THE USE OF AN INTRAVAGINAL TRIPTORELIN GEL TO INDUCE OVULATION IN THE MARE

by

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Abstract

The objective of these studies was to investigate the efficacy of an intravaginal triptorelin acetate (TA) gel as an ovulation-inducing agent in mares. In Exp 1, 24 mares were stratified by parity and age and randomly assigned to 3 treatment groups receiving either: 5 mL TA gel (500 μg TA; TA5), 10 mL TA gel (1,000 μg TA; TA10), or 5 mL vehicle gel only (CON). Following the appearance of a follicle ≥ 25 mm, blood samples were obtained every 24 h until treatment administration for measurement of luteinizing hormone (LH) concentrations. Once a follicle ≥ 35 mm in diameter was detected, treatment was administered intravaginally. Following treatment, blood samples were collected and ovaries were scanned via transrectal ultrasonography every 12 h until 48 h post-ovulation. Both TA5 and TA10 tended ($P = 0.08$) to experience a brief surge in LH by 12 h post-treatment. Regarding LH concentrations, there was a significant ($P < 0.005$) treatment by time interaction. The interval from treatment to ovulation was not different ($P > 0.05$) between groups, nor was there a difference ($P > 0.05$) in the percentage of mares ovulating within 48 h of treatment administration. We hypothesized that LH was not staying elevated long enough for ovulation to occur in a greater percentage of mares. Furthermore, more frequent sampling and scanning was needed to get a more robust characterization of the effect of TA on LH and a more accurate timeframe for when ovulation was occurring. Experiment 2 involved the same CON and TA5 treatment groups; however, the TA10 treatment was split into two 5-mL doses of TA gel, administered 24 h apart (two 500-μg doses of TA; TA5x2). Blood collection and ultrasonography occurred every 12 h upon detection of a follicle ≥ 25 mm in diameter. Once a follicle ≥ 35 mm was detected, treatment was administered and ultrasonography and blood collection occurred every 6 h until 48 h post-ovulation. Both TA5 and TA5x2 had a significant increase ($P < 0.05$) in LH by 6 h post-treatment, which was declining by 12 h post-treatment.

The second dose administered to TA5x2 failed to elicit an increase in LH ($P > 0.05$). Overall, the treatment by time interaction was significant ($P < 0.005$) in regard to LH and the interval from treatment to ovulation was shorter ($P < 0.01$) in TA5 and TA5x2 compared with CON. In conclusion, TA gel increased LH concentrations and hastened the interval from treatment to ovulation in mares in Exp. 2, but not Exp. 1, without an advantage in the timing of ovulation noted between the 5 or 10-mL doses, or administration of two 5-mL doses given 24 h apart. The results of these studies suggest that further testing is needed to effectively evaluate the efficacy of TA gel as an ovulation-inducing agent in mares.

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Dedication

I dedicate this thesis and the work I have done to my maternal grandmother and my paternal great-grandparents. Marilyn “Jo” Sabin was the most caring, encouraging, and beautiful human being I have ever known. I had the great fortune to know my great-grandparents, Raymond and Evelyn Drumwright, who were kind, loving, and supportive. I don’t know who or where I would be without the influence of these three amazing people in my life. I hope I am making the three of you proud. Until we meet again…

Chapter 1 - General Review of Literature

Reproductive Anatomy of the Mare

The reproductive tract of the mare consists of a pair of bilateral ovaries, a uterus, vagina, and several other related structures. However, these organs cannot function without neural and hormonal input from the hypothalamus, hypophysis (pituitary gland), and pineal gland (McKinnon and Voss, 1992). Beginning with the external genitalia, the vulva consists of 2 labia (major and minor) and the clitoris. Labia are comprised of adipose tissue and constrictor vulvae muscles, which keep the labia in apposition to prevent foreign material from entering the vagina (McKinnon and Voss, 1992; Senger, 2004). The clitoris is homologous to the penis, being composed of erectile tissue and housing many sensory nerve endings (Senger, 2004). Additionally, the clitoris contains 3 sinuses which can harbor bacteria related to venereal diseases, such as contagious equine metritis. Therefore, the clitoris is typically swabbed during breeding soundness examinations of mares to test for venereal diseases, particularly in live cover breeding practices (Davies-Morel, 2008).

Moving internally beyond the vulva lies the vagina, which can be subdivided into the cranial and caudal vagina. The cranial portion of the vagina originates from the paramesonephric ducts, while the caudal portion of the vagina originates from the urogenital sinus (Senger, 2004). The apposition of the vaginal walls due to its position above the pelvic girdle results in the formation of a vaginal seal which aids in protecting the reproductive tract from foreign material (Davies-Morel, 2008). The caudal portion of the vagina is also referred to as the vestibule and contains the urethral opening, thus making it common to the urinary and reproductive tracts (Senger, 2004). The mucosal layer of the caudal portion of the vagina is comprised of stratified squamous epithelium, which thickens around the time of estrus to protect itself from the

physicality of copulation and to prevent microorganisms from breaching the submucosal layer (Davies-Morel, 2008; Senger, 2004). The mucosal layer of the cranial portion of the vagina is highly secretory in nature and consists of columnar epithelium (Senger, 2004). In addition to mucus, the mucosal layer produces highly acidic secretions, which are both bacteriocidal and spermicidal, making it necessary for semen to be deposited at the cranial end of the vagina near the cervix during natural breeding or through the cervix and into the uterus during artificial insemination (Ginther, 1992).

The cervix protrudes into the cranial end of the vagina and is attached to the most caudal portion of the uterus. This structure is composed of collagenous connective tissue and a smooth muscle sphincter (Davies-Morel, 2008, McKinnon and Voss, 1992). The lining of the cervix consists of a series of longitudinal folds, which are continuous with the endometrium in the body of the uterus, allowing for gross expansion during parturition (Ginther, 1992; McKinnon and Voss, 1992). When the mare is in diestrus, the cervix is highly constrictive and serves as a barrier; however, during estrus the cervix is relaxed and produces copious amounts of mucus which facilitates passage of semen through the cervix and into the uterus (Davies-Morel, 2008; Senger, 2004).

The mare has a simplex bipartite uterus that is divided into a body and 2 horns (Davies-Morel, 2008). The body of the uterus is suspended from the lumbar region of the spine by the mesometrium portion of the broad ligament. The uterus consists of 3 layers: the perimetrium, myometrium, and endometrium (Ginther, 1992). The perimetrium is continuous with the broad ligament. The myometrium is composed of an external longitudinal muscle cell layer, a central vascular layer, and an internal circular muscle layer. The structure of the myometrium allows for expansion during pregnancy and is responsible for contractions necessary for expulsion of the

fetus during parturition (Davies-Morel, 2008). The endometrium consists of longitudinal folds composed of collagenous connective tissue and is the site of placental attachment during pregnancy (McKinnon and Voss, 1992). The epithelium of the uterine endometrium contains mucus-secreting and ciliated cells, forming a mucopolysaccharide blanket. These features are also located in the respiratory tract and aid in the clearance of foreign material. Therefore, it has been suggested that the endometrium serves as another form of defense against uterine infection (Causey, 2007). The horns of the uterus lie above or intermingle with intestinal folds. Diameter increases progressively from the tips of the horns to the convergence with the uterine body (McKinnon and Voss, 1992).

At the tips of the uterine horns lie utero-tubular junctions (UTJ) consisting of sphincters that separate the uterine horns from the oviducts (Davies-Morel, 2008). It has been demonstrated that fertilized ova secrete prostaglandin E (PGE) beginning around d 5 post-fertilization, which is necessary for passage of the ova through the utero-tubular junction (Ball et al., 1993; Weber et al., 1991). Unfertilized ova remain in the ampullary-isthmic junction (AIJ) where they eventually deteriorate (Ginther, 1992). Oviducts are comprised of 3 layers, similar to those of the uterus but thinner; furthermore, the oviducts are divided into 3 continuous sections: the isthmus, the ampulla, and the infundibulum. The isthmus and the ampulla are approximately the same length; however, the isthmus is closest in proximity to the UTJ and is smaller in diameter compared with the ampulla. The ampulla is the site of fertilization and is lined with small, hair-like projections called fimbriae, which retain the ovulated ova. The infundibulum is a funnel-like structure that also contains fimbriae and lies in close proximity to the ovulation fossa on the ovary (Davies-Morel, 2008; Ginther, 1992; McKinnon and Voss, 1992).

The bilateral ovaries in mares are situated below the fourth and fifth lumbar vertebrae and are supported by the mesovarium of the broad ligament; however, the exact position is variable because the mesovarium allows a wide range of passive movement (Davies-Morel, 2008; McKinnon and Voss, 1992). The size of the ovaries varies with season and reproductive state. The ovaries are smaller during seasonal anestrous compared with the breeding season due to a lack of large antral follicles (Davies-Morel, 2008). Equine ovaries are bean-shaped, with the convex outer surface attached to the mesovarium with entrance points for nerves and blood supply, while the concave surface is not attached and is the location of the ovulation fossa.

The mare's ovary is unique compared to that of other domestic species because the cortex (gamete-producing layer) is located internally and the medulla (supportive layer containing vasculature, nerves, and lymphatics) is located externally on the ovary. In other domestic species, such as cows and sows, the medulla lies internally on the ovary (Ginther, 1992; McKinnon and Voss, 1992). While developing follicles on the mare's ovary can be easily palpated, corpora lutea are more difficult to feel because they penetrate into the ovarian tissue, rather than protruding from the ovarian surface, as in other domestic species (Senger, 2004). The tunica albuginea surrounds and protects the entire ovary, with the exception of the ovulation fossa, which is the single anatomical location from which a follicle can ovulate in mares. Other domestic species also ovulate from the ovarian cortex, but in non-equine species ovulation can occur from nearly any location on the ovarian surface (Davies-Morel, 2008; Senger, 2004).

Estrous Cycle of the Mare

Mares are seasonally polyestrous with a natural breeding season from approximately April through October in the Northern hemisphere. The onset of reproductive competence in mature mares is primarily dictated by photoperiod; however, other factors such as nutritional

status and ambient temperature play a minor role (Ginther et al., 1972; Hughes et al., 1972). The average date of first ovulation following winter anestrous and the vernal transition is in early April, regardless of indigenous weather conditions (Sharp, 1980). Approximately 14 to 16 h of daylight are needed for the mare to begin cycling (Nagy et al., 2000). Photoperiod is perceived through the retinas of the eyes and conveyed through a complex network of neurons to the suprachiamsmatic nucleus (SCN) in the hypothalamus. The SCN then relays signals to the pineal gland, where melatonin is synthesized from serotonin. The rate-limiting enzyme, serotonin N-acetyltransferase, reaches peak concentrations during dark hours and is in very low concentration during daylight hours (Rudeen et al., 1975). Because horses are classified as long-day breeders, greater concentrations of melatonin (i.e. during the winter months when day-length is short) suppress reproductive cyclicity. Melatonin indirectly suppresses gonadotropin-releasing hormone (GnRH) secretion, thereby suppressing follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion, both of which are necessary for follicular development and ovulation (Davies-Morel, 2008; Senger, 2004). The exact mechanisms that allow melatonin to regulate GnRH secretion are not well-understood and are not well-researched in the horse. In sheep and other species, there is evidence to suggest that melatonin may act through dopaminergic pathways, among others, to influence GnRH secretion (Malpaux et al., 1999).

