group compared to the Day 0 and 16 groups, and intermediate in the Day 8 and 12 groups ($P<0.001$).

Table 9. 1. Effects of a letrozole-containing intravaginal device in combination with PGF and GnRH on ovarian function in heifers (mean±SEM).

Treatment group	Dominant follicle diameter (mm)		CL diameter (mm) at 9 days post-ovulation
	At device placement	At day before ovulation	
Day 0 (n=10)	5.7 ± 0.35^{a}	12.8 ± 0.58^{a}	20.9 ± 1.09^{a}
Day 4 (n=9)	11.9 ± 0.59^{b}	16.4 ± 0.78^{b}	25.2 ± 1.65^{ab}
Day 8 (n=8)	7.2 ± 1.76^{abc}	14.5 ± 0.83^{ab}	20.8 ± 1.88^{ab}
Day 12 (n=11)	10.1 ± 0.74^{bc}	14.2 ± 0.48^{ab}	21.7 ± 0.99^{ab}
Day 16 (n=10)	6.0 ± 1.56^{ac}	11.7 ± 0.59^{a}	26.8 ± 2.69^{b}

^{abc} Within columns, values with no common superscript are different ($p<0.05$)

The percentage of heifers that ovulated after GnRH treatment was higher when a letrozole-releasing intravaginal device was added to the PGF plus GnRH (control) protocol ($P = 0.05$, Table 9. 2). No effect of group on synchrony of ovulation was detected; hence data were combined into a single letrozole group. The interval from GnRH treatment to ovulation was longer in the control group compared to the combined letrozole-treated groups ($P = 0.008$, Table 9. 2). The degree of ovulation synchrony was greater in the combined letrozole-treated groups than in the control group ($P = 0.01$, Table 9. 2 and Table 9. 3). The distribution of the ovulations in the letrozole-treated and control groups is summarized in Table 9. 3.

Table 9. 2. Effect of addition of letrozole to a PGF-GnRH-protocol on ovulation in heifers.

	Control	Letrozole	P-value
Ovulation percentage	69.4% (34/49)	87.1% (42/48)	0.05
Days from GnRH treatment to ovulation	2.4 ± 0.18	1.9 ± 0.08	0.01
Degree of synchrony	0.68 ± 0.13	0.24 ± 0.07	0.01

Table 9. 3. Distribution of ovulation in heifers given a letrozole-releasing intravaginal device for 4 days beginning on different days of the estrous cycle, followed by a luteolytic dose of prostaglandin and an ovulation-inducing dose of GnRH.

	Ovulation in relation to GnRH		
Treatment group	Before*	Synchronized*	Ovulation failure*
Day 0	0	10	0
Day 4	3	7	0
Day 8	0	7	1
Day 12	0	10	1
Day 16	3	5	1
Letrozole total	6 (12%)[a]	39 (81%)[a]	3 (6%)[a]
Control total	7 (14%)[a]	28 (57%)[b]	14 (28%)[b]

*Before = ovulation between PGF and GnRH treatments; Synchronized = ovulation within 48 hours after GnRH treatment; Ovulation failure = no ovulation between PGF treatment and 48 hours after GnRH treatment. [ab] Within columns, values with no common superscript are different ($p<0.05$)

Plasma estradiol concentrations at the time of device removal in Groups 0, 4, 8, and 12 were compared to samples collected from heifers in Group 16 at equivalent time points before letrozole treatment in that group. Group 16 lacked a comparable control group due to the experimental design. Estradiol concentrations at device removal were lower in Groups 0 and 4, compared to their respective controls (P = 0.02 and P = 0.05, respectively; Figure 9. 2), and estradiol concentration tended to be lower following letrozole treatment in Group 8 (P = 0.1,

Figure 9. 2). Estradiol concentrations were low in Group 12 and not different from Day 12 controls (P = 0.9, Figure 9. 2).

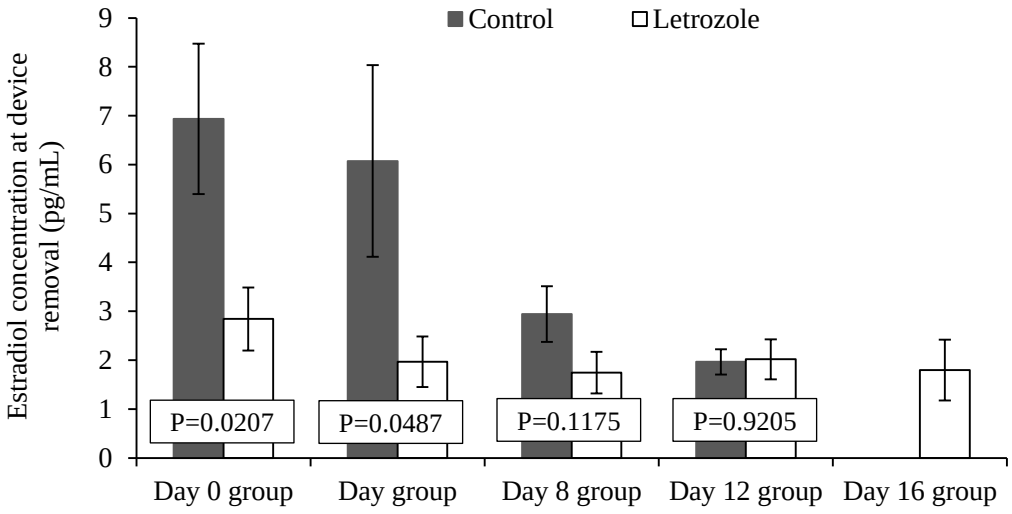


Figure 9. 2. Plasma estradiol concentration (mean ± SEM) at letrozole device removal in heifers treated with a 4-day regimen of letrozole intravaginally compared to untreated controls. Devices were placed on Days 0 (n=10), 4 (n = 10), 8 (n = 8), 12 (n=11) or 16 (n=9; Day 0 = ovulation). Control samples were obtained from heifers in the Group 16 at Days 4, 8, 12 and 16, prior to treatment with letrozole on Day 16. Hence, Day 16 group lacked of a control group at device removal.

Corpus luteum diameter profiles were not different among treatment groups (P = 0.45, Figure 9. 3). Similarly, progesterone concentrations were not different among treatment groups during the 9 days following ovulation (P = 0.33, Figure 9. 4).

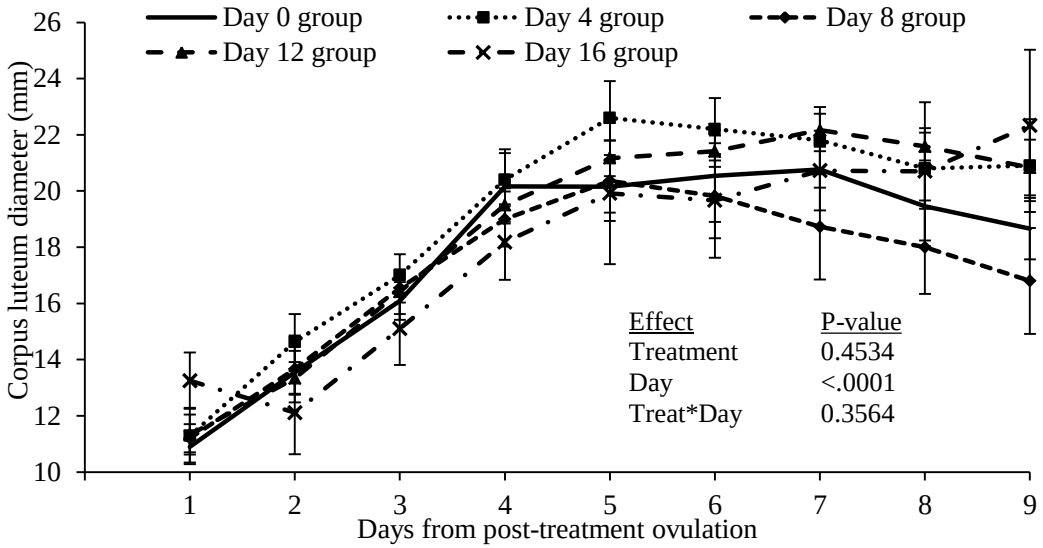


Figure 9. 3. Corpus luteum diameter profiles (mean ± SEM) in heifers after treatment with letrozole-releasing intravaginal device for 4 days followed by PGF at device removal and GnRH 24 h later. Devices were placed on Days 0 (n=10), 4 (n = 10), 8 (n = 8), 12 (n=11) or 16 (n=9; Day 0 = ovulation).

