ABSTRACT

WOCK, JILLENE MARIE. Effect of GnRH compared to Estradiol-17β at beginning of Superovulation Protocol on Superovulatory Response and Embryo Quality. (Under the direction of Dr. Mitchell Hockett).

Success of a superovulatory protocol is largely determined by the ability to avoid the influence of the dominant follicle. The objective of this study was to determine if GnRH plus CiDR administration at the onset of the superovulatory protocol was a viable alternative to the traditional injection of Estradiol/Progesterone with CiDR. Animals were divided into lactating cows, dry cows, and heifers. All animals did not receive the same FSH dose, therefore animals were further subclassified as HIGH FSH (≥300mg) or LOWFSH (<300mg). There were no heifers on high FSH. Animals receiving GnRH had a CiDR inserted at a random time in the estrous cycle (day 0) and received i.m. injection of GnRH on day 3. A commercial gonadotropin, FSH was adminstered in decreasing doses on days 5-8. The CiDR was removed on day 8, and $PGF_{2\alpha}$ was administered AM and PM of day 8. Animals treated with E_2/P_4 were injected with 2.5 mg E_2/50 mg of P_4 by intramuscular (IM) injection and received a CiDR on a random day of estrous cycle (day 0). FSH was administered in decreasing doses on days 4-7. The CiDR device was removed on day 7, and 25 mg of $PGF_{2\alpha}$ was administered AM and PM of day 7. All animals were artificially inseminated at standing heat, and at 6 hour intervals during estrus. Animals not displaying estrus were inseminated 48 hours following CiDR removal. Seven days following estrus, embryos were collected. Total number of embryos recovered, the percent of total embryos fertilized, the total number of embryos recovered that were IETS grade 1 & 2 embryos, and the percent of total embryos recovered that were IETS grade 1 & 2 were recorded. Analysis of data resulted in no statistical differences ($P>0.05$) between E_2 and GnRH treatments for

number embryos recovered (10.86 ± 0.97 vs 11.14 ± 0.99), the number of total embryos recovered that were grade 1 and 2 embryos (5.65 ± 0.66 vs 5.64 ± 0.68), percentage fertilized (59.09 ± 4.15% vs 56.43 ± 4.38%), and percentage of grade 1 and 2 embryos (50.54 ± 4.02% vs 50.53 ± 4.2%), respectively.

Superovulatory protocols utilizing GnRH to turn over the dominant follicle is an effective protocol compared to protocols utilizing E_2/P_4.

Effect of GnRH compared to Estradiol-17β at beginning of Superovulation Protocol on Superovulatory Response and Embryo Quality

by
Jillene Marie Wock

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APPROVED BY:

Charlotte E. Farin	William L. Flowers	C. Scott Whisnant

Mitchell E. Hockett
Chair of Advisory Committee

BIOGRAPHY

Born November 12, 1982 in Dickinson, North Dakota, Jillene Marie Wock is the eldest daughter of James Wock and Jill Kuchenski. Jillene has two younger brothers, Brandon (18) and Jeremy (16) Wock, whom are both very important to Jillene.

Jillene grew up mostly in North Carolina, moving from various places, but finally spent most of her adolescent years in Selma, North Carolina. She attended Selma Elementary, Selma Middle School and finally finished at Smithfield-Selma High and North Johnston High (last year of high school) in 2001. Overall, a well-rounded student, she played soccer, ran cross country and ran indoor track. In addition to sports, she maintained a high academic standing and volunteered through her church. Even as a young girl, Jillene loved animals and knew she wanted to work with them in some way. She applied and was accepted to North Carolina State University, where she concentrated on exploring her options in the world of animal science. After taking Dr. Mitch Hockett's reproductive physiology course, she decided not only that she did quite well in the class, but she really enjoyed all aspects of reproduction, including endocrinology and assisted reproductive techniques. She continued to further enhance her knowledge in that concentration by taking Dr. Charlotte Farin's Advanced Reproductive Physiology class. She worked with equine enthusiasts such as Liv Sandberg, assisting in the Equine Management course and Dr. Carlos Pintos at the NCSU Veterinary School on a research project examining equine semen extenders. With an intent to go to vet school, her ambitions quickly turned towards graduate school because she was interested in doing research in the field of reproduction. She was accepted into the

graduate school in Animal Science at North Carolina State University. Jillene worked with Dr. Mitch Hockett in the Department of Animal Science with a concentration in reproductive physiology towards a Master of Science degree. She worked for two years on a project involving superovulation and the comparison of protocols using estradiol or GnRH. At this point in her life, Jillene is searching for her dream job to work with reproductive techniques in humans either as research or in a commercial setting. Eventually, she would like to go back to school at Colorado State University to obtain her doctorate in reproductive physiology.

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A person's accomplishments are not only achieved through one person, but the many people that are there for support, motivation, and encouragement. While there are countless helpful hands that deserve my appreciation, I can mention a few individuals that have been supportive in my program.

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I would like to thank all the other graduate students that have shared the same struggles and accomplishments as I have. We all have taught our share of great labs and bad; we all have had our share of late nights or early mornings in the lab; and we have all had our struggles in maintaining a high academic standard. Seeing those struggles and successes has encouraged me to continue to push forward and look upward.

Within the past two years, I have met some of the most supportive and understanding people that I call friends. Even a life as busy as mine, my friends still manage to be encouraging during the times of hardship as well as during the times of celebration. My motorcycle friends have also given me something to take my mind away from school through group rides. My significant other, Troy Brooks has undoubtedly supported everything that I have accomplished as well as encouraged me when times were hard. The little things he has done such as take care of my dog and bring me food while writing means a lot to me.

I want to express my appreciation for my family: my mom, my dad and my two brothers for their faith in me. Without that, it would be hard to continue to focus on what is really important: my education and my career. As a woman, my mom has always taught me that I need to work hard for what I want and do it on my own. And to this day, I have always lived by that standard. My parents' love and compassion has helped drive my success.

Finally, I make my greatest attribution to God. It's through Him I find strength to do anything. I have always lived through this belief and to this day, I feel I could never be more successful.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

Introduction

While dairy numbers continue to decrease nationwide, milk production continues to increase. The USDA reported that milk production doubled from 11,206 pounds per cow per year in 1977 to 20,267 in 2007 (USDA, 2008). Cow numbers declined from about 10.9 million cows in 1977 to 9.2 million cows in 2007 (USDA, 2008).

Much improvement has been made in herd management practices such as feeding, milking procedures and stress management strategies (Foote, 1996). These improvements have led to an increase in herd size. Improvements in basic management practices have included improvements in reproductive management practices (Foote, 1996). The goal in reproductive management is to maximize the genetic output, production, and productive life of superior animals.

Assisted reproductive techniques (ART) have been instrumental for increasing genetic potential of milk production in the dairy cattle industry. These techniques are useful in increasing the genetic superiority in female animals and greatly increase the number of offspring of superior animals. These technologies are used to artificially manipulate the length of estrous cycles, time of ovulation, or numbers of follicles that can potentially grow and ovulate (Roche et al., 1999; Ireland et al., 2000). Multiple ovulation and embryo transfer (MOET) has become popular for producing many embryos from a superior animal and transferring them into less valuable recipient animals. Effective superovulation protocols are required for an efficient embryo transfer program in dairy cattle.

The purpose of superovulation is to maximize the growth of subordinate follicles and

obtain fertilized and transferable embryos that are able to produce pregnancies (Armstrong, 1993). Superovulation protocols attempt to synchronize follicular waves by the use of ablation of the dominant follicle or hormonal manipulation and optimize the super stimulatory response by engaging treatments that support follicular growth in the absence of the dominant follicle (Adams et al., 1994).

Embryo transfer is a technique that allows for the transfer of embryos from a donor animal to a recipient animal. According to Bo et al. (2002), over 500,000 embryos were transferred in 2002 and that number continues to grow each year. Embryo transfer can be performed on farms with the most basic technology to farms with more advanced technology. However, high variability still remains in ovulation response in cattle and embryo quality in the most advanced reproductive programs (Lerner et al., 1986; Mapletoft, 2002; Bo et al., 2002).

Understanding follicular dynamics allows for the manipulation of the estrous cycle in dairy cattle. Bo et al. (2002) reported that the use of Estradiol-17β or synthetic estrogen (E2) in combination with progesterone has been practiced for about 3 decades. Estradiol-17β is a compound given to animals to turn over the dominant follicle. Unfortunately, estradiol-17β is a steroid hormone and recently has shown to have environmental effects in rivers, lakes and other areas where animals can contaminate the environment (Hanselman et al., 2003). Estrogenic compounds such as estradiol, estrone and estriol are all found in livestock feces (Hanselman et al., 2003). Livestock feces can potentially contaminate the ground water and surface resources (Raman et al., 2001; Hanselman et al., 2003). According to Raman et al.

(2001), dairy production of environmental estrogens amount to 1000µg d^{-1} per cow and a mass flux of excreted estrogen as high as 9kg d-1. There is also concern that estrogenic compounds may enter milk and meat infected for human consumption. The Food and Drug Administration (FDA) states that no estrogenic compounds are to be given to lactating animals (FDA, 2008). This conundrum has lead to the development of alternative protocols to turn over the dominant follicle. Thompson et al. (1999) reported in beef cattle that gonadotropin-releasing hormone (GnRH) will induce ovulation or luteinization of the largest follicle at the time of treatment in estrus synchronization protocols. This property of GnRH would make it useful in superovulation to induce turnover of the dominant follicle. Additionally, GnRH is approved for the use in reproductive management in lactating dairy cows. The objective of this study was to develop a superovulation protocol alternative that utilized GnRH to replace estrogenic compounds for turnover of the dominant follicle. A thorough understanding of the hormonal profile, reproductive endocrinology and the physiology and the bovine estrous cycle enables reproductive specialists to practice assisted reproductive techniques (ART) and more specifically, superovulation in dairy cattle.

Literature Review

The Hormonal Profile of the Estrous Cycle

The normal estrous cycle length of dairy cattle averages 21 days with a normal range of 17 to 25 days (Bartol et al., 1981; Tucker, 1982). The length of the estrous cycle is determined by the number of follicular waves in the cycle (Fortune, 1994). A shorter estrous cycle will have fewer follicular waves, and a longer estrous cycle will have more follicular waves (Fortune, 1994; Evans, 2003). The estrous cycle is characterized by a series of events of hormonal regulation leading to changes in the female reproductive tract in order to establish an ideal environment for a conceptus (Bartol et al., 1981; Tucker, 1982). The estrous cycle is divided into 4 stages: Proestrus; Estrus; Metestrus; and Diestrus and 2 phases, including luteal and follicular (Goodman and Karsch, 1980). The corpus luteum (CL) is the dominant ovarian structure, and progesterone (P_4) is the dominant hormone for most of the luteal phase (Goodman and Karsch, 1980; Rahe et al, 1980). The concentration of P_4 gradually increases from day 4 after ovulation, reaches and plateaus at day 12 and drops between days 15 and 20 (Henricks et al., 1971; Schams et al., 1977; Rahe et al., 1980). By Day 16 of the estrous cycle, plasma P_4 concentration is well above 4ng/ml; but by day 17, plasma P_4 concentrations drop below 3 ng/ml (Henricks et al., 1971; Snook et al., 1971). Progesterone concentrations must decrease before concentrations of estrogen can increase (Henricks et al., 1971).

