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Developmental Competence of Oocytes Derived from Seasonally Anovulatory Mares Treated with Estradiol and Sulpiride

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**DEVELOPMENTAL COMPETENCE OF OOCYTES DERIVED
FROM SEASONALLY ANOVULATORY MARES TREATED
WITH ESTRADIOL AND SULPIRIDE**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The School of Animal Sciences

by
Michelle O. Vetter
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ABSTRACT

Over the course of two years (2021-2022), two experiments were conducted during the nonbreeding seasons to evaluate the in vitro developmental potential of oocytes derived from seasonally anovulatory mares treated with ECP-sulpiride. The objective of experiments 1 and 2 were to compare pooled recovery and maturation rates of oocytes from mares treated with estradiol cypionate (ECP) and sulpiride with oocytes collected from naturally transitional mares (controls). Cleavage rates of oocytes subjected to intracytoplasmic sperm injection (ICSI) were compared only in experiment 2. In experiments 1 and 2, a total of 36 light horse and pony-cross anovulatory mares were used. Treatment was administered intramuscularly and consisted of 50 mg ECP followed by 3 g sulpiride (n = 18) in sucrose acetate isobutyrate (SAIB), while controls received vehicle only (n = 18). Jugular blood samples were collected prior to treatment and continued daily for 12 days in order to characterize plasma LH and prolactin concentrations. All mares were subjected to ovum pick up (OPU) when the first 30 mm follicle of the season was detected. All follicles \geq 8 mm were targeted for OPU and aspirates from three follicle diameter categories (8-19 mm, 20-29 mm, and > 30 mm) were pooled within treatment groups. In experiments 1 and 2, 7/7 and 10/11 ECP-sulpiride treated mares responded within 15 days of treatment. Mean day to first 30 mm follicle of ECP-sulpiride treated mares was advanced in experiments 1 (P = 0.005) and 2 (P = 0.0007) by 23 and 19 days, respectively. In both experiments, plasma prolactin and LH increased (P \leq 0.0001) in all mares treated with ECP-sulpiride for at least 7-10 days after treatment. The recovery and maturation rates of oocytes from ECP-sulpiride and vehicle only treated mares did not differ (41.6 vs 45%; P = 0.77 and 55.6 vs 65.4%; P = 0.47, respectively) and there was no significant effect on follicle diameter.

Cleavage rates of oocytes subjected to ICSI did not differ between ECP-sulpiride and vehicle only treated mares (71.4 vs 61.3%; $P = 0.99$, respectively).

CHAPTER 1. INTRODUCTION

Many breed associations consider January 1 as the official birth date for foals born in the same season. There is considerable competitive and economic incentive to induce early cyclicity in mares, so they foal as close to the January 1 birth date as possible. In the Northern hemisphere, the breeding season begins in late spring and ends in early fall, with a period of anovulation during the winter. The seasonal nature of the mare's reproductive cycle has led to the development of techniques to manipulate cyclicity to satisfy the universal birthday standard. The most common method is the use of artificial lighting, considering the mare's reproductive cycle is dictated by photoperiod. However, this process has proven to be costly and does not effectively bypass the erratic ovarian activity associated with the spring transition. Another promising approach is through stimulating the release of the hypophyseal hormone prolactin using dopamine antagonists, such as sulpiride. Pretreatment with ECP one day prior to a single injection of sulpiride in SAIB vehicle is sufficient to stimulate most seasonally anovulatory mares to cycle as early as February, significantly advancing the first ovulation of the year, given that most mares normally begin to cycle in early April. Assisted reproductive technologies (ART) such as ovum pick up and intracytoplasmic sperm injection, can be used to collect oocytes from mares to produce embryos in vitro regardless of season. While these technologies were initially used to address fertility problems of sub-fertile mares or stallions with poor reproductive performance in vivo, it is becoming commonplace for owners to enroll horses of normal fertility in OPU-ICSI programs.

While clinical OPU-ICSI programs in horses has gained popularity in recent years, optimization is still needed. Efficiency of OPU-ICSI programs is greatly influenced by an oocyte's developmental competence, which is the germ cell's ability to mature, cleave following

fertilization, develop to the blastocyst stage, and generate a viable offspring (Sirard et al., 2006). Current success rates of commercial equine ICSI procedures are relatively low, yielding an average of 1.2 blastocysts per OPU session (Hinrichs et al., 2014).

Inducing early cyclicity in seasonally anovulatory mares with ECP-sulpiride results in follicular activity similar to that of an unstimulated, cycling mare. Ovulations from ECP-sulpiride stimulated follicles are fertile and ECP-sulpiride treated mares are capable of sustaining morphologically normal pregnancies with cardiac activity (Oberhaus et al., 2017). In vitro developmental competence of oocytes obtained from stimulated follicles of anovulatory mares has yet to be determined. If oocytes derived from seasonally anovulatory mares treated with ECP-sulpiride have equal developmental potential to oocytes from naturally occurring follicles in the spring, then they could be utilized for the in vitro production of embryos through OPU and ICSI.

CHAPTER 2. REVIEW OF LITERATURE

2.1. The Hypothalamic-Hypophyseal-Ovarian Axis

Regulation of mammalian reproduction begins at the level of the brain in the hypothalamus. The hypothalamus is a neuroendocrine organ that sits just above the adenohypophysis in the brain, connected by the hypophyseal stalk. Communication between the hypothalamus and hypophysis is achieved through a system of blood vessels located between the two organs referred to as the hypothalamic-hypophyseal portal system. Gonadotropin-releasing hormone (GnRH), a 10 amino acid neuropeptide, is released in a pulsatile fashion into portal circulation, eventually binding to GnRH receptors on gonadotropes of the adenohypophysis to signal the synthesis and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Follicle stimulating hormone and LH are glycoproteins that exert their actions on various cell types in the ovary. Follicle stimulating hormone promotes ovarian follicular development and estradiol synthesis while increasing concentrations of LH stimulate ovulation, followed by the formation of the corpus luteum (CL) and subsequent progesterone secretion.

Interactions of gonadotropins with theca and granulosa cells promotes steroid synthesis and secretion. Testosterone, estradiol, and progesterone are the main sex steroids produced by the ovary that influence reproductive tract development and sexual behavior. Androgen production in theca interna cells is mediated by LH and is aromatized into estradiol in neighboring granulosa cells through the action of FSH. Steroids produced by the ovary are required for proper stimulation and regulation of the hypothalamic-hypophyseal axis and its regulation of the estrous cycle and reproductive behavior.

Regulation of GnRH and subsequent gonadotropin secretion in the mare is dictated by season and phase of the estrous cycle. Among other environmental cues such as day length and

temperature, it has been postulated that melatonin plays a role in alteration of GnRH production in respect to the changing seasons (Aurich, 2011). Secretion patterns of gonadotropins in a cycling mare are differentially regulated according to GnRH pulse frequency. Higher GnRH pulse frequency favors LH secretion while lower pulse frequency favors FSH secretion, with frequency varying with stage of cycle. Steroids such as estradiol and progesterone are also involved in regulation of GnRH secretion through positive and negative feedback mechanisms (Clarke and Pompolo, 2005). Negative and positive feedback on GnRH by progesterone and estradiol, respectively, fluctuates with respect to the phases of the mare's estrous cycle. Upstream negative feedback by progesterone on GnRH also reduces LH secretion. Estradiol has a stimulatory effect on LH while progesterone is inhibitory. Together, hypothalamic, adenohipophyseal, and ovarian hormones create an interconnected axis where action upon or regulation of one part greatly affects the other. The mechanisms elucidated herein provide the appropriate endocrine environments needed for proper modulation of the mare's estrous cycle.

2.2. Estrous Cycle of the Mare

The estrous cycle of the mare occurs over an average period of 21 days and consists of two main phases, the follicular phase (estrus) and the luteal phase (diestrus; Raz and Aharonson-Raz, 2012).

2.2.1. Follicular Phase

The estrous cycle is defined as the period of time between two consecutive ovulations, accompanied with behavioral estrus and sexual receptivity to a stallion (Satué and Carlos Gardon, 2021). GnRH from the hypothalamus stimulates release of FSH, the adenohipophyseal gonadotropin responsible for follicular recruitment and development. The ovaries of a cycling

mare are highly active during the breeding season and are characterized by the presence of multiple smaller antral follicles and a single, large dominant follicle that is ovulated. The process of ovarian follicular dynamics occurs continuously throughout the estrous cycle. In the female, antral follicles emerge from a reservoir of small antral follicles located within the ovary that are recruited into follicular waves primarily by FSH (Monniaux et al., 2014). Post-emergence, antral follicles enter the common growth phase, increasing in size at the same rate, about 3 mm per day, for 6-7 days. At the end of the common growth phase, deviation occurs, where the follicle selected for dominance continues to grow while the remaining cohort of antral follicles regress. Follicle deviation involves inhibin, a glycoprotein hormone produced by granulosa cells in the ovaries. Inhibin decreases synthesis and secretion of FSH from the gonadotropes, forcing subordinate follicles to cease further development. Concomitantly, increasing LH concentrations and LH receptor content within the largest follicle contribute to the acquisition of dominance and post-deviation follicle growth (Gastal et al., 2000). The dominant follicle continues to grow until reaching a preovulatory size of 35-40 mm in diameter (Satué and Gardon, 2021). Throughout this process, granulosa cells of the dominant follicle are producing estradiol. Follicle-derived estradiol concentrations peak during estrus, about 2 days before ovulation. Through positive feedback on GnRH, increasing estradiol concentrations stimulate a preovulatory surge of LH, resulting in ovulation of the dominant follicle.

2.2.2. Luteal Phase

The luteal phase of the estrous cycle immediately follows ovulation and is characterized by minimal concentrations of estradiol, increasing concentrations of progesterone, and the presence of a CL (Satué and Gardon, 2021). Formation of the CL involves remodeling of granulosa and theca cells from the ruptured follicle into luteal tissue. Luteal cells are the primary

source of progesterone during diestrus, reaching its peak at 8 days post-ovulation (Aurich, 2011). In the pregnant mare, the CL remains for the duration of gestation, providing the reproductive tract with a progesterone-dominant environment to allow for sustainment of pregnancy. In the non-pregnant mare, the CL will undergo luteolysis, which is the degradation of the corpus luteum. Similar to other species, luteolysis in the mare is initiated by an oxytocin (OT) stimulus, most likely originating from the neurohypophysis and endometrium (McCracken et al., 1999; Aurich, 2011). Oxytocin stimulates the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium, initiating the degradation of the CL. Diestrus comes to an end upon the completion of luteolysis and is coupled with a decline in progesterone. Reduction in progesterone and rising estradiol and FSH concentrations allow a new follicular wave to develop, and the cycle is repeated every 21 days throughout the breeding season.

2.3. Seasonality of Reproduction

The mare experiences seasonal reproductive activity with recurring ovulatory periods during the spring and summer months and an anovulatory period during the winter. The seasonal nature of the mare is an evolutionary tactic to ensure foals are born when conditions are most favorable. Phases of the annual reproductive rhythm include the breeding season, autumnal transition, winter anestrus, and spring transition. Hypothalamic GnRH secretion previously absent for the duration of anestrus is restored during the spring transition and is paralleled with the increase in photoperiod and environmental temperatures in early spring. The first ovulation of the year marks the end of the spring transition and the beginning of the breeding season. The breeding season in the northern hemisphere begins in April or May and continues until the autumnal transition in early fall. The mare will experience recurring periods of estrus

accompanied by an average of 7 ovulations during the breeding season, with each estrual period lasting a range of 4-7 days out of the 21-day cycle (Ginther, 1974; Raz and Aharonson-Raz, 2012). Nearing the autumnal transition, days begin to shorten and gonadotropin secretion from the adenohypophysis is reduced, resulting in decreased ovarian activity (Nagy et al., 2000). The transition into and out of the breeding season is a gradual process and most mares do not transition in synchrony.