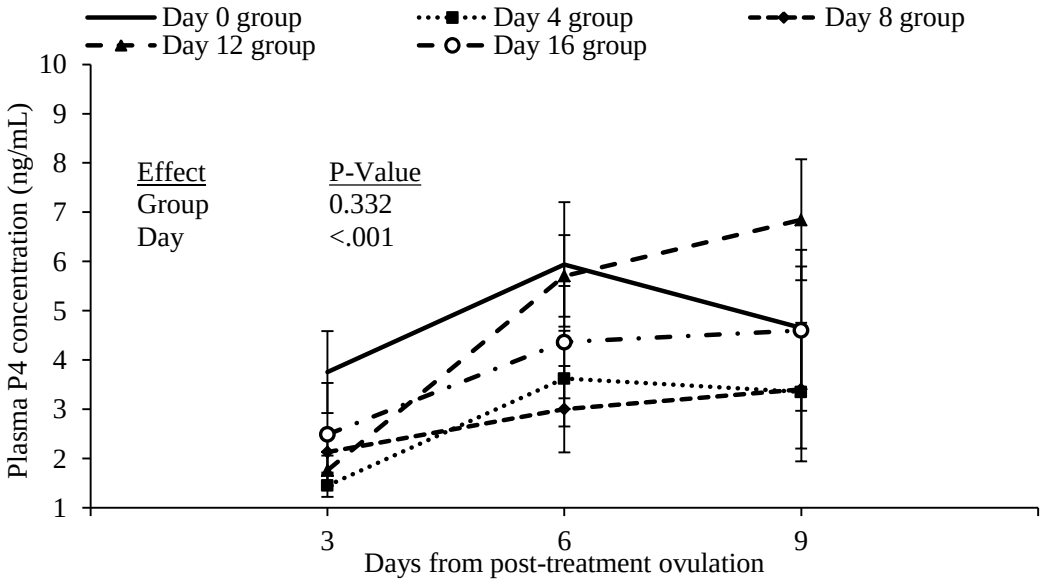


Figure 9. 4. Plasma progesterone (P4) profiles (mean ± SEM) in heifers after treatment with letrozole-releasing intravaginal device for 4 days followed by PGF at device removal and GnRH

24 h later. Devices were placed on Days 0 (n=10), 4 (n = 9), 8 (n = 8), 12 (n=11) or 16 (n=10; Day 0 = ovulation).

Two subsets of animals were identified based on lifespan of the CL resulting from the post-treatment ovulations. One group included heifers with CL of normal lifespan which were actively growing or static 9 days after the post-treatment ovulation (7/10 in Group 0, 4/10 in Group 4, 4/8 in Group 8, 10/11 in Group 12, and 5/9 in Group 16). The remaining heifers had CL of short lifespan which were either regressing or had already regressed by 9 days after the post-treatment ovulation [263]. Corpus luteum diameter profiles were not different among treatment groups within the two sub-populations (P = 0.62 and P = 0.41, for normal and short lifespan CL, respectively). Similarly, progesterone concentrations were not different among treatment groups within each sub-population during the observational period (P = 0.88 and P = 0.40, for normal and short lifespan CL, respectively).

9.4.2 Experiment 2 - Artificial insemination trial

The percentage of heifers that ovulated following treatment in the artificial insemination trial was 80% (36/45). Two heifers were not inseminated because it was not possible to reach the uterine lumen in order to deposit the semen. From the total number of heifers inseminated (n=43), three were confirmed pregnant 35 days post-AI.

9.5 Discussion

Studies of the effect of different letrozole treatment regimens on ovarian function in cattle have provided evidence of the potential of aromatase inhibitors as a tool to manipulate ovarian function in this species ([39, 40] Chapter 5, 6 and 8). The data presented herein further support

this notion by confirming our hypothesis that extended letrozole treatment using intravaginal devices, combined with a single PGF and GnRH treatment, increases the percentage of heifers that ovulated and the synchrony of ovulation, regardless the stage of the estrous cycle at initiation of the protocol. However, letrozole-PGF-GnRH-based protocol for ovulation synchronization resulted in poor pregnancy rates

The percentage of heifers that ovulated was increased by the addition of a 4-day regimen of letrozole to a PGF plus GnRH protocol as compared to PGF plus GnRH alone (87.1% vs 69.4%, respectively). This increase in ovulatory response and synchrony of ovulation after the addition of letrozole may involve more follicles responding to the GnRH treatment and a decrease in early ovulations (ovulations that occurred prior to GnRH treatment). We have reported previously that letrozole induced growth and prolonged the lifespan of dominant follicles by increasing circulating plasma LH concentrations ([39, 40] Chapters 5 and 6). We speculated that the addition of letrozole to the PGF plus GnRH protocol would allow for smaller, less competent follicles, which otherwise would not have responded to GnRH treatment, to reach the necessary diameter and LH receptor populations to acquire ovulatory capacity and ovulate within 48 h post GnRH treatment. In addition to promoting follicular growth, letrozole treatment likely prolonged the lifespan of the static dominant follicles that otherwise would have become atretic by the time of GnRH treatment ([39, 40], Chapter 8). The reduction in early ovulations is supported by the observation that letrozole prevents estradiol secretion (Chapters 5, 6 and 8) thus minimizing the occurrence of a pre-ovulatory rise in estradiol concentration and a LH surge prior to administration of GnRH. The improved ovulatory response and synchrony of ovulation after GnRH indicated that letrozole could be applied for the development of a FTAI protocol.

Pregnancy rates were low in the small breeding trial; only 3 of 43 inseminated heifers were pregnant 35 days post-AI. The effect of letrozole treatment on fertility was unexpected. We have reported that letrozole treatment affects the plasma hormone profile and steroidogenesis, thus it is also expected to alter other aspects of reproductive function such as the duration of follicle dominance, oocyte age and activation, the process of fertilization, embryo quality, and CL lifespan. However, it is possible that other factors unrelated with the non-steroidal aromatase inhibitor treatment may also have influenced the pregnancy outcome obtained in this study.

The impact of duration of dominance of the pre-ovulatory follicle on timing of ovulation and fertility has been reported [264, 265]. Prolonged dominance of the ovulatory follicle has been associated with reduced pregnancy rates. The decrease in fertility was more profound after 9 days of dominance (35 to 70% reduction in pregnancy rates) compared to after 2 days of dominance [264]. Duration of dominance is related to increased LH levels [266] and early activation of oocytes (resumption of meiosis) has been associated with decreased fertility [264, 266-268].