The follicular phase is marked by an increase in E_2 secreted by the dominant follicle,

which occurs between days 15 and 20 (Henricks et al., 1971; Goodman & Karsch, 1980; Rahe et al., 1980). Estrogen is usually present during proestrus, estrous (Henricks et al., 1971; Ireland et al., 1984) and the beginning of the early luteal phase (Hansel & Echernkamp, 1972; Ireland et al., 1984). Concentrations of E_2 are significantly higher on Day 5 when dominance is established in the first wave of follicles compared to the level at Day 3 (Lucy et al., 1992; Fortune et al, 2001). Concentrations of E_2 and P_4 are regulated through feedback systems, which allow for ovulation to occur (Goodman & Karsch, 1980; Hansel & Convey, 1983). Ovulation is induced by the luteinizing hormone (LH) surge (Goodman & Karsch, 1980; Hansel and Convey, 1983) which occurs approximately 30 hours after the onset of estrus (Rahe et al., 1980). Gonadotropin-Releasing Hormone is released from the hypothalamus to stimulate the release of LH from the anterior pituitary (Clarke & Cummings, 1982; Lucy et al., 1992). Clarke and Cummings (1982) reported that when jugular samples were assayed for GnRH and plasma LH from ewes, LH and GnRH pulsatility always occurred in the same sampling interval. As a result, LH pulsatility is parallel with GnRH pulsatility (Clarke & Cummings, 1982). Concentrations of E_2 and P_4 also work to control LH pulsatility (Goodman & Karsch, 1980). Luteinizing Hormone pulsatility increases, allowing for an increase in E_2 concentration (Silvia et al., 1991; Lucy et al., 1992). According to evidence supported by the work of Henricks et al. (1971), in order for a preovulatory rise in LH, there must be a rise in E_2 and a fall in P_4. Luteinizing Hormone is released at a high amplitude and low frequency pulses when P_4 is high during the luteal phase (Goodman & Karsch, 1980). Luteinizing Hormone is released in low amplitude and

high frequency pulses when P_4 is low and this occurs during the follicular stage (Goodman & Karsch, 1980; Rahe et al., 1980). The dominant follicle of the first, and sometimes the second wave, becomes atretic because of the low frequency pulsatile release of LH (Rahe et al, 1980; Lucy et al., 1992).

A slight decline in P_4 allows for the secretion of the hormone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which is responsible for luteal regression (McCracken et al., 1972; Silvia et al., 1991). The CL then regresses and P_4 concentrations decline (Silvia et al., 1991). A decrease in P_4 concentration causes an increase in LH pulsatility and this leads to an increase in E_2 secreted by ovulatory follicles (Henricks et al., 1971; Holst et al., 1972).

Reproductive Endocrinology

Hormones are bioactive molecules that control a wide variety of functions in the body including, but not limiting to, reproductive processes such as puberty, gametogenesis and other sexual functions (Chapin et al., 1996, Crisp et al., 1998). Hormones are secreted in small amounts and target specific binding receptors that induce some biological function (Crisp et al., 1998). Regulation of these hormones begins in the hypothalamus of the brain, and extends to the anterior and posterior pituitary (Harris, 1948; Schally, Arimura & Kasin, 1973). The pars distalis, the pars tuberalis and the pars intermedia make up the adenohypophysis, which means glandular lobe (Harris, 1948). Hormones from the hypothalamus reach the anterior pituitary via the release of synthesized hormones from the nerve endings in the hypothalamic nerve fibers into the capillaries in the median eminence

(Harris, 1948; Schally, Arimura & Kasin, 1973). The capillaries run into portal vessels that carry the hormones to the anterior pituitary, where other hormones are released into the body via the blood (Harris, 1948; Schally, Arimura & Kasin, 1973). Endocrine glands such as the ovary, testis, adrenal cortex and the thyroid are all affected by the release of anterior pituitary hormones (Harris, 1948; Schally, Arimura & Kasin, 1973). The posterior pituitary or the "neurohypophysis" includes the infundibulum and infundibular process which connect a neural supply of hormones from the hypothalamus to the posterior pituitary (Harris, 1948). Only vasopressin and oxytocin are released from the posterior pituitary (Harris, 1948; Schally, Arimura & Kasin, 1973). Reproductive hormones of importance include GnRH, the gonadotropins, LH, and Follicle Stimulating Hormone (FSH); the steroid hormones, E_2 and P_4; and prostaglandin ($PGF_{2\alpha}$). The study of these reproductive hormones and their origin, chemical makeup, synthesis, and their function has been critical in clinical and commercial use.

Gonadotropin-Releasing Hormone

A highly conserved neuro-decapeptide, luteinizing hormone-releasing hormone (LHRH), more recently referred to as GnRH is a hormone that controls reproduction in all vertebrates (Wetsel et al., 1993; Peng et al., 1994; Schneider et al., 2006). From an evolutionary stand point, the molecule is present in all vertebrates and some invertebrates, indicating that this is an ancient hormone (Carolsfeld et al, 2000). A relatively small molecule (Nikitovitch-Winer et al, 1963), GnRH is 10 amino acids in length (Carolsfeld et al,

2000). There are 12 known forms of the peptide among different species with identical residues in positions 1,4,9, and 10 (Carolsfeld et al, 2000). The main form of GnRH found in mammals is classified as mGnRH (Carolsfeld et al, 2000). Gonadotropin-releasing hormone was partially purified by Nikitovitch-Winer and others (1963) through a series of experiments designed to classify the size and the stability of GnRH. Gonadotropin-releasing hormone is released from neurons scattered along the basal forebrain, which are localized primarily in the septum, preoptic area and anterior lobe in the hypothalamus (Wetsel et al, 1993). From the hypothalamus, GnRH travels to the anterior pituitary via the portal system to stimulate the release of the gonadotropins, LH and FSH (Peng et al, 1994; Yasin et al, 1995). Gonadotropin-releasing hormone is released in a pulsatile fashion and its effects are determined by amplitude and frequency (Yasin et al, 1995; Evans et al, 1994; Terasawa, 1998). According to Evans et al. (1994), a preovulatory increase in estradiol causes a surge-like release in GnRH, which is followed by an ovulatory surge of LH. An increase in GnRH pulsatilty causes an increase in the GnRH receptor number and this leads to the pre-ovulatory LH surge (Yasin et al, 1995). Baranov et al. (1967) concluded that the activity of GnRH in the median eminence and the pituitary stalk of rats are coordinated with the activity of LH in the anterior pituitary. The maximum content of GnRH in the median eminence and the pituitary stalk and LH in the pituitary was shown in the proestrus stage (Baranov et al., 1967).

Luteinizing Hormone

Luteinizing Hormone is a glycoprotein released from the anterior pituitary (Milvae & Hinckley, 1996) and is regulated through the negative feedback mechanisms of estrogen and progesterone (Kinder et al; 1996; Milvae & Hinckley, 1996; Fortune, 1994). Luteinizing Hormone activity is expressed through interaction of an alpha and beta subunit (Pierce & Parsons, 1981; Baeziger & Greene, 1988). The alpha subunit is the same among all other glycoproteins and the beta subunit defines the biological activity of the hormone (Pierce & Parsons, 1981; Baeziger & Greene, 1988). Luteinizing Hormone is secreted at low levels throughout the bovine estrous cycle, except for the large ovulatory surge at the end of the last follicular wave (Milvae & Hinckley, 1996). At this point, the follicle swells, ovulates and forms the CL (Milvae & Hinckley, 1996) According to Lucy et al. (1992), "the dominant follicle in the first wave becomes atretic because of the decrease in LH that occurs in the middle of the estrous cycle." There is an increase in estradiol in these follicles and decreased androgen in follicular fluid compared to that of subordinate follicles (Fortune, 1994). If progesterone remains at basal levels, then these follicles cannot continue to release estrogen and will regress allowing for the next surge of FSH and the development of a new wave (Fortune, 1994; Lucy et al, 1992). The corpus luteum that is formed is lysed by the release of pulses of $PGF_{2\alpha}$ from the uterus (Milvae & Hinckley, 1996; Adams et al., 2008). During luteolysis of the ovulatory follicle, progesterone falls, causing a loss in the negative feedback of the LH regulating hormones (Milvae & Hinckley, 1996). Luteinizing Hormone slowly rises, causing an increase in estrogen from the mature pre-ovulatory follicle (Fortune,

1994; Milvae & Hinckley, 1996). The increase in estrogen leads to a positive feedback between E_2 and LH and causes the LH surge, which leads to ovulation of the follicle, (Milvae & Hinckley, 1996; Adams et al., 1999).

Follicle Stimulating Hormone

Follicle Stimulating Hormone is a glycoprotein synthesized in the anterior pituitary and secreted to support the growth of immature ovarian follicles and granulosa cell development (Catt & Pierce, 1986; Dahl & Stone, 1992; Leeuw et al, 1996; O'Shaughnessy, Dudley & Rajapaksha, 1996). Follicle stimulating hormone is responsible for reproductive processes such as gametogensis, follicular development and ovulation (Dahl & Stone, 1992; Leeuw et al, 1996). Follicle stimulating hormone is found in lower vertebrates such as teleost fish and all mammals (Pierce & Parsons, 1981). Follicle stimulating hormone is a non-covalently linked heterodimer of α and β subunits (Pierce & Parsons, 1981; Baeziger & Greene, 1988; Dahl & Stone, 1992; Leeuw et al, 1996). The highly conserved α subunit is the same amino acid sequence for each glycoprotein, and the hormone specific β subunit indicates the biological activity of the hormone (Pierce & Parsons, 1981; Baeziger & Greene, 1988; Dahl & Stone, 1992; Leeuw et al, 1996). Follicle stimulating hormone isoforms have been isolated and characterized in the anterior pituitary, glands, serum, and urine (Leeuw et al., 1996). Follicle stimulating hormone works through intracellular second messenger systems such as adenylate cyclase in response to various hypothalamic and gonadal factors to target specific binding receptor sites on granulosa cells (Dahl & Stone, 1992; Leeuw et al.,

1996). Receptor binding activity, biological activity and metabolic activity are affected by the pH differences of the FSH iso-forms (Leeuw et al., 1996). More acidic FSH iso-forms have lower binding affinity; however, because of the lower clearance rate, they have greater in-vivo biological activities (Leeuw et al., 1996). Follicle stimulating hormone secretion is influenced by the release of gonadal proteins, inhibin and activin (Dahl & Stone, 1992). Inhibin is released in response to FSH, feeds back on the pituitary and inhibits the release of FSH (Dahl & Stone, 1992). Activin, however, works to stimulate FSH release (Dahl & Stone, 1992). Follicle stimulating hormone works with LH to develop follicular waves and cause ovulation (Turzillo & Fortune, 1990). A rise in FSH in the cycle indicates that a new wave has emerged (Turzillo & Fortune, 1990). Follicle stimulating hormone can be used to super-stimulate ovaries to produce many ovulatory follicles for collection by preventing atresia of subordinate follicles (Mapletoft et al. 2002).

Estrogens

Estrogens are important for a number of physiological processes, including reproductive events such as growth, differentiation and function of tissues in the reproductive tracts of females. (Katzenellenbogen, 1996; Osborne et al, 2000). Estradiol targets female reproductive tissues such as the mammary glands, uterus, vagina and ovaries (Katzenellenbogen, 1996; Osborne et al, 2000). Estrogens are a part of the steroid hormone family and are mediated through the estrogen receptor (ER), which has a hormone binding site of 250 amino acids (Katzenellenbogen, 1996; Parker et al, 1993; Osborne et al, 2000).

They function as a ligand-activated transcriptional complex that shares a common structural and functional organization of ligand binding, protein dimerization and transcriptional activation (Katzenellenbogen, 1996; Parker et al, 1993; Osborne et al, 2000). Estrogens influence gene expression by diffusing through the cell and binding to the nucleus which causes a dimerization of the receptors and facilitates the promoter region of the gene to begin gene transcription (Katzenellenbogen, 1996; Osborne et al, 2000). There are many sources of estrogen release; however, from a reproductive standpoint in the female tract, the ovarian follicle is the major source of estradiol synthesis (Spicer & Echternkamp, 1985; Richards & Hedin, 1988). Estrogen synergizes with LH and FSH in granulosa cells by phosphorylation of the estrogen receptor (Richards & Hedin, 1988). This mechanism is required for the emergence and development of follicular waves and finally the development of the preovulatory follicle (Richards & Hedin, 1988; Richards et al., 2002). Estrogen has been shown to increase GnRH receptor mRNAs for GnRH synthesis (Yasin et al., 1995). It has been proven that estrogen will also induce follicle atresia (Hutz et al, 1988; Bo et al, 1993).