During the winter, most mares are anovulatory, ceasing opportunities for breeding until spring. Ovarian activity is significantly reduced or absent during winter anestrus. Ovaries often become small and inactive due to suppression of GnRH, preventing the prolonged LH surge needed to achieve regular ovulations. Follicular waves may occur during winter anestrus due to uninterrupted FSH concentrations, but ovulation typically does not follow. Steroidal activity is greatly reduced during winter anestrus. Circulating estradiol is present at basal concentrations while progesterone concentrations are less than 1 ng/mL.

Industry desire for mares to foal as early in the year as possible motivates equine breeders to ensure mares conceive during the limited breeding season. Therefore, understanding of the endocrine mechanisms influencing equine seasonality is imperative for efficient breeding management and manipulation of the estrous cycle.

2.4. Prolactin

Prolactin is a short chain polypeptide hormone synthesized in and secreted by lactotropes in the adenohypophysis (Freeman et al., 2000). Prolactin is primarily recognized for its role in mammary growth, development, and lactogenesis in mammals (Freeman et al., 2000; Hadley and Levine, 2007). However, prolactin has a diverse range of functions in reproduction in a multitude of different species. In rats, prolactin is a luteotropic factor, aiding in CL development and

progesterone production (Rothchild, 1981). Luteolysis of the murine CL is also induced by prolactin secretion (Rothchild, 1981). In livestock such as horses and goats, increased prolactin concentrations are a stimulant of seasonal hair shedding (Thompson et al., 1997; Donadeu and Thompson, 2002; Celi et al., 2003). Prolactin receptor expression is also present in testicular tissues and spermatogenic cells of male rodents, signifying a role in the regulation of spermatogenesis (Hondo et al., 1995; Ishida et al., 2010). Hyperprolactinemia is known to cause irregular ovarian activity in women, frequently leading to menstrual disturbance and infertility (Crosignani, 2012). The myriad of physiological effects of prolactin on the mammalian reproductive system is efficiently regulated by neuroendocrine mechanisms.

Regulation of prolactin secretion occurs through a short loop feedback system where prolactin itself stimulates its own inhibitory factor, dopamine. Dopamine, a catecholamine neurotransmitter, is released from dopaminergic neurons located in the arcuate nucleus of the hypothalamus (Grattan, 2015). Three sub-populations of dopamine neurons serve as regulators of prolactin secretion: the tuberoinfundibular (TIDA), tuberohypophyseal (THDA), and periventricular hypophyseal (PHDA) neurons. Elevated levels of endogenous prolactin stimulate dopamine synthesis by interaction with these dopamine neurons. The TIDA neurons of the arcuate nucleus are responsible for the release of dopamine in a pulsatile pattern directly into the hypothalamo-hypophyseal portal system, making TIDA neurons the major regulator of hypophyseal prolactin secretion (Freeman et al., 2000; Grattan, 2015). Dopamine sourced from TIDA neurons binds to D2 and D4 dopamine receptors on lactotrophs in the adenohypophysis, inhibiting calcium from entering the cell (Grattan, 2015). Interruption of this calcium influx results in membrane hyperpolarization, which reduces prolactin secretion from lactotropic cells (Grattan, 2015). Dopamine also suppresses adenylate cyclase activity within the lactotrope cell,

reducing prolactin gene expression (Grattan, 2015). Estradiol has a role in the regulation of prolactin secretion (Grattan, 2015). Ovarian estradiol interacts with TIDA neurons to decrease dopamine release, enabling prolactin secretion. Estradiol can also interact with lactotropes to increase prolactin gene expression and therefore increases prolactin levels (Grattan, 2015). Inhibitory activity of dopamine neurons on prolactin is dependent on prolactin concentrations in circulation.

Circulating prolactin binds to the prolactin receptor (PRLr) located within the membrane of target cells. King et al. (2010) validated that equine prolactin and its receptor were present within ovarian tissues. Using immunohistochemistry, Oberhaus et al. (2015) further elucidated the presence of PRLr within follicular tissue and corpora lutea of summer cycling, winter cycling, and anestrus mares. PRLr staining intensity increased with follicle diameter and was highest in antral follicles of summer cycling mares. Positive PRLr staining was also observed in luteal cells and oocytes plus surrounding granulosa cells. Peaks in prolactin concentrations right before or immediately after luteolysis have been reported in the mare (Irvine et al., 2000). Presence of prolactin in follicular fluid in addition to a periovulatory prolactin surge in the mare has also been reported (King et al., 2008). The authors described a positive association between prolactin and follicle diameter, suggesting that the amount of follicular prolactin fluctuates in relation to the growth of the follicle (King et al., 2008). Associations of prolactin concentrations with stage of cycle and follicle diameter, in addition to the presence of prolactin and its receptor within the equine ovary, suggests that prolactin plays a possible role in follicle growth, development, and luteal function (Oberhaus et al., 2018).

2.5. Estrous Cycle Manipulation

Historically, the most common method of cycle manipulation is the use of artificial lighting (Burkhardt, 1947; Kooistra and Ginther, 1975). However, this process has been proven to be costly and often does not entirely bypass the erratic ovarian activity associated with the spring transition. Obstacles associated with artificial lighting has led to the development of pharmacological methods to manipulate the mare's estrous cycle. Administration of GnRH and GnRH agonists have been used to stimulate gonadotropins, mainly LH, to induce ovulations in seasonally anovulatory mares (Johnson, 1987; Hyland and Jeffcott, 1988; Fitzgerald et al., 1993; Williams et al., 2007). Still, responses to treatment with GnRH and its agonists have produced variable success as the response to treatment is dependent on the depth of anestrus and ovarian activity before treatment (Fitzgerald et al., 1993; Mumford et al., 1994; Nagy et al., 2000). Lapin and Ginther (1977) found success in inducing single and multiple ovulations in seasonally anovulatory pony mares using equine pituitary extract; however, these products are not commercially available, making clinical application impractical (Nagy et al., 2000).

Researchers noticed a correlation between seasonal reproductive rhythm of the mare and prolactin concentrations. Hypophysis and serum prolactin concentrations are highest in summer cycling mares and lowest during winter anestrus and are directly associated with changes in photoperiod and environmental temperature (Johnson, 1986; Thompson et al., 1986). This discovery led to the administration of exogenous and stimulation of endogenous prolactin to manipulate the estrous cycle. Thompson et al. (1997) administered recombinant porcine prolactin (rpPRL) to seasonally anestrous mares which resulted in a hastened date to first ovulation of the year. While an increase in prolactin response to rpPRL treatment was observed, use of cross-species prolactin resulted in antibody production against the hormone (Thompson et al., 1997).

Additionally, sources of equine prolactin are not readily available for use in research. Hindrances associated with the use of exogenous prolactin has led to the development of treatment protocols to stimulate prolactin using dopamine antagonists.

2.5.1. Estradiol-Dopamine Antagonists

Dopamine antagonists such as domperidone, perphenazine, and metoclopramide have been administered to increase prolactin concentrations in mares (Becker and Johnson, 1990; Bennett-Wimbush et al., 1998; Brendemuehl and Cross, 2000; Kelley et al., 2006). It was also reported that increasing endogenous prolactin concentrations by administration of a dopamine antagonist (fluphenazine) can induce follicular growth and activity in anestrus mares (Nequin et al., 1993). Utilization of the dopamine antagonist, sulpiride, to hasten the date of the first ovulation of the year in mares has also shown promising results.

The first documented use of sulpiride in the horse was reported by Johnson and Becker (1987). Sulpiride antagonizes dopamine secretion by competitively inhibiting dopamine from binding to its D2 receptor on lactotropes. In this initial experiment, administration of sulpiride resulted in an increase of serum prolactin concentrations in nonpregnant mares (Johnson and Becker, 1987). In addition, sulpiride injections were able to advance the date to first ovulation in anestrus and transitional mares (Besognet et al., 1997; Panzani et al., 2011).

Estradiol has been reported to increase hypophyseal prolactin content (Thompson et al., 1991) as well as the prolactin response to sulpiride (Kelley et al., 2006; Clavier et al., 2012). Kelley et al. (2006), administered every other day injections of estradiol benzoate (EB) in vegetable oil for a total of 10 injections, followed by daily sulpiride injections 11 days later. Pretreatment of seasonally anovulatory mares with EB enhanced the prolactin response to sulpiride and subsequent LH concentrations, with treated mares ovulating an average of 45 days

earlier than controls (Kelley et al., 2006). Still, such treatment needed to be simplified for practical application. Thompson et al. (2008) compared the efficacy of two different single-injection estradiol deliveries to every other day EB injections in geldings. In comparison to the results seen with every other day EB administration, a single injection of 100 mg ECP resulted in the most similar response to treatment (Thompson et al., 2008).

Success of estradiol-sulpiride treatment depends on the protocol used. The duration of endogenous prolactin stimulation by sulpiride administered subcutaneously (Thompson and DePew, 1997) or intramuscularly (Besognet et al., 1997) in oil is brief. It has been suggested that the duration of prolactin stimulation is most likely contributing to an ovarian response of treated mares (Oberhaus et al., 2018). The estradiol-sulpiride treatment protocol could be further simplified by combining the dopamine antagonist with a long-acting vehicle that promotes sustained elevated prolactin concentrations with a single injection. Sucrose acetate isobutyrate (SAIB) is a hydrophobic polymer that is used to sustain the release of the GnRH analogue, deslorelin, in the commercial product SucroMate™ Equine (Ferris et al., 2012; Thorn Bioscience, Louisville, KY). In a study by Oberhaus, et al. (2022), the efficacy of SAIB as a vehicle for both EB and sulpiride was assessed; prolactin and LH were stimulated for 10 and 14 days, respectively, supporting the use of SAIB as a vehicle.

Ovulations from ECP-sulpiride stimulated follicles are indeed fertile as demonstrated by Oberhaus et al. (2017). In that study, 8 of the 9 responding mares treated with ECP-sulpiride were artificially inseminated and induced to ovulate and 6 mares (75%) conceived and carried morphologically normal pregnancies with cardiac activity up to 22 days. In a follow up study, it was reported that ovulatory-sized follicles from seasonally anovulatory mares stimulated with ECP-sulpiride appeared to have follicular fluid steroid hormone production and LH receptor

expression equivalent to naturally occurring follicles in the spring (Oberhaus et al., 2018). Concentrations of estradiol and progesterone, along with IGF-I, in the follicular fluid were similar between groups. An interesting result to note is that prolactin concentrations in follicular fluid were increased in ECP-sulpiride treated mares. The gene expression analysis of LH receptors revealed that both stimulated and non-stimulated follicles shared similar LH receptor expression levels (Oberhaus et al., 2018). Looman (2020) administered ECP-sulpiride to anestrus, transitional, and progestin (altrenogest)-treated mares. Mean day to first ovulation of all 3 ECP-sulpiride treated groups was advanced by 32 days when compared to mares that received vehicle only. Ten embryos (4 frozen, 4 fresh, and 2 cloned) were transferred to mares in each treatment group, and an overall pregnancy rate of 70% was achieved (Looman, 2020).