During the present study, we attempted to design a minimal duration of follicle dominance in the pre-ovulatory follicles by adjusting the length of letrozole treatment to 4 days. The duration of dominance of the pre-ovulatory follicles among groups was estimated based on the average day of emergence of the follicle that became the ovulatory follicle - follicles typically reach dominant status by 3 days post-wave emergence (2.8 days) [65]. It would follow that the duration of dominance in Day 0 and 4 groups were 4 and 8 days, respectively. Day 8 group contained heifers that would have had follicles from the first follicular wave (n=4) and heifers with follicles originating from the second follicular wave (n=4). Hence, heifers in this group

would have had follicles in which dominance would have been 4 days or 12 days at the time of ovulation. Day 12 group should have had follicles that originated from only the second wave, and would have been dominant for about 4 days before ovulation. Day 16 group would have had follicles originating from either the second or third wave of follicular growth and the duration of dominance in this group would have been 4 and 12 days (4 and 6 heifers, respectively). As evidenced by these numbers, heifers between Days 7 and 9 (Day 8 ± 1) and between Days 15 and 17 (Day 16 ± 1; Day 0 = ovulation) would be at risk of developing an ovulatory follicle that had been dominant for approximately 12 days and would likely have an aged and/or prematurely activated oocyte. However, we must consider the possibility that increased LH secretion (caused by letrozole treatment [39, 40]) may also cause premature activation of oocytes and a reduction in fertility, even if duration of dominance of the pre-ovulatory follicle was within normal range (1 to 5 days, [268]).

Estradiol concentrations were reduced in heifers in Groups 0 and 4, tended to be reduced in Group 8, while Group 12 did not differ from their respective controls. Considering that estradiol concentration at device removal did not differ among letrozole-treated groups and averaged 2.1 ± 0.24 pg/mL, the differences noted between letrozole-treated heifers relative to their controls were attributed to changes in estradiol concentration in the control samples. Estradiol concentration in the control heifers Days 4, 8, 12 and 16 were consistent with those obtained previously: basal estradiol concentrations have been reported to be around 2 pg/mL, with a small rise between Days 4 and 7 post-LH peak and no significant changes in estradiol concentration thereafter until the next pre-ovulatory estradiol rise [55]. It is possible, however, that the presence of a newly recruited wave of follicular development (third wave) was responsible for the low estradiol

concentration observed in the control heifers on Day 16. In other words, the high estradiol concentration expected with the presence of a growing (estrogen-active) follicle from the second wave (potentially an ovulatory wave) may have been obscured by the coexistence of atretic (estrogen-inactive) follicles from the second follicular wave with newly recruited follicles which have not yet reach their maximal estrogen production potential.

There is some controversy regarding the requirement of estradiol for final follicular maturation and the presence of fertilizable oocytes in mammals. In rhesus monkeys, aromatase inhibitor treatment during the late follicular phase did not alter the number nor the pattern of growth of follicles although oocyte activation and *in vitro* fertility was reduced [139]. While some studies reported that addition of estradiol in *in vitro* maturation protocols impaired bovine oocyte nuclear maturation and subsequent embryo development [269, 270], others found that estradiol was essential for normal *in vitro* maturation [271], especially of early antral follicle-derived oocytes [272]. However, data on the effects of estradiol deprivation on bovine oocyte maturation *in vivo* are not available, most likely due to the lack of an efficient treatment regimen to mimic such a condition. Although not directly assessed in this study, we can presume that follicular environment has been affected by treatment, affecting oocyte quality by disturbing meiosis. However, treatment with anastrozole, another non-steroidal aromatase inhibitor, did not impair follicular growth, ovulation nor fertilization *in vivo* and embryo development *in vitro* using a mouse model [273]. In addition, there are several important extra-gonadal effects of the estradiol produced by growing pre-ovulatory follicles, such as the development of receptive endometrium, the production of the cervical mucus, and sperm transport [274]. All these processes could be negatively affected by letrozole-induced estradiol inhibition. This notion is

further supported by the observation that heifers showed normal signs of estrus after initial PGF and GnRH treatment (control ovulations) but did not exhibit any estrous behaviour (i.e., mounting, standing to be mounted, vaginal mucus) following PGF and GnRH treatment after letrozole treatment.

The effect of letrozole treatment on CL lifespan in this study was also unexpected. In previous studies, letrozole treatment resulted in larger CL which secreted higher levels of progesterone [39, 40] (Chapters 5 and 6). Thirty out of 48 (62.5%) heifers treated had CL considered to be of normal diameter at last observation (9 days post-letrozole treatment ovulation), while 18 (37.5%) heifers underwent luteolysis prior to the last observation at 9 days after ovulation. Progesterone production was not affected by group and its profile corresponded to CL lifespan (i.e., normal vs short lifespan). The reason for these differences on CL lifespan within groups remains unclear. There appeared to be no relationship between duration of follicular dominance and lifespan of the resulting CL. Short-lived CL have been described following hCG-induced ovulation of the dominant follicle of the first follicular wave in cattle, suggesting that pre-ovulatory changes intrinsic to the treatment may be responsible for the abnormal CL function [275].

Another possible explanation for the observed short lifespan CL is related with the occurrence of early luteolysis. Short luteal phases in 33% of cows [276] and 47 % of heifers have been reported [277] when GnRH treatment was given 24 h after PGF. The short luteal phases were related to early release of $PGF_{2\alpha}$ from the endometrium [278]. Reduced estradiol concentration during the proestrus has also been linked to short luteal lifespan. It has been hypothesized that high estradiol concentrations during proestrus are needed in order to induce an

adequate number of progesterone receptors, thus allowing progesterone to regulate the uterine secretion of PGF [263, 279]. Therefore, low estradiol concentration induced by letrozole treatment may impair the inhibitory effect that progesterone has on PGF secretion by allowing the increase in number of estrogen and oxytocin receptors in the endometrium and early release of $PGF_{2\alpha}$ [280].

An important limitation of the present AI trial is the lack of a letrozole-free control group. Therefore, it is difficult to determine the impact that factors such as AI technician, semen quality and semen handling had on pregnancy rates. Another important factor to consider is the timing of the inseminations. Heifers were inseminated 24 h after GnRH based on an earlier study in which it was reported that ovulations occurred between 24 and 32 h after GnRH treatment [94, 98]. However, it is unknown if letrozole treatment alters the window of time between GnRH treatment and ovulation. Daily ultrasound examinations did not allow the determination of the time of ovulation precisely in the present study. Finally, the presence of the short lifespan CL during the AI trial also needs to be considered, which would reduce the proportion of heifers that could have remained pregnant. Post-AI ultrasound examinations were not performed until pregnancy check; hence, no information on CL lifespan is available for this set of animals.

In summary, the addition of a letrozole-impregnated intravaginal device for 4 days, combined with PGF treatment at device removal and GnRH 24 h post-device removal increased the percentage of ovulations and synchrony of ovulation in cattle, regardless the stage of the estrous cycle at initiation of treatment. Reduced luteal lifespan after letrozole treatment was unexpected and requires further investigation in order to elucidate the mechanism responsible for this observation. Although the results obtained in the AI trial are unfavorable, we consider that

this portion of the study needs to be repeated with the inclusion of control group and in vitro fertilization trials in order to draw more meaningful conclusions on the impact that letrozole treatment has on oocyte competence and fertility. Adjustments of timing of AI, interval from PGF to GnRH treatment and even *in vitro* assessment of oocyte fertizability after estradiol deprivation may be considered. Finally, the letrozole treatment regimen presented herein has potential as a model to investigate the effect of estradiol deprivation on oocyte maturation and fertility *in vivo*.

We conclude that the addition of letrozole to a GnRH plus PGF protocol can be used to increase the number of animals ovulating and the synchrony of ovulation, but additional studies are needed in order to elucidate the mechanisms related to reduced fertility observed herein.