During the vernal transition period between winter anestrous and the breeding season, mares will develop follicles greater than 30 mm in diameter and show signs of estrus; however, these follicles often regress as a result of insufficient LH concentrations. Once the mare successfully produces an antral follicle that responds to an LH surge and ovulates following the transitional period, estrous cycles will remain regular until the autumnal transition period. At this time, estrous cycles become less regular and fewer mares ovulate as winter approaches

(McKinnon and Voss, 1992). From December through February most mares will become anovulatory and are considered seasonally anestrous. A small population of mares may never experience winter anestrous, particularly those that are kept stabled with high planes of nutrition, although cycles are generally considered to be less consistent during the winter months (Hughes et al., 1972).

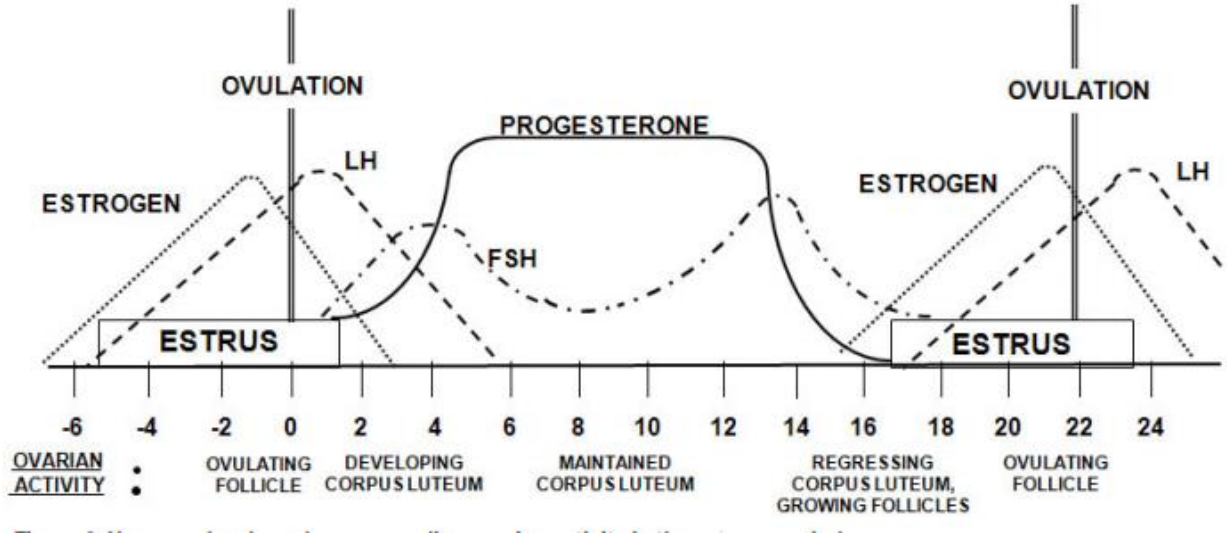

Figure 1.1 - Plasma hormone concentrations and corresponding ovarian activity throughout the estrous cycle of the mare (Taylor-MacAllister and Freeman, 2013).

The estrous cycle of the mare is defined as the period between 2 subsequent ovulations, which can be detected by rectal palpation, transrectal ultrasonography, or by measuring plasma progesterone (P4) concentrations (Towson and Ginther, 1989). The average length of the estrous cycle is 21 d; however, reports in the literature show that equine estrous cycles typically vary in length between 19 and 22 d. During estrus, which lasts between 5 and 7 d, the mare displays signs of heat and is receptive to a stallion (Ginther et al. 1972; Hughes et al., 1972; McKinnon and Voss, 1992). Ovulation typically occurs 24 to 36 h before the end of estrus (Ginther, 1992).

Diestrus lasts 14 to 15 d and can be divided into a luteal phase and a follicular phase. Following ovulation (d 0), the remaining follicular tissue from the site of ovulation undergoes structural changes and forms a corpus hemorrhagicum, which then becomes a corpus luteum. The corpus luteum (CL) is fully functional approximately 5 d post-ovulation and secretes P4, which has a negative feedback on GnRH and selectively inhibits LH secretion. Under high P4 concentrations, the frequency of LH pulses is reduced. This causes the dominant follicle produced by the first FSH wave, that is now LH-dependent, to regress. Occasionally, a preovulatory follicle will ovulate during the luteal phase and is believed to be fertile (Hughes and Stabenfeldt, 1977; Vandeplassche et al., 1979). If pregnancy recognition does not occur within approximately 13 d post-ovulation, the CL is lysed by the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium of the uterus, marking the end of the luteal phase of diestrus (Davies-Morel, 2008; Senger, 2004). Without the negative feedback of P4 on the hypothalamus, increasing amounts of GnRH stimulate the release of LH and FSH from the anterior pituitary gland. This phase of diestrus is referred to as the follicular phase, during which rapid follicular growth occurs (Ginther, 1992; McKinnon and Voss, 1992).

A cohort of small follicles (2 to 5 mm) is observable around the time of ovulation. These follicles continue to grow at a rate of approximately 3 mm/d during the luteal phase until they reach approximately 25 to 30 mm in diameter. When luteolysis occurs, 1 or 2 follicles become dominant and continue to grow while the subordinate follicles undergo atresia (Palmer, 1987). The primary ovulatory follicle typically grows from 30 mm 6 d before ovulation to approximately 40-45 mm around the time of ovulation; however, ovulation of follicles with diameters between 30 and 60 mm in diameter have been reported (McKinnon and Voss, 1992; Palmer, 1987). Season may have an impact on the size of the ovulated follicle. Ginther and

Pierson (1989) found that follicles ovulated in April and May had mean diameters of 46 and 48 mm, respectively, compared with 40 mm in July.

Hormonal Regulation

Estrous Cycle

The endocrine system essentially controls all events of the estrous cycle. The gatekeepers of the hypothalamo-pituitary-ovarian axis are the kisspeptin (Kp) family of neuropeptides, primarily located in the arcuate nucleus and preoptic area of the hypothalamus (Seminara, 2007). Kisspeptins are involved in sexual differentiation, puberty, seasonality, and GnRH secretion. Kisspeptin neurons make contact with both GnRH nerve terminals and GnRH cell bodies within the median eminence to stimulate GnRH secretion (Decourt et al., 2014; Seminara, 2007). Following stimulation by Kp, GnRH, a decapeptide, is released from a larger 56 amino acid peptide known as gonadotropin-releasing hormone-associated peptide. Gonadotropin-releasing hormone then travels through neurons that extend from the hypothalamus down through the pituitary stalk. The hormone then travels through the hypothalmo-hypophyseal portal plexus system, after which it is released into the anterior pituitary where it binds to its receptors on gonadotrophic cells (Decourt et al., 2014). The primary function of GnRH is to stimulate the synthesis and release of glycoprotein hormones, namely LH and FSH, from the gonadotropes located in the anterior pituitary (Ginther, 1992; McKinnon and Voss, 1992; Senger, 2004; Davies-Morel, 2008). These glycoprotein hormones are heterodimeric, meaning they share a common α-subunit and have unique β-subunits which determine their biological specificity. Secretion of GnRH is pulsatile and the pulse frequency and amplitude can preferentially stimulate the synthesis and secretion of either FSH or LH β-subunits. Higher frequency, lower amplitude pulses of GnRH stimulate a preferential synthesis and release of LH, whereas lower

frequency, higher amplitude pulses stimulate preferential synthesis and release of FSH (Garza et al., 1986). There are currently 23 isoforms of GnRH that have been identified in vertebrate species (Pawson and McNeilly, 2005). For the sake of clarity, GnRH described in this review refers to the type I GnRH isoform, unless otherwise specified.

Both LH and FSH travel to the gonads through systemic circulation. Receptors for FSH are found on granulosa cells, which are somatic cells that form layers around the oocyte. In the preantral follicle, FSH stimulates P4 production, which is rapidly metabolized to androstenedione, a precursor for androgens. Stimulated by FSH, a follicle develops a fluid-filled antrum. Once the antrum has formed, granulosa cells develop LH receptors (LHr), whereas LHr were previously confined to theca cells. Follicle-stimulating hormone also increases the vascularity of follicles. Increased perfusion helps meet the metabolic demands of the follicle and disperses follicular steroidal products into the bloodstream. Another role of FSH is to facilitate ovulation by stimulating the secretion of plasminogen activator from granulosa cells into the follicular fluid. Plasminogen activator converts plasminogen to plasmin, a protease that weakens the tensile strength of the follicular wall, aiding in follicular rupture during ovulation. Concentrations of LH remain relatively low during the luteal phase of the estrous cycle; however, LH becomes increasingly important during estrus and after ovulation has occurred. In conjunction with FSH, LH has a major role in the final maturation of the dominant follicle (Davies-Morel, 2008; McKinnon and Voss, 1992). The involvement of LH and FSH in steroidogenesis will be discussed further in the next section. The role of LH during ovulation will be discussed in greater detail later in this chapter.

Estradiol-17β (E2) is secreted by the cells of the follicle and is responsible for sexual behavior surrounding estrus, including the receptivity of the mare to the stallion (Davies-Morel,

2008; Senger, 2004). Estradiol-17β plays a dual role in reproduction, acting as both a stimulator and inhibitor of GnRH, depending on the concurrent concentration of P4. During the luteal phase of the estrous cycle, when P4 concentrations are high, E2 from the developing antral follicle works synergistically with P4 to elicit a negative feedback on GnRH at the level of the hypothalamus. Once $PGF_{2\alpha}$ is released from the endometrium of the uterus and the CL is lysed, P4 concentrations begin to decline. At this time, E2 works independently of P4 at the level of the hypothalamus to upregulate the synthesis and secretion of GnRH, leading to increased gonadotropin secretion which is necessary for final maturation of the dominant follicle and ovulation (Davies-Morel, 2008; McKinnon and Voss, 1992; Senger, 2004).

Steroidogenesis

The collaboration of FSH and LH in steroidogenesis is described as the 2-cell, 2-gonadotropin theory (Fig. 2). The secretion of androgens is enhanced by LH alone, whereas production of E2 requires both LH and FSH (Griffin and Ojeda, 1992). Cholesterol is the precursor for steroidogenesis within theca and granulosa cells. Cholesterol can be synthesized from acetate, derived from high-density (HDL) or low-density (LDL) lipoproteins, or made from the hydrolysis of cholesterol esters (Senger, 2004). Cholesterol is transported from the outer to the inner mitochondrial membrane by steroidogenic acute-regulatory (StAR) protein, where enzymes act on cholesterol to form intermediate products, such as pregnenalone (P5) (Belin et al., 2000; Griffin and Ojeda, 1992).