2.6. Oocyte Growth and Maturation

Oogenesis begins during early fetal life and ends in adulthood. During fetal development, primordial germ cells (PGCs) migrate from the embryonic epiblast to the genital ridge and eventually arrive at the gonad. Once in the developing ovary, PGCs enter mitosis, giving rise to oogonia that undergo an incomplete cytokinesis, leading to the formation of germ cell nests (Sánchez and Smits, 2012). Oogonia then cease mitosis and enter meiosis where they are considered primary oocytes (Sánchez and Smits, 2012). The oocyte will progress through the stages of prophase I of meiosis (leptotene, zygotene, pachytene) where a series of key events occur, including the pairing of homologous chromosomes and genetic recombination (Pepling, 2013). At this time, the oocyte is enclosed in a single layer of pre-granulosa cells, creating the primordial follicle, and remains arrested in the diplotene stage of prophase I (also known as the germinal vesicle (GV) stage) until puberty (Pepling, 2013). At the start of the estrous cycle, a limited number of primordial follicles enter the initial recruitment phase and develop into

primary and secondary (preantral) follicles. A small cohort of preantral follicles are selected to develop to the antral stage, after which a single follicle gains dominance and advances to the preovulatory stage. Throughout the progression of folliculogenesis, the diplotene arrested oocyte grows and accumulates RNA and other cellular machinery needed for further growth and maturation (Holt et al., 2013). After the preovulatory surge of LH, the oocyte will resume meiosis and undergo germinal vesicle breakdown (GVBD). The oocyte progresses through the stages of meiosis until arresting for a second time in metaphase II (MII), where the cell remains until fertilization (Holt et al., 2013).

Oocytes gradually and sequentially acquire developmental competence during folliculogenesis (Sánchez and Smitz, 2012). To participate in fertilization and embryo development, an oocyte must have the ability to resume meiosis and mature to metaphase II (Sirard et al., 2006). Meiotic competence, or the ability to resume meiosis, is acquired during the transition from the preantral to antral follicle stages and involves incremental nuclear and cytoplasmic changes accomplished using RNA stored during the growth phase (Pan and Li, 2019). Maturation promoting factor (MPF), a heterodimer of p34^{cdc2} (or CDK1) and cyclin B, must be activated for the oocyte to resume germinal vesicle breakdown (GVBD) and enter metaphase I (Kanatsu-Shinohara et al., 2000). Meiotic events following the LH surge involve cell-cell communication between the oocyte and the surrounding somatic cells, chromatin condensation during GVBD, chromosome segregation, and polar body extrusion (Coticchio et al., 2014; Chavez et al., 2021). In addition, cytoplasmic and molecular maturation must be completed for the oocyte to develop competently. Cytoplasmic maturation can be described as the morphological changes that occur within the cytoplasm throughout oocyte growth and concurrently with nuclear maturation to prepare the oocyte for fertilization and early embryonic

development (Sirard et al., 2006). During early stages of follicle growth, the oocyte has high transcriptional activity. As follicles grow to ovulatory size, RNA and protein synthesis is gradually reduced until the oocyte reaches a dormant state, a process known as oocyte capacitation (Hyttel et al., 1997; Gilchrist and Thompson, 2007). Nucleolus condensation and ribosome depletion, due to reduced transcriptional and translational activities, are indicative of cytoplasmic competence (Fair et al., 1995; Sirard, 2006). The redistribution of cellular organelles, such as mitochondria and cortical granules, also occurs within the cytoplasm (Ferreira et al., 2009). Molecular maturation involves the storage of undefined molecules within the oocyte, most likely specific mRNAs and proteins that are necessary to promote the molecular events needed to activate the embryonic genome (Sirard et al., 2006). Achievement of meiotic competence along with cytoplasmic and molecular maturation is essential for oocyte growth and crucial for the oocyte's ability to develop into a viable embryo after fertilization (Sánchez and Smitz, 2012).

2.7. In vitro Embryo Production

In vitro embryo production is an important technology used in the assisted reproduction of domestic animals that aims to produce offspring outside of the maternal environment. Production of embryos in this manner involves in vitro maturation (IVM) of recovered oocytes, in vitro fertilization (IVF) or intracytoplasmic sperm injection of in vitro matured oocytes, and in vitro culture (IVC) of presumptive zygotes up to the blastocyst stage (Paramio and Izquierdo, 2014). Success varies between species, with blastocyst rates ranging from 30-40% in bovine, 20-50% in ovine, 40-50% in caprine, and 20% in equine (Camargo et al., 2006; Zhu et al., 2018; Stout, 2020; Souza-Fabjan et al., 2021). Efficiency of IVEP is dictated by an oocyte's

developmental competence, which is described as the germ cell's ability to complete nuclear and cytoplasmic maturation, fertilize normally, develop to the blastocyst stage, and produce a healthy offspring (Sirard et al., 2006; Gilchrist and Thompson, 2007).

Application of ART in the horse, such as OPU and ICSI, has made efficient IVEP possible. Advances in ART in the horse have progressed relatively slowly when compared to other livestock species, such as ruminants. Researchers cannot utilize abattoir-derived equine ovaries to advance research as they have done in ruminants, but post-mortem collection of oocytes for IVEP is an option to produce offspring from animals that die unexpectedly (Hinrichs et al., 2012).

2.7.1. Ovum Pick Up

Another approach to oocyte recovery is OPU, which involves the ultrasound-guided aspiration of follicles on the ovary using a 60-cm long, 12-gauge double lumen (Cook et al., 1993). One option is to aspirate a single dominant, stimulated follicle to recover an in vivo matured oocyte (Hinrichs, 2018). Administration of GnRH analogs or human chorionic gonadotropin (hCG) 24-36 hours before aspiration stimulates the resumption of meiosis of the oocyte within the dominant follicle, with the goal of collecting a mature oocyte just before ovulation (Hinrichs, 2018). The MII oocyte may be transferred into the uterine tube of an inseminated mare (Carnevale, 2004), subjected to ICSI and transferred surgically to the uterine tube of a recipient (Choi et al., 2002), or cultured in vitro up to the blastocyst stage for transcervical embryo transfer (Hinrichs et al., 2014). The recovery rates of pre-ovulatory follicles are high, and these in vivo matured oocytes are developmentally competent (Hinrichs et al., 1990). Growth of the dominant follicle needs to be monitored frequently and precise timing of hCG/GnRH trigger needs to be coordinated with the ICSI laboratory (Hinrichs, 2018). Vigorous

repeated flushing and scraping with the needle is needed to dislodge the tightly attached COC from the follicle wall, which can be problematic due to the large size of the dominant follicle. Because of the inconsistencies associated with superovulation in the mare (Alvarenga et al., 2008), only one, or sometimes two, dominant follicles are available for aspiration in each estrus during the breeding season (Hinrichs, 2018). Another approach to OPU is aspiration of all visible subordinate follicles to recover immature oocytes for in vitro maturation. In this manner, OPU can be performed year-round, on average once every 14 days, without stimulation or monitoring of ovarian activity (Jacobson et al., 2010). Repeated OPU sessions have not been found to have any detrimental effects on mare fertility (Mari et al., 2005; Vanderwall et al., 2006). From a clinical standpoint, a population of multiple small to medium sized antral follicles (> 10 mm) on the ovary is desired for OPU (Galli et al., 2014).

Earlier studies in the mare report recovery rates of immature oocytes ranging from 12.3% to 84% varying with stage of cycle, follicle diameter, and technique used (Bracher et al., 1993; Cook et al., 1993). Bracher et al. (1993) reported that aspirations from unstimulated, immature follicles yielded higher oocyte recovery rates than hCG or PGF_{2α} stimulated follicles. Stage of estrous cycle has been reported to affect recovery rates, with higher oocyte recovery associated with aspirations performed in cyclic, estrus mares when compared to aspirations performed during diestrus (Cook et al., 1993). This study revealed an effect of follicle diameter on oocyte recovery, where follicles < 15 mm produced higher (31%) recovery rates when compared to follicles > 20 mm (8%) (Cook et al., 1993). Additionally, Purcell et al. (2007) compared recovery rates of oocytes between transitional, cycling, and pregnant mares, based on small (10-20 mm) and large (> 20 mm) follicle diameter. While there was no difference in rates between cycling and transitional mares, the authors found that recovery from small follicles (21.6%) was

greater than that of large follicles (11.7%; Purcell et al., 2007). It has been demonstrated that cycling, transitional, and anestrus mares are suitable oocyte donors for OPU (Colleoni et al., 2007; Iacono et al., 2014). Follicles during the non-breeding season are smaller, but the number of follicles available for OPU does not differ significantly from that of the breeding season, and oocytes collected during both seasons are capable of normal maturation and fertilization (Hinrichs and Schmidt, 2000; Mak et al., 2018).

When targeting immature follicles, one may recover an average of 2 to 10 oocytes per mare if adequate flushing of the follicle is achieved (Goudet et al., 1997; Galli et al., 2007; Jacobson et al., 2010). ICSI programs are more efficient when all immature follicles are aspirated rather than a single pre-ovulatory follicle (Hinrichs, 2018). This can be observed in a study by Jacobson et al. (2010), which reported that aspiration of immature follicles yielded more blastocysts per OPU session. Follicles ≤ 10 mm are more abundant on the ovary than larger ones, so the increased blastocyst yield in this study is likely due to a higher number of oocytes being available for manipulation, not their inherent developmental capacity. Developmental potential of recovered equine oocytes is influenced by the size and maturity of the follicle it was derived from and has been reported to increase with increasing follicle size (Hinrichs and Schmidt, 2000). This can be observed in other species including the bovine (Lonergan et al., 1994), porcine (Marchal et al., 2002), buffalo (Raghu et al., 2002), and human (Rosen et al., 2008). Nevertheless, immature equine oocytes from smaller follicles can acquire meiotic competence and reach MII when matured in vitro (Hinrichs, 1998).

2.7.2. *In Vitro Maturation*

In vitro maturation may be achieved by placing immature oocytes into a controlled culture environment to facilitate the completion of nuclear and cytoplasmic maturation. Upon retrieval, oocytes may be placed directly into IVM or held overnight at room temperature. Holding immature equine oocytes to delay maturation allows for overnight shipment of oocytes to the ICSI laboratory (Galli et al., 2016). This practice also ensures that ICSI and other manipulations can be performed at more convenient working hours (Hinrichs, 2020).

The earliest successful report of in vitro matured equine oocytes was that of Fulka and Okolski (1981), followed by Zhang et al. (1989), whose work resulted in the first production of equine embryos derived from IVM oocytes. Matured oocytes were transferred to the uterine tubes of inseminated mares, recovered as day 7 blastocysts by uterine flushing, and transferred into a recipient mare, resulting in one pregnancy (Zhang et al., 1989). Squires et al. (1996) reported the first successful establishment of pregnancy accomplished using in vitro matured oocytes subjected to ICSI using oocytes collected from abattoir derived ovaries. Choi et al. (2004) also demonstrated that IVM oocytes subjected to ICSI were capable of blastocyst formation in vivo. Furthermore, Hinrichs et al. (2005) reported the birth of two live foals derived from IVM equine oocytes that underwent ICSI and cultured to the blastocyst stage in vitro. These studies provide evidence that in vitro matured equine oocytes are capable of normal maturation and embryonic development.

Equine cumulus oocyte complexes (COC) available for in vitro maturation are classified as compact (Cp), expanded (Ex), or denuded (Dn). Most oocytes will have Cp or Ex cumuli, and their meiotic competence may be correlated to their cumulus cell morphology. In other species, such as the bovine, oocytes with multiple layers of healthy, compact cumuli are selected for IVM

and appear to have the highest meiotic competence; however, this is not necessarily the case for the equine (Kakkassery et al., 2010). In a study by Hinrichs et al. (1997), Ex oocytes were most likely to originate from atretic follicles (≤ 10 mm) while Cp oocytes were predominantly present in growing follicles (> 20 mm). Cumulus expansion was associated with degree of atresia. In several studies, Ex oocytes matured in vitro were found to reach MII at a faster rate and in higher proportions when compared to Cp oocytes (Hinrichs et al., 1993; Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000; Galli et al., 2007).