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CHAPTER 10: GENERAL DISCUSSION

The ban of the use of estrogens in livestock and lack of commercially available estrogenic preparations negatively impacts the implementation of reproductive biotechnologies that depend on the use of estrogens in cattle production systems, limiting potential reproductive efficiency and genetic improvement provided by the use of AI or MOET programs [24]. In this context, the development of alternative methods for controlling ovarian function and improvement of fertility in cattle, with efficacy and predictability comparable with that of estrogen plus progesterone treatments [5], and with no harmful effects on human and animal health is needed. Non-steroidal aromatase inhibitors, such as letrozole, prevent the body from producing estrogens by reversibly binding to the heme group of the $P450_{arom}$. Letrozole, have been administered to premenopausal women for the purpose of follicular stimulation, induction of ovulation, and ovarian superstimulation [36, 37]. We hypothesized that letrozole could be used to manipulate ovarian follicular wave dynamics, synchronize ovulation and improve post-breeding and embryo transfer fertility in cattle. Five experiments were conducted to test this hypothesis. We examined: 1) the effect of route and vehicle of letrozole treatment on ovarian function in cattle (Chapters 5, 6, and 7), 2) the effect of type of aromatase inhibitor on ovary function in cattle (Chapter 7), 3) the effect of duration of aromatase inhibitor treatment (short vs prolonged) on ovarian follicles in cattle (Chapters 5, 6 and 8), and 4) the efficacy of an aromatase inhibitor-based protocol to synchronize ovulation in cattle (Chapters 8 and 9). Finally, a small breeding trial was conducted, in an attempt to obtain preliminary data and understanding of the effects of aromatase inhibitors treatment on pregnancy outcomes (Chapter 9).

To the best of our knowledge, these are the first studies on non-steroidal aromatase inhibitors for control of reproduction in cattle. Although studies on the effects of letrozole on ovarian function in women suggested that aromatase inhibitor-stimulated follicular growth was driven by increased FSH secretion as a result of removal of the negative feedback effects of estrogen [34, 141], current results in cattle suggest that it was LH rather than FSH that was responsible for the effects of letrozole [39, 40]. The objective of this general discussion is to summarize and integrate the results obtained from the experiments presented in Chapters 5, 6, 7, 8 and 9 of this thesis to current information available in the literature.

10.1 Effects of non-steroidal aromatase inhibitor in estradiol concentration

Plasma estradiol concentrations were significantly reduced by letrozole administration *in vivo* (Chapters 5, 6, 8 and 9) and *in vitro* (Chapter 7). The duration and magnitude of the reduction in estradiol concentrations after letrozole treatment was affected by route and vehicle of administration (*in vivo*) and by type and dose of aromatase inhibitor (*in vitro*). The vehicle formulated for intramuscular administration of letrozole (Chapter 5) appeared to have a depot effect, delaying and prolonging drug release from the injection site. Hence, plasma estradiol concentrations in heifers treated intramuscularly took 24 h longer than in those treated intravenously to reach minimum values, and remained low 6 days after treatment. In the intravenous letrozole group, estradiol levels reached the minimum concentration rapidly (between 12 and 24 h after treatment) but returned to placebo-treated control levels within 2 days. After insertion of letrozole-impregnated gel-based intravaginal devices (Chapter 6), estradiol concentrations were reduced, and the preovulatory rise in estradiol concentrations occurred 24 h later than in the control group. However, the duration of estradiol inhibition was

relatively short and was related with the rapid release of letrozole from the devices and the early drop in letrozole plasma concentration. Addition of a wax matrix to the formulations in order to prolong the release of letrozole from the intravaginal devices (Chapter 7) affected the extent and duration of estradiol inhibition as discussed in Chapters 8. Both letrozole formulations (Wax plus gel coat and Wax alone) significantly reduced plasma estradiol concentrations to a similar extent in treated heifers, and estradiol concentrations differed from the control group by 12 h after device insertion. In Chapter 9, estradiol concentrations were reduced after insertion of the wax plus gel coat letrozole-containing intravaginal device in heifers treated from Days 0 to 4, and from Days 4 to 8, and tended to be reduced in heifers treated from Days 8 to 12 (Days 0, 4 and 8 groups, respectively. Day 0 = ovulation).

As compared to their respective controls, estradiol concentration were reduced by 56% and 45% with im and iv letrozole administration, respectively (Chapter 5), by 45% with the gel-based letrozole intravaginal device (Chapter 6) and by 60% with the wax-based letrozole intravaginal devices (Wax plus gel coat and Wax alone combined, Chapters 8 and 9). These levels of inhibition in estradiol production following the administration of letrozole in cycling heifers were similar to that reported for cycling females in other species. Reduction in estradiol concentration of about 50% were reported in premenopausal bonnet monkeys treated with 3.5 mg/day of letrozole over 7 days (from Days 7 to 14 of the menstrual cycle) [281], and in premenopausal women treated with 5 mg/day of letrozole from Days 3 to 7 of the menstrual cycle [225]. It appears, however, that estradiol inhibition by letrozole treatment is more profound in males and in post-menopausal women than in cycling cattle, and premenopausal women and non-human primates. When given to boars at a dose of 0.1 mg/kg body weight, letrozole

treatment reduced estrogen levels by 90% at 24 hours and levels remained low for at least 168 hours post-treatment [282]. In post-menopausal women, estrogen suppression was 97 to 99% [33, 187], and 30% with the lowest (0.02 mg) and 90% with the highest (30 mg) dose of letrozole tested in healthy human males [182]. It is recognized that differences in doses and regimens of administration of letrozole, as well as methods used for the determination of estradiol levels in plasma or serum must be taken into consideration in the interpretation of these findings. Furthermore, the inherently low circulating estradiol concentration in non-pregnant cattle, compared to females of other species such women and the mare, represents an obstacle for the determination of an effect of letrozole treatment on estradiol secretion in cattle.

Our *in vitro*-generated data on the effects of type of aromatase inhibitor (letrozole, anastrozole and fenbendazole) using bovine granulosa cells in culture revealed that letrozole and anastrozole were efficacious in reducing estradiol production to levels found in non-FSH-stimulated granulosa cells (Chapter 7). Fenbendazole, however, did not reduce estradiol concentrations significantly. These observations are consistent with reports in which letrozole and anastrozole were found to be strong and selective inhibitors of the $P450_{arom}$ [181, 255]. In comparing relative potencies, estradiol synthesis was reduced to that of non-FSH-stimulated cells at a lower dose with letrozole than with anastrozole (20 ng/mL vs 200 ng/mL, respectively) which is in agreement with a previous report that letrozole was several orders of magnitude more potent than anastrozole *in vitro* using cell culture from different tissues such as hamsters ovarian tissue fragments, normal human adipose tissue fibroblasts and the JEG-3 human choriocarcinoma cell line [259]. Albendazole, a benzimidazole anthelmintic drug closely related to fenbendazole, inhibited the activity of cytochrome P450 enzymes (CYP enzymes; a family of

enzymes of which aromatase is a member) *in vitro* in rat and mouflon sheep [256-258].[256, 257], [258]. Therefore, fenbendazole may have had a direct, although mild, effect on $P450_{arom}$ activity, or it may have affected estradiol production indirectly, through non-specific inhibition of other P450 enzymes in the bovine granulosa cells *in vitro.*

10.2 Effects of non-steroidal aromatase inhibitor treatment on follicular dynamics

The timely control of wave emergence and ovulation has been instrumental for the successful application of reproductive technologies in cattle and other species. The outcome of a superstimulation treatment is strongly influenced by the stage of follicular development at the time of initiation of treatment, with optimal ovarian responses being obtained when superstimulatory treatments were initiated at the time of follicular wave emergence [5, 109]. Similarly, FTAI eliminates the need for estrus detection but requires synchronous growth and ovulation of a viable dominant follicle in order to be successful. The effect of non-steroidal aromatase inhibitors on non-ovulatory follicle growth and wave emergence in cattle has been assessed previously [39, 40] and in Chapters 5 and 8 of the present thesis. The effect of letrozole treatment on pre-ovulatory follicles and ovulation was the subject of Chapter 6 and 9.