Progesterone

Progesterone is a steroid hormone responsible for the maintenance of the female reproductive tract (Graham & Clarke, 1997; Williams & Sigler, 1998; Mulac-Jericevic, 2000; Rueda et al, 2000). Progesterone actions are mediated through specific intracellular progesterone receptors (PR) that are members of the transcriptional nuclear receptor superfamily (Graham & Clarke, 1997; Williams & Sigler, 1998; Mulac-Jericevic, 2000;

Rueda et al, 2000). In mammals, progesterone functions to support the uterus and ovary in releasing mature oocytes, facilitate implantation and maintenance of pregnancy, to assist in mammary development, and milk secretion and mediation in sexual behavior in the brain (Graham & Clarke, 1997; Rueda et al, 2000). Progesterone receptors are influenced by the release of estrogen in most target tissues (Graham & Clarke, 1997; Williams & Sigler, 1998). Progesterone is synthesized and secreted from the ovary as the primordial follicles are the main site for cyclic hormonal changes in the estrous cycle (Graham & Clarke, 1997; Rueda et al, 2000). Once the follicle ruptures, the corpus luteum is formed and becomes the major endocrine organ for the release of progesterone (Graham & Clarke, 1997; Rueda et al, 2000). A number of hormones such as LH, $PGF_{2\alpha}$, FSH and Prolactin (PRL) all influence progesterone's release from the corpus luteum (Graham & Clarke, 1997; Rueda et al, 2000). The estrous cycle can be lengthened by progesterone by extending the luteal phase and this prolongs both morphological and functional follicular dominance (Sirios & Fortune, 1990).

Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ is made from arachidonic acid and causes luteal regression in non-pregnant ruminants (Basu & Kindahl, 1987; Silvia et al., 1991; Skarzynski & Okuda, 1999). Prostaglandin $F_{2\alpha}$ is released in a pulsitile fashion from the uterus in intervals of about 6-8 hours and reaches the ovary through the counter current mechanism in the utero-ovarian broad ligament (Basu & Kindahl, 1987; Silvia et al, 1991; Skarzynski & Okuda, 1999). Pulsatile release of $PGF_{2\alpha}$ occurs when a pulse generator is present (Silvia et al., 1991). The

pulse generator is the source of signal that "initiates each episode of secretion (pulse)" and it requires a uterus capable of responding to $PGF_{2\alpha}$ release (Silvia et al., 1991). There are several proposed hypotheses that may account for the responsiveness to $PGF_{2\alpha}$ (Silvia et al., 1991). Prostaglandin $F_{2\alpha}$ is stimulated by pulses of oxytocin (Roberts et al, 1990; Silvia et al., 1991). The rapid effect of oxytocin on $PGF_{2\alpha}$ causes the pulsitile nature of the hormone (Flint et al., 1986). Several changes that take place in the uterine tissue make it responsive to oxytocin (Silvia et al., 1991). The uterine response to oxytocin may be due to prostaglandin H (PGH) endoperoxide synthase (PGH synthase; cyclooxygenase) enzyme (Silvia, 1991). According to Basu & Kindahl (1987), there is a decrease in the intracellular inhibitor of PGH synthase late in the estrous cycle which is between days 14 and 17 at the same time of $PGF_{2\alpha}$ release. Luteal oxytocin and $PGF_{2\alpha}$ are involved in a positive feedback in which oxytocin is released from the corpus luteum and is initiated with high concentrations of each (Roberts et al, 1990; Silvia et al., 1991). Oxytocin receptors are found in both the uterus and the mammary gland (Soloff, 1975; Sheldrick & Flint, 1985) and receptor numbers in the endometrium are high around the time that luteal regression occurs (Sheldrick & Flint, 1985; Flint et al., 1986; Fuchs et al., 1990). The uterus can properly respond when there are receptors for the pulse generator's signal (Silvia et al., 1991). The signal is responsible for the intracellular signaling system by linking the pulse generator's receptor to the effector enzymes responsible for the movement of stored arachidonic acid or the metabolism of free arachidonic acid to $PGF_{2\alpha}$ (Silvia et al., 1991). An increase in estrogen concentration and a decline in progesterone concentration results in an increase oxytocin receptors (Soloff, 1975,

Sheldrick & Flint, 1985). Flint et al. (1986) raised the oxytocin receptor numbers through estradiol and progesterone treatments and this stimulated the hydrolysis of phosphoinositides to produce inositol phosphate, diacylglycerol. Diacyglycerol is then further hydrolysed to form arachidonic acid, and this encourages $PGF_{2\alpha}$ synthesis (Flint et al., 1986; Fuchs et al., 1990).

Reproductive Physiology

A comprehensive understanding of the reproductive hormones is necessary to understand the reproductive physiology of the processes needed to prepare the reproductive system for ovulation and finally pregnancy if an animal becomes pregnant. Changes in the estrous cycle of animals allow for the growth of follicular waves; follicular dynamics such as the recruitment, selection and dominance of follicular waves; ovulation and finally the development of the embryo before it is collected for embryo transfer.

Follicular Waves

The purpose of the female gonad is to support the differentiation, growth and release of the mature oocyte in preparation for fertilization (McGee et al., 2000.) The gonads act as chemical support for maintenance of the reproductive cycle of the female such as pregnancy support, placentation, gestation, parturition and regular maintenance of the estrous cycle, (McGee et al., 2000.) Folliculogenesis is defined as "formation of Graafian (mature, provulatory) follicles from a pool of primordial (non-growing) follicles" (Spicer and

Echternkamp, 1986). Follicular waves develop during the bovine estrous cycle leading up to ovulation. A complete follicular wave constitutes as the synchronous development of about 3-6 small follicles, beginning at the size of 4 to 5 mm, with the selection of a dominant follicle (Pierson & Ginther,1984; Ginther, Kastelic & Knopf, 1989; Lucy et al., 1992; Bo et al., 1993; Adams et al., 1999; Hendriksen, et al., 2000; Fortune et al., 2001; Evans, 2003; Lucy, 2007). Sirois and Fortune (1988) showed through a constant observation of follicular waves via ultrasound, that bovine have anywhere from one to four waves (Sirois and Fortune, 1988; Ginther, Kastelic & Knopf, 1989). Furthermore, studies have shown that 95% of bovine estrous cycles contain two or three waves (Rajakoski, 1960; Ginther, Kastelic & Knopf, 1989; Barros et al., 2001; Lucy et al., 1992; Adams et al., 1999; Evans, 2003; Lucy, 2007). Follicular waves usually last about 8 to 10 days for each wave (Sirois and Fortune, 1988; Lucy et al., 1992; Barros et al., 2001). An animal with 2 follicular waves will have emergence of waves at approximately day 2 and day 10 (Rajakoski, 1960; Sirois and Fortune; 1988; Ko et al., 1991; Evans, 2003). An animal with 3 waves will have wave emergence at around days 2, 9, and 16 (Sirois and Fortune, 1988; Evans, 2003). Adams et al. (2008) suggested that a 2 versus 3 wave animal is dependent on the development of the dominant follicle in Wave 1. In three- wave animals, the dominant follicle in the first wave lasts three days longer than a two-wave animal (Adams et al., 2008). A fourth wave is observed and is characterized by delayed luteolysis or failure to ovulate (Adams, 1999; Evans, 2003).

Follicular Dynamics

Follicles develop through a series of stages, noted as "Follicular Dynamics" (Lucy et al., 1992). A life history of follicles includes endowment and maintenance, initial recruitment, maturation, atresia or cyclic recruitment, ovulation, and exhaustion (Hodgen, 1982; McGee et al., 2000; Fortune et al., 2001). When the follicle resumes development, the follicle will either undergo ovulation or atresia (Spicer and Echternkamp, 1985; Fortune, 1994; Fortune et al., 2001). This cycle begins at the onset of puberty and continues until the pool of resting follicles lead to ovarian exhaustion (Spicer and Echternkamp, 1985; Mc Gee et al., 2000). The oocyte, at this point, is finished growing and is mature enough to undergo germinal vesicle breakdown (Mc Gee et al., 2000). "Follicular Dynamics" is described in three steps: 1) Recruitment of antral follicles, 2) Selection of potential ovulatory follicles and 3) Dominance of ovulatory follicles (Lucy et al., 1992).

Recruitment of Primordial Follicles

Recruitment is the first step in "Follicular Dynamics" and is the process of continual growth and regression of antral follicles that leads to the development of the pre-ovulatory follicle (Lucy et al., 1992; Fortune, 1994; Ireland et al., 2000). Recruitment of the follicles includes two stages: initial recruitment and the cyclic recruitment (Mc Gee et al., 2000). Initial recruitment includes endowing primordial follicles early in life (133,000 on average in bovine) and maintaining them in a resting state (Erickson, 1966; Spicer & Echternkamp, 1985; Avery et al., 1998; Mc Gee et al., 2000). Follicles will develop through primordial, primary and secondary stages before entering the cyclic recruitment (Lucy, 2007). Initial

recruitment may be due to a release from inhibitory stimuli that maintain the resting follicles in stasis or prophase I of meiosis (Fortune, 1994; Avery et al., 1998; Mc Gee et al., 2000). The resting follicles will consist of a flat layer of granulosa cells (Fortune, 1994; Avery et al., 1998; Mc Gee et al., 2000). The germinal vesicle is the resting nucleus (Avery et al., 1998). The hormones responsible for the initiation of growth are still unknown (Savio, Boland & Roche, 1990; Mc Gee et al., 2000; Ireland et al., 2000). Often primordial follicles undergo atresia, or follicle death (Spicer & Echternkamp, 1985; Lucy, 2007). Even though this is a dormant state for follicles, it is continuous throughout life and begins after follicle formation (Fortune, 1994; Spicer & Echternkamp, 1985). The oocyte at this point begins to grow, but is still immature for germinal vesicle breakdown (Mc Gee et al., 2000). If gonadal stimulation is optimal, follicles are then "rescued" into cyclic recruitment (Fortune et al., 2001; Spicer & Echternkamp, 1985). During cyclic recruitment, a cohort of follicles is chosen to progress to a pathway of ovulation depending on the role of gonadotropins (Lucy et al., 1992; Fortune, 1994; Ireland et al., 2000: Fortune et al., 2001). At this point the recruited follicle becomes dependant on gonadotropins for both the emergence and the growth of follicular waves after the dominant follicle turns over (Ireland et al., 2000; Fortune, 1994; Mc Gee et al., 2000). Follicle Stimulating Hormone (FSH) is a gonadotropin that is required for all subordinate follicles to grow (Adams, 1999; Ireland et al., 2000; Fortune et al., 2001). Concentrations of FSH surge on the day of ovulation (day 0), as well as precede the emergence of any of the waves (Dodson, 1978; Walters & Shallenberger, 1984; Adams et al., 1992; Adams et al., 2007).

Selection of Recruited Follicles

Selection is the process of reducing the recruitment of follicles to a "species-specific ovulatory quota" (Lucy et al., 1992; Ireland et al., 2000). Selected follicles begin to secrete estrogen, which begin feedback mechanisms that regulate the gonadotropins FSH and LH during the cycle (Fortune, 1994; Fortune et al., 2001). Follicle stimulating hormone is crucial for follicular development and LH is crucial for ovulatory follicles (Fortune, 1994; Fortune et al., 2001). Towards the end of a wave, when ovulation or follicle turn over occurs, circulating FSH concentrations increase 1.5 to 2 times over the following 2 days and peak about 12 to 24 hours before the emergence of a follicular wave (Adams et al., 2008). This corresponds with the development of the dominant follicle size at 4-5 mm (Adams et al., 2008) and ends with the selection phase of follicular dynamics (Ireland et al., 2000).