Commonly used media for equine IVM are tissue culture medium 199 (TCM 199) and Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12), which are occasionally supplemented with fetal bovine serum (FBS), gonadotropins LH and FSH, and antibiotics (Galli et al., 2007). Other components such as preovulatory follicular fluid and the growth factors insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) have been added to improve IVM conditions with variable success (Dell'Aquila et al., 1997; Aguilar et al., 2001; Carneiro et al., 2001; Lorenzo et al., 2002). High quality commercial media are available, and the formulation is generally similar, but the exact medium composition is kept confidential.

Equine oocytes are generally matured at 38.2°C in a 5-6% CO₂ environment in atmospheric O₂ (~21%) for 30 ± 2 hours (Squires, 1996). During oocyte maturation, cumulus cells surrounding the oocyte will extrude extracellular matrix, causing separation of the cells from one another, a process known as cumulus cell expansion (Nevoral et al., 2014). Expansion of cumulus cells can be observed just before ovulation in vivo and during meiotic maturation in vitro (Salustri et al., 1989). Metaphase II oocytes with an extruded first polar body present in the perivitelline space are selected for fertilization by ICSI.

2.7.3. Intracytoplasmic Sperm Injection

ICSI is a substantial technology applied in the assisted reproduction of domestic animals, including the equine. Fertilization by ICSI involves the injection of a single spermatozoon into the cytoplasm of a mature oocyte by means of micromanipulation under an inverted microscope (Hinrichs, 2018). Since only one spermatozoon is required for fertilization, ICSI is useful in cases where the stallion of choice has poor reproductive performance *in vivo* or when quantities of semen are scarce and/or expensive (Carnevale et al., 2007). Mares unable to establish pregnancy due to reproductive tract abnormalities, such as oviduct occlusion, chronic uterine infection, and uterine fibrosis are also candidates for ICSI (Cuervo-Arango et al., 2019). In some cases, clinical application of ICSI is also practiced with animals of normal fertility, since OPU can be performed year-round requiring less time out of training and competition, no hormonal stimulation, and a minimal amount of sperm.

The application of ICSI in the equine has improved the production of embryos *in vitro*, unlike conventional *in vitro* fertilization in the horse. Two foals have been born as a result of conventional IVF; however, in both instances, *in vivo* matured oocytes were used (Palmer et al., 1991; Bezard, 1992). In other studies, fertilization of *in vitro* matured oocytes by conventional IVF has resulted in the production of blastocysts, but a successful pregnancy was never established (Meintjes et al., 1995; Hinrichs et al., 2002). Failure of IVF in the horse may be attributed to inadequate sperm capacitation *in vitro* (Alm et al., 2001).

The first ICSI foal produced using oocytes derived from slaughterhouse material was reported by Squires et al. (1996). Cochran et al. (1998) reported the first successful use of immature oocytes collected from live mares via OPU for ICSI. The early cleavage stage embryos were transferred to the uterine tubes of recipient mares resulting in the birth of 2 live foals

(Cochran et al., 1998). The first equine blastocysts produced entirely in vitro and transferred into the uteri of recipient mares resulting in a successful pregnancy was reported by Li et al. (2001). Several other studies report successful production of equine embryos by ICSI, demonstrating their developmental competence both in vivo (Choi et al., 2004) and in vitro (Hinrichs et al., 2005). In a study by Choi et al. (2002), the authors established that both fresh and frozen-thawed spermatozoa may be used for ICSI, with no significant differences in embryo development observed. Stallion semen subjected to two freeze-thaw cycles is also capable of producing normal fertilization and blastocyst rates when used for ICSI (Choi et al., 2006). Equine ICSI embryos cryopreserved on days 7 and 8 of culture are capable of normal embryonic development after transfer (Lazzari et al., 2020). A considerable effect of season on the likelihood of success of an OPU-ICSI program and the developmental potential of such embryos has not been reported. Equine oocytes collected during the nonbreeding season are capable of fertilization by ICSI and embryo development in vitro (Choi et al., 2016; Mak et al., 2018). Additionally, Colleoni et al., (2007) described that in vitro embryo production can be performed during the breeding season as well as the fall and spring transition periods, with no effect on maturation, cleavage, and blastocyst rates. In other words, OPU-ICSI procedures may proceed year-round, yielding similar results in both the breeding and nonbreeding seasons.

The success of an ICSI procedure is expressed as the rate of blastocyst development. Likelihood of success of a commercial OPU-ICSI program can be affected by many factors including the identity, age, and antral follicle count of the mare (Cuervo-Arango et al., 2019). In 2014, it was reported that in a commercial equine ICSI program, an average of 1.2 blastocysts were produced per aspiration (Hinrichs et al., 2014). In a later study by Lazzari et al. (2020), an average of 1.7 to 2 blastocysts per OPU-ICSI session were produced solely from warmblood

mares. Most recently, Claes and Stout (2022) advertised a mean of 2.12 blastocysts per session in their clinical IVEP program, with a pregnancy rate > 70% and a subsequent foaling rate of approximately 60%.

Two major techniques investigated by researchers over the years are conventional ICSI and Piezo-driven assisted ICSI (cICSI and PDAI, respectively). In cICSI, a sharp microtool is used to break the sperm tail to immobilize the sperm and disrupt its plasma membrane. The sharp microtool penetrates both the zona pellucida and the oocyte plasma membrane by mechanical force and deposits the sperm directly into the cytoplasm (Kimura and Yanagimachi, 1995). In PDAI, the plasma membrane of the sperm is disrupted by piezoelectric pulses. A blunt microtool is advanced through the zona and into the cytoplasm driven by several rapid pulses made by the piezo drill, almost like a microscopic jackhammer (Kimura and Yanagimachi, 1995). It has been reported that cICSI rates can be quite variable from lab to lab, but it has been described that cICSI can achieve blastocyst rates comparable to PDAI (Choi et al., 2005; Leisinger et al., 2016). Moreover, Salgado et al. (2018) did not discover any differences in blastocyst rates of oocytes subjected to either cICSI or PDAI (39 vs 40%, respectively). In terms of blastocyst quality, it was reported that nuclear fragmentation rate was higher, and cell number was lower in blastocysts produced by cICSI (Salgado et al., 2018). Nevertheless, satisfactory fertilization and blastocyst rates can be achieved with either cICSI or PDAI (Leisinger et al., 2016; Salgado et al., 2018). Laser-assisted ICSI (LAI) is another modified injection technique that involves the creation of several tiny holes in the zona with a laser device to aid in the mechanical penetration of the oocyte by a sharp microtool (Smits et al., 2012). Employment of LAI has not been reported to significantly improve cleavage or blastocysts rates of injected equine oocytes when compared to PDAI (Smits et al., 2012).

2.7.4. *In Vitro Culture*

Immediately following ICSI, presumptive zygotes are placed into an in vitro culture environment to facilitate the early stages of embryonic development. Fertilized equine oocytes are generally cultured in vitro up to the blastocyst stage in a defined medium at 38.2°C in a mixed gas atmosphere of 5% CO₂, 5% O₂, and 90% N₂ (Hinrichs et al., 2005). Equine embryos remain in IVC for 7-10 days post fertilization, after which they may be transferred to a recipient or cryopreserved for later use (Claes and Stout, 2022). Components of culture media vary, but often include macromolecules, serum, vitamins, amino acids, antioxidants, antibiotics, and energy substrates (lactate, pyruvate, or glucose; Morbeck et al., 2014).

In both in vivo and in vitro environments, the fertilized oocyte undergoes multiple morphological and metabolic changes to eventually arrive at the blastocyst stage. This period of growth is referred to as preimplantation embryo development. The timing of equine embryo development in vitro is similar to that observed in vivo, but there are slight differences (Carnevale and Metcalf, 2019). The presence of two pronuclei (one maternal and one paternal) in the cytoplasm and two polar bodies in the perivitelline space after ICSI is indicative of successful in vitro fertilization; however, this cannot be easily visualized in the equine zygote due to the high amount of dark lipids present (Veeck and Zaninovic, 2003; Carnevale and Metcalf, 2019). After DNA replication, the zygote progresses through the first mitotic cell division and is divided into two blastomeres (Veeck and Zaninovic, 2003). A 2-cell equine embryo is often observed by 24 hours, a 4-cell by 48 hours, and an 8-cell around 72 hours post fertilization (Bezard et al., 1989). In vivo, compaction is initiated in the equine embryo around day 4 and results in the establishment of an outer layer of cells that later form the trophectoderm, and a congregation of inner cells that will become the inner cell mass (ICM; Betteridge et al.,

1982; Veeck and Zaninovic, 2003). The trophectoderm will later give rise to the placenta, and the ICM will give rise to the embryo proper (McCue, 2014). Following compaction, the cavitation process begins, and a blastocoele cavity of fluid forms (Veeck and Zaninovic, 2003). In the mare, the blastocyst migrates from the uterine tube to the uterus at days 5 or 6 of development (Betteridge et al., 1982). By day 7, a protective capsule is present in between the zona pellucida and the layer of trophoblast cells and persists until the blastocyst reaches about 34 mm in diameter (around 21 days of pregnancy; Betteridge et al., 1982). Quality of equine IVP embryos is often graded on a scale of 1 to 4. Grade 1 embryos are considered excellent (spherical, cells of uniform size and shape, no fragmentation), grade 2 embryos are considered fair (moderate abnormalities; irregular shape, cytoplasmic fragmentation), grade 3 embryos are classified as poor (major abnormalities; high degree of fragmentation, many extruded or degenerate cells), and grade 4 embryos are degenerate or dead (McKinnon and Squires 1988).

It is important to note that aberrant development of IVP equine embryos has been observed in culture. In comparison to in vivo derived embryos, in vitro produced equine blastocysts have lower cell numbers, higher rates of apoptosis, an indistinct separation of the trophectoderm and ICM cells, as well as a smaller or absent blastocoele cavity (Tremoleda et al., 2003). Additionally, blastomeres often leak out of the zona through the hole made during ICSI (Carnevale and Metcalf, 2019).

Various approaches have been taken to achieve acceptable blastocyst rates from in vitro cultured equine zygotes, including co-culture with somatic cells (Battut et al., 1991; Li et al., 2001) and in vivo culture of oocytes that underwent ICSI (Galli and Lazzari, 2001; Choi et al., 2004). Several culture media and culture conditions have been described for the culture of presumptive equine zygotes. Galli et al. (2002) reported that addition of bovine serum albumin

(BSA) and amino acids to synthetic oviductal fluid (SOF) culture media resulted in a 14% blastocyst development rate. In addition, blastocyst rates of 15% were obtained when fertilized oocytes were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (Choi et al., 2004). Culture in DMEM/F-12 medium supplemented with either BSA or FBS under a mixed gas atmosphere (5% CO₂, 5% O₂, 90% N₂) resulted in blastocyst rates similar to those observed with in vivo culture of ICSI embryos (~ 27%; Hinrichs et al., 2005; Choi et al., 2006). Utilization of a modified commercial human embryo culture medium (LifeGlobal[®]) for the culture of equine oocytes yielded blastocyst rates of 20% (Hinrichs et al., 2014). The use of a complete human culture system (Sage[™], Origio) by Leisinger et al. (2016) yielded blastocyst rates > 20% using cICSI. More recently, utilization of a modified SOF-IVC medium supplemented with BSA and amino acids in a commercial OPU-ICSI program has led to a significant increase in the number of freezable and transferrable embryos (Lazzari et al., 2020). In summation, media composition and culture conditions employed by different ICSI laboratories vary widely, and improvements can always be made to optimize the culture environment for IVC of equine embryos.