We were able to determine the changes in ovarian dynamics after letrozole treatment during experiments reported on in Chapters 5, 6, 8 and 9. In general terms, letrozole had a stimulatory effect on the growth and lifespan of the non-ovulatory dominant follicle which was consistent with earlier reports [39, 40]. Furthermore, intravaginally administered letrozole treatment during the pre-ovulatory follicular wave also resulted in a greater diameter and prolonged lifespan of the ovulatory follicle, delaying ovulation by 24 h (Chapter 6). Likewise, it has been shown that women treated with letrozole from Days 3 to 7 of the menstrual cycle had larger ovulatory

follicles than untreated controls [224]. However, formulation and route of delivery of letrozole affected the extent of this effect. For example, letrozole given im or iv (Chapter 5) did not result in significantly larger dominant follicles. However, despite the effect of aromatase inhibitor treatment on dominant follicle diameter, the prolonged interval from treatment to new wave emergence (Chapter 5 and 8) and from treatment to ovulation (Chapter 6), indicates that letrozole administration prolonged the functionality of the dominant follicle, which may be even more meaningful. This observation was also supported by the outcomes of the synchronization trial (Chapter 9). The higher proportion of ovulations observed in the letrozole-treated group was attributed to smaller, less competent follicles (which otherwise would not have responded to GnRH treatment) reaching the necessary diameter and LH receptor populations to acquire ovulatory capacity and ovulate within 48 h post GnRH treatment. In addition to promoting follicular growth, letrozole treatment likely prolonged the lifespan of the dominant follicle (as documented in Chapters 5 and 8, and in [39, 40]) making it possible to maintain the viability of static follicles that otherwise would have become atretic by the time of GnRH treatment. As a result, the addition of letrozole to a PGF plus GnRH protocol increased ovulatory response (87.1% vs 69.4%, respectively) and the synchrony of ovulation. The prolonged dominance resulting after letrozole treatment resembles that observed in beef cows following exogenous pulsatile administration of LH [283] or that obtained after increased LH pulsatility by induction of sub-luteal progesterone concentration in cattle [16].

The effect of letrozole treatment on follicles prior to selection was assessed in experiments described in Chapters 6 and 9. We hypothesized that letrozole treatment initiated prior to the onset of pre-ovulatory dominant follicle selection would result in the development of multiple

dominant follicles. This hypothesis was based on earlier research in cattle [40] and results obtained from women undergoing ovarian stimulation [36, 37, 216]. In cattle, the initiation of a 3-day regimen of letrozole given iv prior to follicular selection resulted in the development of two co-dominant follicles, one of which regressed immediately after interruption of letrozole administration [40]. Similarly, a mild superovulatory response was described in women following the use of letrozole in increasing doses (step-up protocol) and after a 5-day letrozole regimen of 5 or 7.5 mg/day, and it was concluded that increased FSH secretion may have been responsible for multi-follicular growth [36, 37, 284]. However, the results obtained in the current studies (Chapters 6 and 9) failed to support our hypothesis. Although non-steroidal aromatase inhibitor treatment was initiated before dominant follicle selection in Chapter 6, it is possible that the low bioavailability of letrozole resulting from the intravaginal formulation accounted for the lack of co-dominance. In the experiments described in Chapter 9, the letrozole formulation used in the intravaginal devices prolonged release and therefore increased letrozole bioavailability. Nevertheless, no superstimulatory response occurred. We speculate that the delay of the increase in plasma letrozole concentration due to absorption through the vaginal mucosa (Chapter 7) may have interfered with the superstimulatory effect. The formerly reported co-dominance was obtained after intravenous letrozole administration; immediate and complete bioavailability of letrozole may have favored the mild ovarian superstimulation observed in that study [40].

10.3 Effects of non-steroidal aromatase inhibitor treatment in CL function

Studies in cattle indicated luteotrophic effects on the existing CL after treatment with a single or a 3-day regimen of letrozole; circulating LH was increased while FSH remained unchanged [39, 40]. Similar findings have been reported in women, where enhanced luteal function was

associated with increased circulating LH concentrations but no difference in FSH concentrations [225]. Non-steroidal aromatase inhibitors exerted a luteotrophic effect when given during the diestrous and proestrous in beef heifers (Chapters 5 and 6, respectively). The effect was evidenced by larger CL diameter profiles and/or increased plasma progesterone concentrations in letrozole-treated heifers. In Chapter 5, the luteotrophic influence on the extant CL appeared to be more evident when letrozole was given intramuscularly than when given intravenously. Intravaginally administered letrozole treatment during the growing phase of the ovulatory wave (Chapter 6) resulted in the ovulation of a larger dominant follicle but this did not result in larger CL as it has been previously reported [116, 118, 285]. Interesting, despite the lack of an increase in CL size, elevated plasma progesterone profiles were observed over the first 12 days post-ovulation in the letrozole-treated group. We speculated that preovulatory letrozole treatment may have affected the number or proportion of large luteal cells (granulosa cell origin) and small luteal cell (thecal cell origin) contained within the CL [230], resulting in an increase in progesterone production per CL volume. Small and large luteal cells are normally present in the bovine CL in a ratio of 7.6:1 [231]. Small luteal cells respond directly to LH stimulus to secrete progesterone [232, 233], while large luteal cells appear to be responsible for sustained secretion of progesterone in the absence of a stimulus [233]. Treatment of cows with equine chorionic gonadotropin resulted in increased density and number of large luteal cells which increased the capacity of the CL to produce progesterone without altering its volume [234]. We interpreted that the stimulatory effects of letrozole treatment during proestrous on the newly formed CL function has the potential of enhancing fertility by increasing circulating progesterone

concentrations during the first 7 days post-ovulation in cattle, which may be specially beneficial in dairy cows.

In the experiments described in Chapter 9, we observed CL dysfunction following letrozole treatment for the first time. In this study, 18 out of 48 heifers (37.5%) ovulating after treatment with a wax plus gel coat letrozole-containing intravaginal device underwent early luteolysis (luteal regression observed during the first 9 days post-ovulation). Progesterone production profile corresponded to CL lifespan (i.e., normal vs short lifespan). This unexpected finding was unrelated to size and lifespan of the pre-ovulatory dominant follicle. Although we cannot rule out a direct effect of letrozole on CL steroidogenic machinery, other factors need to be taken into consideration in order to interpret this finding. Reduced estradiol concentration during the proestrus in cattle has been linked to short luteal lifespan, and it has been hypothesized that high estradiol concentrations during proestrus are needed in order to induce an adequate number of endometrial progesterone receptors, thus allowing progesterone to regulate the uterine secretion of PGF [44, 45]. Therefore, low estradiol concentration induced by letrozole treatment may have impaired the inhibitory effect that progesterone has on PGF secretion, allowing for an increase in the number of estrogen and oxytocin receptors in the endometrium and early release of PGF2α [46]. However, due to the lack of a contemporary untreated control group, we cannot fully determine whether the presence of short-lived CL in the non-steroidal aromatase inhibitor-treated group was entirely due to letrozole or there were other factors involved. Corpora lutea with a shortened lifespan have also been described following hCG-induced ovulation of the dominant follicle of the first follicular wave in cattle, suggesting that pre-ovulatory changes intrinsic to the treatment may be responsible for the abnormal CL function [40]. Short luteal phases in 33% of

cows [41] and 47 % of heifers have been reported [42] when GnRH treatment was given 24 h after PGF. The short luteal phases were related to early release of PGF2α from the endometrium [43]. Therefore, adjusting the timing of GnRH treatment in relation to PGF administration in our letrozole-based protocol may help to minimize the incidence of early luteolysis. Although letrozole-mediated estradiol deprivation early in the luteal phase and its impact of CL functionality has not been critically assessed, our observation of luteotrophic effects of aromatase inhibitor on the developing CL ([39, 40] and Chapter 5) indicate that letrozole treatment during this period may be the key to improving CL size and/or progesterone production.