Dominance of Ovulatory Follicles

The final stage of follicular dynamics is dominance (Fortune, 1994). At this point, there is an emergence of the largest follicle that is 4 mm or 5 mm in diameter (Fortune, 1994; Ginther et al., 2000; Ireland et al., 2000; Barros and Nogueira, 2001; Fortune et al., 2001; Mapletoft et al., 2002). The second largest follicle emerges about 6 to 7 hours after the first largest follicle and they continue to grow in synchrony with each other until the largest follicle reaches about 8.5mm (Ginther et al., 2000). The mean size of the dominant follicle in 2 wave animals has been reported to be about 13mm and in three wave animals, the dominant follicles in each respective wave grow to about 12mm, 10mm and 13mm (Sirois & Fortune; 1988; Evans, 2003). Ireland et al. (2000) state that the dominant follicle is defined by these

criteria: “1) the dominant follicle in a wave is at least 1 to 2 mm larger than the next largest follicle, and 2) growth of all subordinate follicles in the same wave ceases.” Divergence occurs when the largest subordinate follicle stops growing (Ginther et al., 1996; Fortune et al., 2001). Ginther et al. (1996) suggested that the largest subordinate follicle, at one time in development, could have been larger. However, the growth of the largest follicle is much faster than the second largest follicle at the time of divergence (Ginther et al., 1996). The atresia of the selected follicles correlates with the assurance of the development of one dominant follicle at the time of ideal hormone levels in circulation (Fortune, 1994; Fortune et al., 2001). For example, the largest subordinate follicle has the potential to become the dominant follicle if the original follicle dies or is destroyed (Adams et al., 1993; Fortune, 1994). Divergence of the dominant follicle is characterized by decreasing FSH concentrations, a decrease in size of the largest subordinate follicle and the dominant follicle’s switch of dependency from FSH to LH. This shift in hormone dependency allows the dominant follicle to prevent the growth of subordinate follicles by releasing inhibin and estrogen, and this has no effect on LH (Lucy et al., 1992; Fortune et al., 2001; O’Shea et al., 1994; Fortune et al., 2001, Lucy, 2007). The increase in inhibin and estradiol inhibits FSH synthesis and secretion from the anterior pituitary (Spicer & Echternkamp, 1986; O’Shea et al., 1994; Ginther et al., 1996). Insulin-like growth factors (IGF) and follistatin are two other products produced by follicles that regulate FSH synthesis (Singh et al., 1999). The combination of these factors causes atresia or a decline in the growth of the subordinate follicles (Ko et al., 1991; O’Shea, 1994; Ginther et al., 1996; Lucy, 2007). Once dominance

has occurred, the dominant follicle will grow linearly for 6 days, which is known as the growing phase (Ko et al, 1991). The dominant follicle will cease to grow and will stay the same size for another 6 days, also known as the static phase (Ko et al., 1991). Finally, if the follicle does not ovulate, the follicle will regress (Ko et al., 1991). The dominant follicle has an active lifespan of 5 to 7 days and after that, the dominant follicle in the first wave will undergo atresia (Lucy et al., 1992). Dominant follicles that undergo atresia are not supported by the correct hormonal levels needed for the final maturation and ovulation of the oocyte (Sirois & Fortune, 1990). Regardless of which wave the cow ovulates, the dominant follicle at the time of luteal regression will ovulate (Fortune, 1994).

Ovulation

Just as hormones control the sequence of events leading to the development of the ovulatory follicle, hormones also support ovulation of the dominant follicle. Changes in progesterone and estradiol concentrations allow for the preovulatory LH surge to occur (Milvae & Hinckley, 1996; Adams et al., 1999). A study performed by Stock and Fortune (1993) demonstrated that maintaining progesterone levels via the use of CIDR inserts prolongs the growth of the dominant follicle and this led to the increase in estradiol-17β concentrations. However, a decrease in progesterone allows for a surge in estradiol and this feeds back on GnRH and releases the LH surge necessary for ovulation (Milvae & Hinckley, 1996; Adams et al., 1999). Estradiol also allows for the increase in oxytocin from the corpus luteum and this feed backs on $PGF_{2\alpha}$ release from the uterus to assist in ovulation (Soloff,

1975, Sheldrick & Flint, 1985). The prevoulatory follicle undergoes a series of changes critical for ovulation once the LH surge has occurred (Park et al., 2004). A major change includes expression of new mRNAs that allow resumption of meiosis through changes associated with the granulosa cells of the follicular wall and the cells surrounding the oocyte also known as the cumulus cells (Park et al., 2004). The resumption of meiosis is indicative of germinal vesicle breakdown of the follicular walls (Park et al., 2004). Where some researchers have shown a variable ovulation time (Brewster & Cole 1940), a later study performed by Walker, Nebel & McGilliard (1996) showed that Holstein cows ovulated at an overall mean of 27.6 ± 5.4 hours after the onset of estrus. This mean includes all ovulations such as those induced by $PGF_{2\alpha}$ and those that were spontaneous.

Bovine Embryo Development

Oogenesis is the process of developing an oocyte or egg through accumulation of populations of maternal RNA and proteins (Teleford & Schultz, 1990; De Sousa et al., 1998). The follicle-enclosed oocytes are arrested in the diplotene stage of meiotic prophase I (De Sousa et al., 1998). The preovulatory LH surge and oocyte release from the follicle will allow for meiosis to resume (Avery et al., 1998; De Sousa et al., 1998). Initiation of follicle growth is characterized by vesicle breakdown, which leads to a resumption of meiosis from metaphase I to metaphase II (Shea, 1981; Avery et al., 1998). Once the first polar body is expelled, ooctyes will remain in a static state until insemination or parthenogenetic activation (Shea, 1981; Avery et al., 1998). The size of the follicle determines the meiotic competence,

or the ability to resume meiosis, of the follicle and the oocyte (Avery et al., 1998). Dairy cattle oocytes and follicles are meiotically competent at a diameter of 110 μm of the oocyte and 2mm follicles (Avery et al., 1998). Folliculogenesis is characterized by an increase in size of the inside zona diameter of the gamete to about 30 μm in the primordial follicle, and 120 μm in the tertiary follicle (De Sousa et al., 1998). Fertilization is characterized by insemination or penetration of the sperm into the oocyte (Teleford & Schultz, 1990). Embryogenesis is defined as the continual growth of the fertilized oocyte, which is termed as "embryo" (Teleford & Schultz, 1990). The average size of a bovine embryo is between 150 to 190μm with a zona pellucida thickness of about 12 to 15μm (Lindner & Wright, 1983). The first three cell cycles, which are the one, two and four-cell stages, develop with a constant pattern of protein synthesis (Crosby et al., 1988; Teleford & Schultz, 1990). Up until the blastocyst stage, the embryo does not vary much in size; however, cell numbers are increased in cleavage divisions from the two cell stage to the 16-cell stage (Lindner & Wright, 1983). The two cell stage has an appearance that shows "a spindle of divisions and these are arranged at right angles of each other" (Hamilton & Laing, 1946). A four cell embryo contains blastomeres that are arranged in pairs so that the figure looks like a cross (Hamilton & Laing, 1946). The eight cell embryos are arranged so that the cells are spherical in shape, uniform in size, and the blastomeres are closely packed (Hamilton & Laing, 1946). Sperm can also still be visible in the zona pellucida (Hamilton & Laing, 1946). A morula is a 5-day embryo with 16 to 32 cells (Shea, 1981; Lindner & wright, 1983) and can be visualized as a "ball of cells" (Lindner & Wright, 1983). Embryos with 16 cells have a centrally placed

blastomere that has its own nucleus, and is surrounded by the other cells (Hamilton & Laing, 1946). The blastomeres are hard to distinguish from one another and the cellular mass of the embryo occupies 60-70% of the perivitelline space (Hamilton & Laing, 1946; Lindner & Wright, 1983). The morula typically compacts at day 6 and is characterized by "coalesced" blastomeres forming a compact mass (Shea, 1981; Lindner & Wright, 1983). A day 7 embryo develops into an early blastocyst (Lindner & Wright, 1983). Early blastocyst embryos have a pronounced outer trophoblast layer with a prominent compact inner cell mass (Hamilton & Laing, 1946; Chang, 1952; Shea, 1981; Lindner & Wright, 1983). The inner cell mass is comprised by a migration of cells to one side of the embryo, while the blastocoel elongates (Shea, 1981). The size of an early blastocyst is about .172mm in diameter (Chang, 1952). The early blastocyst will develop into a blastocyst, which is characterized by a pronounced differentiation of the outer trophoblast and a tightly compact inner cell mass (Chang, 1952; Lindner & Wright, 1983). Flat and elongated cells known as trophoblastic cells (Chang, 1952; Shea, 1981) are distributed thinly along the zona (Hamilton & Laing, 1946). A day 8 embryo develops into an expanded blastocyst, where the embryo dramatically begins to increase in size 1.2 to 1.5 times its original size (Lindner & Wright, 1983). The cells in day 10 embryos begin to differentiate into endodermal cells, ectodermal cells and mesodermal cells and the zona has disappeared (Chang, 1952). Embryos are typically collected on day 7 following estrus when the embryo should be considered a late morula or early blastocyst (Shea, 1981). According to data collected by Shea (1982), 57% of embryos collected on day 7 are early blastocysts and 36% are late morula. Shea's data also

support that there is a 58% pregnancy rate following 366 seven-day transfers (1981). Embryo collection on day 5 results in embryos at the morula stage of development, and determining the number of cells and morphology at this stage is difficult (Shea, 1981). If embryos are collected at day 9, the embryo is more likely to be "hatched" from the zona pellucida (Shea, 1981). The zona pellucida is a distinctive characteristic for identifying embryos and once hatched, the embryo can be difficult to find (Shea, 1981). Collecting embryos at day 12, when elongation has begun, can be done with the naked eye, but damage to the trophoblast wall is likely (Shea, 1981).

Morphology of embryos can vary from an appearance of ideally spherical ooplasm to a degenerate appearance of decreasing ooplasm (Shea, 1981). Embryos are graded based on the quality of embryo development based on the standards of Linder & Wright (1983) which was later modified by the International Embryo Transfer Society (Robertson & Nelson, 1998). Embryos that are graded as "excellent" are "spherical, symmetrical and have cells of uniform size, color and texture" (Lindner & Wright, 1983). "Good" embryos have trivial imperfections such as a few extruded blastomeres, irregular shape, and few vesicles" (Lindner & Wright, 1983). "Excellent" and "good" embryos were combined to form a numerical quality grade of "1" (Robertson & Nelson, 1998; Hasler, 2001). Fair embryos are classified as being "definite, but not severe problems, presence of extruded blastomeres, vesiculation, few degenerated cells" (Lindner & Wright, 1983). Embryos receiving a condition of "fair" were designated as the numerical quality grade as "2" (Robertson & Nelson, 1998; Hasler, 2001). Finally, poor embryos or IETS grade 3 embryos have "severe

problems, numerous extruded blastomeres, degenerated cells, cells of varying sizes, large numerous vesicles but a viable-appearing embryonic mass" (Lindner & Wright, 1983; Robertson & Nelson, 1998). An 8 to 12 cell embryo collected on day 7 indicates that the embryo is dead, the embryo is degenerated or ovulation was delayed (Shea, 1981). Degenerated embryos typically produce poor results in pregnancies (Shea, 1981). According to a study performed by Lindner & Wright (1983), pregnancy rate is less likely to be affected by developmental stage than quality. Poor quality embryos yielded the lowest pregnancy rates, while embryos rated as excellent or good yielded the highest pregnancy rates (Lindner & Wright, 1983).