2.8. Prolactin and the Oocyte

Prolactin receptors have also been identified in the ovaries of many other species including mouse, rat, rabbit, bovine, porcine, and human (Poindexter et al., 1979; Waters et al., 1984; Shirota et al., 1990; Słomczyńska et al., 2001; Kiapekou et al., 2009). Various roles for prolactin in steroidogenesis, ovulation, and oocyte maturation have been suggested. The effect of prolactin on oocyte maturation and embryonic development appears to differ with respect to in vivo and in vitro environments. The addition of human prolactin to granulosa and luteal cell culture at high concentrations resulted in the suppression of progesterone and estradiol secretion,

while low concentrations of prolactin increased basal progesterone levels (McNatty et al., 1974; Lee et al., 1986; Alila et al., 1987). Plus, prolactin levels are higher during the ovulatory period and luteal phase of the menstrual cycle and transiently increase in women undergoing gonadotropin treatment for IVF (Vekemans et al., 1977; Oda et al., 1991). Earlier studies claim that estradiol-induced hyperprolactinemia negatively affected pregnancy outcomes in IVF patients since treatment with bromocriptine, a dopamine agonist, was reported to improve fertilization rates of oocytes in hyperprolactinemic patients (Reinhaller et al., 1988). Conversely, other studies provided evidence that high prolactin was not associated with infertility (Hofmann et al., 1989; Anthony Pattinson et al., 1990). Laufer et al. (1984) reported that mature fertilized oocytes associated with a successful pregnancy were retrieved from follicles with higher prolactin concentrations. In addition, Oda et al. (1991) reported that fertilization and cleavage rates of oocytes from hyperprolactinemic patients were significantly higher than those with hypoprolactinemia.

When prolactin antiserum was added to organ culture of porcine antral follicles, it appeared that meiotic resumption was induced in these oocytes (Baker and Hunter, 1978). This suggests that prolactin may be involved in the maintenance of meiotic arrest of porcine oocytes. Using an *in vitro* perfused rabbit ovary model, Yoshimura et al. (1991) reported that addition of prolactin (1000 ng/ml) to the perfusion medium greatly inhibited hCG-stimulated ovulations in a dose-dependent manner. Increased activity of gonadotropin-stimulated plasminogen activator (PA), which is produced by granulosa cells, is believed to be involved in follicle rupture during ovulation (Reich et al., 1985). Activity of PA was significantly inhibited in mature follicles upon exposure to prolactin, which may explain why ovulations were hindered in prolactin-treated follicles. When evaluating the developmental capacity of these oocytes, a greater percentage of

blastocysts were produced by oocytes that were matured in the medium with prolactin. Perhaps the delay in ovulation caused by high prolactin levels is beneficial for meiotic and cytoplasmic maturation in this species.

Interestingly, homozygous PRLr gene knockout mice proved to be infertile, with multiple reproductive abnormalities, such as irregular ovulation and deficient preimplantation embryo development (Ormandy et al., 1997). Kiapekou et al. (2009) elucidated the expression of PRLr in murine preantral follicles, COCs, and germinal vesicle stage oocytes. In this study, culture of preantral mouse follicles with or without prolactin was executed to evaluate the effect of prolactin on early embryonic development *in vitro*. The maturation rate of oocytes and cleavage rate of subsequent zygotes post fertilization were significantly higher in the groups cultured with prolactin than without (64.5 vs 34.5% and 61.9 vs 43%, respectively). In addition, the authors reported an advanced rate of development to the morulae and blastocyst stages in the prolactin culture group. Finally, increased blastocyst rates were observed in groups of cumulus-enclosed bovine oocytes matured in the presence of prolactin (Lebedeva et al., 2008). Together, the studies reviewed herein suggest that prolactin is involved in the mechanisms associated with the completion of meiotic and cytoplasmic maturation that is imperative to the acquisition of developmental competence.

2.9. Rational for Present Experiments

While it has been established that administration of ECP-sulpiride to seasonally anovulatory mares reliably hastens the date to first ovulation of the year, considerable research is still needed to investigate the effects of this stimulation protocol on the outcomes of the *in vitro* production of embryos. Ovulations from ECP-sulpiride stimulated follicles are fertile *in vivo*, but

the in vitro developmental potential of oocytes from these stimulated follicles has yet to be explored (Oberhaus et al., 2017; Looman, 2020). To the author's knowledge, this study is the first to evaluate the meiotic and developmental competence of oocytes collected via OPU from ECP-sulpiride treated mares through subjection to in vitro maturation, ICSI, and in vitro culture. The objectives of the experiments described herein were conducted to determine if oocytes from seasonally anovulatory mares induced to cycle with ECP-sulpiride would have equivalent in vitro developmental potential to oocytes from naturally occurring follicles of transitional mares in the spring. We hypothesize that oocytes derived from ECP-sulpiride stimulated follicles will have similar in vitro developmental competence to oocytes from follicles of naturally transitioning mares.

CHAPTER 3. MATERIALS AND METHODS

3.1. Animals

Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center. All mares used in these experiments resided at the LSU Reproductive Biology Center in St. Gabriel, Louisiana and represented Quarter Horse, Thoroughbred, and light horse-pony cross breeds. Mares were maintained outdoors with ad libitum access to Bermuda grass pasture and automatic waterers. Hay prepared from the same pasture was supplemented starting early November. Beginning in January, mares were limit-grazed on winter Ryegrass. Protein lick tubs were also available ad libitum (18% All Natural, Positive Feed, Ltd., Sealy, TX).

Beginning in early December 2020 and 2021, all non-pregnant mares were subjected to ovarian ultrasound once a week and jugular blood samples were collected approximately every four days to determine progesterone concentrations. Mares were deemed seasonally anovulatory when no significant ovarian structures were observed (follicles > 20 mm and corpora lutea), and plasma progesterone concentrations were consistently ≤ 1 ng/mL for at least three weeks prior to the start of the experiment.

3.2. Experiment One

Treatment

Fourteen light horse and pony-cross anovulatory mares were blocked by breed and age before random assignment to treatment or control groups. On February 23, 2021, mares assigned to treatment ($n = 7$) received 50 mg ECP (Sigma Aldrich, St. Louis, MO) in 1 ml SAIB (Eastman Chemical Company, Kingsport, TN) while mare assigned to controls ($n = 7$) received 1 mL SAIB. One day later, treatment mares received 3 g sulpiride (racemic mixture; Sigma Aldrich) in

5 mL SAIB while control mares received 5 mL SAIB only. All treatments were administered intramuscularly. Blood samples were collected via jugular venipuncture into evacuated tubes containing sodium heparin (Vacurette; Greiner Bio-One, Monroe, NC) one day prior to treatment with sulpiride and continued daily for 10 days post-treatment to characterize prolactin and LH. Plasma was frozen at -20 °C until assays were performed. Ovarian follicular activity was monitored via ultrasonography (Aloka 550V with 5-Mhz linear-array transducer; Hitachi-Aloka, Wallingford, CT) daily and continued until May 1. Size and number of ovarian follicles were recorded.

Ultrasound-Guided Transvaginal Ovum Pick Up

Upon detection of the first 30 mm follicle, the mare was subjected to ultrasound-guided transvaginal OPU. For the procedure, mares were restrained in palpation stocks and sedated with detomidine (0.005-0.01 mg/kg body weight (BW); Dormosedan, Zoetis, Parsippany, NJ) and butorphanol (0.005-0.01 mg/kg BW; Torbugesic, Zoetis, Parsippany, NJ), administered intravenously, to minimize movement during the procedure. Mares also received 0.3 mg/kg BW of N-butylscopolammonium bromide (Buscopan, Boehringer Ingelheim, Duluth, GA), intravenously, to decrease rectal tone. After aseptic preparation of the perineal area, a transvaginal probe (Hitachi-Aloka, Wallingford, CT) was inserted into the vagina. The ovary was then manipulated and fixed by rectal palpation against the vaginal wall. A 60 cm, 12-gauge double lumen needle (MILA international, Inc, Florence, KY), connected to a collection bottle, was advanced through the vaginal wall to aspirate follicles using a vacuum pump (Cook Medical, Bloomington, IN). All follicles ≥ 8 mm were targeted for OPU, and aspirates from each session were collected and pooled into a separate set of collection bottles based on treatment group and follicle diameter (8-19 mm, 20-29 mm, ≥ 30 mm). Follicles were flushed four to eight

times with commercial embryo flush media (ViGRO Complete Flush Solution, Vetoquinol, Fort Worth, TX) supplemented with 5 USP units/mL heparin sodium (Sagent Pharmaceuticals, Schaumburg, IL). Following the procedure, mares received flunixin meglumine (0.5 mg/kg BW; Banamine; Merck Animal Health, Madison, NJ) and procaine penicillin (20,000 units/kg BW; Agri-Cillin; AgriLabs, St. Joseph, MO).

Oocyte Recovery and Maturation

Throughout the following steps of the experiment, oocytes were grouped separately based on follicle diameter. Aspirates were filtered through a filter containing a 75-micron stainless steel screen (EmCon; Agtech, Manhattan, KS) to isolate oocytes. Oocytes were identified using a stereomicroscope (SMZ1500, Nikon Instruments Inc, Melville, NY) and collected into 2 mL cryovials (reference). Oocytes were held 14 to 20 hours (overnight) at room temperature (approximately 22°C) in holding media (Leisinger et al., 2016; EQ-Hold; ivf Biosciences, Cornwall, UK) before being placed in maturation media (EQ-IVM; ivf Biosciences) and incubated at 38.2°C at 6% CO₂ for 30 hours.

In vitro matured oocytes were fixed and mounted onto microscope slides to visualize the MII plate. Mounting of oocyte was achieved by placing oocyte onto a microscope slide within a drop of maturation media, depositing 4 small spots of paraffin wax and petroleum jelly mixture onto the edges of a cover slip, placing the cover slip over the oocyte on the microscope slide, and tapping gently with a utensil until a bubble formed around the oocyte, indicating that the oocyte is adequately pressed onto the slide. Microscope slides were placed in 45% acetic acid in 95% ethanol mixture in a Coplin jar for 3 days to remove lipids from the cytoplasm of the oocyte(s). Slides were then dried by blotting and a single drop of orcein stain (10 mg/mL water and acetic acid, 60:40 vol:vol) was placed on one open side of the coverslip. A disposable wipe was placed

on the side of the coverslip opposite from the stain to draw the stain across the slide. Stained oocytes were viewed using brightfield microscopy under 400X magnification to visualize a MII plate.

Radioimmunoassay

Frozen plasma samples were thawed, and concentrations of prolactin and LH determined by radioimmunoassays previously developed and validated in our laboratory (Cartmill et al., 2003; Colborn et al., 1991; Thompson, Godke, et al., 1983; Thompson, Reville, et al., 1983). Intra- and inter-assay coefficient of variation and levels of detection were 7%, 12%, and 0.2 ng/mL for prolactin and 6%, 9%, and 0.2 ng/mL for LH.

3.3. Experiment Two

Animals

Mares for this experiment were housed at one of two Louisiana Agricultural Experiment Station farms: the Central Research Station Horse Unit on the Ben Hur Plantation and the Reproductive Biology Center. The two farms were located approximately 9 miles apart south of the Louisiana State University main campus in Baton Rouge. Nutritional management and determination of seasonal anovulation was identical to experiment one.