10.4 Effect of non-steroidal aromatase inhibitor treatment on gonadotropin secretion

We have reported that letrozole induces an increase in plasma LH concentrations [39, 40]. However, the exact nature of the increase in LH concentration (i.e. increase basal secretion and/or increase LH pulsatility) after letrozole treatment was not evident from our data. None of the studies presented herein were designed to characterize the influence of letrozole on LH pulsatility. Although measurement of LH and FSH concentrations were attempted in Chapter 5 and 6, daily and 12 h sampling frequency has limitations when measuring gonadotropin concentrations and attempting to correlate them to ovarian dynamics. Several studies in which analysis of LH pulse frequency was their main endpoint used sampling frequencies of 10 to 15 minutes [286-289]. Sampling intervals of 20 min have been considered inadequate to consistently detect changes in LH pulsatility [13].

The findings summarized in this thesis further support the hypothesis that treatment with aromatase inhibitor resulted in an increase in LH secretion which in turned resulted in greater

dominant follicle diameter and lifespan, and increased CL diameter and/or increased progesterone secretion. In Chapter 5, a surge on LH concentration was observed on the day of wave emergence in the letrozole im group while no such increase was detected in the letrozole iv or combined placebo-treated control groups. We were unable to detect an increase in LH concentration in Chapter 6, although the delay in ovulation led us to speculate that the LH surge was also delayed by letrozole treatment. The effect of aromatase inhibitor treatment on gonadotropin secretion was not included among the endpoints studied in the experiments reported in Chapters 8 and 9.

Several research groups have generated information on the aromatase inhibitor-induced changes in gonadotropin secretion in other species. Our results are in agreement with the study by Cortínez et al. [225] in which a larger follicular size in letrozole-treated women was attributed to the higher levels of LH found in the circulation. Similarly, long-term letrozole treatment in the stallion resulted in a 9-fold increase in LH secretion, while FSH concentrations did not differ from that of control stallions [290]. Increased LH concentration after letrozole treatment, without significant changes in FSH, were also reported using a rat model [291]. Kisspeptin-secreting neurons contain ER and other sex steroid receptors and are located in close proximity to GnRH-secreting cells in the hypothalamus [59, 60]. Reducing circulating E2 concentrations have been correlated with higher kisspeptin (Kp) mRNA levels and increased GnRH output [58]. Administration of 100 pmole/k of Kp in cattle, resulted in increased circulating LH concentrations during diestrous and proestrus, but not during estrous [61]which may assist in the interpretation of our findings: letrozole treatment (or reduced estradiol) resulted in increased follicular growth during diestrous (Chapter 5 and 8) and proestrous (Chapters 6 and 9), possibly

through increased Kp output and increased GnRH secretion, while during estrous (Chapter 6), letrozole treatment may have delayed the Kp increase and therefore the preovulatory LH surge. Regarding FSH, it is known that follicular factors other than estradiol influence the secretion of this gonadotropin [52, 53]. Inhibin secretion has been positively correlated with follicular growth and it has been associated with the drop in FSH secretion that encompasses follicular selection [54]. Therefore, levels of inhibin secreted by the dominant follicle may increase during letrozole administration and diminish the expected rises in FSH concentration. The potential action of inhibin on FSH secretion in part would explain the lack of a superstimulatory effect when letrozole treatment was initiated prior to selection (Chapters 6 and 9). Additional studies will be needed in order to clarify this concept. Measurement of inhibin concentration during letrozole treatment or combination of an aromatase inhibitor plus inhibin-blocking treatment (or using animals immunized against inhibin) may have to be considered for this purpose.

10.5 Effect of non-steroidal aromatase inhibitor treatment on fertility in cattle

A small breeding trial was included as part of the study reported in Chapter 9. Unexpectedly low pregnancy rates were obtained; only 3 of 43 inseminated heifers were pregnant 35 days post-AI. Although we were tempted to conclude that non-steroidal aromatase inhibitor treatment has a direct negative impact on oocyte competence, other factors inherent to the experimental design and resulting from the altered gonadotropin profiles obtained after letrozole treatment need to be considered.

The requirement of estradiol for final follicular maturation and development of oocyte competence in mammals is not fully understood. While the inclusion of estradiol in *in vitro* maturation protocols impaired bovine oocyte nuclear maturation and subsequent embryo

development in some studies [269, 270], others report that estradiol is essential for normal *in vitro* maturation [271], especially of early antral follicle-derived oocytes [272]. In rhesus monkeys, aromatase inhibitor treatment during the late follicular phase did not alter the number nor the pattern of growth of follicles, although oocyte activation and *in vitro* fertility was reduced [139]. However, data on the effect of estradiol deprivation on bovine oocyte maturation *in vivo* are not available, most likely due to the lack of an efficient animal model or treatment regimen to mimic such a condition. Although not directly assessed in this study, we can presume that follicular environment may have been affected by letrozole treatment, potentially affecting oocyte quality by disturbing meiosis. However, a mouse model demonstrated that treatment with anastrozole did not impair follicular growth, ovulation nor fertilization *in vivo* and embryo development *in vitro* [273]. In addition, non-steroidal aromatase inhibitor treatment may have had several important extra-gonadal effects; estradiol produced by the growing pre-ovulatory follicle has been shown to be associated with the development of receptive endometrium, the production of the cervical mucus, and sperm transport [274]. Clinical studies in humans, however, denote that the main advantage of the use of letrozole over other estradiol suppressors used in ovarian stimulation (such as clomiphene citrate, CC) is that uterine maturity does not seem to be compromised [200, 201, 203]. The improved endometrial thickness obtained after letrozole treatment compared to that of CC-treated patients has been attributed to the relatively short half-life of letrozole and the reversible nature of letrozole interaction with the $P450_{arom}$ [200, 201, 203]. An interesting observation made during the experiments included in Chapter 9 supports, in part, the notion of alteration in reproductive organs other than the ovaries after non-steroidal aromatase inhibitor treatment in cattle. Heifers showed normal signs of estrus after

initial PGF and GnRH treatment (control ovulations), but failed to exhibit estrus signs or behaviour (i.e., mounting, standing to be mounted, vaginal mucus discharge) once letrozole was included to the PGF-GnRH protocol.

Other factors that need to be examined to understand the pregnancy outcomes presented in Chapter 9 include duration of dominance, and incidence of oocyte aging and/or activation. The impact of duration of dominance of the pre-ovulatory follicle on timing of ovulation and subsequent fertility has been reported [265, 292]. Prolonged dominance of the ovulatory follicle has been associated with increased LH pulsatility [266]. Additionally, early activation of oocytes (resumption of meiosis) driven by increased LH pulsatility has been considered the main cause of decreased fertility following the use of progestogens in synchronization protocols [268, 292-294]. During the breeding trial, heifers in which treatment was initiated between Days 7 and 9 and between Days 15 and 17 (Day 0 = ovulation) may have been at risk of developing an ovulatory follicle which was dominant for approximately 12 days and would likely have had an aged and/or prematurely activated oocyte (Chapter 9). We must also consider the possibility that increased LH secretion (potentially caused by letrozole treatment [39, 40]) may have led to premature activation of oocytes and a reduction in fertility, even in those heifers in which duration of dominance of the pre-ovulatory follicle was within normal range (1 to 5 days, [268]). Finally, another factor which could have accounted for the low pregnancy rates is the occurrence of short-lived CL, previously discussed. Unfortunately, post-AI ultrasound examinations were not performed until pregnancy check 35 days later; hence, no information on CL lifespan was available for this set of animals.