Assisted Reproductive Technology (ART)

Understanding the events of the bovine estrous cycle has led to the development of technologies that allow for increased production of bovine embryos from superior animals (Mapletoft, 1984). These technologies aid in advancing our knowledge in reproduction, cellular and molecular biological processes, and genomic techniques (Mapletoft, 1984; Galli, 2003). While there are a number of reproductive techniques, estrous synchronization, follicle aspiration, artificial insemination, embryo transfer and superovulation are used commercially to maximize milk and meat production at lower costs and help prevent transmission of diseases (Mapletoft, 1984).

The Use of GnRH in an Estrus Synchronization Protocol

The main goal of an estrus synchronization protocol is to control luteal and follicular function in order to minimize estrus detection (Folman et al, 1990; Pursley et al, 1995; Bo et al, 2000). Protocols can utilize either progestogens and/or prostaglandins to either extend or restrict the luteal phase (Bo et al, 1995; Pursley et al, 1995; Pursley et al, 1997b). Some protocols utilize only $PGF_{2\alpha}$, while other protocols utilize both $PGF_{2\alpha}$ and GnRH (Pursley, 1997b). These hormones are more efficient in eliminating estrus detection (Pursely, 1997b). The "Ovsynch" protocol has become a popular and successful tool in estrous synchronization (Pursley et al, 1997). The Ovsynch protocol consists of an injection of GnRH which is given at a random point in the estrous cycle (Pursely et al, 1995; Stevenson, Kobayashi, & Thompson, 1999; Moreira et al., 2000). This allows luteinization of the dominant follicle and a new follicular wave emerges (Pursely et al, 1995; Stevenson, Kobayashi, & Thompson, 1999; Moreira et al., 2000). An injection of $PGF_{2\alpha}$ 7 days later regresses the corpus luteum (Schmitt et al., 1996) and a second injection of GnRH is given 48 hours later and causes ovulation of the dominant follicle (Pursely et al, 1995; Moreira et al., 2000). Synchrony through this method allows for a timed artificial insemination about 16 hours after GnRH injection (Moreira et al., 2000).

Follicle Aspiration

Follicle aspiration is a mechanical way of abolishing the dominant follicle (Wikland, Enk & Hamberger, 1985; Bennet et al, 1988; Pieterse et al, 1988; Bergfelt, Lightfoot &

Adams, 1994). The first attempt to collect oocytes during *in vitro* fertilization was Wikland, Enk & Hamberger in 1985. By 1987, 80% of all collections were done by this technique (Bennet et al, 1993). Most follicle aspirations are performed by an ultrasound and require adequate training and expertise (Wikland, Enk & Hamberger, 1985). Most follicle aspiration protocols can are similar to the one described by Pieterse & Kappen (1988). The animals were sedated with an intravenous injection of Detomidine hydrochloride to relax the intestines (Pieterse & Kappen 1988). An ultrasound machine with a vaginal transducer and a grip at the end is used. This helps with the manipulation of the transducer which is equipped with a needle guide (Wikland, Enk & Hamberger, 1985; Pieterse & Kappen, 1988). The transducer, covered with sterile plastic, is lubricated with non toxic contact gel that will not damage the oocyte (Piedsterse & Kappen,1988; Wikland, Enk & Hamberger, 1985). The needle guide contains two sterilized needles of different sizes (1.2 mm and 1.5 mm) with a length of 23cm to 25cm (Wikland, Enk & Hamberger, 1985; Pieterse & Kappen, 1988). The needle is sharp and beveled at 60º and there are shallow tracks that allow enhancement of the contrast of the needle (Wikland, Enk & Hamberger, 1985). The other end of the needle contains a suction apparatus to collect the oocyes (Pieterse & Kappen, 1988). The transducer is guided into the vagina and is detected as far as possible to one side of the cervix (Pieterse & Kappen, 1988; Bergfelt, Lightfoot & Adams, 1994). The transducer is used to locate the ovary with the follicles (Wikland, Enk & Hamberger, 1985) and is positioned so that the puncture line on the ultrasound monitor transects the follicle to be aspirated (Pieterse & Kappen, 1988). The needle enters a canal in the transducer for guidance and punctures the

follicle once the follicle is positioned on the puncture line (Wikland, Enk & Hamberger, 1985; Pieterse & Kappen 1988; Bergfelt, Lightfoot & Adams, 1994). Once the follicle is punctured, draws in the follicular fluid (Pieterse & Kappen, 1988; Bergfelt, Lightfoot & Adams, 1994). Oocytes can then be reviewed (Pieterse & Kappen, 1988).

While the technique is a powerful tool for eliminating follicles, there are a number of complications as a result of introducing a needle into the vaginal wall (Bennet et al, 1993). Complications include hemorrhage, as a result of trauma to the vagina and/or ovaries and other related structures; and finally the risk of infection due to the introduction of the tools necessary for oocyte collection (Bennet et al, 1993). Barros & Nogueira (2001) suggest that the procedure is also expensive and requires the use of an ultrasound which should be done by an experienced technician.

Artificial Insemination

Artificial insemination (AI) began in bees, progressed to plants, and finally more recently to animals (Foote, 2002). The history of AI can be traced back to 1678 when Leeuwenhoek and Hamm first identified semen as "animolecules," but the actual first successful insemination was performed by Spallanzani in a dog (Willet, 1956; Foote, 2002). Artificial Insemination in dairy cattle became popular in the United States in the 1930s and a basic procedure was developed by the 1940s (Foote, 2002). Artificial Insemination is another reproductive technique that allows for genetic selection for milk production and quality (Foote, 2002; Thibier & Wagner, 2002). Factors such as sire selection, testicular

evaluation, semen collection, evaluation and processing and fertility testing must be considered (Foote, 2002). A good microscope is needed to measure the motility, concentration, viscosity, structure and the numbers of sperm cells present (Willet, 1956; Foote, 2002). In the past, the volume was measured by a graduated cylinder; however, recently, there are more accurate forms of measuring volume by weight (Foote, 2002). Semen quality is measured through motility and morphology tests such as the hypo-osmotic swelling test, mucus or gel penetration, and integrity of the DNA (Graham, 1978; Foote, 2002). Shipment of semen is also popular and can be done when the semen is extended and cooled or frozen (Foote, 2002). Salisbury and others (1941) first used sodium citrate and di-hydrogen phosphate, which were both added to egg yolk and found that the yolk-citrate mixture was the best method for extending the semen (Salisbury, Fuller & Willet, 1941; Foote, 2002). Both methods showed to be good extenders for up to 5 days (Salisbury, Fuller &Willet, 1941; Foote, 2002). Milk extender was also shown to provide effective results when added to glycerol when freezing (Foote, 2002). Artificial insemination has the best results when used with estrous synchronization protocols such as Ovsynch (Moreira et al., 2000).

Embryo Transfer

Embryo transfer is a reproductive technique that allows the collection of ova from a donor cow and the implantation of that ovum into a recipient cow (Mapletoft, 1984). Embryo transfer was first performed in rabbits in 1890 as reviewed by Mapletoft (1984).

The first embryo recovered in bovine was done in 1930 by Hartman, Lewis, Miller, and Swett (1930) and the first calf born as a result of embryo transfer was in Wisconsin in 1951 (Willett et al, 1951.) Every year about 500,000 embryos are recovered and transferred or frozen (Thibier, 2000; Hasler, 2001). Commercial application of embryo transfer did not become popular until after 1972 (Seidel, 1981). Multiple Ovulation and Embryo Transfer (MOET) programs take advantage of superovulation and utilize embryo transfer techniques to move the embryos collected from the donor cow into recipient cows (Barros & Nogueira 2001). The development of non- surgical embryo transfer has allowed a non invasive method of collecting embryos and transferring them into a recipient animal (Brand & Akabwai, 1978). Advantages of embryo transfer include genetic improvement, controlling diseases, importing and exporting embryos, salvage of reproductive function, research, and cost and succession rates (Brand & Akabwai, 1978; Mapletoft, 1984; Galli et al., 2003). Genetics are improved by increasing selection intensity and shortening generation intervals (Mapletoft, 1984) since a large number of offspring may be produced from superior cows using MOET (Seidel, 1981; Mapletoft, 1984). Embryo transfer plays an important role in minimizing disease transfer and allows for movement of embryos across the country whereas the transport of live adult animals is much more expensive and stressful on the animal (Mapletoft, 1984). Offspring from terminally ill animals or animals that cannot otherwise produce offspring naturally can be placed into a recipient cow (Mapletoft, 1984). Finally, embryo transfer allows for an extension in research such as in twin preganancies, uterine capacity, endocrine control of uterine environment, maternal recognition of pregnancy and

other reproductive issues (Mapletoft, 1984).

An important factor in an embryo transfer protocol is being able to synchronize the donor and the recipient cow (Brand & Akabwai, 1978; Lindner & Wright, 1983; Mapletoft, 1984). Without this synchrony, pregnancy rates would be low (Brand & Akabwai, 1978). Prostglandin $F_{2\alpha}$ is administered to the recipient 12-18 hours before the donor cow so that synchronization can be accomplished between donor and recipient cows (Mapletoft, 1984). A donor cow observed in heat is artificially inseminated and the embryo can be collected 6 to 8 days after estrus (Mapletoft, 1984). According to Lindner and Wright (1983), synchrony protocols can be out of synch +2 days (recipient in estrus before the donor) and -2 days (recipient in estrus after the donor) without a significant decrease in pregnancy rates (Lindner & Wright, 1983; Mapletoft, 1984). A study performed by Lindner and Wright (1983) shows that synchrony of recipients and donors are more important in synchrony of cell stage rather than age of embryos. The embryo is collected through a non-surgical procedure. A rubber cuffed catheter is inserted through the cervix and is inserted through one of the horns (Mapletoft (1984). Embryos can be collected through a closed or interrupted flow system or by an uninterrupted syringe technique (Mapletoft, 1984). The embryos are filtered into a plankton filter and transferred into a dish to be searched through a stereo dissecting microscope (Mapletoft, 1984). Embryos can be cultured or transferred right after collection (Mapletoft, 1984). Bovine blastocysts can be stored in a refrigerator in 4ºC for up to two days and transferred with some success (Lindner & Wright, 1983).

Superovulation

The objective of superovulation is to continue the growth of the subordinate follicles that would become atretic naturally by overcoming the effects of the dominant follicle (Bergfelt et al., 1997; Mapletoft, Steward & Adams, 2002). Different combinations of hormone treatments such as GnRH and $PGF_{2\alpha}$ (McDougall, S., N.B. Williamson & K.L. Macmillan. 1995; Pursley et al., 1995), P_4 and E_2 (Bo et al., 1995, Bo et al., 1996) and follicle aspiration (Bergfelt, Lightfoot & Adams, 1994; Bergfelt et al., 1997) are used to induce follicle turnover (Bergfelt et al., 1997). Gonadotropin treatments increase the number of embryos recovered (Bergfelt et al, 1997). The source of the gonadotropin, the batch and the biological activity are all important to consider when administering exogenous hormones (Murphy et al, 1984; Kanitz, 2002). Currently there are many types of gonadotropins that have been tested in superovulation protocols such as equine chorionic gonadotropin (eCG) (Murphy et al., 1984; Donaldson, 1989; Mapletoft, Steward & Adams, 2002), human menopausal gonadotropin (hCG) (Mapletoft, Steward& Adams, 2002) and ovine (Donaldson, 1989) porcine (Murphy et al, 1984; Donaldson, 1989; Mapletoft, Steward & Adams, 2002) and bovine (Bellows et al., 1991) pituitary FSH.