ECP-Sulpiride Treatment

Twenty-two light horse and pony-cross anovulatory mares were identified as seasonally anovulatory and blocked by breed and age before random assignment to treatment or control. Dose and source of ECP and sulpiride and treatment schedule were identical to experiment one. On February 12, 2022, mares assigned to treatment (n = 11) received 50 mg ECP followed by 3 g sulpiride, both suspended in SAIB, one day later while controls (n = 11) received SAIB only.

Intracytoplasmic Sperm Injection and In Vitro Culture

An equine ICSI media suite (ivf Biosciences) was used for the following procedures. Oocyte holding and maturation procedures were identical to those utilized in experiment 1. Throughout the experiment, oocytes were matured, subjected to ICSI, and cultured separately based on follicle diameter. Semen from the same ejaculate was used for all ICSI procedures. Frozen semen extended with ButoCrio (Butopharma USA, Pheonix, AZ) from a single stallion of proven ICSI fertility was prepared in EQ-SemenPrep media. Cumulus-expanded oocytes were stripped of cumulus cells in 100 μ L EQ-Strip, then transferred to EQ-ICSI and evaluated for extrusion of the first polar body. Oocytes with polar bodies were transferred to 10 μ L drops of EQ-ICSI in preparation of sperm injection. A single sperm was selected, and the microinjection pipette was used to break the tail to immobilize the sperm. The sperm was then aspirated tail first into the microinjection pipette and injected into the oocyte at the 3 o'clock position while the oocyte was held in place by a holding pipette. The sperm was then delivered into the oocyte cytoplasm. Presumptive zygotes were then transferred to 500 μ L EQ-IVC1 with a 400 μ L oil overlay and incubated at 38.2°C at 6% O₂, 6% CO₂, and 88% N₂ for 3 days. On day 3 of culture, presumptive zygotes were evaluated for cleavage.

Statistical Analyses

Data were analyzed by GraphPad Prism 9.3.1 (350) (GraphPad Software, Inc, San Diego, CA). All variables were analyzed for normal distribution using Shapiro-Wilk Test. Single-point data (days to ovarian response and number of follicles) were analyzed by two-tailed Student's t-test. Recovery, maturation, and cleavage rates were first analyzed based on follicle diameter using a Fisher's exact test. When no statistical differences were detected, recovery and maturation rates of oocytes from experiments 1 and 2 were pooled and analyzed by Fisher's

exact test. Cleavage rates from experiment 2 were also compared using Fisher's exact test. Concentrations of prolactin and LH were analyzed by mixed-model ANOVA with repeated sampling. For all tests, significance was set a $P \leq 0.05$. For data sets in which the variance tended to be proportional to the mean (multiplicative effects), parallel ANOVA were performed both on the raw data and on log-transformed data. When log transformation had little effect on the overall analyses or our interpretation of the results, we chose to present non-transformed data in the figures. When appropriate, means were separated by Fisher's Least Significant Difference test.

CHAPTER 4. RESULTS

4.1. Ovarian Responses

An ovarian response was defined as the presence of a follicle at least 30 mm in diameter within 25 days after treatment with sulpiride. Mean day to first 30 mm follicle after treatment with ECP and sulpiride in experiment 1 and 2 are presented in Figure 1. All seven ECP-sulpiride-treated mares in experiment 1 responded within 14 days of treatment with a mean day of 11.6 ± 1 d (Figure 1). Mean day of calendar year to first 30 mm follicle was advanced ($P < 0.01$) by 23 days in mares treated with ECP-sulpiride compared to mares that received vehicle only (March 5 ± 2.7 d vs March 27 ± 17 d). Of the 11 mares in experiment 2 treated with ECP-sulpiride, 10 mares responded within 15 days of treatment with a mean day of 12.4 ± 2.6 d (Figure 1). Mean calendar day of year to first 30 mm follicle was advanced ($P < 0.001$) by 19 days in mares treated with ECP-sulpiride compared to mares administered vehicle only (February 25 ± 8.5 d vs March 15 ± 12 d).

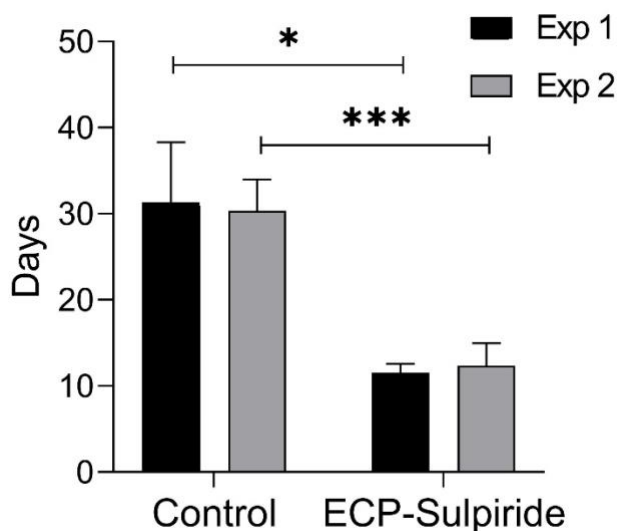


Figure 1. Mean day to first 30 mm follicle after treatment with 50 mg ECP and 3 g sulpiride or vehicle only in experiments 1 and 2. Means differ at $P < 0.05$ (*) or < 0.001 (***)

There were similar numbers of ovarian follicles present upon detection of the first 30 mm follicle in Exp 1; however, a greater ($P < 0.001$) number of follicles were observed on the ovaries of control mares than on mares treated with ECP-sulpiride in Exp 2 (Figure 2).

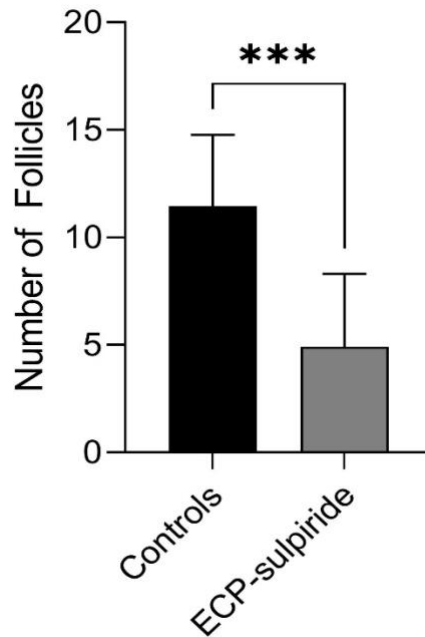


Figure 2. Mean number of ovarian follicles present upon detection of the first 30 mm follicle in experiment 2 in both mares treated with 50 mg ECP and 3 g sulpiride ($n = 11$) and mares treated with vehicle only ($n = 11$). Means differed at $P < 0.001$ (***).

4.2. Endocrine Responses of Responding and Nonresponding Mares

Mean prolactin concentrations of mares treated with ECP-sulpiride or vehicle only in experiments are presented in Figure 3. Prolactin was stimulated ($P < 0.0001$; Figure 3A) in mares treated with ECP-sulpiride for at least 7 days after sulpiride treatment. There was an interaction ($P < 0.0001$) between treatment and day for mean plasma prolactin with concentrations being higher on days 0.5 through 7. Plasma prolactin concentrations of mares treated with ECP-sulpiride or vehicle only in Experiment 2 are presented as geometric means in Figure 3B. Prolactin was stimulated ($P < 0.0001$) in mares treated with ECP-sulpiride for at least 10 days

after sulpiride treatment. There was an interaction ($P < 0.0001$) between treatment and day for mean plasma prolactin with concentrations being higher on days 1 through 10.

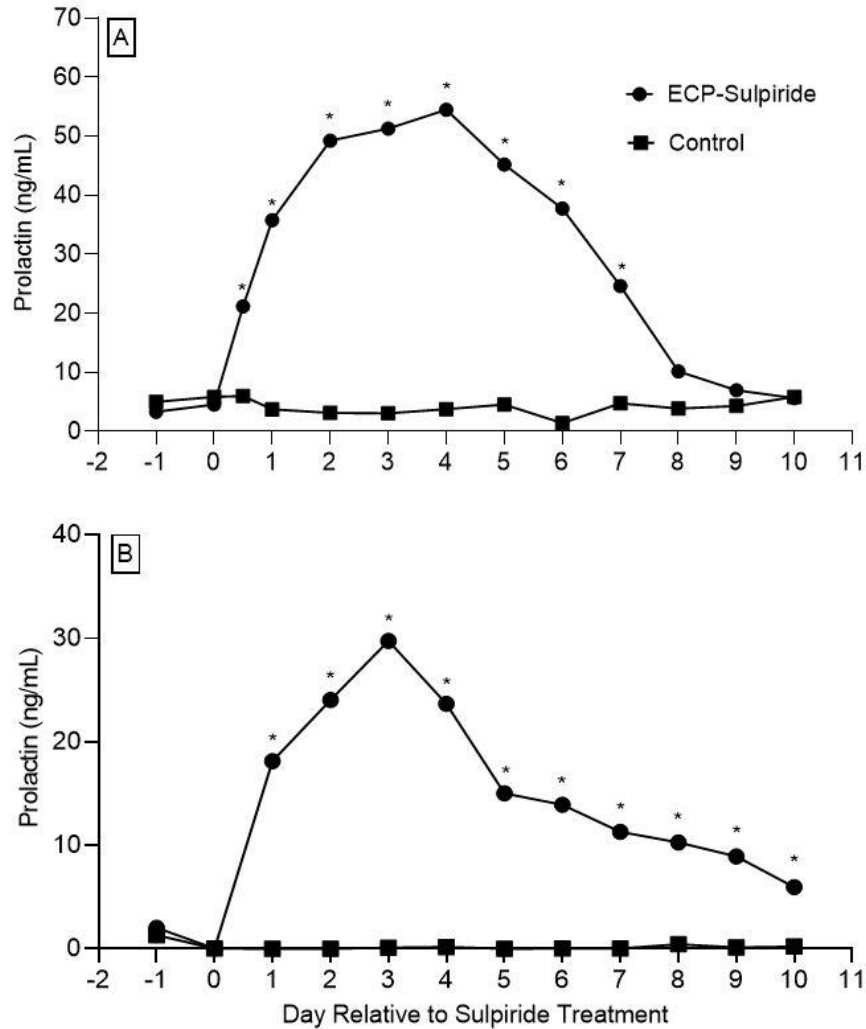


Figure 3. Mean plasma prolactin concentrations of seasonally anovulatory mares treated with 50 mg ECP and 3 g sulpiride or vehicle only in Experiment 1 (panel A) and Experiment 2 (panel B; geometric means). Asterisks indicate differences at $P < 0.001$. Pooled SEM was 5.75 ng/mL for panel A and 0.67 ng/mL for panel B.

Mean plasma LH concentrations of mares treated with ECP-sulpiride or vehicle in both experiments are presented in Figure 4. In experiment 1, LH was stimulated ($P = 0.03$; Figure 4A)

in mares treated with ECP-sulpiride when compared to mares that received vehicle only. There was an interaction ($P < 0.0001$) between treatment and day for plasma LH with concentrations greatest in ECP-sulpiride treated mares at days 9 and 10 post treatment. Mean plasma LH concentrations of mares treated with ECP-sulpiride vs mares treated with vehicle only in Experiment 2 are presented in Figure 4B. The LH response to treatment did not differ ($P = 0.06$) between groups; however, there was an interaction ($P < 0.0001$) between treatment and day with plasma LH concentrations greatest in ECP-sulpiride treated mares at days 9 and 10 post treatment.