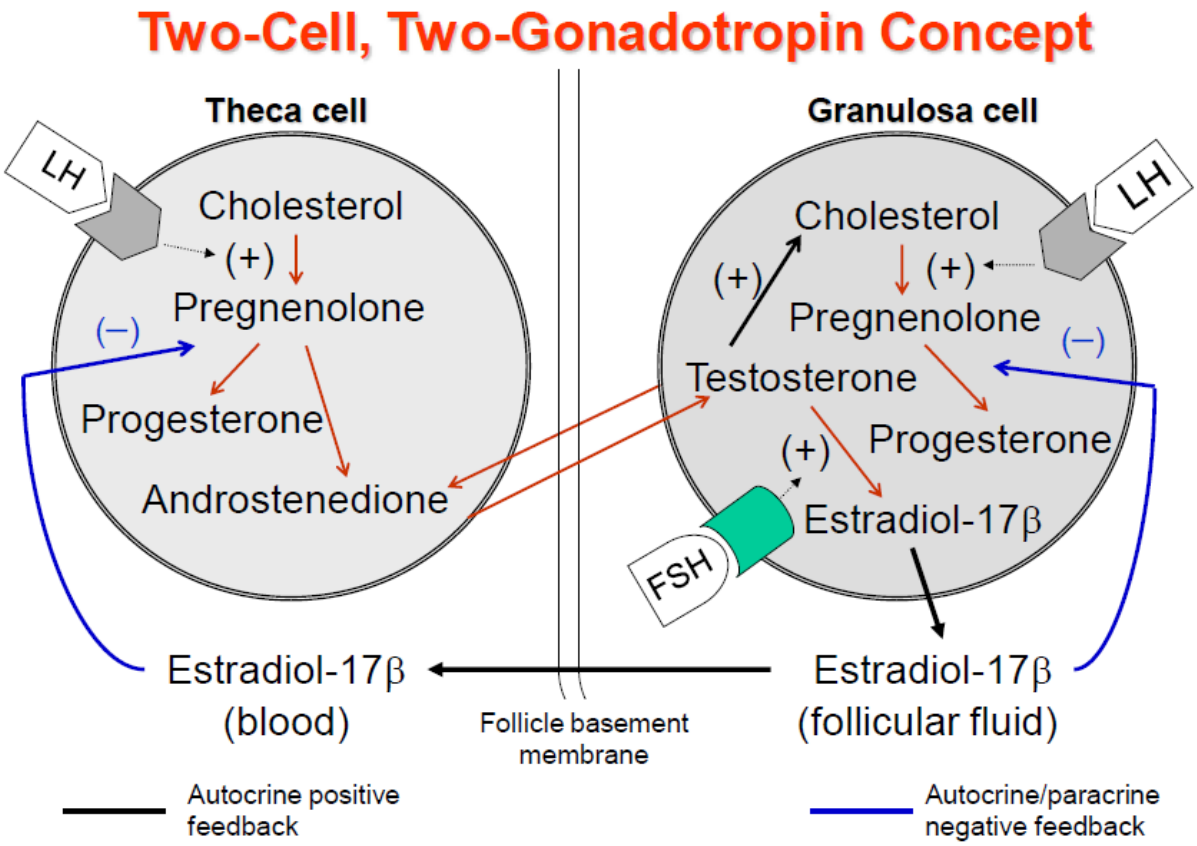

Figure 1.2 - Steroidogenesis as explained by the two-cell, two-gonadotropin concept (Fortune and Quirk, 1988).

Theca cells always have LHr, whereas granulosa cells do not develop LHr until the follicle has developed an antrum. Once LH binds to its G-protein coupled receptor on the surface of theca and granulosa cells, it activates P-450 side-chain cleavage (P-450_{scc}) enzyme, which acts on cholesterol to release isocaproic acid to form P5. Pregnenalone is converted to P4 in both theca and granulosa cells by 3β-hydroxysteroid dehydrogenase (3β-HSD). No further conversion occurs in granulosa cells because they lack 17α-hydroxylase and 17, 20 lyase (P-$450_{17\alpha}$) enzymes. Theca cells contain P-$450_{17\alpha}$ enzymes; therefore, P5 can be converted to androstenedione in the endoplasmic reticulum of these cells (Belin et al., 2000). Pregnenalone is

converted to dehydroepiandrosterone (DHEA) via P-$450_{17\alpha}$ enzymes, which is then converted to androstenedione by 3β-HSD enzymes (Erickson et al., 1985; Sirois et al., 1991).

Once androstenedione has been produced in the theca cells, it diffuses across the basement membrane of the follicle to granulosa cells (McKinnon and Voss, 1992). Once inside granulosa cells, androstenedione is transported to the mitochondria where 17β-hydroxysteroid dehydrogenase (17β-HSD) converts it to testosterone (Belin et al., 2000). Luteinizing hormone binds to its receptor on the surface of granulosa cells and stimulates aromatase activity, which converts testosterone to estrogens, specifically estrone and E2 (McKinnon and Voss, 1992). Estradiol-17β travels out of the granulosa cells into follicular fluid in the antrum of the follicle where it works through autocrine and paracrine mechanisms to provide negative feedback on the conversion of P5 to P4 in the granulosa cell (Senger, 2004). Some E2 from follicular fluid crosses the basement membrane of the follicle into the bloodstream and provides negative feedback on the conversion of P5 to P4 within theca cells (Erickson et al, 1985; Senger, 2004).

Folliculogenesis

The mare, like other domestic species, is born with all of the oogonia that she will ever have. Each oocyte contains 64 chromosomes and is surrounded by a single layer of squamous cells. The structure that houses the oocyte and supportive cells is called a primordial follicle (Davies Morel, 2008; Senger, 2004). Development of these primordial follicles is gonadotropin-independent and begins at varying rates shortly after birth. Oocytes within the primordial follicles undergo the first stages of meiosis and then enter nuclear arrest until puberty. During the peripubertal period, increasing pulses of GnRH stimulate the synthesis and secretion of LH and FSH. This increase in circulating pituitary hormones causes the layer of cells surrounding the

oocyte to become cuboidal, at which point it is referred to as a primary follicle (Fortune et al., 2004).

Subsequent stages of folliculogenesis are gonadotropin-dependent and begin to occur at puberty. After puberty, the final stages of meiosis occur in waves so that a continuous supply of developing follicles is available for ovulation throughout the mare's lifetime (Ginther, 2001). Primary follicles either undergo atresia or develop into secondary follicles. Secondary follicles have 2 or more layers of cells and a zona pellucida, a thick, translucent membrane. At this stage, the oocyte within the secondary follicle is haploid, having 32 chromosomes (Ginther, 1992). Driven by gonadotropins, the epithelial cells surrounding the oocyte differentiate into follicular cells that produce and secrete follicular fluid. The oocyte becomes situated towards the edge of the follicle and the surrounding follicular cells become either an outer layer of thecal cells or an inner layer of granulosal cells. Granulosal cells are separated from the thecal cells by a basement membrane. The thecal cell population is further divided into the theca externa (outer layer) and the theca interna, which is the vascularized inner layer of thecal cells (Davies-Morel, 2008; Senger, 2004). The theca externa is composed of connective tissue, while the theca interna produce androgens under the influence of LH from the anterior pituitary. Granulosal cells produce estrogens, inhibins, and follicular fluid and are thought to govern maturation of the oocyte (Fortune et al., 2004). As follicular fluid accumulates, the follicle develops a fluid-filled antrum and is now referred to as either a tertiary follicle or antral follicle. Once the follicle establishes dominance and becomes preovulatory in nature, it is referred to as a Graafian follicle (Ginther et al., 2003; Senger, 2004).

Follicular development occurs in major and minor waves in some monovular species, such as the cow and mare. Dominant follicles are only produced by major waves (Ginther et al.,

2003). Folliculogenesis can be described by 5 phases: recruitment, emergence, selection, deviation, and dominance (Evans, 2003). Recruitment is defined as the phase in which a cohort of primordial follicles begins to grow and become increasingly dependent on gonadotropins (Evans, 2003; Ginther et al., 2003). Emergence is defined retrospectively as the last day before the future dominant follicle reached 4 mm (cow) or 6 mm (horse) in diameter (Evans, 2003; Ginther et al., 2003). On average, the future dominant follicle in mares emerges approximately 1 d before the largest future subordinate follicle (Gastal et al., 1997). Emergence marks the beginning of a follicular wave; therefore, the duration of a follicular wave is defined as the period from emergence in the first follicular wave to emergence of the second follicular wave (Ireland et al., 2000). Selection is a common-growth phase lasting approximately 3 (cow) or 6 (mare) d, during which between 7 to 11 follicles continue to grow in response to FSH (Evans 2003; Ginther et al., 2001a; Ginther et al., 2003). The beginning of deviation corresponds with the end of selection. Near the end of selection, if the largest follicle is ablated, the second largest follicle will establish dominance; therefore, all follicles in a cohort during selection are capable of establishing dominance. The future dominant follicle is the one that reaches the greatest diameter by the end of the common-growth phase (Ginther et al., 2003).

Peak FSH concentrations are generally observed when a follicle reaches approximately 13 mm in diameter (Ginther et al., 2003). There is typically a 3 d period between peak FSH concentrations and the beginning of deviation. During this pre-deviation period, all follicles in the cohort contribute to increasing concentrations of E2 and inhibin, which results in a decline in FSH concentrations. This leads to a “survival of the fittest” situation, whereby the largest follicle has the advantage because of its increased responsiveness to lesser concentrations of FSH during the decline compared with smaller follicles, thus enabling its survival while the subordinate

follicle are starved (Ginther et al., 2003). Ginther et al. (2003) defined the beginning of deviation as a retrospective concept, being the observation period prior to an apparent change in diameter between the first and second largest follicle. On average, this change in diameter occurs when the largest and second largest follicles are 22.5 and 19 mm in diameter, respectively. Ginther et al. (2003) note that the beginning of deviation may be difficult to define if all subordinate follicles in the cohort are small. Furthermore, defining deviation may be even more difficult in the mare because of overlapping non-ovulatory waves. Periodic ablation of follicles that are not participating in the ovulatory wave makes it easier to define the occurrence of deviation; however, ablation is not a technique used by industry professionals and is generally conducted in a research setting.

Approximately 1 d before deviation, the largest follicle begins secreting increasing quantities of E2. Estradiol-17β works both independently and synergistically with inhibin, a dimeric protein secreted by granulosa cells, to reduce FSH secretion. Ablation of the largest follicle at the expected onset of deviation results in a reduction in E2 concentrations and a resultant increase in circulating FSH, whereas ablation of only the second largest follicle fails to engender this response (Ginther et al., 2003). Donadeu and Ginther (2001) found that total inhibin concentrations increase just before the decline in FSH. The authors concluded that inhibin likely suppresses FSH secretion during the first 2 d of the FSH decline following a wave-inducing FSH surge, with E2 playing an increasingly greater role in FSH suppression starting 1 d before deviation.

Luteinizing hormone also plays an important role in deviation, as physically evidenced by the appearance of LHr on granulosa cells around the time of diameter deviation. Bergfelt et al. (2001) found that experimentally reduced concentrations of LH in mares around the time of

deviation produced dominant follicles with smaller diameters and decreased circulating concentrations of E2 and inhibin compared with follicles under the influence of normal physiological LH concentrations. Luteinizing hormone activates P-450_{scc}, the enzyme responsible for conversion of cholesterol to P5. Belin et al. (2000) reported an increase in StAR protein, P-450_{scc}, and 3β-HSD protein content of granulosa cells during and after deviation. Intrafollicular factors such as E2, IGF-1, activin-A, and inhibin-A all play a role in increasing sensitivity of the dominant follicle to LH and FSH, facilitating deviation (Ginther et al., 2003).

Dominance is the phase whereby the dominant follicle grows at a rate such that it creates an environment unfit for subordinate follicles to continue growing (Ireland et al., 2000). During dominance, gap junctions between the oocyte and the granulosa cells deteriorate in response to increasing concentrations of LH in the periphery. Once the granulosal projections are no longer in direct contact with the oocyte, cyclic adenosine monophosphate (cAMP) is no longer able to sustain meiotic arrest; therefore, resumption of meiotic division occurs, which is necessary for final maturation of the oocyte (Senger, 2004).