Deficiencies in the experimental design and limited resources (number of heifers available) introduced additional confounding factors to the interpretation of the results of the AI trial included in Chapter 9. The lack of a letrozole-free control group made it difficult to determine the impact that factors such as AI technician, semen quality and semen handling may have had on pregnancy rates. Although semen quality was deemed to be satisfactory in terms of post-thaw motile cells and progressive motility (49% and 44%, respectively) ensuring the present of at least 12 million progressively motile sperm cells per insemination dose, previous evidence of the fertility of the semen was not available. Another important factor to consider is the timing of the inseminations. Heifers were inseminated 24 h after GnRH and this was based on an earlier study in which it was reported that ovulations occurred between 24 and 32 h after GnRH treatment [94, 98]. However, it is unknown if letrozole treatment alters the timing between GnRH treatment and ovulation. Daily ultrasound examinations did not allow the determination of the time of ovulation precisely during the studies included in Chapter 9. In the Ovsynch protocol, the second GnRH treatment is normally administered 48 h post PGF, and cows are inseminated 16 to 18 h later [295]. A study showed that GnRH treatment 24 h post-PGF resulted in lower pregnancy rates that when GnRH was given 48 h post-PGF, with both groups being inseminated 24 h post-second GnRH [94]. As discussed previously, it is conceivable that increasing the interval between PGF and GnRH from 24 h to 48 h could be beneficial not only to prevent short-lived CL as previously discussed, but also for oocyte competence and fertility. Furthermore, adjusting the timing of insemination using a letrozole-PGF-GnRH based protocol may also result in improved pregnancy rates.

10.6 Pharmacokinetics of letrozole in cattle

The pharmacokinetic parameters estimated during this thesis work are in accordance with those reported previously in cattle [40], and differ to some extent from those described humans [184, 185]. Letrozole appears to have a shorter half-life in cattle (on average, 33 h) than in humans (48 h). Mean residence time (MRT) value represents the average duration of persistence of the drug in the body and, although it was numerically similar to that reported in humans (60.5 h in heifers (Chapter 6) vs 58.7 h in women [184]), direct comparisons cannot be made since two different methods where used for the calculation of this parameter in each study. Finally, the volume of distribution observed in the heifers (8 L/kg) was higher than that reported in women (2 L/kg) [184], suggesting that letrozole has higher tissue distribution in this species as compared to humans. Since the half-life of a drug not only depends on elimination, the difference in tissue distribution may be in part responsible for the shorter half-life of letrozole estimated in cattle as compared to humans.

Bioavailability can been defined as the amount of a drug given by any route, other than intravenously, that reaches general circulation and is available at the site of action [227]. In this thesis, bioavailability was calculated using AUC_{last} data obtained using the intravenous route of administration of letrozole in Chapter 5. In Chapter 6, the low bioavailability of letrozole obtained with the intravaginal devices (16%) may be explained by the melting point of the gel-vehicle used. This gel-based vehicle is commonly used for intravaginal suppositories for women, in which body temperature is lower than that of cattle (37° vs 39° C) [228, 229]. As previously discussed, rapid liquefaction and release of the formulation from the intravaginal devices may have caused the loss of most of the formulation through the vulvar opening. After *in vivo* testing

of newly formulated intravaginal devices in Chapter 7, we concluded that the wax plus gel coat device provided the highest bioavailability (63%), followed closely by the wax-only device (58%). The addition of DOPE as a absorption enhancer to the device formulation may have decreased its melting point causing loss of the device contents to the outside a decreasing its bioavailability (43%).

The increasing interest in aromatase inhibitor-based protocols to control ovarian function in mammals created the need for the development of effective routes and vehicles of administration to ensure the desired biological effects are achieved. Our hypothesis that the formulation and route of administration used to provide letrozole treatment in heifers will have an important influence on the changes in ovarian function was supported by the experiments presented in this thesis. Since half-life and volume of distribution are inherent to the chemical characteristics of drug of interest and, in our studies these parameters were relatively independent of vehicle, variations in the ovarian effects among experiments were attributed to differences in the absorption pattern among formulation and routes of administration. In Chapter 5, the oil-based intramuscular vehicle used for letrozole administration appeared to act as a depot, releasing letrozole from the injection site at a slow and steady rate, and the effects on ovarian function were more consistent and reliable that those obtained with following intravenous administration. In another example, the profile of letrozole concentration over time reported in Chapter 6 was affected primarily by the rapid liquefaction and release of the formulation from the intravaginal devices. This rapid release may have interfered with absorption of letrozole, due to most of the formulation being lost through the vulvar opening. Therefore, letrozole was released from the intravaginal devices for 24 h post-insertion, but elimination took place locally and absorption and

plasma clearance took place thereafter. The duration of estradiol inhibition may have been influenced by the pharmacokinetic characteristics of this intravaginal formulation, and could account for the 24 h delay in the estradiol rise observed in the letrozole-treated group. The intravaginal devices tested in Chapter 7 indicated that, although the total amount of letrozole delivered (AUC_{last}) did not differ between the wax plus gel coat device and wax-only device, the characteristics of the delivery during both the first 12 hours and 12 days did differ. The wax plus gel coating formulation resulted in an early rise in plasma letrozole concentrations, reaching a steady plasma concentration by 24 h after device insertion. Plasma letrozole concentrations did not reach values similar to that in the wax plus gel coat group until 60 h after treatment following insertion of wax-only intravaginal devices (Chapter 7). Although these pharmacokinetic differences were expected, their biological relevance was confirmed in Chapter 8. Both letrozole formulations significantly reduced plasma estradiol concentrations to a similar extent in treated heifers, and estradiol concentrations differed from the control group by 12 h after device insertion. However, the lesser efficacy of the wax only group in affecting ovarian function may indicate that the amount of letrozole required to reduce estradiol concentrations is not necessarily the same as that required to affect ovarian function, especially in the first few hours after device insertion.

Drug administration by the intramuscular route in large animals is commonly used. However, the fact that withdrawal of the circulating concentration of the active compound depends entirely on absorption and clearance makes it is difficult to manipulate the length of the effect. This remains to be an important limitation for this route of administration, particularly when the length of exposure to a given drug greatly impacts the success of the treatment, as is

the case for synchronization of ovulation for breeding purposes. When it comes to prolonged treatment in farm animals for reproductive management, the intravaginal route of administration is preferable because it is well-tolerated by the animals, reduces handling and stress, is user-friendly and easily applied, there is a high retention rate (which varies with device design), and it enables controlled withdrawal [243].

10.7 Summary

In summary, our results demonstrate that route of administration, or more precisely, the nature of the vehicle used for the administration of letrozole (intravenous, intramuscular depot, short release intravaginal or prolonged release intravaginal) has an impact on the effects of letrozole on hormonal profiles and ovarian dynamics. The intramuscular route appeared to provide a prolonged release of letrozole from the injection site which had a marked effect on estradiol production, dominant follicle lifespan, and CL form and function (Chapter 5). Letrozole treatment during the ovulatory follicle wave by means of a gel-based intravaginal releasing device resulted in more rapidly growing dominant follicles and a larger ovulatory follicles, delayed ovulation (by 24 h) of a single follicle and formation of a CL that secreted higher levels of progesterone (Chapter 6). A wax-based vehicle, with higher melting point than the polymer-based vehicle used in Chapter 6, allowed for a steady and continuous delivery of the active compound over the treatment period. The addition of a letrozole-containing gel coating increased the rate of initial absorption and hastened the increase on plasma concentrations of the active ingredient, while the letrozole-containing wax-based vehicle prolonged drug-delivery from the intravaginal device (Chapter 7). When tested *in vivo,* we confirmed that letrozole-impregnated intravaginal devices formulated with a wax base plus a gel coat vehicle was most suitable for the

application of a letrozole-based protocol for the synchronization of ovulation in cattle, since it effectively delivered elevated concentrations of letrozole, reduced estradiol production resulting in increased follicular growth and lifespan, without adversely affecting progesterone production (Chapter 8). The addition of a letrozole-impregnated intravaginal device for 4 days, combined with PGF treatment at device removal and GnRH 24 h post-device removal increased the percentage of ovulations and synchrony of ovulation in cattle, regardless the stage of the estrous cycle at initiation of treatment (Chapter 9). As observed in previous studies [39, 40], the effects observed could be associated with an increase in circulating LH concentrations. However, the effects of treatment on gonadotropin concentrations are inconclusive, possibly due to inadequate sampling frequency. The impact of letrozole treatment of oocyte fertility remains unknown.