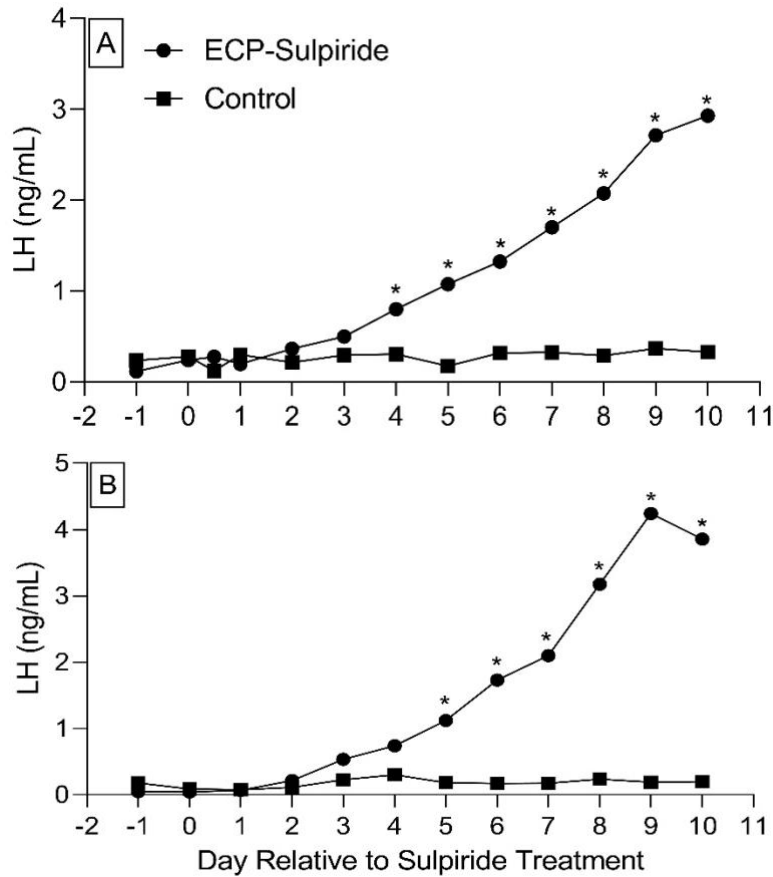


Figure 4. Mean plasma LH concentrations of seasonally anovulatory mares treated with 50 mg ECP and 3 g sulpiride or vehicle only in Experiment 1 (panel A) and Experiment 2 (panel B). Asterisks indicate differences at $P < 0.0001$. Pooled SEM was 0.44 ng/mL for panel A and 0.62 ng/mL for panel B.

4.3. Recovery, Maturation, and Cleavage Rates

Recovery and maturation rates of oocytes derived from seasonally anovulatory mares treated with ECP-sulpiride or vehicle only were pooled between both experiments. Overall rates from both experiments are presented in Table 1. Rates from experiments 1 and 2 are presented separately in Tables 2 and 3, respectively. Oocytes were pooled within treatments based on follicle diameter (8-19 mm, 20-29 mm, and ≥ 30 mm). Recovery rates were calculated based on the number of oocytes obtained from the number of follicles successfully aspirated and flushed. Pooled recovery rates of oocytes derived from ECP-sulpiride treated mares and mares administered vehicle only were 41.6% (27/65) and 45.1% (55/122), respectively, and did not differ (Figure 5).

Table 1. Overall recovery, maturation, and cleavage rates of oocytes derived from seasonally anovulatory mares treated with ECP and sulpiride.

Percentage	ECP-Sulpiride	Control	P-value
Recovery	41.6% (27/65)	45.1% (55/122)	0.77
Maturation	55.6% (15/27)	65.4% (36/55)	0.47
Cleavage*	71.4% (5/7)	63.1% (19/31)	0.99

Abbrev: ECP = estradiol cypionate

*Experiment 2 only

Table 2. Recovery and maturation rates of oocytes derived from seasonally anovulatory mares treated with ECP and sulpiride in experiment 1.

Percentage	ECP-Sulpiride	Control	P-value
Recovery	46.4% (13/28)	33.3% (7/21)	0.39
Maturation	61.5% (8/13)	71.4% (5/7)	0.99

Abbrev: ECP = estradiol cypionate

Table 3. Recovery, maturation, and cleavage rates of oocytes derived from seasonally anovulatory mares treated with ECP and sulpiride in experiment 2.

Percentage	ECP-Sulpiride	Control	P-value
Recovery	40.5% (15/37)	52.4% (53/101)	0.25
Maturation	46.6% (7/15)	58.4% (31/53)	0.55
Cleavage	71.4% (5/7)	63.1% (19/31)	0.99

Abbrev: ECP = estradiol cypionate

Pooled maturation rates of oocytes derived from seasonally anovulatory mares treated with ECP-sulpiride and mares that received vehicle only based on follicle diameter are presented in Figure 6. In experiment 1, maturation rates were calculated based on the number of oocytes that contained a metaphase II plate at the time of staining from the number of follicles recovered. In experiment 2, maturation rates were calculated based on the number of oocytes that contained an extruded first polar body in the perivitelline space at the time of ICSI from the number of

follicles recovered. Maturation rates of oocytes derived from ECP-sulpiride treated mares did not differ from mares that were administered vehicle only (55.6 vs 65.4%, respectively).

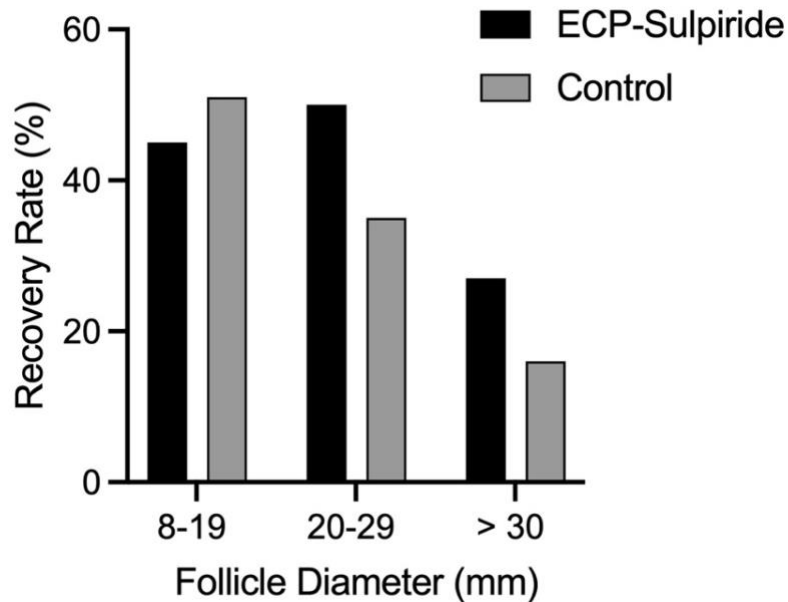


Figure 5. Pooled recovery rates of oocytes derived from seasonally anovulatory mares treated with 50 mg ECP and 3 g sulpiride or vehicle only. No differences were detected.

Cleavage rates of embryos derived from oocytes from ECP-sulpiride treated mares vs mares that received vehicle only based on follicle diameter in Experiment 2 are presented in Figure 7. Cleavage rates were calculated based on the number of embryos that cleaved from the number of oocytes that underwent ICSI. Cleavage rates of embryos derived from ECP-sulpiride treated mares vs mares administered vehicle only were 71.4% (5/7) and 61.3% (19/31), respectively. Based on follicle diameter, cleavage rates of embryos from ECP-sulpiride treated mares and mares that received vehicle only did not differ.

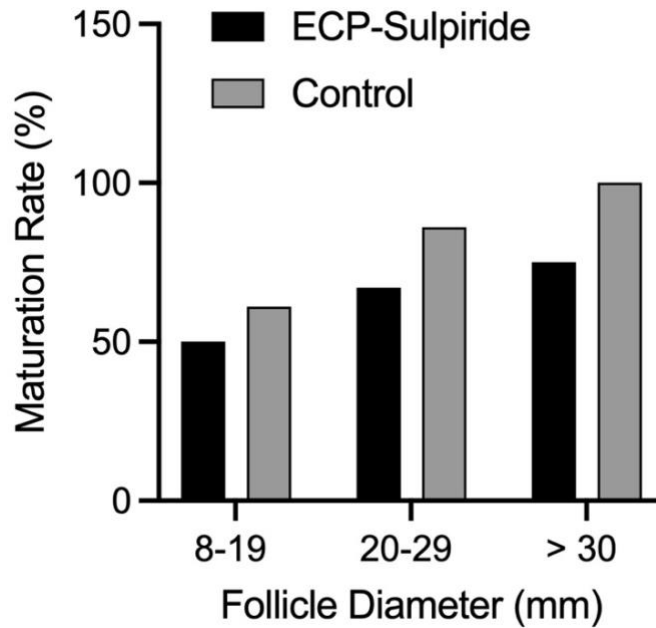


Figure 6. Pooled maturation rates of oocytes derived from seasonally anovulatory mares treated with 50 mg ECP and 3 g sulpiride or vehicle only. No differences were detected.

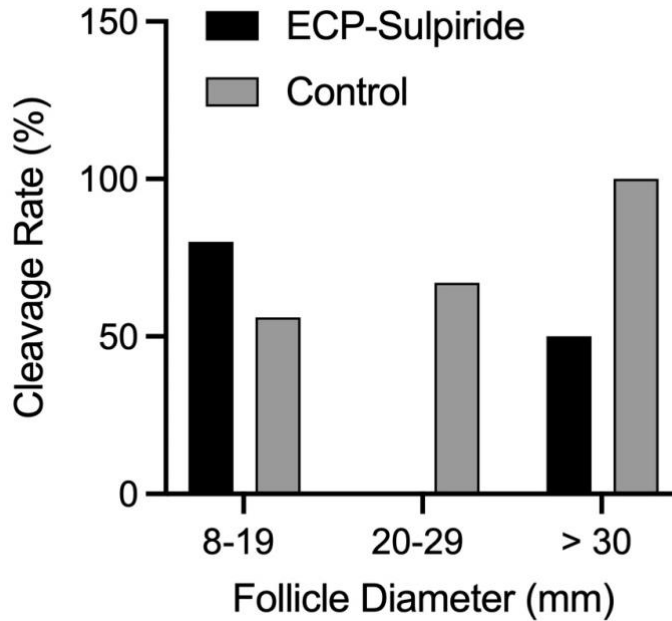


Figure 7. Cleavage rates of oocytes derived from seasonally anovulatory mares treated with 50 mg ECP and 3 g sulpiride or vehicle only. No differences were detected.

CHAPTER 5. DISCUSSION

The use of dopamine antagonists to induce early cyclicity in seasonally anovulatory mares has been the subject of much research; however, to date, this is the first study to evaluate the *in vitro* developmental potential of oocytes derived from seasonally anovulatory mares treated with a dopamine antagonist, namely sulpiride. In experiments 1 and 2, a treatment success rate, defined as a 30 mm follicle within 25 days of treatment with sulpiride, of 100% and 90%, respectively, was observed in mares administered 50 mg ECP followed by 3 g sulpiride. All responding mares produced an ovulatory sized follicle in an average of 11 days after sulpiride treatment. Other studies utilizing estradiol and sulpiride to hasten the date to first ovulation reported success rates ranging from 39-89%, depending on the vehicle and number of injections used (Kelley et al., 2006; Mitcham, 2012; Oberhaus et al., 2017, 2018; Looman, 2020). In the present study, mean day to first 30 mm follicle of the year was advanced in anovulatory mares administered ECP-sulpiride by an average of 21 days, which is comparable to previous reports (Oberhaus et al., 2018; Looman, 2020). The overall success rate of 94% observed in the present study utilizing only one ECP injection one day prior to a single sulpiride injection is the most successful response to treatment thus far.