Ovulation

Unlike other domestic livestock species, the mare does not experience an abrupt LH surge prior to ovulation, but rather a prolonged increase in LH concentrations beginning 6 to 7 d prior to ovulation. This rise in LH concentrations is in response to increasing E2 production by the dominant follicle (McKinnon and Voss, 1992; Yoon, 2012). Peak LH concentrations occur 1 to 3 d post-ovulation (Ginther, 1979; Whitmore et al., 1973). The exact threshold of LH required for ovulation to occur remains unknown and probably varies from mare to mare, but increased LH concentrations trigger a cascade of events through the cAMP-mediated protein kinase system that result in ovulation (McKinnon and Voss, 1992).

The first effect of the LH surge is an increase in prostaglandin E2 (PGE_2) production that causes hyperemia of the ovary and, more specifically, of the dominant follicle. This increase in PGE_2 , along with an increase in histamines, bradykinins, and mast cell infiltration of the tissues surrounding the follicle causes significant edema, which increases intrafollicular pressure (McKinnon and Voss, 1992; Senger, 2004; Yoon, 2012). Additionally, angiogenic factors, which promote the growth of new blood vessels, have been found in the follicular fluid of dominant follicles, indicating that the follicle itself may have some control over blood flow (Senger, 2004). Concurrently, $PGF_{2\alpha}$, as well as other prostaglandins (thromboxane and leukotriene), cause local vasoconstriction at the apex of the follicle and contractions of the smooth muscle components of the ovary, both of which increase follicular pressure and decrease the tensile strength of the follicular wall (McKinnon and Voss, 1992; Senger, 2004; Yoon, 2012).

The preovulatory LH surge causes a shift from E2 to P4 production, although not to the magnitude seen during the luteal phase (Yoon, 2012). Theca interna cells begin to produce P4 instead of testosterone (Senger, 2004). Progesterone is important to ovulation as the use of P4 inhibitors interfere with the ability of LH to induce ovulation *in vivo* in rats (Brannstrom and Janson, 1989). Rising P4 concentrations lead to an increase in the production of collagenase. Although not to the magnitude seen during luteolysis, $PGF_{2\alpha}$ is released to stimulate the release of lysosomal enzymes (Senger, 2004). Increasing LH concentrations also stimulate an increase in plasminogen activator in the follicular fluid, which converts plasminogen to plasmin. Plasmin, along with collagenase, proteases, and lysosomal enzymes cause the proteolysis of collagen, which decreases the tensile strength of the follicular wall (McKinnon and Voss, 1992; Senger, 2004; Yoon, 2012). In addition to the actions of the aforementioned factors, $PGF_{2\alpha}$ causes contraction of the smooth muscle layer of the ovary which causes mechanical rupture of the

follicle. Mechanical rupture involves evacuation of the majority of follicular fluid from the antrum of the follicle through the ovulation fossa. This process can take between 5 and 90 s, although some residual fluid may remain in the antrum (Townson and Ginther, 1989). The time required for complete apposition of the follicular wall ranges from 30 min to 5 h after the initial evacuation of follicular fluid (Carnevale et al., 1988; Townson and Ginther, 1987).

Induction of Ovulation

Inducing ovulation is an important aspect of breeding management. From an industry perspective, it is important to get mares bred as early in the breeding season as possible. Many breed organizations, such as the American Quarter Horse Association, American Paint Horse Association, Jockey Club, and The Arabian Horse Association define any registered horse's birthday as January 1 of the year of birth, regardless of when the foal was actually born. A foal that is born earlier has a competitive advantage over its cohorts born later in the same year, particularly when competing in age-specific events, such as races, performance futurities, and in-hand classes (Langlois and Blouin, 1998). For this reason, many producers use artificial lighting which results in decreased melatonin secretion, thereby hastening the vernal transition. Once mares are no longer transitional, ovulation is induced to ensure proper timing of ovulation in relation to insemination in an attempt to achieve pregnancy on the first non-transitional estrous cycle. Coupled with methods to hasten the vernal transition, ovulation induction allows for more efficient and timely breeding, thereby resulting in earlier foaling dates.

Depending on the fertility of individual stallions, fresh semen is viable for approximately 48 h within the reproductive tract of the mare. In contrast, the ovum is only viable for 6 to 12 h after ovulation (Yoon, 2012). Mares are generally inseminated prior to ovulation in an effort to have viable semen present at the AIJ when the ovulated ova arrives after moving through the

infundibulum and ampulla. For this reason, ovulation-inducing agents are often used to ensure proper timing of insemination (Campbell, 2012; Yoon, 2012). Doing so reduces the number of inseminations required, which reduces the inherent insult to the uterus and the risk of breeding-associated endometritis due to the inflammatory response induced by intrauterine insemination (Kotilainen et al., 1994; Troedsson, 2006). Gaining control over the timing of ovulation also allows for more timely shipments of cooled or frozen semen, thereby reducing costs for mare owners and requiring fewer semen collections, which can lessen the burden on stallions (Campbell, 2012; Yoon, 2012).

Human Chorionic Gonadotropin

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone produced by the cytotrophoblasts of the chorionic villi of the human placenta (Yen et al., 1968; Yoon, 2012). The hormone is composed of a common α subunit shared with other glycoprotein hormones, and a β subunit that confers specificity. Although the β subunit of hCG differs from the β subunit of LH, hCG binds to LHr and elicits LH-like activity in the mare. Additionally, hCG demonstrates a longer half-life once injected compared with LH after endogenous release (Cole and Kardana, 1992). Day (1939) was the first to report using hCG to induce ovulation in mares. He used an intravenous injection of 1,000 "mare's units" of hCG in mares with follicles of "sufficient size." Mares that were in estrus at the time of injection ovulated 22 to 30 h post-injection, compared with 30 to 60 h for mares with follicles of "sufficient size" that were not displaying signs of estrus at the time of injection. Follow-up studies demonstrated that intravenous administration of 1,500 to 3,300 i.u. hCG shortens the duration of estrus and results in a greater percentage of mares ovulating 24 to 48 h post-treatment when compared with

untreated controls (Butterfield et al., 1964; Davison, 1947; Kilicarslan et al., 1996; Loy and Hughes, 1966; Nishikawa, 1959; Voss et al., 1974; and Webel et al., 1977).

Sullivan et al. (1973) were the first to report adverse effects in response to hCG administration. Repeated use of hCG over successive cycles resulted in reduced responses. Treatment with hCG on the third successive cycle resulted in a failure to shorten estrus and induce ovulation within a precise time interval. The authors postulated that an immunologic response could be the cause for diminished success of hCG in inducing ovulation. Roser et al. (1979) investigated this hypothesis and found that the mares did, in fact, develop antibodies against hCG; however, these antibodies did not cross-react with equine LH and there was no reduction in efficacy following successive treatments. Co-administration of corticoids did not prevent or reduce the formation of antibodies (Duchamp et al., 1987).

There is considerable debate in the literature whether the repeated use of hCG results in a reduction in efficacy. Barbacini et al. (2000), Blanchard et al. (2003), and Gastal et al. (2006) all reported no differences in the efficacy of hCG after repeated use during successive estrous cycles. In contrast, Wilson et al. (1990) and McCue et al. (2004) reported a reduction in efficacy regarding the percentage of mares ovulating between 24 and 48 h post-treatment following the use of hCG during successive estrous cycles. Furthermore, the findings of McCue et al. (2004) indicated that hCG was not as effective at inducing ovulation in older mares; however, this has yet to be confirmed by any other research. Another study reported that the use of hCG resulted in a greater occurrence of twin pregnancies compared with untreated controls, although this has not been reported elsewhere in the literature (Veronesi et al., 2003). The exact mechanism that may result in some mares not responding to hCG has not been fully elucidated. A consensus regarding repeated administration of hCG has not been reached; however, hCG is cost-effective and

reliable, therefore it continues to be the most widely used method of ovulation induction in the U.S. for mares (McKinnon and Voss, 1992; Brinsko et al., 2010; Yoon, 2012).

Prostaglandin

Exogenous $PGF_{2\alpha}$ is commonly used to "short-cycle" mares by lysing the CL which results in a decrease in P4 production, thus hastening the return to estrus. As mentioned previously, $PGF_{2\alpha}$ plays an important role in ovulation, as well. In addition to reducing P4 concentrations, a $PGF_{2\alpha}$ analog also results in an acute increase in circulating LH and FSH when given on d 8 of diestrus (Nett et al., 1979; Witherspoon et al., 1975). Furthermore, fenprostalene, a $PGF_{2\alpha}$ analog, shortens estrus and the interval from treatment to ovulation without having any negative effects on diestrus or P4 secretion during the subsequent cycle (Savage and Liptrap, 1986). The authors of this study also indicated that $PGF_{2\alpha}$ caused a greater percentage of mares to ovulate within 48 h of treatment administration compared with controls that received saline. In contrast, results of a study that compared hCG, the $PGF_{2\alpha}$ analogue luprositol, and the GnRH agonist buserelin, indicated that $PGF_{2\alpha}$ failed to shorten the interval between treatment administration and ovulation compared with hCG and buserelin (Harrison et al., 1991). The differing results may be due to the formulation of the 2 products. Perhaps fenprostalene is more effective than luprositol for inducing ovulation in mares; however, further research is needed to compare the products directly. Endogenous $PGF_{2\alpha}$ does aid in the process of ovulation; however, PGE_2 and P4 are also contributing factors and ovulation cannot occur without sufficient LH concentrations. Perhaps co-administration of $PGF_{2\alpha}$ with a proven ovulation-inducing agent could enhance efficacy, resulting in an even greater percentage of mares ovulating within 48 h of treatment.

Pituitary Hormones and Extracts

Equine pituitary extracts have also been used to induce ovulation in mares. After purification and reprecipitation, crude equine gonadotropin (CEG) contains approximately 8 to 10% LH and 4 to 6% FSH by volume (McKinnon and Voss, 1992). The administration of CEG reliably induces ovulation with 48 h of administration (Duchamp et al., 1987; Lapin and Ginther, 1977). At this time, CEG is not commercially available. There is considerable expense associated with extraction and the LH to FSH ratio is inconsistent. Furthermore, CEG is contaminated with other pituitary hormones (McKinnon and Voss, 1992; Yoon, 2012).

The use of single chain recombinant equine LH (reLH) is one of the newer agents being tested for inducing ovulation in the mare. It was first noted that reLH *in vitro,* inoculated with Chinese hamster ovary (CHO) cells, in equine Leydig cells increased testosterone (Jablonka-Shariff et al., 2007). Because reLH elicited the same response as endogenous LH on Leydig cells, the next hypothesis was that perhaps it would induce ovulation in the mare by binding to LHr on granulosa cells. Administration of 0.75 mg reLH successfully induces ovulation in mares within 48 h. Use of reLH does not affect concentrations of P4, E2, or FSH and the effect on LH cannot be determined because it is measured along with native LH in radioimmunoassays. (Yoon et al., 2007). Although reLH appears to be a reliable method of ovulation-induction, the technology and reagents involved are expensive and it is not commercially available.

Native GnRH

Because GnRH causes the release of LH and FSH from the anterior pituitary, native and synthetic GnRH have been tested as ovulation-inducing agents. Wallace et al. (1977) used 4.5 mg of native equine GnRH but was unsuccessful at inducing ovulation or shortening estrus. On the other hand, pulsatile infusion of native GnRH was successful at hastening ovulation

(Johnson, 1986). A similar trend has been noted with synthetic GnRH. Irvine et al. (1975) reported that a single injection of 2 mg of synthetic GnRH fails to induce ovulation, whereas daily injections of 2 mg synthetic GnRH from d 2 of estrus until ovulation hastens the time to ovulation and shortens estrus. Because pulsatile infusion is not practically feasible for breeding operations, native GnRH is not a commercially applicable method of ovulation induction in the mare.