FSH is responsible for inducing super stimulation of the follicles (Mapletoft, Steward & Adams, 2002). Because the half life of the purified commercial FSH when injected into a dairy cow is 5 hours, injections should be given twice daily (Monniaux et al, 1983; Mapletoft, 2002). However, other studies suggest that only one injection per day is as efficient as giving two injections per day (Kanitz et al, 2002). A popular commercial FSH

analogue is Folltropin-®V; (Bioniche Animal Health, Bellville, ON, Canada) and is an efficient analogue for super stimulating follicles (Mapletoft et al, 2001). Using Folltropin-®V has produced more corpora lutea and fertilized ova than prepared LH and FSH solutions at different concentrations (Mapletoft et al, 2002). Kanitz et al. (2002) showed that it is not necessary to have LH contamination in FSH solutions to induce superovulation; however, LH contamination, to an extent, will not alter the effects of the supoerovulatory protocol. An abstract written by Linsell et al. (1986) reported that in order to have FSH activity, FSH concentrations have to be significantly higher than LH concentrations in order for there to be FSH activity. An injection of $PGF_{2\alpha}$ is given on the last day of gonadotropin treatment to regress the CL (Prado Delgado, Elsden & Seidel, 1989).

Superovulation Protocols

Superstimulatory treatments should be given when the dominant follicle is not present, whether the dominant follicle has turned over in the previous wave or the dominant follicle is ablated or turned over due to hormonal stimulation (Wikland, Enk & Hamberger, 1985; Bennet et al, 1988; Pieterse et al, 1988). Two-wave animals have a dominant follicle that is not functional 30% of the estrous cycle or 6 out of 20 days (Bergfelt et al, 1997). Three-wave animals have a dominant follicle that is not functional 35% of the estrous cycle or 8 of 23 days (Bergfelt et al, 1997). Only a small window (20% of the estrous cycle) exists to initiate superovulatory treatment at the time of wave emergence (Bergfelt et al, 1997). In addition to turning the dominant follicle over, commercial uses of superovulation require that

the estrous cycles of animals are synchronized as well to minimize labor intensive management (Bo et al., 1995). Synchronization can be achieved through progestins administered through an intravaginal progesterone-releasing device (CIDR) (Macmillan & Peterson, 1993) or prostaglandins (Bo et al., 1995).

Studies performed by Bo and et al. (1993) used estradiol valerate (EV) and a combination of progesterone and estradiol-17β with the use of a progesterone implant to cause turnover of the dominant follicle and allow for the emergence of a new wave (Bo et al., 1993; Bo et al., 1994; Bo et al., 1995). An intravaginal progesterone-releasing device insertion and estradiol together induces premature luteolysis (Macmillan & Petterson, 1993). The objectives of the studies were to apply treatment at random times in the development of the follicular waves and cause one of two outcomes: 1) Regression of the dominant follicle and accelerate the emergence of a new wave, or 2) delaying the emergence of a new wave (Bo et al., 1993; Bo et al., 1994; Bo et al., 1995). The outcomes of treatment depend on the timing during the estrous cycle. Treatment given in the early (Day 1) or mid (Day3) growing phase of the follicular wave will terminate the growth of the dominant follicle and shorten the period of follicular dominance as well as allow an early surge of FSH for new wave development (Bo et al, 1993; Bo et al., 1995). Treatment in the late growing phase of follicular development (Day 6) caused a delay in emergence of a new wave that is "probably associated with suppression of the pre-wave FSH surge" (Bo et al. 1993). Bo and others (1995) proceeded to treat animals in the early follicular growing phase of the next wave (Day 9) and observed the same results as treatment with the early growing phase of the first wave:

the dominant follicle regressed and accelerated new follicular wave growth. The new wave is predicted to emerge approximately 4 days after treatment (Bo et al., 1995).

Superstimulatory treatments are only effective when the dominant follicle is not present, but are most effective at the "time of the endogenous prewave surge in FSH or wave emergence" (Bo et al. 1995). A study performed by Nasser and others (1993) concluded that more follicles are recruited and the suppression of subordinate follicles is prevented when superovulation is iduced during the pre-wave surge. Follicle stimulating hormone was administered between days 8 and 12 of the estrous cycle (Lindsell, Murphey & Mapletoft, 1986) and given in decreasing doses in twelve hour intervals for five days (Lindsell, Murphey & Mapletoft, 1986; Nasser, 1993). The last day of gonadotropin treatment was accompanied by an injection of $PGF_{2\alpha}$ (Prado Delgado, Elsden & Seidel, 1989) and CIDR removal (Macmillan & Peterson, 1993). Estrus was detected 48 hours later and insemination is performed at that time (Macmillan & Peterson, 1993). The percentage of estrus detections at 72 hours and 96 hours later was much lower than at 48 hours, suggesting artificial insemination was best performed at 48 hours after PGF injection and CIDR removal (Macmillan & Peterson, 1993).

Summary

Estradiol has been shown (Bo et al., 1993; Bo et al., 1994; Bo et al., 1995) to successfully turn over the dominant follicle and this protocol has been used for a number of years by many technicians. However, environmental contamination by estradiol products

from the feces of treated animals has become a concern (Hanselman, Graetz & Wilkie 2003). Estradiol products have been found in water sources and this affects endocrine functions of aquatic wildlife (Hanselman, Graetz & Wilkie 2003). The FDA has indicated that estrogen compounds are not for the use "in animals intended for subsequent breeding or in dairy animals." (FDA, Code of Regulations, 2008). There is some concern that estrogenic compounds injected into meat and dairy animals will contaminate products intended for human consumption. An alternative superovulation protocol must be developed to decrease the amount of estrogens in the environment. Follicle aspiration has been practiced for many years; however, it is more invasive and causes reproductive complications for animals. As a result, an alternative hormonal protocol must be developed to decrease the amount of estrogens in the environment, while minimizing the amount of stress induced on animals.

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EFFECT OF GNRH COMPARED TO ESTRADIOL-17β AT BEGINNING OF SUPEROVULATION PROTOCOL ON SUPEROVULATORY RESPONSE AND EMBRYO QUALITY

Introduction

The primary objective of a superovulation protocol is to maximize the number of growing follicles available for ovulation from one animal at a given time (Mapletoft, Steward & Adams, 2002). Ovulation and fertilization of multiple oocytes allows for transfer of the resulting embryos to recipient females. This technique enables the animal industry to select and breed superior animals Superovulation protocols aim at synchronizing the growth of many follicles via administration of super-stimulatory treatment in the absence of a dominant follicle (Adams et al., 1994). A variety of protocols utilize estrogenic compounds to turn over the dominant follicle in preparation for super-stimulatory treatments with FSH (Bo et al., 1993; Bo et al., 1994; Bo et al., 1995). However, estrogenic compounds such as estradiol, estrone and estriol have been found to contaminate surface and ground water (Raman et al., 2001; Hanselman, Graetz & Wilkie, 2003). These estrogenic compounds, especially estradiol-17β, are found in animal wastes including waste from dairy cattle (Raman et al., 2001; Hanselman, Graetz & Wilkie, 2003). This influx of estrogenic compounds has lead to reproductive complications in wildlife and aquatic animals (Raman et al., 2001; Hanselman, Graetz & Wilkie, 2003). Estrogenic contamination causes endocrine disruption in the body by interactions with the hormone receptor (Crain et al., 1998). The FDA has indicated that estrogen compounds are not for the use "in animals intended for subsequent breeding or in dairy animals." (FDA, Code of Regulations, 2008). There is concern that estrogenic compounds injected into meat and dairy animals will contaminate

food products intended for human consumption. The need to minimize the amount of estrogenic compounds being transferred into the environment is cause to find an alternative method of turning over the dominant follicle in superovulation protocols. Thompson and others (1999) reported for beef cattle that GnRH will induce ovulation or luteinization of the largest follicle at the time of treatment in estrus synchronization protocols. A similar study performed by McDougall, Williamson & Macmillan (1995) shows that a single injection of GnRH administered in the presence of a 10mm or larger follicle caused ovulation in dairy cows. A superovulation protocol utilizing GnRH would provide an alternative protocol and thereby eliminate the need to use of estrogen in superovulation protocols. Therefore, the objective of this study was to compare the conventional superovulatory protocol utilizing estradiol-17β with a novel superovulation protocol that utilized GnRH for turnover of the dominant follicle.

Method and Materials

Holstein heifers (n=22), dry (n=23) and lactating cows (n=55) cows ranging in age from 11 to 140 months were used in this study which was performed from September, 2005 to September, 2007. Animals were fed a balanced grain and hay diet and kept either on pasture or under a shelter at a commercial embryo transfer facility in Durham, North Carolina.

A method for turning the dominant follicle over was compared by using two different superovulation protocols. A total of 394 collections were performed with animals superovulated using either the conventional estradiol-17β/progesterone protocol (n=205) or a novel GnRH protocol (n=189). A switchback method was used on all animals in the study assuring that each animal received each treatment at least once, but animals may have been utilized on each treatment multiple times. Embryos were recovered by one of two experienced technicians.

Treatment I: Estradiol-17β/ Progesterone Protocol

On day 0, animals had a Controlled Internal Drug Releasing Insert (CIDR®), containing 1.38 g of Progesterone (P_4), (Eazi-Breed^{TM}; Pfizer Animal Health, Kalamazoo, MI) inserted intravaginally and received an intramuscular injection of estradiol (2.5 mg) and progesterone (50 mg) similar to the protocol described by Bo et al., (1995). On days 4 through 7, a commercial follicle stimulating hormone, (Folltropin®-V; Bioniche Animal

Health, Belleville, Ont, Canada) was injected intramuscularly in decreasing doses depending on the status and sensitivity of the animal to the drug. Typically, heifers were administered with 200 mg of FSH and cows received 300 mg of FSH. Based on their response, treatment was increased or decreased in subsequent flushes. On day 7, the CiDR was removed and 25mg of $PGF_{2\alpha}$ (Lutalyse®; Pfizer Animal Health, Kanamazoo, MI) was administered intramuscularly. Animals were artificially inseminated using commercially available semen at the onset of estrus and at 12 hour intervals until animals stopped displaying estrus. All animals were inseminated a minimum of two times. At the time of last insemination, each cow was administered with 100µg of GnRH (Cystorellin®; Merial Limited, Athens, GA). On day 16, the animals were flushed for viable embryos (Figure 1).

Treatment II: GnRH Protocol

On day 0, animals had a CIDR®, containing 1.38 g of P4, (Eazi-Breed™; Pfizer Animal Health, Kanamazoo, MI, 49001) inserted intravaginally. On day 3, 100µg of GnRH (Cystorellin®; Merial Limited, Athens, GA) was injected intramuscularly to cause turnover of the dominant follicle. On days 5 through 8, a commercial follicle stimulating hormone, (Folltropin®-V; Bioniche Animal Health, Belleville, Ont, Canada) was injected intramuscularly in decreasing doses depending on the status and sensitivity of the animal to the drug. Typically, heifers were administered with 200 mg of FSH and cows received 300 mg of FSH. Based on their response, treatment was increased or decreased in subsequent flushes. On day 8, the CiDR was removed and 25mg of $PGF_{2\alpha}$ (Lutalyse®; Pfizer Animal

Health, Kanamazoo, MI) was administered intramuscularly. Animals were artificially inseminated using commercially available semen at the onset of estrus and at 12 hour intervals until animals stopped displaying estrus. All animals were inseminated a minimum of two times. At the time of last insemination, each cow was administered with 100µg of GnRH (Cystorellin®; Merial Limited, Athens, GA). On day 17, the animals were flushed for viable embryos (Figure 2).

Collection and Grading of Embryos

Embryo collection was performed by flushing the uterine body by one of two experienced technicians as described by Foote (1970). Briefly, an epidural anesthetic (5ml of Lidocaine) was used to minimize pain and relax the rectum. A Foley catheter was guided into the vagina and through the uterus by rectal palpation as first described by Kuzan and Seidel (1986). A Foley catheter with a balloon was used to allow inflation beyond the cervix to maintain the catheter in that position and to allow each uterine horn to be flushed simultaneously. ViGro Complete Flush Solution (Bioniche, Athens, GA, 30601) was used as the medium to evacuate embryos.