One observation made in experiment 2 was that naturally transitioning mares (controls) appeared to have more follicles available for aspiration in comparison to mares treated with ECP-sulpiride; however, this was not an unexpected result. The ovaries of an anestrus mare are small and inactive in terms of follicle development. In comparison, the ovaries of a naturally transitioning mare have an increased number of follicles present, often resembling a cluster of grapes (M. Vetter, personal observation). This statement agrees with that of Donadeu and Pedersen (2008), who observed that follicular waves of the spring transition produced more

follicles than waves of both the anovulatory and ovulatory seasons. The dominant, stimulated follicle of most treated mares occupied a majority of the ovary, ultimately reducing the number of subordinate follicles available for aspiration. Contrarily, the ovaries of control mares often contained multiple subordinate follicles in addition to the ovulatory sized follicle. The marked difference in ovarian characteristics (i.e., number of follicles) of ECP-sulpiride treated and naturally transitioning mares at the time of OPU suggests that seasonally anovulatory mares would serve as the superior control group, therefore providing a more precise comparison of ovarian activity and oocyte competence with and without treatment.

It is important to note is that while 90% of all mares in both groups eventually produced a follicle ≥ 30 mm, the large follicle(s) present on the ovaries of control mares were unlikely to ovulate due to the low levels of circulating gonadotropins and steroidogenic incompetence of follicles that are typically present during the spring transition. In contrast, anestrus mares administered ECP-sulpiride in previous studies are capable of achieving an advanced first ovulation of the year well before a naturally transitioning mare (Oberhaus et al., 2017; Looman, 2020). In experiment 1, follicular activity of ECP-sulpiride treated mares subjected to OPU was monitored in the weeks following the procedure, and most mares resumed regular cyclicity and achieved normal, second ovulations. It appears that treatment with ECP-sulpiride induces seasonally anovulatory mares into an advanced transition-like state, or in some cases, entirely bypasses the erratic ovarian activity often associated with the spring transition.

It is understood that the competence of equine oocytes is positively correlated with follicle size (Goudet et al., 1997; Hinrichs and Schmidt, 2000). Recovery, maturation, and cleavage rates were analyzed based on follicle diameter to determine if ECP-sulpiride treatment had an effect on oocyte competence according to these parameters; however, no statistical

differences were detected. Though, upon observation, it appeared that a greater number of oocytes were collected from follicles 8-19 mm in diameter, but their ability to mature and cleave following ICSI seemed inferior to oocytes collected from larger follicles (20-29 mm and ≥ 30 mm). This effect has been described previously, where recovery rates from follicles < 20 mm were greater, but these oocytes lacked meiotic competence when compared to those originating from follicles > 20 mm (Hinrichs and Schmidt, 2000).

A correlation between stimulated prolactin concentrations and ovarian response has not been observed, neither has a threshold concentration of prolactin been determined. In essence, a prolactin response does not guarantee an ovarian response. All sulpiride-treated mares had elevated prolactin concentrations after treatment. Mares responding to treatment displayed prolactin concentrations ≤ 60 ng/mL. There is a possibility that threshold prolactin concentrations must be reached in order for a mare to respond, but the exact value correlated with response has yet to be identified and could vary between individual mares (Oberhaus et al., 2017). Most unstimulated cycling mares have prolactin concentrations around 10-15 ng/mL in the summer (Thompson and Oberhaus, 2015). Therefore, it is likely that induction of cyclicity in anovulatory mares with ECP-sulpiride requires stimulation of prolactin to at least those concentrations, which were achieved in the present experiments.

Pretreatment with ECP produced elevated LH concentrations starting approximately 5 days after treatment with ECP which is consistent to the timing reported in other studies (Oberhaus et al., 2017; Looman, 2022; Oberhaus et al., 2022). Similar to prolactin, a threshold concentration of LH after stimulation with ECP has not been determined, although failure of either endocrine response typically results in no ovarian response.

The mechanism by which prolactin induces such rapid follicular growth in mares treated with ECP-sulpiride has yet to be elucidated. It can be postulated that stimulation of angiogenic factors such as vascular endothelial growth factor (VEGF) could promote rapid folliculogenesis as VEGF induces vascular growth in follicles and increases permeability of endothelial cells (Reynolds and Redmer, 1998). Prolactin present in follicular fluid or added to culture has been found to promote endothelial cell proliferation by inducing expression of VEGF, which is expressed in many cell types, including theca, granulosa, luteal, and cumulus cells of many species (Yamamoto et al., 1997; Barboni et al., 2000; Watson and Al-zi'abi, 2002; Al-zi'abi et al., 2003; Goldhar et al., 2005; Castilla et al., 2009). Highly vascularized preovulatory follicles are more competent than transitional follicles with less vascularity; enhanced blood flow allows for transport of oxygen, nutrients, substrates, and circulating gonadotropins to aid in follicular growth and steroidogenesis (Watson and Al-zi'abi, 2002). The promotion of angiogenesis by prolactin and the establishment of rich blood flow to the growing follicles could be contributing to the rapid follicular growth and maturation repeatedly observed in ECP-sulpiride treated mares.

The late-stage follicular growth induced by ECP-sulpiride treatment may be attributed to an upregulation in LH receptors. Failure of follicles to ovulate during early spring can be attributed to inadequate follicular fluid and circulating estradiol concentrations (Davis and Sharp, 1991; Watson et al., 2004). Larger follicles during the breeding season have higher LH receptor mRNA content and follicular steroidogenic activity when compared to large follicles that emerge during the spring transition (Watson et al., 2004). Increase in follicular steroidogenic activity approaching the breeding season results in an elevation of estradiol in circulation, which has been suggested to be a key factor in stimulating the release of LH prior to the first ovulation of the year (Sharp et al., 2001). It has been established that stimulated follicles from these

responding mares have LH receptor content and steroid production equivalent to naturally occurring follicles in the spring (Oberhaus et al., 2018). Such follicles have the ability to respond to LH input in the same way an unstimulated ovulatory-sized follicle would. We propose that treatment with ECP-sulpiride prompts an increase in estradiol and a subsequent upregulation in LH receptor expression, endowing the follicle with gonadotropic and steroidogenic competence sufficient to achieve late-stage follicular growth and ovulation.

In bovine, a single OPU session usually results in recovery rates $\geq 70\%$ (Galli et al., 2014). In contrast, an overall recovery rate of 41.6% observed in treated mares of the present study could be considered acceptable in the equine. The recovery rates observed in the present study resemble those of Mak et al. (2018), who reported an oocyte recovery rate of 51% in the nonbreeding season and 39% in the breeding season. In a large scale clinical OPU-ICSI program, a total of 515 OPU sessions yielded an average recovery rate of 53%, which is not far off from the 41.6% recovery rate achieved in the present study, despite a lesser total of OPU sessions performed (Claes and Stout, 2022). Other studies report rates closer to 60% (Galli et al., 2007; Choi et al., 2016). Aspirations performed on treated mares in different aspiration sessions produced recovery rates ranging from 20-66%. This variability could be due to several factors: the mare's identity (age, breed), her behavior during the procedure, the number and size of follicles present on the ovaries at the time of OPU, or whether all punctured follicles were adequately flushed and scraped during aspiration.

Maturation rates of oocytes derived from ECP-sulpiride stimulated follicles did not differ from those derived from naturally occurring follicles in the spring. It has been previously observed that maturation rate of oocytes collected during both the breeding and nonbreeding seasons do not differ (Mak et al., 2018). Maturation rates of both treatment groups observed in

the present study are lower than the near 80% maturation rates reported by Mak et al. (2018) and the > 80% maturation rate reported by Leisinger et al. (2016). In contrast, the rate of oocyte maturation achieved in the current experiments is comparable to earlier studies that report rates of approximately 60% (Colleoni et al., 2007; Jacobson et al., 2010; Choi et al., 2016; Claes and Stout, 2022). No detrimental effects on maturation were observed in oocytes derived from stimulated mares in the present study. Perhaps the favorable in vitro maturation conditions (e.g., culture medium) facilitated sufficient nuclear maturation and contributed equally to the acquisition of meiotic competence of oocytes from both stimulated and unstimulated mares. In essence, oocytes from induced follicles of seasonally anovulatory mares appeared to mature equally to those from naturally occurring follicles.

Cleavage rate of oocytes collected from anovulatory follicles stimulated with ECP-sulpiride did not differ from those collected from unstimulated transitional follicles. The 70% cleavage rate of treatment oocytes observed in this study resemble those of a clinical or commercial equine IVP program using immature oocytes collected via OPU for ICSI (Colleoni et al., 2007; Galli et al., 2013; Leisinger et al., 2016; Cuervo-Arango et al., 2019). Conventional ICSI is simple and repeatable, and utilization of this technique in the present study resulted in a reasonable cleavage rate. Induction of folliculogenesis through stimulation of prolactin and LH appeared to facilitate growth of treatment oocytes similarly to what would take place during a normal cycle. Acceleration of follicle growth induced by treatment did not appear to negatively affect the acquisition of meiotic and cytoplasmic competence of oocytes from ECP-sulpiride stimulated follicles. Based on the results of the present study, oocytes recovered from stimulated follicles acquired the ability to reach maturity and undergo cleavage similarly to oocytes from naturally transitioning mares.

A limitation of the current study was the small number of oocyte observations for statistical analysis, but that was to be expected given the average number of oocytes collected from a single mare by OPU. Despite this, the current study was very practical and most resembled the real-world, unpredictable results often received in an equine IVP program. The ultimate determination of developmental competence is the ability of the oocyte to mature to MII, undergo cleavage, form a blastocyst, and develop to term, producing a healthy offspring (Sirard et al., 2006). Based on the observations made in the present study, oocytes derived from ECP-sulpiride stimulated follicles are able to reach MII in culture and cleave following ICSI. Using blastocyst development as an endpoint in future studies to evaluate the competence of ECP-sulpiride oocytes would aid in further characterization of their developmental potential. In addition, transcriptomic analysis of follicular cells and oocytes from ECP-sulpiride treated mares is currently underway in order to further understand how ECP-sulpiride treatment affects the follicular environment and subsequent oocyte developmental competence at the transcript level.

SUMMARY AND CONCLUSIONS

In conclusion, follicle development and consequent oocyte growth can be induced by treating seasonally anovulatory mares with ECP-sulpiride. Utilization of this treatment allows anovulatory mares to cycle earlier in the year, providing additional opportunities to produce offspring from valuable horses, both in vivo and in vitro. The disinhibition of prolactin through the antagonistic effects of sulpiride on dopamine and the stimulation of LH by ECP are requirements for an anovulatory mare to achieve early cyclicity. Generation of elevated prolactin and LH concentrations by ECP-sulpiride facilitates growth of follicles comparable to those observed in the natural breeding season. The growth of oocytes and accumulation of molecules needed to support fertilization and cleavage is not compromised by the rapid follicular growth induced by treatment. In addition, recovery of immature oocytes via OPU following induction of cyclicity with ECP-sulpiride is not affected by treatment. Overall, treatment with ECP-sulpiride is an efficient method to stimulate ovarian activity in anovulatory mares without compromising the quality of oocytes within stimulated follicles. Utilization of this protocol could potentially provide more opportunities for positive outcomes in ART such as the in vitro production of embryos through OPU and ICSI.

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VITA

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