CHAPTER 11: GENERAL CONCLUSIONS

The general objectives of this thesis included the determination of the effect of route and vehicle of letrozole treatment on ovarian function in cattle (Chapters 5, 6, and 7), the effect of type of aromatase inhibitor on ovary function in cattle (Chapter 7), the effect of duration of aromatase inhibitor treatment (short vs prolonged) on ovarian follicles in cattle (Chapters 5, 6 and 8), and the efficacy of an aromatase inhibitor-based protocol to synchronize ovulation in cattle (Chapters 8 and 9). The chapter-specific conclusions are summarized as follows:

1) Chapter 5: Effects of vehicle and route of administration of a non-steroidal aromatase inhibitor on ovarian function in a bovine model.
    a. Route of administration and the nature of the vehicle used for the administration of letrozole (depot vs intravenous) have an impact on the effects of letrozole on ovarian dynamics and hormonal profiles.
    b. Intramuscular route provides a prolonged release of letrozole from the injection site.
    c. Letrozole treatment given intramuscularly on Day 3 post wave emergence lengthens the lifespan of the dominant follicle, delays post-treatment wave emergence, and has a luteotrophic effect as evidenced by a larger CL and higher circulating progesterone concentrations. However, the same dose of letrozole given intravenously is not effective in altering ovarian function.
    d. The effects of letrozole on ovarian function are associated with an increase in circulating LH concentrations.

e. Due to the extremely prolonged release of letrozole from the injection site when given im, am alternative route of administration that allows for controlled termination of letrozole exposure may be needed to effectively control estradiol production and ovarian function in cattle.

f. Results further support the working hypothesis that letrozole has potential as a steroid-free option for the control of ovarian function for the purposes of fixed-time artificial insemination and embryo transfer.

2) Chapter 6: Non-steroidal aromatase inhibitor treatment with an intravaginal device and its effect on pre-ovulatory ovarian follicles in a bovine model.

a. Letrozole treatment during the ovulatory follicular wave results in more rapidly growing dominant follicles and larger ovulatory follicles.

b. Letrozole treatment during the ovulatory follicular wave by means of a gel-based intravaginal device delays ovulation of a single follicle by 24 h.

c. Letrozole treatment during the ovulatory follicular wave by means of a gel-based intravaginal device results in the formation of a CL that secreted higher levels of progesterone.

d. Letrozole treatment during the ovulatory follicular wave by means of a gel-based intravaginal device significantly reduces estradiol secretion and delays pre-ovulatory surge in estradiol.

e. A sustained-release intravaginal device has potential in the development of an aromatase inhibitor-based protocol for control of ovulation for herd synchronization.

f. The stimulatory effects of letrozole treatment on CL function has the potential of enhancing fertility by increasing circulating progesterone concentrations during the first 7 days post-ovulation in cattle.

3) Chapter 7: Formulation and testing of an intravaginal device for aromatase inhibitor delivery in cattle.
    a. Among the aromatase inhibitors tested (letrozole, anastrozole and fenbendazole) letrozole has greatest potency, as determined by inhibition of estradiol production *in vitro*.
    b. A wax-based vehicle, with higher melting point than the polymer-based vehicle used in Chapter 6, allows for a steady and continuous delivery of the active compound over the treatment period.
    c. Addition of a letrozole-containing gel coating improves initial absorption and hastens the increase on plasma concentrations of the active ingredient, while the letrozole-containing wax-based vehicle prolonged drug-delivery from the intravaginal device.
4) Chapter 8: Effect of aromatase inhibitor intravaginal devices on ovarian function in cattle.
    a. The vehicle used to deliver letrozole with an intravaginal device (wax plus gel coat vs wax alone) in cattle differentially affects ovarian function.
    b. Letrozole-impregnated intravaginal devices formulated with a wax base plus a gel coat vehicle effectively delivers elevated concentrations of letrozole in heifers and

reduces estradiol production resulting in increased follicular growth and lifespan, without adversely affecting progesterone production

c. Letrozole-impregnated intravaginal devices formulated with a wax base plus a gel coat vehicle is most suitable for the application of a letrozole-based protocol for the synchronization of ovulation in cattle.

5) Chapter 9: Synchronization of ovulation in cattle with an aromatase inhibitor-based protocol: a pilot study.

a. Addition of a letrozole-impregnated intravaginal device for 4 days, combined with PGF treatment at device removal and GnRH 24 h post-device removal increases the percentage of ovulations and synchrony of ovulation in cattle, regardless the stage of the estrous cycle at initiation of treatment.

b. Reduced luteal lifespan after letrozole treatment is reported for the first time. Further investigation is needed in order to elucidate the mechanism responsible for this observation.

c. Although the results obtained in the AI trial are disappointing, additional studies are needed in order to elucidate the mechanisms related with reduced fertility after letrozole-based synchronization of ovulation.

d. Adjustments of timing of AI and interval from PGF to GnRH treatment may need to be considered in order to improve pregnancy rates after letrozole-based synchronization protocols.

e. Letrozole treatment regimen presented in Chapter 9 has potential as a model to investigate the effect of estradiol deprivation on oocyte maturation and fertility *in vivo*.

The results of the experiments included in this thesis and the related literature lead us to conclude that the general hypothesis that letrozole has potential as a steroid-free option for the control of ovarian function for the purposes of fixed-time artificial insemination and embryo production was supported. However, further research will be needed in order to elucidate the effects of estradiol deprivation and letrozole treatment during the proestrous on oocyte competence and fertility of the resulting ovulations in cattle.

CHAPTER 12: FUTURE STUDIES

Based on the conclusions drawn and the limitations identified during the work presented in this thesis, I raise the following research questions for future consideration:

- Which non-steroidal follicular factors, if any, are preventing the increase in FSH secretion before and after follicular selection? Is inhibin involved in the lack of FSH surge after letrozole-induced estradiol deprivation in cattle?
- Is there a critical timing for the luteotrophic effects of letrozole to become evident? Is letrozole, given at different stages on the estrous cycle, detrimental to luteal function?
- Can a long-term treatment with letrozole stimulate the growth of subordinate follicles to ovulatory size? Can these subordinate follicles be induced to ovulate?
- Can aromatase inhibitors be used in order to reduce the amount of exogenous gonadotropin needed for ovarian superstimulation in cattle?
- Is estradiol required for the normal growth and maturation of the dominant follicle? Are the concentration of intrafollicular factors different between letrozole treated and untreated animals?
- How does estradiol deprivation alter gonadotropin secretion patterns? Does it affect it at hypothalamic level? At pituitary level?
- Does inhibition of estradiol secretion during proestrus affect oocyte competence? Are those oocytes matured *in vivo* under low estradiol fertilizable *in vitro?*
- Does inhibition of estradiol secretion during proestrus affect sperm transport post-service?

- Does inhibition of estradiol secretion during proestrus alter uterine environment and embryo survival? Does pre-ovulatory treatment with letrozole in recipient cows affect embryo survival?
- Can these results obtained in heifers be replicated in other farm animals or women?

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