The embryos were flushed into a 5µm screen Embryo Collection Filter (Bioniche, Athens, GA) and rinsed into a gridded Petri dish. Each dish was carefully searched under a stereomicroscope and embryos were placed in ViGro Holding Plus (Bioniche, Athens, GA) medium in 4 to 6-well petri dishes. Collected embryos were graded for quality and stage of development by a licensed veterinarian certified by the American Embryo Transfer

Association who was blinded with regards to treatment. Embryos were graded based on a scale developed by the International Embryo Transfer Society (IETS). Excellent or IETS grade 1 embryos are "spherical, symmetrical with cells of uniform size, color and texture" (Lindner & Wright, 1983). Fair or IETS grade 2 embryos are classified as being "definite, but not severe problems, presence of extruded blastomeres, vesiculation, few degenerated cells" (Lindner & Wright, 1983). Finally, poor embryos or IETS grade 3 embryos have "severe problems, numerous extruded blastomeres, degenerated cells, cells of varying sizes, large numerous vesicles but a viable-appearing embryo mass" (Lindner & Wright, 1983; Robertson & Nelson, 1998). Degenerated embryos usually have stunted growth for an embryo collected on day 7 (Shea, 1981). Embryos of quality grade of 1 or 2 were considered high quality embryos.

Statistical Analysis

A preliminary analysis was performed on the first estradiol-17β treatment and GnRH treatment of each animal. The results were compared to the results obtained when using the entire dataset to show that utilizing one animal for multiple observations on one treatment did not bias the data. No differences were observed; therefore the complete dataset was used for all analyses.

Results were analyzed utilizing the analysis of variance using a Frequency and Mixed procedures of the SAS program (SAS 9.1.3 w/SP4, SAS Institute, Cary, NC) to compare the effects of the estradiol-17β protocol to the alternative treatment using a GnRH protocol in an ovulation synchronization regimen to turn over the dominant follicle. Mean number of total embryos recovered, the total number of embryos recovered that were IETS grade 1 & 2, the percent of fertilized embryos and the percent of total number of embryos recovered that were IETS grade 1 & 2 embryos were determined in the model that included Treatment, Stage, Treatment by Stage interaction and Technician.

Treatments, estradiol-17β/ progesterone (n= 205) and GnRH (n= 189) were the total number of embryos recovered that were IETS grade 1 & 2, the percent of fertilized embryos and the percent of total number of embryos recovered that were IETS grade 1 & 2 embryos analyzed for differences in means of the total number of embryos recovered.

Follicle stimulating hormone dose and lactation status were also compared. Animals were divided into lactating cows (n=215), dry cows (n=120) and heifers (n=59). Each status

was then divided into high (≥300mg) and low (<300mg) FSH dose. The following groups: Lactating cows on High FSH dose (n=175) and Lactating Cows on Low FSH dose (n=40), Dry cows on High FSH dose (n=60), Dry cows on low FSH dose (n=60), and Heifers on Low FSH dose (n=59) were created.

Finally, effects of technician 1 (n= 361) and technician 2 (n = 33) were included in the model to compare the means of total number of embryos recovered.

Results

Technician 1 was responsible for performing 361 flushes which accounted for 91.62% of the total flushes performed. Technician 2 was responsible for performing 33 flushes which accounted for 8.38% of the flushes performed. There were no differences in the number of embryos recovered per flush (10.55 ± 0.67 vs 11.46 ± 1.45; P=0.53), the number of IETS grade 1 and 2 embryos collected per flush (5.09 ± 0.45 vs 6.19 ± 0.99; P=0.26), or the percent of fertilized embryos recovered (52.24 ± 2.59% vs 63.29 ± 6.64%; P=0.10) for technician 1 and technician 2, respectively. A higher percentage of embryos collected by technician two (56.96± 6.28%) were IETS grade 1 and 2 compared to technician 1 (44.11 ± 2.63%; P=0.04).

There were no differences in the number of embryos recovered (10.86 ± 0.97 vs 11.14 ± 0.99; P= 0.75), the number of grade 1 and 2 embryos collected (5.65 ± 0.66 vs 5.64 ± 0.68; P= 0.98) the percent of fertilized embryos (59.9 ± 4.15% vs 56.43 ± 4.38%; P=0.52) and grade 1 and 2 embryos (50.54 ± 4.02 vs 50.53 ± 4.20; P=0.99) for estradiol and GnRH respectively. (Table 1, Figure 3, Figure 4)

Differences were observed between the numbers of embryos recovered from animals with different lactation status. The number of embryos recovered from Dry Cows on High (14.8 ± 1.44) and Low FSH (13.2 ± 1.57) doses were significantly higher (P< 0.0001) than the number of embryos recovered from Lactating Cows on High (9.2 ± 1.01) and Low (9.5 ± 1.59) FSH dose or from heifers (n= 8.30 ± 1.34). Dry Cows on High (46.37% ± 6.2) and Low (43.07% ± 6.39) FSH also had a significantly lower (P=0.0004) percentage of fertilized

embryos compared to other groups (Table 2). However, the number of IETS-grade 1 & 2 embryos collected from dry cows was not significantly different from the number of IETS-grade 1 & 2 collected from the other lactating cows or heifers (P= 0.6). The percent of embryos of IETS-grade 1 & 2 collected from Dry Cows were significantly lower (P=0.0016) than all other groups (Table 2).

Discussion

The alternative protocol using GnRH to turn over the dominant follicle during superovulation utilized in this study produced results similar to the conventional protocol using estradiol-17β/progesterone. Endpoints analyzed demonstrated that no differences existed, but the E_2/P_4 and GnRH superovulatory protocols on the total number of embryos recovered, the number of fertilized embryos, the number of IETS-grade 1 & 2 embryos, and percentages of fertilized and IETS-grade 1 & 2 embryos are consistant with the conclusion that use of GnRH for dominant follicle turnover is an efficient alternative superovlation protocol. Moenter et al. (1990) reported that estrogen acts on GnRH to increase its secretion and therefore induces the LH surge. A study performed by Bo et al. (1993) used estradiol valerate to turn over the dominant follicle at a random stage in the estrous cycle. Estradiol treatment alone suppressed dominance in the growing phase hastened atresia when a dominant follicle was present, and accelerated the emergence of the next wave (Bo et al., 1993). A subsequent study performed by Bo et al. (1994) used estradiol-17β with and without progesterone treatment in beef cattle. Animals treated with a progesterone implant had an earlier wave emergence and had a less variable emergence of the second wave than animals without a progesterone insert (Bo et al., 1994). Estradiol is an effective treatment to turn over the dominant follicle. Estradiol can induce follicle regression, allowing emergence of a new follicular wave (Hutz, Diershke & Wolf, 1988). Concern for exogenous estrogens entering the environment through animal feces and entering milk and meat for human consumption led to a discontinuation of the use of estrogenic compounds for superovulation

protocols. Under 556.240 in the FDA Code of Regulations (2008), estradiol should not be used "in animals intended for subsequent breeding or in dairy animals." An alternative protocol must be used to turn over the dominant follicle in superovulatory protocols. Sources in beef (Thompson et al., 1999) and dairy (McDougall, Williamson & Macmillan, 1995) cows show that GnRH induces turnover of the dominant follicle. Gonadotropin-releasing hormone administration triggers the release of LH (McDougall, Williamson & Macmillan, 1995) similar to the surge of LH following estradiol-17β administration.

The estradiol-17β and GnRH protocols used in the current study resulted in similar numbers of embryos collected as previously reported in the literature. Gouveia et al. (2002) recovered a mean of 10.89 embryos and a mean of 7.61 fertilized embryos using a CIDR and estradiol benzoate in beef cows. Another study by Merton et al. (2003) reported that with dominant follicle aspiration, the mean total number of embryos recovered was 7.0 in heifers and 7.6 in cows of unknown lactation status. The number of IETS-grade 1 & 2 embryos collected was 4.3 in heifers and 5.4 in cows of unknown lactation status (Merton et. al., 2003). These results are comparable to those obtained in this study for both estradiol-17β/progesterone and GnRH treatment in dairy animals.

Also evaluated in this study is the lactation status and FSH dose. Dry cows produced a similar number of IETS-grade 1 & 2 embryos to animals within other lactation groups, but the percentage of IETS-grade 1 & 2 embryos was much lower. Results in the high and low dose dry cows are high compared to the other animals in the study, which indicated that these animals may have been overstimulated. Heifers from the study produced a much higher

percentage of fertilized and grade 1 & 2 embryos than the other groups of animals. Goulding et al. (1990) reported that 8.5 embryos were recovered from heifers treated with FSH on Day 10, which was comparable to the results we received in this study. Heifers in that study produced 4.1 IETS grade 1 & 2 embryos, again comparable to the results in the present study. Pursley et al. (1997) showed that heifers also had a pregnancy rate of 82% compared to cows with a 46% pregnancy rate following a second injection of $PGF_{2\alpha}$ in estrous synchronization protocols. The difference in preganacy rates between cows and heifers were also noted in other studies (Lee et al., 1983). Higher fertilization rates of embryos collected from heifers was expected.

Treatment with higher doses of FSH showed no benefit with respect to increasing the number of embryos collected. Therefore, we recommend practitioners consider using low doses (< 300mg) for effective superovulation while allowing a cost savings from reduced use of FSH.

Technician 2 recovered a higher percentage of grade 1 and 2 embryos. However, 42% of flushes performed by Technician 2 were heifers which produced more IETS grade 1 & 2 embryos than cows. 89% of the flushes performed by Technician 1 were Lactating and Dry Cow flushes. Therefore it was not surprising that a higher percentage of embryos recovered by technician 2 were grade 1 and 2 embryos.

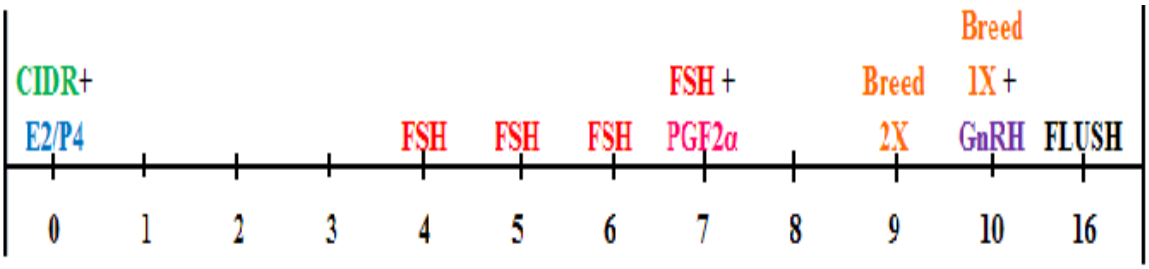
The superovulatory protocol that utilizes GnRH to turn over the dominant follicle was shown to be an effective alternative to use of our estradiol-17β treatment. Commercially, costs associated with administration will also benefit animal producers because estradiol-

17β/progesterone treatment costs on average $5.00 per treatment while GnRH only cost $4.00 per treatment. Additionally, administration of estradiol-17β to dairy animals is no longer permitted by law. The GnRH protocol tested here is a 17-day protocol compared to the 16 day conventional protocol. Future studies may be needed on testing the timing of GnRH or FSH treatment within the present superovulatory protocol to best maximize efficiency in producing high quality embryos.

Conclusions

Two superovulatory protocols, one utilizing estradiol-17β/progesterone and one utilizing GnRH to turn over the dominant follicle were compared using lactating, dry and heifer cows. Results showed that both treatments produced similar results in the number of embryos recovered, the number of quality embryos collected and the percent of the embryos fertilized between the two treatments. As a result, GnRH was determined to be an efficient alternative for turning over the dominant follicle in a superovulatory protocol.

Treatment I: Estradiol-17β/ Progesterone



Days of Experiment

Figure 1: Estradiol-17β/ Progesterone treatment protocol. Treatment with Estradiol-17β/ Progesterone and CiDR insert began on Day 0. On Days 4-7, animals received either a high (≥ 300mg) or low (< 300mg) FSH dose followed by $PGF_{2\alpha}$ on Day 7. Animals were bred twice, once in the A.M. and once in the P.M. on Day 9. Animals were bred once in the A.M. followed by GnRH on Day 10. Embryos were recovered on Day 16 (Gestational Day 7).