GnRH Analogues

A GnRH analogue, leuprolide, was tested as an ovulation-inducing agent by Bott et al. (1996). Time from treatment to ovulation was reduced and mean duration of estrus was shorter in the treatment group compared with untreated controls. While leuprolide may be an effective method of inducing ovulation in the mare, follicle size at the time of treatment is an important consideration. The average follicle size at time of treatment was 37 mm; however, follicle size ranged from 28 to 48 mm in diameter in this study. If treatment was administered to a mare with a large follicle, it is logical to assume that she would ovulate naturally, rather than a result of treatment alone, within a shorter timeframe compared with a control mare whose follicle was smaller.

Another GnRH analogue, gonadorelin (Cystorelin™), was used by Ingwerson et al. (2007) in an attempt to induce ovulation. The mean number of d from treatment to ovulation was reduced among Cystorelin™- treated mares when compared with saline-treated controls. However, there was no reduction in the variability of time to ovulation among the treated mares, making gonadorelin unsuitable for ovulation induction in the mare. Similar results were obtained from the use of another GnRH analogue, fertirelin acetate (Marchiori et al., 2009). Hastening ovulation is not the only goal of ovulation induction, a suitable agent must also decrease the

variability of time between treatment and ovulation, allowing for more precise timing of insemination.

GnRH Agonists

Another method of ovulation induction is the use of GnRH agonists, which are synthetic peptides that interact with GnRH receptors (GnRHr) to stimulate the release of pituitary gonadotropins. When given continuously, GnRH agonists cause a down-regulation of the pituitary, more specifically they cause a down-regulation of GnRHr, decreased sensitivity of the pituitary to endogenous GnRH, and a decrease in pulsatile release of LH from the pituitary (Schneider et al., 2006). Three GnRH agonists include buserelin acetate, deslorelin acetate, and triptorelin acetate.

Buserelin Acetate

A single injection of 20 or 40 μg buserelin acetate resulted in 73.6 and 86.6% of mares ovulating within 48 h of treatment (Humke and Beaupoil, 1979. Multiple injections of buserelin acetate in 12 h intervals induce LH surges of compounding magnitudes after each subsequent injection and hastens the interval from treatment to ovulation (Barrier-Batut et al., 2001). Squires et al. (1988) reported that buserelin acetate was as effective as hCG at hastening the interval from treatment to ovulation. Although buserelin has been proven effective for ovulation induction, the necessity of multiple injections makes it less practical in an applied setting.

Deslorelin Acetate

Deslorelin acetate was first offered commercially as a controlled-release subcutaneous implant, better known as Ovuplant (Zoetis, Kirkland, QC, Canada). Ovuplant is effective at hastening ovulation (Farquhar et al., 2000); however, an increase of 3 to 7 d has been reported for interovulatory intervals of mares treated with Ovuplant, with some mares experiencing

interovulatory intervals exceeding 30 d (Morehead and Blanchard, 2000; Vanderwall et al., 2001). Furthermore, follicular growth and FSH concentrations were suppressed in mares treated with Ovuplant, leading authors to suspect that a down-regulation of endogenous pituitary hormones was occurring (Johnson et al., 2000; Farquhar et al., 2001). McCue et al. (2002) reported that removal of the implant 48 h after insertion allowed mares to have normal interovulatory intervals. However, due to the concerns regarding pituitary down-regulation, along with the increased costs associated with implantation and removal, Ovuplant is no longer commercially available in the United States (Yoon, 2012).

A slow-release injectable form of deslorelin acetate was approved and released in the U.S. in 2010 and is commercially known as SucroMate (Thorn Bioscience, LLC, Louisville, KY). Deslorelin acetate can also be compounded and purchased through licensed pharmacies. Both Sucromate (Ferris et al., 2012) and compounded deslorelin (McCue et al., 2007) are as effective as hCG for ovulation-induction in mares, with 89.9 and 90.1% of mares ovulating within 48 h of administration, respectively. Although effective, the cost of Sucromate may be prohibitive for some producers. Compounded deslorelin acetate is less expensive than Sucromate, but still not as cost-effective as hCG. In situations where mares are being bred with shipped semen to highly valuable stallions, the reliability of Sucromate may be well-worth the additional cost.

Triptorelin Acetate

Triptorelin acetate (TA) is a GnRH agonist that differs structurally from native mammalian GnRH by the replacement of glycine with D-tryptophan. This amino acid replacement results in an increased half-life and a greater binding affinity to GnRH receptors. While the use of TA in animal and zoological science for ovulation-induction and super-

ovulation has been primarily limited to the sow, it has been added to semen extender to induce ovulation following artificial insemination in rabbits (Viudes-de-Castro et al., 2007). Triptorelin acetate was also used by Schneider et al. (2006) in an injectable controlled-release microencapsulated formulation in dairy heifers to induce pituitary down-regulation. Heifers receiving TA experienced a more uniform preovulatory LH surge; however, the number of post-ovulatory LH pulses was reduced. Furthermore, the proportion of degenerated cumulus oocyte complexes and number of immature oocytes from small follicles were increased. The authors concluded that continuous use of the GnRH agonist beyond 1 wk may impair the development of bovine follicles and oocytes.

For ovulation-induction, TA is integrated into an intravaginal gel vehicle and is commercially available under the product name, OvuGel (Pennatek, Radnor, PA, USA). Each mL of OvuGel contains 100 μg of TA in a 1.2% methylcellulose gel. This product is designed for intravaginal use because many swine producers have gone needle-free, thus necessitating a different delivery method for ovulation-inducing agents. OvuGel induces the release of LH between 4 and 8 h post-treatment, resulting in a surge comparable to the magnitude of spontaneous surges experienced in untreated controls (Stewart et al., 2010). Taibl et al. (2008) first tested the use of OvuGel in sows and found that a greater percentage of sows ovulated within 48 h of OvuGel administration (78.2%) compared with both placebo-treated (37.8%) and negative controls (45.8%). The authors concluded that OvuGel successfully advanced ovulation and may be useful in fixed-time artificial insemination (FTAI) protocols. Furthermore, FTAI eliminates the need for heat-detection which reduces labor costs associated with breeding.

Taibl et al. (2009) reported that OvuGel-treated sows had a greater pregnancy rate, number of CL and fetuses, and percentage of embryo survivability compared with placebo-

treated sows. Furthermore, the use of OvuGel reduced the number of inseminations required per pregnancy, allowing for maximization of the genetic value of a small number of high-index boars, which may be used as part of a strategy to improve overall efficiency of the swine production industry (Johnston et al., 2010). Additionally, because there is great concern among consumers regarding the use of chemicals or hormones, tissue harvested from OvuGel-treated sows was investigated for safety. No endocrine, pathology, toxicology, or biological effects were reported in either males or females following the use of OvuGel; therefore, edible meat from OvuGel-treated sows was deemed safe for human consumption (Francisco et al., 2013).

Because of the efficacy seen in sows, there was considerable interest in investigating the use of OvuGel to induce ovulation in mares. An unpublished pilot study was conducted in 2014 to test the efficacy of OvuGel for inducing ovulation (Dr. Steve Webel, personal communication). Mares received either 5 mL OvuGel, 10 mL OvuGel, or 5 mL methylcellulose gel intravaginally when follicles measured between 34 and 42 mm in diamter. Both OvuGel-treated groups had a greater percentage of mares ovulating within 48 h of treatment. While noteworthy results were achieved from this trial, the study was conducted in October, nearing the end of the physiological breeding season. Furthermore, statistical analysis was not possible due to the unequal numbers assigned to treatment groups and the large amount of variation in follicle size at the time of treatment. A larger, more controlled study was needed in order to evaluate the efficacy of OvuGel in mares. Furthermore, its effect on LH concentrations were of particular interest, especially considering the unique nature of the protracted LH surge that begins prior to ovulation and peaks post-ovulation in mares.

Summary

Induction of ovulation is a technique frequently used by equine producers and has been the focus of a vast amount of research in the last 50 yr. While hCG and deslorelin acetate are very effective for this purpose, the use of OvuGel warranted further investigation following positive results in sows and the pilot study conducted in mares. Furthermore, OvuGel could serve as a useful alternative for mares that do not respond reliably to hCG and it may be more cost-efficient than commercially available deslorelin acetate. Thus, the following experiments were conducted to determine the effect of OvuGel on LH concentrations and evaluate its efficacy as an ovulation-inducing agent in mares.

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Chapter 2 - Use of intravaginal Triptorelin gel to induce ovulation in mares

Introduction

Producers often use ovulation-inducing agents in the equine breeding industry to more accurately time insemination relative to ovulation, thereby reducing the number of inseminations required for conception. While hCG is commonly used to induce ovulation, some mares respond less reliably if hCG has been administered multiple times within a season (McCue et al., 2004). Deslorelin, a GnRH agonist, is an effective ovulation-inducing agent in mares and is available commercially, or it can be compounded and purchased through a licensed pharmacy (Yoon, 2012). Another GnRH agonist, triptorelin acetate (TA), has been packaged into an intravaginal methylcellulose gel (OvuGel, Pennatek, Radnor, PA) for use in commercial sows as part of fixed-timed artificial insemination (FTAI) protocols. Administration of TA gel 96 h post-weaning results in ovulation within 48 h post-treatment, thereby allowing for improved timing of insemination without the need for labor-intensive estrus detection (Taibl et al., 2008). Furthermore, ovulation induction using TA gel in sows reduced the number of inseminations required per pregnancy while having no adverse effects on the number of ovulations, number of pigs born alive, or litter size (Johnston et al., 2009). Because of its efficacy for ovulation induction in sows, TA gel was used in an attempt to induce ovulation in mares in a small pilot study (Dr. Steve Webel, personal communication). Administration of TA gel resulted in a greater percentage of mares ovulating within 48 h of treatment, warranting a larger trial to evaluate its efficacy. Therefore, it was our objective to investigate its efficacy as an ovulation-inducing agent in mares. We hypothesized that administration of TA gel would result in an increase in

peripheral LH concentrations, thereby hastening the interval from treatment to ovulation compared with placebo-treated controls.

Materials and Methods

All procedures described herein were approved by the Kansas State University Animal Care and Use Committee prior to initiation of the trials.

Animals

Stock-type, non-lactating mares between 3 to 18 yr old were used. Mares were group-housed in dry lots with ad libitum access to water and brome hay. Purina Strategy (Purina Mills, Inc., St. Louis, MO) was fed on an as-needed basis in order to maintain weight. Mares weighed on average 493 ± 8.33 kg. Follicular development was monitored via transrectal ultrasonography beginning in March and continuing through at least one complete estrous cycle for each mare to ensure that normal estrous cycles were occurring prior to the treatment cycle.