Treament II: GnRH

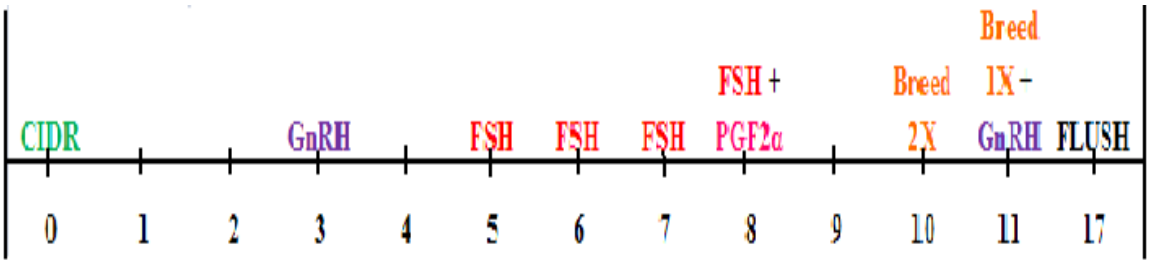


Figure 2: Gonadotropin-Releasing Hormone treatment protocol. CiDR was inserted on Day 0. GnRH treatment began on Day 3. On Days 5-8, animals received either a high (≥ 300mg) or low (< 300mg) FSH dose followed by $PGF_{2\alpha}$ on Day 8. Animals were bred twice, once in the A.M. and once in the P.M. on Day 10. Animals were bred once in the A.M. followed by GnRH on Day 11. Embryos were recovered on Day 17 (Gestational Day 7).

Table 1: Effect of Estradiol-17β vs GnRH Treatment on Total Number of Embryos Recovered, Number of IETS Grade 1 & 2 Embryos, Percent of IETS Grade 1 & 2 and Percent Fertilized

Estradiol vs GnRH			
Factor	***Results***		
	Estradiol	**GnRH**	**P-Value**
Number of Embryos Recovered	10.9 ± 1.0	11.1 ± 1.0	P=0.75
Number of IETS-Grade 1 & 2 Embryos	5.7 ± 0.7	5.6 ± 0.7	P=0.98
% IETS-Grade 1 & 2 Embryos	50.5 ± 4.0	50.5 ± 4.2	P=0.99
% Fertilized	59.1 ± 4.2	56.43± 4.4	P=0.52

Table 2: The Effect of FSH dose and Lactation Status on Embryo Recovery and Embryo Quality

Milk Status[1]	Lactating Cows		Dry Cows		Heifer
FSH Dose[2]	High[3] (n= 175)	Low[4] (n= 40)	High (n=60)	Low (n=60)	Low (n=59)
Number Embryos Recovered	9.2[c]	9.5 [bc]	14.8[a]	13.2[ab]	8.3[c]
Number IETS-Grade 1 & 2 Embryos	4.8[g]	5.92[g]	5.85[g]	5.63[g]	5.95[g]
% IETS Grade 1 & 2 Embryos	51.16[h]	60.29[i]	39.88[j]	37.80[j]	63.55[i]
% Fertilized	58.73[e]	68.42[d]	46.37[f]	43.07[f]	72.23[d]

[abc] Means within rows without common superscripts differ (P< 0.0001)
[def] Means within rows without common superscripts differ (P= 0.0004)
[g] Means within rows did not differ (P= 0.6)
[hij] Means within rows without common superscripts differ (P= .0016)

[1] Milk status is classified into Lactating Cows, Dry Cows and Heifer Cows
[2] Each milk status of animals is further classified into High and Low Dose of FSH.
[3] High is defined as FSH Dose > 300mg
[4] Low is defined as FSH Dose < 300mg

Figure 3: Effect of Estradiol-17β vs GnRH Treatment on Embryos Recovered

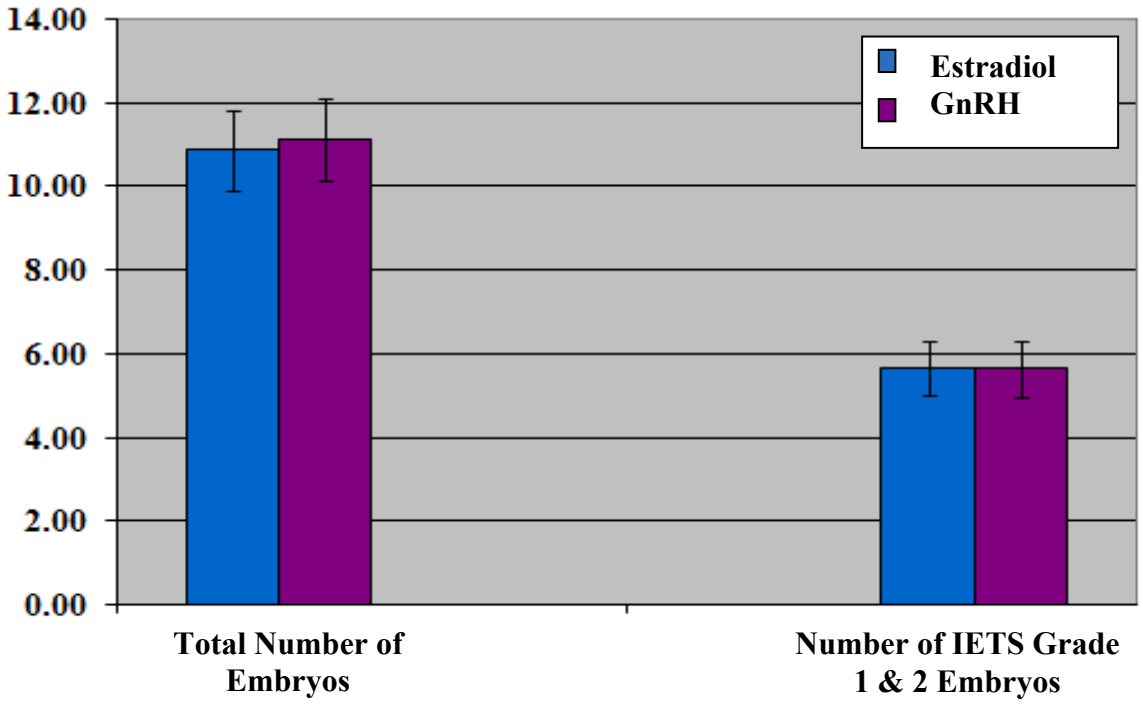


Figure 3: The number of embryos and the number of IETS-grade 1 & 2 embryos are represented for the Estradiol-17β protocol and the GnRH protocol. There were no significant differences in the number of embryos recovered (P=0.75) and IETS-grade 1 & 2 embryos (P=0.98).

Figure 4: Effect of Estradiol-17β or GnRH on Percent Fertilized and IETS-graded 1 & 2 Embryos

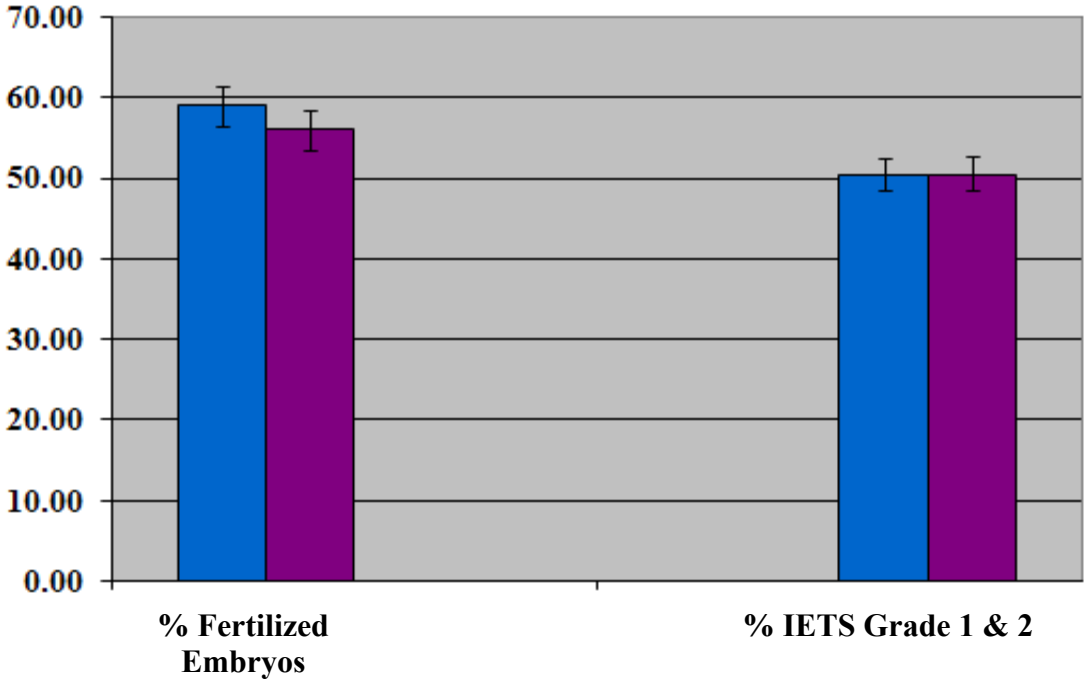


Figure 4: The percentages for fertilized and grade 1 & 2 embryos are represented for the Estradiol-17β protocol and the GnRH protocol. There were no significant differences for percent of fertilized embryos (P= 0.52) and percent of grade 1 & 2 Embryos (P= 0.99).

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APPENDIX

SAS Code

```
/*--------------------------
|  Import wizard is not picking up
  characters in Daily_milk
| e.g DRY becomes . (missing)
 ----------------------------*/

libname save '.';
options pageno=1;

/*
proc contents data=save.wock1;
run;
proc print data=save.wock1;
*where cow="Ashlyn Clone";run;


proc gchart data=wock;
vbar Milk; run;
*/
/*
proc contents data=clean0 varnum;
run;
proc contents data=save.wock1 varnum;
run;

proc freq data=clean0 nlevels;
   tables _all_  / noprint;
   tables fshDose_;
run;


proc freq data=save.wock1;
   tables milk*fsh / missing;
run;
proc freq data=clean0;
   tables milk*fshDose_ / missing;
run;
proc print data=clean0;
```

```
   where milk=' ';
run;
*/
PROC IMPORT OUT= WORK.CLEAN0
            DATAFILE= "C:\Documents and Settings\donaghy\My Documents\sa
ndysas\JilleneWock\408Obsevations.xls"
            DBMS=EXCEL REPLACE;
     SHEET="'Complete data$'";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
RUN;


data clean; set clean0;
if Technician = 'M' or  Technician = 'S';

if Milk ne "." ;

Fsh = (fshdose_ > 14);

if Milk="DRY" and fsh=1 then  stage = "DH";
else if Milk="DRY" and fsh=0 then stage = "DL";
else if Milk="L" and fsh=1 then stage = "LH";
else if Milk="L" and fsh=0 then stage = "LL";
else if Milk="Heifer" and fsh=1 then stage = "HH";
else if Milk="Heifer" and fsh=0 then stage = "HL";

grade12=g1+g2;

proc freq data=clean;
   tables milk*stage /list missing;
   tables stage treatment;
   tables Stage*treatment technician*stage;
run;

%macro repeat(var);
proc mixed data=clean;
  where stage ne "HH";
  class Cow Treatment stage Technician;
```

```
  model &var = Treatment stage Treatment*stage Technician/ddfm=satterthwaite;
  random cow;
  lsmeans  Treatment;
  lsmeans  Technician;
  lsmeans stage / pdiff;
  Title "Variable= &var";
run; quit;
%mend var;


%repeat(recov)
%repeat(PctFert)
%repeat(Grade12)
%repeat(PctGrade12)
```