Experimental Design

Experiment 1

Experiment 1 took place between April and June, 2014. After stratifying by parity and age, 24 mares were assigned to 3 treatment groups (n = 8 / group): mares received either 5 mL 1.2% methylcellulose gel only (CON), 5 mL TA gel (500 μg TA; TA5), or 10 mL TA gel (1,000 μg TA; TA10). Follicular development was monitored via transrectal ultrasonography using a Medison Sonovet SV-600 ultrasound machine (Universal Medical Systems, Inc., Bedford Hills, NY) with a Medison 65 mm, 5 mHz probe (Universal Medical Systems, Inc., Bedford Hills, NY). Once a follicle measuring ≥ 25 mm in diameter was observed, blood sampling and ultrasonography occurred every 24 h between 0700 and 0800 h until the dominant follicle

reached ≥ 35 mm. When a dominant follicle measuring ≥ 35 mm in diameter was noted, the assigned treatment was administered approximately 2 to 3 cm posterior to the cervix using a modified AI pipette. A sterile 10 mL luer lock syringe with an 18 ga needle was used to draw up the assigned treatment from 52.5-mL vials. The needle was then replaced with a luer lock AI pipette, approximately 15 cm in length. Prior to treatment administration, vulvas and surrounding areas were washed to reduce the likelihood of vaginal contamination. Following treatment, ultrasonography and blood sampling occurred every 12 h (0700 and 1900 h) until 48 h post-ovulation, which marked the endpoint for mares on the study.

Experiment 2

Experiment 2 took place between March and June, 2015. After blocking for parity and age, 23 mares were assigned to 3 treatment groups with 8 mares in each TA gel-treated group and 7 mares in the control group. The groups received either 5 mL of 1.2% methylcellulose gel only (CON), 5 mL TA gel (500 μg TA; TA5), or two 5-mL doses of TA gel, administered 24 h apart (1,000 μg; TA5x2). Follicular development was monitored via transrectal ultrasonography using a Medison Sonovet SV-600 ultrasound machine (Universal Medical Systems, Inc., Bedford Hills, NY) with a Medison 65 mm, 5 mHz probe (Universal Medical Systems, Inc., Bedford Hills, NY). Once a follicle measuring ≥ 25 mm in diameter was observed, blood sampling and ultrasonography occurred every 12 h at 0700 and 1900 h until a follicle ≥ 35 mm was detected. When a dominant follicle measuring ≥ 35-mm in diameter was observed, assigned treatment was administered intravaginally, as described in Exp. 1. The TA5x2 mares received the second dose 24 h after the first dose was administered. Once treatments were administered, ultrasonography and blood sampling occurred every 6 h (0000, 6000, 1200, and 1800 h) until 48 h post-ovulation, which marked the endpoint for mares on the study.

Blood Sampling and Analysis

For each time point in which blood was collected, a 10 mL sample of whole blood was collected at the time of ultrasonography via jugular venipuncture using heparinized Vacutainers (Becton, Dickson and Company, Franklin Lakes, NJ) containing 142 USP units of freeze-dried sodium heparin. Following collection, whole blood was refrigerated. After the 1900 h collection period each day in Exp. 1 and the 0600 and 1800 h collection periods each day in Exp. 2, whole blood was centrifuged at 1,400 x *g* at room temperature for 15 min to separate plasma. Plasma was pipetted into 1.5 mL microcentrifuge tubes and frozen at -18° C for later analysis. Analysis of LH concentrations were performed in Dr. Don Thompson's laboratory at Louisiana State University using established RIA techniques (Thompson et al., 1983). The intra- and interassay coefficients of variation were 6 and 9%, respectively, with a sensitivity of 0.2 ng/mL.

Statistical Analysis

All statistical analyses were performed using SAS v. 9.3 (SAS Inst. Inc., Cary, NC). Experiments 1 and 2 were analyzed using the same statistical procedures. Interval from treatment to ovulation was analyzed using ANOVA. Number of mares ovulating within 48 h of treatment administration was analyzed using PROC FREQ with an exact statement to account for small sample size. PROC MIXED was used to analyze LH concentrations. Of repeated measures, autoregressive 1 (AR-1), and Toeplitz (TOEP 2) covariance structures, AR-1 yielded the lowest Akaike information criterion (AIC) and was therefore used to analyze LH concentrations. In Exp. 2, LH data were normalized and reanalyzed because LH concentrations tended to be different at 0 h ($P = 0.06$). Area under the curve (AUC) analysis was performed on LH data from 0 to 36 h in Exp. 1 on all 3 treatment groups. In Exp. 2, AUC was performed on LH data from 0 to 24 h and from 24 h to 48 h. Data for LH concentrations were reported as least squares means.

Intervals from treatment to ovulation were reported as the mean ± SE. Data were considered significant if $P \leq 0.05$ and a tendency was noted if $0.05 \leq P \leq 0.10$.

After performing analyses for normality in Exp. 1, a mare in the CON group was found to be an outlier because her LH concentrations were 3 to 10-fold greater than her cohorts throughout the study; therefore, she was removed from the data. Normality tests for Exp. 2 did not identify any outliers. There were no differences when comparing mares from the TA5 and TA10 groups in Exp. 1, so the groups were pooled for analysis of LH concentrations.

Results

Experiment 1

Follicle size at time of treatment was not different ($P = 0.16$) between the groups (Table 2.1). Diameter of the follicle during the observation prior to ovulation was not different ($P = 0.94$) between the groups (Table 2.1). The treatment to ovulation interval was not different ($P = 0.92$) between any of the groups (Table 2.1; Figure 2.1). The number of mares ovulating within 48 h of treatment administration were not different ($P = 0.5$; Table 2.1). There were no differences ($P = 0.77$) in LH concentrations at -24 and 0 h prior to treatment (Fig. 2.2*)*. Mares in the TA5 and TA10 treatment groups tended ($P = 0.09$) to experience a surge in LH within 12 h of treatment (Fig. 2.2). Mares in the CON group had greater ($P = 0.03$) LH concentrations compared with mares in the TA5 treatment group 60 h post-treatment (Fig. 2.2). For LH, overall treatment effect was not significant ($P = 0.50$); however, a treatment by time interaction was identified ($P < 0.001$). The AUC between 0 and 24 h was not different for TA5 compared with CON ($P = 0.60$), TA10 compared with CON ($P = 0.31$) or TA5 compared with TA10 ($P = 0.61$). No negative side effects were noted following treatment.

Table 2.1 - Follicle size at time of treatment (TRT) and the effect of TA gel administration in Exp. 1 and 2 upon detection of a follicle ≥ 35 mm in diameter on follicle size at ovulation (OV), interval from treatment to ovulation, number of mares within each group ovulating within 48 h of treatment (# OV ≤ 48 h), and percentage of mares ovulating within 48 h of treatment (% OV ≤ 48 h) compared with placebo-treated controls.

	n	Follicle Size at TRT (mm)	Follicle Size at OV (mm)	Interval from TRT to OV (h)	# OV ≤ 48 h	% OV ≤ 48 h
Experiment 1						
CON[1]	7	35.6 ± 0.5	42.9 ± 2.2	73.7 ± 14.9	2	28.6%
TA5[2] & TA10[3]	16	36.3 ± 0.4	41.9 ± 2.0	72.8 ±14.0	9	56.3%
Experiment 2						
CON[1]	7	35.3 ± 0.3	47.0 ± 1.6	123.1 ± 21.7[a]	0[a]	0.0%[a]
TA5[2]	8	36.0 ± 0.3	42.6 ± 1.5	61.5 ± 8.8[b]	6[b]	75.0%[b]
TA5x2[4]	8	36.0 ± 0.3	42.8 ± 1.5	61.5 ± 9.6[b]	4[a]	50.0%[a]

[a,b] Values within a column with differing superscripts are different ($P < 0.05$).
[1] CON = mares receiving 5-mL of methylcellulose only, without GnRH agonist (TA).
[2] TA5 = mares receiving 5-mL TA.
[3] TA10 = mares receiving 10-mL TA.
[4] TA5x2 = mares receiving two 5-mL doses of TA, 24 h apart.

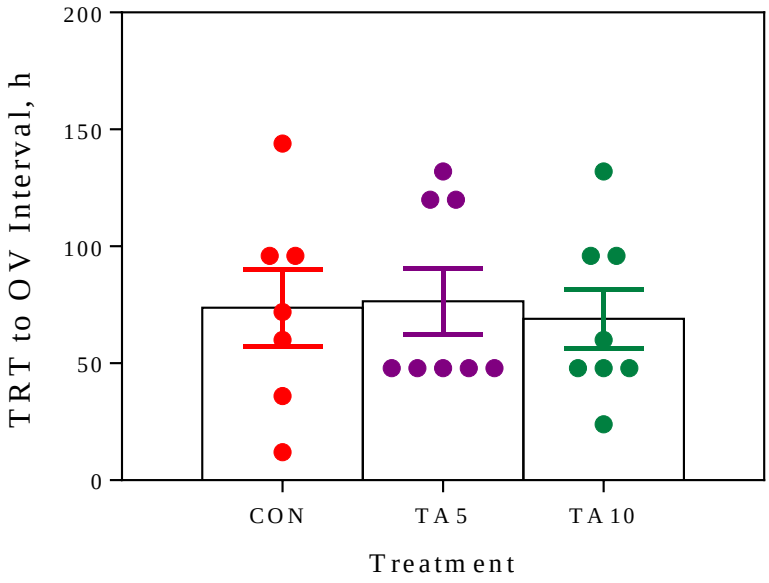

Figure 2.1 - Interval from treatment to ovulation in Exp. 1 following intravaginal administration of 5 mL TA gel (TA5), 10 mL TA gel (TA10), or 5-mL methylcellulose gel only (CON) to mares when a follicle ≥ 35-mm in diameter was observed. Bars represent SE and dots represent each mare within a treatment group.

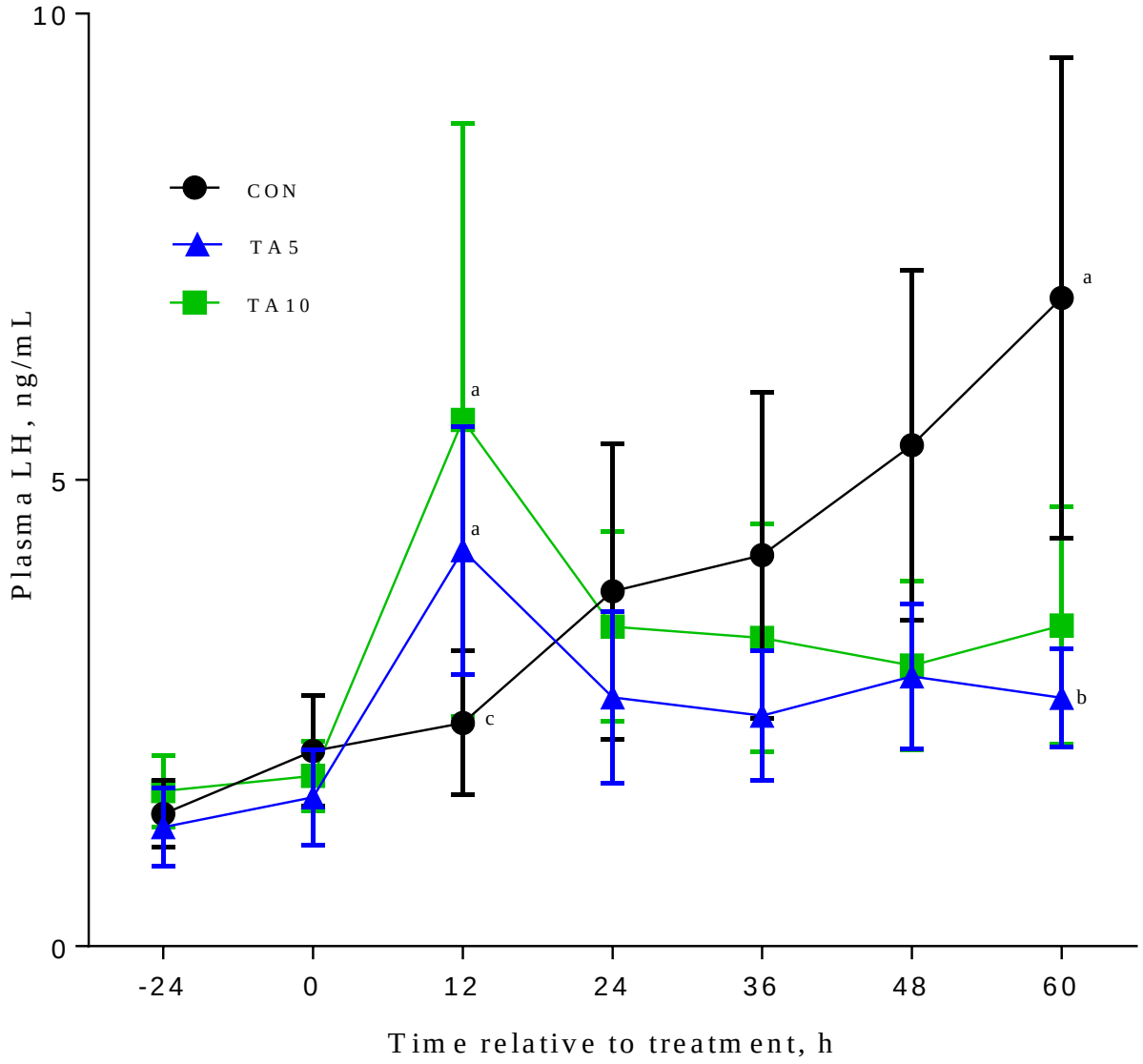

Figure 2.2 - Peripheral concentrations of LH (ng/mL), reported as least squares means, in Exp. 1 following intravaginal administration of 5 mL TA (TA5), 10 mL TA (TA10), or 5 mL methylcellulose gel (CON) to mares when a follicle ≥ 35 mm in diameter was observed. Blood samples were obtained every 24 h prior to treatment and every 12 h post-treatment until 48 h post-ovulation. [a,b] $P < 0.05$. [a,c] $0.05 < P < 0.10$.

Experiment 2

Follicle size at the time of treatment was not different ($P = 0.14$; Table 2.1). Diameter of the ovulatory follicle during the observation before ovulation tended ($P = 0.06$) to be greater among the CON mares compared with both TA5 and TA5x2 mares (Table 2.1). The treatment to ovulation interval was shorter for mares in the TA5 and TA5x2 treatment groups compared with CON mares ($P = 0.006$); however, there was no difference ($P = 1.00$) in the treatment to ovulation interval between TA5 and TA5x2 (Table 2.1; Fig. 2.3). The number of mares ovulating within 48 h of treatment administration was greater ($P = 0.02$) among TA5 mares compared with TA5x2 and CON mares (Table 2.1). There were no differences in LH concentrations at -24 or -12 h prior to treatment ($P > 0.05$; Fig. 2.4); however, there was a tendency for LH concentrations to differ between the groups at 0 h ($P = 0.06$). Prior to normalization, concentrations of LH at 6 ($P = 0.03$) and 12 h ($P = 0.04$) post-treatment were different, with TA5 and TA5x2 having greater LH concentrations compared with CON (Fig. 2.4); however, after normalization of the LH data there were no differences at 6 or 12 h post-treatment. The second dose of TA gel given to TA5x2 mares did not result in an increase in LH concentrations ($P = 0.44$; Fig. 2.4). Luteinizing hormone concentrations were greater among TA5 and TA5x2 mares compared with CON mares at 72, 78, and 84 h post-treatment ($P < 0.05$; Fig. 2.4); however, these differences were no longer significant after the data were normalized (Fig. 2.5). In regard to LH concentrations, the overall treatment effect was not significant ($P = 0.1$); however, the treatment by time interaction was significant ($P < 0.001$). After normalization, the treatment by time interaction remained significant ($P < 0.001$) but there were no significant treatment effects at specific times. The AUC from 0 to 24 h was not different for TA5 compared with CON ($P = 0.11$), TA5x2 compared with CON ($P = 0.33$), or TA5 compared with TA5x2 ($P = 0.49$).

Furthermore, the AUC for TA5x2 at 0 to 24 h and 24 to 48 h was not different (P = 0.25). Upon ultrasonographic examination 6 h after administration, 1 TA5x2 mare retained fluid following the second TA gel dose, but this was resolved by 12 h post-treatment. No negative side effects of treatment were observed.

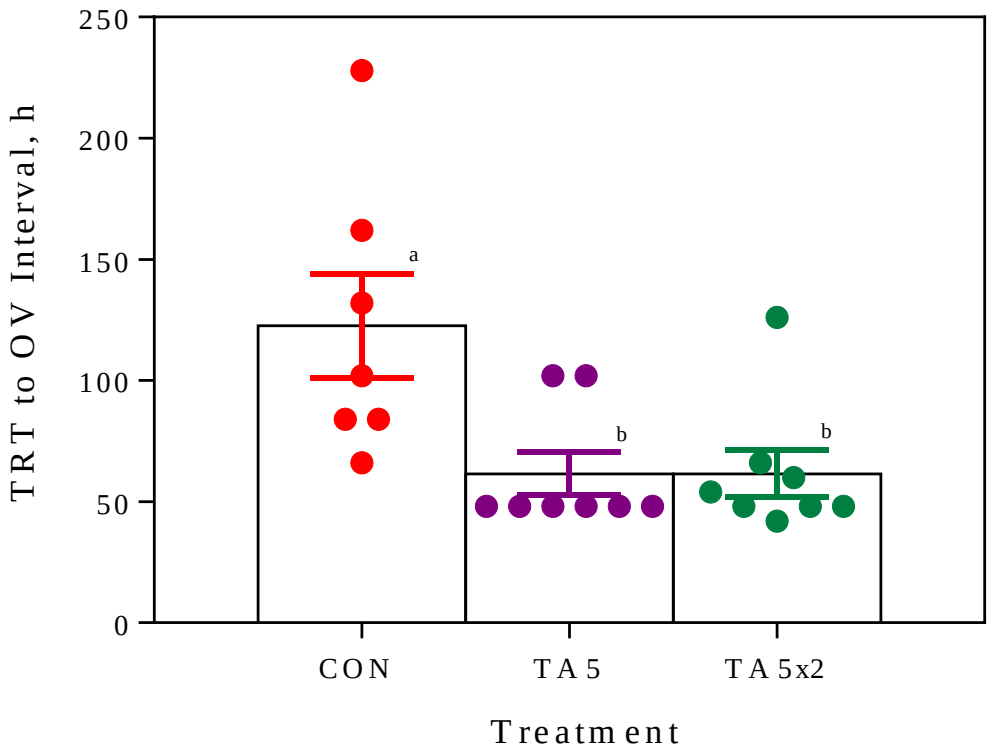

Figure 2.3 - Interval from treatment to ovulation in Exp. 2 following intravaginal administration of 5 mL TA gel (TA5), two 5-mL doses of TA gel given 24 h apart (TA5x2), or 5-mL methylcellulose gel only (CON) to mares when a follicle ≥ 35-mm in diameter was observed. Bars represent SE and dots represent individual mares. [a,b] $P < 0.05$.

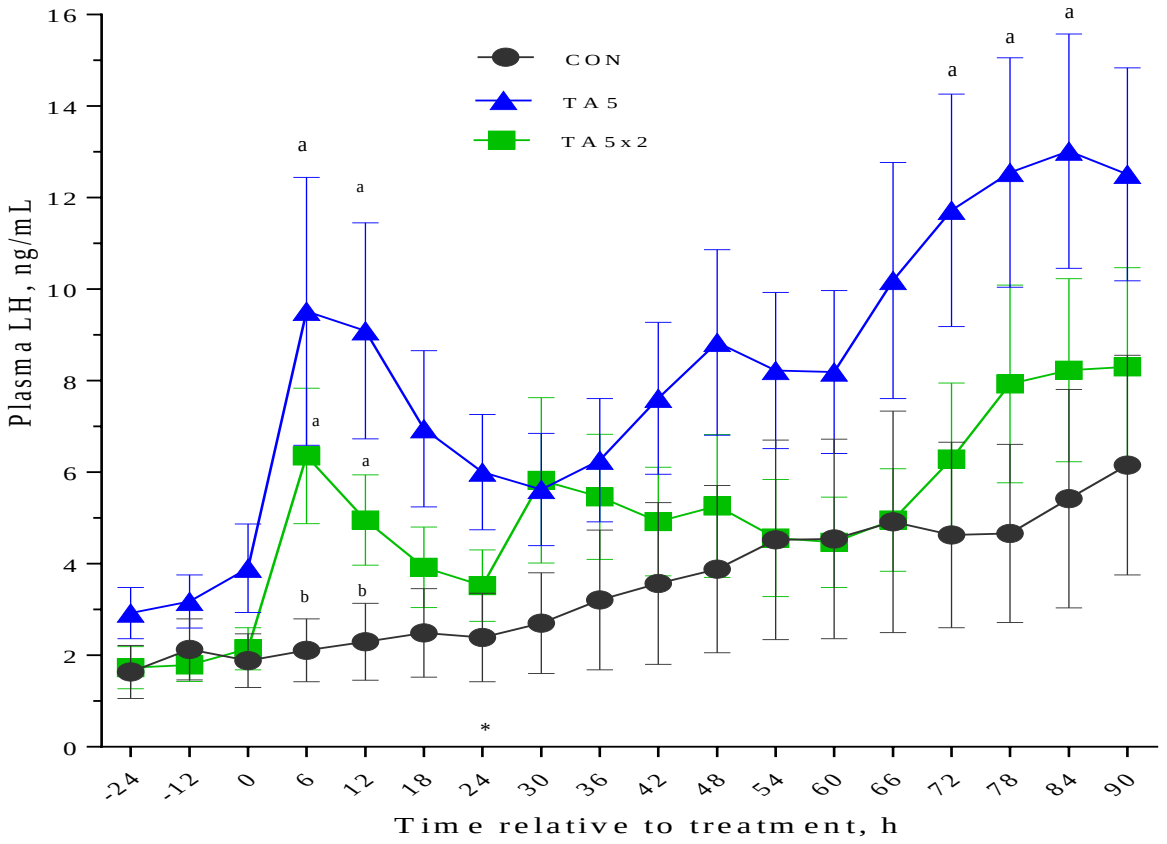

Figure 2.4 - Peripheral concentrations of LH (ng/mL) in Exp. 2 following intravaginal administration of 5 mL TA gel (TA5), two 5-mL doses of TA gel, given 24 h apart (TA5x2), or 5 mL methylcellulose gel only (CON) to mares when a follicle ≥ 35-mm in diameter was observed. * Indicates administration of second 5-mL dose of TA to TA5x2 mares. [a,b] Time points with differing superscripts differ, $P < 0.05$.

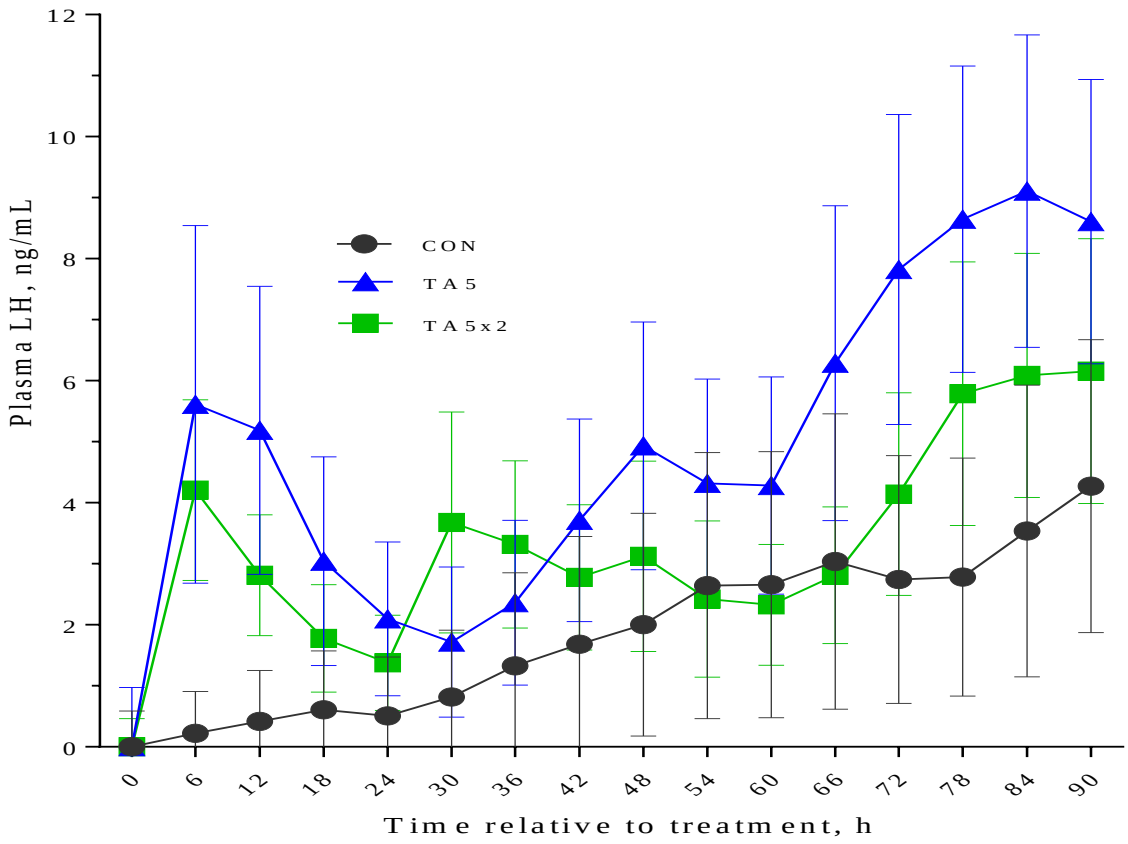

Figure 2.5 - Normalized data for peripheral concentrations of LH (ng/mL) in Exp. 2 following intravaginal administration of 5 mL TA gel (TA5), two 5-mL doses of TA gel, given 24 h apart (TA5x2), or 5 mL methylcellulose gel only (CON) to mares when a follicle ≥ 35-mm in diameter was observed. Blood samples for LH measurement were obtained every 12 h prior to treatment and every 6 h post-treatment until 48 h post-ovulation.

Discussion

The initial pilot study involving the use of TA gel to induce ovulation in mares resulted in 100.0 and 66.7% of mares treated with 5 and 10 mL TA gel, respectively, ovulating within 48 h of treatment administration (Dr. Steve Webel, personal communication). Although a lesser percentage of mares ovulated within 48 h of treatment in Exp. 1 compared with results from the pilot study, this is possibly due to the difference in follicle size at the time of treatment administration between the 2 studies. The mean diameters of the dominant follicle at the time of treatment in the pilot study were 40.0 and 38.7 mm for mares in the 5 and 10 mL TA gel treatment groups, respectively, compared with 36.3 mm for TA5 and TA10 mares in Exp. 1. Once the preovulatory follicle reaches 40 mm in diameter, ovulation generally occurs within 2 d (Ginther et al., 2000); therefore, it is not clear whether ovulation occurred naturally or as a result of TA gel treatment in the pilot study.

In Exp. 1, TA gel failed to cause a greater number of mares to ovulate within 48 of TA gel administration nor was the interval from treatment to ovulation hastened by TA gel administration. All 5 mares in the TA5 group that ovulated within 48 h of treatment ovulated between 36 and 48 h of treatment administration. On the other hand, of the TA5 mares that failed to ovulate within 48 h of treatment administration, all 3 ovulated between 120 and 132 h post-treatment. Similarly, of the 4 TA10 mares that failed to ovulate within 48h of treatment administration, 3 of them ovulated between 96 and 132 h post-treatment. The longer treatment to ovulation interval for the non-responding mares in the TA5 and TA10 treatment groups compared with the more evenly distributed treatment to ovulation interval observed in CON mares explains the lack of difference between the mean treatment to ovulation intervals of the 3 groups.

The greatest peripheral LH concentrations were observed 12 h post-treatment among TA5 and TA10 mares in Exp. 1. This tendency for the TA5 and TA10-treated mares to experience a surge in peripheral LH concentrations within 12 h post-treatment was followed by a decline to nearly pre-treatment values by 24 h post-treatment. The LH response in mares was consistent with the response observed in sows following intravaginal administration of 200 μg TA 96 h post-weaning. The LH surge in TA-treated sows was observed between 4 and 12 h after treatment was administered, followed by a decline to pre-treatment concentrations by 24 h post-treatment (Stewart et al., 2010). Sows experience an abrupt LH surge with peak values being reached just before ovulation, whereas the endogenous LH pattern surrounding ovulation in mares is slower, with a more protracted peak. In mares, LH begins to increase approximately 4 days before ovulation and peak values are not reached until 1 to 3 d post-ovulation (Ginther, 1979). The LH surge elicited by TA gel in both species is more characteristic of the endogenous LH surge experienced by sows, rather than the pattern experienced by mares.

Available literature in the sow reports between 75 and 78.2% of sows ovulating within 48 h of TA gel administration (Taibl et al., 2008; Taibl et al., 2009). The percentage of TA gel-treated mares in Exp. 1 ovulating within 48 h of treatment administration is not comparable to the percentages reported in sows. Because of the differences in periovulatory LH secretion patterns between the 2 species, it was postulated that perhaps peripheral LH concentrations were not being sustained long enough to cause ovulation to occur in a comparable percentage of mares. In Exp. 1, the greatest LH concentrations for TA5 and TA10 mares were detected at 12h, the first time point after TA gel administration. It is certainly possible that more frequent blood sampling would have allowed more accuracy and precision in describing the mare's LH response following treatment. Had blood samples been obtained more frequently, we may have noted a

significant increase in LH following TA gel administration. Because TA10 mares did not respond to treatment more favorably than TA5 mares in Exp. 1, the 10-mL dose of TA gel was separated into two 5-mL doses administered 24 h apart in an attempt to maintain elevated LH concentrations for a longer period of time in Exp. 2. The 24-h period between doses was chosen because it would be practical in many commercial settings. In addition to the change in dosing regimen, Exp. 2 included more frequent blood collections and ultrasonography in order to more precisely characterize the responses elicited by TA gel administration.

In Exp. 2, a greater number of TA5 mares ovulated within 48 h of treatment compared with CON mares, whereas TA5x2 failed to cause a greater number of mares to ovulate within that timeframe compared with CON mares. Because only 1 additional mare in the TA5 group ovulated within 48 h of treatment administration in Exp. 2 compared with Exp. 1, the difference in number of TA5 mares ovulating within 48 h compared with CON mares in Exp. 2 versus Exp. 1 is likely due to no CON mares ovulating within 48 h in Exp. 2, whereas 2 CON mares ovulated within that timeframe in Exp. 1. The interval from treatment to ovulation was shorter among TA5 and TA5x2 mares compared with CON mares in Exp. 2. The difference in the intervals from treatment to ovulation between both TA5 and TA5x2 compared with CON in Exp. 2 may be attributable to the longer treatment to ovulation interval observed in CON mares in Exp.2 compared with the ovulation interval of CON mares in Exp.1. In general, the average length of time between the development of a 35 mm follicle and ovulation during natural cycles in mares is approximately 4 d, or 96 h (Ginther et al., 2000). The CON mares in Exp. 1 had a shorter interval from treatment at a $\geq$ 35 mm follicle to ovulation compared with averages reported in the literature, perhaps leading to the lack of difference between the 2 TA gel-treated groups and the CON group. On the other hand, the CON mares in Exp. 2 had a longer interval from treatment at

a ≥ 35 mm follicle to ovulation compared with reported averages, potentially explaining the differences observed in the ovulation intervals between the 2 TA gel-treated groups and the CON group. Because mares were housed at the same location and managed similarly between experiments, there is no definitive explanation for the difference in treatment to ovulation intervals between the CON groups in Exp. 1 and Exp. 2, other than individual variation.

Results of Exp. 2 indicated that peak LH concentrations were reached around 6 h after TA gel administration and were already beginning to decline by 12 h post-treatment. This observation indicates that peak LH concentrations may have been reached prior to the first post-treatment blood collection in Exp. 1, possibly explaining why the LH increase at 12 h post-treatment was not significant. After normalization in Exp. 2, there were no differences in LH concentrations at any time point. The loss of significance is likely due to small sample sizes and the variation in LH concentrations between mares.

In sows, increasing doses of TA gel have caused greater percentages of sows to ovulate within 48 h of treatment administration, with no reduction in efficacy as the dose increased (Knox et al., 2014); therefore, there is no evidence in the literature to explain why TA10 did not perform as well, or better than, TA5 in Exp. 1. In mares, 2.5 mL TA gel (250 μg TA) failed to induce ovulation within 48 h of treatment administration in an unpublished study (Dr. Steve Webel, personal communication). It is possible that the optimum dose for TA gel in mares to induce ovulation could be greater than 5 mL (500 μg TA) and less than 10 mL (1,000 μg TA). Perhaps a 7.5 mL (750 μg TA) dose would be more effective at inducing ovulation in mares compared with 5 mL (500 μg TA).

Although TA gel administration induced a surge in peripheral LH concentrations in Exp. 2 and tended to do so in Exp. 1, the percentage of mares ovulating within 48 h of TA gel

administration was less than percentages reported in recent literature for existing products widely used in the equine industry. Ferris et al. (2012) reported that ovulation occurs within 48 h in 82.8 and 89.9% of mares following administration of hCG or deslorelin acetate, respectively. Inclusion of more mares in the current study could have yielded more favorable data. Furthermore, intravaginal administration of TA gel is more time-consuming and labor intensive than IV or IM administration of hCG or deslorelin acetate. An alternate route of administration, such as an injectable method, could be investigated to determine if efficacy of TA at inducing ovulation in mares would improve. Additionally, pregnancy rates following TA gel-induced ovulations, efficacy in lactating mares, and safety of product use during lactation would need to be evaluated before the product becomes available commercially for use in horses. There is no data in the sow regarding the efficacy and safety in lactating sows because they are typically anovulatory while lactating.

Between Exp. 1 and 2, of the 19 mares that did respond to TA gel treatment, regardless of dose, 18 ovulated between 36 and 48 h which indicates that the product is quite accurate in the timing of ovulation, given that the mares respond to treatment; however, the percentages of mares ovulating within 48 h of TA gel administration in our experiments are substandard compared with current products, such as hCG and deslorelin acetate. Although administration of 5 mL of TA gel resulted in 75% of mares ovulating within 48 h of treatment administration in Exp. 2, the results were not consistent between Exp. 1 and 2. In conclusion, because of the inconsistent results between these 2 experiments, further testing would be needed to fully evaluate the efficacy of TA gel as an ovulation-inducing agent in mares.

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